Ca^{2+} and Phosphoinositides Regulations in α-actinin-4 F-actin Binding

by

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Summary

α-actinin-4 is a non-muscle isoform of α-actinin that belongs to the spectrin superfamily. It comprises three functional regions: an N-terminal actin-binding region that consists of two calponin homology (CH) domains, a central region that consists of four copies of the spectrin-like repeat domain and a C-terminal calmodulin-like domain that is predicted to bind Ca^{2+}. α-actinin-4 is organised as an antiparallel homodimer formed by the interaction of four spectrin-like repeats between the two monomers, giving a rod-like shape, with actin binding regions at both ends.

α-actinin-4 is an abundant actin-bundling protein, which provides a direct link between actin filaments and integrins, and is believed to play an important role in stabilising cell shape and adhesion and regulating cell migration. It also acts as a tumor suppressor and influences the metastatic potential and invasiveness in human cancers. A cluster of three actin binding motifs have been identified in the CH domains (2X CH) from other members of the spectrin superfamily, utrophin and dystrophin. Two of them reside in the CH1 domain and the third resides in the first α-helix of the CH2 domain. In addition, a PIP2 binding site has been mapped on a region adjacent to actin-binding site-3. These observations imply the F-actin binding activity would be regulated by phosphoinositides. Five mutations of α-actinin-4, K122N, an alternative splice variant, K255E, T259I and S263P, have been reported to be involved in three human diseases, non-small lung cancer (NSCLC), small cell lung cancer (SCLC) and focal segmental glomerulosclerosis (FSGS). The mutation site within these mutants is located on the actin binding region. Therefore, the actin binding region is presumed to be associated with the progression of human disease.

The aims of this thesis focused on the regulation of the F-actin binding activity of α-actinin-4 by phosphoinositides (PIP2 and PIP3), the calmodulin-like domain and Ca^{2+}, determination of the three-dimensional structure of the CH2 domain in solution and identification of the phosphoinositide binding site on the CH2 domain. In order to investigate the F-actin binding activity quantitatively, a novel *in vitro* F-actin binding assay (solid phase) was established to replace the semi-quantitative actin bundling assay.

Using this novel solid phase F-actin binding assay, Ca^{2+} was shown to enhance the F-actin binding activity of α-actinin-4 in a concentration dependent manner. The presence of 10 mM Ca^{2+} results in a two-fold increase in the F-actin binding activity. Both PIP2 and PIP3 inhibited the F-actin-binding activity of α-actinin-4 in a concentration dependent manner with an approximate IC_{50} of 75 and 45 μM, respectively.

In order to characterise how phosphoinositides regulated the F-actin binding activity of α-actinin-4, the solution structure of α-actinin-4 CH2 domain was determined and the phosphoinositide binding residues within the CH2 domain were identified using NMR spectroscopy. The solution structure of α-actinin-4 CH2 domain contained six α-helices and was similar to that of other spectrin superfamily members. The strategy used in identification of the phosphoinositide binding site was an NMR-based 2D ^{1}H-^{15}N HSQC ligand titration assay to replace the traditional semi-quantitative protein-lipid overlay assay. Using the NMR-based ligand titration assay, the recognition site for the inositol head group resides in residues Trp 172, Tyr 265 and His 266 and the binding region of acyl chains resides in the first α-helix structure which is one of the putative F-actin binding sites. In order to examine the interaction of phosphoinositides with this site, Y265A and H266E mutants of α-actinin-4 CH2 domain were generated using site-directed mutagenesis and verified the interaction with phosphoinositides and the inositol head group using an NMR-based ligand titration assay. These results confirmed the phosphoinositide binding site on the CH2 domain and residues, Tyr 265 and His 266, are critical for interacting with phosphoinositides.

Wildtype and mutants (Y265A and H266E) of α-actinin-4 were expressed in mammalian cells as EGFP-fusion proteins. Wildtype α-actinin-4 was shown to be co-localised with focal adhesions and actin stress fibres. However, Y265A and H266E mutants of α-actinin-4 were co-localised with actin stress fibres but poorly co-localised with focal adhesions. Moreover, both Y265A and H266E mutants of α-actinin-4 were co-localised with actin in the cytoplasm rather than localised along the cell membrane after EGF stimulation for 30 minutes. These results suggested that PIP2 assists the co-localisation of α-actinin-4 with focal adhesions.

Taken together, the results described in this thesis concluded that Ca^{2+} enhanced the F-actin binding activity of α-actinin-4 *in vitro*. However, phosphoinositides (PIP2 or PIP3) inhibited the F-actin binding activity *in vitro*. Moreover, the results described in this thesis provided a phosphoinositide binding site on α-actinin-4 CH2 domain. Binding to PIP2 is important to the localisation of α-actinin-4 in focal adhesions.

Declarations

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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List of Abbreviations

2D	two-dimensional
3D	three-dimensional
4D	four-dimensional
ABD	Actin binding domain
Amp	ampicillin
ARIA	Ambiguous Restraints of Iterative Assignment
Arp 2/3	actin-related protein 2/3
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C-	carboxyl-
CamLD	calmodulin-like domain
CCPNMR	Collaborative Computing Project for the NMR
cDNA	complementary DNA
CH	Calponin Homology
CTLs	cytotoxic T lymphocytes
COSY	Correlation Spectroscopy
CSI	chemical shift index
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOL	degree of labeling
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
ECM	extra-cellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	Epidermal growth factor

EGFP	enhanced green fluorescent protein
ENTH	epsin N-terminal homology
ER	endoplasmic reticulum
Erk 1/2	extracellular signal-related kinase 1/2
ES	embryonic stem
EtBr	ethidium bromide
F-actin	filamentous actin
FAK	focal adhesion kinase
FBS	fetal bovine serum
FERM	4.1-ezrin/radixin/moiesin
FITC	fluorescein isothiocyanate
FP	fluorescence polarisation
FRET	forster resonance energy transfer
FSGS	focal segmental glomerulosclerosis
FYVE	the first letters of four proteins, Fab1p, YOTB, Vac1p and EEA1
G-actin	globular actin
GLB	gel loading buffer
GST	glutathione-S-transferase
HSQC	Heteronuclear Single Quantum Coherence
ILK	integrin-linked kinase
IP3	inositol 1,4,5-trisphosphate
IP4	inositol 1,3,4,5-tetraphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
kb	kilobase pair
Kd	equilibrium dissociation constant
kDa	kilo Dalton
LB	Luria broth
LCC	large cell lung cancer
LPA	lysophosphatidic acid
mA	milli-amperes

MEKK 1	mitogen-activated protein/extracellular signal-related kinase kinase 1
N-	amino-
NMR	nuclear magnetic resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
NSCLC	non-small cell lung cancer
OD_{600nm}	optical density at 600 nm wavelength
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
PDGF	Platelet-derived growth factor
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP 5K	phosphatidylinositol-4-phosphate 5-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonylfluoride
PPD	*p*-Phenyldiamine
ppm	parts per million
PTB	phosphotyrosine binding
PX	Phox homology
RNA	ribonucleic acid
SCLC	small cell lung cancer
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Src homology
SV40	simian virus 40
TAE	Tris-acetate EDTA
TBS	Tris buffered saline
TE	Tris-EDTA

TILs	tumor infiltrating lymphocytes
TOCSY	Total Correlation Spectroscopy
Tris	Tris(hydroxymethyl)aminoethane
TRITC	Tetramethylrhodamine isothiocyanate
TTBS	Tris buffered saline/0.1% Triton X-100
Tween 20	polyethylene-sorbitan monolaurate
UV	ultra violet
V	volts
v	volume
w	weight
X-GAL	5-bromo-4-chloro-3-indoyl-galactopyranoside

CHAPTER 1

General Introduction and Literature Review

1.1 Introduction

α-actinin, a member of the spectrin superfamily, is an ubiquitously expressed filamentous actin (F-actin) cross-linking protein and co-localises with integrin and F-actin in focal adhesions. It is thought to be an important protein in stabilising cell morphology and adhesion and regulating cell migration (Burridge and Chrzanowska-Wodnicka, 1996). In addition, α-actinin acts as a tumor suppressor (Nikolopoulos *et al.*, 2000) and mutation is associated with the metastatic potential and cancer invasiveness (Honda *et al.*, 1998). Five mutations of α-actinin-4 have been reported to be associated with three human diseases, non-small cell lung cancer (NSCLC) (Mami-Chouaib *et al.*, 2002), small cell lung cancer (SCLC) (Honda *et al.*, 2004) and focal segmental glomerulosclerosis (FSGS) (Kaplan *et al.*, 2001). The mutation site of α-actinin-4 mutants (K255E, T259I and S262P) and natural spliced variant is located at the C-terminal end of the CH2 domain and that of K122N mutant resides on the CH1 domain which plays a significant role in F-actin binding and its regulation. The actin-binding region is proposed to be important in the progression of human disease. Hence, this study focuses on the characterisation of the structure, function and regulation of α-actinin-4.

1.2 Structure of α-actinin

1.2.1 Spectrin Superfamily

Spectrin superfamily proteins are composed of the actin binding calponin homology (CH) domains and either as single copy or in tandem arrangements of multiple spectrin repeats. These proteins include the actin cross-linking proteins, such as α-actinin and filamin, and the membrane associated actin binding proteins, such as spectrin, dystrophin and urophin. α-actinin is an important structural protein in focal adhesions; it dynamically cross-links actin filments to determine the mechanical properties of the actin filament network. In addition to its structural role, α-actinin has been reported to be involved in the suppression of tumorigenicity in human neuroblastoma cells (Nikolopoulos *et al.*, 2000) and be involved in the metastatic potential and invasiveness of human cancer (Honda *et al.*, 1998).

1.2.2 α-actinin

α-actinin is a ~110 kDa cytoskeletal rod-shaped protein and is a member of the spectrin superfamily. α-actinin has been proposed to cross-link actin filaments and connect the actin cytoskeleton to the cell membrane (Jockusch *et al.*, 1995). α-actinin is composed of three functional domains: an actin binding region consisting of two calponin homology (CH) domains (hereafter called CH1 and CH2 domains) at the N-terminus, a central region consisting of four spectrin-like repeats and a calmodulin-like domain at the C-terminus. The calmodulin-like domain consists of two functional EF-hand motifs, which bind Ca^{2+} and regulate the actin binding activity in non-muscle isoforms of α-actinin (Witke *et al.*, 1993). α-actinin is organised as an antiparallel homodimer formed by the interaction of four spectrin-like repeats between two molecules to give a rod-like shape with actin binding regions at both ends (Figure 1.1). This structure allows α-actinin to cross-link actin filaments into tight bundles; however, α-actinin does not bind to G-actin, the actin monomer. The three-dimensional structure of actin filaments decorated with the actin binding region of α-actinin indicated that the CH domains of α-actinin are in contact with subdomain 1 on two actin monomers along the long-pitch helix of the F-actin at a site centered at subdomain 2 (McGough *et al.*, 1994).

In addition to F-actin and Ca^{2+}, α-actinin has been found to interact with several cytoplasmic proteins in focal adhesions including vinculin, integrin β1, zyxin, extracellular signal-related kinase 1/2 (Erk 1/2), mitogen-activated protein/extracellular signal-related kinase kinase 1 (MEKK1), protein kinase N and the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) (Schwartz *et al.*, 1995). Moreover, a phosphoinositide binding site has been identified at the N-terminus of the CH2 domain, suggesting that these protein-protein interactions could be regulated by cell signaling. Under platelet-derived growth factor (PDGF) stimulation, PI3-kinase is activated to catalyse the conversion of PIP2 to PIP3. α-actinin bound PIP3 is dissociated from focal adhesions to the cytoplasm. It is presumed that PIP3 disrupts the interaction between α-actinin and integrin directly or indirectly (Greenwood *et al.*, 2000) (more detailed description in section 1.3.1.2.3).

1.2.3 Isoforms of α-actinin

Four isoforms of α-actinin have been described in vertebrates (α-actinin-1, -2, -3 and -4).

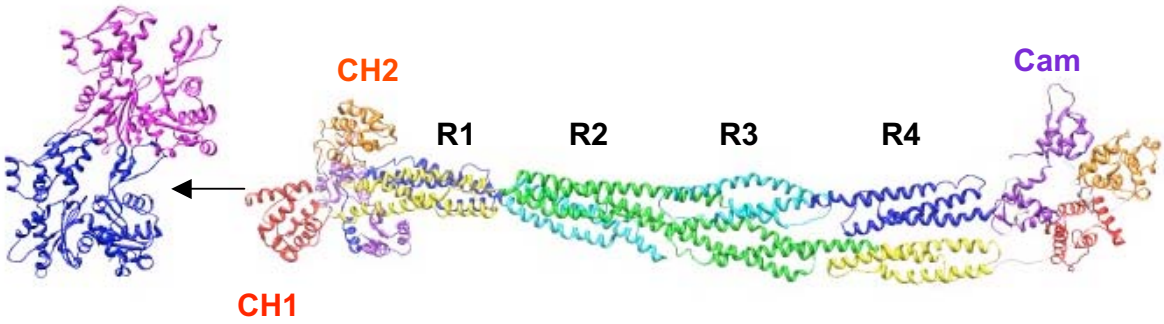


Figure 1.1 The three-dimensional structure of chicken skeletal muscle α-actinin (pdb.1SJJ). α-actinin contains three functional domains: an actin binding region consisting of two calponin homology domains (CH1 and CH2) at the N-terminus, a central region consisting of four spectrin-like repeats and a calmodulin-like domain at the C-terminus. The calmodulin-like domain consists of two Ca^{2+} binding EF-hand motifs and thereby regulate the actin binding activity in non-muscle isoforms. α-actinin is organised as an antiparallel homodimer formed by the interaction of four spectrin-like repeats between two molecules giving a rod-like shape with actin-binding regions at both ends. The α-actinin binding site on F-actin is located on the cleft between two molecules of actin monomer. Actin monomers indicated in pink and blue.

These isoforms are divided into two groups: non-muscle cytoskeletal (calcium-sensitive) isoforms and muscle sarcomeric (calcium-insensitive) isoforms. α-actinin-2 and α-actinin-3, the muscle isoforms, are expressed in cardiac and/or skeletal muscles, and are localised to Z-discs where they cross-link actin filaments with adjacent sarcomeres to form the contractile machinery of the cell (Young *et al.*, 1998). α-actinin-1 and α-actinin-4, the non-muscle isoforms, are expressed ubiquitously. Non-muscle isoforms of α-actinin have been reported to be associated with focal adhesion molecules, such as integrin β1 (Kelly and Taylor, 2005), and are believed to play important roles in stabilising cell adhesion and regulating cell shape and cell migration (Gluck and Ben-Ze'ev, 1994; Gluck *et al.*, 1993; Knudsen *et al.*, 1995). Using an *in vitro* F-actin bundling analysis, the calmodulin-like domain bound Ca^{2+} and diminished the binding activity of α-actinin to F-actin (Witke *et al.*, 1993). These results suggested Ca^{2+} might negatively regulate the F-actin binding activity of α-actinin. Based on the structural information prediction, the calmodulin-like domain could undergo a conformational change which allows it to bind the linker peptide between the CH1 and the CH2 domains in the presence of Ca^{2+}, resulting in interference of actin binding ability (Tang *et al.*, 2001) (details described in chapter 3).

1.2.4 Calponin Homology Domains

The calponin homology (CH) domain is approximately 100 amino acid residues and is involved in actin binding. Some signaling and cytoskeletal proteins contain the CH domain and are classified into three groups. Proteins containing a single N-terminal CH domain (1X CH), such as calponin, Vav, IQGAP and Cdc24. Proteins with an actin-binding domain (ABD) composed of two CH domains (2X CH), including spectrin, dystrophin, utrophin, filamin and α-actinin. Finally, proteins contain two ABDs in tandem (4X CH), such as fimbrin and plastin (Korenbaum and Rivero, 2002; Gimona *et al.*, 2002). The N-terminus of α-actinin has an actin binding region of 240-residues, which contains two CH domains (2X CH) in a tandem arrangement. The three-dimensional structures of these two CH domains are similar and consist of four main α helices and two shorter helices, which are connected by long or short loops (Figure 1.2). However, the protein sequence homology (Figure 1.3) and biological function of these two CH domains are not identical (Banuelos *et al.*, 1998). The F-actin binding affinity of these two CH domains (CH1 or CH2) has been investigated using an *in vitro* F-actin co-sedimentation assay by Way *et al.* in 1992. The results indicated

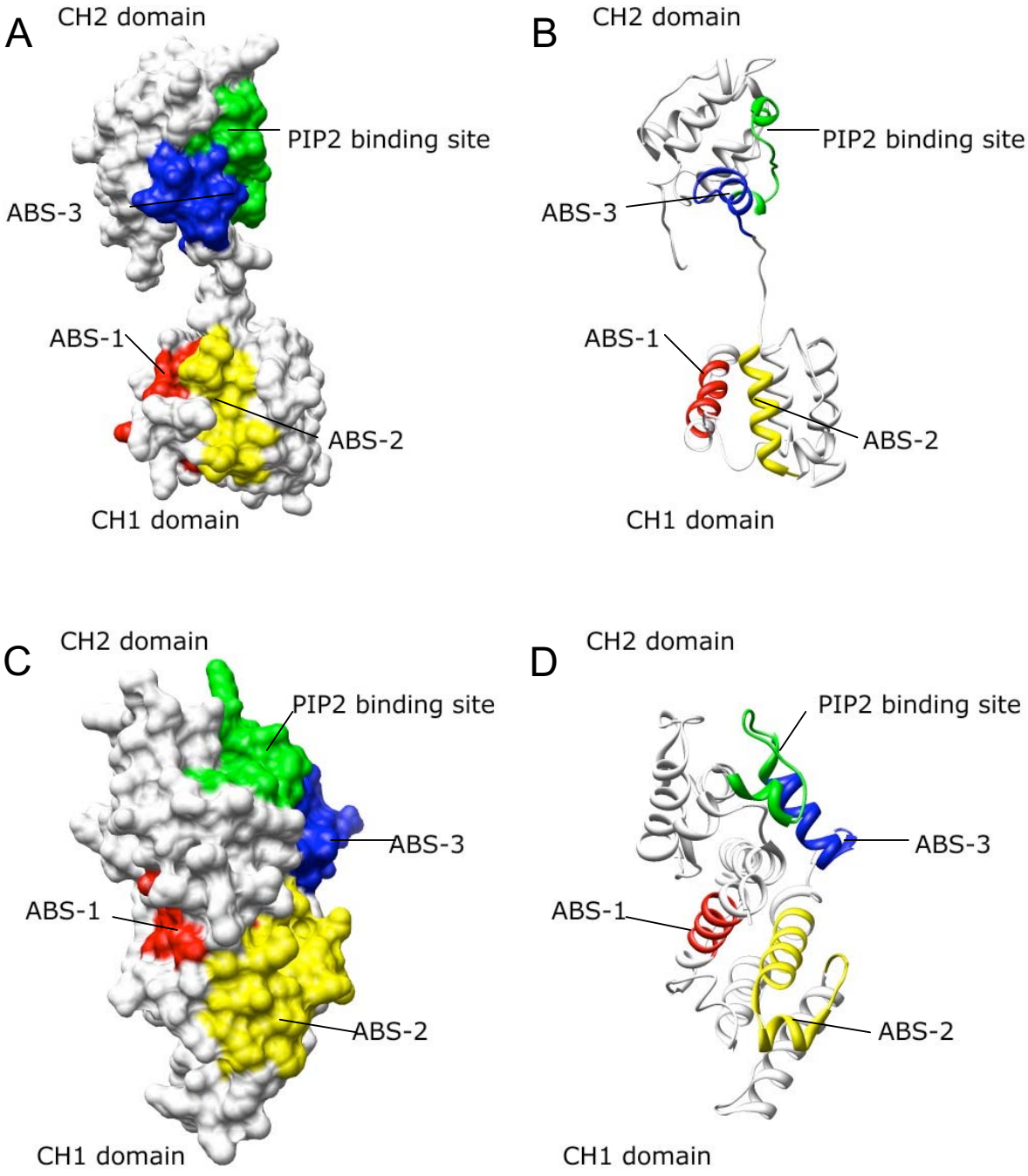
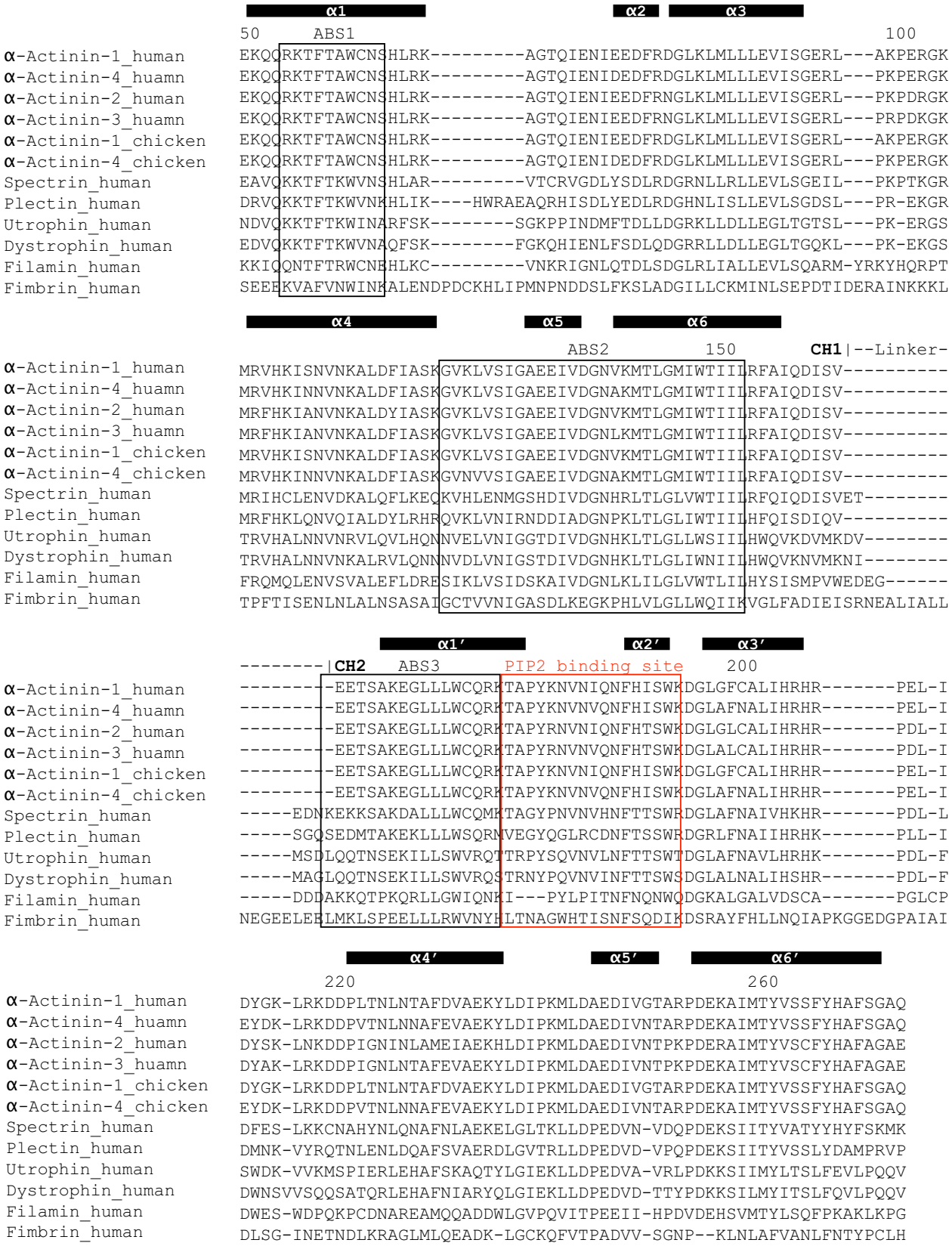


Figure 1.2 The surface and ribbon diagrams of chicken skeletal muscle α-actinin (pdb.1SJJ) and human α-actinin-1 (pdb.2EYI) actin binding domains (2X CH). The putative actin binding site (ABS)-1, 2 and 3 and the region of PIP2 binding are indicated in red, yellow, blue and green, respectively. The three-dimensional structures of the CH domains are shown a open (extended) conformation for chicken skeletal muscle α-actinin (A and B) and a closed (compacted) conformation for human α-actinin-1 (C and D), respectively. The images were generated by Chimera software (Pettersen, 2004).

Figure 1.3 Amino acid sequence alignment of the CH domains (2X CH). The positions of actin binding site 1-3 and the PIP2 binding site are indicated by black boxes and red box, respectively. Secondary structure elements are indicated by black cylinders. Accession codes are: human α-actinin-1, P12814; human α-actinin-4, O43707; human α-actinin-2, P35609; human α-actinin-3, Q08043; chicken α-actinin-1, P05094; chicken α-actinin-4, Q90734; human spectrin, O15020; human plectin, G02520; human utrophin, P46939; human dystrophin, P11532; human filamin, O75369; and human fimbrin, AAH31083.


α1
α2
α3
50
ABS1
100
α-Actinin-1_human EKQQRKTFTAWCNSHLRK---------AGTQIENIEEDFRDGLKLMLLLEVISGERL---AKPERGK
α-Actinin-4_huamn EKQQRKTFTAWCNSHLRK---------AGTQIENIDEDFRDGLKLMLLLEVISGERL---PKPERGK
α-Actinin-2_human EKQQRKTFTAWCNSHLRK---------AGTQIENIEEDFRNGLKLMLLLEVISGERL---PKPDRGK
α-Actinin-3_huamn EKQQRKTFTAWCNSHLRK---------AGTQIENIEEDFRNGLKLMLLLEVISGERL---PRPDKGK
α-Actinin-1_chicken EKQQRKTFTAWCNSHLRK---------AGTQIENIEEDFRDGLKLMLLLEVISGERL---AKPERGK
α-Actinin-4_chicken EKQQRKTFTAWCNSHLRK---------AGTQIENIDEDFRDGLKLMLLLEVISGERL---PKPERGK
Spectrin_human EAVQKKTFTKWVNSHLAR---------VTCRVGDLYSDLRDGRNLLRLLEVLSGEIL---PKPTKGR
Plectin_human DRVQKKTFTKWVNKHLIK----HWRAEAQRHISDLYEDLRDGHNLISLLEVLSGDSL---PR-EKGR
Utrophin_human NDVQKKTFTKWINARFSK--------SGKPPINDMFTDLLDGRKLLDLLEGLTGTSL---PK-ERGS
Dystrophin_human EDVQKKTFTKWVNAQFSK--------FGKQHIENLFSDLQDGRRLLDLLEGLTGQKL---PK-EKGS
Filamin_human KKIQQNTFTRWCNEHLKC---------VNKRIGNLQTDLSDGLRLIALLEVLSQARM-YRKYHQRPT
Fimbrin_human SEEEKVAFVNWINKALENDPDCKHLIPMNPNDDSLFKSLADGILLCKMINLSEPDTIDERAINKKKL
α4
α5
α6
ABS2
150
CH1|--Linker-
α-Actinin-1_human MRVHKISNVNKALDFIASKGVKLVSIGAEEIVDGNVKMTLGMIWTIILRFAIQDISV----------
α-Actinin-4_huamn MRVHKINNVNKALDFIASKGVKLVSIGAEEIVDGNAKMTLGMIWTIILRFAIQDISV----------
α-Actinin-2_human MRFHKIANVNKALDYIASKGVKLVSIGAEEIVDGNVKMTLGMIWTIILRFAIQDISV----------
α-Actinin-3_huamn MRFHKIANVNKALDFIASKGVKLVSIGAEEIVDGNLKMTLGMIWTIILRFAIQDISV----------
α-Actinin-1_chicken MRVHKISNVNKALDFIASKGVKLVSIGAEEIVDGNVKMTLGMIWTIILRFAIQDISV----------
α-Actinin-4_chicken MRVHKINNVNKALDFIASKGVNVVSIGAEEIVDGNAKMTLGMIWTIILRFAIQDISV----------
Spectrin_human MRIHCLENVDKALQFLKEQKVHLENMGSHDIVDGNHRLTLGLVWTIILRFQIQDISVET--------
Plectin_human MRFHKLQNVQIALDYLRHRQVKLVNIRNDDIADGNPKLTLGLIWTIILHFQISDIQV----------
Utrophin_human TRVHALNNVNRVLQVLHQNNVELVNIGGTDIVDGNHKLTLGLLWSIILHWQVKDVMKDV--------
Dystrophin_human TRVHALNNVNKALRVLQNNNVDLVNIGSTDIVDGNHKLTLGLIWNIILHWQVKNVMKNI--------
Filamin_human FRQMQLENVSVALEFLDRESIKLVSIDSKAIVDGNLKLILGLVWTLILHYSISMPVWEDEG------
Fimbrin_human TPFTISENLNLALNSASAIGCTVVNIGASDLKEGKPHLVLGLLWQIIKVGLFADIEISRNEALIALL
α1′
α2′
α3′
--------|CH2
ABS3
PIP2 binding site
200
α-Actinin-1_human --------EETSAKEGLLLWCQRKTAPYKNVNIQNFHISWKDGLGFCALIHRHR-------PEL-I
α-Actinin-4_huamn --------EETSAKEGLLLWCQRKTAPYKNVNVQNFHISWKDGLAFNALIHRHR-------PEL-I
α-Actinin-2_human --------EETSAKEGLLLWCQRKTAPYRNVNIQNFHTSWKDGLGLCALIHRHR-------PDL-I
α-Actinin-3_huamn --------EETSAKEGLLLWCQRKTAPYRNVNVQNFHTSWKDGLALCALIHRHR-------PDL-I
α-Actinin-1_chicken --------EETSAKEGLLLWCQRKTAPYKNVNIQNFHISWKDGLGFCALIHRHR-------PEL-I
α-Actinin-4_chicken --------EETSAKEGLLLWCQRKTAPYKNVNVQNFHISWKDGLAFNALIHRHR-------PEL-I
Spectrin_human -----EDNKEKKSAKDALLLWCQMKTAGYPNVNVHNFTTSWRDGLAFNAIVHKHR-------PDL-L
Plectin_human -----SGQSEDMTAKEKLLLWSQRNVEGYQGLRCDNFTSSWRDGRLFNAIIHRHK-------PLL-I
Utrophin_human -----MSDLQQTNSEKILLSWVRQTTRPYSQVNVLNFTTSWTDGLAFNAVLHRHK-------PDL-F
Dystrophin_human -----MAGLQQTNSEKILLSWVRQSTRNYPQVNVINFTTSWSDGLALNALIHSHR-------PDL-F
Filamin_human -----DDDAKKQTPKQRLLGWIQNKI---PYLPITNFNQNWQDGKALGALVDSCA-------PGLCP
Fimbrin_human NEGEELEELMKLSPEELLLRWVNYHLTNAGWHTISNFSQDIKDSRAYFHLLNQIAPKGGEDGPAIAI
α4′
α5′
α6′
220
260
α-Actinin-1_human DYGK-LRKDDPLTNLNTAFDVAEKYLDIPKMLDAEDIVGTARPDEKAIMTYVSSFYHAFSGAQ
α-Actinin-4_huamn EYDK-LRKDDPVTNLNNAFEVAEKYLDIPKMLDAEDIVNTARPDEKAIMTYVSSFYHAFSGAQ
α-Actinin-2_human DYSK-LNKDDPIGNINLAMEIAEKHLDIPKMLDAEDIVNTPKPDERAIMTYVSCFYHAFAGAE
α-Actinin-3_huamn DYAK-LRKDDPIGNLNTAFEVAEKYLDIPKMLDAEDIVNTPKPDEKAIMTYVSCFYHAFAGAE
α-Actinin-1_chicken DYGK-LRKDDPLTNLNTAFDVAEKYLDIPKMLDAEDIVGTARPDEKAIMTYVSSFYHAFSGAQ
α-Actinin-4_chicken EYDK-LRKDDPVTNLNNAFEVAEKYLDIPKMLDAEDIVNTARPDEKAIMTYVSSFYHAFSGAQ
Spectrin_human DFES-LKKCNAHYNLQNAFNLAEKELGLTKLLDPEDVN-VDQPDEKSIITYVATYYHYFSKMK
Plectin_human DMNK-VYRQTNLENLDQAFSVAERDLGVTRLLDPEDVD-VPQPDEKSIITYVSSLYDAMPRVP
Utrophin_human SWDK-VVKMSPIERLEHAFSKAQTYLGIEKLLDPEDVA-VRLPDKKSIIMYLTSLFEVLPQQV
Dystrophin_human DWNSVVSQQSATQRLEHAFNIARYQLGIEKLLDPEDVD-TTYPDKKSILMYITSLFQVLPQQV
Filamin_human DWES-WDPQKPCDNAREAMQQADDWLGVPQVITPEEII-HPDVDEHSVMTYLSQFPKAKLKPG
Fimbrin_human DLSG-INETNDLKRAGLMLQEADK-LGCKQFVTPADVV-SGNP--KLNLAFVANLFNTYPCLH

that the F-actin binding affinity of the CH1 domain is stronger than that of the CH2 domain but is weaker than that of the entire actin binding region (the CH1 + CH2 domains) (Way *et al.*, 1992). These results suggested that the CH1 domain plays a critical role in F-actin binding but the CH2 domain might also interact with F-actin and enhance the overall binding ability of the tandem domains.

1.2.4.1 F-Actin Binding Sites on the CH Domains (2X CH)

Three clusters of actin binding sequences have been identified in the CH domains of α-actinin by biochemical analysis (Kuhlman *et al.*, 1992; Hemmings *et al.*, 1992) and crystal structure predictions (McGough *et al.*, 1994). They are actin binding site 1 (ABS-1), ABS-2 and ABS-3, which are found on the α1 helix of the CH1 domain, the α5-α6 of the CH1 domain and the α1′ helix of the CH2 domain, respectively (Figure 1.2 and Figure 1.3 black boxes). The actin binding regions described above also have been verified on the other members of the spectrin superfamily, such as dystrophin (Levine *et al.*, 1992) and utrophin (Winder *et al.*, 1995). The actin binding regions derived from spectrin superfamily members were classified into two types, open (extended) and closed (compacted) conformations (more details referred to section 1.2.5). The structural images of extended and compacted conformations of the CH domains (2X CH) were shown in Figure 1.2. The crystal structure of human α-actinin-1 showed that ABS-2 and ABS-3 form a contiguous region on the surface of the CH domains (Borrego-Diaz *et al.*, 2006), but ABS-1 is buried in the interface between the CH1 and the CH2 domains (Figure 1.2 C and D). Therefore, rearrangement of the CH domains would be expected when binding to F-actin and could be regulated by interacting with the calmodulin-like domain (from the opposite molecule of α-actinin in the dimer) and Ca^{2+} or phosphoinositides. The three-dimensional structures of the CH domains (2X CH) are described in section 1.2.5.

1.2.4.2 Phosphoinositide Binding Site on the CH2 Domain

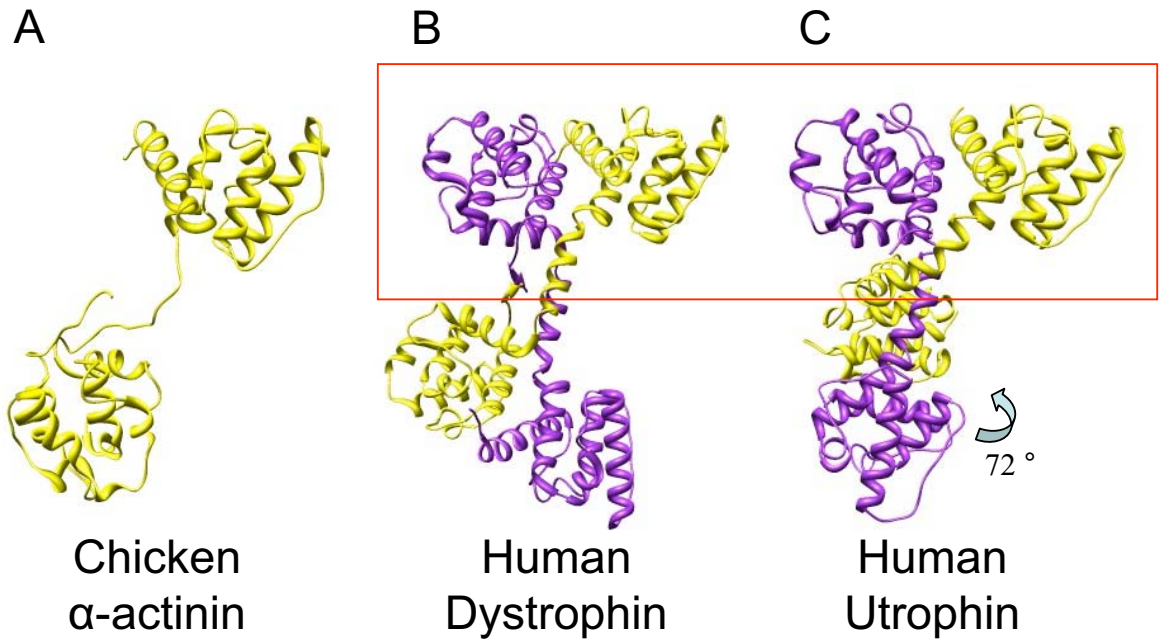
The CH2 domain of α-actinin contains a highly conserved PIP2 binding region, which is located on a region adjacent to the ABS-3 (Figure 1.3 red box). By using Western blot analysis with a specific PIP2 antibody, the PIP2 binding site has been mapped on the residues 177-193, the loop connecting α1′ and α2′ helices of the CH2 domain, adjacent to the ABS-3 (Fukami *et al.*, 1996) (for details see chapter 5). It has been proposed that the

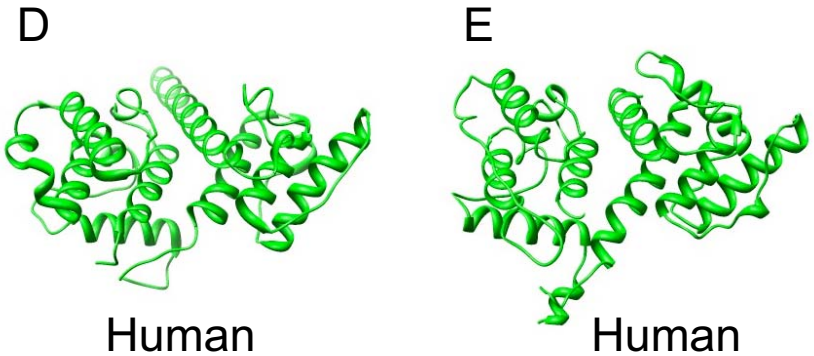
phosphate groups at positions 4 and 5 of PIP2 interact with the positively charged amino acid residues and the acyl chains are in contact with the hydrophobic region of the CH2 domain for performing its function. Therefore, residues Lys 181, Lys 193 and His 189 were predicted to be involved in the binding of α-actinin to PIP2 (Fukami *et al.*, 1996). (Within this thesis, the residues numbers correspond to that of full length α-actinin-4). In addition, Fraley *et al.* have generated three human α-actinin-1 mutants, K181I, K193I and a triple mutant K181I/H189L/K193I, and verified their phosphoinositide (including PIP2 and PIP3) binding using a protein-lipid overlay assay. The results indicated that all these α-actinin mutants have a reduced ability to bind PIP2 or PIP3 (Fraley *et al.*, 2003). However, the crystal structure evidence from α-actinin-3 showed that residue Lys 193 was almost buried inside of the protein (Franzot *et al.*, 2005). Hence, Franzot *et al.* predicted that three residues, Arg 175, Arg 204 (which are outside the previously mapped region) and Lys 181, might be a possible phosphoinositide binding site (Franzot *et al.*, 2005) (more details are described in chapter 5).

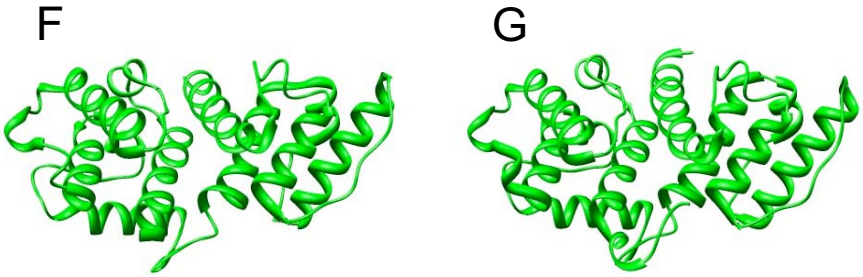
1.2.5 The Three-Dimensional Structure of the CH Domains (2X CH)

The three-dimensional structures of a number of the CH domains derived from other spectrin superfamily members have been reported (Djinovic Carugo *et al.*, 1997; Keep *et al.*, 1999b; Norwood *et al.*, 2000; Garcia-Alvarez *et al.*, 2003; Goldsmith *et al.*, 1997; Franzot *et al.*, 2005; Borrego-Diaz *et al.*, 2006). These CH domains (2X CH) are highly conserved but have different properties that define them into two categories, open (Figure 1.2 A and B) and closed (Figure 1.2 C and D) (or extended and compacted) conformations. For example, the CH domains (2X CH) of plectin and fimbrin fold back upon themselves to form a compact globular structure (Figure 1.4 D and E) (Goldsmith *et al.*, 1997; Klein *et al.*, 2004; Sevcik *et al.*, 2004; Garcia-Alvarez *et al.*, 2003). Compared with the CH domains of fimbrin and plectin, the CH domains (2X CH) of dystrophin and utrophin were shown to be in an extended conformation. As shown in Figure 1.4 B and C, the CH1 and the CH2 domains were separated by a long central α-helix. Two molecules of the CH domains (2X CH) form an antiparallel homodimer by the interaction of a long central α-helix between the CH1 and the CH2 domains (Keep *et al.*, 1999a; Norwood *et al.*, 2000). Within the structure of human dystrophin and utrophin, a compacted conformation is displayed between two molecules of the CH domains (Figure 1.4 red box). In addition, compared with the CH domain dimer of

Figure 1.4 Structure comparisons of actin binding domains (2X CH domains) from the spectrin superfamily. The three-dimensional structures of human dystrophin (B), utrophin (C) and chicken skeletal muscle α-actinin (A) show the open (extended) conformation and form antiparallel homodimers (one chain in yellow and the other chain in purple). The structures of human plectin (D), fimbrin (E), α-actinin-1 (G) and -3 (F) show the closed (compacted) conformation (indicated in green). However, within the structure of human dystrophin and utrophin, a compacted conformation is displayed between two molecules of CH domains (indicated in red box). PDB codes are: chicken α-actinin-1, 1SJJ; human dystrophin, 1DXX; human utrophin, 1QAG; human plectin, 1MB8; human fimbrin, 1AOA; human α-actinin-1, 1EYN; human α-actinin-3, 1WKU.


A
B
C
72 °
Chicken
α-actinin
Human
Dystrophin
Human
Utrophin


D
E
Human
Plectin
Human
Fimbrin


F
G
Human
α-actinin-3
Human
α-actinin-1

dystrophin, the utrophin CH domain dimer reveals a change of 72° in the orientation of one pair of the CH1 and the CH2 domains (from different monomers) relative to the other pair (Figure 1.4 B and C). The crystal structure of α-actinin-3 CH domains (2X CH) has been reported to be a closed conformation (Figure, 1.4 F) and a conformational change within the CH domains is required for efficient binding to F-actin (Franzot *et al.*, 2005; Liu *et al.*, 2004). The crystal structure of α-actinin-1 CH domains (2X CH) also has been reported and shows a typical compact conformation (Figure 1.2 C and D and Figure 1.4 G). Rearrangement of the compacted and extended conformation could enhance the F-actin binding ability of the actin-binding domain (Borrego-Diaz *et al.*, 2006). According to the structural information of α-actinin-1 CH domains (2X CH), there is a strong hydrophobic interaction between residue Trp 147 on α6 helix of the CH1 domain and residue Lys 255 on α6′ helix of the CH2 domain to maintain the compacted conformation of the actin-binding domain. Within this compacted conformation, the ABS-1 is buried between the CH1 and the CH2 domains as shown in Figure 1.2 C and D. In the presence of F-actin, the interface between the CH1 and the CH2 domains was proposed to be disrupted to alter the conformation of the CH domains slightly from a compacted to an extended conformation, resulting in enhancement of the F-actin binding ability (Borrego-Diaz *et al.*, 2006). Based on these two conformations, closed and open, two models of F-actin binding have been described (Sutherland-Smith *et al.*, 2003; Moores *et al.*, 2000) (Figure 1.5). For a model described by Sutherland-Smith *et al.* in 2003, the CH domains form a compacted conformation to bind a cleft between two molecules of actin monomer. For a model described by Moores *et al.* in 2000, the CH domains form an extended conformation; however, a compacted conformation shows between two molecules of the CH domains (Figure 1.5 B purple and green). Although the actin-binding model of the CH domains is controversial and uncertain (Lehman *et al.*, 2004), the rearrangement of two conformations should be an important criteria in the binding on F-actin, further influencing the rearrangement of the cytoskeleton. Hence, the conformational change between open and closed conformations within the CH domains of α-actinin was proposed to be induced by the calmodulin-like domain (with bound Ca^{2+}) or phosphoinositide interaction.

1.2.6 Mutation on α-actinin-4 CH Domains in Human Diseases

Recently, four α-actinin-4 mutants, K122N, K255E, T259I and S262P, and a natural spliced

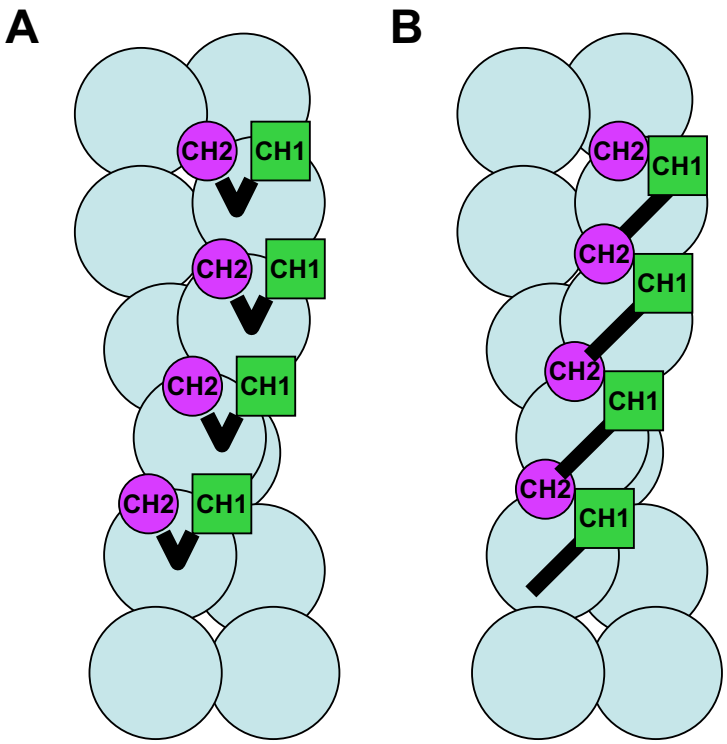


Figure 1.5 Two models of actin binding domain on F-actin. (A) The model proposed by Sutherland-Smith *et al* with the CH domains in closed conformation. (B) The model proposed by Moores *et al* with the CH domains in open conformation. The diagrams were adapted from Lehman, 2004.

variant resulting in three amino acid substitutions have been reported to be associated with three human diseases, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC) and focal segmental glomerulosclerosis (FSGS). The position of these mutations locates on the C-terminal of the CH2 domain, except K122N that is within the CH1 domain (Figure 1.6). Amino acid sequence observations implied that the F-actin binding or bundling activity would be influenced by these residue changes. Indeed, the F-actin bundling activity of these mutants as well as the spliced variant has been investigated using an *in vitro* F-actin sedimentation assay and the results indicated that the F-actin bundling activity of these mutants and the spliced variant is stronger than that of wildtype (Menez *et al.*, 2004; Honda *et al.*, 2004; Kaplan *et al.*, 2001).

1.3 The Biological Function of α-actinin in Mammals

α-actinin-4 has been reported to be involved in the following biological functions, a structural role in focal adhesions, tumor suppression, metastasis and invasion and normal podocyte adhesion.

1.3.1 Structure and Function of Focal Adhesions

During embryogenesis, morphogenesis and wound healing, tissue formation in animals depends on the ability of many cell types to develop specific contacts with each other and with extra-cellular matrix (ECM) (Jockusch *et al.*, 1995; Burridge and Chrzanowska-Wodnicka, 1996). These contacts are highly dynamic and sensitively respond to signals either in-side-out or out-side-in, resulting in a reversible structural organisation. Focal adhesion has been defined as a site of tight structure, which contains the interaction between a cell and its surrounding ECM (Jockusch *et al.*, 1995; Burridge and Chrzanowska-Wodnicka, 1996) (Figure 1.7). The functions of focal adhesions may include regulating cell morphology, migration, proliferation and differentiation. Typically focal adhesions are mainly found in cell culture (Abercrombie *et al.*, 1971) and are rarely found *in vivo*. Therefore, some speculated that focal adhesions were artificial structures found in the cell culture. However, Fuchs *et al.* have reported in 1997 that focal adhesions are clearly present *in vivo* at cell matrix junction by immunoelectron microscopy (Fuchs *et al.*, 1997). These cell-ECM contacts provide a convenient model to analyse the molecular basis of cell adhesion and migration.

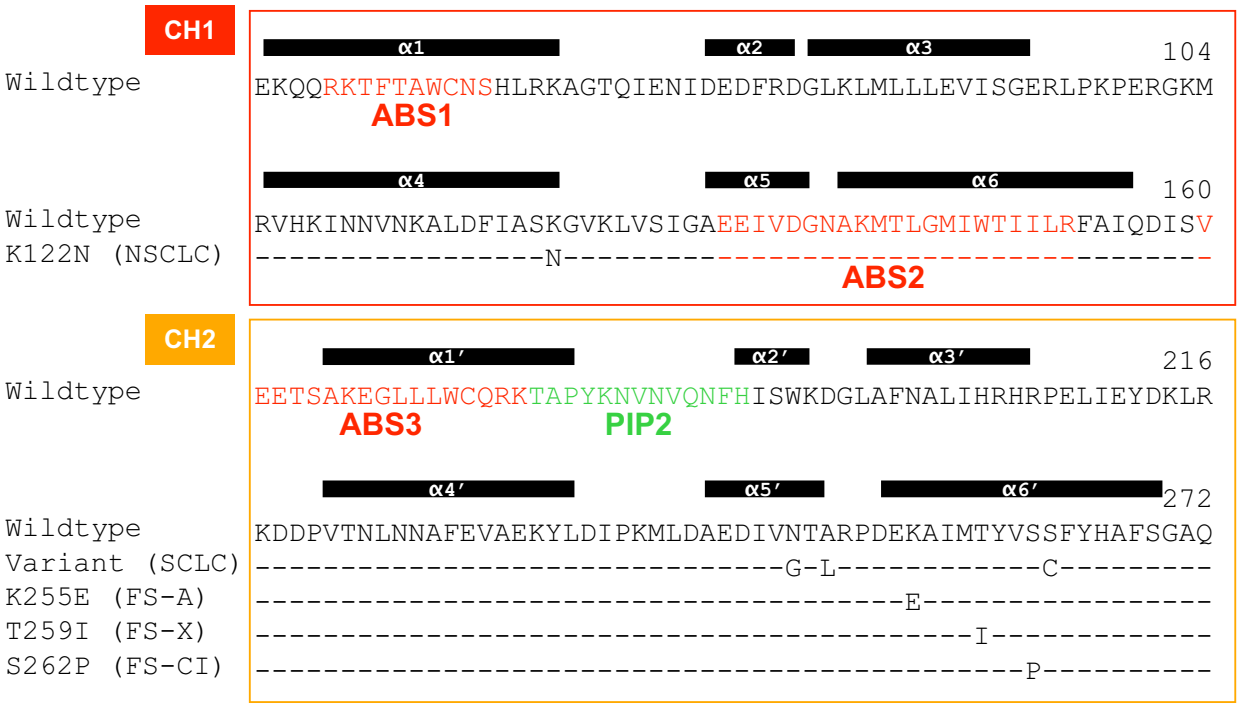


Figure 1.6 Amino acid sequence alignment of the CH domains of wildtype, four mutants and a natural spliced variant. The CH domains consist of six α-helices, which are connected by long or short loop. Three actin-binding sites (ABS1-3) have been identified. They reside on the α1 helix of the CH1 domain, the α5 and α6 helix of CH1 domain and the α1´ helix of the CH2 domain, respectively (red). The CH2 domain contains a putative PIP2 binding site which is located near its N-terminus (green). The CH1 domain is indicated by red box and the CH2 domain is indicated by orange box. Four mutants and a natural spliced variant of α-actinin-4 have been identified to be involved in three human diseases, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC) and focal segmental glomerulosclerosis (FSGS). Secondary structure elements are indicated by black box.

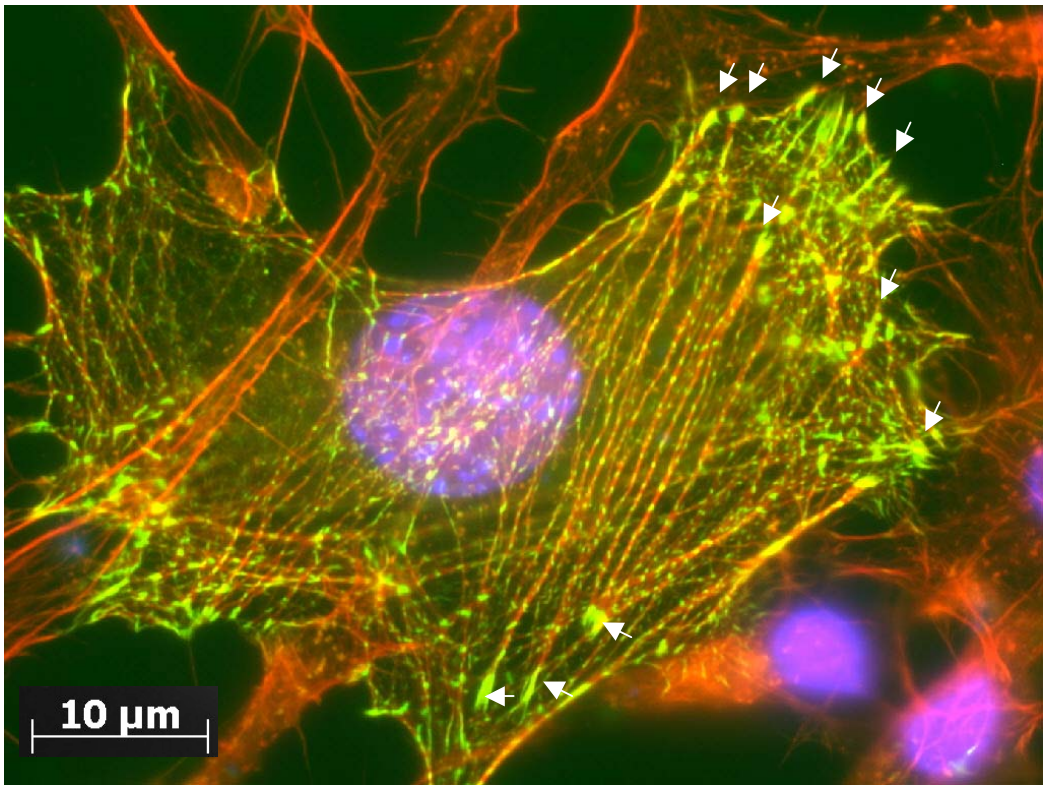


Figure 1.7 Focal adhesions. EGFP-α-actinin-4 fusion protein was transiently expressed in NIH3T3 cells. α-actinin-4, a focal adhesion associated protein, is co-localised with integrin on focal adhesions (green greater spot with arrows) and actin stress fibres (green fine spot). F-actin and nucleus stained with TRITC-conjugated phalloidin (red) and DAPI (blue), respectively. Bars, 10 µM.

1.3.1.1 Structural Organisation of Focal Adhesions

The basic structure of focal adhesions consists of three parts: a cytoplasmic face, an extra-cellular face and a transmembrane connecting region (Figure 1.8). A number of proteins have been reported to be involved in those three parts (Table 1.1) (Lo, 2006).

The major transmembrane components in focal adhesions are integrins, which are composed of multiple types of α and β subunits (Schwartz *et al.*, 1995). Both subunits of integrins are composed of three regions: a large extra-cellular region, which is responsible for heterodimeric association and ECM protein recognition; a cytoplasmic region, which plays an important role in structural stability and signal transduction of focal adhesions; and a transmembrane region. Integrins must be activated by interacting with ECM to serve as structural links between ECM and microfilament proteins at the cytoplasmic face of focal adhesions.

Parallel F-actin rods are assembled into bundles by interacting with bundling proteins, which belong to a large family of actin-binding proteins, such as filamin, tenuin, vinculin and α-actinin. This bundled F-actin does not directly link to the integral plasma membrane components, such as integrin, but through the actin filament-capping proteins, such as radixin, tensin, talin and α-actinin. For example, talin forms a rod shaped homodimer with two polypeptides arranged in antiparallel orientation (Nayal *et al.*, 2004). The N-terminal portion of talin can interact directly with cell membranes, whereas the large C-terminal portion interacts with β1 integrin, actin and vinculin. α-actinin also forms a rod shaped antiparallel homodimer to provide two F-actin binding regions at both ends. Intergrin interacts with α-actinin on the region between the spectrin-like repeats 1 and 2 (Kelly and Taylor, 2005).

1.3.1.2 Regulatory Signaling of Focal Adhesions

In contrast to the structural proteins, the function of regulatory proteins in focal adhesions is thought to modulate the formation of microfilaments. These regulatory proteins include actin filament regulators, proline motif proteins, LIM proteins (named from the Lin-11, Isl-1 and Mec-3 genes), proteases, phospholipases, kinases (serine/threonine and tyrosine kinase) and GTP-binding proteins (Table 1.1) (Burridge and Chrzanowska-Wodnicka, 1996; Lo,

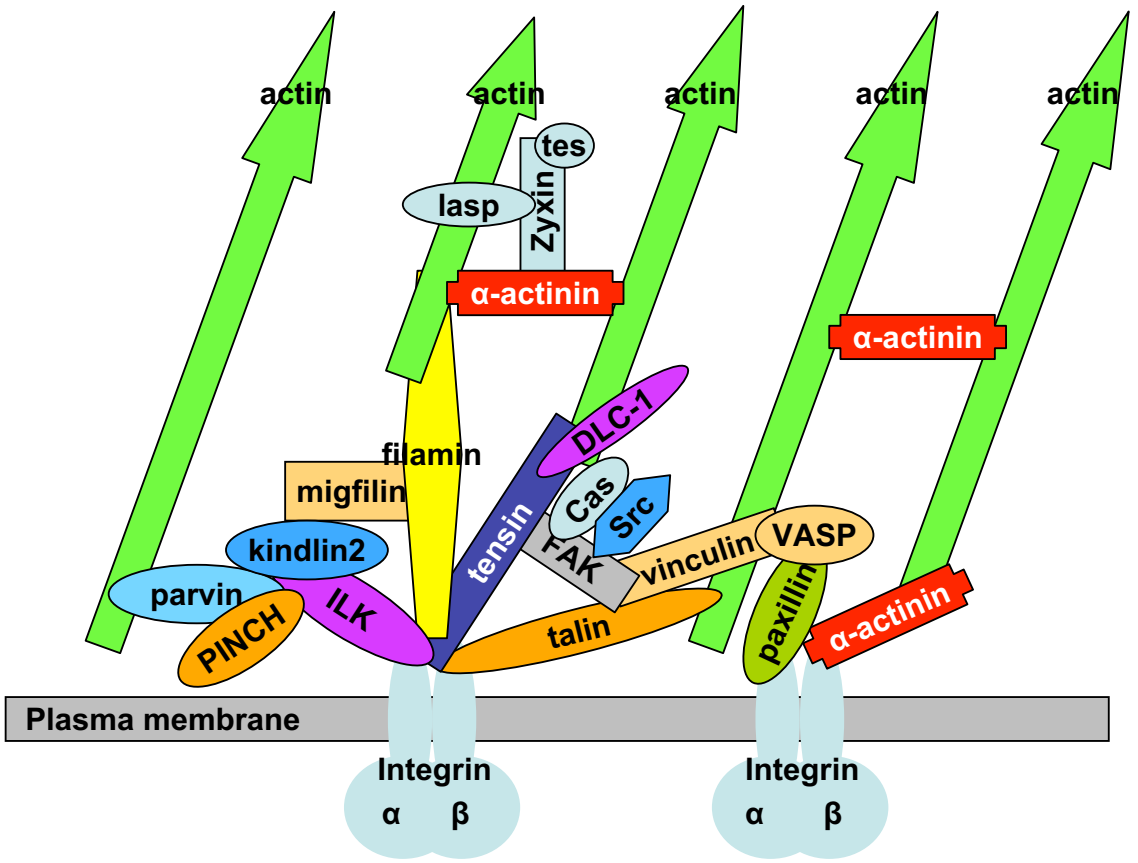


Figure 1.8 Structure of focal adhesions. The basic structure of focal adhesions consists of three parts: a cytoplasmic face, an extra-cellular face and a transmenbrane connecting region. Once the integrin is activated by interaction of extra-cellular matrix (ECM), such as fibronectin, a number of proteins, including structural and regulatory proteins, would be recruited to form a complex to perform downstream signaling. The function of focal adhesions is involved in regulating cell morphology, migration, proliferation and differentiation. The diagram was adapted from Lo, 2006.

Table 1.1 Focal adhesion proteins (Lo, 2006)

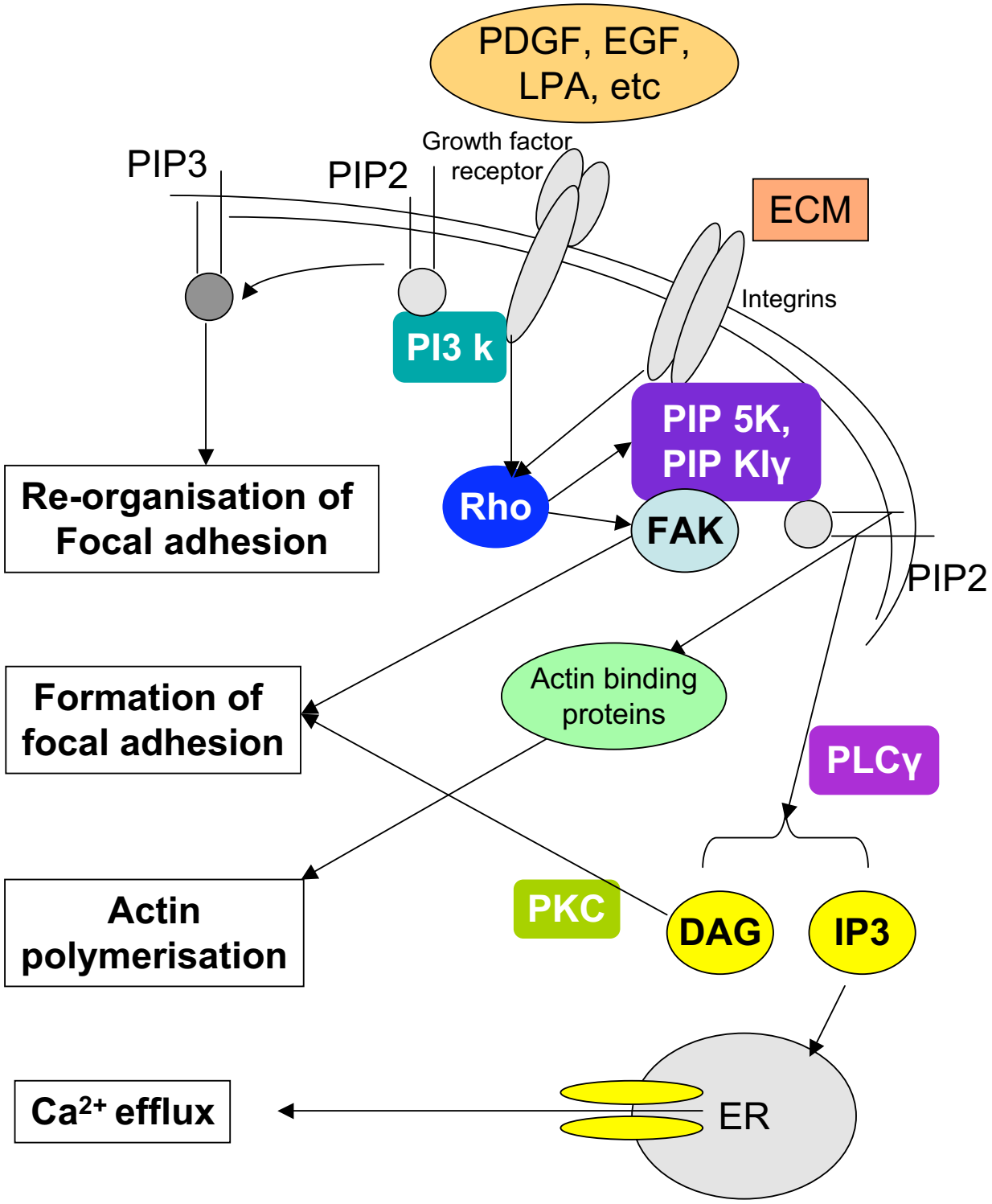
Location		Focal adhesion proteins
Extracellular		Collagen, Fibronectin, Heparan sulfate, Laminin, Proteoglycan, Vitronectin
Transmembrane		Integrins 18α and 8 β (24 combinations in humans), LAR-PTP receptor, Layilin, Syndecan-4
Cytoplasmic	Structural	Actin, α-actinin, ESAT, Ezrin, Filamin, Fimbrin, Kindling, Lasp-1, LIM nebulette, MENA, Meosin, Nexilin, Paladin, Parvin, Profilin, Ponsin, Radixin, Talin, Tensin, Tenuin, VASP, Vinculin, Cinexin
	Enzymatic	Protein tyrosine kinase: Abl, Csk, FAK, Pyk2, Src Protein serine/threonine kinase: ILK, PAK, PKC Protein phosphatase: SHP-2, PTP-1B, ILKAP Modulators of small GTPase: ASAP1, DLC-1, Graf, PKL, PSGAP, RC-GAP72 Others: Calpain II, PI3-K, PLCγ
	Adapters	p130Cas, Caveolin-1, Crk, CRP, Cten, DOCK180, DRAL, FRNK, Grb 7, Hic-5, LIP.1, LPP, Mig-2, Migfilin, Paxillin, PINCH, Syndesmos, Syntenin, Tes, Trip 6, Zyxin

2006). Other proteins may participate in signal transduction pathways, including the integrin mediated signaling pathway that is thought to be involved in cell adhesion and migration and the receptor mediated signaling pathway that is proposed to be associated with cell proliferation and differentiation. These two signal transduction pathways are involved in focal adhesion assembly (Figure 1.9) (more details are discussed below).

1.3.1.2.1 Integrin Mediated Signaling Pathway

It is known that integrins are the transmembrane proteins in focal adhesions. In the integrin mediated signaling pathway, integrin binds at least three actin binding proteins, talin, α-actinin and filamin and then recruits at least three known tyrosine kinases, c-Src, focal adhesion kinase (FAK) and integrin-linked kinase (ILK), to regulate cell adhesion and cell division (Brakebusch and Fassler, 2003) (Figure 1.8). Biochemical evidence showed that talin is phosphorylated by serine/threonine kinase and tyrosine kinase to regulate integrin activation. Talin, along with vinculin and α-actinin, is also structurally and functionally regulated by interactions with phosphatidylinositol 4-phosphate 5-kinase (PI(4)P 5-Kinase) or PI(5)P 4-kinase which catalyses the conversion of PI(4)P or PI(5)P to phosphatidylinositol 4,5-bisphosphate (PIP2). FAK is a tyrosine kinase that localises specifically on focal adhesions. Upon integrin activation, FAK is autophosphorylated in response to occupancy of integrin receptors by ligands, as well as clustering of integrins without occupancy of ligand-binding sites. Autophosphorylation of FAK at Tyr 397 creates a binding site for the Src homology (SH) 2 domain containing proteins, such as Src-like kinases, Grb7, phosphatidylinositol (PI) 3-kinase, Shc and phospholipase Cγ (PLCγ), forming an integrin-activated signaling complex. This complex may recruit a number of focal adhesion associated proteins, such as talin, vinculin, α-actinin, and F-actin, to form the stable focal adhesion and to stimulate actin stress fibre assembly (Figure 1.9). On the other hand, c-Src may activate phospholipase Cγ (PLCγ) (Finkelstein and Schwartzberg, 2004). PLCγ hydrolyses PIP2 to generate two secondary messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), which have been shown to mobilise Ca^{2+} from the endoplasmic reticulum (ER) and to activate protein kinase C (PKC), respectively. PKC is localised on focal adhesions of filopodia in macrophages and is thought to regulate the formation of actin stress fibres and contributes to cytoskeleton organisation (Tang *et al.*, 1997).

Figure 1.9 Overview of the focal adhesions associated signaling pathways. Two signaling pathways are involved in the focal adhesion assembly. Both integrins and growth factor receptors regulate the activity of Rho. Rho activates the PIP 5-kinase to increase the PIP2 levels, promoting actin polymerisation. PIP2 also recruits a growth factor dependent enzyme, PLCγ, to generate DAG and IP3. PI 3-kinase also is activated, in the mean time, by receptor-mediated activation to elevate PIP3 levels, resulting in reorganisation of actin filaments and focal adhesions.


PDGF, EGF, LPA, etc
Growth factor receptor
PIP3
PIP2
ECM
Integrins
PI3 k
PIP 5K, PIP KIγ
Rho
FAK
PIP2
Re-organisation of Focal adhesion
Formation of focal adhesion
Actin binding proteins
PLCγ
Actin polymerisation
PKC
DAG
IP3
Ca2+ efflux
ER

1.3.1.2.2 Receptor Mediated Signaling Pathway

Two types of receptor mediated signaling pathways are involved in focal adhesion assembly, the growth factor induced signaling pathway, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, bombesin or bradykinin, and the lysophosphatidic acid (LPA) induced signaling pathway. The critical proteins for the growth factor induced signaling pathway are small GTP-binding protein Rho superfamilies, which include RhoA, B and C, Rac 1 and 2, and Cdc42. Activation of RhoA is necessary and sufficient for focal adhesion assembly and stress fibre formation. Biochemical evidence indicated that Rac 1 is directly responsible for membrane ruffling and extension of lamellipodia, and Cdc42 controls the formation of filopodia (Nobes and Hall, 1995). RhoA has three known activated targets, phospholipase D, PI(4)P5-kinase and PI3-kinase, and all of which are involved in phospholipid metabolism and phosphoinositide generation. The role of these phosphoinositides in focal adhesion assembly is discussed below. On the other hand, RhoA may activate two known tyrosine kinases, c-Src and FAK. Hence, the regulation mechanism for forming focal adhesions and stress fibres may operate through integrin mediated signaling (Figure 1.9).

The LPA induced signaling pathway is thought to be involved in modulating cell growth and survival (Brindley, 2004). In the LPA induced signaling pathway, the LPA receptor couples to two G proteins, Gi and Gq. Activation of the Gi-coupled receptor by LPA treatment stimulates the Ras to MAPK cascade, resulting in gene expression and mitogenesis. Activation of the Gq-coupled receptor by LPA treatment stimulates phospholipase Cβ (PLCβ), resulting in linkage to RhoA signal transduction pathway as described above.

1.3.1.2.3 Regulation of Focal Adhesions by Phosphoinositides

Phosphatidylinositol 4,5-bisphosphate (PIP2) plays an essential role in regulating actin polymerisation and depolymerisation through its action on actin regulatory proteins. These proteins include profilin, cofilin, gelsolin, gCap and α-actinin (Kuhlman *et al.*, 1992). The function of these proteins, except that of α-actinin could be suppressed by PIP2 binding. For example, biochemical evidence showed that profilin bound PIP2 dissociated from actin monomers, resulting in actin polymerisation and stabilising the cytoskeleton (Lassing and

Lindberg, 1985) (as described in section 1.3.1.2.4). Fukami *et al.* have reported that PIP2 is required for α-actinin to realise its maximum gelating activity in striated muscles (Fukami *et al.*, 1992). They have also reported that a decrease in the amount of PIP2 that is bound to α-actinin and vinculin upon platelet-derived growth factor (PDGF) stimulation was suggested to be involved in actin depolymerisation (Fukami *et al.*, 1994). These results implied that PIP2 participates in the formation of actin stress fibres by its binding to α-actinin. However, an opposite opinion on the PIP2 regulation in F-actin binding ability of α-actinin has been reported (Fraley *et al.*, 2003). Fraley *et al.* have reported that an increase in the amount of both PIP2 and phosphatidylinositol 3,4,5-triphosphate (PIP3) inhibits α-actinin bundling activity by blocking the interaction of the actin-binding domain with F-actin *in vitro*. They have also reported that PIP2 and PIP3 differentially regulate α-actinin function by modulating the structure and flexibility of the protein (Corgan *et al.*, 2004).

The metabolic pathway of PIP2 has three possible routes: conversion of PIP2 to PIP3 by PI 3-kinase, hydrolysis from PIP2 to IP3 and DAG by PLCγ, and hydrolysis from PIP2 to phosphatidylinositol 4-phosphate (PI(4)P) by PIP2 phosphatase. All of these phosphoinositides and their converting enzymes may participate in regulating of focal adhesions. Greenwood *et al.* have reported that PIP2 bound α-actinin was converted to PIP3 by PI3-kinase and disrupted the interaction between α-actinin and integrin β subunit, resulting in activation of F-actin re-organisation in PDGF-stimulated rat embryonic fibroblast cells (Greenwood *et al.*, 2000). These results suggested that the activation of PI3-kinase induced remodeling of focal adhesions in PDGF-treated rat embryonic fibroblasts. In addition, Sakisaka *et al.* have reported that the activation of PIP2 phosphatase by EGF hydrolysed PIP2 bound α-actinin and decreased the PIP2 dependent F-actin cross-linking activity of α-actinin (Sakisaka *et al.*, 1997). These results suggested that PIP2 phosphatase participated in the rearrangement of actin filaments. However, the amount of PIP in cells is significantly lower than that of PIP2 (McLaughlin *et al.*, 2002), therefore, PIP might not play a critical role in regulating focal adhesions. Based on these studies, the roles of phosphoinositides in regulating focal adhesions remain controversial and further investigation is required to clarify this issue.

1.3.1.2.4 Regulation of Actin Assembly by Integrins

The major protein involved in the structure of the cytoplasmic face in focal adhesions is globular-actin (G-actin). G-actin is an essential component of the cytoskeleton in eukaryotic cells. The actin cytoskeleton plays a crucial role in the maintenance of cell morphology and polarity, in endocytosis and intracellular trafficking, in contractility, migration and cell division. In the presence of ATP, G-actin polymerises into filamentous actin (F-actin). Actin polymerisation is highly regulated by actin filament regulators, such as profilin and gelsolin. For example, profilin is a small G-actin-binding protein, which comprises an actin-binding region, the lipid phosphoinositide PIP2 binding region and the poly-L-proline interaction region. Biochemical studies suggest that profilin uses these three binding regions in regulating actin polymerisation (Lassing and Lindberg, 1985). In low concentrations of PIP2, profilin interacts with G-actin to prevent actin polymerisation. Once the amount of PIP2 is raised by activation of PI(4)P-kinase or PI(5)P-kinase, PIP2 dissociates profilin-actin complexes and promotes actin polymerisation (Lassing and Lindberg, 1985).

Another key nucleator of actin polymerisation is the actin-related protein 2/3 (Arp2/3) complex. Biochemical studies showed that Arp2/3 complex was recruited into the integrin associated focal adhesions by vinculin to induce the polymerisation of actin monomer (DeMali *et al.*, 2002). This interaction is transient and requires both PIP2 binding to vinculin and activation of the Arp2/3 complex by Rac1.

1.3.2 Cytoskeletal Proteins as Tumor Suppressors

Malignant transformation of the cell is characterised by disruption of cytoskeletal organisation, decreased adhesion and altered adhesion-dependent responses (Mizejewski, 1999). When cells become transformed or immortalised, the cytoskeletal and junctional proteins are re-organised, and the expression of several actin-associated proteins, such as tropomyosin, gelsolin, vinculin and α-actinin, are reduced under microscope observations and from biochemical studies (Ben-Ze'ev, 1997; 1985). Gluck *et al.* have reported that restoration of α-actinin-1 expression in tumorigenic simian virus 40 (SV40)-transformed 3T3 cells, which express a diminished level of α-actinin-1, results in the suppression of the tumorigenic and metastatic ability of these cells (Gluck and Ben-Ze'ev, 1994; Gluck *et al.*, 1993). These results suggested that α-actinin-1 played a role as a tumor suppressor in the

tumorigenic cells. Like α-actinin-1, α-actinin-4 is abundant in non-tumorigenic, substrate-adherent human neuroblastoma cell variants but is absent or only weakly expressed in malignant, poorly substrate-adherent neuroblasts (Nikolopoulos *et al.*, 2000). To investigate whether α-actinin-4 plays a role in determining the suppression of tumorigenicity of neuroblastoma cells, α-actinin-4 has been over-expressed in a highly tumorigenic cell line, BE(2)C, that expressed low levels of the endogenous α-actinin-4. These α-actinin-4 over-expressing cells revealed to be dramatically less malignant than mock transfected control cells in the *in vitro* transformation assay (Nikolopoulos *et al.*, 2000). The malignant potential of the α-actinin-4 over-expressed cells also has been verified *in vivo* by inoculation of these cells into nude mice. The results showed that no tumors or colonies were detected in nude mice during the 3 month observation or at autopsy. Moreover, the expression level of a proto-oncogene, *N-myc*, was also shown to be decreased. These results suggested that α-actinin-4 decreases or abrogates the tumorigenic potential of malignant neuroblastoma cells *in vitro* and *in vivo*.

The expression level of α-actinin-4 in normal and cancerous prostate tissue has been investigated using immunofluorescence histochemistry stain (Hara *et al.*, 2007). The authors state that the expression level of α-actinin-4 was significantly lower in prostate cancer than in normal prostate tissue, and restoration of α-actinin-4 expression inhibited cell proliferation in the prostate cancer cell line 22RV1. These results demonstrated that the expression level of α-actinin-4 in cells was associated with their tumorigenic potential. In order to investigate which domain was involved in the inhibition of cell proliferation, a series of deletion mutants derived from α-actinin-4 were transfected into HeLa cells to measure their effect on colony forming activity *in vitro*. The results indicated that the actin binding region (residue 28-269) and the spectrin repeats (residue 270-752) were responsible for the inhibition of cell proliferation (Hara *et al.*, 2007). These results supported that α-actinin-4 played a role as a tumor suppressor in the tumorigenic cells.

However, some research reports have shown conflicting data. Honda *et al.* have reported that α-actinin-4 enhances cell motility and cancer invasion by regulating the actin cytoskeleton (Honda *et al.*, 1998). The localisation of α-actinin-4 was detected using immunofluorescence histochemistry staining with a specific anti-α-actinin-4 antibody in

primary normal uterine endometrial fibroblasts and most cancer cell lines, including squamous cell lung cancer, vulvar epidermoid cancer, breast cancer, colorectal cancer (Honda *et al.*, 1998), esophageal cancer (Fu *et al.*, 2007) and ovarian cancer (Yamamoto *et al.*, 2007). The results indicated that α-actinin-4 was co-localised with actin stress fibres in primary normal uterine endometrial fibroblasts but poorly co-localised with actin stress fibres in cancer cell lines. In MCF7, a breast cancer cell line, α-actinin-4 was localised specifically in the nucleus (Honda *et al.*, 1998). These results implied that α-actinin-4 might be involved in the regulation of transcription activity in cancer cells. In addition, α-actinin-4 was revealed to be diffusely dispersed in the cytoplasm in most cancer cell lines, including PC10 (lung cancer), A431 (vulvar epidermoid cancer), SW480 (colon cancer), TE4 (esophageal cancer) and R27 (breast cancer) cells (Honda *et al.*, 1998). These results implied that α-actinin-4 might be involved in different functions in cancer cells. In order to investigate how α-actinin-4 influences the migration ability in cancer cells, α-actinin-4 was detected using immunofluorescence histochemistry staining with anti-α-actinin-4 antibody in the wound healing assay. The results showed that α-actinin-4 was remarkably expressed in cultured cancer cell lines along the edges of the wound and migrating into the wound. These results suggest that α-actinin-4 influences the cell motility in human cancers.

Honda *et al.* have also reported that α-actinin-4 promotes lymph node metastasis of colorectal cancer (Honda *et al.*, 2005). The expression level of α-actinin-4 was investigated in 26 clinical cases of colorectal cancer using quantitative immunofluorescence histochemistry with anti-α-actinin-4 antibody. The results showed that the expression of α-actinin-4 was increased in 19 cases, compared with the normal intestinal epithelium. These results were not consistent with the expression level of α-actinin-4 in prostate cancer (as described above). In order to investigate whether α-actinin-4 was involved in cancer metastasis, a cell line whose expression level of α-actinin-4 can be induced by tetracycline was established from colorectal cancer. The cells were inoculated into nude mice to verify the role of α-actinin-4 in cancer metastasis *in vivo*. Upon the induction of α-actinin-4 expression by tetracycline, the cell metastasised into regional mesenteric lymph nodes. These observations were similar to the behavior of clinical cancers (Honda *et al.*, 2005).

In addition, α-actinin-4 has been reported to be associated with β-catenin, which plays an

important role in suppression of cancer invasion. E-cadherin and its cytoplasmic binding protein, β-catenin, act as an invasion suppressor system of epithelial malignancies (Hirohashi, 1998). Both E-cadherin and α-actinin-4 interact with β-catenin and share the same binding site on β-catenin (residues 1-249). Biochemical evidence showed that when the expression level of E-cadherin was reduced by RNA interference in cancer cells, β-catenin interacted with α-actinin-4 to promote cell movement and mediate cancer invasion and metastasis (Hayashida *et al.*, 2005). These results imply that α-actinin-4 is associated with the invasiveness and metastatic potential on human cancers (Hayashida *et al.*, 2005).

To sum up, the expression level and the subcellular localisation of α-actinin-4 were inconsistent in different types of cancers. Moreover, α-actinin-4 was proposed to be a suppressor of tumorigenicity and cancer malignancy but also to be an inducer of cancer invasion and metastatic potential. Therefore, the roles of α-actinin-4 in cancers are uncertain.

1.3.2.1 Point Mutation of α-actinin-4 in Non-Small Lung Cancer (NSCLC)

A large number of cancer specific antigens have been identified and these antigens can be recognised by cytotoxic T lymphocytes (CTLs) derived from blood lymphocytes or tumor infiltrating lymphocytes (TILs). Some of these antigens are strictly tumor specific, and therefore constitute promising targets for anti-cancer immunisation. Mami-Chouaib *et al.* have reported that anti-tumor CTL clones were generated by stimulating lymphocytes that originated from a large cell lung carcinoma (LCC) patient (Mami-Chouaib *et al.*, 2002; Echchakir *et al.*, 2001) and these CTL clones could be helpful for the isolation of the LCC specific antigenic peptide. In order to identify the antigenic peptide recognised by the CTL clones, a cDNA library was prepared using poly $(A)^+$ RNA extracted from the LCC cells and screened by anti-tumor CTL clones. A cDNA clone was isolated and its sequence corresponded to that of gene *ACTN4*. However, the protein sequence of α-actinin-4 identified from LCC cells had a single amino acid residue replacement (K122N) located adjacent to the putative actin binding site-2 in the CH1 domain (Figure 1.6). α-actinin-4 K122N mutant derived from the LCC is essential for recognition by CTLs, therefore, the K122N mutant could be a potential immunotherapeutic target in lung carcinoma. Although the mutation does not take place within one of the actin binding sites, it is close to the

ABS-2 and the question arises of whether the K122N mutant could influence actin-bundling activity. The F-actin bundling activity of α-actinin-4 K122N mutant was measured using an *in vitro* sedimentation assay. The results indicated that K122N enhanced the bundling activity for F-actin (Menez *et al.*, 2004).

As described in section 1.3.2, α-actinin-4 exhibits tumor suppressing activity (Nikolopoulos *et al.*, 2000). In order to investigate whether the K122N mutant promotes tumorigenicity in cells, a highly tumorigenic neuroblastoma cell line that expressed only low levels of the endogenous α-actinin-4 (as described in section 1.3.2) have been used as an experimental system (Menez *et al.*, 2004). The colony forming and migration activity of the cells that over-expressed wildtype and K122N mutant of α-actinin-4 have been investigated, respectively. In comparison with cells over-expressing wildtype α-actinin-4, K122N over-expressing cells showed 5-10 fold higher colony forming efficiency and 5-fold lower migration capacity. These two cell lines were also inoculated into athymic nude mice to investigate their malignant potential, respectively. In comparison with that of wildtype over-expressing cells, the tumor growth rate of K122N over-expressing cells was considerably reduced during the 45-day observation period. These results suggested that the α-actinin-4 K122N mutant resulted in a higher tumorigenic activity *in vitro* and *in vivo* (Menez *et al.*, 2004). These results assumed that point mutations in *ACTN4* can abrogate its tumor suppression ability. However, the precise mechanism of tumor suppression is not well characterised.

1.3.2.2 Alternative Splicing of α-actinin-4 in Small Cell Lung Cancer (SCLC)

In addition to the α-actinin-4 K122N mutant reported in NSCLC, a natural variant type of α-actinin-4 has also been reported to be involved in small cell lung cancer (SCLC) (Honda *et al.*, 2004). Honda *et al.* have identified a novel alternatively spliced variant mRNA of α-actinin-4 in SCLC. In the variant mRNA of α-actinin-4, the 83-bp exon 8 is replaced by a new exon of the same size which exists within intron 8. The polypeptide encoded by this alternative transcript results in changes in three amino acid residues, N248G, A250L and S263C at the C-terminal of the CH2 domain (Figure 1.6). The expression of variant transcripts was investigated using RT-PCR in normal human tissues, lung cancer tissues and cancer cell lines, including fibroblast, keratinocyte, leukemia, mesothelioma, lung cancer,

gastric cancer, vulvar cancer, colorectal cancer and ovarian cancer (Honda *et al.*, 2004). The results showed that the variant transcript was detected in the normal testis and brain tissues. In human cancer, the variant transcript was detected specifically in SCLC, but not in NSCLC or other cancer cell lines. The F-actin bundling activity of the variant was investigated using an *in vitro* sedimentation assay. Similar to that of the K122N mutant in NSCLC, the F-actin bundling activity of the variant was stronger than that of wildtype α-actinin-4 (Honda *et al.*, 2004). These results demonstrated that the natural spliced variant α-actinin-4 bound F-actin tighter than wildtype α-actinin-4 and implied that the actin cytoskeleton re-organisation could be influenced when cells were stimulated by growth factors, such as PDGF and EGF (referred to section 1.3.1.2.3). In order to investigate the subcellular localisation of the variant, EGFP-variant α-actinin-4 fusion protein was transiently over-expressed in NIH3T3 cells. As described in section 1.3.2, wildtype α-actinin-4 was co-localised with focal adhesions and actin stress fibres. The variant, however, was co-localised mainly with actin stress fibres but poorly with focal adhesion (Honda *et al.*, 2004). These results suggested that the natural spliced variant α-actinin-4 could be involved in a different function in SCLC. However, the critical role of the variant α-actinin-4 in SCLC is not well characterised.

1.3.3 Required for Normal Glomerular Function

As described in section 1.2.4.3, three α-actinin-4 mutants, K255E, T259I and S262P, have been reported to be associated with human focal segmental glomerulosclerosis (FSGS). FSGS describes a pattern of primary glomerular lesions. The clinical hallmarks of FSGS routinely include proteinuria, hypertension, and progression to end-stage renal disease (ESRD) (Conlon *et al.*, 1995). These observations presumed that α-actinin-4 is required for normal renal function. The α-actinin-4 knock-out mice have been established using homologous recombination in embryonic stem (ES) cells (Kos *et al.*, 2003). The results indicated that the proportion of the homozygous null mice (*Actn4*$^{-/-}$), observed from heterozygous matings was significantly less than expected under mendelian law, approximately 8% and 10% in 0-4 day-old and 7-12 day-old mice, respectively. However, the normal proportion of the *Actn4*$^{-/-}$ mice was observed in late embryos at approximately 24.5% in E16.5-E18.8. The authors stated that the *Actn4*$^{-/-}$ mice do not survive at the perinatal period. The heterozygous mice, *Actn4*$^{+/-}$, showed no obvious ill effect. Compared

with the heterozygous mice, the homozygous mice, *Actn4*$^{-/-}$, showed progressive proteinuria, glomerulosclerosis, kidney failure, and typically death by several months of age. Histologic examination of the homozygous mice indicated abnormalities only in the kidney (Kos *et al.*, 2003). These results suggested that α-actinin-4 is essential for normal kidney function. Under microscope observations, podocyte cells generated from the *Actn4*$^{-/-}$ mice showed a loss of adhesion ability at approximately 60% to glomerular basement membrane components collagen IV and laminin 10 and 11 (Dandapani *et al.*, 2007). In addition, phosphorylation of integrin β1 on podocyte cells generated from the *Actn4*$^{-/-}$ mice was reduced (Dandapani *et al.*, 2007). These results concluded that α-actinin-4 plays an important role in stabilising glomerular architecture and preventing disease.

1.3.3.1 Mutation of α-actinin-4 in Focal Segmental Glomerulosclerosis (FSGS)

As described in section 1.3.3, α-actinin-4 is essential for normal glomerular function in mammals (Dandapani *et al.*, 2007). The phenotype of the α-actinin-4 deficient mice reveals progressive proteinuria, severe glomerular disease, and typically death by several months of age (Kos *et al.*, 2003). Indeed, three mutants of α-actinin-4 have been identified and linked to a human disease, focal segmental glomerulosclerosis (FSGS). FSGS is a common renal injury characterised by regions of sclerosis in the renal glomeruli from a wide range of primary disorders, such as diabetes, HIV infection and hypertension (Ichikawa and Fogo, 1996; Somlo and Mundel, 2000). It has been shown that mutations of α-actinin-4 cause focal segmental glomerulosclerosis (FSGS), which is inherited in an autosomal dominant pattern (Kaplan *et al.*, 2001). Three α-actinin-4 mutants, FS-A, FS-X and FSCI have been identified from three families by Kaplan *et al.* in 2001, and the cDNA sequences of these mutants have been investigated. Point mutations take place in A682G, C695T and T703C, and the encoded polypeptides reveal a single amino acid residue replacement in K255E, T259I or S262P within FS-A, FS-X and FS-CI, respectively (Figure 1.6). Similar to the K122N in NSCLC and the spliced variant in SCLC, biochemical evidence indicated that these mutants of α-actinin-4 bind F-actin tighter than wildtype α-actinin-4 (Kaplan *et al.*, 2001). In order to investigate the biological role of these mutants in FSGS, the α-actinin-4 K255E mutant (FS-A) has been over-expressed in podocytes. The subcellular localisation of the K255E mutant showed an aggregated appearance in the cytoplasm (Yao *et al.*, 2004). This K255E mutant was detected predominantly in the Triton-insoluble fraction and

localised almost exclusively along stress fibres rather than in focal adhesions (Michaud *et al.*, 2006). Yao *et al.* have developed a knock-in mouse by replacing α-actinin-4 with the K255E mutant to observe the behavior of α-actinin-4 K255E mutant (Yao *et al.*, 2004). The results indicated that the K255E mutant degraded much faster than wildtype α-actinin-4 and the rapid degradation of the K255E mutant was reversed by lactacystin, a specific proteasome inhibitor. These results suggested that α-actinin-4 K255E mutant was rapidly degraded through the ubiquitin-proteasome pathway.

1.4 Aims and Approaches

Every protein in the cells has its own normal functions; however, if one protein becomes mutated, it might have a gain or loss of function, resulting in an abnormal influence on the behavior and fate of the cells. Although the role of α-actinin-4 in the cells is not well understood, some studies have implied that α-actinin-4 plays a crucial role in stabilising cell shape and adhesion and regulating cell migration (Burridge and Chrzanowska-Wodnicka, 1996) and is involved in the normal kidney function (Kos *et al.*, 2003; Dandapani *et al.*, 2007). However, how the CH domains bind F-actin is not well characterised. Although two conformations of CH domains have been proposed (Sutherland-Smith *et al.*, 2003; Moores *et al.*, 2000), the rearrangement mechanism of these two conformations is not well understood.

It is well know that the structure of the cytoskeleton is highly dynamic and tightly regulated by signals from inside or outside of the cells, resulting in stimulation of cellular processes, such as proliferation, differentiation and migration. Upon normal physiological conditions, once PDGF or EGF binds its receptor on the cell membrane, it activates a signal transduction cascade and induces reorganisation of focal adhesions. PIP2 was converted by PI3-kinase to PIP3, resulting in the actin filaments reorganisation (Greenwood *et al.*, 2000). In addition, PIP2 would be broken down by PLCγ into two secondary messages, DAG and IP3. DAG would be involved in the PKC associated signaling pathway to reconstruct the focal adhesions. IP3 would release Ca^{2+} from the endoplasmic reticulum to the cytoplasm. However, the roles of PIP2, PIP3 and Ca^{2+} in regulating reconstruction of focal adhesions remain unclear. Although the PIP2 binding site has been identified on the CH2 domain from

chicken skeletal muscle α-actinin (Fukami *et al.*, 1996), the structural information from human α-actinin-1 and -3 suggested the region 177-193 does not correspond to the site of interaction with phosphoinositides (Borrego-Diaz *et al.*, 2006). These observations imply that the biochemical and structural evidence are not consistent.

The specific objectives included:

1. Establishing a novel *in vitro* F-actin binding assay to replace the traditional semi-quantitatively sedimentation assay.
2. Investigating how Ca^{2+}, the calmodulin-like domain or phosphoinositides (PIP2 and PIP3) regulate F-actin binding activity of α-actinin-4 using a novel solid phase F-actin binding assay.
3. Determining the three-dimensional structure of α-actinin-4 CH2 domain using NMR spectroscopy.
4. Identifying the phosphoinositide binding site on α-actinin-4 CH2 domain using NMR-based ligand titration assay.
5. Verifying the critical phosphoinositide interaction residues on the CH2 domain *in vitro*.
6. Investigating the subcellular localisation of α-actinin-4 in the resting and EGF-stimulating cells.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

The major chemicals and reagents and their main suppliers are listed below. All chemicals and regents were of analytical grade or of the highest purity available.

1,2-Dioleoyl-sn-glycerol (DAG)	Cayman chemical
^{13}C-glucose	Aldrich
$^{15}NH_4Cl$	Aldrich
Acetic acid	BDH-AnalaR
Adenosine triphosphate (ATP)	Sigma
Agarose, DNA grade	Progen
Ampicillin	Sigma
Bacto-agar	Difco
Bacto-tryptone	Difco
BIGDYE™	Applied Biosystems
Bovine serum albumin (BSA)	Sigma
Bradford reagent	BioRad
Bromophenol blue	Sigma
$CaCl_2$	Sigma
Casein	Sigma
Coomassie brilliant blue	Sigma
D_2O	Aldrich
Deoxynucleotide triphosphates (dNTPs)	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Scimar
D-myo-Inositol 1,3,4,5-tetraphosphate (IP4)	Cayman chemical
D-myo-Inositol 1,4,5-trisphosphate (IP3)	Sigma
Epidermal growth factor (EGF)	Sigma
Ethanol	BDH-AnalaR
Ethidium bromide	Sigma
Ethylene diamine tetraacetic acid (EDTA)	Sigma

Fluorescein-5-isothiocyanate (FITC)	Sigma
Glacial acetic acid	Sigma
Glutathione agarose resin	Bioserve
Glutathione, reduced form	Sigma
Glycerol	Sigma
H_2NaPO_4	Sigma
HNa_2PO_4	Sigma
Hydrochloric acid	BDH-AnalaR
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Scimar
Kanamycin	Sigma
KCl	BDH-AnalaR
Lipofectamine^{TM}2000	Invirogen
Methanol	BDH-AnalaR
$MgCl_2$	BDH-AnalaR
Mineral oil	Sigma
Na_2CO_3	Sigma
NaCl	BDH-AnalaR
NaOH	BDH-AnalaR
Non-muscle actin	Cytoskeleton
Paraformaldehyde	Merck
Phalloidin-TRITC	Sigma
Phenylmethylsulfonylfluoride (PMSF)	Sigma
Propan-2-ol (Isopropanol)	BDH-AnalaR
PtdIns-(3,4,5)-P3-1,2-dipalmitoyl (PIP3)	Cayman chemical
PtdIns-(4,5)-P2-1,2-dipalmitoyl (PIP2)	Cayman chemical
Restriction endonucleases	New England Biolabs
Skim milk powder	Diploma
Sodium azide	Sigma
Sodium dodecyl sulfate (SDS)	Sigma
T4 DNA Ligase	New England Biolabs
Thrombin	Sigma
Triton X-100	Sigma

Trypsin	Sigma
Turbo *Pfu* DNA polymerase	Stratagene
Tween-20	Sigma
XGAL	Progen
Xylene cyanol FF	Sigma
yeast extract	Difco
β-mercaptoethanol	Sigma

2.1.2 Solutions

All buffers and solutions were made up in Milli-Q® water and sterilised by autoclaving or filtering with 0.45 μM filter. The following solutions were used:

Actin polymerisation buffer	500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP
Antifade reagent	9.3 mM *p*-Phenyldiamine (PPD), 0.1X PBS, 80% (v/v) glycerol pH 9.0. Stored in the dark at -20°C
Blocking solution	1% (w/v) saturated casein in TBS
Coomassie blue	0.1% (w/v) Coomassie brilliant blue, 30% (v/v) methanol, 10% (v/v) acetic acid
Cracking solution	50 mM NaOH, 0.5% (w/v) SDS, 5 mM EDTA
Destain solution	50% (v/v) methanol, 5% (v/v) glacial acetic acid
ECL solution 1	2.5 mM Luminol, 0.4 mM courmaric acid, 100 mM Tris (pH8.5)
ECL solution 2	0.0192% (v/v) H_2O_2, 100 mM Tris (pH 8.5)
Freezing solution	10% (v/v) DMSO, 90% (v/v) fetal bonvine serum
General actin buffer	5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$
GLB (10X)	50% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol
PBS	8% (w/v) NaCl, 0.02% (w/v) KCl, 0.02% (w/v) KH_2PO_4, 0.115% (w/v) Na_2HPO_4
SDS-PAGE loading buffer	0.05 M Tris (pH 6.8), 4% (w/v) SDS, 12% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) Coomassie brilliant blue
TAE	40 mM Tris, 20 mM NaAc, 10 mM EDTA (pH 8.2)
TBS	20 mM Tris pH 7.4, 150 mM NaCl

TBST	TBS, 0.1% (v/v) Tween 20
TTBS	TBS, 0.1% (v/v) Triton X-100
Western Blocking solution	TBS, 0.1% (v/v) Tween-20, 4% (w/v) skim milk
Western transfer solution	190 mM glycine, 25 mM Tris and 15% (v/v) methanol
XGAL	20 mg/mL in DMF, stored at -20°C

2.1.3 Bacterial Strains

Stock cultures of these *E. coli* strains (and transformants) were stored as glycerol stocks at -80°C.

DH5α	The *E. coli* DH5α strain was used in transformations and was a host for all recombinant plasmids. DH5α: *sup*E44, Δ*lac*U169 (phi80 *lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*1, *rel*A1.
BL21	The *E. coli* BL21 strain was used in transformations and was a host for recombinant protein expression plasmids. BL21: B F^- *dcm*, *omp*, *hsd*S (rb^-mb^-) *gal*.

2.1.4 Mammalian Cell Line

NIH3T3, mouse fibroblast cells, were obtained from American-Type Tissue Culture Collection (ATCC). This cell line is suitable for DNA tensfection studies.

2.1.5 Bacterial Growth Media

Bacterial growth media were made up in Milli-Q® water and sterilised by autoclaving. Ampicillin (100 μg/mL) or Kanamycin (25 μg/mL) was added after the media had cooled to 55°C when required.

Luria broth (LB)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl. The pH was adjusted to 7.0 with NaOH
LB agar plate	LB medium supplemented with 1.5% (w/v) bacto-agar
MinA	60 mM K_2HPO_4, 33 mM KH_2PO_4, 1.7 mM Na_3Citrate, 15 mM NH_4Cl. Autoclave. Then add 0.005% (w/v) thiamine, 0.2% (w/v) glucose, 0.8 mM $MgSO_4$. (Miller, 1972)

SOC medium	2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose

2.1.6 Mammalian Cell Growth Media

Dulbecco's modified eagles medium (DMEM)	Invitrogen
Fetal bovine serum (FBS)	JRH
Penicillin and Streptomycin	Invitrogen

2.1.7 Kits

QIAGEN Plasmid Midi	QIAGEN
QIAprep Spin Miniprep	QIAGEN
QIAquick Gel Extraction	QIAGEN
QIAquick PCR Purification	QIAGEN
Quikchange™ site-directed mutagenesis	Stratagene

2.1.8 DNA and Protein Molecular Weight Standards

100 bp DNA Ladder	Band sizes (bp): 1517, 1200, 1000, 900, 800, 700, 600, 500/517, 400, 300, 200, 100. (New England Biolabs)
1 kb DNA Ladder	Band sizes (kb): 10, 8, 6, 5, 4, 3, 2, 1.5, 1, 0.5. (New England Biolabs)
SDS-7	Approximate MW (kDa): 66, 45, 36, 29, 24, 20.1, 14.2. (Sigma)
BenchMark™ (Pre-stained)	Approximate MW (kDa): 190, 120, 85, 60, 50, 40, 25, 20, 15, 10 (Invitrogen)

2.1.9 Plasmid Vectors

pGEX-4T2	4900 bp cloning and bacterial expression vector; used to generate a glutathione-S-transferase (GST) fusion protein. (Amersham)
pEGFP-C2	4700 bp cloning and mammalian cell expression vector; used to generate a enhanced green fluorescent protein (EGFP) fusion protein. (Clontech)

2.1.10 Antibodies

anti-GST, raised in mouse was prepared from hybridoma cells.

anti-mouse-HRP conjugate, raised in rabbit (Sigma).

2.1.11 Oligonucleotides

Synthetic DNA primers were synthesised by Geneworks. Shown 5' to 3'.

Number	name	RE site	Nucleotide sequence
215	EGFP-N	-	CGTCGCCGTCCAGCTCGACCAG
284	pGEX-5'	-	GGGCTGGCAAGCCACGTTTGGTG
285	pGEX-3'	-	CCGGGAGCTGCATGTGTCAGAG
349	hA4_M1_5'	*Eco*RI	GAGGAATTCGAATGGTGGACTACCACGCGGCG
350	hA4_N291_5'	*Eco*RI	TCAGAATTCAGAACGAGCACCTGATG
351	hA4_Q766_5'	*Eco*RI	GCCGAATTCAGATGCAGGAGTTCC
352	hA4_E290_3'	*Xho*I	ACGCTCGAGTTACTCTTGGTTGACAGC
353	hA4_E765_3'	*Xho*I	CTCCTCGAGCTACTCCTGGCTGATGCC
354	hA4_L911_3'	*Xho*I	TCTCTCGAGTCACAGGTCGCTCTCGCC
430	hA4_V284_5'	*Eco*RI	ATCGAATTCGTGCTGGCTGTCAACC
431	hA4_E409_5'	*Eco*RI	CGCGAATTCGAGCGGCTCGACCAC
432	hA4_Q522_5'	*Eco*RI	ACAGAATTCCAGCTGGAGGCCATCG
433	hA4_S646_5'	*Eco*RI	AAGGAATTCTCCAACGAGCACCTGCGC
434	hA4_L408_3'	*Xho*I	GTCCTCGAGTTACAGCCTGCGGATCTC
435	hA4_K521_3'	*Xho*I	GGCCTCGAGTTACTTCTCTGTTTTCTCC
463	ACTN4-CH1-F1	*Bam*HI	AAAGGATCCGAGAAGCAGCAGCGCAAG
464	ACTN4-CH1-R1	*Eco*RI	GGAATTCTCAGGAGATGTCCTGGATGGC
465	ACTN4-CH2-F1	*Bam*HI	GCAGGATCCGAGACCTCGGCCAAGGAA
466	ACTN4-CH2-R1	*Eco*RI	GGAATTCCTACTGCGCTCCTGAAAAGGC
490	hA4_Cam_F1	*Eco*RI	GACGAATTCCAGATGCAGGAGTTCCGG
491	hA4_S159_R2	*Xho*I	CAGCTCGAGTTAGGAGATGTCCTGGATGGC
493	hA4_E765_R2	*Xho*I	GAGCTCGAGCTACTCCTGGCTGATGCCCTTGGC
532	hA4_M1_F2	*Eco*RI	GAGGAATTCATGGTGGACTACCACGCG
533	hA4_L911_R2	*Xba*I	TCTTCTAGATCACAGGTCGCTCTCGCC

534	hA4_Q645_R1	*Xho*I	CACCTCGAGCTACTGCTGCTTGCTCTGCTC
535	ACTN4-W172A-F	-	GAAGGGCTCCTTCTCGCGTGCCAGAGAAAGACA
536	ACTN4-W172A-R	-	TGTCTTTCTCTGGCACGCGAGAAGGAGCCCTTC
537	ACTN4-S263D-F	-	ATGACCTATGTGTCCGACTTCTACCATGCCTTT
538	ACTN4-S263D-R	-	AAAGGCATGGTAGAAGTCGGACACATAGGTCAT
539	ACTN4-H266E-F	-	GTGTCCAGCTTCTACGAGGCCTTTTCAGGAGCG
540	ACTN4-H266E-R	-	CGCTCCTGAAAAGGCCTCGTAGAAGCTGGACAC
541	hA4_R771_R1	*Xho*I	GTCCTCGAGTTACCGGAACTCCTGCATCTG
542	hA4_N403_F1	*Eco*RI	AAGGAATTCAATGAGATCCGCAGGCTG
543	ACTN4-Y265A-F	-	TATGTGTCCAGCTTCGCCCATGCCTTTTCAGGA
544	ACTN4-Y265A-R	-	TCCTGAAAAGGCATGGGCCAAGCTGGACACATA
551	ACTN4-L169N-F	-	TCGGCCAAGGAAGGGAACCTTCTCTGGTGCCAG
552	ACTN4-L169N-R	-	CTGGCACCAGAGAAGGTTCCCTTCCTTGGCCGA

2.1.12 Miscellaneous Materials

96-well plate	Falcon
Biomax™ MR X-ray film	Kodak/Integrated Sciences
Centricon concentrators, 3, 10 and 30 kDa molecular weight cut-off	Amicon
Greiner Lumitrac 600 white 96 well plates	Stennick Scientific
Minisart 0.45 and 0.8 μM filters, syringe top	Sartorius
NMR tubes	Wilmad Glass co
NuPage 4-12% Bis-Tris Gels	Invitrogen
PD10 column	Amersham
Scalpel blades	Swann Morton
Superdex 75™	Amersham
Syringes	Becton Dickinson
Tissue culture plastic: T75 flasks, 6-well trays	Falcon

2.2 Methods

2.2.1 Molecular Biology Techniques

2.2.1.1 Mini-Preparation of Plasmid DNA

The plasmid DNA was prepared using QIAprep Spin Miniprep Kit from QIAGEN Inc. A 4 mL of overnight culture was harvested by centrifugation at 3,000 x *g* for 5 minutes and the supernatant was removed. The cell pellet was resuspended in 100 µL of buffer P1 and lysed by 100 µL of buffer P2. After addition of 150 µL buffer P3 contaminants were removed by centrifugation at 10,000 x *g* for 10 minutes. The plasmid samples were loaded into the QIAquick columns and the columns were centrifuged at 10,000 x *g* for 1 minute. The plasmid samples were washed with 0.75 mL of buffer PE and eluted in 50 µL of buffer EB by centrifugation at 10,000 x *g*.

2.2.1.2 Midi-Preparation of Plasmid DNA

The plasmid DNA was prepared using QIAGEN Plasmid Midi Kit from QIAGEN Inc. A 50 mL of overnight culture was harvested by centrifugation at 3,000 x *g* for 5 minutes and the supernatant was removed. The cell pellet was resuspended in 4 mL of buffer P1 and lysed by 4 mL of buffer P2. After addition of 4 mL buffer P3 contaminants were removed by centrifugation at 20,000 x *g* for 30 minutes. The plasmid DNA was purified by filtration through a disposable column, QIAGEN-tip, prepared by equilibrating with 4 mL of buffer QBT. The plasmid DNA attached to the column was washed with 2X 10 mL of buffer QC and eluted with 4 mL of buffer QF. The plasmid DNA was precipitated with isopropanol, washed with 70% ethanol and resuspended in TE buffer.

2.2.1.3 Polymerase Chain Reaction (PCR)

PCR reactions contained 1X reaction buffer, 1 mM dNTPs, 10 ng each primer, 50 ng template DNA and 2.5 U *Pfu* DNA polymerase in a total volume of 100 µL. Reactions were performed in a PTC-100 Programmable Thermal Cycler (MJ Research Inc.) with the following steps: initial hot start denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at appropriate temperature depending on the Tm value of primer pair for 1 min and extension at 68°C for 1 min. After final extension at 68°C for 10 min, PCR samples were stored at 4°C until further processing.

2.2.1.4 Restriction Enzyme Digestion

The plasmid DNA or PCR products were digested with appropriate restriction enzyme in either 20 µL or 100 µL reactions at 37°C for 16 to 18 hours. The extent of digestion was analysed by agarose gel electrophoresis (see section 2.2.1.13).

2.2.1.5 Purification of DNA Fragment from Agarose Gel

The kit used for preparation of DNA fragments from an agarose gel was QIAquick Gel Extraction Kit purchased from QIAGEN Inc. DNA fragments were excised from agarose gel with a clean scalpel blade. To avoid UV light-induced DNA damage, preparative gels were visualised using long wave UV light. The gel slices were weighed and added 3 volumes of buffer QC to 1 volume of gel in Eppendorf tubes. For example, to the 100 mg of gel slices were added 300 µL of buffer QC. The samples were incubated at 50°C for 10 minutes until the gels dissolved. The DNA samples were purified using the QIAquick columns by centrifugation at 10,000 x *g* for 1 minute. The DNA samples were washed with 0.75 mL of buffer PE and eluted in 50 µL of buffer EB by centrifugation at 10,000 x *g*.

2.2.1.6 Purification of DNA Fragment from PCR Reaction

The kit used to prepare the DNA fragments from a PCR reaction was QIAquick PCR Purification Kit from QIAGEN Inc. The PCR product was mixed with buffer PBI with the ratio 1:5 and incubated for 1 minute at room temperature. The DNA samples were loaded into the QIAquick columns and centrifuged at 10,000 x *g* for 1 minute. The DNA samples were washed with 0.75 mL of buffer PE and eluted in 50 µL of buffer EB by centrifugation at 10,000 x *g*.

2.2.1.7 Ligation

Ligation reactions contained 1X ligation buffer, 1 U of T4 DNA ligase and a 3:1 molar ratio of insert to vector DNA fragments in a 10 µL volume and processed at room temperature for 3 hours or 16°C overnight.

2.2.1.8 Preparation of Competent Cells

A 5 mL bacterial culture was set up in LB medium from a single bacterial colony and grown overnight at 37°C with shaking. The following morning, 5 mL bacterial culture was added

to 500 mL of LB medium and the culture was expanded to an optical density at 600 nm wavelength (OD_{600nm}) of 0.4-0.5. Bacterial pellets were harvested by centrifugation at 3,000 x *g* at 4°C for 10 minutes. Cell pellets were then washed in 100 mL ice cold $CaCl_2$ solution (0.06 M $CaCl_2$, 15% (v/v) glycerol), centrifuged at 3,000 x *g* at 4°C for 15 minutes and resuspended in 20 mL ice cold $CaCl_2$ solution. Approximately 200 µL aliquots in Eppendorf tubes were snap frozen in a dry ice/ethanol bath and stored at -80°C for up to six months.

2.2.1.9 Heat Shock Transformation

Plasmid DNA or 20 µL ligation reaction was added into 200 µL $CaCl_2$ competent cells and incubated on ice for 30 minutes. Cells were then incubated at 42°C for 90 seconds and chilled on ice for 2 minutes. 0.8 mL of SOC medium was immediately added to the cells and incubated at 37°C for 1 hour with rolling. Cells were plated onto LB agar plates with appropriate selection (Ampicillin or Kanamycin) and incubated overnight for 37°C.

2.2.1.10 Colony Cracking Screening

A transformant colony was picked with a sterile toothpick, dipped in 20 µL cracking buffer (50 mM NaOH, 0.5% SDS, 5 mM EDTA) and incubated at 55°C for 30 minutes. The samples were analysed by agarose gel electrophoresis (see section 2.2.1.13).

2.2.1.11 Automated DNA Sequencing

DNA sequencing reaction contained 0.5-0.1 µg of plasmid DNA, 100 ng of sequencing primer and 0.5 µL of BIGDYE™ ready reaction mix (Applied Biosystems) in a total volume of 10 µL. PCR reactions were performed in a PTC-100 Programmable Thermal Cycler (MJ Research Inc.) with the following steps: initial hot start denaturation at 94°C for 5 min, 26 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 4 minutes. The sequencing products were precipitated with 80% (v/v) isopropanol at room temperature for 10 minutes followed by centrifugation at 20,000 x *g* for 10 minutes. Pellets were washed with 500 µL 75% (v/v) isopropanol and dried before being processed at the IMVS DNA sequencing facility which uses an ABI sequencer (Applied Biosystems).

2.2.1.12 Site-Directed Mutagenesis by PCR

The Quikchange™ site-directed mutagenesis kit (Stratagene) was used for mutagenesis PCR reactions according to the manufacturer's recommendation. Mutagenesis PCR reactions contained 1X reaction buffer, 1 mM dNTPs, 10 ng each primer, 50 ng template DNA and 2.5 U *Pfu* DNA polymerase in a total volume of 50 µL. Reactions were performed in a PTC-100 Programmable Thermal Cycler (MJ Research Inc.) with the following steps: initial hot start denaturation at 94°C for 5 min, 16-20 cycles of denaturation at 94°C for 1 min, annealing at 55-60°C for 1 min and extension at 68°C for 10 min. After final extension at 68°C for 10 min, PCR samples were stored at 4°C until further processing. PCR samples were digested by *Dpn*I to remove parental DNA and then transformed into DH5α competent cells using heat shock method (Section 2.2.1.9).

2.2.1.13 Agarose Gel Electrophoresis

DNA agarose gels were prepared by pouring the melted 1.0% or 1.5% agarose/1X TAE gel solution onto a glass slide with a comb inserted to form wells. DNA samples were mixed with DNA loading buffer (1X GLB in final), loaded into a well and electrophoresed at 100 V for 40-45 minutes. The DNA was visualised by staining with ethidium bromide (EtBr, 5 µg/mL) followed with exposure to short wavelength ultra violet (UV) light on a Chromato-Vue transilluminator (Ultra-Violet Products Inc, San Gabriel, CA). Images were captured on photoprint paper with a Mitsubishi Video Copy Processor (Mitsubishi Electric Corporation).

2.2.2 Protein Chemistry Techniques

2.2.2.1 Induction and Purification of GST-fusion Proteins

A 10 mL bacterial culture was set up in LB medium containing 100 µg/mL Amp from a single *E. coli* BL21 colony and grown overnight at 37°C with shaking. The following morning, 10 mL bacterial culture was added to 1 L of LB medium containing 100 µg/mL Amp and the culture was expanded to an optical density at 600 nm wavelength (OD_{600nm}) of 0.5-0.6. Expression of GST-fusion protein was induced with 0.2 mM IPTG at 30°C for 4 hours with shaking. Bacterial cells were harvested by centrifugation at 3,000 x *g* at 4°C for 15 minutes and the supernatant was removed. Bacterial pellets were lysed by lysozyme (0.5

mg/mL final) and sonication (four times of 30 seconds burst) in a Triton X-100 containing TBS buffer (TTBS) supplemented with the protease inhibitor, PMSF (1 mM final) and EDTA (1 mM). Cell debris was removed by centrifugation at 22,000 x *g* at 4°C for 30 minutes and the supernatant was filtered through 0.8 and 0.45 μM filters. The GST-fusion proteins were purified by GST affinity chromatography. Samples were passed through a glutathione agarose column. After washed with TTBS and TBS buffers, the GST-fusion proteins were digested with 100 units of thrombin on the column in 1X TBS containing 2.5 mM $CaCl_2$ for 18 hours at room temperature. The GST-fusion proteins were eluted in TBS buffer and the GST-tag was washed out with 1X TBS containing 10 mM glutathione buffer. Samples from each step of the purification process were analysed by SDS-PAGE (see section 2.2.2.4).

2.2.2.2 Determination of Protein Concentration Using Bradford Assay

Protein concentration was determined by Bradford assay (Bradford, 1976). 10 μL of BSA standards (0 to 1 mg/mL) or protein samples were mixed with 200 μL of 25% (v/v) Bradford reagent (BioRad) in wells of a 96-well microtiter plate at room temperature for 10 minutes. Absorbance at 600 nm was measured in an absorbance plate reader (Molecular Dynamics). Samples and BSA standards were carried out in triplicate and averages were used in further calculations. A standard curve was plotted and the sample concentration was determined from the standard curve.

2.2.2.3 Concentration and Buffer Exchange of Recombinant Proteins

Recombinant proteins were concentrated by Amicon Ultra Filter (Amicon). 3, 10 or 30 kDa molecular weight cut-off filter units were used. Proteins were concentrated by centrifugation in Amicon Ultra Filter Unit at 3,000-4,000 x *g* at 4°C. PD10 columns (GE Healthcare) were used to buffer exchange proteins according to the manufacturer's directions.

2.2.2.4 SDS-PAGE

Denaturing SDS-polyacrylamide gels (NuPage 4-12% Bis-Tris Gel) were purchased from Invitrogen Inc. Protein samples were mixed with 1X load buffer and heated at 95°C for 5 minutes before loading onto the gel. The gels were electrophoresed at 200 V in MOPS or MES buffer (Invitrogen). The gels were run until the loading dye reached the bottom of the

gel and either stained with Coomassie blue for protein content observation or transfered onto nitrocellulose membrane for Western blot assay. The gels were stained with Coomassie blue for 1-2 hours at room temperature. Stain was removed by extensive washing in Destain buffer overnight. Digital images were recorded using a Canon laser scanner and UMAX MagicscanII software.

2.2.2.5 Size Exclusion Chromatography

The protein samples were separated by size exclusion chromatography using a Superdex-G75 column (Pharmacia) with bed volume of 20 mL connected to a Pharmacia pump P-50, lamp LKB-UV-MII, GradiFrac and chart recorder LKB-REC102. The column was initially prepared by washing with 0.5 M NaOH at a flow rate of 1 mL/min for 2 hours and then equilibrated extensively in the appropriate buffer (1X TBS). Protein samples were loaded onto the column with 1X TBS buffer. A flow rate of 1 mL/min was used and 1 mL fractions were collected. Protein was detected by measuring A_{280nm}. Bradford assay (section 2.2.2.2) and SDS-PAGE (section 2.2.2.4) were used to identify fractions containing proteins, quantify the amount of eluted protein and analyse the protein size(s).

2.2.2.6 Actin Polymerisation

Non-muscle actin was purchased from Cytoskeleton Inc. Non-muscle actin was resuspended in General Actin Buffer (5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$) to the concentration with 1 mg/mL and incubated on ice for 1 hour to depolymerise actin oligomers that formed during storage. The actin sample was mixed with $1/10^{th}$ volume of Polymerisation Buffer (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP) and incubated at room temperature for 1 hour.

2.2.2.7 Preparation of the F-actin Coated 96-well Plates

The 96-well plates were coated with 100 µL of F-actin solution (10 µg/mL) overnight at 4°C and blocked with 200 µL of 1% saturated casein at 37°C for 2 hours. The 96-well plates were washed with 1X TBS and stored at 4°C.

2.2.2.8 FITC Labeling Reaction

2 mg of protein (dissolved in 0.1 M sodium carbonate) was mixed with 1 mg of FITC (dissolved in 50 µL of DMSO) and incubated at room temperature for 4 hours with vigorous

shaking. Unreacted FITC was removed using a PD10 column (Section 2.2.2.3). The concentration of fluorescein-labeled proteins was measured using Bradford assay (Section 2.2.2.2) and the Degree of Labeling (DOL) was calculated by following equation.

$DOL=A_{max}$ X MW / [protein] X ε_{dye}

Where MW = the molecular weight of the protein, ε_{dye} = the extinction coefficient of the dye at its absorbance maximum and the protein concentration is in mg/mL.

2.2.2.9 Solid Phase F-actin Binding Assay

The fluorescein-labeled recombinant proteins were added into the 96-well plates which have been coated with F-actin and incubated at room temperature for 2 hours. The plates were washed three to five times with TBST (0.1% (v/v) Tween 20 in TBS) and air-dried for 20-30 minutes. The fluorescence intensity was measured using FLUOstar Galaxy plate reader. The Kd values were calculated by non-liner regression analysis.

2.2.2.10 Western Blot

Proteins were separated using SDS-PAGE (section 2.2.2.4) and transferred onto Hybond-C membrane (Amersham) at 40 mA at room temperature for 90 minutes in western transfer buffer (190 mM glycine, 25 mM Tris and 15% methanol) using a Hoefer SemiPhor Western Transfer apparatus (GE Healthcare). Membranes were blocked overnight at 4°C in western blocking solution (TBS, 0.1% (v/v) Tween-20, 4% (w/v) skim milk) and washed three times in TBST (0.1% (v/v) Tween 20 in TBS). Primary antibody was diluted at 1 in 2000 in western blocking solution and added to the membranes for overnight incubation at 4°C. Membranes were washed three times in TBST buffer for 20 minutes in each. Secondary antibody diluted at 1 in 2000 in western blocking solution and added to the membranes at room temperature for 2 hours before washing the membrane with 3 x 20 minute washes in TBST buffer. Enhanced chemiluminescence (ECL) detection was used as follows. Membranes were developed for 1 minute in freshly prepared mixture of equal volumes of ECL solutions 1 and 2 and exposed to X-ray film that was developed using a CURIX 60 X-Ray developer.

2.2.3 NMR Spectroscopy Techniques

2.2.3.1 NMR Sample Preparation

Unlabeled and isotope labeled samples of the CH2 domain were produced by growing the bacterial cells on LB medium and minimal medium (MinA) containing ^{13}C-glucose and $^{15}NH_4Cl$ as sources of carbon and nitrogen, respectively. For protein purification procedures see section 2.2.2.1. The purified proteins were concentrated using an Amicon stirred cell with 5 kDa filter (Amicon) to a final volume of 1 to 2 mL. Fast desalting PD10 column (GE Healthcare) was used to buffer exchange the protein into 10 mM phosphate buffer containing 0.01% (w/v) NaN_3 at pH 6.8. Protein samples with the unlabeled, ^{15}N labeled or $^{13}C/^{15}N$ double labeled were prepared in concentrations of 3 to 4 mM. These samples with 500 µL in 10 mM phosphate buffer containing 0.01% (w/v) NaN_3 with pH 6.8 were supplemented with 10% (v/v) D_2O and placed in a 5 mm high-resolution thin-walled glass NMR tube. Samples for D_2O experiments were prepared by buffer exchange of the protein into the D_2O preparing 10 mM phosphate buffer using PD10 column.

2.2.3.2 NMR Experiments and Resonance Assignments

NMR experiments were performed on a Varian Inova 600 MHz spectrometer using the BioPack package. All data sets were recorded at 25°C using a 5 mm inverse triple resonance $^{1}H/^{13}C/^{15}N$ pfg probe. The carrier frequency was centred on the H_2O signal. Backbone and sidechain assignments were made using 3D ^{1}H-^{15}N NOESY-HSQC, 3D ^{1}H-^{15}N TOCSY-HSQC, HNCA, HN(CO)CA, HNCO, HN(CA)CO, HBHA(CBCACO)NH, CBCANH, CBCA(CO)NH and HCCH-TOCSY. Aromatic proton assignments were made using 2D-TOCSY, 2D-NOESY recorded by the unlabelled samples in D_2O, and HBCBCGCDHD and HBCBCGCDCEHE. All spectra were processed using NMRPipe (Delaglio *et al.*, 1995) and analysed using CCPNMR (Vranken *et al.*, 2005). ^{1}H, ^{13}C and ^{15}N chemical shifts were referenced to TSP at 0 ppm (Wishart *et al.*, 1995).

2.2.3.3 Structural Restraints

Distance restraints were derived from 2D ^{1}H-^{1}H NOESY and 3D ^{1}H-^{15}N NOESY-HSQC as well as 2D NOESY recorded in D_2O, with a mixing time of 100 ms. Unambiguous and ambiguous restraints were generated automatically by ARIA 2.0. Dihedral angle restraints

were derived from ^{1}H-^{15}N HMQC-J spectrum. The $^{3}J_{HNH\alpha}$ values were determined from F1 and F2 cross peak line widths in a ^{1}H-^{15}N HMQC-J spectrum (Wishart and Wang, 1998). Dihedral angle restraints were set the φ-angles of -120 ± 40° with the $^{3}J_{HNH\alpha}$ value greater than or equal to 8 Hz and -60 ± 30° with the $^{3}J_{HNH\alpha}$ value less than or equal to 5 Hz. Hydrogen bond restraints were determined by deuterium exchange experiments. Hydrogen bond donors were determined when the amide group had not exchanged from ^{1}H to ^{2}D within 1 hour at 25°C after resuspension of a lyophilised sample from H_2O to D_2O. The hydrogen-acceptor distance was set between 1.7 and 2.2 Å and the donor-acceptor distance was set between 2.7 and 3.2 Å.

2.2.3.4 Structure Calculations

The structure calculations were performed using the program ARIA 2.0 (Linge *et al.*, 2003) with the assistance of Mr. Iain Murchland. An ensemble of 20 structures was calculated at iteration 0 to 7 and seven of the lowest energy conformers were selected for the next iteration. A total of 200 structures were calculated at the final iteration. The 20 structures with the lowest energy were selected for the refinement step and 200 structures were performed in water solvent using the OPLSX non-bond parameter set (Linge and Nilges, 1999). The 20 structures with the lowest overall energies were selected as the final ensemble.

2.2.3.5 Structure Analyses

The quality of the ensemble was validated using PROCHECK (Laskowski *et al.*, 1993). The hydrogen bond and the secondary structure were analysed using MOLMOL (Kordadi *et al.*, 1996) and Ramachandran plot was generated using PROCHECK (Laskowski *et al.*, 1993).

2.2.3.6 NMR-based Ligand Titration Experiments

The 0.2 mM ^{15}N-labeled NMR samples were titrated with increasing concentration of ligands (IP3, IP4, PIP2, PIP3 or DAG) in 10 mM phosphate buffer containing 0.01% (w/v) NaN_3, pH 6.8 or 1X TBS containing 0.01% (w/v) NaN_3, pH 7.0. 2D ^{1}H-^{15}N HSQC spectra were recorded on Varian Inova 600 spectrometer with spectral widths of 8000 Hz in the ^{1}H dimension and 2000 Hz in the ^{15}N dimension. The number of increments was 64 and the number of transients was 32. The number of points was 2048 with the acquisition time of

0.128 seconds. All data sets were recorded at 25°C using the ghsqc experiment from Varian. All spectra were processed using NMRPipe (Delaglio *et al.*, 1995) and analysed using CCPNMR (Vranken *et al.*, 2005). The ligands were lyophilised prior to the NMR experiment and resuspended in the protein solution in each concentration point avoiding the changes of sample volume and concentration during NMR experiments. These ligands used in the NMR experiments are water soluble with the maximum solubility of IP3 and IP4 of 100 mM, PIP3 of 10 mM and PIP2 and DAG of 1 mM.

The amino acid residues involved in the ligand binding were identified when the values of chemical shift deviations Δ^1H_N or $\Delta^{15}N$ were greater than the values of $|\delta_{mean} + \delta_{std}|$ (Chen *et al.*, 2008; Chang *et al.*, 2006). For Kd evaluation, chemical shift deviations from 2D ^1H-^{15}N HSQC spectra were plotted as a function of ligand concentrations and the Kd values were calculated by non-liner regression analysis.

2.2.4 Tissue Culture Techniques

2.2.4.1 Passage of Mammalian Cells

Adherent NIH3T3 cells were treated with trypsin (1 mL per 75 cm^2) at 37°C for 2 minutes. Detached cells were resuspended in DMEM containing 10% (v/v) FBS, centrifuged at 290 x *g* for 3 minutes and resuspended in fresh DMEM containing 10 % (v/v) FBS. NIH3T3 cells were incubated at 37°C, 5% CO_2 in an incubator for 48-36 hours.

2.2.4.2 Transfection

Mammalian cells were transiently transfected with Lipofectamine™ 2000 (Invitrogen) reagent according to manufacturer's recommendation. Briefly, the Lipofectamine™ 2000 reagent and plasmid were each mixed with DMEM at room temperature for 5 minutes, then combined and incubated at room temperature for 20 minutes. The mixtures were added to NIH3T3 cells plated onto coverslips the previous day and incubated at 37°C for 4-6 hours. Medium was changed to DMEM containing 10% FBS. NIH3T3 cells were incubated at 37°C, 5% CO_2 in an incubator for 48-36 hours.

2.2.4.3 Fixing and Staining

NIH3T3 cells were cultured on coverslips in a 6-well tray for 24 hours. Cells were washed twice with PBS, fixed with cold 4% (w/v) paraformaldehyde in PBS for 20 minutes, rehydrated in PBS for 15 minutes and permeabilised in 0.1% (v/v) Triton X-100 in PBS at room temperature for 10 minutes. After washing three times with PBS, cells were stained with phalloidin-TRITC (50 μg/mL in PBS) for 40 minutes and with DAPI (100 ng/mL in PBS) at room temperature for 2 minutes. After washing three times with PBS, coverslips were mounted onto microscope slides with antifade reagent. Cells were analysed by fluorescence microscopy.

CHAPTER 3

Regulation of F-actin Binding Activity of α-actinin-4 by Ca^{2+}

3.1 Introduction

α-actinin-4 is a Ca^{2+} sensitive protein that cross-links actin filaments and is thought to be associated with stabilising cell morphology and adhesion and regulating cell migration. The Ca^{2+} binding region has been identified as the C-terminal calmodulin-like domain which contains two EF hand motifs. The structure of the EF hand motif from Ca^{2+} binding proteins, such as calmodulin has been well characterised as a helix-loop-helix structure composed of 12 amino acid residues (Wilson and Brunger, 2000). The chelating residues within the helix-loop-helix structure have been well defined and labeled as X, Y, Z, -Y, -X and -Z at the positions of 1, 3, 5, 7, 9 and 12 (Figure 3.1) (Gifford *et al.*, 2007). The amino acid sequences of the EF hand motif are highly conserved especially at the positions 1, 3, 6 and 12 and the number of residues within this helix-loop-helix structure is extremely restricted. Once these critical residues are replaced or single amino acid insertion or deletion occurs, the EF hand does not coordinate with Ca^{2+}. The calmodulin-like domain of Ca^{2+} sensitive α-actinin isoforms (α-actinin-1 and -4) contain four EF-hand motifs but only two of them coordinate with Ca^{2+} (EF hand 1 and 2 at the N-terminus of calmodulin-like domain). The other two do not bind Ca^{2+} because of a single residue deletion between position Z and -Y in the EF hand 3 and a single residue insertion between position Y and Z in the EF hand 4 (Figure 3.2).

The role of Ca^{2+} in the function of non-muscle α-actinin has been studied in *Dictyostelium discoideum* (Witke *et al.*, 1993). Ca^{2+} binding to the two EF hand motifs of α-actinin has been detected using a Ca^{2+} overlay assay. Briefly, α-actinin was coated on the nitrocellulose membrane and incubated with isotope-labeled $^{45}CaCl_2$. Signals were observed on the membrane when $^{45}Ca^{2+}$ bound α-actinin. The results indicated that both EF hand motifs bound Ca^{2+} but with a higher affinity in EF hand 2 and a lower affinity in EF hand 1. Interestingly, the Ca^{2+} binding affinity of two EF hand motifs (2X EF hand) were 2-fold stronger than that of the EF hand 2 alone. In addition, how Ca^{2+} regulates the F-actin cross-linking activity of α-actinin has been investigated using F-actin viscosity assay. Briefly, α-actinin was incubated with F-actin with or without the presence of Ca^{2+} and the viscosity of the solutions was measured. The results showed that the F-actin cross-linking activity of α-actinin was inhibited in the presence of Ca^{2+} in the range of 50 nM to 1 μM

A

EF-loop position	1	2	3	4	5	6	7	8	9	10	11	12
coordinating ligand	X sc		Y sc		Z sc		-Y bb		-X sc*			-Z sc2
most common	Asp 100%	Lys 29%	Asp 76%	Gly 56%	Asp 52%	Gly 96%	Thr 23%	Ile 68%	Asp 32%	Phe 23%	Glu 29%	Glu 92%
also frequently observed		Ala Gln Thr Val Ile Ser Glu Arg	Asn	Lys Arg Asn	Ser Asn		Phe Lys Gln Tyr Glu Arg	Val Leu	Ser Thr Glu Asn Gly Gln	Tyr Ala Thr Leu Glu Lys	Asp Lys Ala Pro Asn	Asp

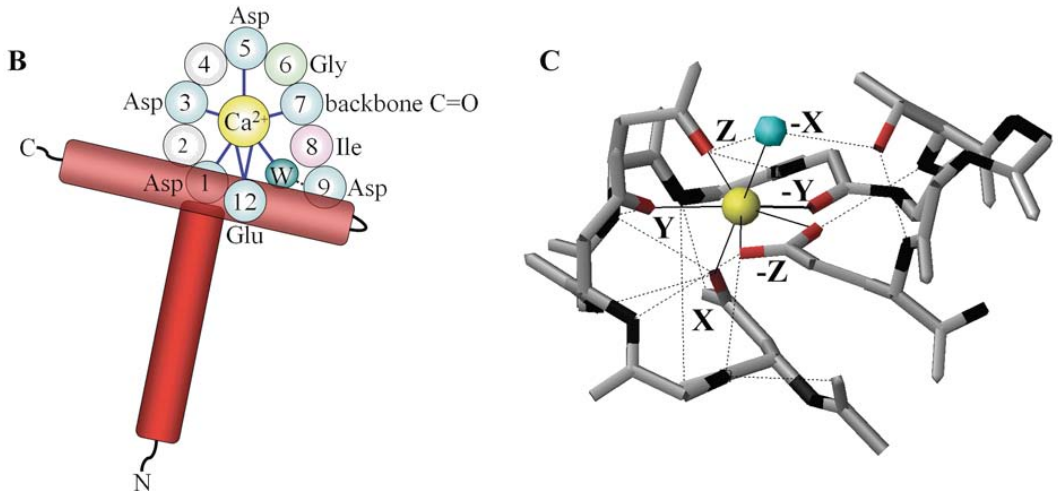


Figure 3.1 The structure of EF hand. (A) The consensus sequence of the EF hand loop. Ca^{2+} coordination in well-defined positions, labeled X, Y, Z, -Y, -X and -Z. Ca^{2+} coordinated with these critical amino acid residues via the side chain (sc) or through the backbone (bb). The * indicates that a water molecule provides an interaction between Ca^{2+} and the side chain of the amino acid residue at position -X by hydrogen bond. (B) A schematic diagram of the Ca^{2+} coordination with two helices in red, the critical amino acid residues in blue and the coordinating water molecule (W) in dark blue. Light green corresponds to the conserved glycine residues that provides the bend in the loop. (C) Ca^{2+} coordination by the EF hand 1 of calmodulin illustrating the interactions between the Ca^{2+} ion and oxygen atoms (continuous lines) and the extensive hydrogen bonding in the loop (broken lines). The backbone NH groups are indicated in black, the oxygen atoms from backbone and side chain in red, the Ca^{2+} ion in yellow and the coordinating water molecule in blue. The diagrams were adapted from Gifford, 2007.

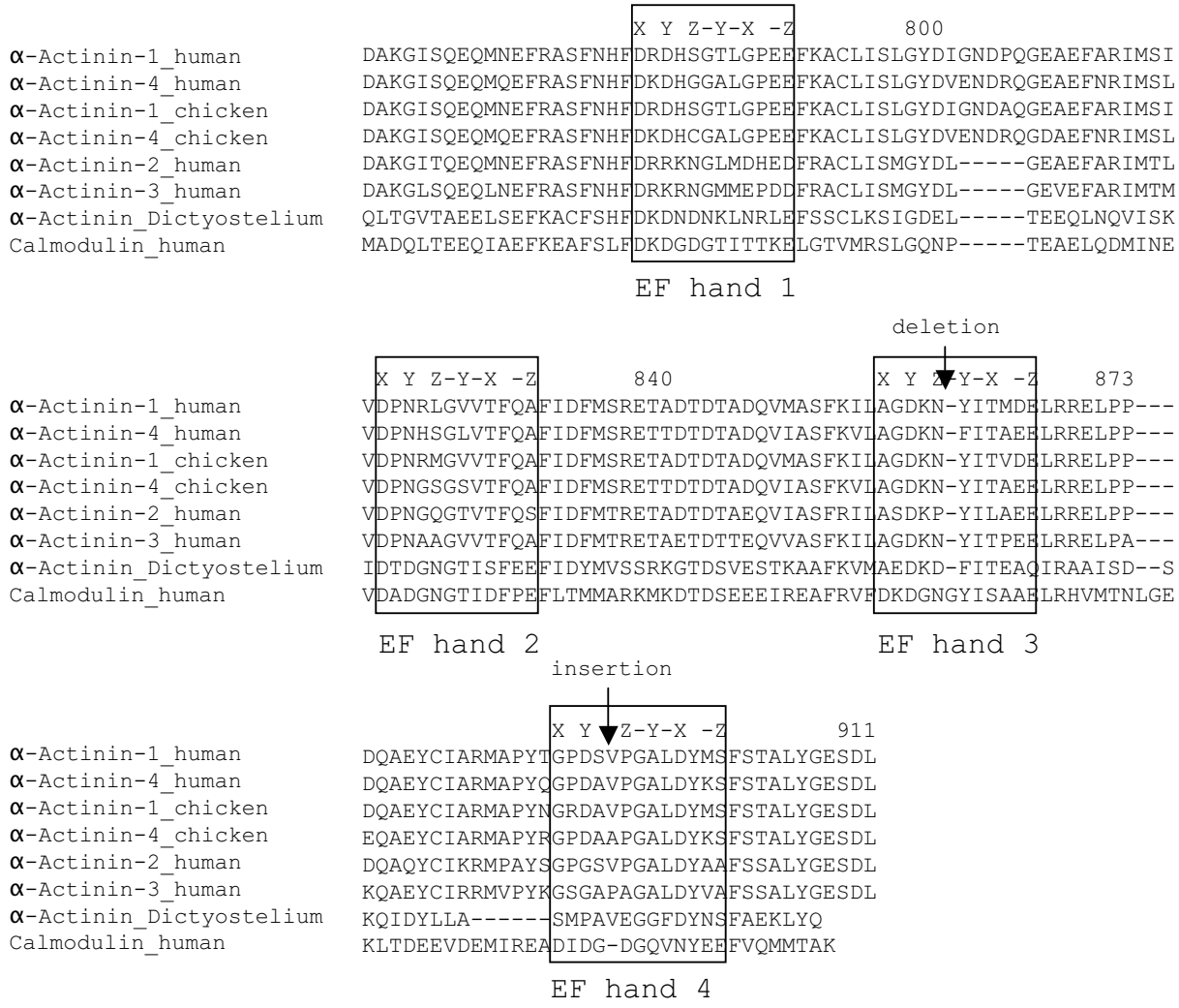


Figure 3.2 Amino acid sequence alignment of α-actinin calmodulin-like domain with calmodulin. Four EF hands are indicated by black boxes. α-actinin contains two functional EF hand motifs (1 and 2) with the proper number of amino acid residues to correspond with calmodulin. The EF hands 3 and 4 are disrupted by an amino acid residue deletion in the EF hand 3 and an amino acid residue insertion in the EF hand 4. Accession codes are: human α-actinin-1, P12814; human α-actinin-4, O43707; chicken α-actinin-1, P05094; chicken α-actinin-4, Q90734; human α-actinin-2, P35609; human α-actinin-3, Q08043; *Dictyostelium discoideum* α-actinin, P05095; and human calmodulin, P62158.

(Witke *et al.*, 1993).

A Ca^{2+} binding model on the calmodulin-like domain of α-actinin has been predicted based on the observation of the α-actinin crystal structure from rabbit skeletal muscle (Tang *et al.*, 2001). In cryoelectron microscopy projections, α-actinin formed an antiparallel homodimer and the end of the molecules showed a six-density ring which was thought to be the CH1, the CH2 and the calmodulin-like domains. These observations presumed that the calmodulin-like domain might regulate the F-actin binding activity of CH domains by a direct interaction in the presence or absence of Ca^{2+} (Tang *et al.*, 2001). The hypothesis they mentioned is as follows; upon the absence of Ca^{2+}, the CH domains (2X CH) form a compacted conformation. In the presence of Ca^{2+}, the calmodulin-like domain could undergo a conformation change which allows it to bind the linker between the CH1 and CH2 domains, resulting in interference of the F-actin binding activity of α-actinin. However, there was no biochemical evidence to support this. These proposals implied that there might be a potential calmodulin-like domain binding site on the CH (2X CH) domains.

A calmodulin binding site on the CH domains of dystrophin and filamin A has been identified and the sequence alignment shows the region is highly conserved within the spectrin superfamily (Jarrett and Foster, 1995; Nakamura *et al.*, 2005). Dystrophin is a member of the spectrin superfamily and binds F-actin through its N-terminal CH domains. Two regions on the CH1 domain, residue 18-42 and 104-125, were reported to be involved in the interaction with calmodulin. Calmodulin bound dystrophin in a Ca^{2+} dependent manner and competitively inhibited F-actin binding (Jarrett and Foster, 1995). Filamin A (FLNa), another member of the spectrin superfamily, is an F-actin cross-linking protein which performs a particular role to support perpendicular branching of F-actin into a three-dimensional “gelled network” (Popowicz *et al.*, 2006). It is composed of a conserved actin-binding region (2X CH domains) at the N-terminus and a string of 24 β-pleated sheet repeats at the C-terminus. How calmodulin and Ca^{2+} regulate the F-actin bundling activity of filamin A has been investigated. In the absence of Ca^{2+}, calmodulin does not influence the F-actin bundling activity of filamin A. In the presence of 2 mM Ca^{2+}, however, calmodulin inhibits the F-actin bundling activity on the *in vitro* F-actin sedimentation assay (Nakamura *et al.*, 2005). Indeed, F-actin is essential for the interaction between calmodulin

and the CH domains of filamin A. Residues 50-96, has been proposed to be the calmodulin binding sites on the CH domains using a GST pull down assay. According to these results, a regulatory model for the F-actin binding of filamin A by calmodulin has been developed (Nakamura *et al.*, 2005) (Figure 3.3). In the absence of F-actin and low or no Ca^{2+}, the CH domains (2X CH) form a compacted conformation by the interaction between residues 87 to 96 (basic region) and the linker between the CH1 and the CH2 domains (acidic region). Once F-actin is present, F-actin may assist the conformational change of the CH domains to an extended conformation and the calmodulin binding site becomes exposed. In high concentrations of Ca^{2+} (in the mM level), calmodulin becomes activated by binding Ca^{2+} and inhibits F-actin binding by interaction with the CH domains. To sum up, the regulation of the conformational change of the CH domains should be very important for the F-actin binding activity of α-actinin. The calmodulin-like domain and Ca^{2+} are proposed to be critical factors for the regulation of the conformational change of the CH domains.

3.2 Aims and Approaches

The aims of this section are to characterised the roles of Ca^{2+} and calmodulin-like domain on the F-actin binding activity of α-actinin-4 using an *in vitro* F-actin binding assay.

There are three conventional methods used for verifying the F-actin binding activity *in vitro*, F-actin viscosity assay, low speed sedimentation assay and co-sedimentation assay. The F-actin viscosity assay is used for measuring the F-actin gel forming ability of the actin binding proteins. F-actin is bundled by actin binding proteins to form a large, strong polymer and the viscosity of the protein solution is increased. Like the viscosity assay, the low speed sedimentation assay measures the F-actin bundling ability of the proteins. Once F-actin is bundled by the actin binding proteins, the large F-actin polymer can be pelleted by centrifugation at 10,000 x *g*, and detected by SDS-PAGE. How strong the F-actin bundling activity of the actin binding proteins is, is dependent on how much F-actin is detected on the SDS-PAGE. The traditional actin binding assay is a co-sedimentation assay. This method is quite similar to that of the low speed sedimentation assay. Briefly, the actin binding proteins are incubated with F-actin and centrifuged at 100,000 x *g*. After appropriate incubation and centrifugation, actin binding proteins from the pellet are quantified by standard

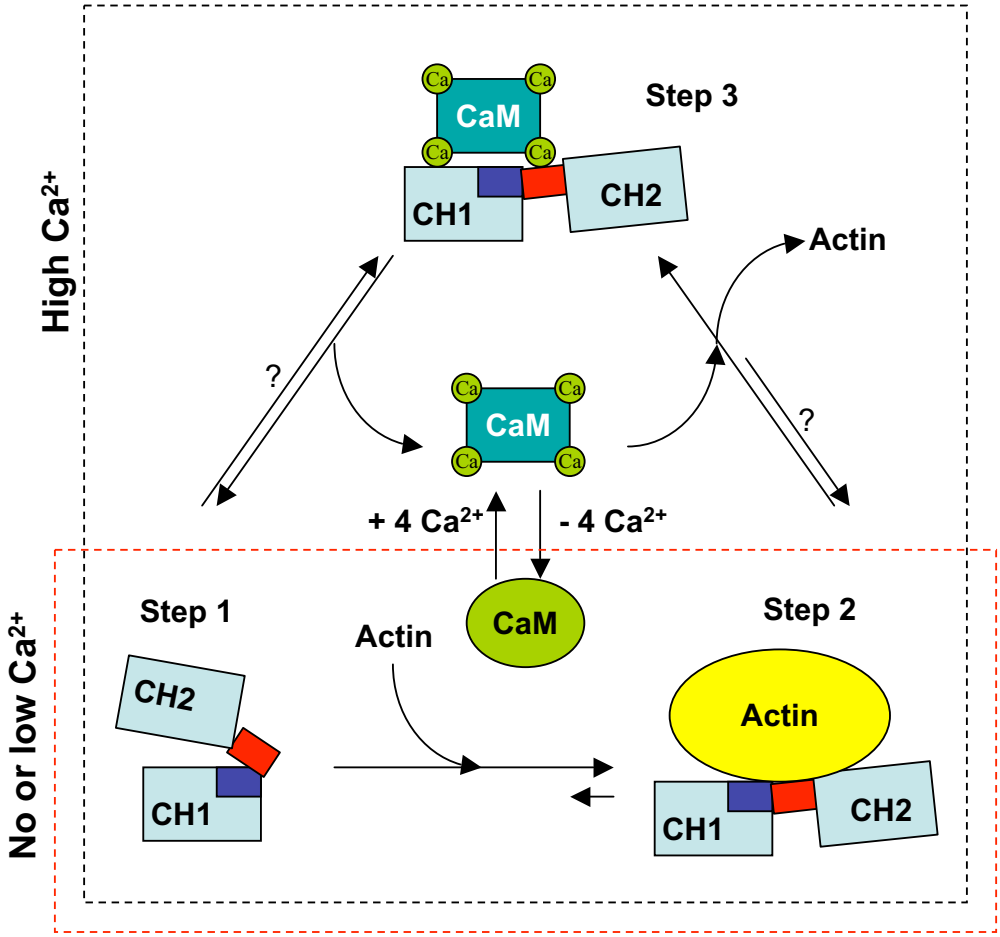


Figure 3.3 A model for the regulation of the F-actin binding of filamin A CH domain by calmodulin. Step 1, the CH domains form a compacted conformation by the interaction between the calmodulin binding site (blue) and linker region (red) in the absence of actin and Ca^{2+}. Step 2, the binding of F-actin exposes the calmodulin binding site. Step 3, Ca^{2+} activates calmodulin (holocalmodulin), and holocalmodulin competes F-actin for filamin A binding, increasing the F-actin bundling activity of filamin A. The diagrams were adapted from Nakamura, 2005.

densitometry. However, these traditional assays have some limitations and shortcomings: all the methods need considerable amounts of F-actin and protein gel materials and the data generated are semi-quantitative rather than quantitative. In order to overcome the limitation, a novel solid phase F-actin binding assay has been established. Polystyrene 96-well plates were coated with F-actin in an appropriate buffer and blocked with 1% saturated casein to avoid non-specific binding. Non-coated wells also were used as a blank. After incubation with a dilution series of fluorescein-labeled actin binding protein, the wells were washed and the fluorescence intensity was detected using a fluorescence plate reader (Figure 3.4). The binding curve is given by plotting the concentration (X) and fluorescence intensity (Y). Based on this binding curve, the Kd values could be calculated using non-linear regression analysis.

3.3 Results

3.3.1 Cloning, Expression and Purification of α-actinin-4

The PCR fragment of the full length α-actinin-4 (911 residues) was subcloned into the expression vector pGEX4T2 (Figure 3.5) using restriction sites, *Eco*RI and *Xho*I. The plasmid encoding the GST-α-actinin-4 fusion protein was sequenced using appropriate primers to ensure expression of a correct protein (data not shown) and transformed into *E. coli* BL21 bacterial cells for protein expression. Samples of the α-actinin-4 were generated according to the method described in section 2.2.2.1. Samples from each step of the purification process were analysed by SDS-PAGE (representative shown in Figure 3.6). An induced protein band at the expected size for the GST-α-actinin-4 fusion protein (~126 kDa) was identified and a protein band at the expected size for α-actinin-4 (100 kDa) was identified in the eluted fraction. In addition to α-actinin-4, there are some light protein bands shown on SDS-PAGE in the eluted fraction (Figure 3.6). These are most likely the break-down products of α-actinin-4. This is maybe a result of α-actinin-4 containing a potential pseudo-thrombin cleavage site or auto-catalysis of thrombin leading to partial loss of specificity. After washing with a glutathione containing buffer, protein band of the expected size for the GST tag (~26 kDa) was identified. The purified α-actinin-4 was collected and prepared for further analysis.

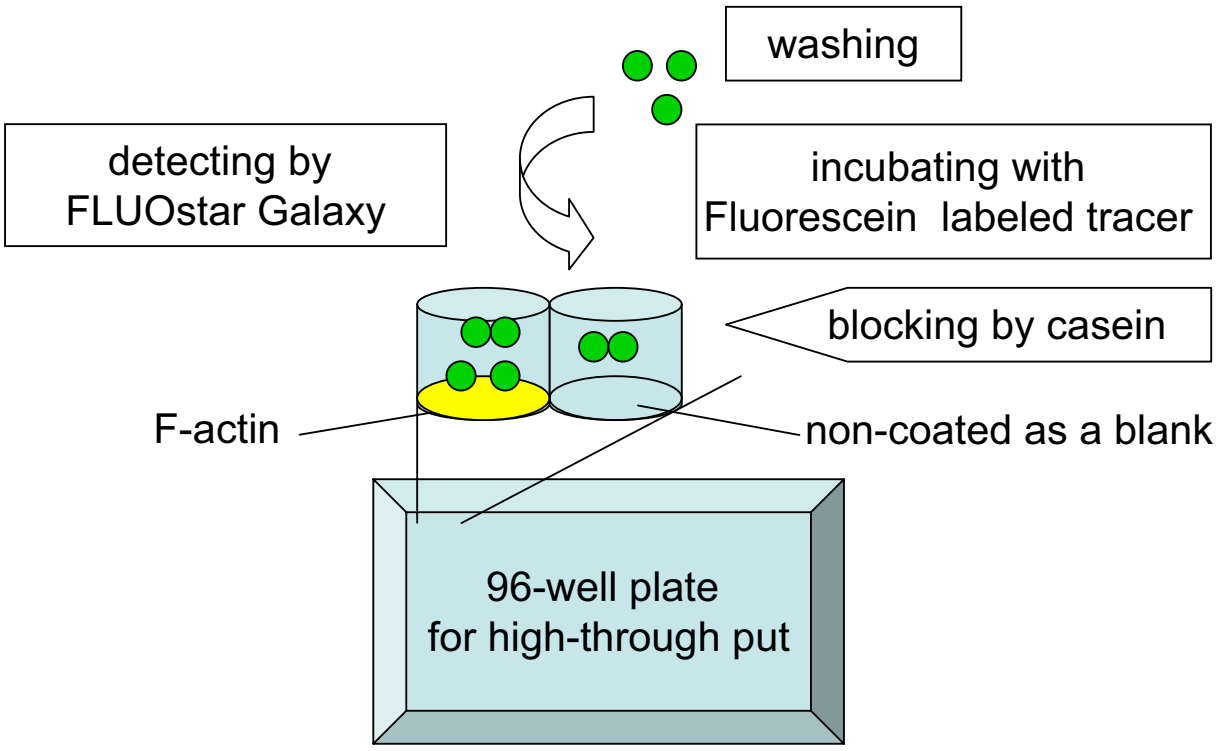


Figure 3.4 The strategy of the solid phase F-actin binding assay. Using a 96-well plate, F-actin was coated on the wells in 1X TBS solution and blocked by 1% saturated casein solution. Non-coated wells also were used as a blank. Fluorescein-labeled proteins were incubated with F-actin on the wells. After an appropriate incubation time, the wells were washed by a Tween 20 containing buffer and fluorescence intensity was detected using a fluorescence reader (FLUOstar Galaxy).

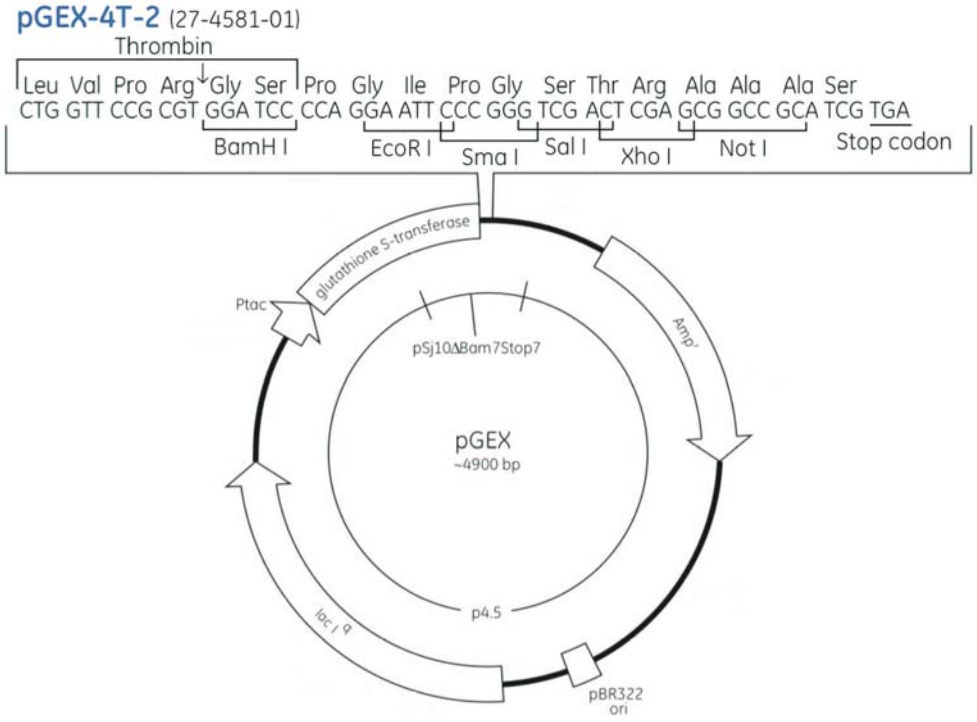


Figure 3.5 Cloning of GST-α-actinin-4 fusion protein expression plasmid. The map of pGEX4T2 vector that expresses the GST-fusion proteins under the IPTG inducible promoter (Ptac) and contains a thrombin cleavage site between the GST tag and the protein of interest. The restriction sites used for the cloning of full length α-actinin-4 are *Eco*RI and *Xho*I.

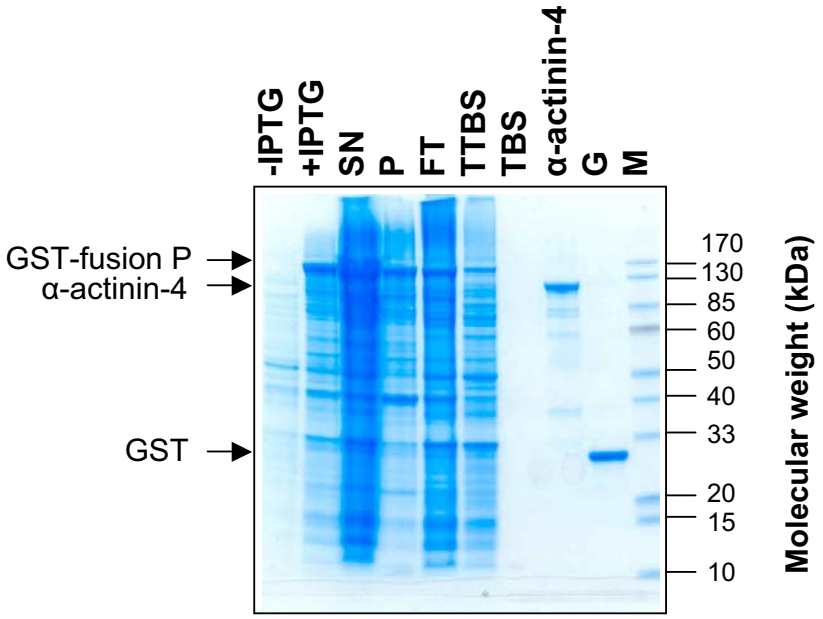


Figure 3.6 Expression and purification of α-actinin-4. The GST-α-actinin-4 fusion protein was over-expressed in *E. coli* BL21 cells and purified by a glutathione agarose column. Cells were harvested after 4 hours with 0.2 mM IPTG treatment by centrifugation. Cell pellets were resuspended in the lysis buffer (1X TBS pH 7.0 containing 0.1% Triton X-100, 1 mM EDTA and 1 mM PMSF) and lysed by 0.5 mg/mL lysozyme and one to four 30 sec bursts of sonication. Cell debris was removed by centrifugation at 22,000 x *g* and the supernatant was passed through a glutathione agarose column. After washing with the washing buffer (1X TBS pH 7.0 containing 0.1% Triton X-100), the GST-fusion protein was digested with thrombin on the column in 1X TBS with 2.5 mM $CaCl_2$ for 18 hours at room temperature. The α-actinin-4 was eluted by 1X TBS buffer and detected by SDS-PAGE. GST-tag was washed out by 1X TBS with 10 mM glutathione buffer. SN, supernatant; P, pellet; FT, flow through; G, glutathione.

3.3.2 Establishment of the Solid Phase F-actin Binding Assay

In the preparation of 96-well plates for the solid phase F-actin binding assay, the ideal amount of F-actin coated on the wells needed to be determined. In order to optimise the amount of F-actin for coating, different amounts of F-actin, 0.5 to 4 μg, were coated on the wells and as a control, the same amount of bovine serum albumin (BSA) was also coated. The sample of α-actinin-4 was labeled with fluorescein isothiocyanate (FITC) according to the method described in section 2.2.2.8 with a degree of labeling (DOL) in the range of 2.0 to 2.5. 1 μM fluorescein-labeled α-actinin-4 was added into the F-actin or BSA coated wells on the 96-well plate and incubated at room temperature for 2 hours. After incubation, each well was washed with TBS buffer containing 0.1% Tween 20 to remove the non-specific binding and the fluorescence intensity was measured. As shown in Figure 3.7 A, the fluorescence intensity increased depending on the amount of F-actin coated on the wells from 0.5 to 4 μg. BSA did not show dose-dependent binding but showed a slight non-specific binding effect. In comparison with the BSA coated group, the F-actin coated group showed a significant difference with p value 0.0009 (p value was calculated by student t-test) (Figure 3.7 A). Approximately 70% of α-actinin-4 bound the F-actin at 1 μg in the linear range with maximum signal, therefore this amount of F-actin was chosen for the solid phase F-actin binding assay.

To investigate the binding specificity on the solid phase F-actin binding assay, G-actin (actin monomer) was coated on the wells as a negative control. Two Ca^{2+} dependent non-muscle isoforms of α-actinins-1 and -4, were generated and labeled with FITC with a DOL in the range of 2.0 to 2.5 (data not shown). Dilution series of fluorescein-labeled α-actinin-1 and -4 ranging from 10^{-9} to 10^{-6} M were added into wells coated with either F-actin or G-actin on the 96-well plate and incubated at room temperature for 2 hours. As shown in Figure 3.7 B and C, both α-actinin-1 and 4 bound F-actin in a concentration dependent manner with similar Kd values of 20±2.6 nM and 35±3.2 nM, respectively. There was no binding effect on the G-actin coated wells. In comparison with the G-actin coated control, the F-actin coated samples showed a significant difference for both α-actinin-1, and -4 with p values 0.0095 and 0.0185, respectively. These results indicated that α-actinins bind F-actin rather than G-actin as previously published and the solid phase F-actin binding assay provides a useful method to quantitatively measure F-actin binding.

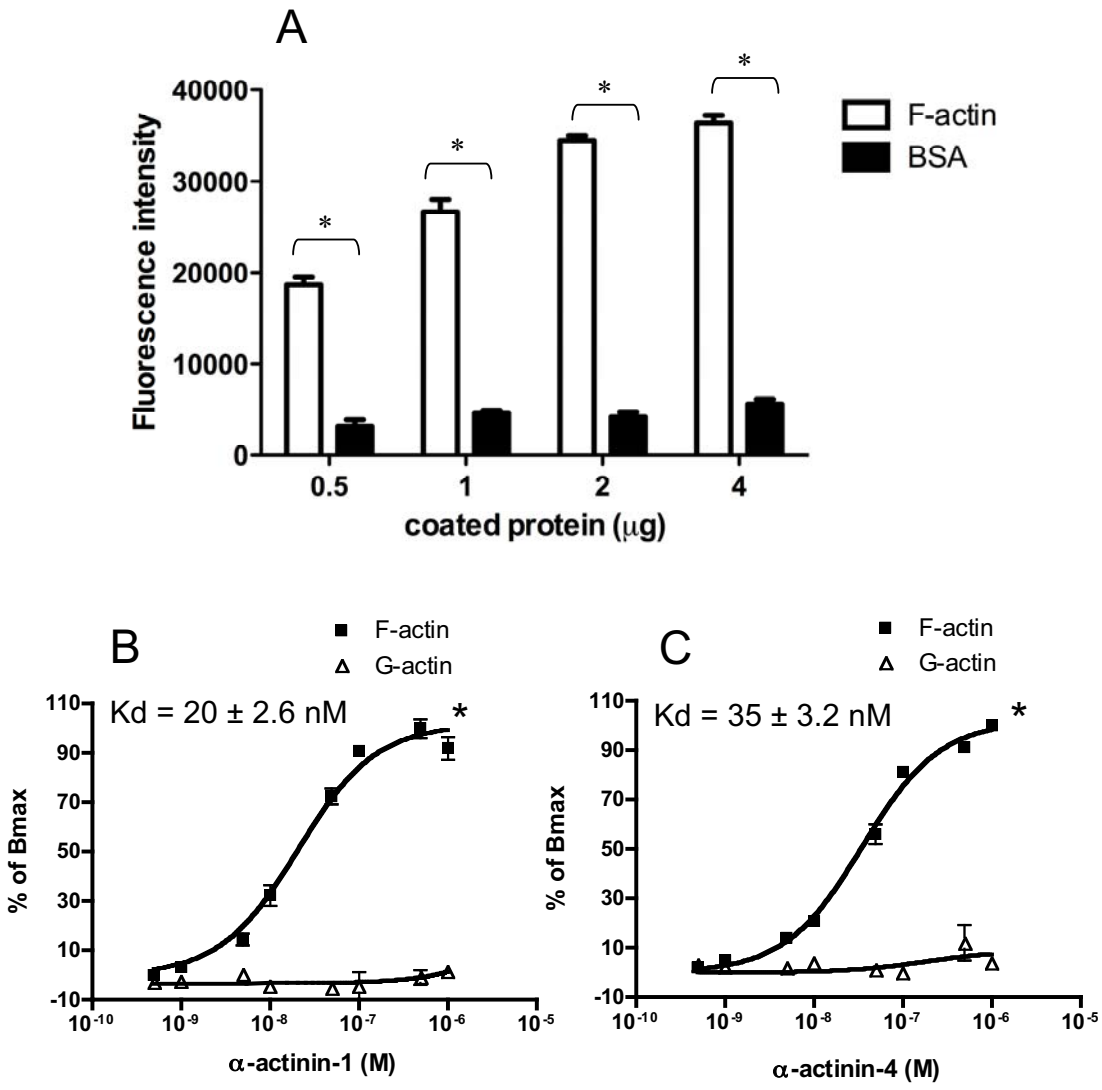


Figure 3.7 (A) The optimisation of the amount of F-actin for the solid phase F-actin binding assay. 1 μM fluorescein-labeled α-actinin-4 was added into the wells coated with different amounts of F-actin and BSA with 0.5, 1, 2 and 4 μg and incubated at room temperature for 2 hours. After incubation, the wells were washed 3 to 5 times with washing buffer, 1X TBS containing 0.1% Tween-20, and fluorescence intensity was measured.

(B and C) Specificity of the solid phase F-actin binding assay. Two non-muscle isoform of α-actinin-1 (B) and -4 (C), were purified and labeled with FITC to verify the specificity of the solid phase F-actin binding assay. Fluorescein-labeled α-actinins from 10^{-9} to 10^{-6} M were added into the wells coated with 1 μg of either F-actin or G-actin and incubated at room temperature for 2 hours, respectively. After incubation, the wells were washed 3 to 5 times with washing buffer, 1X TBS containing 0.1% Tween 20 and fluorescence intensity was measured. The p value was calculated by student t-test. (* indicates $p < 0.05$).

3.3.3 Ca^{2+} Enhances the F-actin Binding Activity of α-actinin-4

α-actinin-4 contains two functional EF-hand motifs for Ca^{2+} binding. The Ca^{2+} effects in the F-actin binding activity of α-actinin-4 were investigated using the solid phase F-actin binding assay. Fluorescein-labeled α-actinin-4 was pre-incubated with the specified concentrations of Ca^{2+} in 0.5 to 20 mM at room temperature for 15 minutes. α-actinin-4 was then added into the F-actin coated 96-well plates at room temperature for 2 hours (detailed description in section 2.2.2.9). As shown in Figure 3.8 A, the F-actin binding activity of α-actinin-4 was enhanced by Ca^{2+} in a concentration dependent manner, saturating at 10 mM (representative shown in Figure 3.8 A). In order to confirm this Ca^{2+} effect, different concentrations of EDTA with 0.1 to 10 mM also were used in the solid phase F-actin binding assay. As shown in Figure 3.8 B, EDTA abolished the 10 mM Ca^{2+} effect in F-actin binding activity of α-actinin-4 (representative shown in Figure 3.8 B). These results indicated that Ca^{2+} positively regulated the F-actin binding activity of α-actinin-4. However, Ca^{2+} could enhance the fluorescence intensity of FITC rather than increase the F-actin binding to α-actinin-4. In order to eliminate this possibility, the F-actin binding curves in the presence and absence of 10 mM Ca^{2+} were generated using the solid phase F-actin binding assay and the Kd values were calculated by non-liner regression analysis. As shown in Figure 3.9, 10 mM Ca^{2+} shifted the F-actin binding curve to the left. These results suggested the F-actin binding activity of α-actinin-4 was enhanced by 10 mM Ca^{2+}. The Kd values were shown to be 37±7.1 nM in the presence of 10 mM Ca^{2+} and 15±4.5 nM in the absence of Ca^{2+} (representative shown in Figure 3.9). Compared with the F-actin binding activity of α-actinin-4 in the absence and presence of 10 mM Ca^{2+}, the F-actin binding activity of α-actinin-4 was 2-fold increased by 10 mM Ca^{2+} with p value 0.01. These results indicated that Ca^{2+} significantly enhanced the F-actin binding activity of α-actinin-4.

3.3.4 Calmodulin-Like Domain Assists the F-actin Binding Activity of α-actinin-4

The putative Ca^{2+} binding sites, two EF-hands, are located at the C-terminus of the α-actinin-4, the calmodulin-like domain. According to the structural observations from Tang *et al.* in 2001, the calmodulin-like domain might directly influence the F-actin binding activity of α-actinin-4 (Tang *et al.*, 2001) (as described in section 3.1). In order to investigate the roles of the calmodulin-like domain on the F-actin binding activity, the CH domains (2X CH) and the calmodulin-like domain deletion mutant of α-actinin-4 (ΔCamLD)

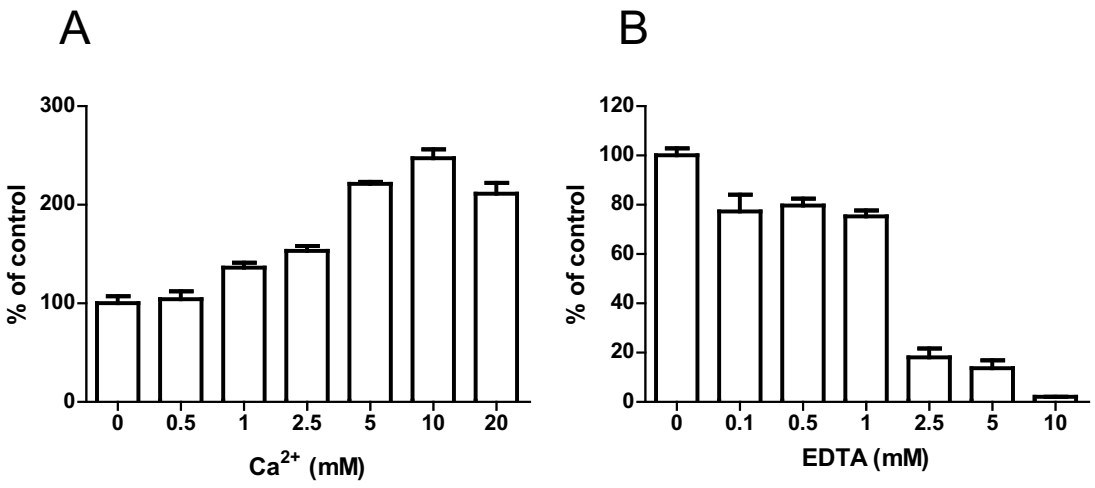


Figure 3.8 (A) The Ca^{2+} effect on the F-actin binding activity. The fluorescein-labeled α-actinin-4 (1 μM) was pre-incubated with Ca^{2+} at concentrations from 0.5 to 20 mM at the room temperature for 15 minutes. α-actinin-4 was then added into wells coated with F-actin and incubated at the room temperature for 2 hours. After incubation, the wells were washed 3 to 5 times with washing buffer, 1X TBS containing 0.1% Tween-20 and fluorescence intensity was measured.

(B) Ca^{2+} effects were abolished by EDTA. The fluorescein-labeled α-actinin-4 (1 μM) was mixed with 10 mM Ca^{2+}, pre-incubated with a dilution series of EDTA with concentrations from 0.1 to 10 mM and incubated at the room temperature for 15 minutes. α-actinin-4 was then added into wells coated with F-actin and incubated at the room temperature for 2 hours. After incubation, the wells washed 3 to 5 times with washing buffer, 1X TBS containing 0.1% Tween-20 and fluorescence intensity was measured.

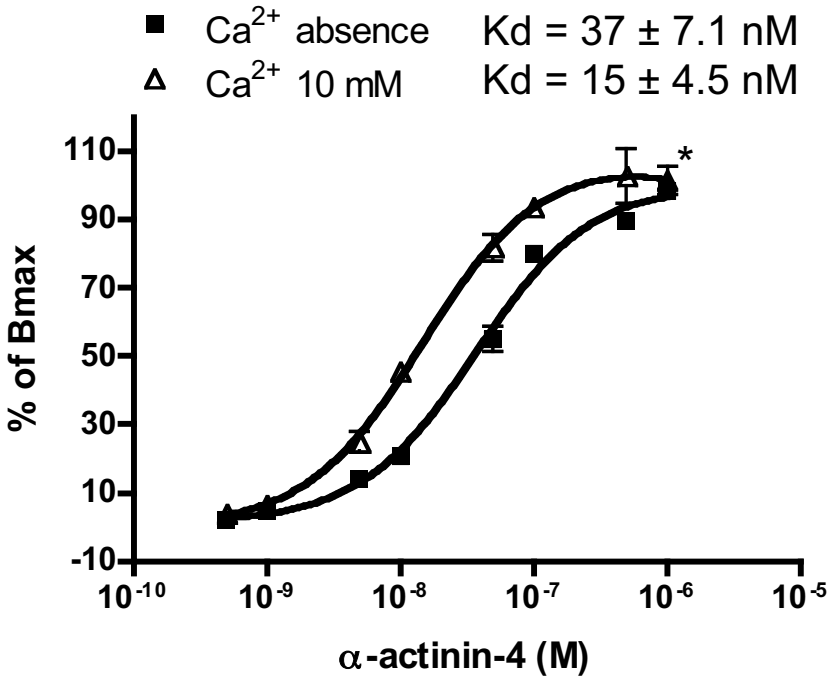


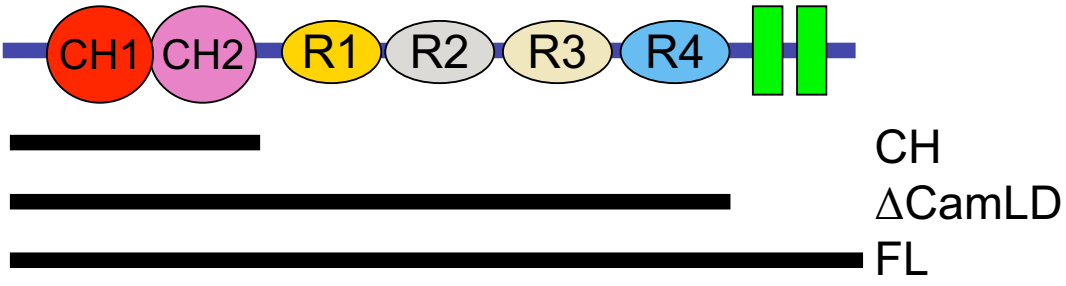
Figure 3.9 Comparison with the F-actin binding curves of α-actinin-4 with or without the presence of Ca^{2+}. Different concentrations of fluorescein-labeled α-actinin-4 was added into the F-actin coated wells in the presence or absence of 10 mM Ca^{2+} and incubated at room temperature for 2 hours. After incubation, the wells washed 3 to 5 times with washing buffer and fluorescence intensity was measured. (* indicates $p < 0.05$).

were generated according to the method described in section 2.2.2.1 (data not shown) and labeled with FITC for the solid phase F-actin binding assay. The regions of the α-actinin-4 protein included in the CH domains (2X CH) and ΔCamLD mutant used in this experiment are shown schematically in Figure 3.10 A. Using the solid phase F-actin binding assay, the F-actin binding activity of the CH domains (2X CH) was shown to be equal to that of the ΔCamLD mutant, with Kd values of 555±77 nM (2X CH) and 545±37 nM (ΔCamLD) in the absence of Ca^{2+} (Figure 3.10 C). The F-actin binding activity of full length α-actinin-4 (FL) was shown to be 10-fold stronger than that of the CH domains and ΔCamLD mutant, with Kd values of 54±5.6 nM in the absence of Ca^{2+} (Figure 3.10 C). These results suggested the calmodulin-like domain assists the F-actin binding activity of α-actinin-4 in the absence of Ca^{2+}. As shown in section 3.3.3, 10 mM Ca^{2+} enhanced the F-actin binding activity of α-actinin-4. The calmodulin-like domain is the presumed Ca^{2+} binding domain of α-actinin-4. Therefore, the F-actin binding activity of the CH domains (2X CH) and ΔCamLD mutant would not be anticipated to be influenced in the presence of 10 mM Ca^{2+}. Using the solid phase F-actin binding assay, the F-actin binding activity of the CH domains (2X CH) was not significantly different to that of the ΔCamLD mutant in the presence of 10 mM Ca^{2+}, with Kd values of 480±27 nM (2X CH) and 590±51 nM (ΔCamLD) (Figure 3.10 C). The F-actin binding activity of the CH domains (2X CH) or ΔCamLD mutant was also not significantly different in the absence and presence of 10 mM Ca^{2+} (Figure 3.10 C). These results suggested that the calmodulin-like domain might directly or indirectly assist the F-actin binding activity of α-actinin-4 and that the F-actin binding activity of full length α-actinin-4 was 20-fold stronger than that of the CH domains (2X CH) and ΔCamLD mutant in the presence of 10 mM Ca^{2+} with $p < 0.05$ (Figure 3.10 C). Hence, these results indicated that the calmodulin-like domain with Ca^{2+} bound assists the F-actin binding activity of α-actinin-4.

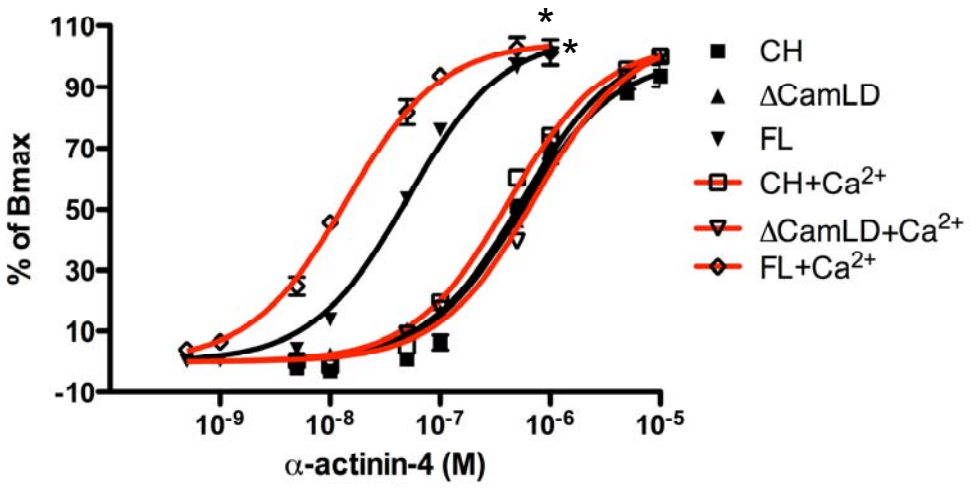
As described above, the calmodulin-like domain assists the F-actin binding activity of α-actinin-4 and Ca^{2+} enhances the assistance of the calmodulin-like domain to the F-actin binding. Because α-actinin-4 is organised as an antiparallel homodimer, the regulatory calmodulin-like domain would be from the opposite molecule of α-actinin-4. According to a report from Nakamura *et al.* in 2005, the F-actin binding activity of filamin A (a member of the spectrin superfamily) was influenced by a direct interaction between the CH domains

Figure 3.10 The calmodulin-like domain assists the F-actin binding activity of α-actinin-4. (A) Diagrammatic representation of full length (FL), the CH domains (CH) and the calmodulin-like domain deletion mutant (ΔCamLD) of α-actinin-4. (B) Comparison of the F-actin binding activities of FL, CH and ΔCamLD of α-actinin-4 in the presence of 10 mM Ca^{2+} (red curves) or the absence of Ca^{2+} (black curves). Different fluorescein-labeled recombinant α-actinin-4 truncated proteins (CH, and ΔCamLD) and FL were added into the F-actin coated wells in the absence or presence of 10 mM Ca^{2+} and incubated at room temperature for 2 hours. After incubation, the wells washed 3 to 5 times with washing buffer, 1X TBS containing 0.1% Tween-20 and fluorescence intensity was measured. The r^2 values of these F-actin binding curves were all greater than 0.98. The Kd values were shown on (C). (* indicates $p < 0.05$).

A



B



C

α-actinin-4	Ca^{2+} (10 mM)	Kd (nM)	
CH	-	555 ± 77	*
	+	480 ± 27	
ΔCamLD	-	545 ± 37	
	+	590 ± 51	*
FL	-	54 ± 5.6	
	+	23 ± 4.1	*

(2X CH) and holocalmodulin (the calmodulin bound Ca^{2+}) and the calmodulin binding region was identified on the CH1 domain using a GST pull down assay (details were referred to section 3.1 and Figure 3.3). Therefore, it was predicted that the regulation of the calmodulin-like domain on the F-actin binding of α-actinin-4 resulted from the direct interaction between the CH domains (2X CH) and the calmodulin-like domain. In order to confirm the roles of the calmodulin-like domain on the F-actin binding, the calmodulin-like domain and the CH domains (2X CH) were purified separately and incubated with F-actin using the solid phase F-actin binding assay. The F-actin binding activity of the CH domains would be increased if the calmodulin-like domain interacted with the CH domains directly. The fluorescein-labeled CH domains were pre-incubated with various concentrations of the calmodulin-like domain and then added into the F-actin coated wells on the 96-well plates (for details see section 2.2.2.9). As shown in Figure 3.11 B, increasing concentrations of the calmodulin-like domain did not enhance the F-actin binding activity of the CH domains in the absence or presence of 10 mM Ca^{2+} (Figure 3.11 B). The reasons could be that fluorescein labeling on the CH domains prevents the interaction between the CH domains and the calmodulin-like domain or the CH domains forms a strong dimer (details are described in section 3.5) to prevent the interaction between the CH domains and the calmodulin-like domain. In order to solve the dimerisation of the CH domains (2X CH) problem, the calmodulin-like domain deletion mutant of α-actinin-4 (ΔCamLD) was generated to verify the calmodulin-like domain regulation on the F-actin binding. The ΔCamLD mutant forms an antiparallel homodimer by the interaction of four spectrin-like repeat domains between two molecules of ΔCamLD mutant to provide two CH domains at both ends. As shown in Figure 3.11 C, an increasing concentration of the calmodulin-like domain enhanced the F-actin binding activity of ΔCamLD mutant in the presence of 10 mM Ca^{2+} with the molar ratio of the calmodulin-like domain to ΔCamLD 5:1, 20:1 and 40:1 ($p < 0.05$) (Figure 3.11 C). However, increasing the concentration of the calmodulin-like domain did not influence the F-actin binding activity of ΔCamLD mutant in the absence of Ca^{2+}. The binding affinity between the CH domains (2X CH) and the calmodulin-like domain might be weak and need to be assist by Ca^{2+}. Therefore, these results suggested that the holocalmodulin-like domain (calmodulin-like domain with bound Ca^{2+}) assists the F-actin binding activity of α-actinin-4 *in vitro*.

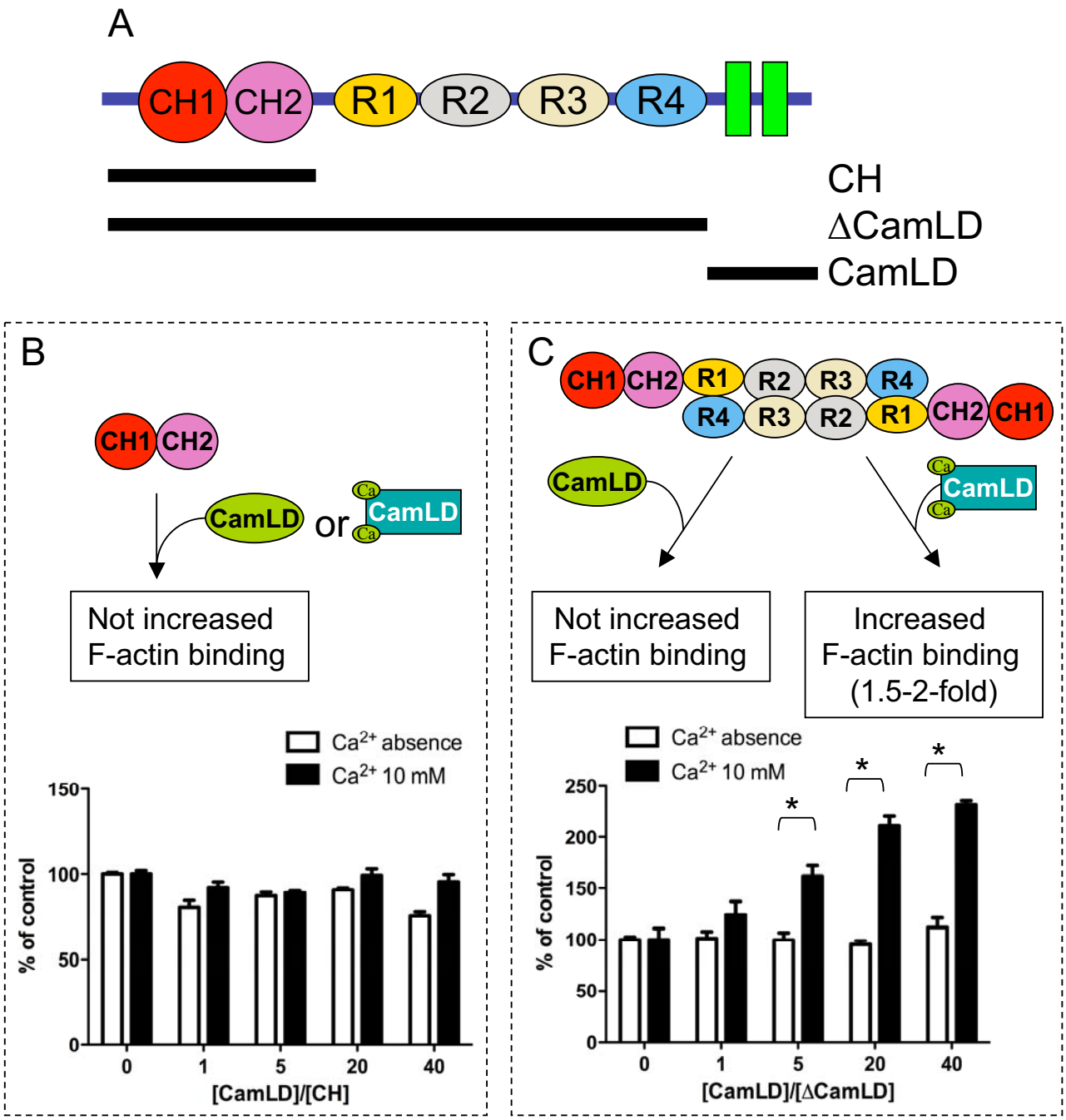


Figure 3.11 The holocalmodulin-like domain enhances the F-actin binding activity of α-actinin-4. (A) Diagrammatic representation of the CH domains (CH), the calmodulin-like domain (CamLD) and the calmodulin-like domain deletion mutant (ΔCamLD) of α-actinin-4. The fluorescein-labeled recombinant CH (B) and ΔCamLD (C) were pre-incubated with the different concentration of CamLD in the presence or absence of 10 mM Ca^{2+} at room temperature for 2 hours and incubated at room temperature for 2 hours. After incubation, the wells washed 3 to 5 times with washing buffer, 1X TBS containing 0.1% Tween-20 and fluorescence. (* indicates $p < 0.05$). Schematic representation of possible CamLD influences to F-actin binding is shown in upper panel of (B) and (C), respectively.

According to the report from Nakamura *et al.* in 2005, a region located on the CH1 domain could be the calmodulin-like domain binding site. Expression of the CH1 domain of α-actinin-4 was attempted using the bacterial recombinant GST-fusion over-expression system for GST pull down assay to verify the interaction between the CH1 domain and the calmodulin-like domain. However, the CH1 domain was insoluble (Figure 3.12). Various induction conditions for protein expression of the CH1 domain were tried, including low concentration of the inducer (IPTG), low induction temperature, less induction time or extension of the CH1 residues at the N terminus (data not shown). These changes did not improve the solubility of the CH1 domain. To sum up, there is no direct evidence for the interaction between the CH1 domain and the calmodulin-like domain.

3.3.5 Dimerisation of the CH Domains

As described in the section 3.3.4, the calmodulin-like domain did not enhance the F-actin binding activity of the CH domains in the absence or presence of Ca^{2+}. One possible explanation might be dimerisation of the CH domains. According to the reports from Keep *et al.* in 1999 and Norwood *et al.* in 2000, the CH domains derived from other members of the spectrin superfamily, utrophin and dystrophin for example, formed antiparallel homodimers in solution (Keep *et al.*, 1999a; Norwood *et al.*, 2000). In order to verify the dimerisation of the CH domains, the recombinant CH domains were separated by size exclusion column with or without DTT and the proteins were identified in each fraction by SDS-PAGE (Figure 3.13 A and B). There are two major peaks shown on these two diagrams with the retention times of 12 and 14 min. In the absence of DTT, the ratio of peak 1 to peak 2 is 3:2. However, the ratio of peak 1 to peak 2 changes to be 1:4 in the presence of 5 mM DTT. Since SDS-PAGE analysis shows that all the fractions contain a single protein that migrates at the same size of 32 kDa, the expected size of the CH domains, the two peaks are likely to represent dimers and monomers, respectively. In order to confirm the dimerisation of the CH domains, different concentrations of the CH domains were separated by SDS-PAGE and stained with Coomassie blue (Figure 3.13 C). The results showed that the CH domains form a dimer at high concentration. These results suggested CH domains form a strong dimer in solution that is resistant to strong reducing agents, such as 5 mM β-mercaptoethanol.

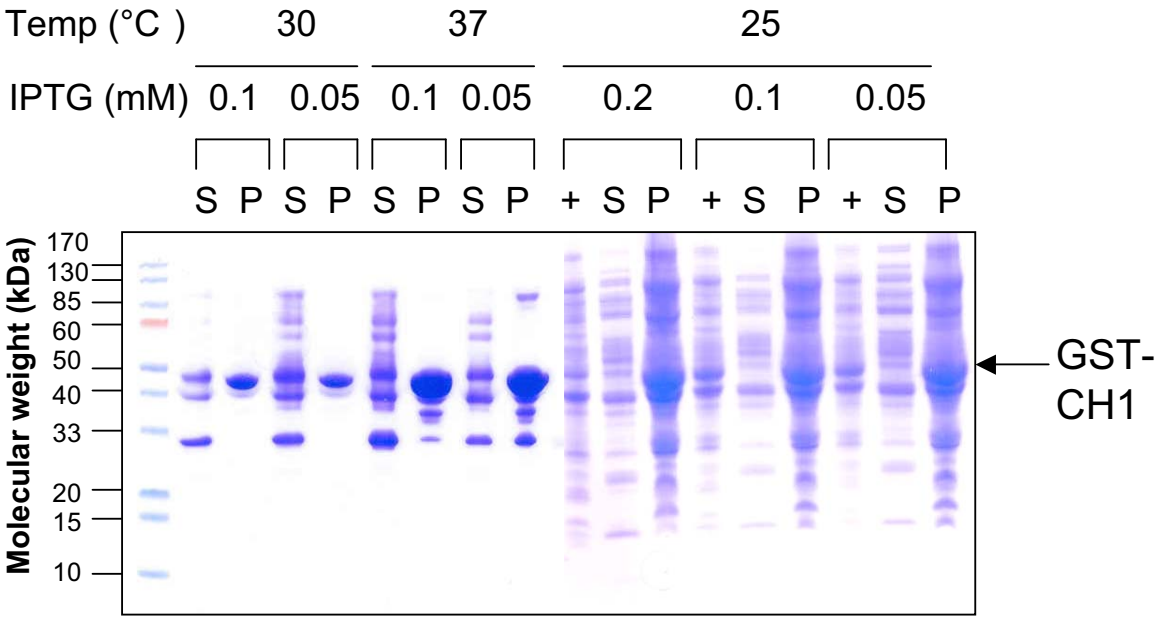
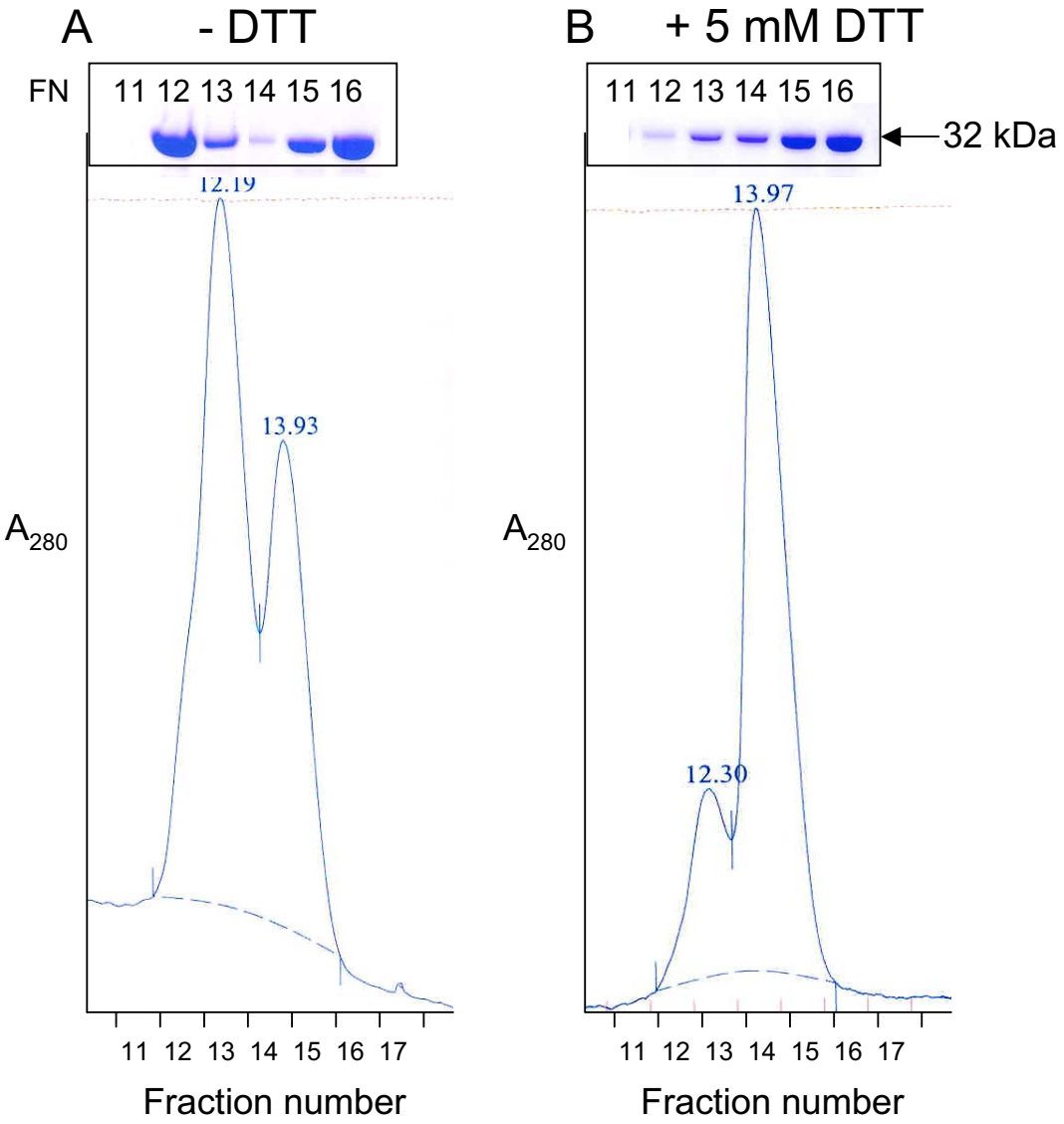


Figure 3.12 The solubility of the α-actinin-4 CH1 domain. The GST-CH1 fusion protein (residue 1 to 159) was expressed in *E. coli* BL21 strain under the different conditions with the concentration of IPTG and expression temperature. Cells were harvested after 4 hours by centrifugation and cell pellets were then resuspended in a Triton X-100 containing lysis buffer (1X TBS pH 7.0 containing 1 mM EDTA and 1 mM PMSF) and lysed by 0.5 mg/mL lysozyme and one to four 30 sec bursts of sonication. Cell lysates were separated into the supernatant (S) and the pellet (P) and detected by the SDS-PAGE.

Figure 3.13 Dimerisation of the α-actinin-4 CH domains. Size exclusion chromatography profiles of CH domains in the absence (A) and the presence of 5 mM DTT (B). A Superdex 75 column was equilibrated in 1 X TBS pH 7.0 with a flow rate 1 ml/min. 1 mg of the CH domains protein was loaded onto the Superdex 75 column and collected fractions with 1 ml/min per each. The protein elution was detected at A280 with a full scale absorbance of 1.0. The proteins in the fraction 11 to 16 were detected by SDS-PAGE (upper panel). (C) Purified recombinant CH domains at concentration from 4 to 0.2 mg/mL were separated by SDS-PAGE and stained with Coomassie blue.


A
- DTT
FN
11 12 13 14 15 16
12.19
13.93
A280
11 12 13 14 15 16 17
Fraction number
B
+ 5 mM DTT
11 12 13 14 15 16
32 kDa
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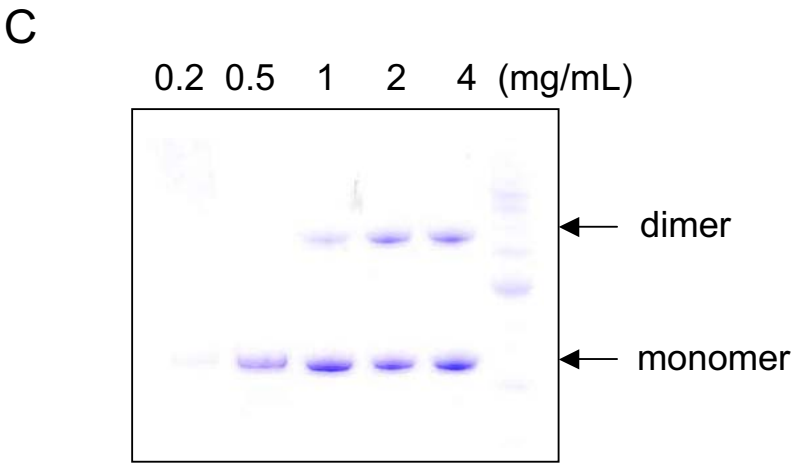
12.30
A280
11 12 13 14 15 16 17
Fraction number


C
0.2 0.5 1 2 4 (mg/mL)
dimer
monomer

3.4 Discussion

3.4.1 The Advantages and Disadvantages of the Solid Phase F-actin Binding Assay

The traditional F-actin binding assays, low speed sedimentation assay and co-sedimentation assay, are good methods for measuring the F-actin bundling and binding activity in solution. In the low speed sedimentation assay, bundled F-actin is pelleted down by centrifugation at 10,000 x *g*, separated by SDS-PAGE, and quantified by a densitometer. The significance of the low speed sedimentation assay is to measure the F-actin bundling activity of actin binding proteins rather than that of the binding itself. In the co-sedimentation assay, actin binding proteins are pelleted down with the F-actin by centrifugation at 100,000 x *g*, separated by SDS-PAGE, and quantified by a densitometer. In order to make the actin binding assay faster and more economical, a novel assay, the solid phase F-actin binding assay was established. In the solid phase F-actin binding assay, F-actin was coated on the wells in the 96-well plates to provide actin binding sites for actin binding proteins. Labeling the actin binding protein is necessary and a fluorescent dye, such as fluorescein is ideal to provide a high sensitively measurement. Therefore, the solid phase F-actin binding assay should be a convenient and quantitative method for measuring the F-actin binding activity of actin binding proteins. However, F-actin bundling by actin binding proteins could not be measured in the solid phase binding assay because F-actin was coated on the wells rather than suspended in solution. Therefore, actin binding proteins may interact with the same strand of F-actin in the solid phase environment rather than with two different strand of F-actin in solution. The F-actin binding activity measured by the solid phase F-actin binding assay of an actin binding protein might be higher than the F-actin bundling activity measured by the sedimentation assay of an actin binding protein. The Kd values of the F-actin bundling affinity of α-actinin measured by the sedimentation assay are around 10^{-7} to 10^{-6} M (Lebart *et al.*, 1995; Fraley *et al.*, 2003; Gimona *et al.*, 2002). The Kd values of the F-actin binding activity of α-actinin-4 measured by the solid phase binding assay, however, are around 10^{-8} to 10^{-7} M. The solid phase F-actin binding assay is to measure molecules interacting with F-actin, but it does not mean that they could make F-actin bundle.

3.4.2 Ca^{2+} enhances the F-actin binding activity of α-actinin-4 *in vitro*

Calmodulin is an important regulator in some signal transduction cascades, including fertilisation, contraction, differentiation, proliferation and apoptosis and one mechanism of this regulation is based on protein-protein interactions with calmodulin. Ca^{2+} is the key switch to control the protein-protein interaction by a Ca^{2+} induced conformational change in calmodulin. Calmodulin contains four functional EF hands and the Kd of Ca^{2+} binding is approximately 10^{-5} M (Gifford *et al.*, 2007). It is consistent with the range of EC_{50} 10^{-6} to 10^{-4} M for α-actinin in *D. discoideum* and filamin A. However, the effective concentration of Ca^{2+} on α-actinin-4 which contains two functional EF hands is higher with the range of 10^{-3} to 10^{-2} M. Ca^{2+} is a critical intracellular signaling ion which is associated with a number of cellular functions. In resting cells, the concentration of cytosolic Ca^{2+} is approximately 10^{-7} M. In the activated cell, the concentration of Ca^{2+} is increased to approximately 10^{-5} M by either the influx of extracellular Ca^{2+} or the release from endoplasmic reticulum (ER) (Berridge *et al.*, 2000; 2003). ER is a major internal reservoir from which Ca^{2+} is released to the cytosol during Ca^{2+} signaling. In the normal physiological condition, the concentration of Ca^{2+} would be unlikely to reach 10^{-3} M. Therefore, it is assumed that Ca^{2+} enhances the F-actin binding activity of α-actinin-4 *in vitro* but that this might not be biologically significant *in vivo*.

As described in section 3.1, α-actinin-4 contains four EF hands in the calmodulin-like domain, but EF hand 3 and 4 are disrupted by an amino acid residue deletion in the EF hand 3 and an amino acid residue insertion in the EF hand 4 (Figure 3.2). On the sequence alignment with calmodulin, α-actinin-4 EF hand 1 has a good match to the consensus sequence, but a Pro residue substitution at position 10. This substitution might influence the Ca^{2+} binding. On the EF hand 1 of α-actinin *D. discoideum*, a critical residue Gly at the position 6 which is important for the loop structure is replaced by Gln. This substitution could influence the Ca^{2+} binding. That could be a reason the EF hand 1 of α-actinin *D. discoideum* does not show Ca^{2+} binding activity in the Ca^{2+} overlay assay (Witke *et al.*, 1993). On the EF hand 2 of α-actinin-4, a critical residue Glu at the position 12 which is important in stabilising the helix-loop-helix structure and function is replaced by Ala. It was assumed that the substitution of this residue might influence the Ca^{2+} binding. These observations implied that EF hand 1 and 2 might not bind Ca^{2+}. Therefore, investigation of the Ca^{2+} binding on EF hand 1 and 2 will be necessary to address this issue further.

3.4.3 Calmodulin-like Domain Assists the F-actin Binding Activity of α-actinin-4

As described in section 3.3.5, the F-actin binding activity of the calmodulin-like domain deletion (ΔCamLD) mutant was equal to that of the CH domains but 10-fold weaker than that of full length α-actinin-4 in the absence of Ca^{2+}. These results suggested that the calmodulin-like domain facilitates the F-actin binding ability of α-actinin-4. It is presumed that the calmodulin-like domain assists the F-actin binding activity by a direct interaction with the CH domains. In order to identify the calmodulin-like domain binding site on the CH domains, I attempted a purification of CH1 domain for a GST pull down assay, but the CH1 domain is insoluble (Figure 3.12). Hence, there is no direct evidence for the interaction between the CH1 domain and the calmodulin-like domain in this thesis.

According to previous reports, the calmodulin-like domain binding site could possibly be located on either a region on the CH1 domain (Nakamura *et al.*, 2005), a linker region between the CH1 and the CH2 domains (Tang *et al.*, 2001) or a region between the CH2 domain and the spectrin-like repeat 1 (Young and Gautel, 2000). Based on the crystal structure model of chicken skeletal muscle α-actinin, the calmodulin-like domain is close to the CH1 domain (Figure 3.14). According to these observations and biochemical evidence described in this chapter, I propose that the calmodulin-like domain assists the F-actin binding activity of α-actinin by a weak interaction with a region on the CH1 domain in the presence of Ca^{2+}.

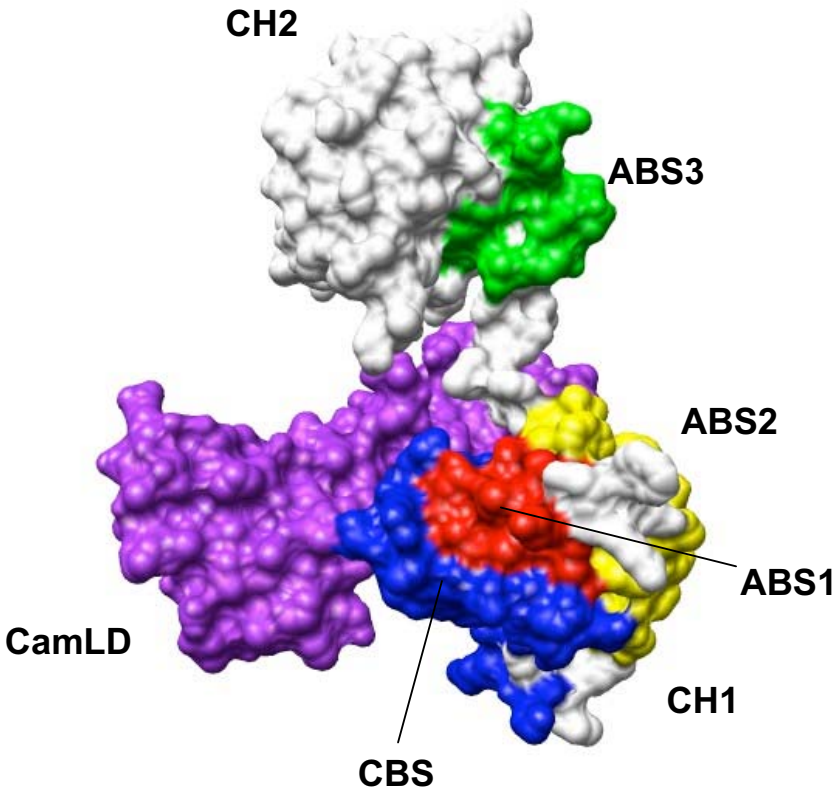


Figure 3.14 The putative calmodulin-like domain binding site on the CH1 domain. In the crystal structure of chicken skeletal muscle α-actinin (pdb.1SJJ), the calmodulin-like domain (CamLD, purple) is close to the putative calmodulin-like domain binding site (CBS, blue) on the CH1 domain from the neighboring molecule. Actin binding site (ABS)-1, -2 and -3 indicated in red, yellow and green, respectively.

CHAPTER 4

Structure Determination of α-actinin-4 CH2 Domain

4.1 The CH2 Domain

The CH domain of α-actinin contains two calponin homology domains (CH1 and 2) involved in F-actin binding. Similar to the CH1 domain, the CH2 domain consists of six α-helices (Figure 4.1). The three-dimensional structure of human β-spectrin CH2 domain has been determined in 1997 by Djinovic Carugo *et al.* and is similar to that of other spectrin superfamily members, such as α-actinin, plectin, utrophon, dystrophin, filamin and fimbrin (Djinovic Carugo *et al.*, 1997). The structure of the β-spectrin CH2 domain contains six α-helices (referred to as αA to αF), connected by two short and four long loops. In addition, two 3_{10} helices (referred to as αXa and αXb) are located in a long loop between αC and αD (Figure 4.1). Three F-actin binding sites have been identified (Winder, 2003), and one of them, ABS-3, is located on helix αA of the CH2 domain. Helix αA resides at the surface of the CH2 domain and interacts tightly with αF by several van der Waals interactions and a salt bridge between residues Lys 166 and Glu 254 which are highly conserved residues in the sepctrin superfamily. The ABS-3 region is highly solvent accessible, allowing the possibility of interacting with F-actin. Beside the ABS-3, a PIP2 binding site has been identified (Fukami *et al.*, 1996). This putative PIP2 binding site is mapped to the loop connecting the first and the second α-helix of the CH2 domain and associated with the regulation of the F-actin binding activity of α-actinin (Fukami *et al.*, 1992; Fraley *et al.*, 2003) (details described in chapter 5).

4.2 Structural Determination by NMR Spectroscopy

4.2.1 The NMR Spectroscopy

The three-dimensional structure of a protein can help us to understand its biological function. There are two main techniques to obtain atomic resolution structure of protein. X-ray crystallography and solution NMR spectroscopy are used to develop structure models of macromolecules for biological and pharmaceutical research. X-ray crystallography can provide structural information for biological macromolecules at atomic resolution but requires the formation of protein crystals. NMR can be used to determine three-dimensional structures of biological macromolecules in solution under near physiological conditions with molecular weights up to 30-40 kDa (Dotsch and Wagner, 1998). NMR spectroscopy

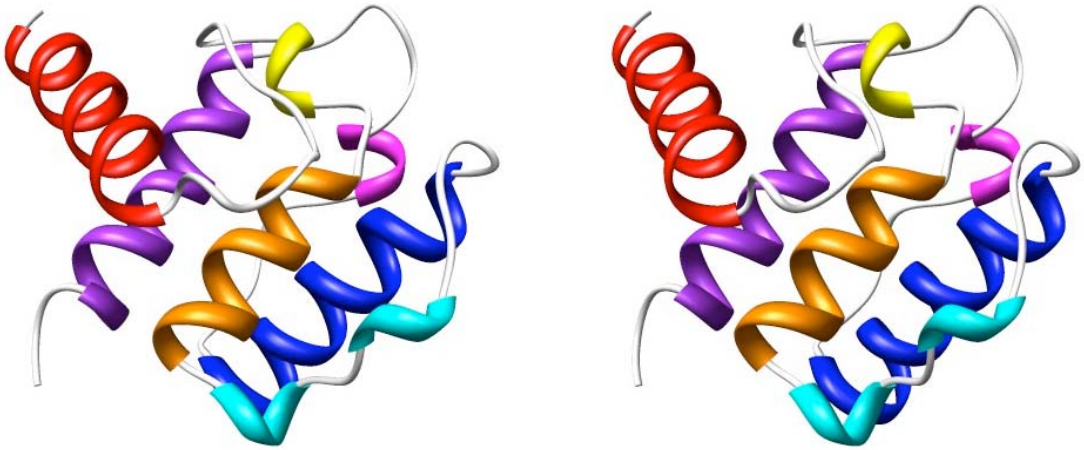


Figure 4.1 The ribbon presentation of human spectrin CH2 domain in stereo. The three-dimensional structure of the CH2 domain contains six helices and three of them, αC, αD and αF (indicated in orange, blue and purple, respectively), form a parallel helix bundle in the central hydrophobic core. αA (indicated in red) packs against αC and αF in a perpendicular orientation. Two 3_{10} helices, αXa and αXb (indicated in cyan), are located in the loop between αC and αD. The diagram is generated by Chimera (Pettersen, 2004).

not only provides structural information but also information about dynamics of biological macromolecules in solution. By the combination of structure and dynamics information, we can have a better understanding of dynamic events of biological molecules, such as folding transitions and intra- and inter-molecular interactions in proteins. Therefore, NMR and X-ray crystallography exists in a symbiotic relationship which allows both fields to gain from the other.

The NMR phenomenon is based on a quantum mechanical property of the nucleus: the spin. NMR spectroscopy employs the magnetic spin properties of atomic nuclei to identify atoms that are close together in space (Howard, 1998). The nuclei of many elemental atoms, including isotopes have a characteristic spin (I). Only some atoms, such as ^{1}H, ^{13}C, ^{15}N, ^{19}F or ^{31}P, have a half-integer spin, which manifest as NMR peaks under an environment of a high field superconductive magnet. When irradiated with pulses of radio frequency electromagnetic radiation, these NMR-active nuclei will resonate at characteristic frequencies. These different frequencies are obtained as NMR peaks and are generally expressed as chemical shift in parts per million (ppm) relative to a reference signal (Roberts, 1993). In NMR spectra different types of nuclei are represented in different ranges of chemical shift. With the same type of nucleus, the chemical shift of a nucleus is dependent on its local molecular environment. For example, in unstructured polypeptide chains, protons within different functional groups resonate at different characteristic frequencies: methyl groups (CH_3) at ~1 ppm; methylene groups (CH_2) at ~2-3 ppm; α-protons at 4-5 ppm; aromatic groups at 6-7.5 ppm; and amide protons at 7-11 ppm (Wuthrich, 1986). These resonances are referred to as random coil chemical shift values. However, when the polypeptide chain is folded or interacts with other molecules, the frequency of proton resonance could be shifted upfield (lower ppm value) or downfield, resulting in change of the chemical shift values. These shifts are due to the interaction between the nuclei and surrounding electrons which affect the local magnetic field experienced by a particular nucleus. Therefore, the chemical shift is highly sensitive to a multitude of structural, electronic, magnetic and dynamic variables and in principle allows an assessment of interaction between macromolecules and various ligands.

One-dimensional NMR spectra of biological macromolecules usually contain hundreds or

even thousands of resonance peaks which overlap heavily and cannot be interpreted because of the resolving power of the technique. To overcome this problem, two or multiple dimensional spectra are required. Multidimensional NMR spectra not only simplify NMR spectra for interpretation but also provide information about the correlations between different nuclei. Two-dimensional NMR spectra are broadly divided into the Homonuclear (eg ^{1}H-^{1}H) and Heteronuclear (eg ^{1}H-^{15}N) NMR experiments. Homonuclear experiments include ^{1}H-^{1}H 2D COSY (COrrelation SpectroscopY), ^{1}H-^{1}H 2D TOCSY (TOtal Correlation SpectroscopY) and ^{1}H-^{1}H 2D NOESY (Nuclear Overhauser Effect SpectroscopY). The 2D COSY and 2D TOCSY experiments display through-bond correlations between different protons (Wuthrich, 1986). The cross signals in the 2D COSY and 2D TOCSY spectra indicate two protons are within three chemical bonds and five chemical bonds, respectively. The TOCSY correlates all protons of an amino acid which are referred to as a spin system. The 2D NOESY experiment displays through-space correlations between different protons (Wuthrich, 1986). Through-space correlations are measured via the nuclear Overhauser effect (NOE), a nuclear relaxation phenomenon, the intensity of which is correlated with the inverse sixth power of the distance between the nuclei (Wuthrich *et al.*, 1991; Wuthrich, 1995). The cross peaks in the 2D NOESY spectrum indicate the distance between two protons is less than approximately 5 Å in the folded molecules. The intensity of the NOESY peak correlates to the distance between two protons and is classified into strong (reflecting protons that are less than 3 Å apart in the molecules), medium (less than 4 Å apart) or weak (less than 6 Å) that reflect their closeness in space (Hinds and Norton, 1994). Therefore, this geometric information is very important for the determination of protein structures.

Heteronuclear experiments can use ^{1}H-^{13}C or ^{1}H-^{15}N through-bond correlations. The natural abundance of ^{13}C and ^{15}N is very low and their gyromagnetic ratio is considerably lower than that of protons. To overcome these problems, specific isotopic (^{13}C or ^{15}N) labeled protein samples are used for increasing the low sensitivity of these nuclei. ^{1}H-^{15}N 2D HSQC (Heteronuclear Single Quantum Correlation) spectrum exhibits correlations between amide nitrogens and the attached protons (amide protons). Each cross peak in the HSQC spectrum represents one amide group (NH-) in the backbone of the polypeptide chain (except proline). In addition, ^{1}H-^{15}N HSQC also contains cross signals from the NH_2 groups of the side

chains of Asn, Gln, Arg and Lys and the NH groups of the aromatic ring of Trp and His. But the NH_2 group of the side chain of Arg and Lys do not show up well on HSQC spectra.

Expanding into a third dimension has several combinations including ^{1}H-^{1}H-^{13}C, ^{1}H-^{1}H-^{15}N or ^{1}H-^{13}C-^{15}N. For example, ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H NOESY experiments can be spreading out in a third ^{15}N (or ^{13}C) dimension (Wuthrich, 1990). This spreadout is achieved by combination of the HSQC and TOCSY or NOESY in a single 3D experiment and the resulting experiments are referred to as 3D TOCSY-HSQC or 3D NOESY-HSQC. Triple resonance experiments display ^{1}H-^{13}C-^{15}N through-bond correlations. They are helpful for the sequential assignment, which is an important step of protein structure determination by NMR. Triple resonance spectra separate signals into different orthogonal planes which usually contain only one or two cross peaks to simplify the spectral analysis. However, double labeled protein samples (^{13}C and ^{15}N) are required, which are more expensive, and require recombinant expression.

4.2.2 The Strategy of Structure Determination by NMR

NMR structure determination includes the following steps (Figure 4.2): preparation of the protein solution, recording NMR spectra, assignment of NMR signals to individual atoms in the molecule, identification of conformational restraints, such as distance restraints, torsion angle restraints and orientational restraints, calculation of the three-dimensional structure based on the experimental restraints and validation of the structure and refinement (Spronk *et al.*, 2004).

The first step of protein structure determination is the preparation of the protein solution. The pH, ionic strength and temperature can influence NMR spectra. Therefore, the protein solution should be stable in the chosen conditions for many weeks. Proteins with a molecular weight larger than 10 kDa should be isotopically labeled in either ^{15}N or ^{13}C or both for 2D or 3D heteronuclear and triple resonance experiments.

The critical step of protein structure determination by NMR is resonance assignment. Each resonance in the NMR spectra must be associated with an individual atom in the protein molecule. The amino acid sequence information and NMR spectra are necessary for this

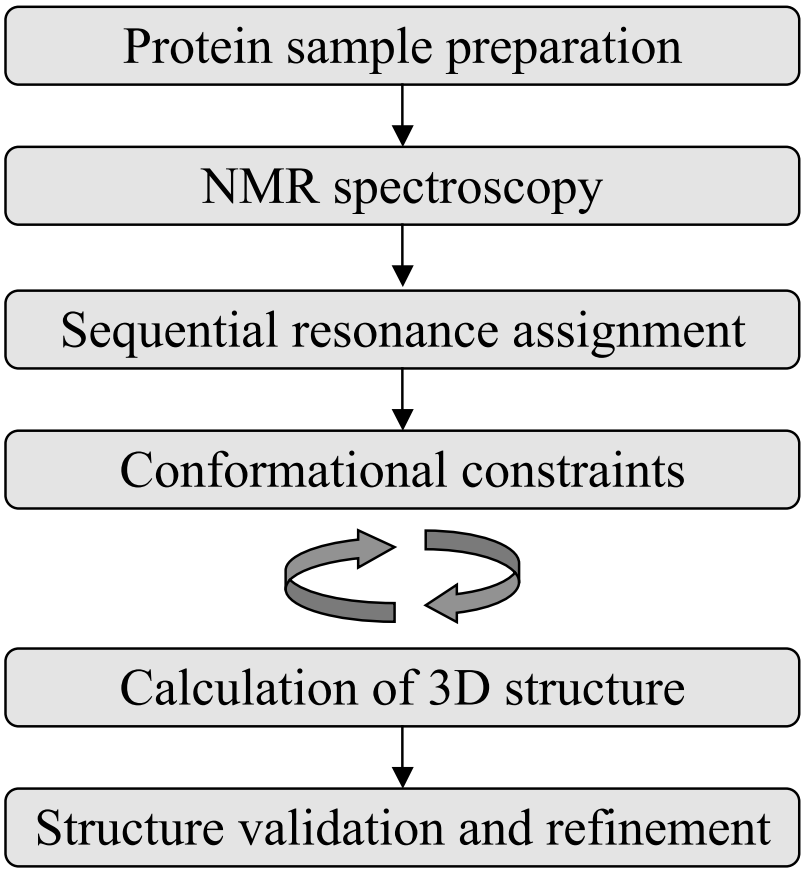


Figure 4.2 Strategy of structure determination by NMR spectroscopy. Outline of the general strategy used in three-dimensional structure determination of biological macromolecules in solution by NMR technique.

task. This process is achieved in two stages (Wuthrich, 1986). The first stage is to identify a spin system which belongs to a particular amino acid residue. This stage is achieved by 2D COSY, 2D TOCSY, 3D TOCSY-HSQC or HCCH-TOCSY, which is a kind of triple resonance spectrum. In this stage, all resonances of protons in the same spin system can be determined. However, the sequence of these spin systems cannot be determined. The second stage is to determine the proper sequential order of these spin systems in the amino acid sequence. This stage is achieved by combination of 2D HSQC and 2D NOESY and 3D NOESY-HSQC as well as some triple resonance spectra, such as a pair of HNCA and NH(CO)CA and a pair of HNCO and HN(CA)CO etc. Because NOESY provides through-space correlations between two protons that are less than approximately 5 Å apart, the amide proton of residue i correlates with the α-proton, β-proton, or γ-proton of residue i-1. By these through-space correlations between the α-proton of residue i-1 and the amide proton of residue i, the order of the amino acid spin systems can be determined. However, the chain of sequential linkages is interrupted by proline residue because of the lack of an amide proton. In addition, these inter-residue cross signals can be applied to identify the secondary structure. In the case of an α-helix, a strong NOE peak is observed between the amide proton of residue i-1 and i. On the other hand, in a β-strand, a strong NOE peak is observed between the α-proton i-1 and the amide proton i.

Triple resonance spectra are helpful for determining the three-dimensional structure of the proteins with a molecular weight larger than 10 kDa. The general strategy is explained using the HNCA and HN(CO)CA spectra as an example (referred to the result section Figure 4.11). HNCA correlates the amide proton i with the α-carbon i and the α-carbon i-1. HN(CO)CA correlates the amide proton i with the α-carbon i-1. At the frequency of each amide proton there are two cross peaks in the α-carbon dimension within the HNCA spectrum and one cross peak in the HN(CO)CA spectrum. The peaks with the interaction between the amide proton i and the α-carbon i-1 will be overlaid if the two spectra shown on the same screen. Using these cross peak chain correlations, the sequential assignment is much easier because of only one or two peaks are shown on the same amide proton frequency.

To build up the protein structure, some geometric conformational information is generally

used for structure calculation, such as distance restraints, dihedral angles restraints, hydrogen bond restraints and residual dipolar couplings. These various restraints can be generated by various NMR experiments. Distance restraints are generated by NOESY experiments. As described above, the intensity of the NOESY peaks represents the distance between two protons and classifies into strong, medium and weak classes. Dihedral angle restraints are obtained from ^{1}H-^{15}N HMQC-J experiment. Hydrogen bond restraints are ascertained from deuterium exchange experiments, which follow the exchange of labile protons with time. Residual dipolar couplings can be measured using Exclusive Correlation SpectroscopY (ECOSY).

4.2.3 Structure Calculation of α-actinin-4 CH2 Domain

Several computer programs are available for the protein structure calculation based on NMR experimental restraints such as CNS (Brunger *et al.*, 1998), X-PLOR-NIH (Schwieters *et al.*, 2003), DYANA (Guntert *et al.*, 1997) and ARIA (Linge *et al.*, 2003). Mainly two approaches are used to build up the 3D structure including interatomic distances and torsion angles of the chemical bonds. These approaches are all based on the experimental restraints, such as NOE distance restraints and dihedral angle restraints etc. The computer programs use a combination of two approaches to define reasonable folds for the proteins. However, the experimental restraints do not illustrate one exact 3D structure because NMR restraints typically describe a range of possible values and many distances cannot be determined (Wider, 2000). Therefore, the structure calculation is repeated several times to determine the ensemble of structures based on the input NMR restraints. A number of statistical values are used to measure quality, for example, restraint violations, root mean square deviation and energies of restraints, etc (Spronk *et al.*, 2004).

The method used for the structure calculation of α-actinin-4 CH2 domain was the ARIA (Ambiguous Restraints of Iterative Assignment) method (Linge *et al.*, 2003). ARIA provides an additional concept which is the application of the ambiguous distance restraints for handling ambiguities in the chemical shift-based NOE cross peak assignments. The ambiguous distance restraint combines alternative assignment possibilities in one restraint. ARIA starts from peak lists and chemical shift lists to generate unambiguous and ambiguous restraint lists and proceeds in cycles of NOE assignment and structure calculation. Several

different restraints also can be added into the calculation processes, such as dihedral angle, J-coupling, residual dipolar coupling, disulfide bridge and hydrogen bond. In each iteration, ARIA calibrates and assigns the NOESY spectra, merges the restraints from different spectra, calculates a ensemble of structures (typically 20 conformers), analyses the violation restraints based on the average distances, and chooses the lowest energy conformers (typically 7 out of 20) for the next iteration. The number of unambiguous restraints is increased and results in reduced ambiguous restraints until a final ensemble of structures is produced.

A new version, ARIA 2.0, provides two features to streamline the structure calculation procedures, a graphical user interface (GUI) and the incorporation of the NMR data model of the Collaborative Computing Project for the NMR community (CCPNMR) (Vranken *et al.*, 2005). The GUI supports the user in setting up and managing a project when performing the structure calculations. The CCPNMR data model is highly efficient for NMR data management and conversion with a variety of other programs. By using the combination of CCPNMR data model and ARIA 2.0 program, the protein structure calculation is becoming more efficient and the time line of structure determination is diminished.

4.3 Aims

Recently, four mutants and a natural splice variant of α-actinin-4 have been reported to be associated with two human diseases, lung cancer and FSGS. These mutations are located on the CH domains (2X CH) of α-actinin-4. However, the actin bundling activities of these mutants are all stronger than that of wildtype α-actinin-4 and the mechanism of this biological effect is not well understood. I attempted to determine the three-dimensional structure of the CH domains (2X CH) using NMR spectroscopy. The CH domains, however, formed a reducing agent resistant dimer in solution and their NMR spectra looked difficult to interpret (Figure 4.3 and 4.4). I also attempted to determine the three-dimensional structure of the CH1 domain using NMR spectroscopy. The CH1 domain, however, was insoluble (referred to Chapter 3 Figure 3.12). The Aim of this chapter was to determine the solution structure of α-actinin-4 CH2 domain using NMR spectroscopy.

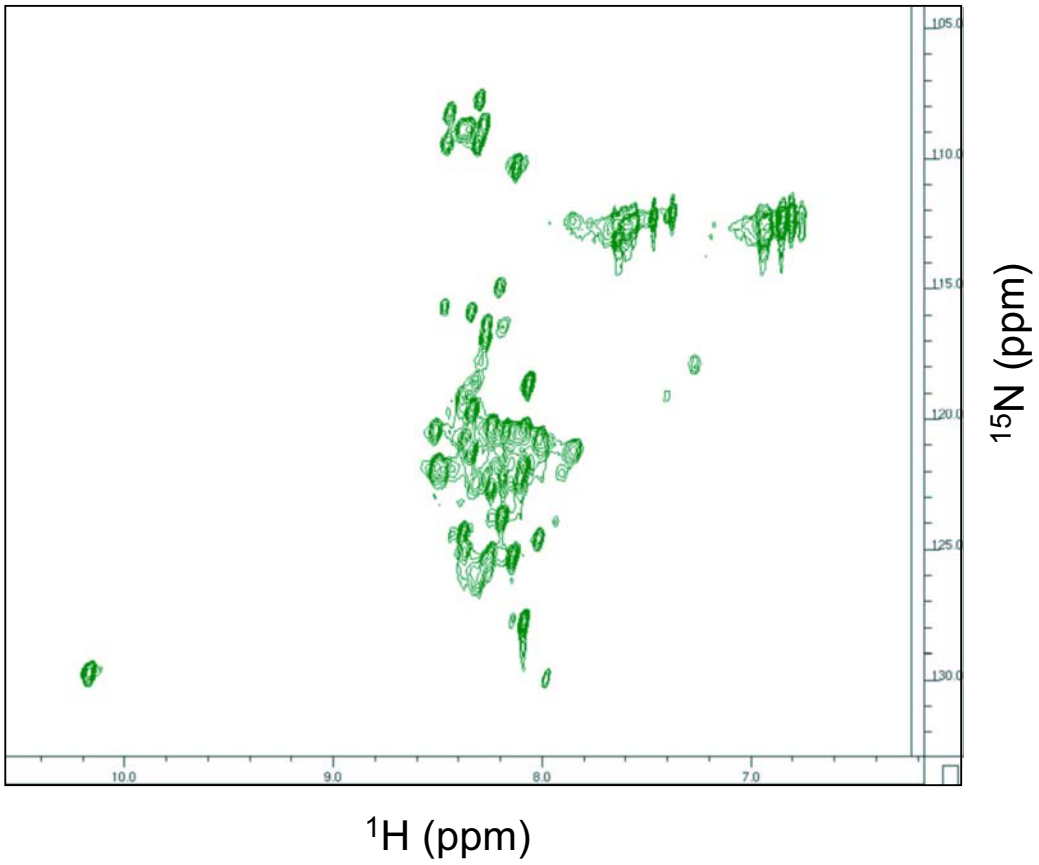


Figure 4.3 2D ^{1}H-^{15}N HSQC spectrum of α-actinin-4 CH domains (2X CH). The HSQC spectrum was recorded using a 3 mM ^{15}N-labeled NMR sample at 25°C. The NMR data were processed by NMRPipe and analysed by CCPNMR software.

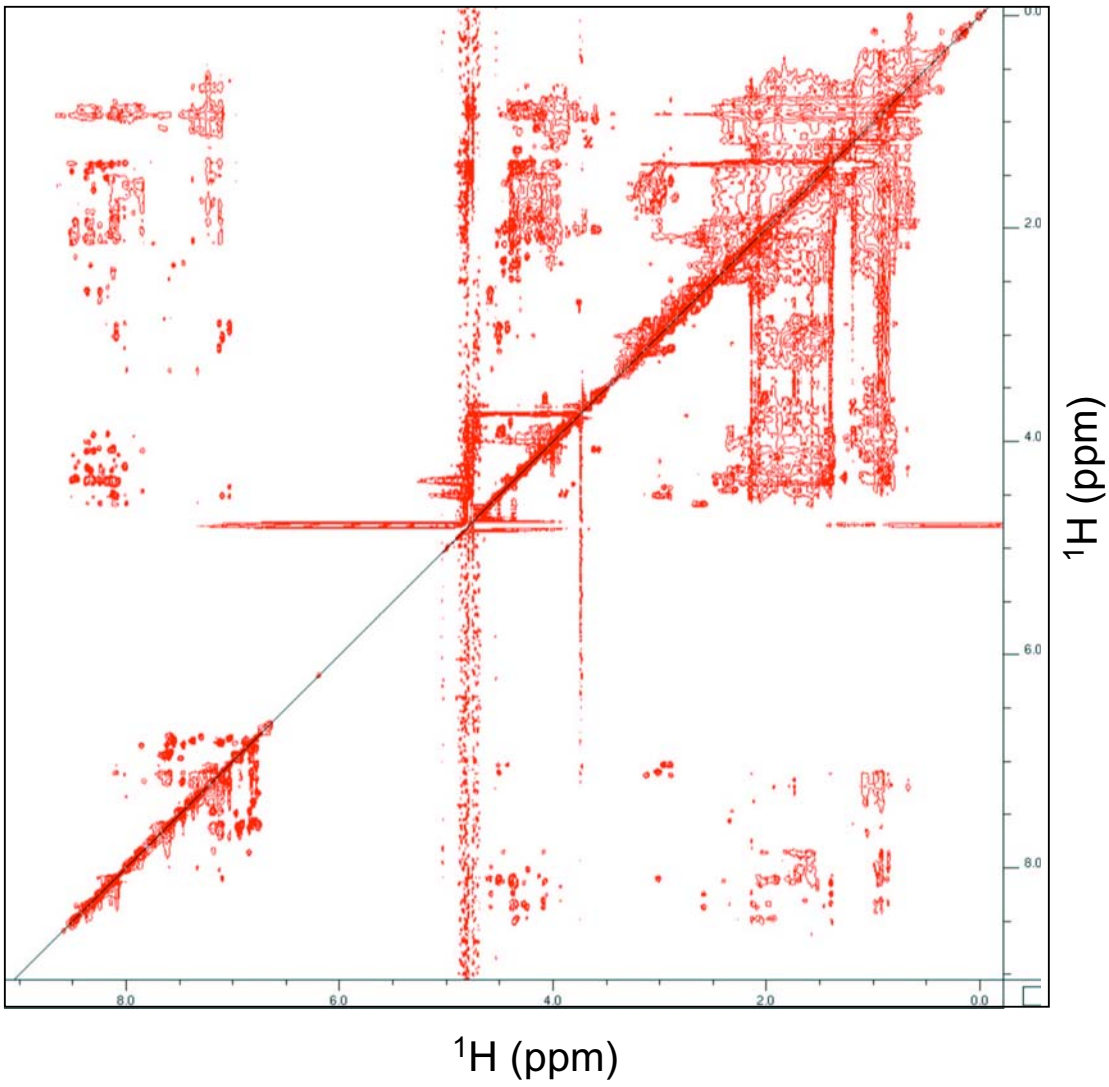


Figure 4.4 2D ^{1}H-^{1}H NOESY spectrum of α-actinin-4 CH domains (2X CH). The 2D NOESY spectrum was recorded using a 3 mM NMR sample at 25°C with a mixing time of 100 ms. The NMR data were processed by NMRPipe and analysed by CCPNMR software. The spectrum was plotted from CCPNMR showing the region from 0 to 10 ppm in both dimensions.

4.4 Results and Discussions

4.4.1 Cloning, Expression and Purification of α-actinin-4 CH2 Domain

The PCR fragment of the α-actinin-4 CH2 domain (residues 162 to 272) was subcloned into the expression vector pGEX4T2 using restriction sites, *Bam*HI and *Eco*RI. The plasmid which encodes the GST-α-actinin-4 CH2 fusion protein was sequenced using appropriate primers to ensure expression of a correct protein (data not shown) and transformed into *E. coli* BL21 bacterial cells for protein expression. Sample of the α-actinin-4 CH2 domain was generated according to the method described in section 2.2.2.1. Samples from each step of the purification process were analysed by SDS-PAGE (representative shown on Figure 4.5). An induced protein band at the expected size for the GST-CH2 (~39 kDa) was identified and a protein band at the expected size for α-actinin-4 CH2 domain (~13 kDa) was identified as well in the eluted fraction. After washing with a glutathione containing buffer, an expected size of protein band for GST tag (~26 kDa) was identified.

For heteronuclear or triple resonance experiments, the ^{15}N labeled or $^{13}C/^{15}N$ double labeled CH2 proteins were generated using bacterial recombinant GST fusion over-expression system. Isotope labeled CH2 proteins were produced by growing the bacteria on minimal medium containing ^{13}C-glucose and $^{15}NH_4Cl$ as sources of carbon and nitrogen.

4.4.2 Sample Preparation for NMR Experiments

NMR sample of the α-actinin-4 CH2 domain was prepared according to the method described in section 2.2.3.1.

4.4.3 Nuclear Magnetic Resonance Data Collection

NMR experiments were performed on a Varian Inova 600 spectrometer using the BioPack package. All data sets were recorded at 25°C using a 5 mm inverse triple resonance $^{1}H/^{13}C/^{15}N$ pfg probe. The carrier frequency was centred on the H_2O signal. All spectra were processed using NMRPipe (Delaglio *et al.*, 1995) and analysed using CCPNMR (Vranken *et al.*, 2005). ^{1}H, ^{13}C and ^{15}N chemical shift were referenced to TSP at 0 ppm (Wishart *et al.*, 1995).

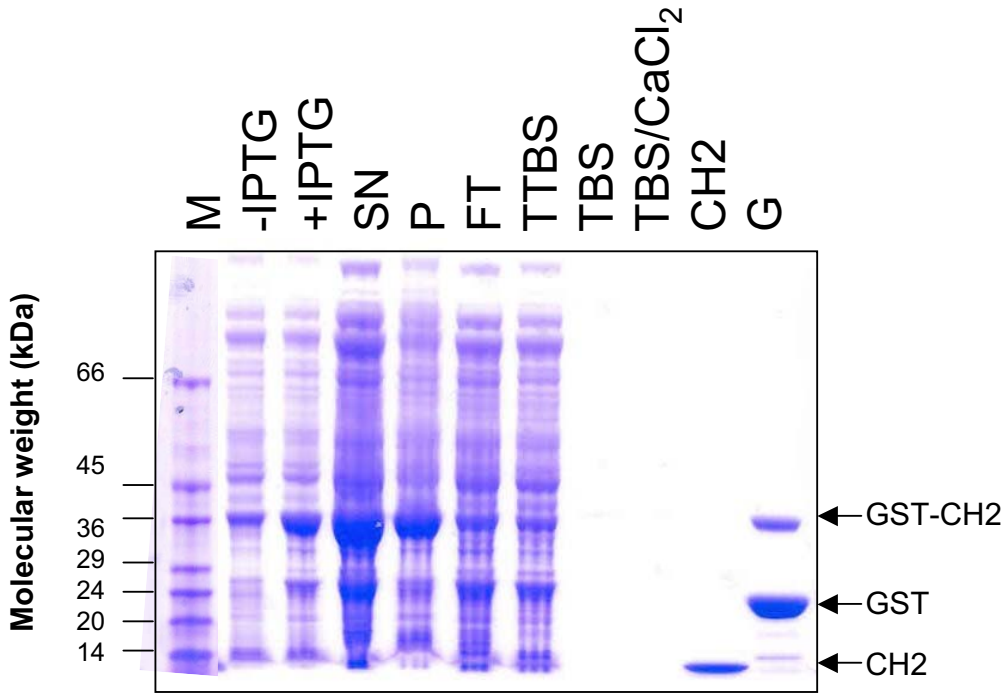


Figure 4.5 Expression and purification of α-actinin-4 CH2 domain. The GST-CH2 fusion protein was expressed in *E. coli* BL21 bacterial cells and purified by a glutathione agarose column. Cells were harvested after 4 hours with 0.2 mM IPTG treatment by centrifugation. Cell pellets were resuspended in the lysis buffer (1X TBS pH 7.0 containing 0.1% Triton X-100, 1 mM EDTA and 1 mM PMSF) and lysed by 0.5 mg/mL lysozyme and one to four 30 sec bursts of sonication. Cell debris was removed by centrifugation with 22,000 x *g* and the supernatant was passed through a glutathione agarose column. After washing with the washing buffer (1X TBS pH 7.0 containing 0.1% Triton X-100), the GST-fusion proteins were digested with thrombin on the column in 1X TBS with 2.5 mM $CaCl_2$ for 18 hours at room temperature. The α-actinin-4 CH2 domain was eluted by 1X TBS buffer and detected by SDS-PAGE. GST-tag was washed out by 1X TBS with 10 mM glutathione buffer. SN, supernatant; P, pellet; FT, flow through; G, glutathione.

Preliminary 1D NMR spectrum was recorded to test the purity and signal strength of the protein sample. The data were processed using VNMR software. As shown in Figure 4.6, the spectrum showed a high signal to noise ratio and wide dispersion in ^{1}H dimension indicating that the CH2 domain is well folded and stable (Figure 4.6). Initial 2D ^{1}H-^{1}H homonuclear experiments, 2D TOCSY and 2D NOESY, were recorded and shown in Figure 4.7 and 4.8. As shown in Figure 4.8, 2D NOESY spectrum showed a large number of HN-HN correlations indicated that the structure of the CH2 domain contains a significant degree of α-helix secondary structure.

4.4.4 Resonance Assignment of α-actinin-4 CH2 Domain

Sequential assignment process was started off with 2D ^{1}H-^{15}N HSQC spectrum referring to 3D ^{1}H-^{15}N TOCSY-HSQC and 3D ^{1}H-^{15}N NOESY-HSQC. The 2D HSQC spectrum is like a fingerprint of a protein. Each cross peak represents an amino acid residue except proline. The 3D TOCSY-HSQC was used to identify spin systems of the different amino acid residues by comparing chemical shift values with those of random coil polypeptide (Wuthrich, 1986). The 3D NOESY-HSQC was used to determine the proper sequential order of these spin systems in the amino acid sequence. As shown in Figure 4.9, the sequential amino acid residues reveal a NOE cross peak with HCα-HN correlation (Figure 4.9). In addition, the 3D NOESY-HSQC spectrum was shown a strong NOE cross peak with HN-HN correlation indicated that the structure of the CH2 domain contains a significant degree of α-helix secondary structure (Figure 4.10). To confirm the sequential assignment, triple resonance spectra were recorded including the combination with HNCA and HN(CO)CA (Figure 4.11) and the combination with HNCO and HN(CA)CO (Figure 4.12). As described in the section 4.2.2, triple resonance spectra are more helpful for the sequential assignment. In addition, the HNCA and HN(CO)CA spectra provide information for α-carbon and the HNCO and HN(CA)CO spectra also provided information for backbone carbonyl assignments. The ^{1}H-^{15}N HSQC spectrum of the CH2 domain is shown in Figure 4.13. All non-proline backbone amide resonances were assigned except the first 6 residues and Ile 190 and Lys 217 which were absent in the HSQC spectrum. The sidechain NH_2 resonances of Asn and Gln residues as well as Hε1 of Trp residues were assigned. The absence of NOE correlations corresponding to the first 6 residues in the 2D NOESY or 3D NOESY-HSQC spectra suggests that this region of the CH2 domain is flexible.

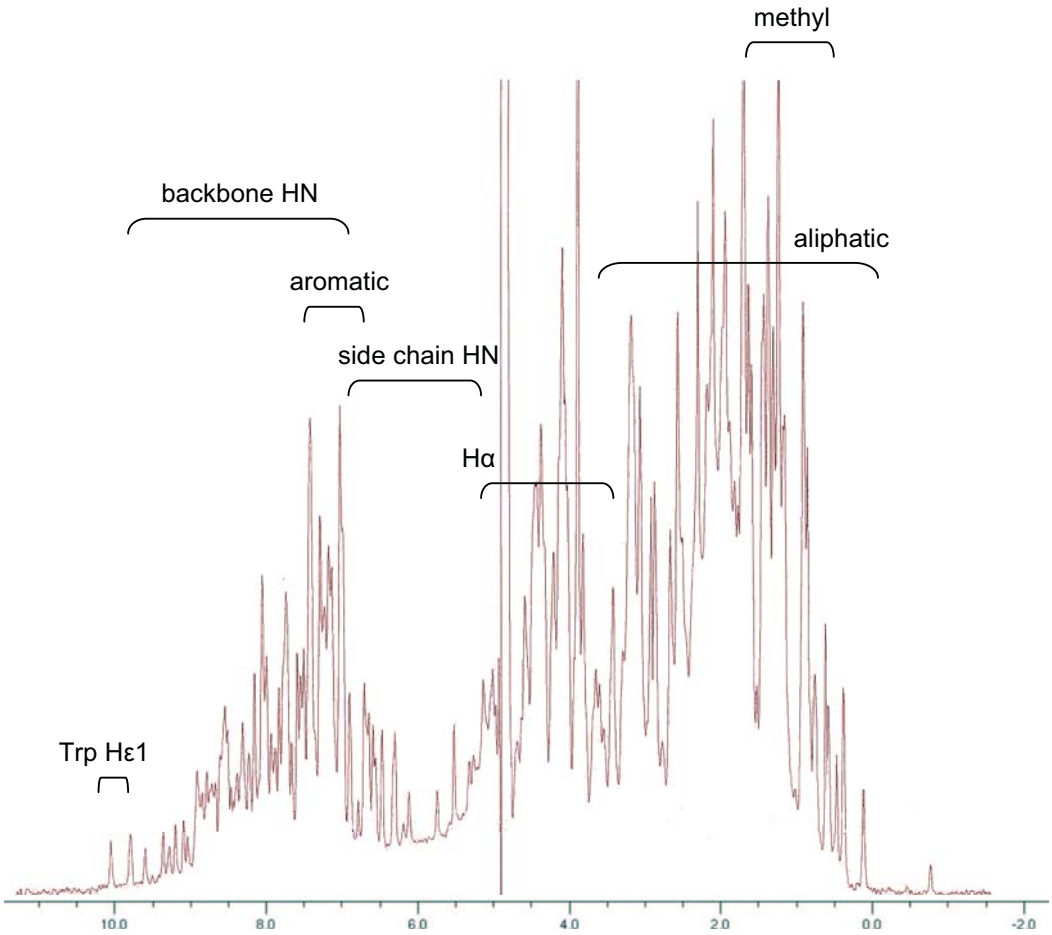


Figure 4.6 1D ^{1}H NMR spectrum of α-actinin-4 CH2 domain. The frequencies of protons in different functional group and backbone or sidechain positions are indicated. The 1D NMR spectrum was recorded using a 3 mM NMR sample at 25°C.

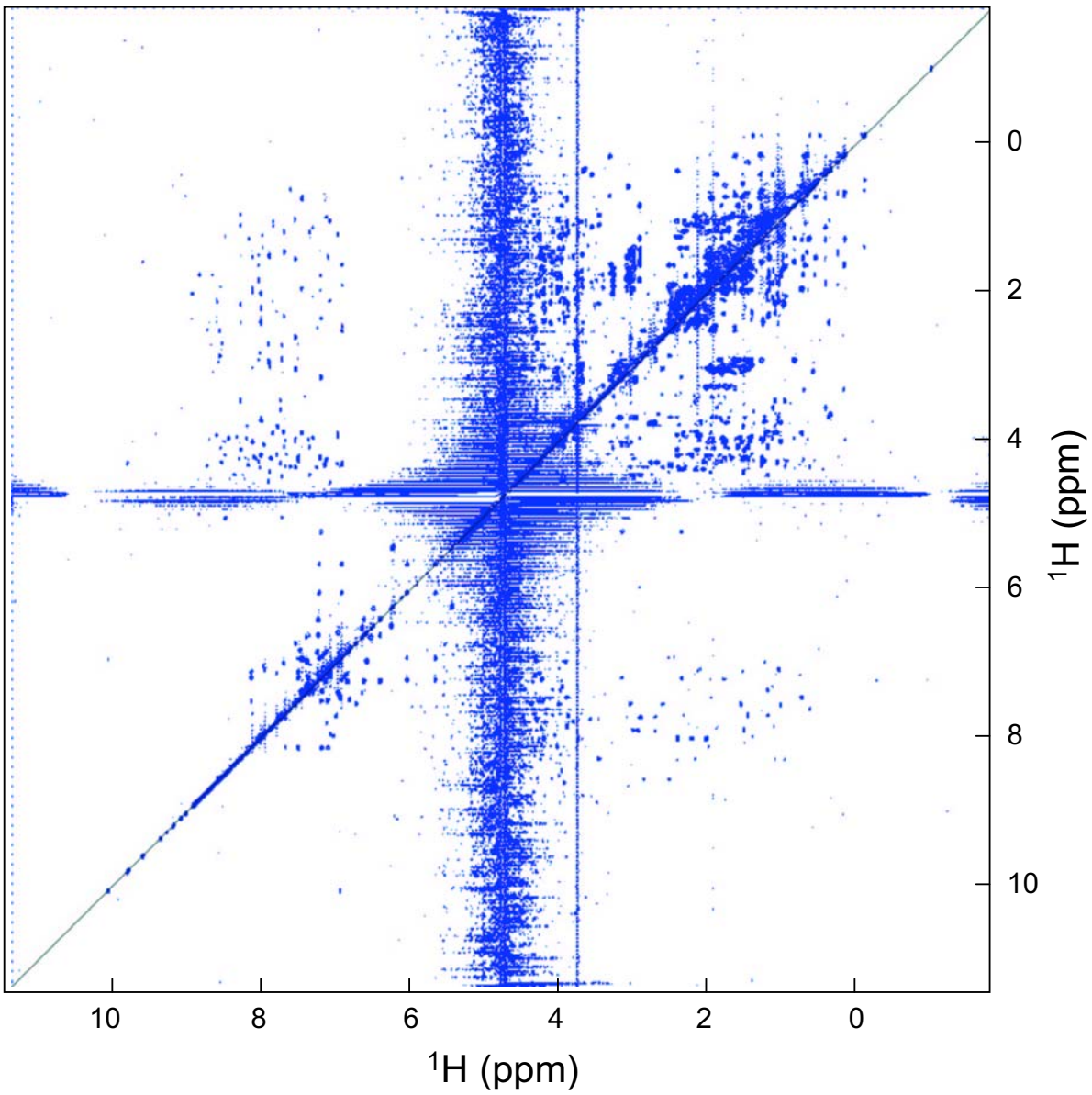


Figure 4.7 2D ^{1}H-^{1}H TOCSY spectrum of α-actinin-4 CH2 domain. The 2D TOCSY spectrum was recorded using a 3 mM NMR sample at 25°C with a mixing time of 80 ms. The NMR data were processed by NMRPipe and analysed by CCPNMR software. The spectrum was plotted from CCPNMR showing the region from 0 to 10 ppm in both dimensions.

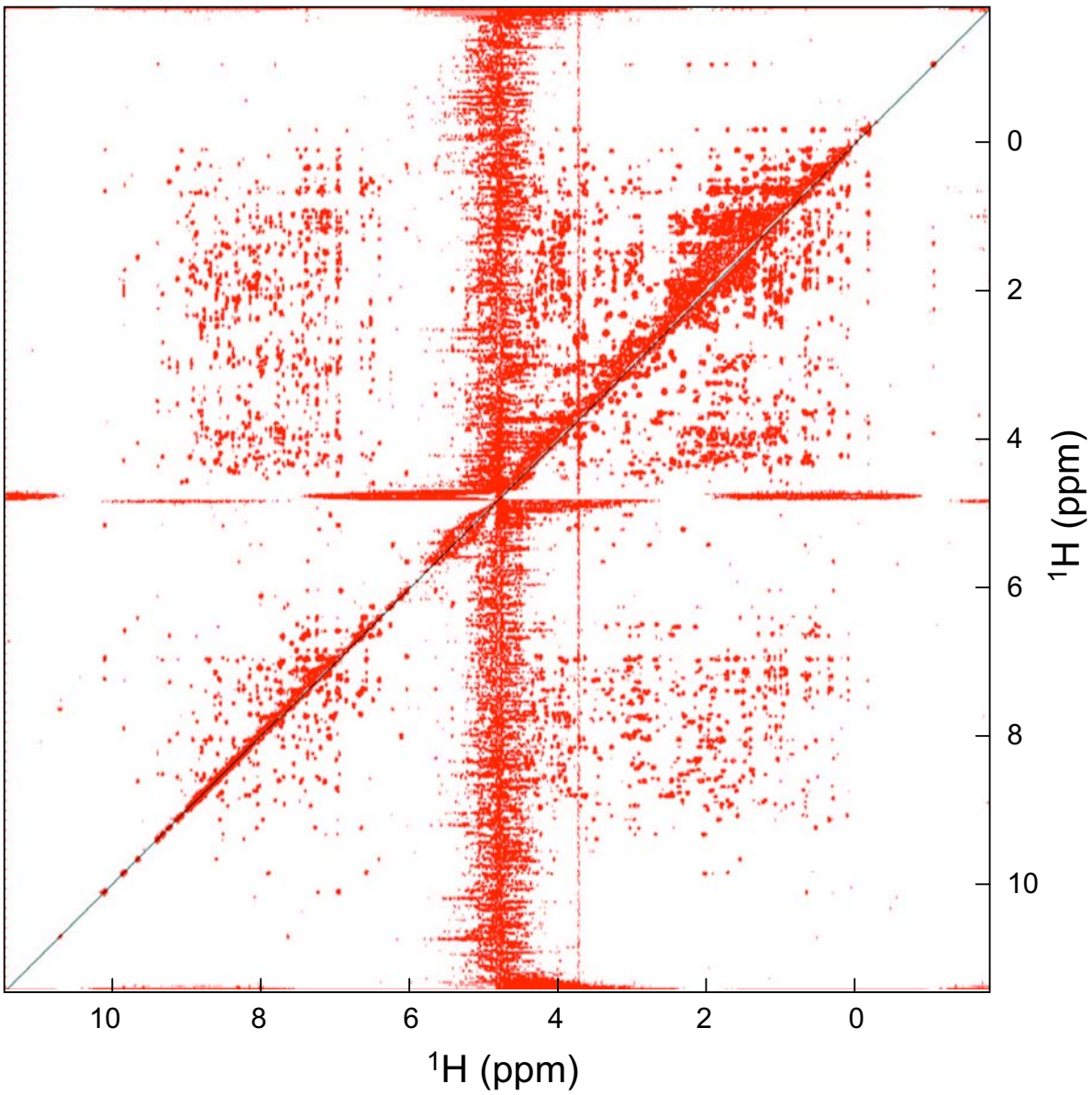


Figure 4.8 2D ^{1}H-^{1}H NOESY spectrum of α-actinin-4 CH2 domain. The 2D NOESY spectrum was recorded using a 3 mM NMR sample at 25°C with a mixing time of 100 ms. The NMR data were processed by NMRPipe and analysed by CCPNMR software. The spectrum was plotted from CCPNMR showing the region from 0 to 10 ppm in both dimensions.

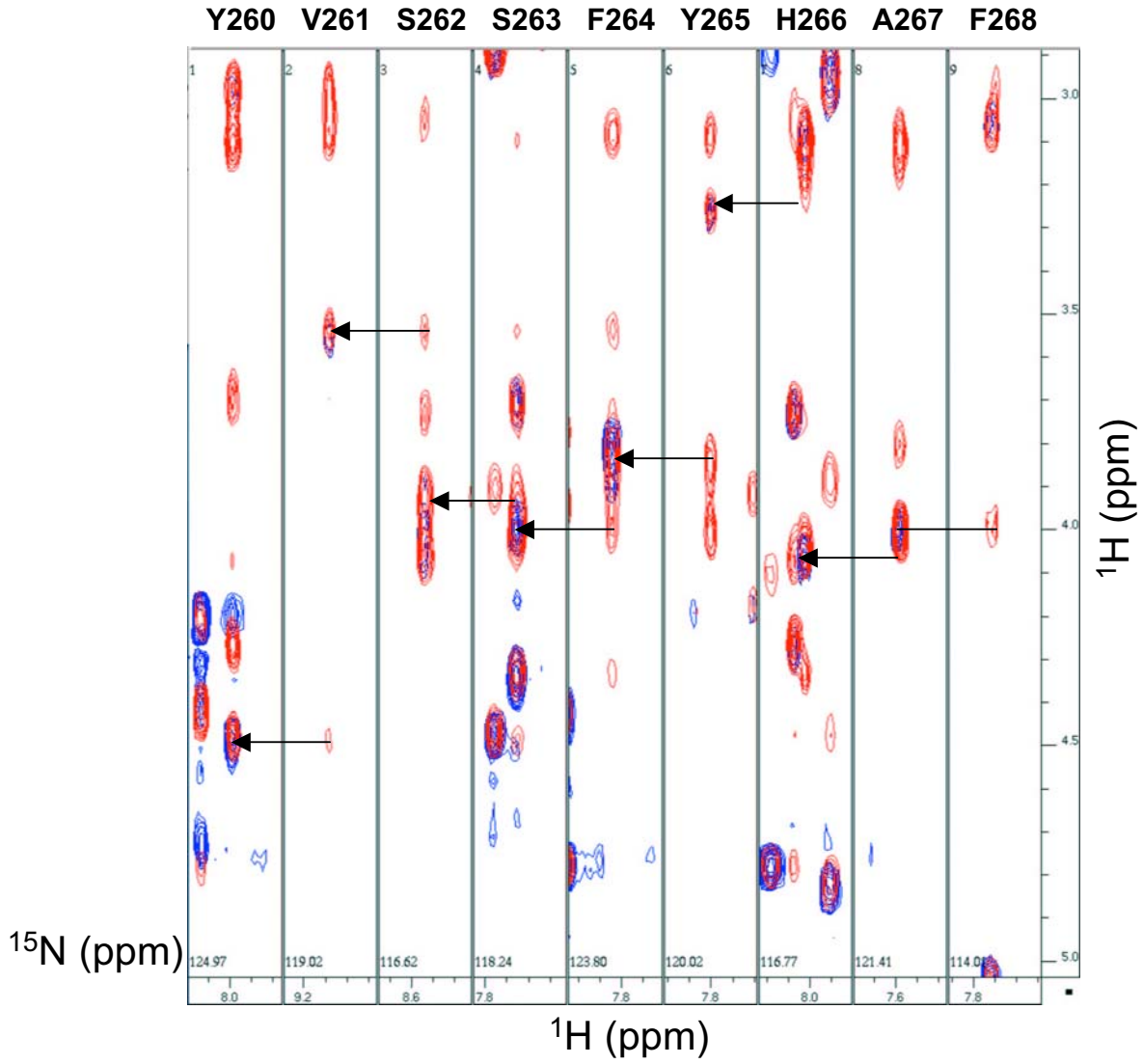


Figure 4.9 Strip plot of residues Y260-F268 of α-actinin-4 CH2 domain with 3D NOESY-HSQC and 3D TOCSY-HSQC spectra. The 3D NOESY-HSQC and 3D TOCSY-HSQC spectra were recorded using a 3 mM ^{15}N labeled NMR sample at 25°C with a mixing time of 100 ms and 80 ms, respectively. The NMR data were processed by NMRPipe and analysed by CCPNMR software. CCPNMR was used to generate the strip plots. Residues Y260 to F268 strips are shown with the NOESY cross peaks in red and the corresponding TOCSY cross peaks overlaid in blue. The residue strips corresponding nitrogen chemical shift values are indicated at the bottom of the strips. The arrows indicate the HCα-HN correlations with the proper sequential order of these amino acid residues.

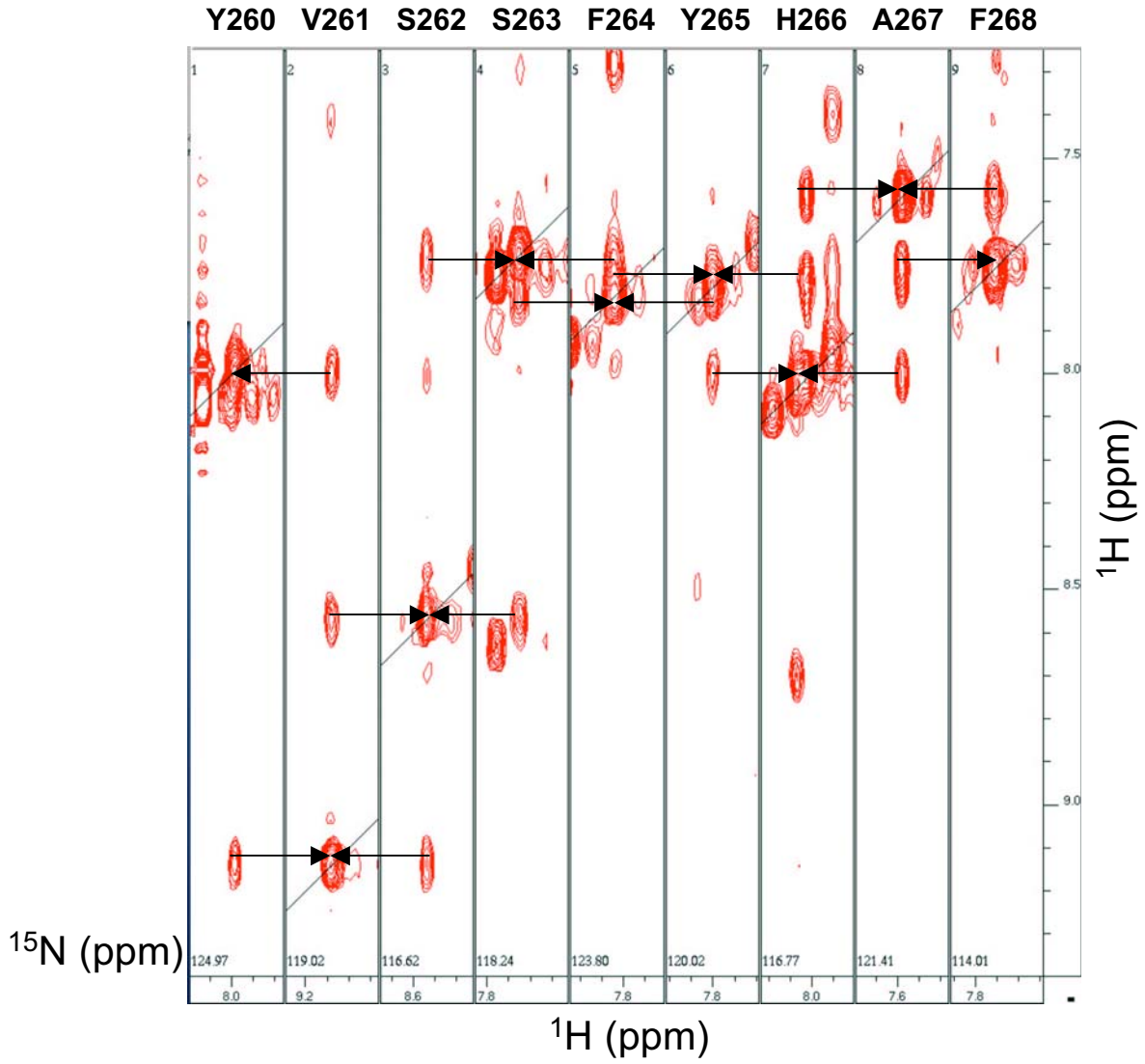


Figure 4.10 Strip plot of residues Y260-F268 of α-actinin-4 CH2 domain with 3D NOESY-HSQC spectra. The 3D NOESY-HSQC spectrum was recorded using a 3 mM ^{15}N labeled NMR sample at 25°C with a mixing time of 100 ms. The NMR data were processed by NMRPipe and analysed by CCPNMR software. CCPNMR was used to generate the strip plots. The residue strips corresponding nitrogen chemical shift values are indicated at the bottom of the strips. The arrows point out the strong HN-HN correlations with the proper sequential order of these amino acid residues indicating a protein with α-helix structure.

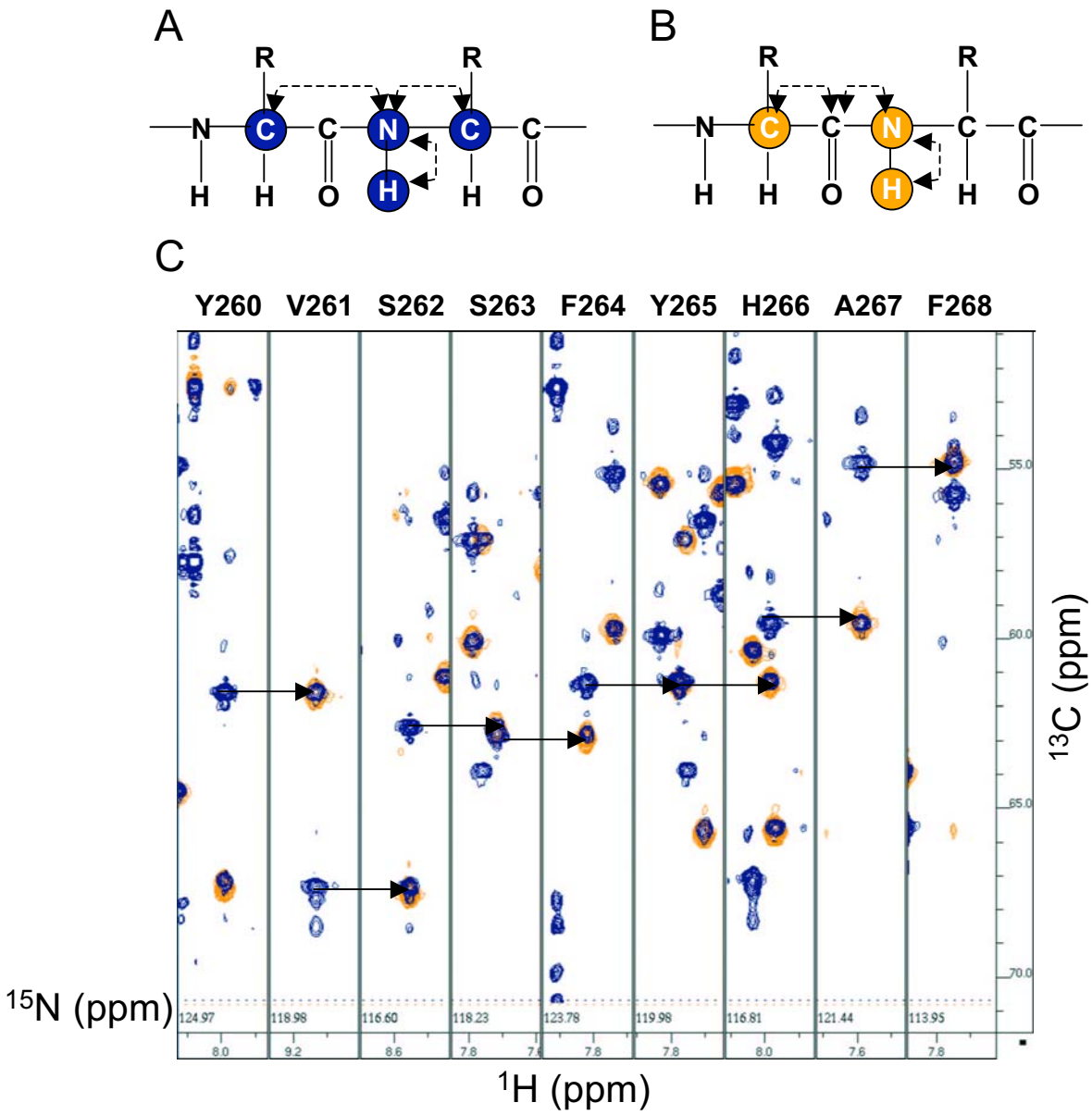


Figure 4.11 Strip plot of residues Y260-F268 of α-actinin-4 CH2 domain with triple resonance HNCA and HN(CO)CA spectra. The correlation diagrams are shown with HNCA (A) and HN(CO)CA (B). (C) The HNCA and HN(CO)CA spectra were recorded using a 3 mM ^{13}C/^{15}N double labeled NMR sample at 25°C. The NMR data were processed by NMRPipe and analysed by CCPNMR software. CCPNMR was used to generate the strip plots. Residues Y260 to F268 strips are shown with the HNCA cross peaks in blue and the corresponding HN(CO)CA cross peaks overlaid in orange. The residue strips corresponding nitrogen chemical shift values are indicated at the bottom of the strips. The arrows point out the strong HN-Cα correlations with the proper sequential order of these amino acid residues.

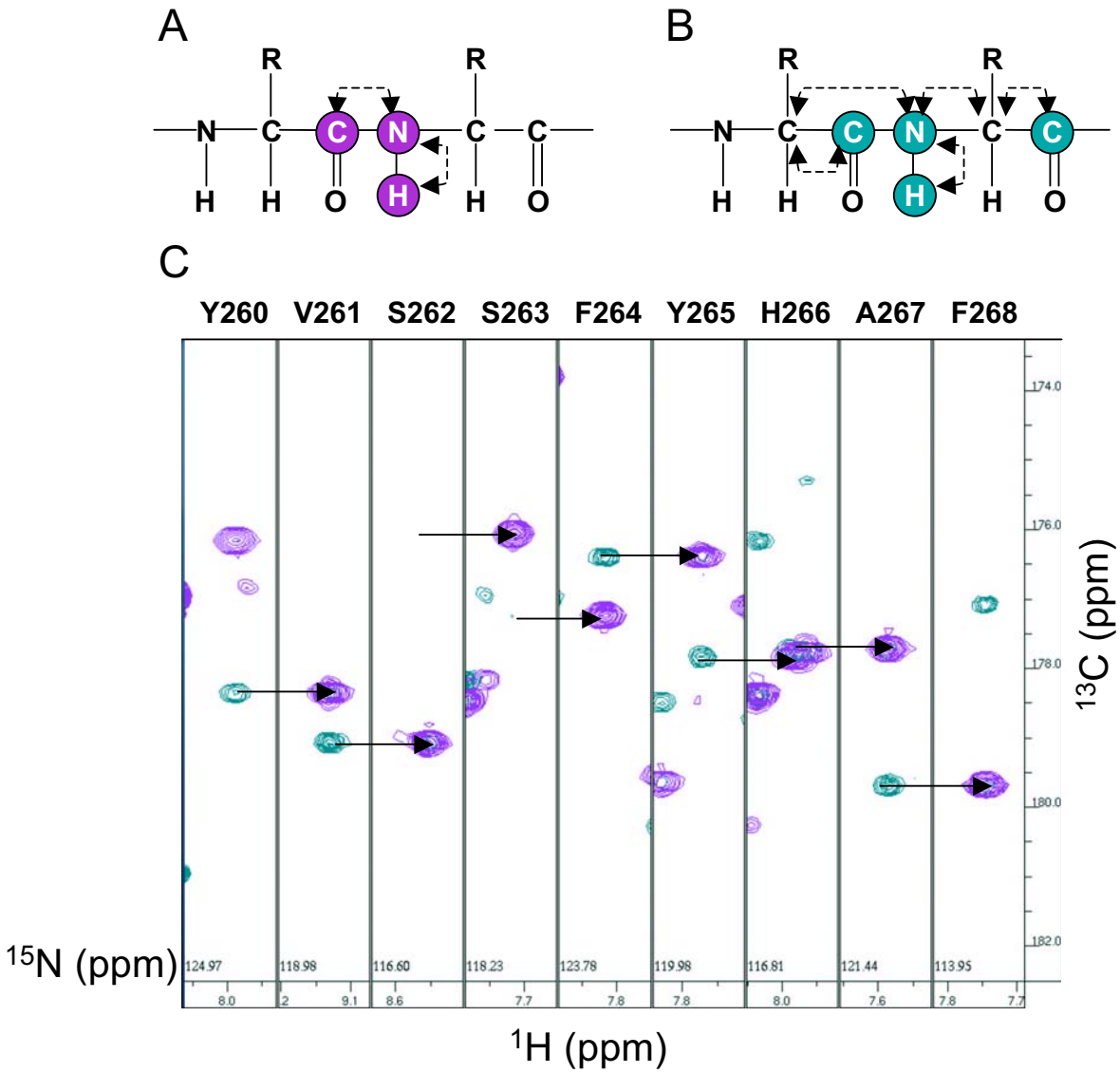


Figure 4.12 Strip plot of residues Y260-F268 of α-actinin-4 CH2 domain with triple resonance HNCO and HN(CA)CO spectra. The correlation diagrams are shown with HNCO (A) and HN(CA)CO (B). (C) The HNCO and HN(CA)CO spectra were recorded using a 3 mM $^{13}C/^{15}N$ double labeled NMR sample at 25°C. The NMR data were processed by NMRPipe and analysed by CCPNMR software. CCPNMR was used to generate the strip plots. Residues Y260 to F268 strips are shown with the HNCO cross peaks in purple and the corresponding HN(CA)CO cross peaks overlaid in green. The residue strips corresponding nitrogen chemical shift values are indicated at the bottom of the strips. The arrows point out the strong HN-CO correlations with the proper sequential order of these amino acid residues.

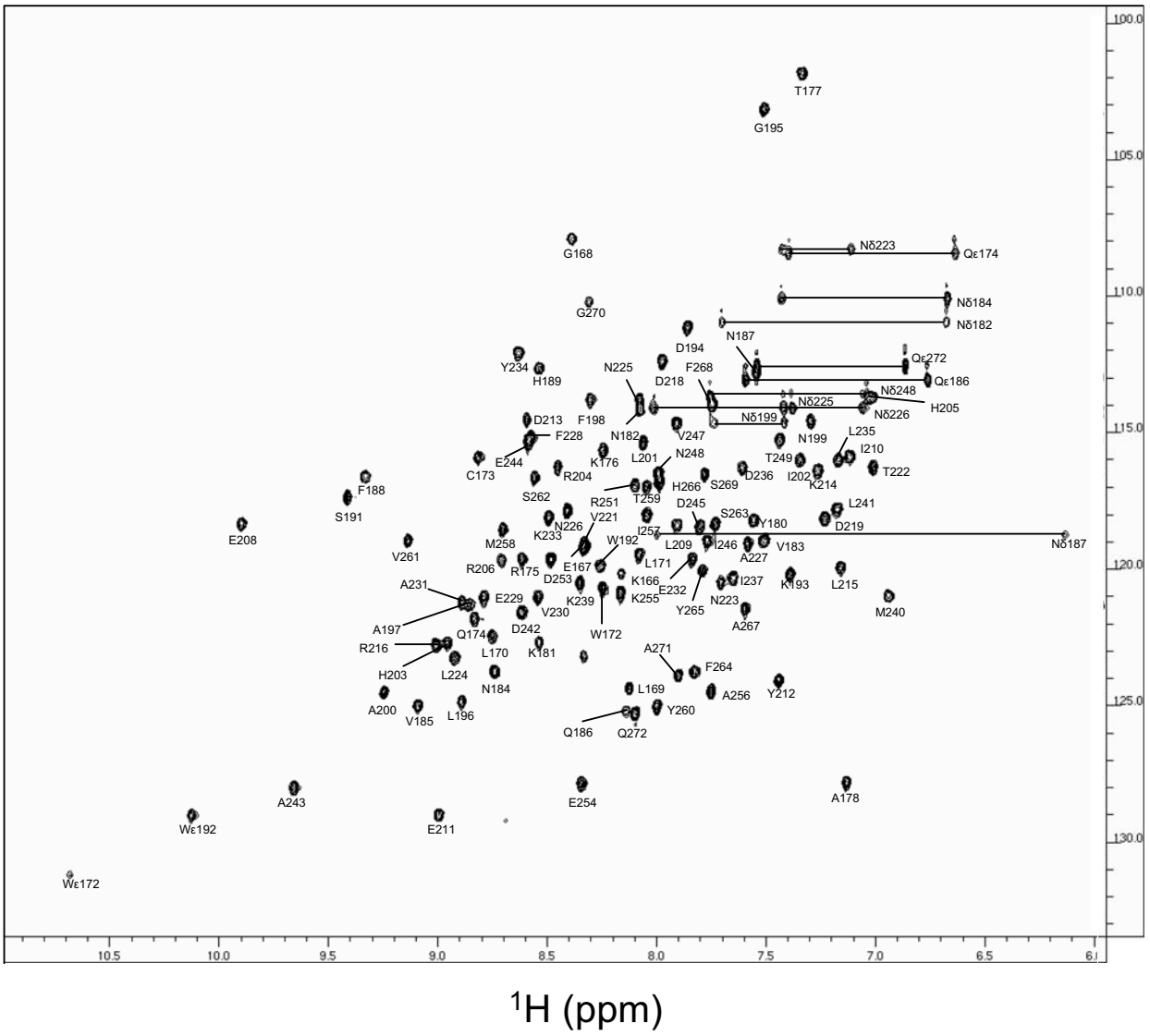


Figure 4.13 Assigned 2D ^{1}H-^{15}N HSQC spectrum of α-actinin-4 CH2 domain. The HSQC spectrum was recorded using a 3 mM ^{15}N-labeled NMR sample at 25°C. The NMR data were processed by NMRPipe and analysed by CCPNMR software. The backbone resonances were assigned and indicated by the one-letter amino acid code and the residue number. Sidechain NH_2 resonances of Asn and Gln are connected by horizontal lines.

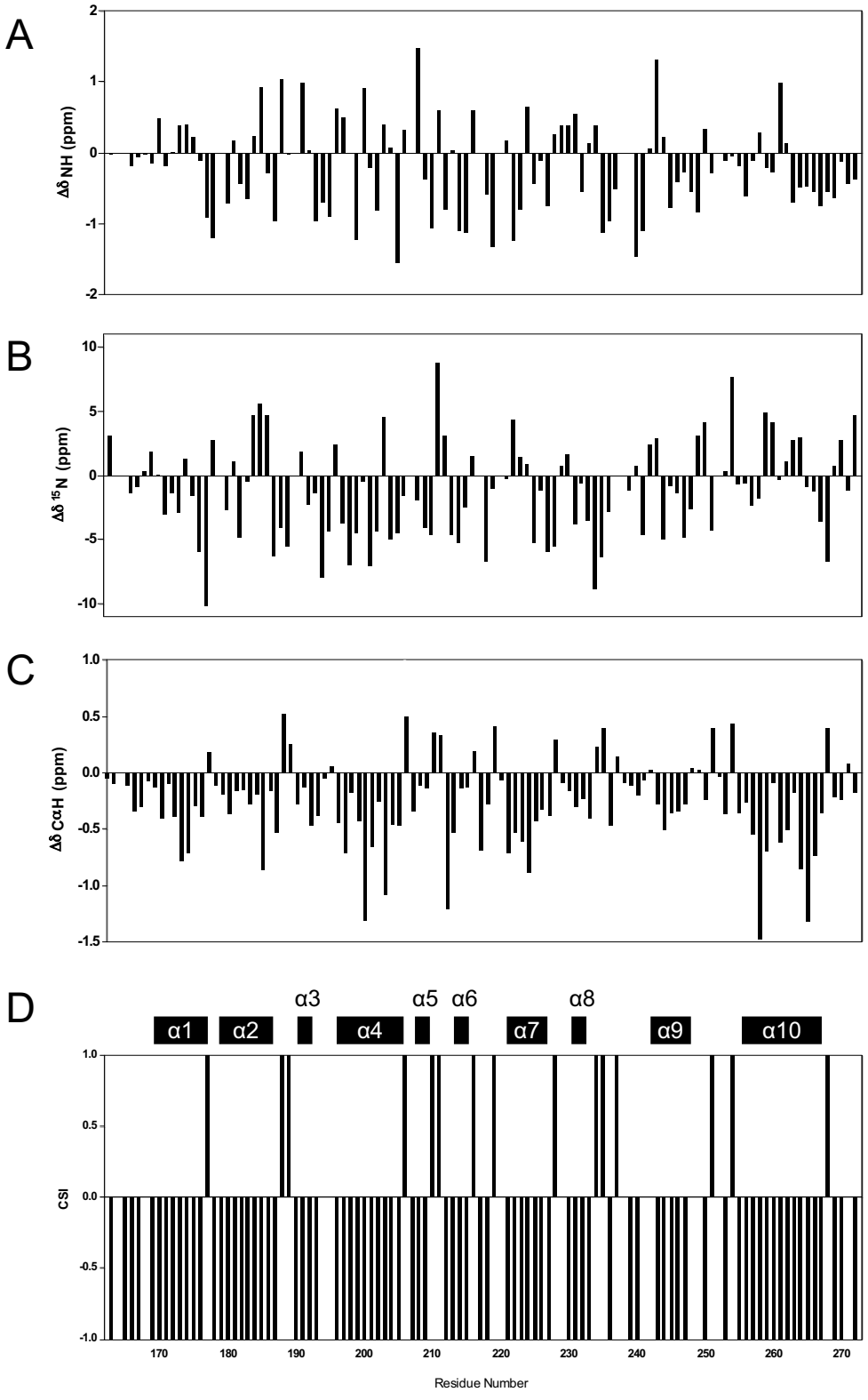
Sidechain assignments were obtained using triple resonances spectra including HBHA(CBCACO)HN, CBCAHN, CBCA(CO)HN and HCCH-TOCSY (data not shown), and confirmed by 3D TOCSY-HSQC and 3D NOESY-HSQC. Aromatic protons were assigned from HBCBCGCDCHD and HBCBCGCDCEHE experiments as well as 2D TOCSY and 2D NOESY experiments that were recorded on unlabeled CH2 sample in D_2O. In total, 94% of backbone ^{1}H, 89% of ^{15}N, 96% of ^{13}CO, 97% of $^{13}C\alpha$, 98% of $^{13}C\beta$ and 96% of sidechain ^{1}H have been assigned. The complete chemical shift assignments for α-actinin-4 CH2 domain are shown in Appendix I.

The chemical shift deviation of ^{15}NH and $^{13}C\alpha H$ from random coil values is a simple and useful method to predict the secondary structure of proteins (Wishart *et al.*, 1995). Deviations in $^{13}C\alpha H$ chemical shift greater than 0.1 ppm are given a value of 1 and those lower than -0.1 ppm are given a value of -1. These values are called the chemical shift index (CSI) (Wishart *et al.*, 1992). Protein regions of three or more residues with values of 1 indicate β-sheet structure, whereas regions of three or more residues with values of -1 indicate α-helix structure. As shown in Figure 4.14, the chemical shift deviation of ^{15}NH, ^{15}N and $^{13}C\alpha H$ from random coil of all assigned residues indicated that the secondary structure of α-actinin-4 CH2 domain is predominantly α-helix and predicts 10 α-helices (Figure 4.14). This structure is consistent with that of chicken skeletal muscle α-actinin CH2 domain (pdb1SJJ) determined by X-ray crystallography.

4.4.5 Structural Determination of α-actinin-4 CH2 Domain

The structure calculations of α-actinin-4 CH2 domain were performed using the program ARIA 2.0 (Linge *et al.*, 2003) with the assistance of Mr. Iain Murchland. Three different groups of restraints were used in the structure calculations, distance, dihedral angle and hydrogen bond restraints. Distance restraints were derived from 2D NOESY and 3D NOESY-HSQC as well as 2D NOESY recorded in D_2O, with a mixing time of 100 ms. Unambiguous and ambiguous restraints were generated automatically by ARIA 2.0. Dihedral angle restraints were derived from ^{1}H-^{15}N HMQC-J spectrum. The $^{3}J_{HNH\alpha}$ values were determined from F1 and F2 cross peak line widths in a ^{1}H-^{15}N HMQC-J spectrum (Wishart and Wang, 1998). Dihedral angle restraints were set the φ-angles of -120 ± 40°

Figure 4.14 Chemical shift index of α-actinin-4 CH2 domain. Deviation of NH (A), ^{15}N (B) and ^{13}CαH (C) chemical shifts from random coil values plotted against residue number. (D) Chemical shift index (CSI) of CH2 domain. The protein regions of three or more residues with values of -1 indicate α-helix structure (Wishart, 1992). Regions of α-helix predicted using the CSI are indicate in black boxes and are labeled as α1 to α10.


A
Δδ NH (ppm)
B
Δδ 15N (ppm)
C
Δδ CαH (ppm)
D
α1
α2
α3
α4
α5
α6
α7
α8
α9
α10
CSI
Residue Number

with the $^{3}J_{HNH\alpha}$ value greater than or equal to 8 Hz and -60 ± 30° with the $^{3}J_{HNH\alpha}$ value less than or equal to 5 Hz. Hydrogen bond restraints were determined by deuterium exchange experiments. Hydrogen bond donors were determined when the amide group had not exchanged from ^{1}H to ^{2}D within 1 hour at 25°C after resuspension of a lyophilised sample from H_2O to D_2O. The hydrogen-acceptor distance was set between 1.7 and 2.2 Å and the donor-acceptor distance was set between 2.7 and 3.2 Å.

An ensemble of 20 structures was calculated at iteration 0 to 7 and seven of the lowest energy conformers were selected for the next iteration. A total of 200 structures were calculated at the final iteration. The final restraints include 1154 unambiguous restraints (including 465 intra-residual NOE, 275 sequential NOE, 132 short range NOE, 55 medium range NOE and 227 long range NOE restraints), 961 ambiguous restraints, 37 hydrogen bond restraints and 100 dihedral angle restraints. The number of unambiguous NOE restraints observed per residue is shown in Figure 4.15. The 20 structures with the lowest energy were selected for the refinement step and 200 structures were performed in water solvent using the OPLSX non-bond parameter set (Linge and Nilges, 1999). The 20 structures with the lowest overall energy were selected as the final ensemble and shown in Figure 4.16. The quality of the ensemble was validated using PROCHECK (Laskowski *et al.*, 1993) and the structural statistics of α-actinin-4 CH2 domain is shown in Table 4.1. The hydrogen bond and the secondary structure were analysed using MOLMOL (Kordadi *et al.*, 1996) and a Ramachandran plot was generated using PROCHECK (Laskowski *et al.*, 1993) (Figure 4.17). The unambiguous restraints list and hydrogen bond list are shown in Appendix II and III.

The final 20 conformers are shown in Figure 4.16. The N-terminal six residues representing Glu 162 to Ala 165 and including -2 Gly and -1 Ser, which remain following thrombin cleavage from the GST tag, are obviously flexible or unstructured. There are no relevant peaks observed on the 2D HSQC spectrum. The C-terminal region, residues from Gly 270 to Gln 272, is also unstructured with few intra-residue and sequential NOE restraints but no medium or long range NOE restraints. The section of the CH2 domain from residues Glu 167 to Phe 268 is well defined with many NOE and hydrogen bond restraints. The RMSD value within this section of backbone atoms is 0.31 ± 0.04 Å (Table 4.1), indicating the CH2

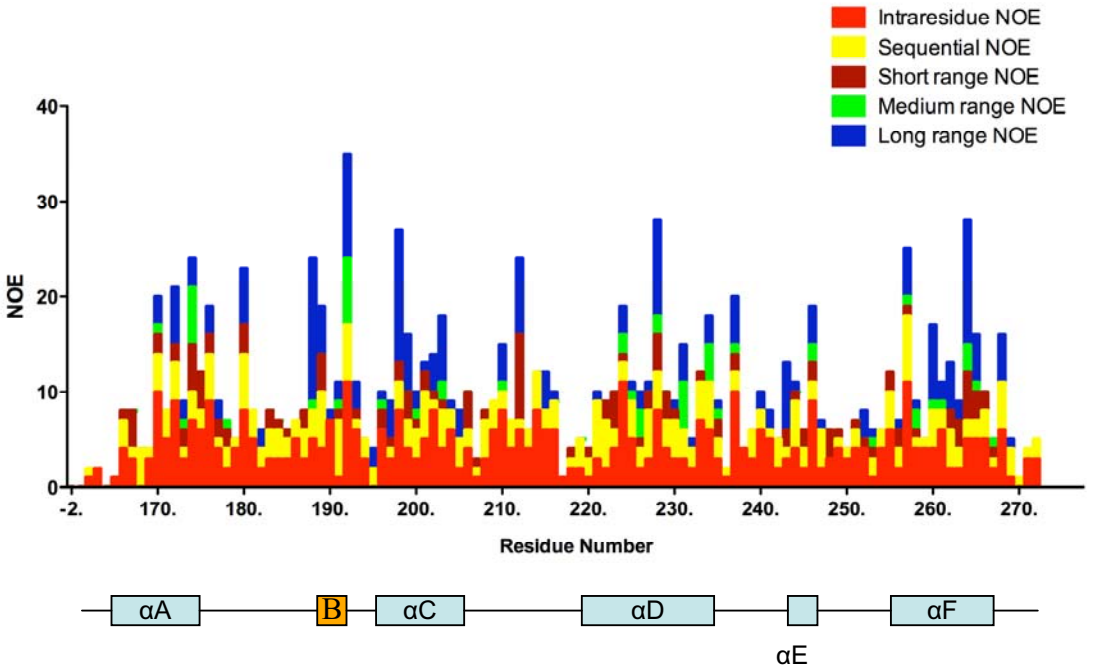


Figure 4.15 Plot of the unambiguous NOE restraints used in the structure calculation of α-actinin4 CH2 domain. Intra-residual, sequential, short range, medium range and long range NOE restraints are shown in red, yellow, brown, green and blue, respectively. The relevant secondary structures are shown as cyan boxes on the bottom. The orange box indicates a 3_{10} helix.

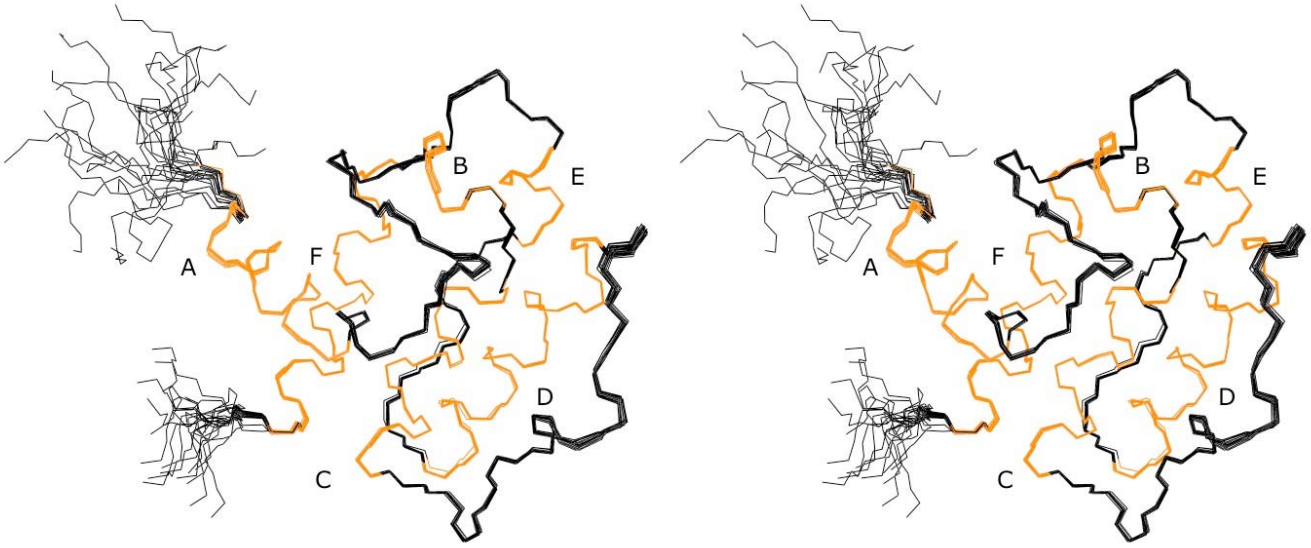
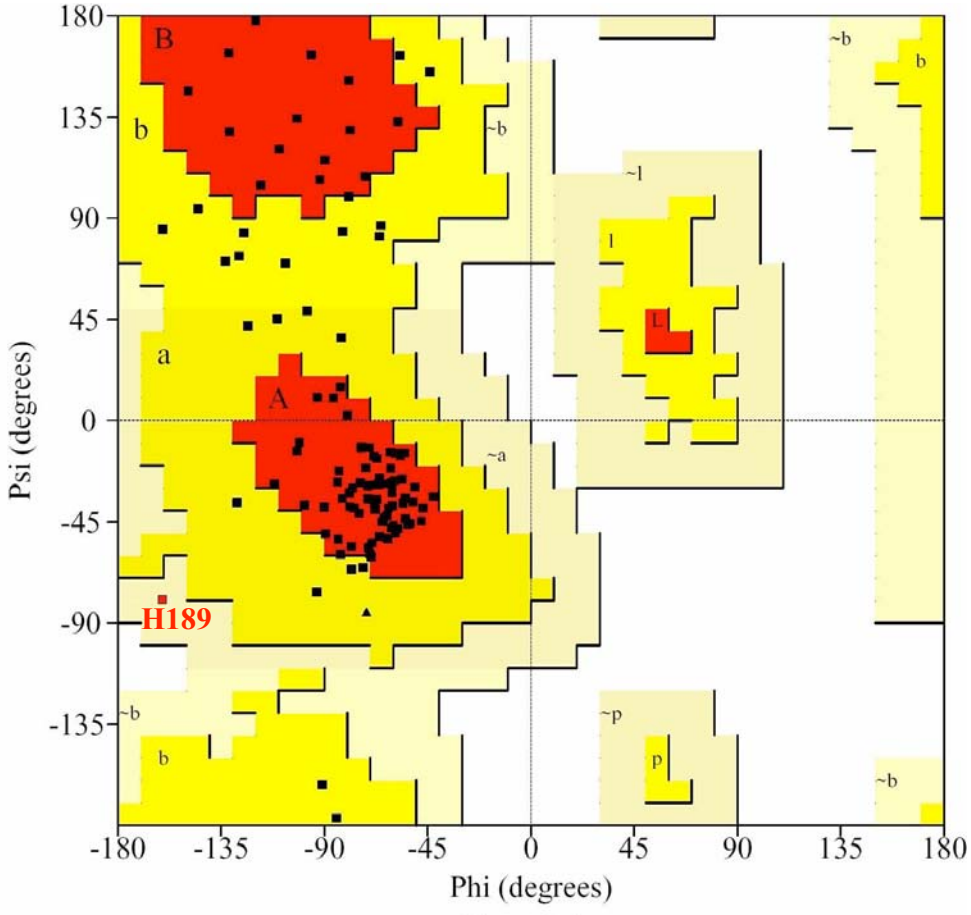


Figure 4.16 The final ensemble structures of α-actinin-4 CH2 domain. Structures were determined using ARIA 2.0 program (Linge, 2003). The ensemble represents 20 conformers with the lowest energy (20 out of 200). Five α-helices are shown in orange. The stereo image was generated by Chimera software.

Table 4.1 Structural statistics for α-actinin-4 CH2 domain

RMSD from experimental distance restraints	
All distance (Å)	$3.42 \times 10^{-2} \pm 1.05 \times 10^{-3}$
All noe distance (Å)	$3.38 \times 10^{-2} \pm 1.18 \times 10^{-3}$
1154 Unambiguous (Å)	$3.30 \times 10^{-2} \pm 1.62 \times 10^{-3}$
961 Ambiguous (Å)	$3.43 \times 10^{-2} \pm 1.12 \times 10^{-3}$
37 Hydrogen bond (Å)	$4.41 \times 10^{-2} \pm 3.67 \times 10^{-3}$
100 Dihedral angle constraints (°)	$5.02 \times 10^{-1} \pm 7.49 \times 10^{-2}$
RMSD from ideal geometry	
Bonds (Å)	$4.06 \times 10^{-3} \pm 1 \times 10^{-4}$
Angles (°)	$5.37 \times 10^{-1} \pm 1.3 \times 10^{-2}$
Improper (°)	$1.49 \pm 7.49 \times 10^{-2}$
Dihedrals (°)	$41.1 \pm 2.01 \times 10^{-1}$
RMSD from the structure with the lowest energy	
All residues (8-109)[a] (2^{nd} structure) (Å)	
Backbone atoms	1.35 ± 0.275
	(0.31 ± 0.04)
	(0.152 ± 0.021)
All heavy atoms	1.39 ± 0.175
	(0.89 ± 0.09)
	(0.427 ± 0.0515)
Non-bonded energies in CSDX/OPLS force field	
vdW	-1165.41 ± 11.75
Elec	-4281.92 ± 108.48
Ramachandran plot (%)	
Most favoured regions	80.1
Additionally allowed regions	18.1
Generously allowed regions	1.3
Disallowed regions	0.5

[a] Calculated with the program MOLMOL (Koradi, 1996).



Plot statistics

Residues in most favoured regions [A,B,L]	82	79.6%
Residues in additional allowed regions [a,b,l,p]	20	19.4%
Residues in generously allowed regions [~a,~b,~l,~p]	1	1.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	103	100.0%
Number of end-residues (excl. Gly and Pro)	1	
Number of glycine residues (shown as triangles)	4	
Number of proline residues	5	
Total number of residues	113	

Figure 4.17 Ramachandran plot of α-actinin-4 CH2 domain. The residue H189 shown in red does not fit the correct Psi and Phi angles. The plot statistics is shown in lower panel. Residues in most favoured regions, additional allowed regions and generously allowed regions are shown in red, yellow and pink, respectively. The diagram was generated using PROCHECK (Laskowski, 1993).

domain is well structured. As shown in Figure 4.16, the CH2 domain contains six α-helices. The RMSD values for the backbone atoms within these secondary structures is 0.152 ± 0.021 Å (Table 4.1), indicating the secondary structures are well defined. The Ramachandran plot is shown in Figure 4.15. The Ramachandran plot is an important indicator for protein structural quality with the combination of the dihedral angles Phi and Psi. The angles Phi and Psi of amino acids in a polypeptide chain are restricted to certain ranges. In the CH2 structure, 80% and 20% amino acid residues fall into the most favoured and additional allowed regions, respectively, exclusive of all Gly and Pro. The only one residue that falls in the generously allowed regions is His 189.

The number of α-helices was inconsistent between the CSI value predictions (Figure 4.14 D) and three-dimensional structure (Figure 4.18). The CSI value predictions showed that the CH2 domain contains six α-helices and four 3_{10} helices. However, the α-actinin-4 CH2 domain consists of five α-helices and one 3_{10} helix which are linked by 5 loops. The stereo ribbon diagram is shown in Figure 4.18. The α-helix structures are composed of αA (Glu 167 to Lys 176), 3_{10} helix B (Ile 190 to Trp 192), αC (Leu 196 to His 205), αD (Pro 220 to Tyr 234), αE (Ala 243 to Val 247) and αF (Glu 254 to Ala 267) (Figure 4.18). In the CSI value predictions, residues from Ala 178 to Asn 187 form an α-helix structure (labeled α2 in Figure 4.14 D) and the α7 helix contains of seven amino acid residues. However, in the three-dimensional α-actinin-4 CH2 structure, the α2 does not form a helix and the α7 is longer than the CSI values predicted at fifteen rather than seven amino acid residues. The region Ala 178 to Asn 187 forms a loop which is consistent with the CH2 structure from other spectrin superfamily members. A single turn 3_{10} helix (labeled in B in Figure 4.15) is formed next to the loop. Three 3_{10} helices shown in Figure 4.14 D, α5, α6 and α8, do not form helices in the three-dimensional structure of the CH2 domain.

As shown in Figure 4.18, the packing of the αA, αC and αF is stabilised by the hydrophobic interactions between Cys 173 and Val 261 as well as Thr 177 and Leu 201. The contacts plot diagram was generated using ARIA 2.0 (Linge *et al.*, 2003) and shown in Figure 4.19. The results indicate that the αC, αD and αF helices form a three-helix bundle and αA contacts to αC and αF. The αC helix is very important to the structure of α-actinin-4 CH2 domain. The αC helix, residues Ala 197 to Ile 202, is entirely buried, building the

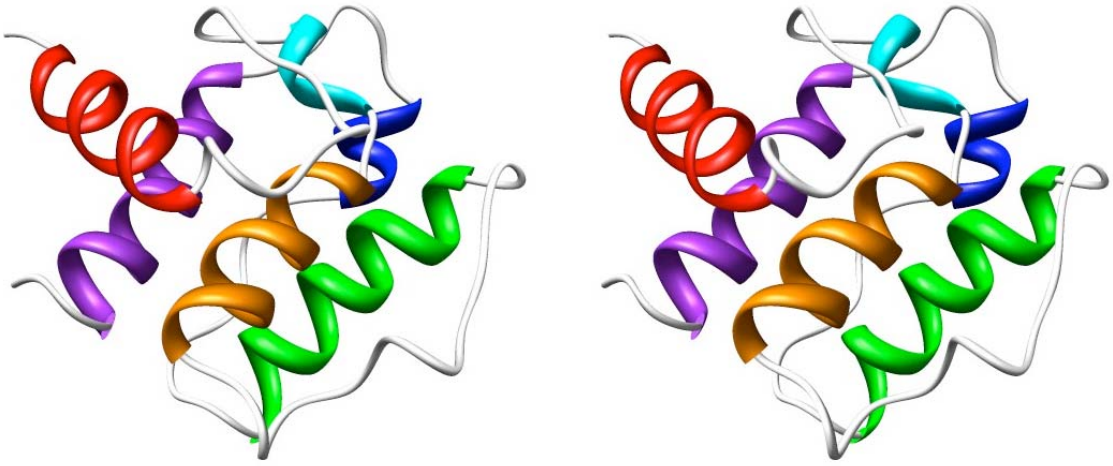


Figure 4.18 The ribbon presentation of α-actinin-4 CH2 domain in stereo. The α-actinin-4 CH2 domain contains five α-helices (indicated in red, orange, green, blue and purple) and one 3_{10} helix (indicated in cyan). The stereo ribbon image was generated using Chimera (Pettersen, 2004).

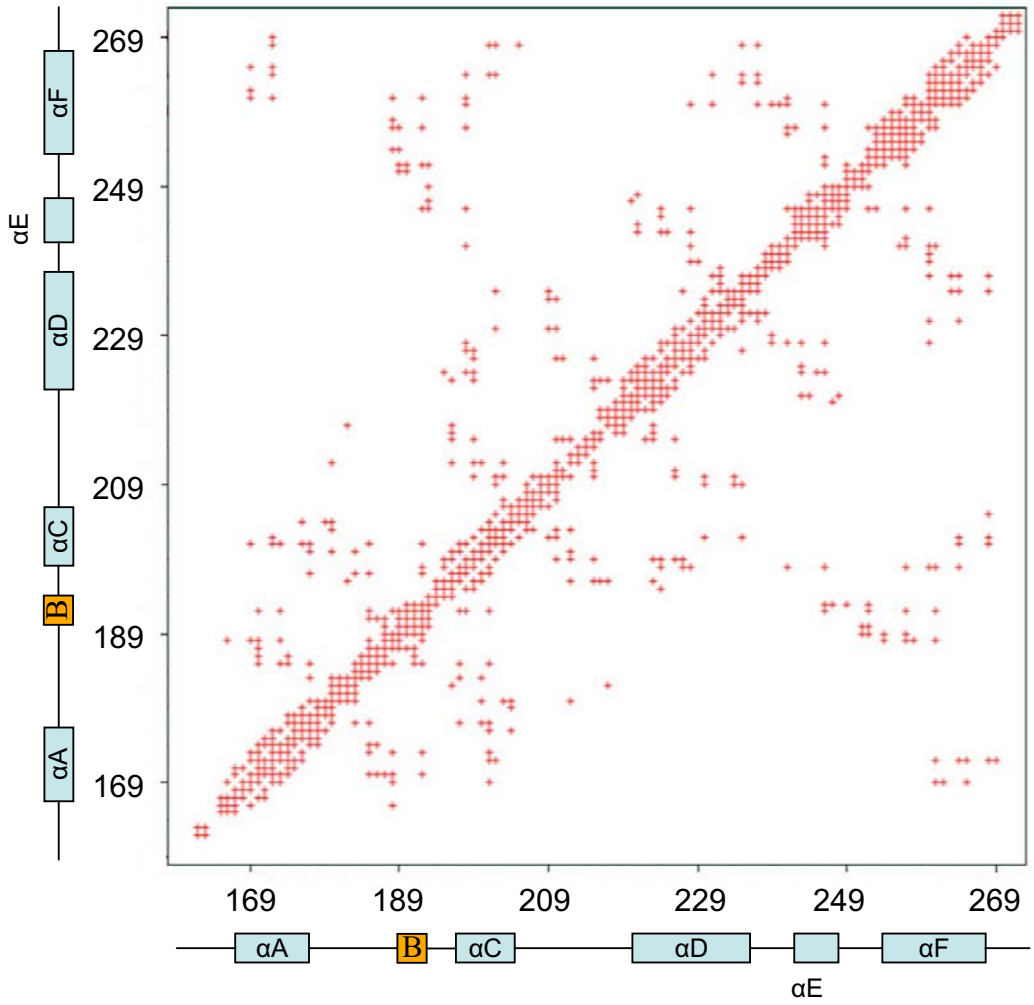


Figure 4.19 Contact plot of α-actinin4 CH2 domain. The relevant secondary structures are shown as cyan boxes on the bottom. The orange box indicates a 3_{10} helix. The diagram was generated using ARIA 2.0 (Linge, 2003).

hydrophobic core of the CH2 domain. The plot diagram of solvent accessibility versus residue number was generated using MOLMOL (Kordadi *et al.*, 1996) and shown in the Figure 4.20. The result indicated that the αC helix is highly hydrophobic. Apart from the αC helix, some other residues participate in formation of the hydrophobic core of the CH2 domain, including Cys 173, Thr 177, Val 185, Trp192, Gly 195, Asn 223, Leu 224, Ala 227, Phe 228, Ala 231, Ile 237, Ile 246, Pro 252, Ile 257, Tyr 260, Val 261 and Phe 264 (Figure 4.21). Extensive aromatic stacking is helpful for the stablisation of the core in the CH2 domain. Figure 4.22 shows the final ensemble of CH2 structures with the hydrophobic aromatic residues, including two Trp, five Tyr and five Phe.

The surface structure of α-actinin-4 CH2 domain is shown in Figure 4.23. The putative ABS-3 which has been identified from utrophin and dystrophin (Winder *et al.*, 1995; Levine *et al.*, 1992; Fabbrizio *et al.*, 1993) resides on the αA helix. This region on the α-actinin-4 CH2 domain is a high solvent accessible area suggesting a role in the interaction with F-actin. The αA helix contains the conserved amino acid residues Leu 169, Leu170, Leu171 and Trp 172 implying that the interaction with F-actin could be through hydrophobic interactions. The PIP2 binding region which has been proposed for chicken α-actinin-1 CH2 domain (Fukami *et al.*, 1996) is located on the loop between αA and αC, including 3_{10} helix B.

4.4.6 Comparison of Other CH2 Domain from Spectrin Superfamily

Structural comparison between the CH2 domain of α-actinin-4 and that of other spectrin superfamily members shows that the strong similarities with six α-helices including four long α-helics (αA, αC, αD and αF) and two short α-helices (αB and αE) (Figure 4.24 and 4.25). However, there are some exceptions. The αC helix of human α-actinin-1, plectin and dystrophin is a long α-helix structure with 20 to 21 amino acid residues. The loop region between αC and αD contains one additional α-helix in human α-actinin-3, chicken α-actinin-1 and human utrophin and two 3_{10} helices in human sepctrin. The CH2 domain of human fimbrin has no αB and a long loop between αC and αD helix.

NMR spectroscopy provides structural information of biological macromolecules in solution. This chapter presents the solution structure of the α-actinin-4 CH2 domain. The three

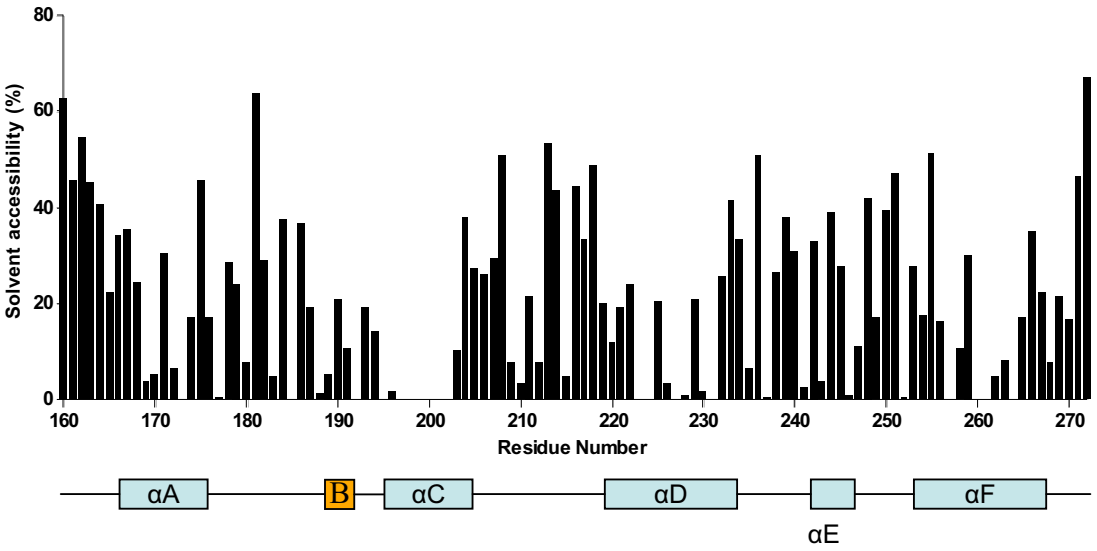


Figure 4.20 Solvent accessibility of residues within α-actinin4 CH2 domain. The relevant secondary structures are shown as cyan boxes on the bottom. The orange box indicates a 3_{10} helix. The diagram was generated using MOLMOL (Koradi, 1996).

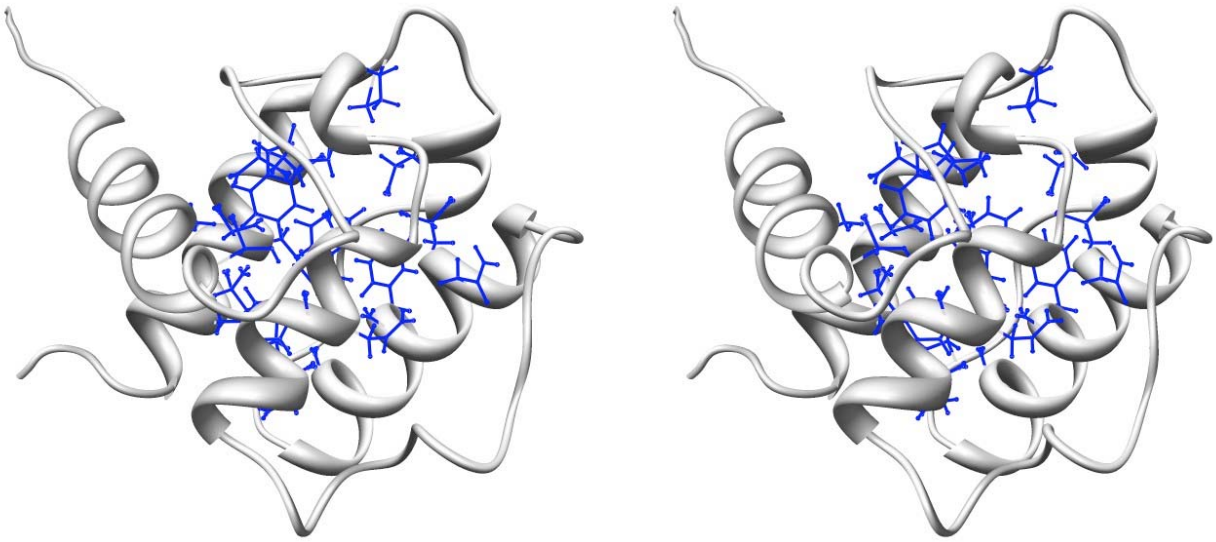


Figure 4.21 The ribbon presentation of α-actinin-4 CH2 domain in stereo along with the side chains participated in the hydrophobic core. The side chains with 0% solvent accessibility are indicated in blue. The stereo image was generated by Chimera software (Pettersen, 2004).

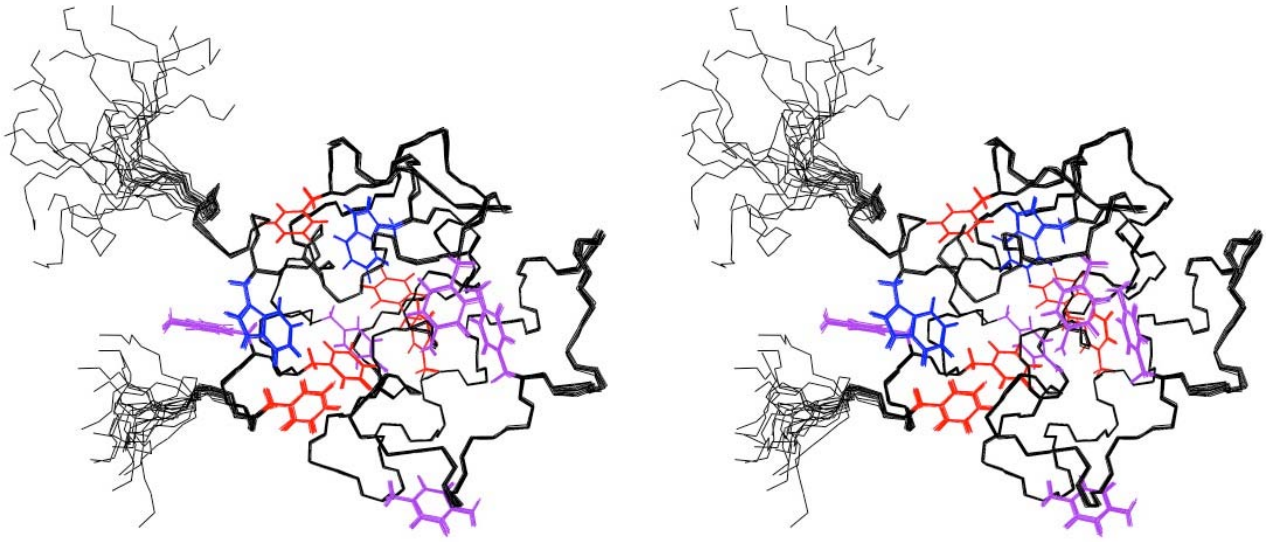


Figure 4.22 The ensemble of 19 structures of α-actinin-4 CH2 domain along with the side chains of aromatic residues. The side chain of Trp, Tyr and Phe are indicated in blue, purple and red, respectively. The stereo image was generated by Chimera software (Pettersen, 2004).

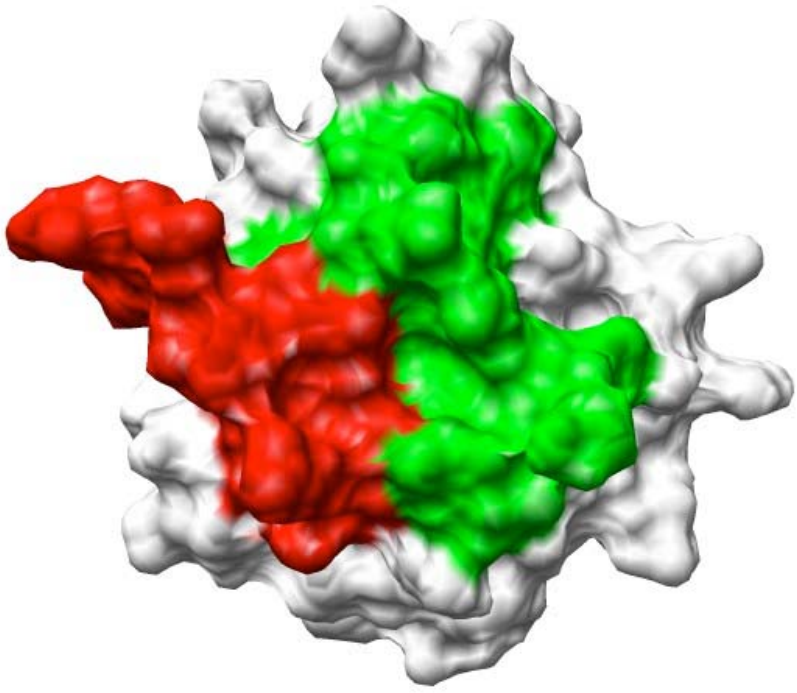


Figure 4.23 The surface structure of α-actinin-4 CH2 domain along with the putative F-actin binding site and PIP2 binding site. The ABS-3 and the region of PIP2 binding are indicated in red and green, respectively. The image was generated by Chimera software (Pettersen, 2004).

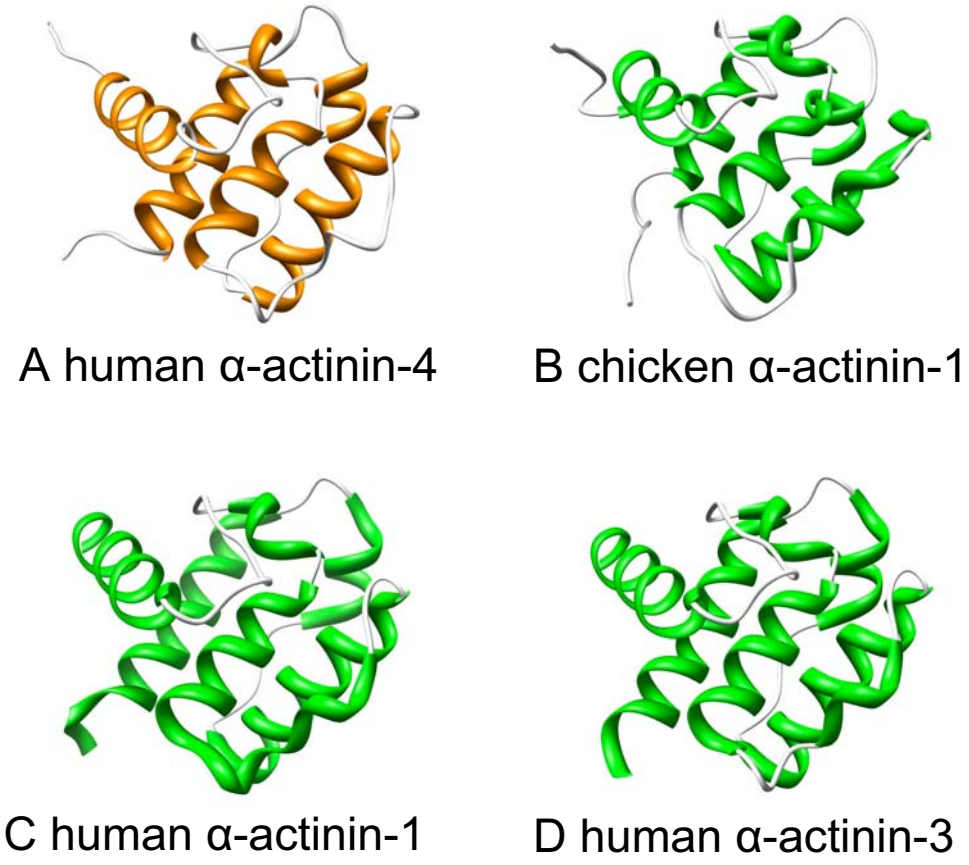
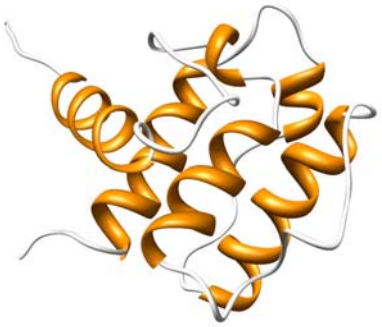
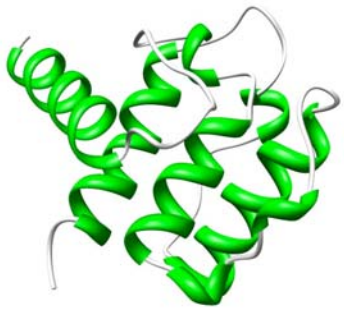


Figure 4.24 The three-dimensional structure comparison of the α-actinin family. The diagram shows the structure of the CH2 domains from human α-actinin-4 (A), chicken α-actinin-1 (B), human α-actinin-1 (C) and human α-actinin-3 (D). The structure of α-actinin-4 is indicated in orange and other members of α-actinin family are indicated in green. The image was generated by Chimera software (Pettersen, 2004). PDB codes are: chicken α-actinin-1, 1SJJ; human α-actinin-1, 1EYN; human α-actinin-3, 1WKU.

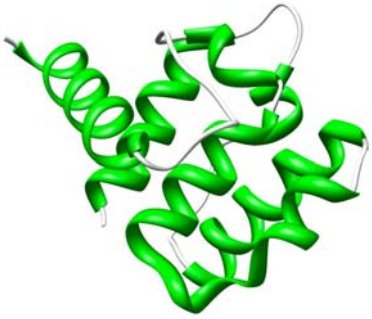
Figure 4.25 The three-dimensional structure comparison of the α-actinin-4 CH2 domain with other members of spectrin superfamily. The diagram shows the structure of the CH2 domains from human α-actinin-4 (A), human spectrin (B), human dystrophin (C), human utrophin (D), human plectin (E) and human fimbrin (F). The structure of the α-actinin-4 CH2 domain is indicated in orange and the CH2 domain from other members of spectrin superfamily is indicated in green. The image was generated by Chimera software (Pettersen, 2004). PDB codes are: human spectrin, 1BKR; human dystrophin, 1DXX; human utrophin, 1QAG; human plectin, 1MB8; human fimbrin, 1AOA.



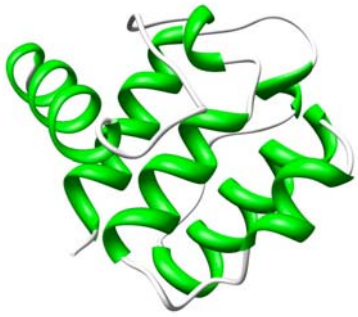
A human α-actinin-4



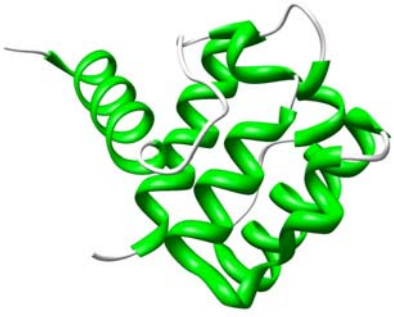
B human spectrin



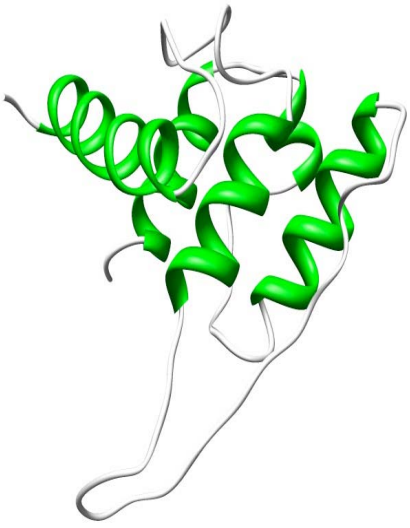
C human dystrophin



D human utrophin



E human plectin



F human fimbrin

dimensional structure of the α-actinin-4 CH2 domain comprises six α-helices and no β-sheet which is similar to that of other members of the spectrin superfamily. The CH2 domain is responsible for F-actin binding in combination with the CH1 domain and also provides a regulatory mechanism for the F-actin binding activity by the interaction with phosphoinositides. The first N-terminal α-helix, referred to one of the F-actin binding sites, shows a high solvent accessibility implying a role in the binding of F-actin. However, the putative PIP2 binding residues, positive charge residues within the region between αA and αC helices, disperse in different orientations, even one of them is buried in the hydrophobic core. Therefore, the location of the phosphoinositide binding is not well understood.

The CH2 domain is important for the regulation of the F-actin binding activity of α-actinin-4. The CH2 domain structure provides information in the investigation of the potential phosphoinositide binding site by a rapid NMR titration technique. The interaction between the CH2 domain and phosphoinositides was investigated and described in the next chapter.

CHAPTER 5

Regulation of the F-actin binding activity of α-actinin-4 by phosphoinositides

5.1 Introduction

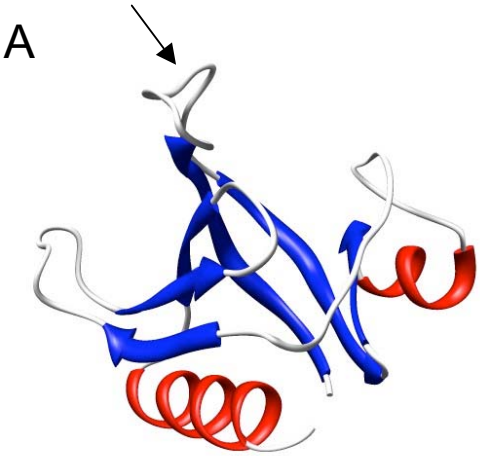
Phosphoinositides play a crucial role not only in producing secondary messengers, such as inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), but also in regulating a variety of cellular functions including cytoskeleton reorganisation and membrane trafficking. As described in section 1.2.2.3, PIP2 is an essential regulator in actin polymerisation and depolymerisation through its action on actin regulatory proteins, such as profilin, cofilin, gelsolin and α-actinin (Kuhlman *et al.*, 1992). Apart from α-actinin, the function of these actin regulatory proteins could be inhibited by the interaction with PIP2. However, the effect of the interaction of PIP2 with α-actinin is controversial. Fukami *et al.* have reported that PIP2 dramatically increased the F-actin bundling activity of smooth muscle α-actinin (Fukami *et al.*, 1992). However, Fraley *et al.* have reported that both PIP2 and PIP3 inhibit the F-actin binding and bundling activity of α-actinin *in vitro* (Fraley *et al.*, 2003).

The PIP2 binding site on the CH2 domain has been identified from chicken skeletal muscle α-actinin by Fukami *et al.* in 1996. α-actinin bound PIP2 was digested with α-chymotrypsin or lysylendopeptidase and detected using a dot-blot assay with a specific PIP2 antibody. The results indicated that the PIP2 binding site is located on the CH2 domain of α-actinin (Fukami *et al.*, 1996). The amino acid sequence of this putative PIP2 binding region is highly conserved and this region comprises residues 177-193 (TAPYRNVNIQNFHLSWK) (the residue numbers correspond to that of human α-actinin-4), mapping to the loop connecting α1′ and α2′ helices of the CH2 domain. It has been proposed that the negatively charged inositol head group of phosphoinositide interacts with the positively charged amino acid residues within the region 177-193 and the acyl chains of phosphoinositides are in contact with the hydrophobic region of the CH2 domain. Therefore, three residues, Arg 181, His 189 and Lys 193, have been predicted to be involved in the interaction with the inositol head group of phosphoinositide. Fukami *et al.* have synthesised two PIP2 binding peptides in which basic residues at position 181 and 193 have been replaced by Ile, giving R181I and K193I, to test the charged interactions between the inositol head group and basic residues by using the phospholipase C inhibition assay (Fukami *et al.*, 1996). The results suggested that the basic residues Arg 181 and Lys 193 play important roles in the interaction between PIP2 and the α-actinin PIP2 binding site (Fukami *et al.*, 1996). Fraley *et al.* also generated three

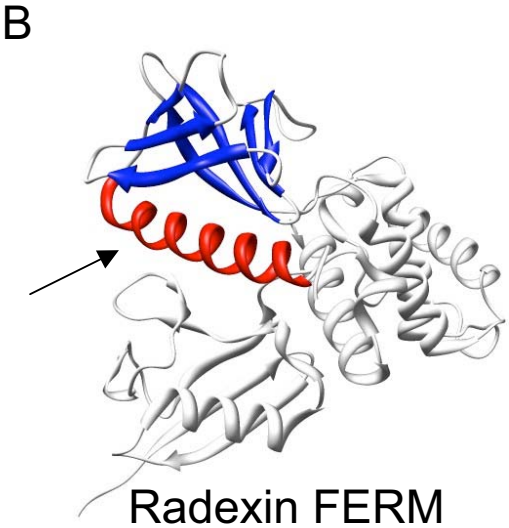
human α-actinin-1 mutants, K181I, K193I and a triple mutant K181I/H189L/K193I, tested phosphoinositide (including PIP2 and PIP3) binding in a protein-lipid overlay assay and showed that all these mutants have reduced PIP2 or PIP3 binding ability (approximately 40%, 30% and 70% for K181I, K193I and K181I/H189L/K193I, respectively) (Fraley *et al.*, 2003). These results suggest that the positively charged residues R/K181 and K193 play important roles in the interaction between phosphoinositides and the CH2 domain of α-actinin. The three-dimensional structures of the CH2 domain have been reported for human α-actinin-1 (Borrego-Diaz *et al.*, 2006), human α-actinin-3 (Franzot *et al.*, 2005) and chicken α-actinin (Liu *et al.*, 2004), which is similar to that of human α-actinin-4 (as illustrated in chapter 4). In the crystal structure of α-actinin-3, Lys 193 is almost buried inside of the CH2 domain. Therefore, Franzot *et al.* predicted that three basic residues, Arg 175, Lys 181 and Arg 204 (two of them are outside the previously mapped region) might be a possible binding site for phosphoinositides (Franzot *et al.*, 2005).

In addition to the CH2 domain, other types of domain have been reported to bind phosphoinositides, including PH (Harlan *et al.*, 1994), FERM (Chishti *et al.*, 1998), PTB (Forman-Kay and Pawson, 1999), FYVE (Gaullier *et al.*, 1998), PX (Ellson *et al.*, 2001; Kanai *et al.*, 2001) and ENTH (Itoh *et al.*, 2001; Ford *et al.*, 2001) domains. The three-dimensional structures of these domains are shown in Figure 5.1. The PH (pleckstrin homology) domain was the first phosphoinositide binding domain discovered in 1994. The PH domain contains 100-120 amino acids and is found in a wide range of signaling proteins such as protein kinases, phospholipases and GTPases. PH domains in several proteins have been described to be involved in interactions with phosphoinositides, including Akt/PKB (Franke *et al.*, 1997; Klippel *et al.*, 1997), PLCδ1 (Garcia *et al.*, 1995; Lemmon *et al.*, 1995), dynamin (Salim *et al.*, 1996; Zheng *et al.*, 1996). The three-dimensional structure of the PH domain consists of seven β-strands and an α-helix forming the β-sandwich structure. This β-sandwich structure is formed by two nearly orthogonal β-sheets that are formed by the first four and the last three β-strands. The top of this barrel-like structure is capped by the C-terminal amphiphilic α-helix. The phosphoinositide binding site is located on the region between the β1-β2 and β3-β4 strands (Figure 5.1 A, indicated in arrow) and the consensus sequences are +XXXX+X++ (+ denotes Arg, Lys or His) as summarised by Janmey *et al.* in 1999 (Janmey *et al.*, 1999). The FERM (4.1-ezrin/radixin/moiesin) domains are composed

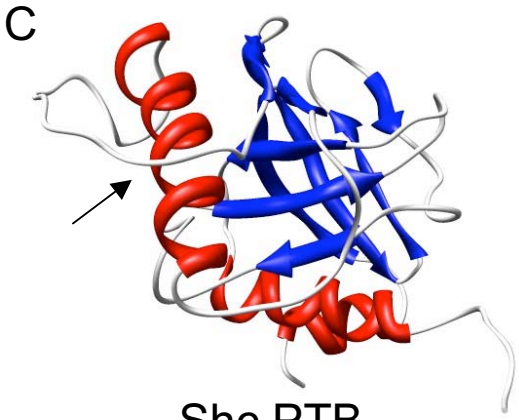
Figure 5.1 The three-dimensional structure of the phosphoinositide binding domains. The diagram shows the structure of the phosphoinositide binding domain from spectrin PH domain (A), radexin C-subdomain of FERM domain (B), shc PTB domain (C), EEA1 FYVE domain (D), $p40^{phox}$ PX domain (E) and espin ENTH domain (F). The α-helix and β-strand are indicated in red and blue, respectively. The phosphoinositide binding sites are indicated in arrows. The image was generated by Chimera software (Pettersen, 2004). PDB codes are: spectrin PH, 1BTN; radexin FERM, 1GC6; shc PTB, 1SHC; EEA1 FYVE, 1JOC; $p40^{phox}$ PX, 1H6H; epsin ENTH, 1H0A.



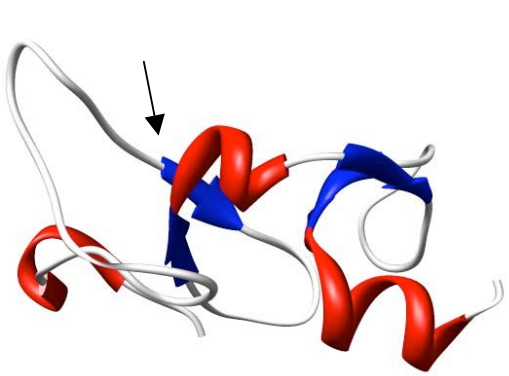
Spectrin PH



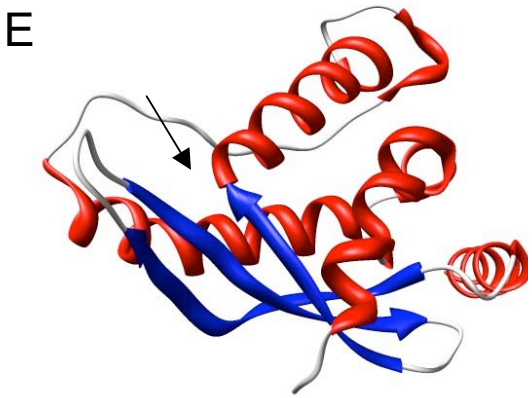
Radexin FERM



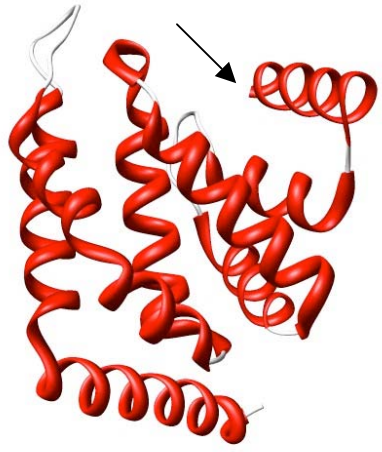
Shc PTB



EEA1 FYVE



p40^{phox} PX



Epsin ENTH

of approximately 310 amino acid residues and are present at the N-terminus of proteins that link the actin cytoskeleton to the plasma membrane, such as radixin (Hamada *et al.*, 2000). The FERM domain contains three subdomains, A, B and C, which interact with each other to fold into a compact globular shape. The three-dimensional structure of subdomain C is similar to that of the PH domain with a standard seven-stranded β-sandwich core and one α-helix. The phosphoinositide binding site is located at the centre of the basic cleft between subdomains A and C (Figure 5.1 B, indicated in arrow). This basic cleft consists of seven Lys and four Arg residues which are responsible for the interaction with the inositiol head group of phosphoinositides. The phosphoinositide binding site is located on a different site from those found in the PH domains, although subdomain C of the FERM domain fold is similar to the PH domain. Another phosphoinositide binding site has been identified by mutagenesis studies in ezrin FERM domain (Barret *et al.*, 2000), indicating that an additional binding site corresponds to the region where PH domains bind phosphoinositides. The PTB (phosphotyrosine binding) domain was originally identified as a domain that bound to a phosphorylated tyrosine within an NPXpY motif (Kavanaugh *et al.*, 1995) and are present in adaptor proteins which are involved in cell signaling, such as IRS-1, Shc (Wolf *et al.*, 1995), and Dab1 (Stolt *et al.*, 2004). The domain structure of the PTB is also similar to that of the PH domain with a standard seven-stranded β-sandwich core and one α-helix and contains a helix inserted between β1 and β2 strands for phosphoinositide binding (Figure 5.1 C, indicated in arrow) (Stolt *et al.*, 2003).

Some proteins contain clusters of basic residues which represent phosphoinositide binding motifs, for example, gelsolin (Feng *et al.*, 2001) and profilin (Chaudhary *et al.*, 1998). Gelsolin and profilin belong to the F-actin capping proteins and their biological function can be regulated by the interaction with PIP2. PIP2 triggers a dissociation of gelsolin and profilin from actin monomers to promote actin polymerisation (Lassing and Lindberg, 1985; Janmey and Stossel, 1987). The consensus sequence of the clusters of basic residues is similar to that of the phosphoinositide binding motif in the PH domain, +XXXX+X++ (+ denotes Arg, Lys or His).

The FYVE (the first letters of four proteins, Fab1p, YOTB, Vac1p and EEA1) domain is a region of approximately 80 amino acids and is found in these regulators that are involved in

membrane trafficking, such as EEA1 (Mu *et al.*, 1995) and actin polymerisation, such as Fgd1 (Olson *et al.*, 1996). The three-dimensional structure of FYVE domain consists of one α-helix and two β-sheets each formed by two β-strands. The phosphoinositide binding site is formed by two positively charged residues in the β1 strand and an additional positively charged residue in the β4 strand (Figure 5.1 D, indicated in arrow) and the consensus sequence, R/SR/KH/YH/RCR/K is highly conserved in all FYVE domains.

The PX (Phox homology) domain consists of 100-140 amino acids and is conserved among a variety of proteins associated with signaling as well as membrane trafficking, such as $p40^{phox}$ and $p47^{phox}$ (Ponting, 1996). The three-dimensional structure of PX domain shows an N-terminal three-stranded β-sheet, followed by an α-helical subdomain made up from four α-helices. The phosphoinositide binding pocket is located between the loop connecting the β1-β2 strands and one of the α-helices (Figure 5.1 E, indicated in arrow).

The ENTH (epsin N-terminal homology) domain is a region of approximately 140 amino acids and is conserved among proteins which associate with clathrin-mediated endocytosis, such as epsin1-3 (Kay *et al.*, 1999) and AP180 (Mao *et al.*, 2001). The three-dimensional structure of ENTH domain consists of six to eight α-helices. The phosphoinositide binding site is located on different positions of the ENTH domains from different proteins, but the interaction with phosphoinositide involves a charged interaction.

Although the three-dimensional structures of these domains are not conserved, all phosphoinositide binding consensus sequences from these phosphoinositide binding domains contain positively charged residues, Arg, Lys or His. These observations suggest proteins bind phosphoinositides through charged interactions.

5.2 Aims and approaches

Although the phosphoinositide binding site has been mapped in a region between helices αA and αC of the CH2 domain (Fukami *et al.*, 1996) and the critical residues, Lys 181, His 189 and Lys 193, have been identified to interact with phosphoinositides in chicken skeletal muscle α-actinin (Fraley *et al.*, 2003), the crystal structure evidence from α-actinin-3

indicated that residue Lys 193 was buried inside of the CH2 domain (Franzot *et al.*, 2005). Based on the NMR structure of human α-actinin-4 CH2 domain determined as part of chapter 4, residue His 189 is buried in the CH2 domain (Figure 5.2 and as shown in Figure 4.20, the relative solvent accessibility of residue His 189 is 5%). These observations imply that the phosphoinositide binding site in human α-actinin-4 could be different to that of chicken skeletal muscle α-actinin. The aim of this chapter is to confirm the phosphoinositide binding site on human α-actinin-4.

The phosphoinositide binding site on human α-actinin-4 CH2 domain was investigated using an NMR-based ^{1}H-^{15}N HSQC (heteronuclear single quantum coherence) ligand titration assay. The HSQC ligand titration assay is an ideal tool for assessing interactions between proteins and ligands. As described in section 4.2.1, each cross peak in the HSQC spectrum represents one amide group (NH-) in the backbone of the polypeptide chain (except proline) as well as the NH_2 groups of the side chains of Asparagine and Glutamine and the NH groups of the aromatic ring of Tryptophan. When a ligand is present, the chemical shift of amide groups that interact with the ligand will be changed due to their altered electronic environment. These perturbations can be used to identify the location of binding sites by mapping the significant shift perturbation on to a known structure. Site-directed mutagenesis was performed upon the critical residues that interact with phosphoinositides as determined by the HSQC ligand titration experiments. To determine if phosphoinositides regulate the F-actin binding activity of α-actinin-4, the F-actin binding activity of α-actinin-4 was investigated using the solid phase F-actin binding assay (referred to chapter 3).

5.3 Results

5.3.1 Sample Preparation for NMR Spectroscopy

Samples of the α-actinin-4 CH2 domain were generated according to the method described in section 2.2.2.1. For ^{1}H-^{15}N HSQC experiments, the ^{15}N-labeled CH2 domain was generated in minimal medium containing $^{15}NH_4Cl$ as a nitrogen source and prepared according to the method described in section 2.2.3.1.

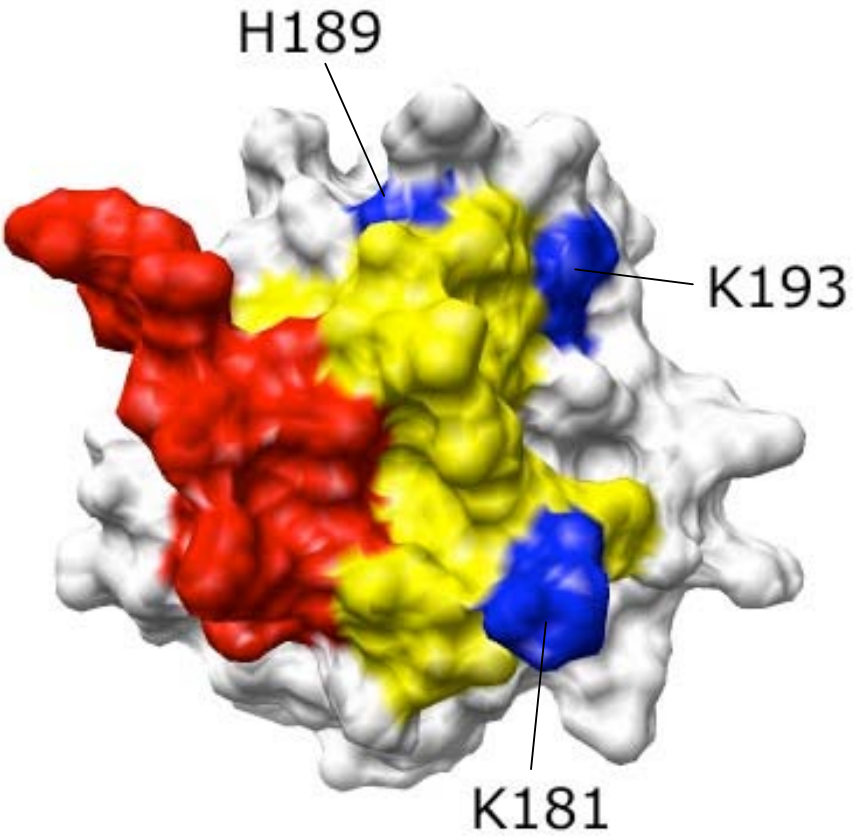


Figure 5.2 The surface structure of α-actinin-4 CH2 domain along with the F-actin binding site and PIP2 binding site. The ABS-3 from utrophin and dystrophin CH domains and the region of PIP2 binding identified by Fukami *et al* are indicated in red and yellow, respectively. The positively charged residues within the PIP2 binding region are indicated in blue. The image was generated by Chimera software (Pettersen, 2004).

5.3.2 NMR-based HSQC Ligand Titration Assay

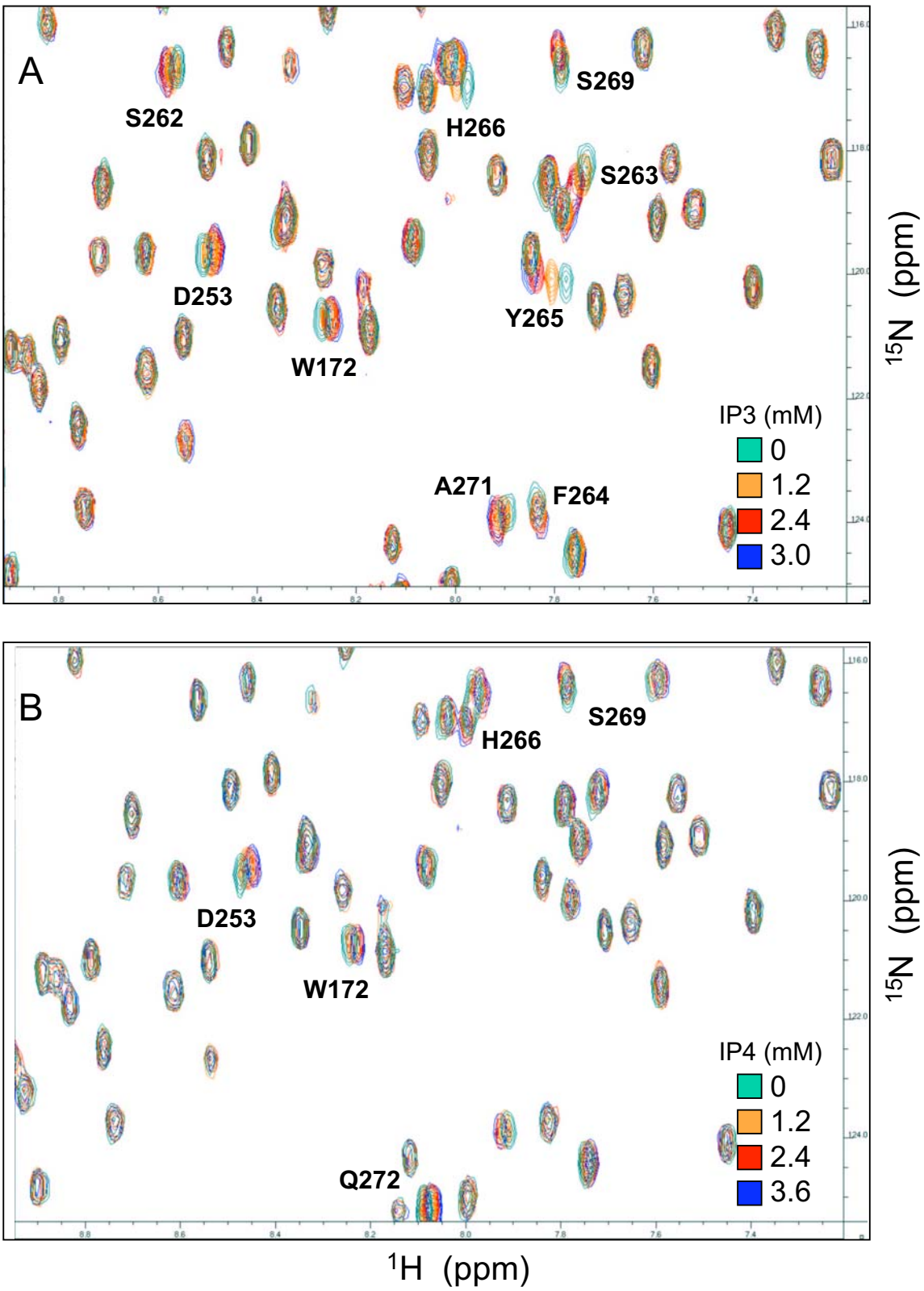
D-myo-Inositol-1,3,4,5-tetraphosphate (IP4), 1,2-dioleoyl-sn-glycerol (DAG), PtdIns-(4,5)-P2 (1,2-dipalmitoyl) (PIP2) and PtdIns-(3,4,5)-P3 (1,2-dipalmitoyl) (PIP3) were purchased from Cayman Chemical (USA). D-myo-Inositol-1,4,5-trisphosphate (IP3) was purchased from Sigma (USA). These chemicals used in the NMR experiments are water soluble with the maximum solubility of IP3 and IP4 of 100 mM, PtdIns(3,4,5)P3 of 10 mM and PtdIns(4,5)P2 and DAG of 1 mM.

The phosphoinositide binding site of the α-actinin-4 CH2 domain was monitored by means of a NMR-based HSQC ligand titration assay according to the method described in section 2.2.3.6. Briefly, 2D ^{1}H-^{15}N HSQC spectra of ^{15}N labeled samples were recorded in the absence and presence of increasing amounts of PIP2, PIP3 or DAG at 50, 100, 150, 200, 250 and 300 μM and IP3 or IP4 in 1.2, 2.4 and 3.6 mM. 2D ^{1}H-^{15}N HSQC experiments were performed on Varian Inova 600 spectrometer at 25°C using the ghsqc experiment from Varian. All spectra were processed using NMRPipe (Delaglio *et al.*, 1995) and analysed using CCPNMR (Vranken *et al.*, 2005).

5.3.3 The Inositol Binding Residues on the α-actinin-4 CH2 Domain

In order to investigate the binding site for the inositol head group of phosphoinositide (IP3 and IP4) on the CH2 domain, a series of 2D ^{1}H-^{15}N HSQC spectra were recorded in the absence and presence of IP3 or IP4. The overlaid 2D HSQC spectra with increasing concentrations of IP3 or IP4 are shown in Figure 5.3. The chemical shift variations in both ^{1}H and ^{15}N dimension on HSQC spectra between the absence and presence of the maximum concentration of IP3 and IP4 (3 mM for IP3 and 3.6 mM for IP4) are shown in Figure 5.4. For resonances that change during the titration, the observed cross peaks maintain intensity, but the chemical shift is a weighted average of the unbound and bound shifts. These changes indicate that the CH2 domain interacts with IP3 or IP4 with fast exchange kinetics on the NMR time scale. The threshold values used to interpret the significant chemical shift changes during ligand titration were values of $\delta_{mean} + \delta_{std}$ (Chen *et al.*, 2008; Chang *et al.*, 2006). In the IP3 titration analysis, the mean value of the chemical shift difference (δ_{mean}) for the 93 completely isolated peaks was 0.007 ppm and the standard deviation (δ_{std}) was 0.012 ppm in proton dimension, and δ_{mean} was 0.03 ppm and δ_{std} was 0.07 ppm in ^{15}N

Figure 5.3 Effect of inositiols addition on the ^{1}H-^{15}N HSQC spectra of the α-actinin-4 CH2 domain. The 0.2 mM CH2 domain was titrated with increasing concentration of IP3 (A) and IP4 (B) with 0 (green), 1.2 (orange), 2.4 (red) and 3.6 (blue) mM in 10 mM phosphate buffer containing 0.01% (w/v) NaN_3, pH 6.8. HSQC spectra were recorded on Varian Inova 600 spectrometer at 25°C as described in section 2.2.3.6. The diagram is shown selected region of the spectra.


A
S262
S269
H266
S263
D253
Y265
W172
A271
F264
IP3 (mM)
0
1.2
2.4
3.0
B
S269
H266
D253
W172
Q272
IP4 (mM)
0
1.2
2.4
3.6
15N (ppm)
15N (ppm)
1H (ppm)

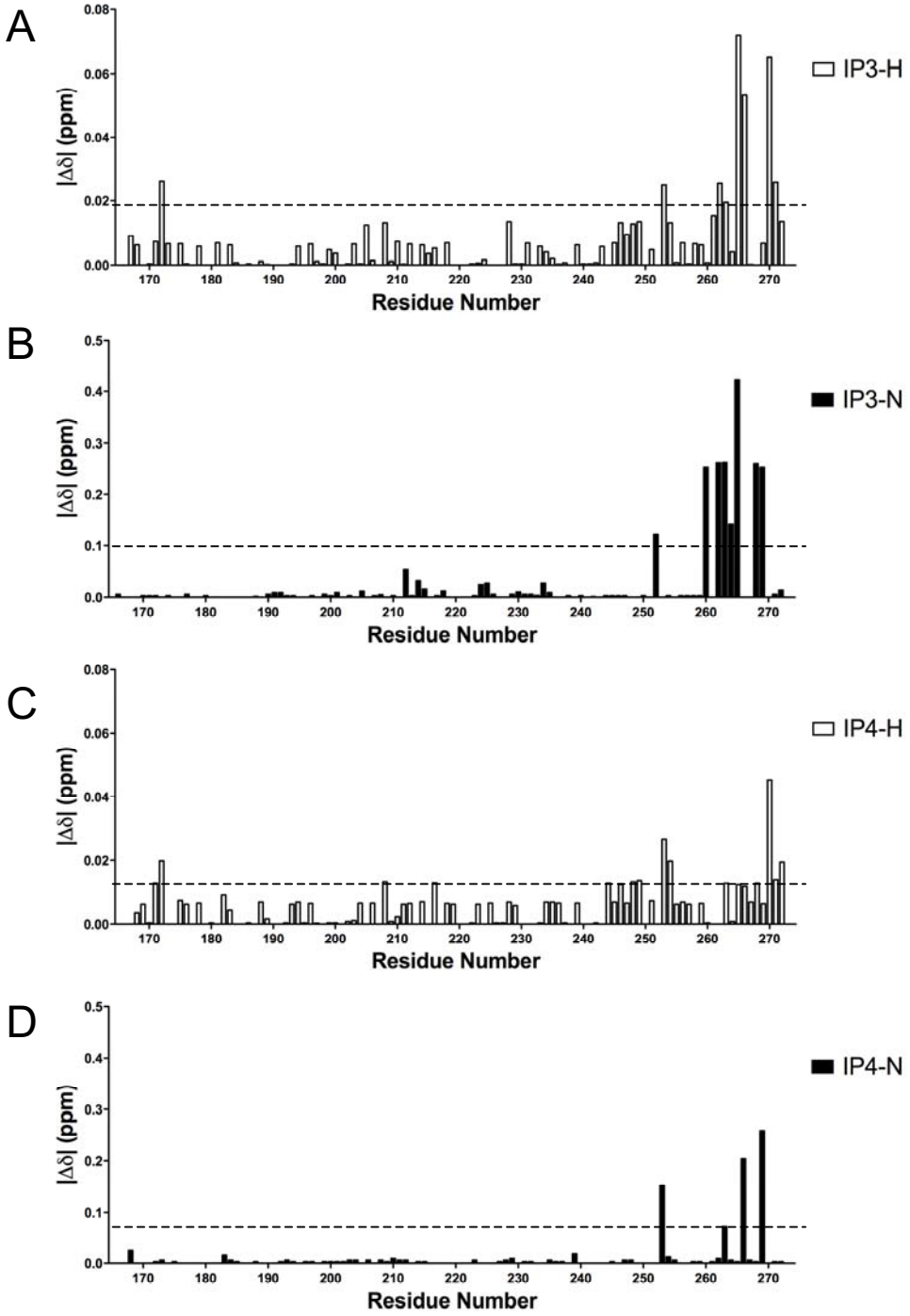


Figure 5.4 Plot of chemical shift perturbation in the α-actinin-4 CH2 domain with inositiols (IP3 or IP4). The magnitude of the ^{1}H (A and C) and ^{15}N (B and D) chemical changes of α-actinin-4 CH2 domain backbone amide residues induced upon the addition of 3 mM IP3 (A and B) or 3.6 mM IP4 (C and D). The threshold ($\delta_{mean} + \delta_{std}$) used to define perturbed residues was set at values of 0.019 ppm for IP3 and 0.015 ppm for IP4 in ^{1}H dimension or 0.1 ppm for IP3 and 0.05 ppm for IP4 in ^{15}N dimension, respectively (denoted by horizontal dashed lines).

dimension. The significant chemical shift changes of backbone amides were set at values of either 0.019 ppm in ^{1}H dimension or 0.1 ppm in ^{15}N dimension and mapped on residues Trp 172, Asp 253, Val 261, Ser 262, Ser 263, Phe 264, Tyr 265, His 266, Ser 269, Gly 270 and Ala 271. As shown in Figure 5.5 A, the structural observations implied that a region mapped on the last C-terminal α-helix was predicted to be involved in IP3 binding. In the IP4 titration analysis, δ_{mean} and δ_{std} based on the 92 residues was 0.006 ppm and 0.007 ppm in proton dimension, and 0.01 ppm and 0.04 ppm in ^{15}N dimension, respectively. The residues implicated to be involved in IP4 binding are Leu 171, Trp 172, Glu 244, Thr 249, Asp 253, Glu 254, Ser 263, Trp 265, His 266, Phe, 268, Ser 269, Gly 270, Ala 271 and Gln 272 (The threshold used to define perturbed residues during IP4 titration was set at values of either 0.013 ppm in ^{1}H dimension or 0.05 ppm in ^{15}N dimension). Similar to that of the IP3 titration, a presumptive IP4 binding region was mapped on C-terminal α-helix of the CH2 domain (Figure 5.5 B).

It is possible to use the concentration dependent changes in chemical shifts to determine dissociated constants (Kd) for ligands. The apparent Kd values for binding of IP3 and IP4 to the CH2 domain were calculated using the residues with significant chemical shift changes (greater than values of $\delta_{mean} + \delta_{std}$ in ^{1}H or ^{15}N dimension) for ligands. As shown in Figure 5.6, the Kd values for the interaction between the CH2 domain and IP3 and IP4 are similar with 3.7 ± 0.6 mM for IP3 and 3.4 ± 0.7 mM for IP4. In addition, the magnitude of chemical shift changes between the absence and presence of the maximum concentration of IP3 (3.0 mM) was larger than that of IP4 (3.6 mM) (Figure 5.4).

5.3.4 The Phosphoinositide Binding Residues on the α-actinin-4 CH2 Domain

In order to investigate the PIP2 and PIP3 binding sites on the CH2 domain, a series of 2D ^{1}H-^{15}N HSQC spectra were recorded in the absence and presence of PIP2 or PIP3. The overlaid 2D HSQC spectra with various concentrations of PIP2 or PIP3 are shown in Figure 5.7. Six backbone amide resonances (Lys 166, Glu 167, Leu 169, Leu 170, Trp 172 and Gly 270) undergo chemical shift changes of more than 0.02 ppm in ^{1}H dimension upon addition of 300 μM PIP2 or PIP3. These observations suggested that these residues are involved in the interaction with phosphoinositides. However, these HSQC spectra were not used to calculate a dissociation constant in the presence of PIP2 or PIP3, since the intensity of all

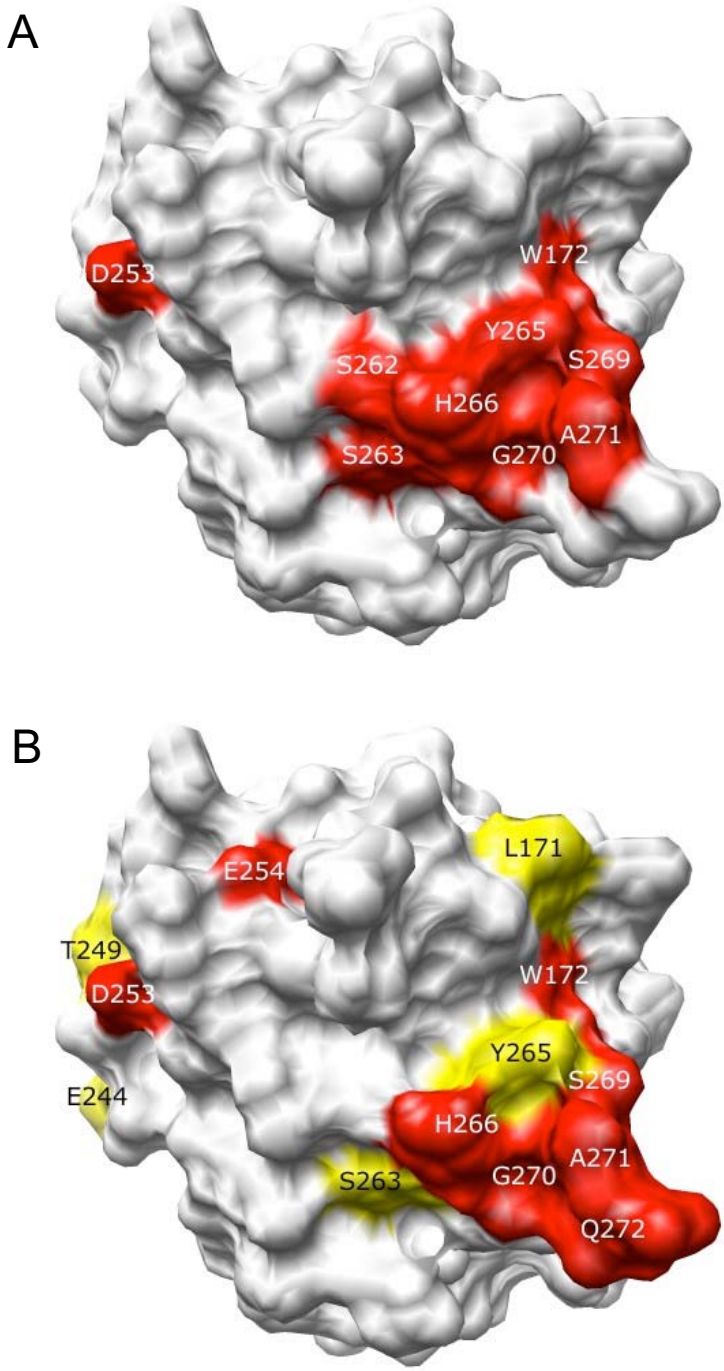


Figure 5.5 Surface diagrams highlighting the residues implicated in inositol binding. The amino acid residues implicated in IP3 (A) and IP4 (B) binding are shown in red (the chemical shift changes greater than $\delta_{mean} + 2X\ \delta_{std}$) and yellow (the chemical shift changes greater than $\delta_{mean} + \delta_{std}$), respectively, and named. The numbering is consistent with the number for full length α-actinin-4. The images were generated by Chimera software (Pettersen, 2004).

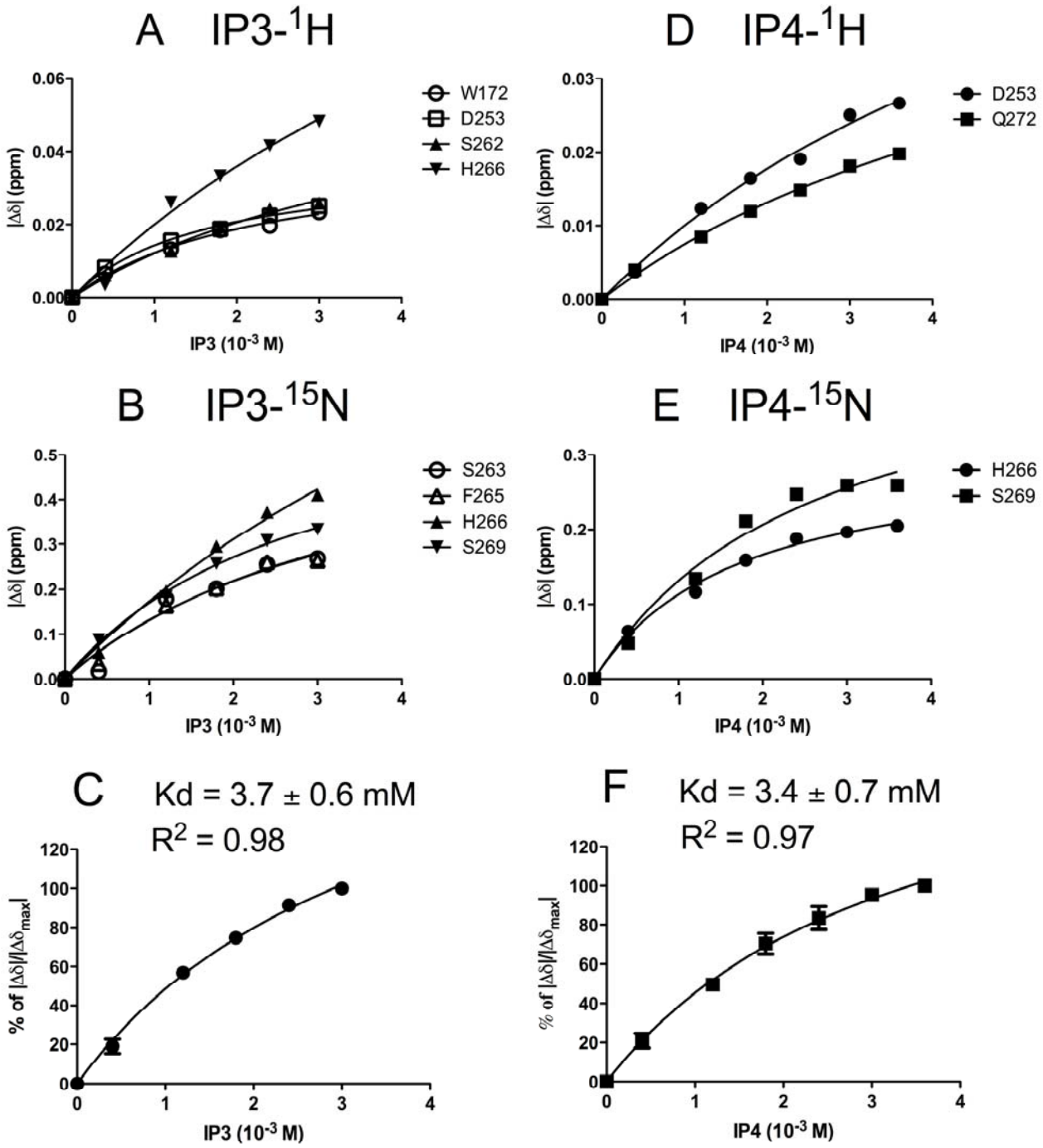
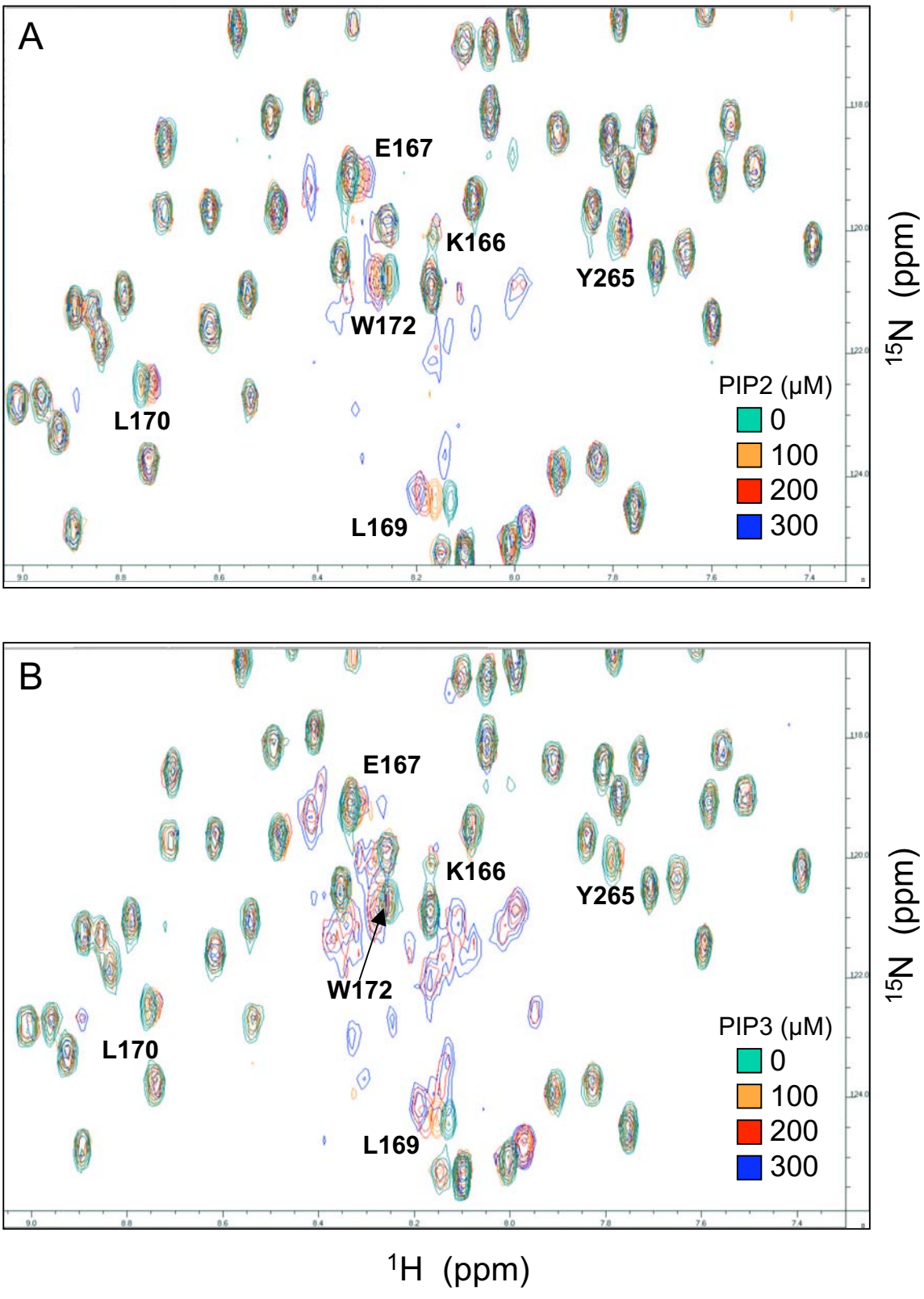


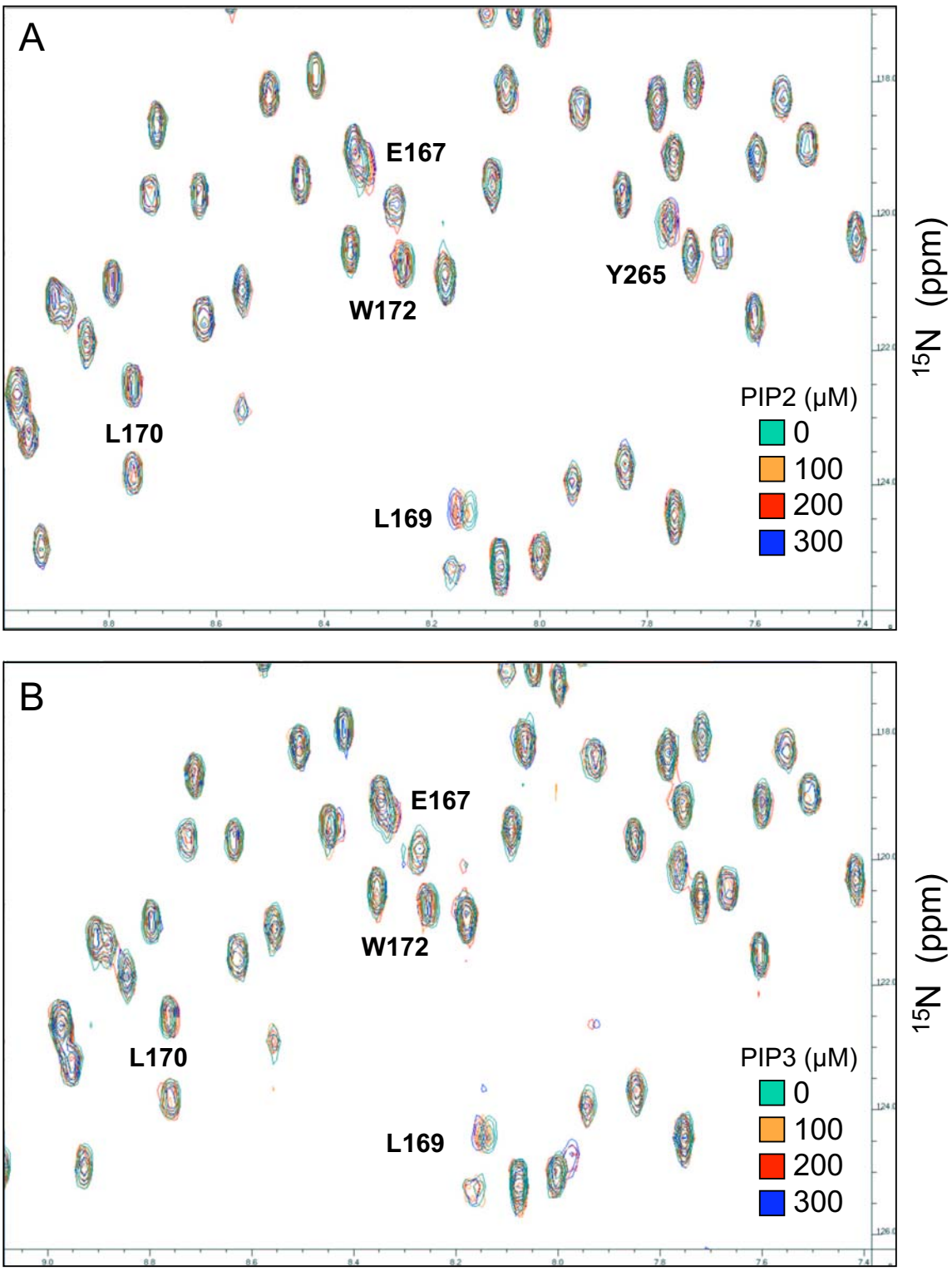
Figure 5.6 Binding isotherm of inositols to the α-actinin-4 CH2 domain. The ^{15}N-labeled CH2 domain (0.2 mM) was titrated with increasing concentration of IP3 (A, B and C) and IP4 (D, E and F) in 10 mM phosphate buffer containing 0.01% (w/v) NaN_3, pH 6.8. HSQC spectra were recorded on Varian Inova 600 spectrometer at 25°C and the Kd values were calculated according to the method described in section 2.2.3.6. The chemical shift titration data used to determine the dissociation constant were shown in (A) and (B) for IP3 and (D) and (E) for IP4. The average titration curves of inositols to the CH2 domain were shown in (C) for IP3 and (F) for IP4.

Figure 5.7 Effect of phosphoinositides addition on the ^{1}H-^{15}N HSQC spectra of the α-actinin-4 CH2 domain. The 0.2 mM CH2 domain was titrated with increasing concentration of PIP2 (A) and PIP3 (B) with 0 (green), 100 (orange), 200 (red) and 300 (blue) μM in 10 mM phosphate buffer containing 0.01% (w/v) NaN_3, pH 6.8. ^{1}H-^{15}N HSQC spectra were recorded on Varian Inova 600 spectrometer at 25°C according to the method described in section 2.2.3.6. The diagram is shown selected region of the spectra.


A
E167
K166
Y265
W172
L170
L169
PIP2 (μM)
0
100
200
300
^{15}N (ppm)
B
E167
K166
Y265
W172
L170
L169
PIP3 (μM)
0
100
200
300
^{15}N (ppm)
^{1}H (ppm)

cross peaks decreased as the concentration of phosphoinositides increased. The exact number of cross peaks with chemical shift changes is difficult to quantify, due to their weak intensity, coupled with the severe spectral overlap. Phosphoinositides generally form micelles in aqueous solution (Flanagan *et al.*, 1997; Sugiura, 1981). When proteins interact with phosphoinositide micelles, the protein may form a large protein-micelle complex, resulting in broadened and low intensity cross peaks shown on the HSQC spectra. Therefore, the dissociation constant for binding of PIP2 or PIP3 is difficult to estimate using these HSQC spectra. In order to overcome this problem, the CH2 domain was dissolved in 1X TBS buffer, pH 7.0 to replace the sodium phosphate buffer in NMR experiments. As shown on Figure 5.8, clear cross peaks were shown on HSQC spectra. However, the magnitude of the chemical shift changes decreased. Nevertheless, the dissociation constant can be estimated using HSQC spectra recorded from protein samples dissolved in TBS buffer. The threshold used to interpret the significant chemical shift changes during phosphoinositide titration were set at values of $\delta_{mean} + \delta_{std}$. The magnitude of the ^{1}H chemical shift changes of the CH2 domain backbone amide residues induced upon the addition of the maximum concentration of PIP2 or PIP3 (300 μM) are shown in Figure 5.9 (The deviations in ^{15}N dimension did not show significant changes). In the PIP2 titration analysis, δ_{mean} and δ_{std} based on the 92 residues was 0.001 ppm and 0.004 ppm in proton dimension, respectively. The residues implicated to be involved in PIP2 binding are Glu 167, Gly 168, Leu 169, Leu 170, Trp 172, Ser 263, Trp 265 and Phe 268 (The threshold used to define perturbed residues during PIP2 titration was set at values of 0.005 ppm in ^{1}H dimension) (Figure 5.10 A). Within the PIP3 titration analysis, δ_{mean} and δ_{std} were calculated based on the 90 residues with 0.001 ppm and 0.003 ppm in ^{1}H dimension, respectively. The significant chemical shift deviations of backbone amides were set at values of 0.004 ppm in ^{1}H dimension and mapped on residues Glu 167, Gly 168, Leu 169, Leu 170, Trp 172, Tyr 265, Ser 269 and Ala 271 (Figure 5.10 B). The amino acid residues implicated in PIP2 or PIP3 binding are similar and are mapped on the first α helix of the CH2 domain. The first α helix of the CH2 domain is highly conserved in the spectrin superfamily and has been reported to be associated with F-actin binding from dystrophin and utrophin (Levine *et al.*, 1992; Winder *et al.*, 1995) (details referred to section 1.2.4.1). These results implied that the F-actin binding activity of α-actinin-4 would be regulated by phosphoinositides.

Figure 5.8 Effect of phosphoinositides titration on the ^{1}H-^{15}N HSQC spectra of the α-actinin-4 CH2 domain. The 0.2 mM CH2 domain was titrated with increasing concentration of PIP2 (A) and PIP3 (B) with 0 (green), 100 (orange), 200 (red) and 300 (blue) μM in 1X TBS containing 0.01% (w/v) NaN_3, pH 7.0. HSQC spectra were recorded on Varian Inova 600 spectrometer at 25°C according to the method described in section 2.2.3.6. The diagram is shown selected region of the spectra.


A
E167
Y265
W172
L170
L169
PIP2 (μM)
0
100
200
300
^{15}N (ppm)
B
E167
W172
L170
L169
PIP3 (μM)
0
100
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^{15}N (ppm)
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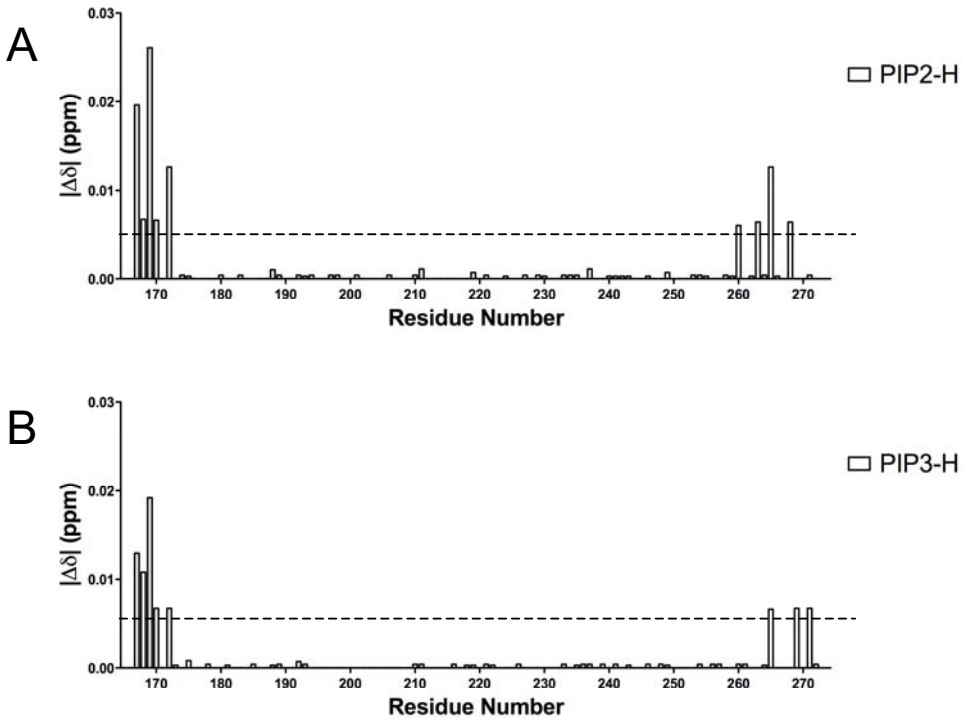


Figure 5.9 Plot of chemical shift perturbation in the α-actinin-4 CH2 domain with phosphoinositides (PIP2 or PIP3). The CH2 domain was dissolved in 1X TBS, pH 7.0 for phosphoinositides titration experiments. The perturbation in ^{1}H dimension of α-actinin-4 CH2 domain backbone amide residues induced upon the addition of PIP2 (A) or PIP3 (B) in 300 μM. The threshold ($\delta_{mean} + \delta_{std}$) used to define perturbed residues, denoted by horizontal dashed lines, was set at values of 0.005 ppm and 0.004 ppm in ^{1}H dimension within PIP2 and PIP3 titration, respectively.

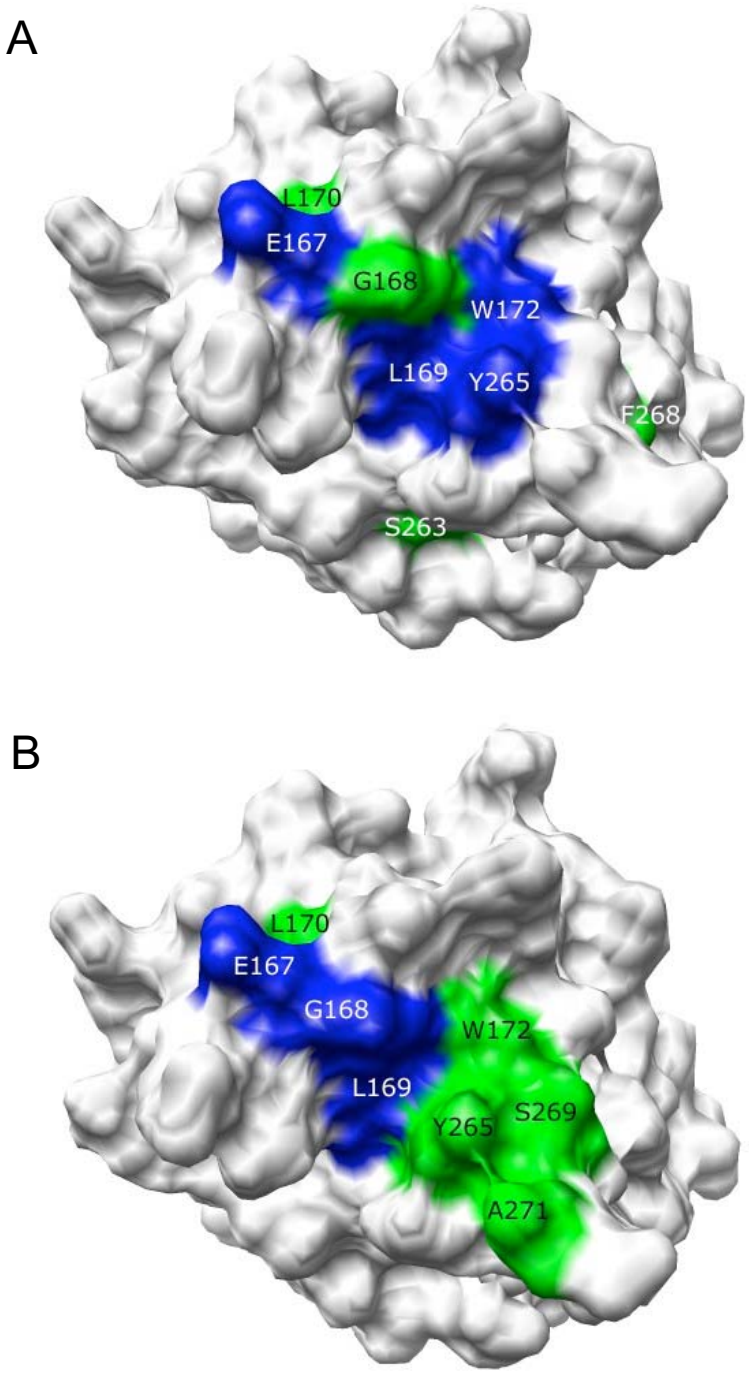


Figure 5.10 Surface diagram highlighting the residues implicated in PIP2 or PIP3 binding. The residues implicated in PIP2 (A) and PIP3 (B) binding are shown in blue (the chemical shift changes greater than $\delta_{mean} + 2X\ \delta_{std}$) and green (the chemical shift changes greater than $\delta_{mean} + \delta_{std}$) and named. The numbering is consistent with the number for full length α-actinin-4. The images were generated by Chimera software (Pettersen, 2004).

The apparent Kd values for binding of PIP2 or PIP3 to the CH2 domain were calculated using the chemical shift changes from residues, Glu 167, Leu 169, Leu 170, Trp 172 and Tyr 265 according to the method described in section 2.2.3.6 (Figure 5.11). The Kd values for interactions between the CH2 domain and PIP2 or PIP3 are 109 ± 16 μM and 127 ± 25 μM, respectively. These results indicated that the binding affinity of PIP2 to the CH2 domain is equal to that of PIP3 binding. These results were consistent with a previous report for skeletal muscle α-actinin to PIP2 and PIP3 binding (Fraley *et al.*, 2003). In addition, these results implied that the phosphate group at position 3 of the inositol within PIP3 (a major difference between the chemical structure of PIP2 and PIP3) might contact a region out side of the CH2 domain.

Compared with the interaction of phosphoinositides (PIP2 and PIP3) and inositiols (IP3 and IP4) with the CH2 domain, the binding affinity of phosphoinositide to the CH2 domain was shown to be 30-fold stronger than that of inositiol to the CH2 domain. It is speculated that the acyl chains of phosphoinositides might be important for their interaction with the CH2 domain. In order to verify the specificity of the acyl chains of phosphoinositides, diacylglycerol (DAG) was used as a ligand with the CH2 domain on HSQC titration analysis. As shown in Figure 5.12, cross peaks in the HSQC spectra did not change with increasing amounts of DAG. These results suggested that the inositiol head group of phosphoinositides is essential to bind the CH2 domain.

5.3.5 Inhibiting the F-actin Binding Activity of α-actinin-4 by Phosphoinositides

According to the results described in section 5.3.4, the phosphoinositide (both PIP2 and PIP3) binding region is located on the first α-helix of the CH2 domain. This region, called ABS-3, has been implicated in F-actin binding from utrophin and dystrophin (Levine *et al.*, 1992; Fabbrizio *et al.*, 1993). Therefore, it is proposed that the F-actin binding activity of α-actinin-4 could be influenced by phosphoinositides. As described in section 1.3.1.2.3, regulation of the F-actin binding activity of α-actinin by phosphoinositides is controversial. Fukami *et al.* have reported that PIP2 significantly increased the F-actin cross-linking activity of smooth muscle α-actinin using the F-actin viscosity assay. Fraley *et al.* have stated that, however, phosphoinositides (both PIP2 and PIP3) inhibited the F-actin bundling activity of skeletal muscle α-actinin using a low speed sedimentation assay. In order to

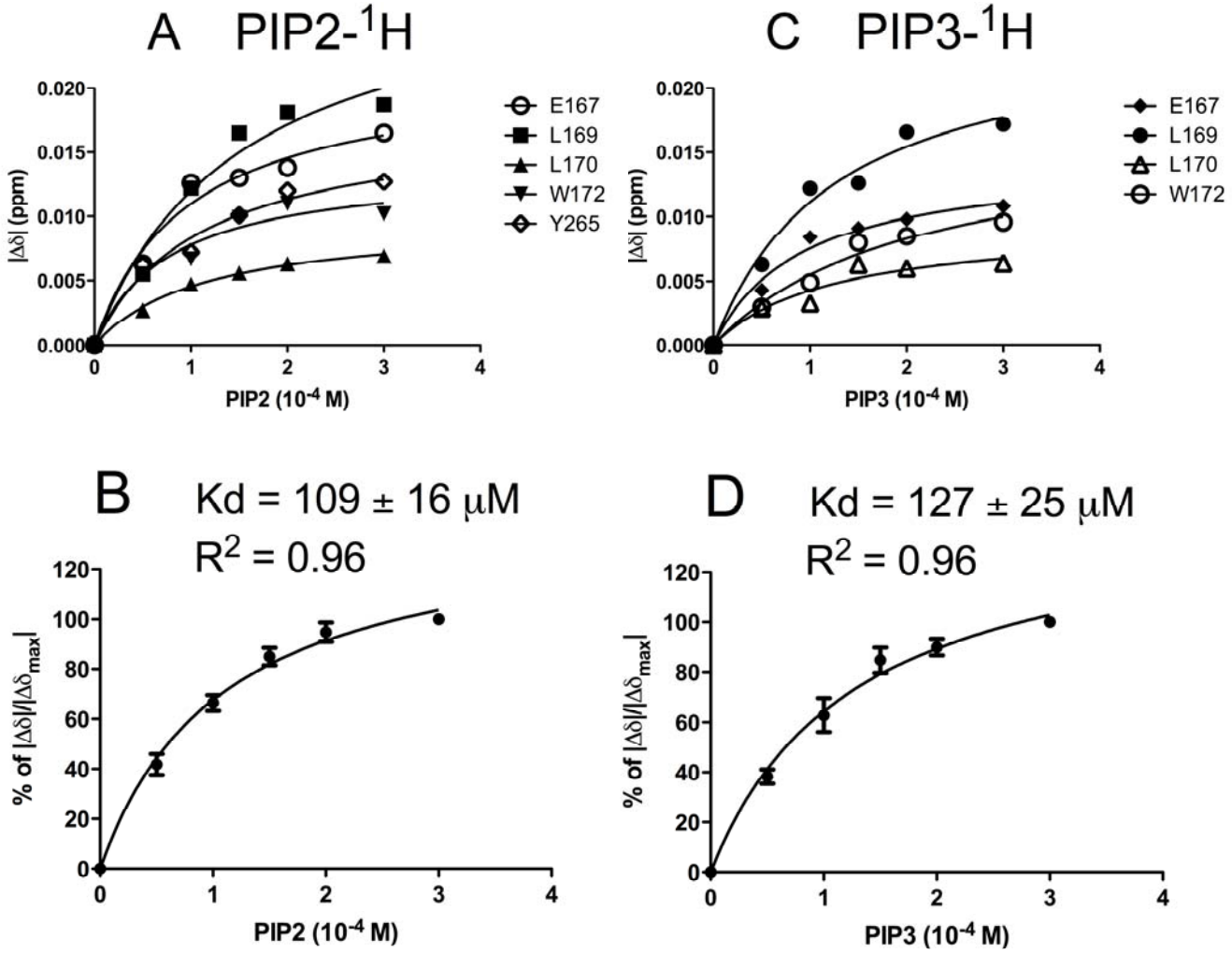


Figure 5.11 Binding isotherm of phosphoinositides to the α-actinin-4 CH2 domain. The 0.2 mM ^{15}N-labeled CH2 domain was titrated with increasing concentration of PIP2 (A and B) and PIP3 (C and D) in 1X TBS containing 0.01% (w/v) NaN_3, pH 7.0. HSQC spectra were recorded on Varian Inova 600 spectrometer at 25°C and the Kd values were estimated according to the method described in section 2.2.3.6. The chemical shift variation data used to determine the dissociation constant were shown in (A) for PIP2 and (C) for PIP3. The average titration curves of phosphoinositides to the CH2 domain were shown in (B) for PIP2 and (D) for PIP3.

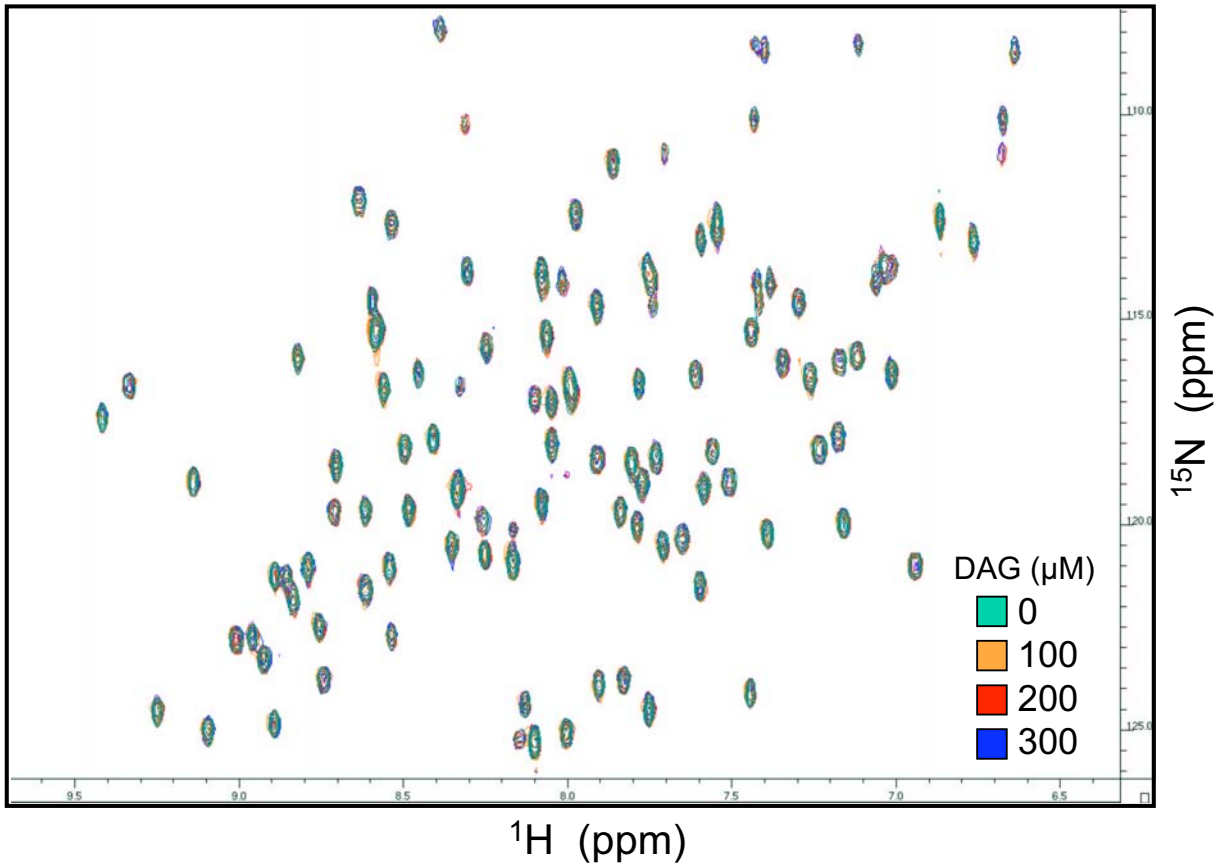


Figure 5.12 Effect of DAG titration on the ^{1}H-^{15}N HSQC spectra of the α-actinin-4 CH2 domain. The 0.2 mM CH2 domain was titrated with increasing concentration of DAG with 0 (green), 100 (orange), 200 (red) and 300 (blue) µM in 1X TBS containing 0.01% (w/v) NaN_3, pH 7.0. HSQC spectra were recorded on Varian Inova 600 spectrometer at 25°C according to the method described in section 2.2.3.6. The diagram is shown selected region of the spectra.

assess the role of phosphoinositides in the regulation of F-actin binding, the F-actin binding activity of α-actinin-4 was investigated in the absence and presence of phosphoinositides (PIP2 or PIP3) using the solid phase F-actin binding assay. Full length α-actinin-4 was generated according to the method described in section 2.2.2.1 and labeled with Fluorescein in the DOL range 2.0 to 2.5 according to the method described in section 2.2.2.8. The Fluorescein labeled α-actinin-4 was pre-incubated with the specified concentration of PIP2 or PIP3 and incubated for 15 minutes at room temperature and then added into the F-actin coated wells for 2 hours at room temperature (for details see section 2.2.2.9). Using the solid phase F-actin binding assay, the inhibition curves were generated and the IC_{50} values were calculated by non-linear regression analysis to be 77.0 ± 3.9 μM for PIP2 and 46.1 ± 1.8 μM for PIP3 (Figure 5.13 A). The results indicated that both PIP2 and PIP3 inhibited the F-actin binding activity of α-actinin-4 in a concentration dependent manner, with PIP3 being more effective.

In order to investigate which part of phosphoinositides (inositiol head group or acyl chains) played an important role in inhibiting F-actin binding to α-actinin-4, DAG and IP3 were used to assess the F-actin binding activity of α-actinin-4 using the solid phase F-actin binding assay. As shown in Figure 5.13 B and C, the F-actin binding activity of α-actinin-4 was not influenced by either DAG or IP3 at concentrations as high as 300 μM for DAG and 4 mM for IP3 (Figure 5.13 B and C). The lack of DAG inhibition of F-actin binding was not surprising, because DAG did not interact with the CH2 domain (as described in section 5.3.4). As shown in section 5.3.3, IP3 binds the C-terminal α- helix of the CH2 domain which does not overlap in ABS-3 (the N-terminal α-helix of the CH2 domain). It is most likely the reason that α-actinin-4 bound IP3 does not influence its F-actin binding activity. These results implied the acyl chains of phosphoinositides were important for inhibition. These results were consistent with a previous report for skeletal muscle α-actinin to PIP2 and PIP3 binding (Fraley *et al.*, 2003).

5.3.6 Site-directed Mutagenesis of Important Phosphoinositide Binding Residues on the CH2 Domain

As described in section 5.3.3 and 5.3.4, residues involved in the interaction between the CH2 domain and phosphoinositides (PIP2 or PIP3) are Glu 167, Gly 168, Leu 169, Trp 172

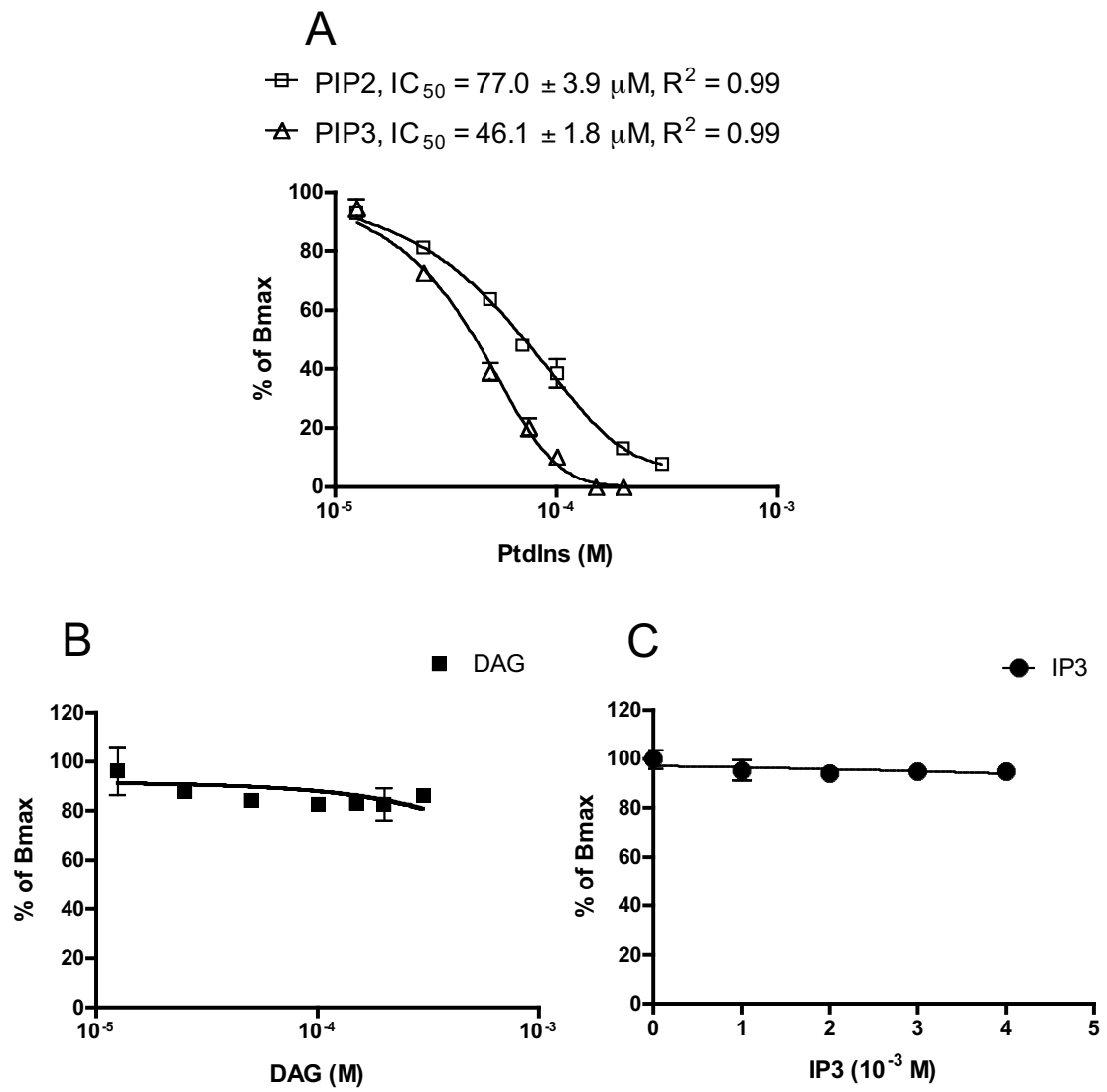


Figure 5.13 Phosphoinositides inhibit F-actin binding activity of α-actinin-4. Fluorescein labeled full length α-actinin-4 was pre-incubated with a dilution series of PIP2, PIP3 and DAG with the concentration from 0 to 200 μM and IP3 with 0 to 4 mM at the room temperature for 15 min. α-actinin-4 was then added in to F-actin coated wells at the room temperature for 2 hours (more details see section 2.2.2.9).

and Tyr 265. A region within the CH2 domain implicated in the binding to inositiols is located on the C-terminal α-helix. Therefore, Trp 172 and Tyr 265 are considered to be critical interaction residues for phosphoinositide binding. His 266 is proposed to be one of the charged residues making interactions with the inositiol head group of phosphoinositides. In order to assess the phosphoinositide binding residues within the CH2 domain, a number of α-actinin-4 CH2 domain point mutants, L169N, W172A, Y265A and H266E, were generated using site-directed mutagenesis according to the method described in section 2.2.1.12. S263D mutant was generated as a negative control because residue Ser 263 is proximal to the presumptive interaction site of phosphoinositides, but not likely to directly interact with phosphoinositides. Those mutants were sequenced using appropriate primers to ensure expression of the correct proteins (Figure 5.14). It is hypothesised that residue His 266 was involved in charged interactions with the inositiol head group of phosphoinositides. If a positively charged residue is substituted by a negatively charged residue, such as Glu or Asp, the charged interaction between residue and phosphate group could be diminished. Trp and Tyr are aromatic residues and their sidechains are hydrophobic but can be involved in hydrogen bond formation. If an aromatic residue is replaced by Ala or Gly, the hydrophobic and hydrogen bond interactions would be diminished. However, Gly is not suitable due to its flexibility. Gly sometimes influences the structure of a protein too much. Based on this concept, if Leu is replaced by polar residues, such as Gln or Ser, the hydrophobic interaction would be abrogated.

The CH2 domain mutants were purified and dissolved in TBS buffer pH 7.0 for ^{1}H-^{15}N HSQC ligand titration experiments by method described in section 2.2.3.5. Mutants, CH2-S263D, CH2-Y265A and CH2-H266E, were expressed as soluble proteins whereas CH2-L169N was poorly soluble and CH2-W172A was insoluble. In order to confirm the lack of solubility of the CH2-W172A mutant, the GST-CH2-W172A fusion protein was analysed using Western blot with anti-GST antibody according to the method described in section 2.2.2.11. As shown in Figure 5.15, the expected size for the GST-CH2-W172A fusion protein (~38 kDa) was detected in the IPTG induced fraction and pellet. These results clearly demonstrated that the CH2-W172A domain was in the insoluble fraction. It was most likely that residue Trp 172 is an important residue in stabilising the structure of the CH2 domain. Based on the solution structure of the CH2 domain illustrated in chapter 4,

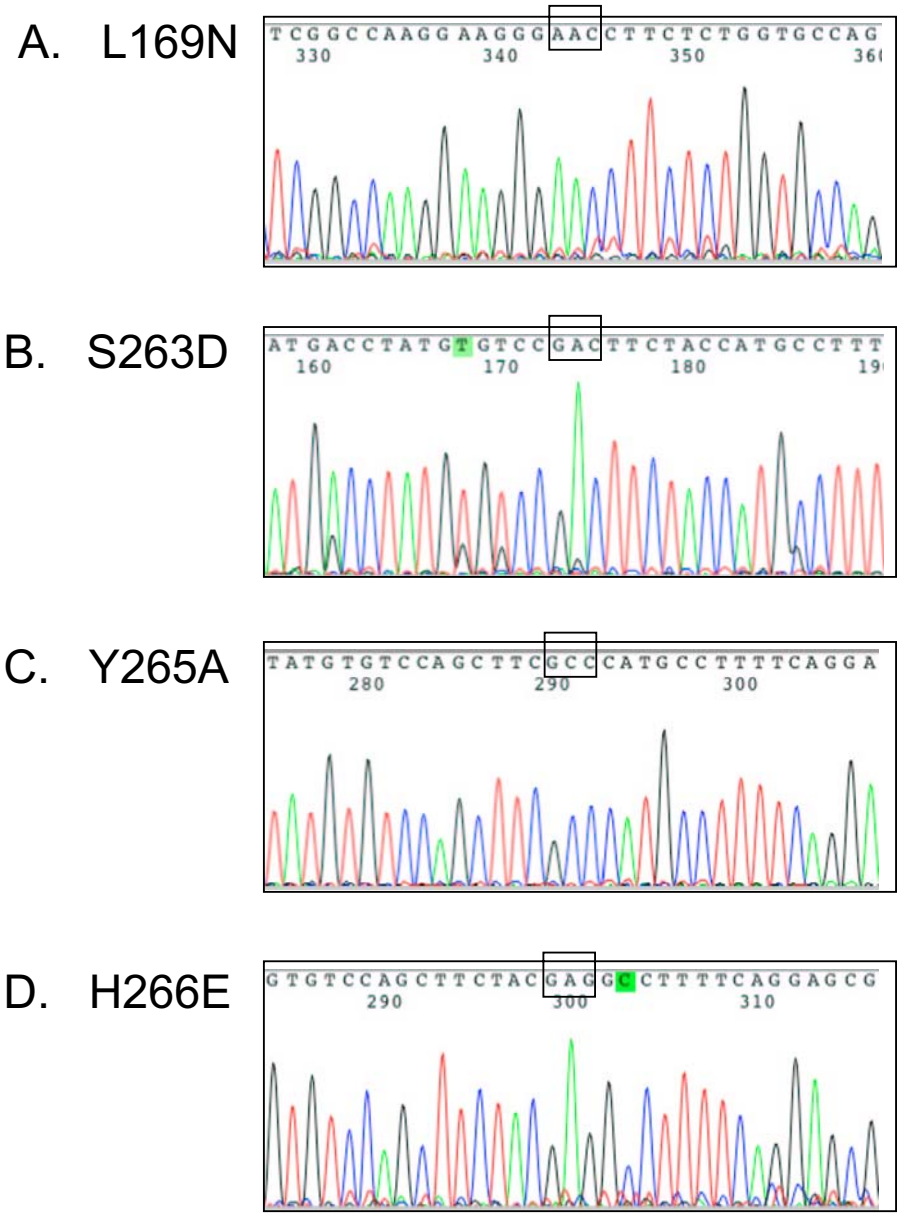


Figure 5.14 The representative electropherogram from DNA sequencing of the α-actinin-4 mutants. Codons for the L169N (A), S263D (B), Y265A (C) and H266E (D) residues are boxed.

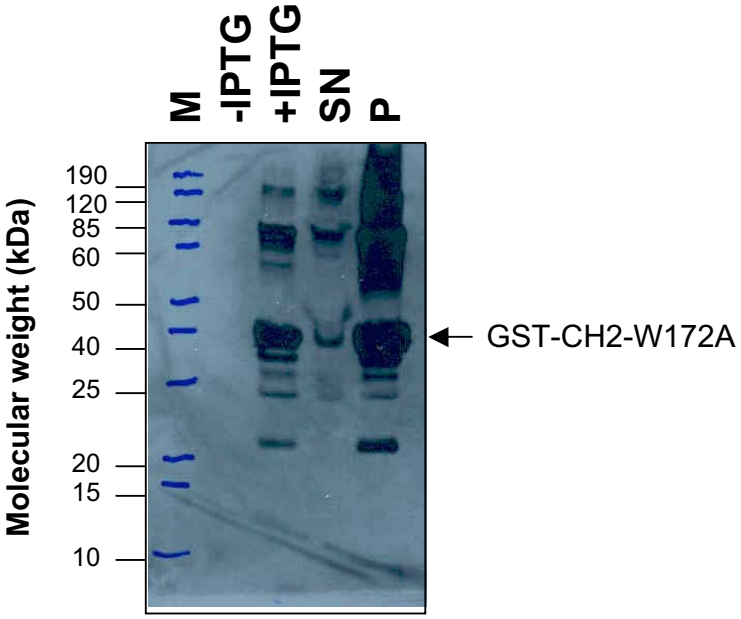


Figure 5.15 Solubility test of the CH2-W172A mutant. The GST-CH2-W172A mutant was induced by 0.1 mM IPTG in *E. coli* BL21 based on the method described in section 2.2.2.1. The bacterial lysate was centrifuged and the supernatant (SN) and pellet (P) were displayed by Western blot with anti-GST antibody.

Trp 172 is one of important residues which participate in formation of the hydrophobic care of the CH2 domain with relative solvent accessibility of 6.4% (referred to Figure 4.20).

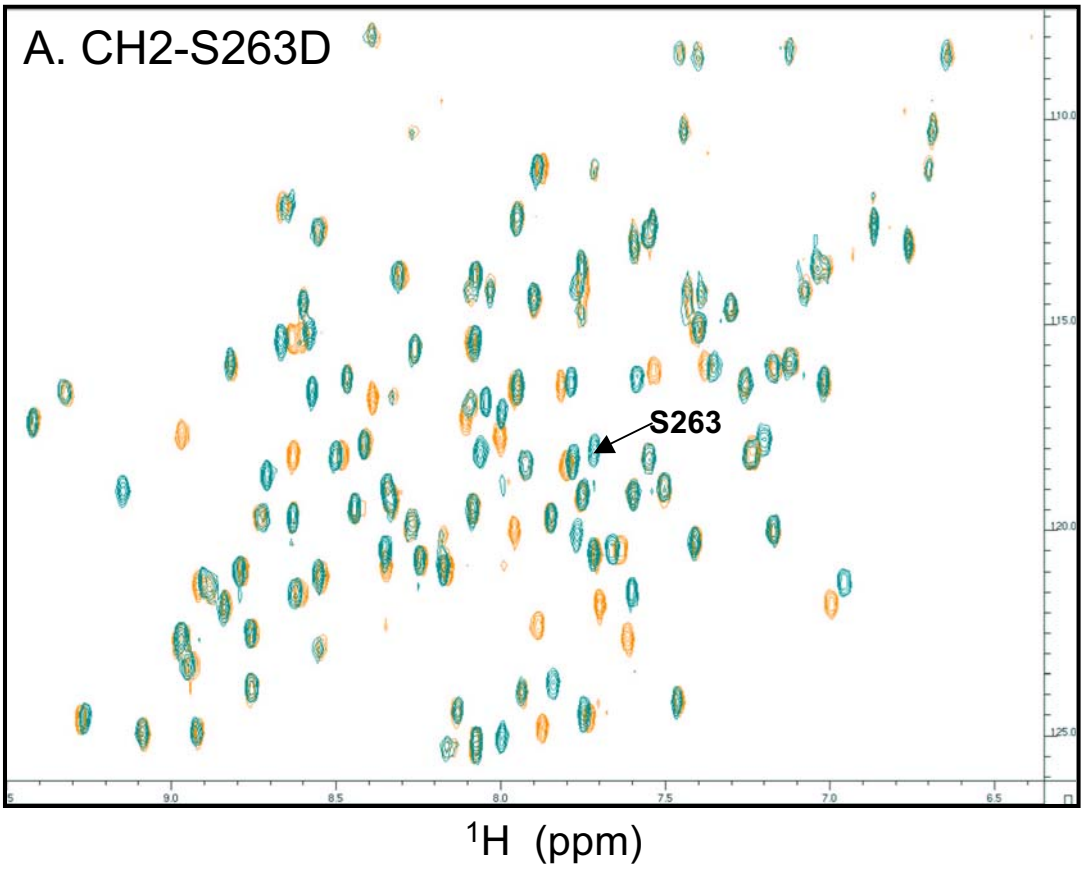
Overlaid HSQC spectra of wildtype and mutants of the CH2 domain including S263D, Y265A and H266E are shown in Figure 5.16 and the chemical shift deviations from that of wildtype CH2 domain were calculated according to the equation $\Delta\delta = [(\Delta HN)^2 + (0.17 \times \Delta^{15}N)^2]^{1/2}$ (Farmer *et al.*, 1996). Compared with that of wildtype, the chemical shift of these mutated residues within CH2 domain mutants was changed due to the different amino acid sidechains. Apart from these residues, the chemical shift of some residues surrounding the mutated residues (S263D, Y265A and H266E) was changed. These spectra indicated that overall these CH2 domain mutants were folded properly in TBS buffer.

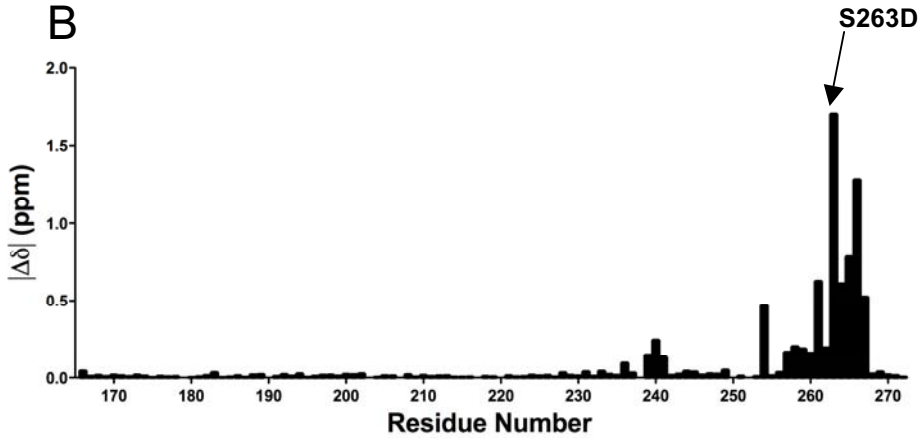
5.3.7 HSQC Ligand Titration Analysis on the α-actinin-4 CH2 Mutants

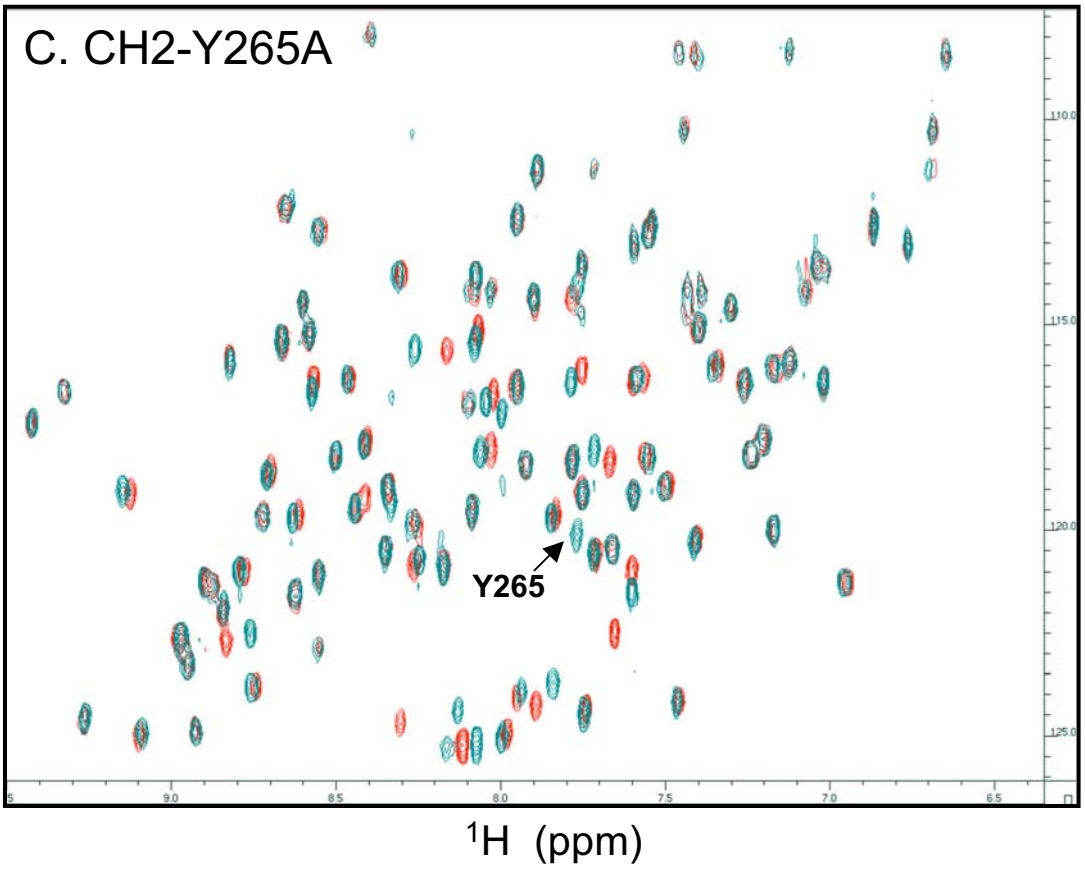
As described in section 5.3.3, residue His 266 was considered to be a critical residue in the charged interaction between the CH2 domain and inositiols (IP3 or IP4). In order to verify this hypothesis, a series of 2D ^{1}H-^{15}N HSQC spectra were recorded on the CH2-H266E mutant in the TBS buffer pH 7.0 in the absence and presence of PIP2 or PIP3 with concentrations of each in 100, 200 and 300 μM. The overlaid 2D HSQC spectra are shown in Figure 5.17. The chemical shift of all residues did not change with increasing concentrations of PIP2 or PIP3, indicating the CH2-H266E did not interact with PIP2 or PIP3. These results support that His 266 is directly involved in the interaction with phosphoinositides.

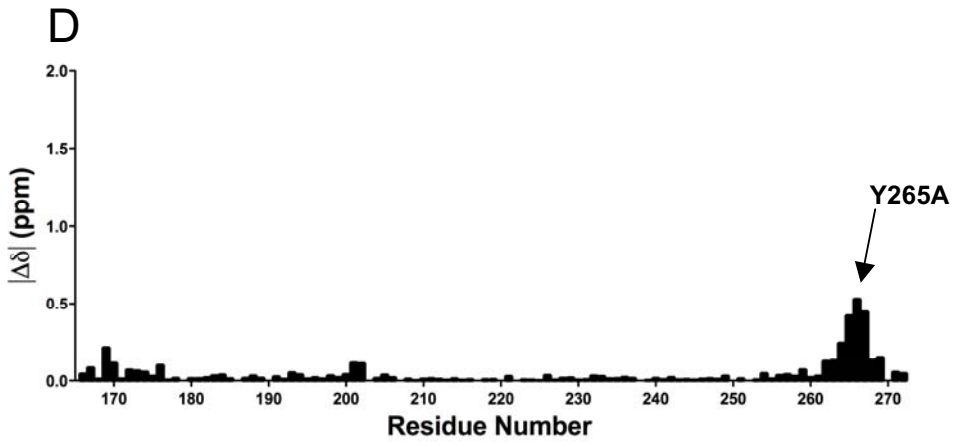
2D ^{1}H-^{15}N HSQC spectra were recorded on the CH2-Y265A mutant dissolved in TBS buffer pH 7.0 in the absence and presence of PIP2 or PIP3 with concentrations of each in 100, 200 and 300 μM. The overlaid 2D HSQC spectra are shown in Figure 5.18. The chemical shift of all residues did not change with increasing amounts of PIP2 or PIP3, indicating the CH2-Y265A did not interact with PIP2 or PIP3. The chemical shift of residue Leu 169 which was the greatest chemical shift changes residue within the wildtype CH2 domain in the titration of phosphoinositides (PIP2 or PIP3) (Figure 5.8) can be used to interpret these NMR data. These results suggested that Tyr 265 is one of the critical residues for the interaction with phosphoinositides presumably by hydrophobic or hydrogen bond interactions.

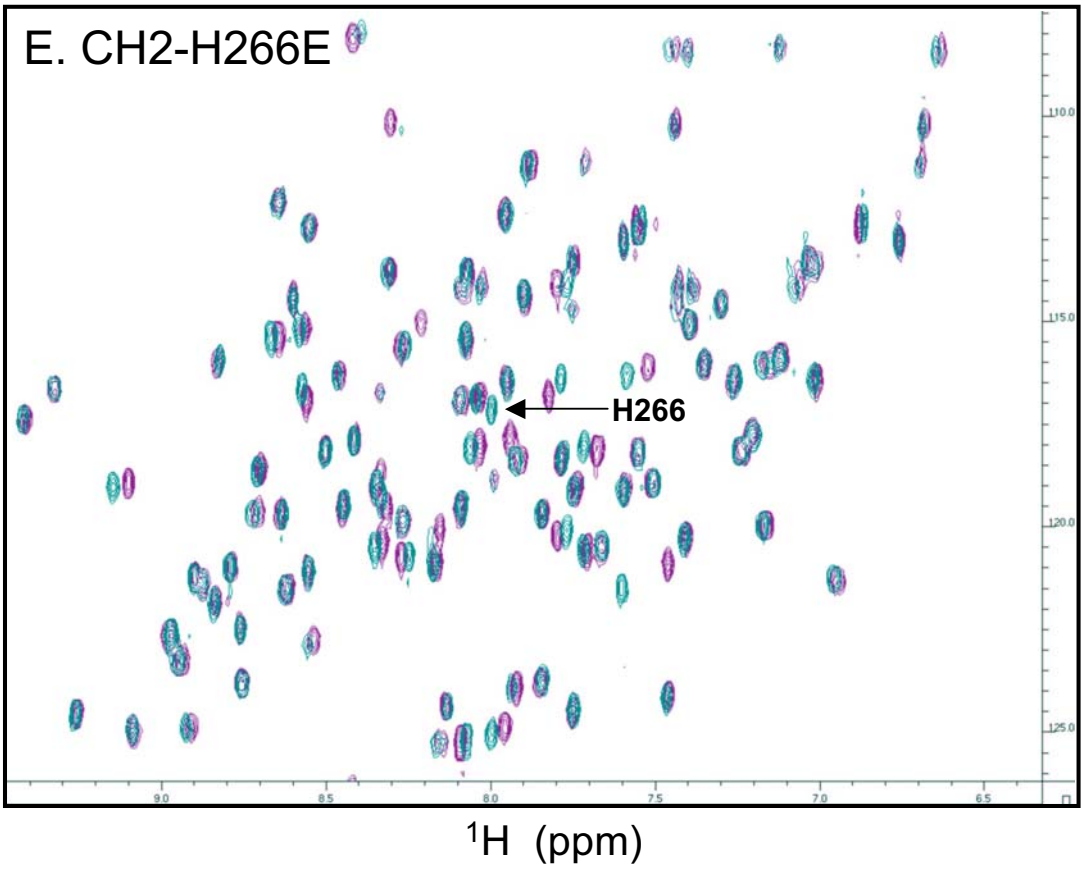
Figure 5.16 The chemical shift changes between wildtype and mutants of α-actinin-4 CH2 domain. Overlaid ^{1}H-^{15}N HSQC spectra of wildtype (green) and S263D (A; orange), Y265A (C; red) and H266E (E; purple) CH2 domain were shown. The HSQC spectrum was recorded using ^{15}N-labeled NMR samples at 25°C. The NMR data were processed by NMRPipe and analysed by CCPNMR software. Chemical shift differences were plot between wildtype and S263D (B), Y265A (D) and H266E (F) CH2 domain. Chemical shift variations were calculated according to the equation $\Delta\delta = [(\Delta HN)^2 + (0.17 \times \Delta^{15}N)^2]^{1/2}$ (Farmer, 1996). Residues Ser 263, Tyr 265 and His 266 for wildtype CH2 domain are indicated in arrows in (A), (C) and (E), respectively.


A. CH2-S263D
S263
^{15}N (ppm)
^{1}H (ppm)


B
S263D
|Δδ| (ppm)
Residue Number


C. CH2-Y265A
Y265
^{15}N (ppm)
^{1}H (ppm)


D
|Δδ| (ppm)
Y265A
Residue Number


E. CH2-H266E
H266
^{15}N (ppm)
^{1}H (ppm)

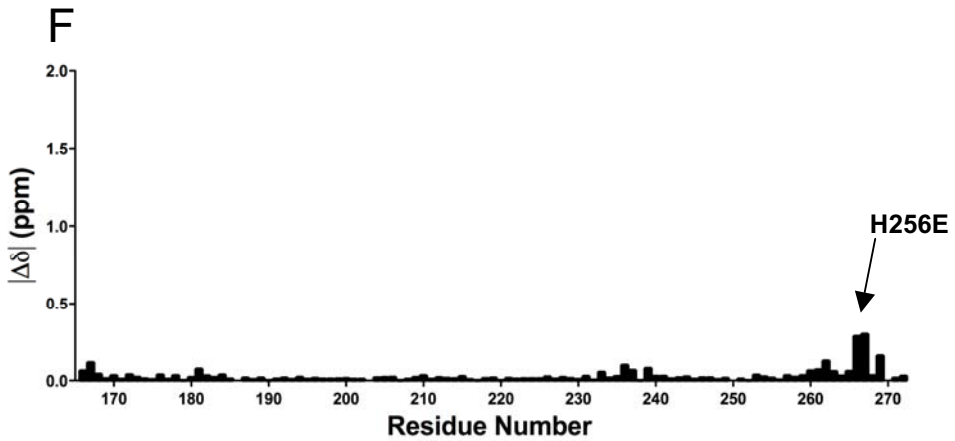

F
|Δδ| (ppm)
H256E
Residue Number

Figure 5.17 Effect of sequential ligands addition on the ^{1}H-^{15}N HSQC spectra of the CH2-H266E mutant. The CH2-H266E mutant (0.2 mM) was titrated with increasing concentrations of PIP2 (A) and PIP3 (B) with 0 (green), 100 (orange), 200 (red) and 300 (blue) μM in 1X TBS buffer containing 0.01% (w/v) NaN_3, pH 7.0. ^{1}H-^{15}N HSQC spectra were recorded on Varian Inova 600 spectrometer according to the method described in section 2.2.3.6. The diagram is shown selected region of the spectra.

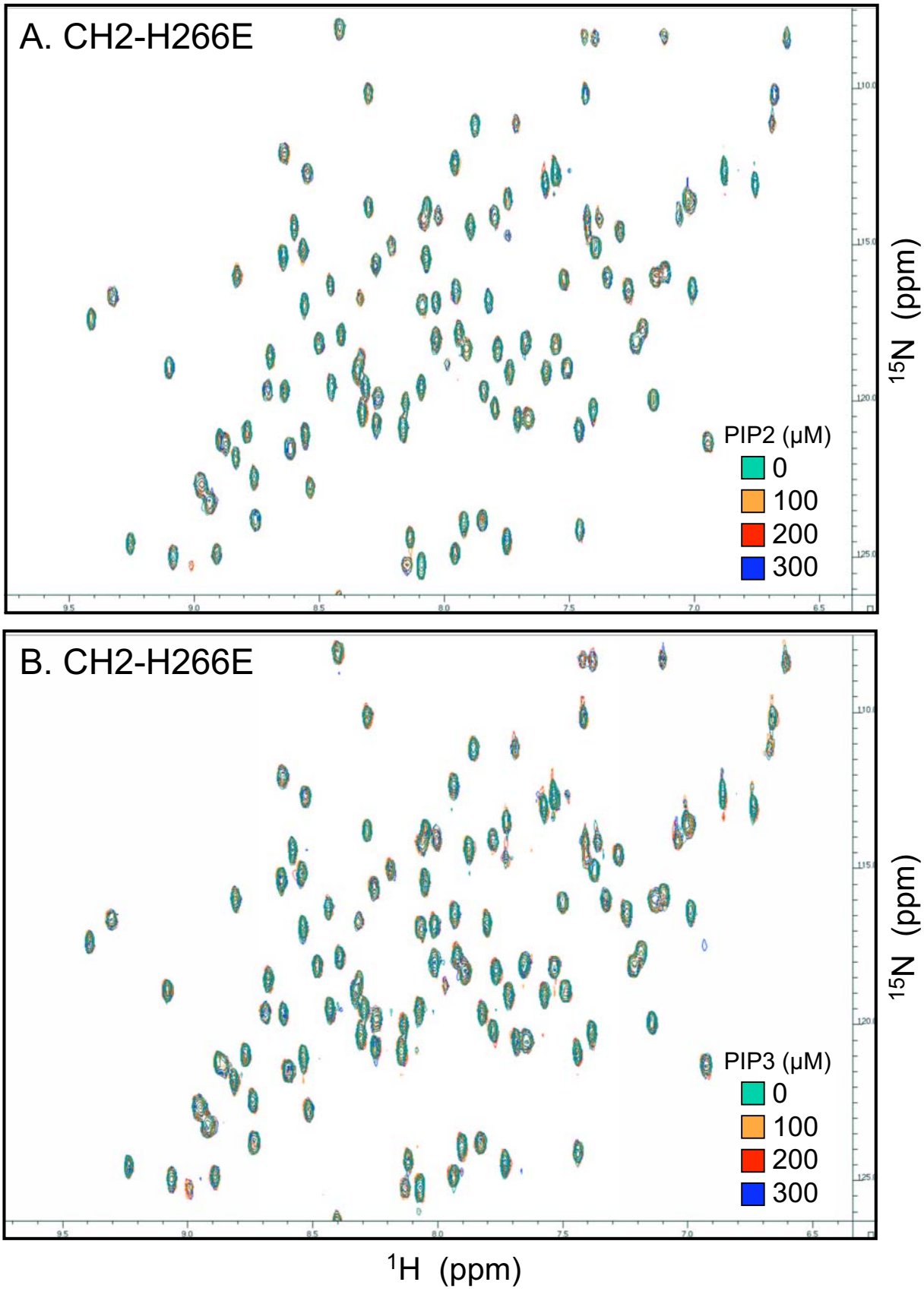
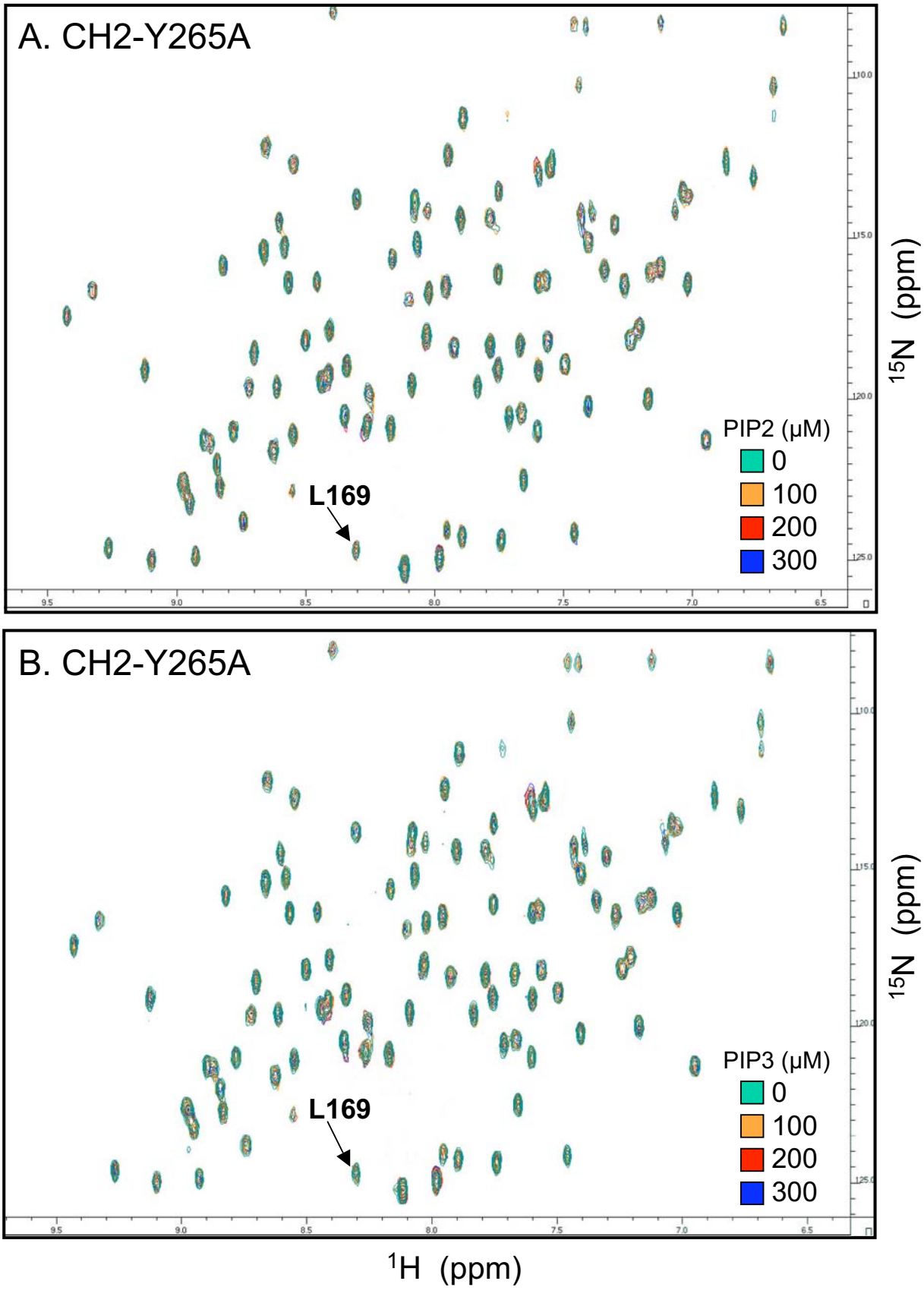

A. CH2-H266E
PIP2 (μM)
0
100
200
300
B. CH2-H266E
PIP3 (μM)
0
100
200
300
15N (ppm)
15N (ppm)
1H (ppm)

Figure 5.18 Effect of sequential ligands addition on the ^{1}H-^{15}N HSQC spectra of the CH2-Y265A mutant. The 0.2 mM CH2-Y265A mutant was titrated with increasing concentrations of PIP2 (A) and PIP3 (B) with 0 (green), 100 (orange), 200 (red) and 300 (blue) μM in 1X TBS buffer containing 0.01% (w/v) NaN_3, pH 7.0. ^{1}H-^{15}N HSQC spectra were recorded on Varian Inova 600 spectrometer according to the method described in section 2.2.3.6. The cross peaks of residue Leu 169 are indicated in arrows. The diagram is shown selected region of the spectra.


A. CH2-Y265A
L169
PIP2 (μM)
0
100
200
300
B. CH2-Y265A
L169
PIP3 (μM)
0
100
200
300
^{15}N (ppm)
^{15}N (ppm)
^{1}H (ppm)

Point mutation on a critical residue within a protein can provide strong evidence for the biological function of this residue. However, point mutation may induce slight conformational changes in a protein to prevent its biological functions. In order to eliminate this possibility, a negative control mutant, CH2-S263D, was generated and performed the HSQC ligand titration analysis with phosphoinositides (PIP2 or PIP3). The overlaid 2D ^{1}H-^{15}N HSQC spectra recorded on the CH2-S263D mutant in the TBS buffer pH 7.0 in the absence and presence of PIP2 or PIP3 with concentrations of each in 100, 200 and 300 μM are shown in Figure 5.19. The results showed that the chemical shifts of the residues (Glu 167, Leu 169, Leu 170, Trp 172 and Tyr 265) which are implicated in the interaction with phosphoinositides were changed. These results were similar to that of wildtype CH2 domain titrated with PIP2 and PIP3 (Figure 5.8), indicating that residue Ser 263 might not be one of residues for phosphoinositide binding.

5.3.8 Abrogation of Phosphoinositide Inhibitory Effect on F-actin Binding Activity of α-actinin-4 Mutants

In order to investigated whether the F-actin binding activity of α-actinin-4 was influenced by point mutation within the phosphoinositide binding site, full length α-actinin-4 containing mutants (L169N, S263D, Y265A and H266E) were generated and labeled with Fluorescein for the solid phase F-actin binding assay. The F-actin binding activity of these α-actinin-4 mutants were investigated by the solid phase binding assay (Table 5.1). As shown in Table 5.1, the Kd value of α-actinin-4 mutants is similar to that of wildtype, suggesting that point mutation within the phosphoinositide binding site did not influence the F-actin binding activity.

As described in section 5.3.5, both PIP2 and PIP3 inhibited the F-actin binding activity of α-actinin-4, with PIP3 being more effective. It was assumed that phosphoinositides bound the actin binding site-3 of the CH2 domain to compete with F-actin binding. Therefore, if the critical residues within phosphoinositide binding were mutated, the F-actin binding activity of α-actinin-4 would not be inhibited by phosphoinositides. In order to test this hypothesis, the F-actin binding activity of α-actinin-4 mutants, L169N, Y265A, H266E and L169N/H266E was investigated in the absence and presence of 100 μM PIP2 or 85 μM PIP3 using the solid phase F-actin binding assay. As described in section 5.3.5, 100 μM

Figure 5.19 Effect of sequential ligands addition on the ^{1}H-^{15}N HSQC spectra of the CH2-S263D mutant. The 0.2 mM CH2-S263D mutant was titrated with increasing concentrations of PIP2 (A) and PIP3 (B) with 0 (green), 100 (orange), 200 (red) and 300 (blue) μM in 1X TBS buffer containing 0.01% (w/v) NaN_3, pH 7.0. ^{1}H-^{15}N HSQC spectra were recorded on Varian Inova 600 spectrometer according to the method described in section 2.2.3.6. The diagram is shown selected region of the spectra.

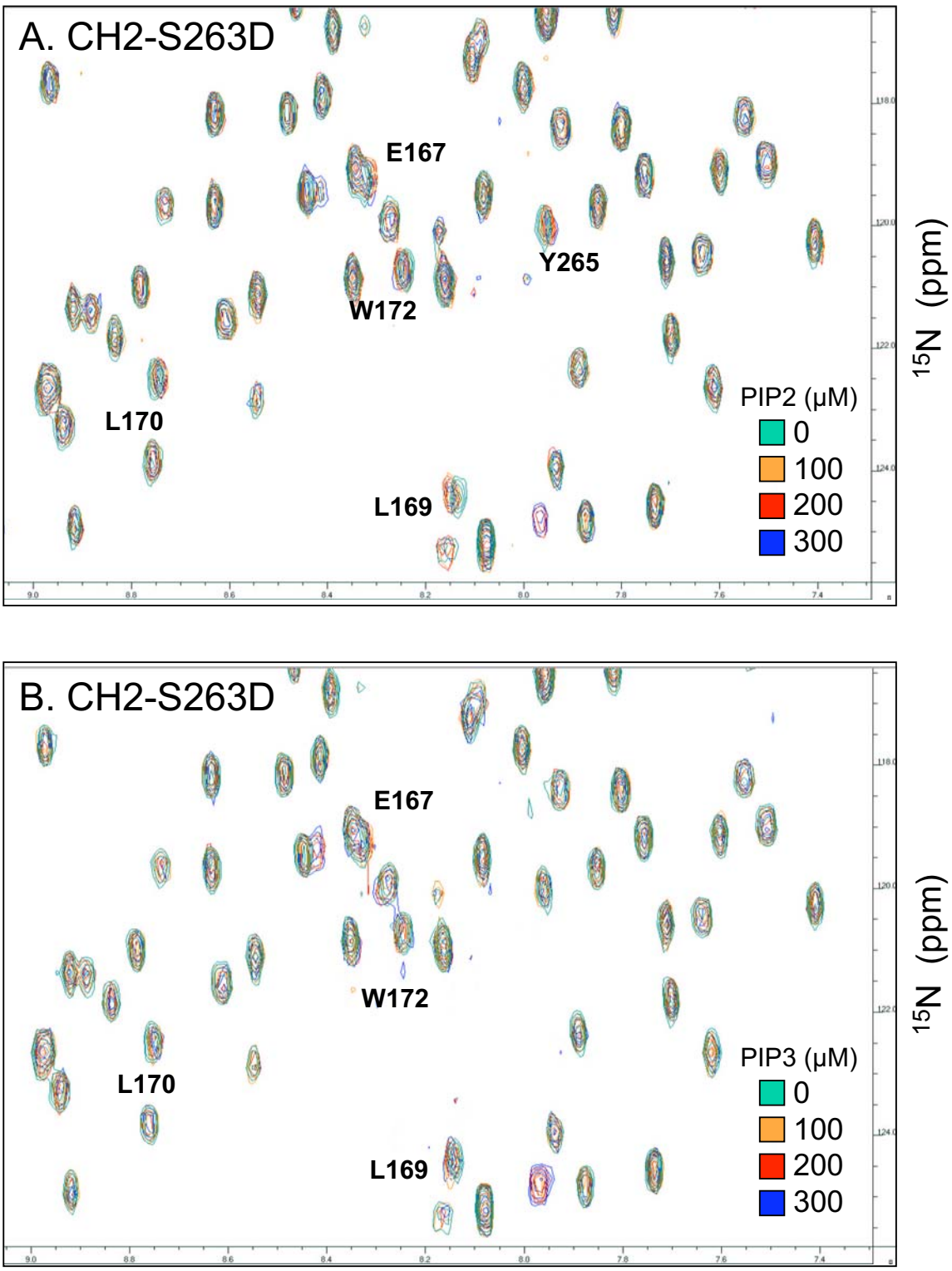

A. CH2-S263D
E167
Y265
W172
L170
L169
PIP2 (μM)
0
100
200
300
^{15}N (ppm)
B. CH2-S263D
E167
W172
L170
L169
PIP3 (μM)
0
100
200
300
^{15}N (ppm)
^{1}H (ppm)

Table 5.1 The F-actin binding activity of α-actinin-4 mutants.

α-actinin	Kd (nM)
WT	36 ± 5.0
L169N	31 ± 5.1
S263D	46 ± 5.6
Y265A	34 ± 3.6
H266E	39 ± 4.7
L169N/H266E	46 ± 4.0

PIP2 and 85 μM PIP3 were sufficient to result in a reduction in F-actin binding to 50%. S263D mutant also was used as a negative control. As shown in Figure 5.20, 100 μM PIP2 and 85 μM PIP3 inhibited the F-actin binding activity of wildtype and S263D mutant of α-actinin-4 approximately 60% for PIP2 and 50% for PIP3. These results confirmed that residue Ser 263 was not involved in the interaction with phosphoinositides. However, the F-actin binding activity of α-actinin-4 mutants, including L169N, Y265A, H266E and L169N/H266E, was not inhibited in the presence of 100 μM PIP2 or 85 μM PIP3. These results confirm that residues, Leu 169, Tyr 265 and His 266, were the critical binding residues for phosphoinositides.

5.4 Discussion

5.4.1 The Phosphoinositide Binding Site on the α-actinin-4 CH2 Domain

As described in sections 5.3.3 and 5.3.4, the phosphoinositide binding site on α-actinin-4 CH2 domain was identified by NMR-based HSQC ligand titration analysis. In the presence of phosphoinositides (PIP2 or PIP3), residues that are implicated in the interaction with phosphoinositides were Glu 167, Gly 168, Leu 169, Trp 172 and Tyr 265 with the Kd values of 109 μM for PIP2 and 127 μM for PIP3. The binding affinity of the CH2 domain to PIP2 and PIP3 are equal. These results are consistent with a previous report from chicken skeletal muscle α-actinin by Fraley *et al.* in 2003 (Fraley *et al.*, 2003). The residues that were involved in the interaction with inositiols were Trp 172, Asp 253, Ser 262, Ser 263, Phe 264, Tyr 265, His 266, Ser 269 and Ala 271 for IP3 and Trp 172, Asp 253, His 266, Ser 269 and Gln 272 for IP4 with the Kd values of 3.7 mM for IP3 and 3.4 mM for IP4. According to NMR-based ligand analysis with the CH2-H265A and CH2-H266E mutants to PIP2 or PIP3, the chemical shift of all backbone amide residues did not change, indicating that residues Tyr 265 and His 266 might be involved in a direct interaction with phosphoinositides. It is most likely that His 266 binds the inositiol head group of phosphoinositides by charged interaction and Tyr 265 interacts with phosphoinositides by hydrophobic or hydrogen bond interaction. Based on the binding affinity of phosphoinositides and inositiols to the CH2 domain, the binding affinity of phosphoinositides to the CH2 domain was shown a 30-fold stronger than that of inositiols. These results implied that the acyl chains of phosphoinositides play a significant role in their binding to the CH2 domain by hydrophobic

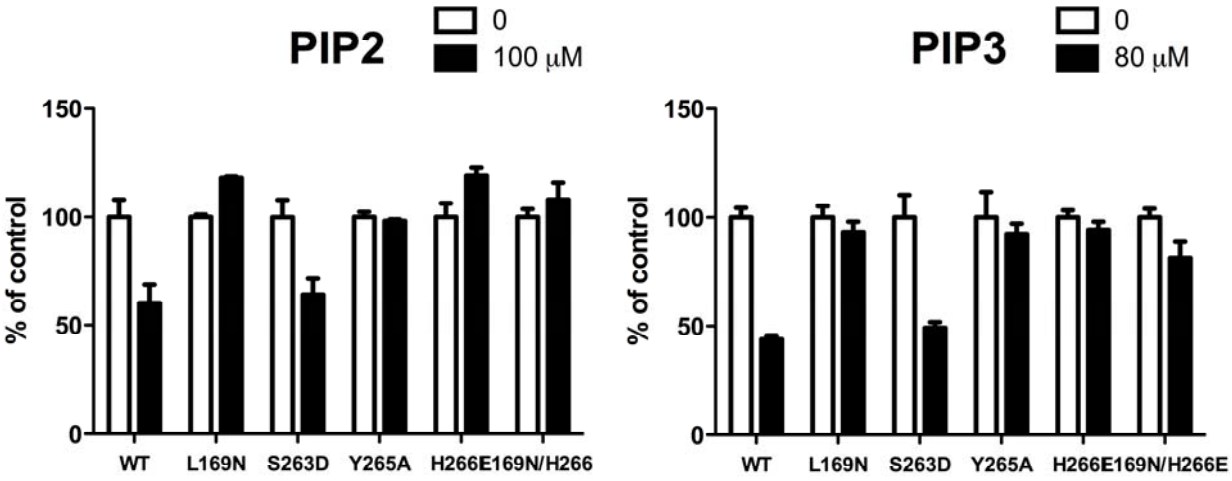


Figure 5.20 The F-actin binding activity of α-actinin-4 mutants in the absence and presence of phosphoinositides. Fluorescein labeled full length α-actinin-4 mutants (L169N, S263D, Y265A, H266E and L169N/H266E) were pre-incubated with PIP2 and PIP3 with the concentration at 100 and 85 μM, respectively, at room temperature for 15 minutes. Samples were then added into F-actin coated wells at room temperature for 2 hours (For details see section 2.2.2.9).

interaction. It is presumed that residues, Leu 169, Leu 170, Trp 172 and Tyr 265, are associated with this interaction.

Hundreds of cellular proteins interact with phosphoinositides by several different functional domains (as described in section 5.1) and the phosphoinositide binding affinity with these domains is generally of low to moderate affinity with the range from μM to nM due to the rapidly reversible dynamic responses to signals (Yin and Janmey, 2003). Even the same domain in a different protein contains different phosphoinositide binding affinities. For example, PH domains are well known domains that have been discovered in a very large number of proteins, from yeast to mammals. 90% of the PH domains from different proteins are of low affinity for phosphoinositides with 10-100 μM (Lemmon *et al.*, 2002). The β-spectrin PH domain and the N-terminal pleckstrin PH domain, for instance, bound PIP2 with the Kd values in the 30-50 μM range (Harlan *et al.*, 1994; Ma and Abrams, 1999). The PI3K SH2 domain interacted with PIP2 and PIP3 with the Kd values of 110 μM and 23 μM, respectively (Ching *et al.*, 2001). The PLC-δ1 PH domain, however, specifically recognised PIP2 with a Kd value of approximately 1.7 μM (Lemmon and Ferguson, 2000). In this study, the α-actinin-4 CH2 domain recognised PIP2 and PIP3 with the Kd values of 109 μM and 127 μM, respectively. The binding affinity of PIP2 and PIP3 to α-actinin-4 CH2 domain was of low affinity and the binding affinity of PIP2 was equal to that of PIP3. These results were similar to that of chicken skeletal muscle α-actinin CH2 domain (Fraley *et al.*, 2003).

The PIP2 binding site on the CH2 domain has been identified from chicken skeletal muscle α-actinin using dot-blot analysis with anti-PIP2 antibody by Fukami *et al.* in 1996. Briefly, skeletal muscle α-actinin bound PIP2 was digested with α-chymotrypsin or lysylendopeptidase, separated on a C18 reverse phase column and detected by dot-blot analysis with anti-PIP2 antibody. The α-actinin fragment recognised by anti-PIP2 antibody was then sequenced (Fukami *et al.*, 1996). This binding site was mapped at the N-terminal of the CH2 domain adjacent to the ABS-3 with the residues 177-193 (TAPYRNVNIQNFHLSWK). The critical residues, R181, H189 and K193, were predicted for the interaction with the inositol head group of phosphoinositide by charged interactions. Compared with other CH2 domains from the spectrin superfamily, the amino acid sequences and three dimensional structures within the residues 177-193 were highly conserved (as

described in chapter 4). In order to investigate whether these critical residues were directly involved in the interaction with phosphoinositides, Fraley *et al.* generated three mutants, K181I, K193I and a triple mutant K181I/H189L/K193I within human α-actinin-1 and tested their phosphoinositide (including PIP2 and PIP3) binding ability using a protein-lipid overlay assay (Fraley *et al.*, 2003). Briefly, α-actinin protein samples (wildtype and three mutants) were incubated with phosphoinositides which have been coated on a nitrocellulose membrane and detected using dot-blot analysis with anti-α-actinin antibody. Signals were than quantified by a densitometer. Their results showed that the PIP2 or PIP3 binding ability was decreased in all mutants (approximately 40%, 30% and 70% for K181I, K193I and K181I/H189L/K193I, respectively) (Fraley *et al.*, 2003). These results suggested that residues Lys 181, His 189 and Lys 193 were implicated in phosphoinositide binding. In this study (α-actinin-4), however, the chemical shift of these three residues did not show significant changes on HSQC spectra in the presence of either phosphoinositides (PIP2 or PIP3) or inositol head group (IP3 or IP4). This biochemical evidence suggested that residues Lys 181, His 189 and Lys 193 within α-actinin-4 are not involved in phosphoinositide binding. These results presented have demonstrated that the human α-actinin-4 CH2 domain contains a specific phosphoinositide binding site located on a region between the first N-terminal and the last C-terminal α-helices (Figure 5.21).

5.4.2 Phosphoinositides Inhibit Binding by Blocking the Binding of α-actinin-4 to F-actin

As shown in Figure 5.13, both PIP2 and PIP3 inhibited the F-actin binding activity of human α-actinin-4 (with the IC_{50} 77 μM for PIP2 and 45 μM for PIP3). These results were similar to that reported for chicken skeletal muscle α-actinin (Fraley *et al.*, 2003). Compared with the binding affinity of the CH2 domain to phosphoinositides (with Kd value 109 μM for PIP2 and 127 μM for PIP3), the inhibitory IC_{50} was shown to be lower than the binding affinity (Kd value). It is presumed that protein-micelle complex might influence the measurement of the dissociation constant for phosphoinositides. Both DAG and IP3 had no any effect on the F-actin binding activity of α-actinin (Figure 5.13), suggesting that the acyl chains were important for inhibition and the inhibitory effect was specific. These results were also similar to that reported for chicken skeletal muscle α-actinin (Fraley *et al.*, 2003). As shown in Figure 5.21, the phosphoinositide binding site within α-actinin-4 CH2 domain

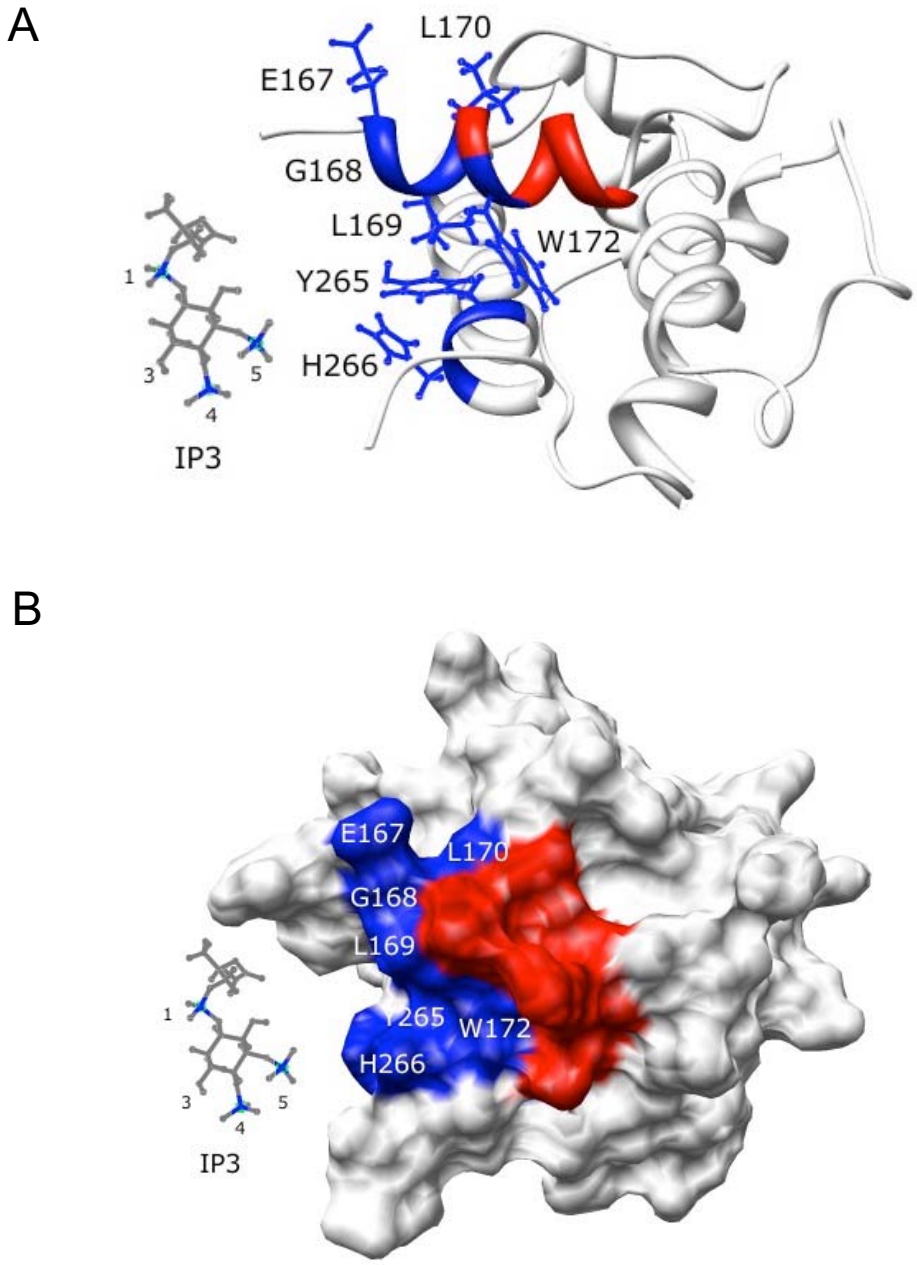


Figure 5.21 A model of phosphoinositide interaction with the α-actinin-4 CH2 domain. The ribbon (A) and surface (B) diagrams were generated by Chimera software (Pettersen, 2004). The ABS-3 which reported from utrophin and dystrophin were indicated in red. The region for phosphoinositide binding was indicated in blue and the critical residues, Glu 167, Gly 168, Leu 169, Leu 170, Trp 172, Tyr 265 and His 266, were named.

overlapped with the actin binding site-3 which reported from utrophin and dystrophin at the first N-terminal α-helix. Therefore, these results demonstrated that phosphoinositides inhibit the F-actin binding activity of α-actinin-4 by blocking the actin binding site-3 to compete with F-actin binding.

The binding affinity of the CH2 domain to IP3 is similar to that of IP4 (with Kd 3.7 mM for IP3 and 3.4 mM for IP4). Moreover, the binding affinity of the CH2 domain to PIP2 is similar to that of PIP3 (with Kd as 109 μM for PIP2 and 127 μM for PIP3). However, the inhibitory effect of PIP3 to the CH2 domain is approximately 2-fold efficient than that of PIP2 to the CH2 domain (with IC_{50} 77 μM for PIP2 and 45 μM for PIP3). These results implied that there might be some other residues within the CH domains to be involved in the interaction with the phosphate group at the position 3 of the inosotiol (the major difference between PIP2 and PIP3). According to the crystal structure of the CH domains of α-actinin-4 K255E mutant (Lee *et al.*, 2008) (shown in Figure 5.22), three positively charged residues within the CH1 domain, His 63, Arg 65 and Lys 66, presumed to be specifically involved in the interaction with PIP3. In order to confirm this hypothesis, α-actinin-4 mutants, H63A, K66A and triple mutant of H63A/R65A/K66A, need to be generated and their sensitivity to phosphoinositides should be measured using the solid phase F-actin binding assay. Time limits have precluded making these mutants as part of this thesis. It is proposed that the phosphate group at the position 3 of the PIP3 inositiol interacts with residues His 63, Arg 65 or Lys 66 within the CH1 domain and the phosphate groups at the position 4 or 5 of the PIP2 or PIP3 inositiol binds residue His 266 within the CH2 domain. Therefore, it is anticipated that the inhibitory effect of PIP3 on the F-actin binding activity of α-actinin-4 mutants (H63A, K66A or triple mutant of H63A/R65A/K66A) would be equal to that of PIP2.

5.4.3 Concentration of Phosphoinositides in Typical Mammalian Cells

As described in this chapter, the phosphoinositide binding affinity of human α-actinin-4 was classified into the low affinity range (Yin and Janmey, 2003; McLaughlin *et al.*, 2002), with Kd values around 50 to 100 μM. A major issue in evaluating the biological significance in regulating cytoskeleton rearrangement is to elucidate what is the concentration of phosphoinositides in typical mammalian cells. PIP2 is not diffusely distributed in cell

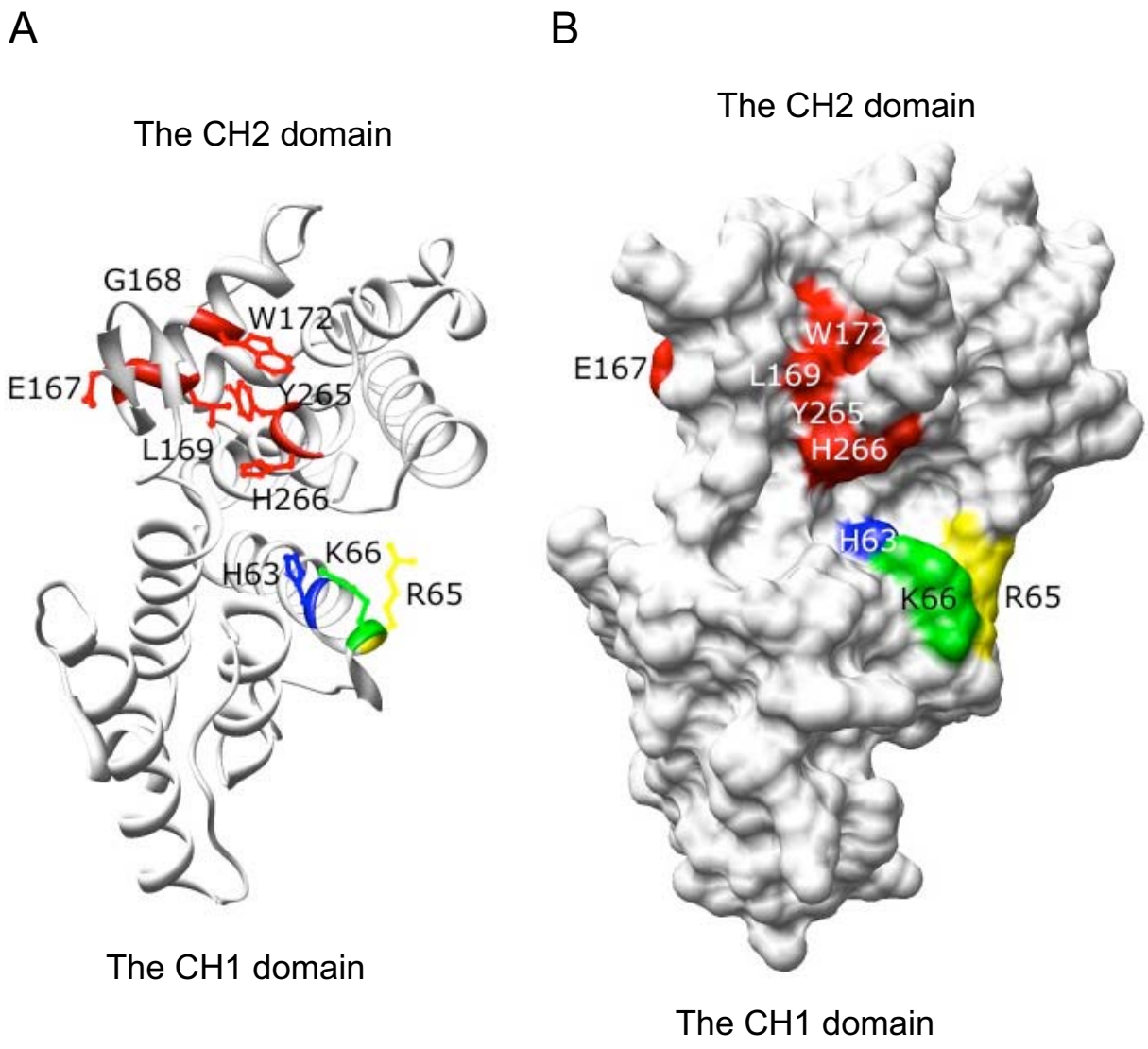


Figure 5.22 A possible interaction model of phosphoinositide with the α-actinin-4 CH domain. The ribbon (A) and surface (B) diagrams were generated by Chimera software (Pettersen, 2004) using crystal structure of α-actinin-4 K255E mutant (pdb.2R0O). The region for phosphoinositide interaction was indicated in red and the critical residues, Glu 167, Gly 168, Leu 169, Trp 172, Tyr 265 and His 266, were named. The possible residues, His 63, Arg 65 and Lys 66, for the phosphate group at position 3 of inositol head group were indicated in blue, green and yellow, respectively.

membrane and could be accumulated at the inner leaflet of the plasma membrane as the cell has been stimulated. The concentration of PIP2 and PIP3 has been measured in neutrophils with or without the stimulation of fMLP (N-formylmethionylleucylphenylalanine) (Stephens *et al.*, 1991). The results indicated that the concentrations of PIP2 and PIP3 are approximately 5 mM and 5 μM, respectively, at the inner leaflet of the plasma membrane in resting cells. Upon the stimulation of fMLP for 10 seconds, concentrations change from approximately 5 mM to 3.5 mM for PIP2 and from 5 μM to 200 μM for PIP3 (Stephens *et al.*, 1991; 1993). As described in this chapter, the inhibitory IC_{50} values of phosphoinositides for α-actinin-4 binding to F-actin are approximately 77 μM for PIP2 and 45 μM for PIP3. Therefore, α-actinin-4 bound phosphoinositides has its biological significance.

CHAPTER 6

Rearrangement of Actin Filament by EGF in NIH3T3 Cells

6.1 Introduction

Cellular interactions with extra-cellular matrix are generally considered to be a key point in the regulation of cell adhesion. These contacts are highly dynamic and tightly regulated by signals either from outside or inside of the cell, resulting in regulation of cellular functions such as proliferation, differentiation and migration. These contacts link bundles of actin microfilaments to extra-cellular matrix via transmembrane proteins, such as integrin, resulting in firm adherence of the cell to the substrate (for details refer to section 1.3.1). Therefore, regulation of the cytoskeleton rearrangement should be a crucial step to cellular functions.

PIP2 plays a key role in regulating the rearrangement of the cytoskeleton. PIP2 regulates actin polymerisation and depolymerisation through its action on actin regulatory proteins, such as profilin (Lassing and Lindberg, 1985) and gelsolin (Janmey and Stossel, 1987). An increase in the amount of PIP2 bound to actin regulatory proteins promotes the formation of actin stress fibres, whereas a decrease leads to actin depolymerisation and rearrangement of actin filament. In PDGF treated Balb/c 3T3 cells, actin stress fibres rearranged into membrane ruffles and α-actinin redistributed with actin. The amount of PIP2 bound to α-actinin was detected using Western blot analysis with anti-PIP2 antibody. The results showed that the amount of PIP2 bound to α-actinin was reduced (Fukami *et al.*, 1994). These results confirmed that PIP2 plays an important role in stabilising actin cytoskeleton. The amount of PIP2 on the cell membrane could be reduced in three ways: hydrolysis to IP3 and DAG by PLCγ, dephosphorylation to PI(4)P by PIP2 phosphatase and conversion to PIP3 by PI3 kinase under the stimulation of factors such as PDGF and EGF. Greenwood *et al.* have demonstrated that focal adhesions and actin stress fibres were rearranged by the activation of PI3 kinase and α-actinin and vinculin were redistributed into the Triton X-100 soluble fraction in PDGF treated rat embryonic fibroblast cells (Greenwood *et al.*, 2000). Once PI3 kinase has been activated, the amount of PIP3 was elevated and induced dissociation between α-actinin and integrin β subunit, resulting in α-actinin reorganisation. These results have been confirmed using wortmannin and LY294002, specific inhibitors for PI3 kinase (Greenwood *et al.*, 2000). Sakisaka *et al.* have reported that a decrease in the amount of PIP2 by PIP2 phosphatase activation resulted in actin filament rearrangement in

EGF treated COS-7 cells (Sakisaka *et al*., 1997). Therefore, activation of these PIP2 metabolic enzymes, such as PLCγ, PI3 kinase and PIP2 phosphatase by PDGF or EGF resulted in actin rearrangement and α-actinin redistribution.

6.2 Aim and Approach

The first aim of this chapter was to investigate the subcellular localisation of α-actinin-4 in both resting cells and stimulated cells. As described in chapter 5, the phosphoinositide binding site was identified on a region between the first N-terminal and the last C-terminal α-helices of the α-actinin-4 CH2 domain. Therefore, the second aim of this chapter was to investigate cellular effects of this phosphoinositide binding site. Two α-actinin-4 mutants, Y265A and H266E, were used in this study due to their critical roles in phosphoinositides binding (chapter 5) and the approach used to investigate the biological function of these two mutants is a mammalian over-expression system. Over-expression of a protein or specific mutants in mammalian cells is a general and efficient method to investigate the function of a protein in cells. Other techniques also can be used to verify the function of a protein in cells, for example, generating knock-in mice or knock-in cells. However, development of an experimental system of knock-in mice or cells is time consuming and outside the scope of this thesis. Therefore, over-expression of a specific mutant protein in mammalian cell lines would be the only feasible way to verify the function of this mutant in cells. Two general techniques, immunohistochemistry staining for the endogenous proteins and over-expression of a protein with a tag at the N- or C-terminus from a foreign plasmid, can be used to investigate the subcellular localisation of a protein in cells. A specific antibody for a protein of interest is required for immunohistochemistry. However, a protein fused with EGFP (enhanced green fluorescnece protein) can be easily detected without an antibody. EGFP can emit fluorescent light under appropriate excitation without any substrate or inducer. It is a general and useful tag for study in the subcellular localisation of a protein in cells. EGFP-fusion proteins can be directly visualised in living cells under the fluorescence microscope. Treatment with EGF in NIH3T3 cells could induce restructuring of focal adhesions and the rearrangement of actin stress fibres by activation of either PI3 kinase or PIP2 phosphatase activity (Wells *et al.*, 1998; Sakisaka *et al.*, 1997). In this chapter, over-expression of EGFP-fusion proteins in NIH3T3 cells was used to verify the

subcellular localisation of α-actinin-4 (including wildtype and two phosphoinositide binding specific mutants) with or without treatment of recombinant human EGF.

6.3 Results

6.3.1 Subcellular Localisation of α-actinin-4

The PCR fragment of the full length α-actinin-4 (911 residues) was subcloned into the pEGFP-C2 (Figure 6.1) expression vector with restriction sites, *Eco*RI and *Xba*I and sequenced using appropriate primers to ensure expression of the correct EGFP-fusion protein in cells. To investigate the subcellular localisation of α-actinin-4, NIH3T3 cells were transiently transfected with plasmids that encoded the EGFP alone and EGFP-α-actinin-4 fusion protein, respectively, according to the method described in section 2.2.4.2. After 48 hours, cells were then fixed with 4% paraformaldehyde and stained with TRITC-conjugated phalloidin to visualise the actin microfilaments. As shown in Figure 6.2, EGFP was shown diffusely throughout the nucleus and the cytoplasm (Figure 6.2 A and C). EGFP-α-actinin-4 was shown to be co-localised with actin at the ends of actin stress fibres (green greater spot), actin stress fibres (green fine spot) and membrane ruffles at the leading edges (indicated in arrows) (Figure 6.2 D and F). As shown in Figure 6.2 E, actin stress fibres were observed throughout the cell body, terminating at the presumptive focal adhesions. Although there was no direct evidence supporting that the ends of the actin stress fibres in these experiments are focal adhesions, the co-localisation of α-actinin was similar to the cellular morphology reported previously (Greenwood *et al.*, 2000; Honda *et al.*, 1998). Immunostaining analysis with the focal adhesion associated protein antibody, such as anti-integrin antibody, could be used to address this issue.

6.3.2 Induction of Actin Filaments and α-actinin-4 Reorganisation by EGF in NIH3T3 Cells

NIH3T3 cells that encode EGFP-α-actinin-4 fusion protein were grown to 80% confluency and serum-starved overnight. Prior to treatment with EGF, the cells were washed and incubated with serum-free medium to remove serum. NIH3T3 cells were then stimulated with EGF for 0, 10 and 30 minutes and fixed, stained and mounted based on the method described in section 2.2.4.3. As shown in Figure 6.3 A, B and C, EGFP-α-actinin-4 was

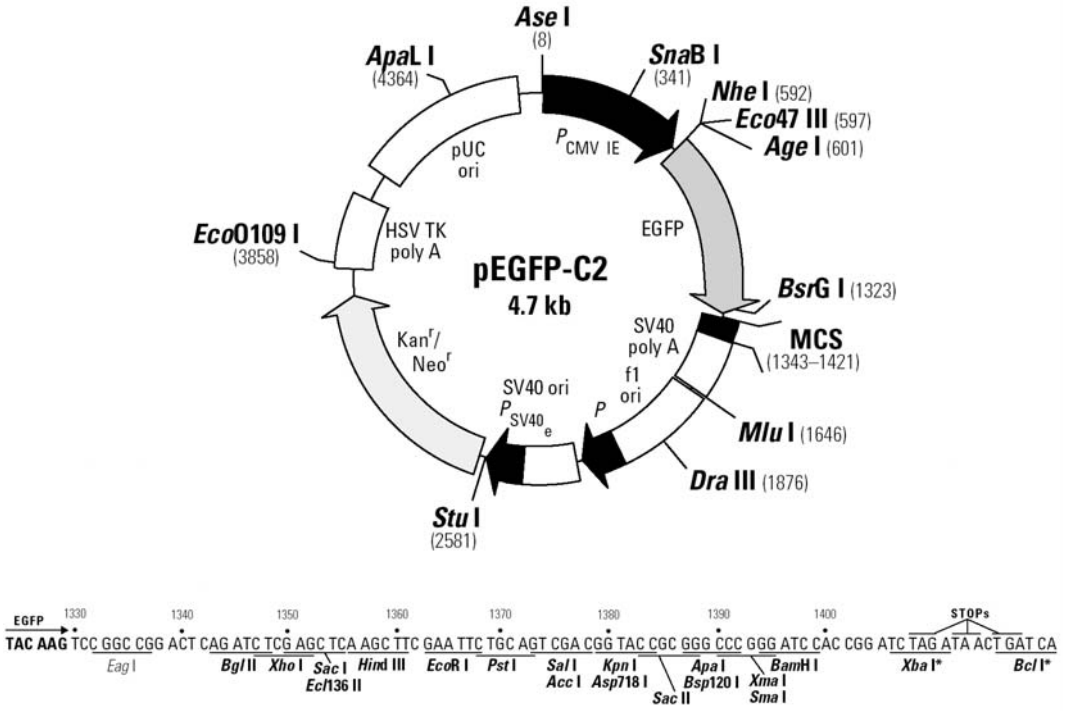


Figure 6.1 Vector map of the plasmid pEGFP-C2. pEGFP-C2 expresses the enhanced green fluorescent protein (EGFP) fusion proteins in mammalian cells (Clontech, Palo Alto, CA, USA).

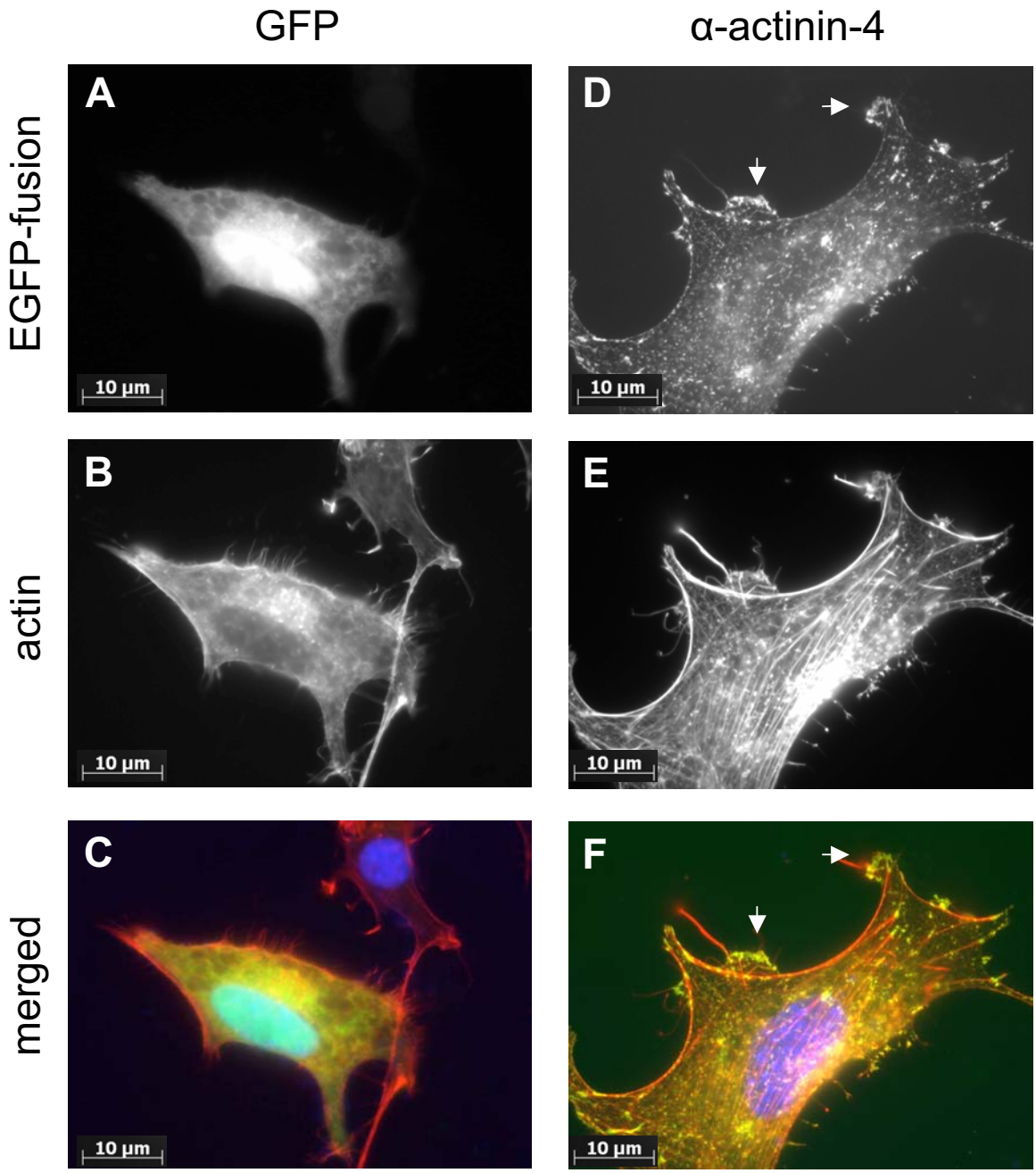
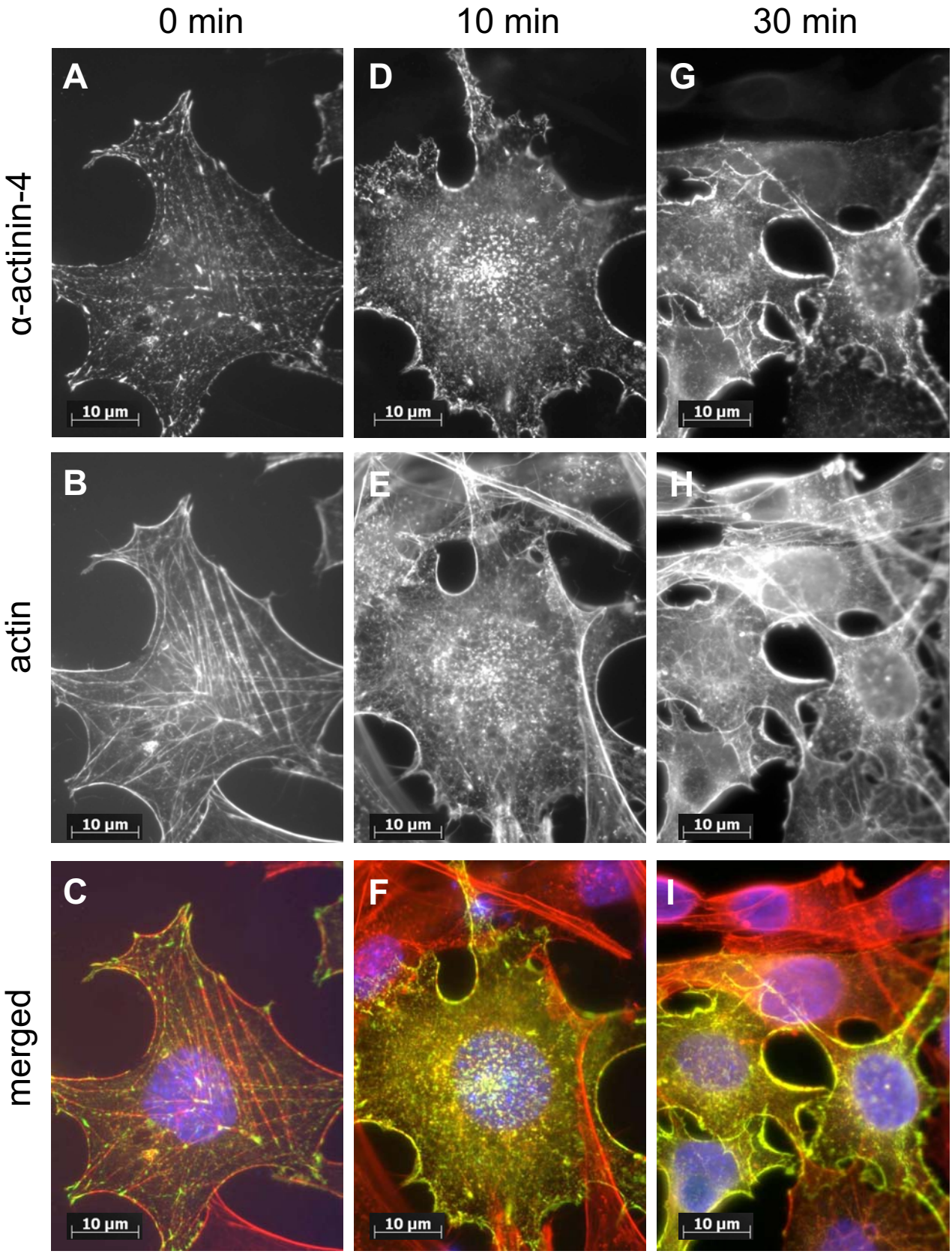


Figure 6.2 Subcellular localisation of EGFP alone and EGFP-α-actinin-4 fusion protein in NIH3T3 cells. NIH3T3 cells were transiently transfected with plasmids that encoded EGFP (A, B and C) and EGFP-α-actinin-4 (D, E and F), respectively, according to the method described in section 2.2.4.2. After 48 hours, cells were fixed with 4% paraformaldehyde, permeabilised with 0.2% Triton X-100 and stained with TRITC-conjugated phalloidin for F-actin and with DAPI for nucleus according to the method described in section 2.2.4.3. The images of each EGFP alone (A) and EGFP-α-actinin-4 (D) and actin stress fibres (B and E) were merged in (C) and (F) (α-actinin-4 in green, actin in red and nucleus in blue). Bars, 10 µM. The images shown are representative of the major fluorescence patterns observed for each clone.

Figure 6.3 Induction of actin filaments and α-actinin-4 re-organisation by EGF in NIH3T3 cells. NIH3T3 cells were transiently transfected with plasmid that encoded EGFP-α-actinin-4 fusion protein according to the method described in section 2.2.4.2. After 48 hours, cells were washed and incubated with serum-free medium for 24 hours. Cells were then treated with 100 ng/mL human recombinant EGF for 0 (A, B and C), 10 (D, E and F) and 30 (G, H and I) minutes. Cells were fixed with 4% paraformaldehyde, permeabilised with 0.2% Triton X-100 and stained with TRITC-conjugated phalloidin for F-actin and with DAPI for nucleus according to the method described in section 2.2.4.3. The images of each EGFP-α-actinin-4 (A, D and G) and actin stress fibres (B, E and H) were merged in (C), (F) and (I) (α-actinin-4 in green, actin in red and nucleus in blue). Bars, 10 μM. The images shown are representative of the major fluorescence patterns observed for each clone.


0 min
10 min
30 min
α-actinin-4
actin
merged
A
B
C
D
E
F
G
H
I
10 µm

co-localised with actin at presumptive focal adhesions and actin stress fibres. When transfected NIH3T3 cells were stimulated with EGF for 10 minutes, the microfilament bundles were absent (Figure 6.3 E) and α-actinin-4 was co-localised with actin dispersed in the cytoplasm (Figure 6.3 D and F). After 30 minutes of EGF treatment, EGFP-fusion α-actinin-4 was co-localised with cell membrane-associated cortical actin cytoskeleton. (Figure 6.3 G and I). These results indicated that α-actinin-4 was co-localised with actin but redistributed from the presumptive focal adhesions and actin stress fibres to the cell membrane. These results were consistent with a previous report from Greenwood *et al.* in 2000 (Greenwood *et al.*, 2000).

6.3.3 Validation of α-actinin-4 Mutants in NIH3T3 Cells

The α-actinin-4 Y265A and H266E mutants were generated from pEGFP-α-actinin-4 wildtype plasmid by site-directed mutagenesis to make single amino acid replacements within the C-terminal region of the CH2 domain. The mutant plasmids were sequenced using appropriate primers to ensure expression of the correct EGFP-fusion proteins (Figure 6.4).

Two plasmids that encode the EGFP-α-actinin-4 mutants, Y265A and H266E, were transiently transfected into NIH3T3 cells for 36 hours. Cells were then fixed with 4% paraformadehyde and stained with TRITC-conjugated phalloidin to visualise the actin microfilaments (Figure 6.5). Similar to those described in section 6.3.1, actin stress fibres were observed throughout the cell body, terminating at presumptive focal adhesions (Figure 6.5 B, E and H). Wildtype α-actinin-4 was co-localised with presumptive focal adhesions (green greater spot) and actin stress fibres (green fine spot) (Figure 6.5 A and C). However, Y265A or H266E α-actinin-4 mutants were co-localised with actin stress fibres but rarely co-localised with the ends of actin stress fibres (Figure 6.5 D and G).

Residues Tyr 265 and His 266 were considered to be two critical residues within the CH2 domain for phosphoinositides binding (referred to chapter 5). As shown in Figure 6.5 D and G, α-actinin-4 mutants, Y265A and H266E, were co-localised with actin stress fibres, suggesting the F-actin binding or bundling activity of these two mutants was not influenced. These results suggested that point mutation within the phosphoinositide binding site did not

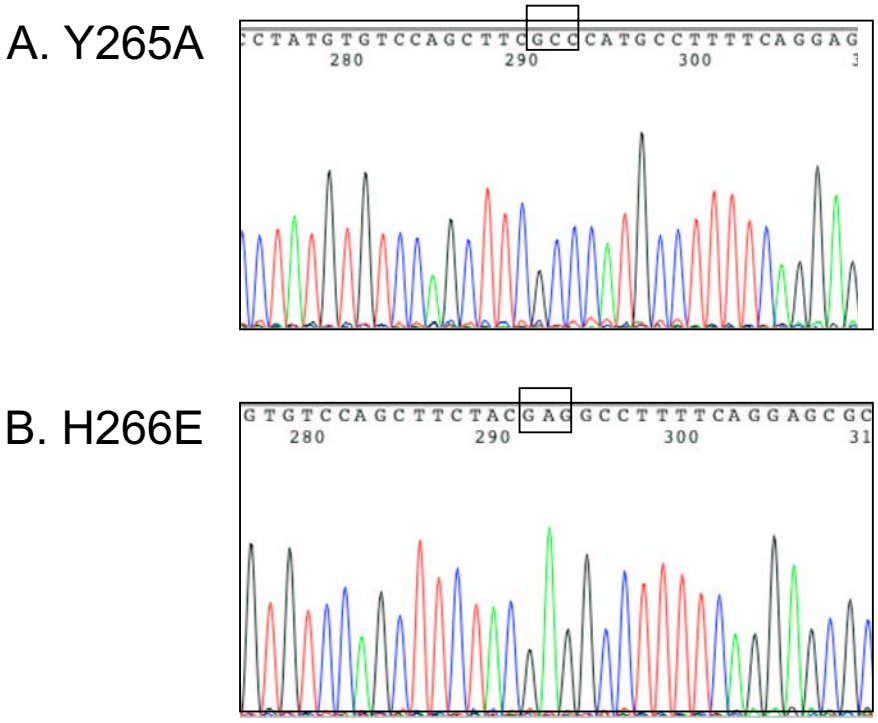
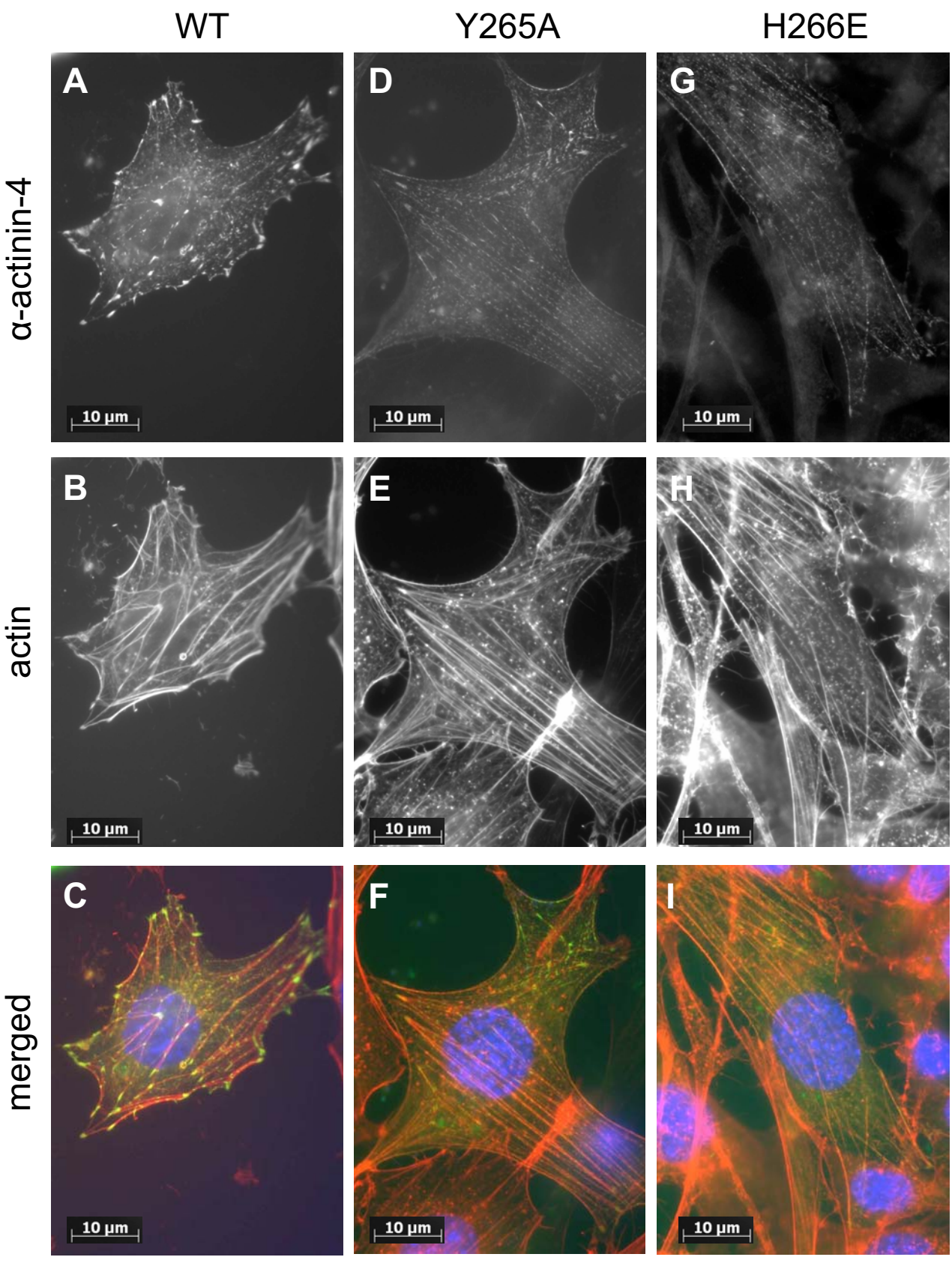


Figure 6.4 The representative electropherogram from DNA sequencing of the α-actinin-4 mutants. Codons for the Y265A (A) and H266E (B) residues are boxed.

Figure 6.5 Subcellular localisation of EGFP-α-actinin-4 mutants Y265A and H266E in NIH3T3 cells. NIH3T3 cells were transiently transfected with plasmid that encoded EGFP-α-actinin-4 wildtype (A, B and C), Y265A (D, E and F) and H266E (G, H and I) according to the method described in section 2.2.4.2. After 48 hours, cells were fixed with 4% paraformaldehyde, permeabilised with 0.2% Triton X-100 and stained with TRITC-conjugated phalloidin for F-actin and with DAPI for nucleus according to the method described in section 2.2.4.3. The images of each EGFP-α-actinin-4 (A, D and G) and actin stress fibres (B, E and H) were merged in (C), (F) and (I) (α-actinin-4 in green, actin in red and nucleus in blue). Bars, 10 μM. The images shown are representative of the major fluorescence patterns observed for each clone.


WT
Y265A
H266E
α-actinin-4
actin
merged
A
B
C
D
E
F
G
H
I
10 µm

influence the F-actin binding activity, consistent with the results described in section 5.3.8 and on Table 5.1. However, the Y265A and H266E α-actinin-4 mutants did not co-localised strongly with the presumptive focal adhesions. These results implied that PIP2 or PIP3 might assist the localisation of α-actinin-4 in focal adhesions.

In order to investigate the action of these α-actinin-4 mutants after EGF stimulation, NIH3T3 cells transiently expressing the α-actinin-4 mutants were treated with EGF according to the method described above. After 30 minutes of EGF treatment, both α-actinin-4 Y265A and H266E mutants were co-localised with actin in the cytoplasm rather than concentrated along the cell membrane (Figure 6.6 D and F for Y265A and 6.6 G and I for H266E). These results suggested that residues Tyr 265 and His 266 are essential for redistribution of α-actinin-4.

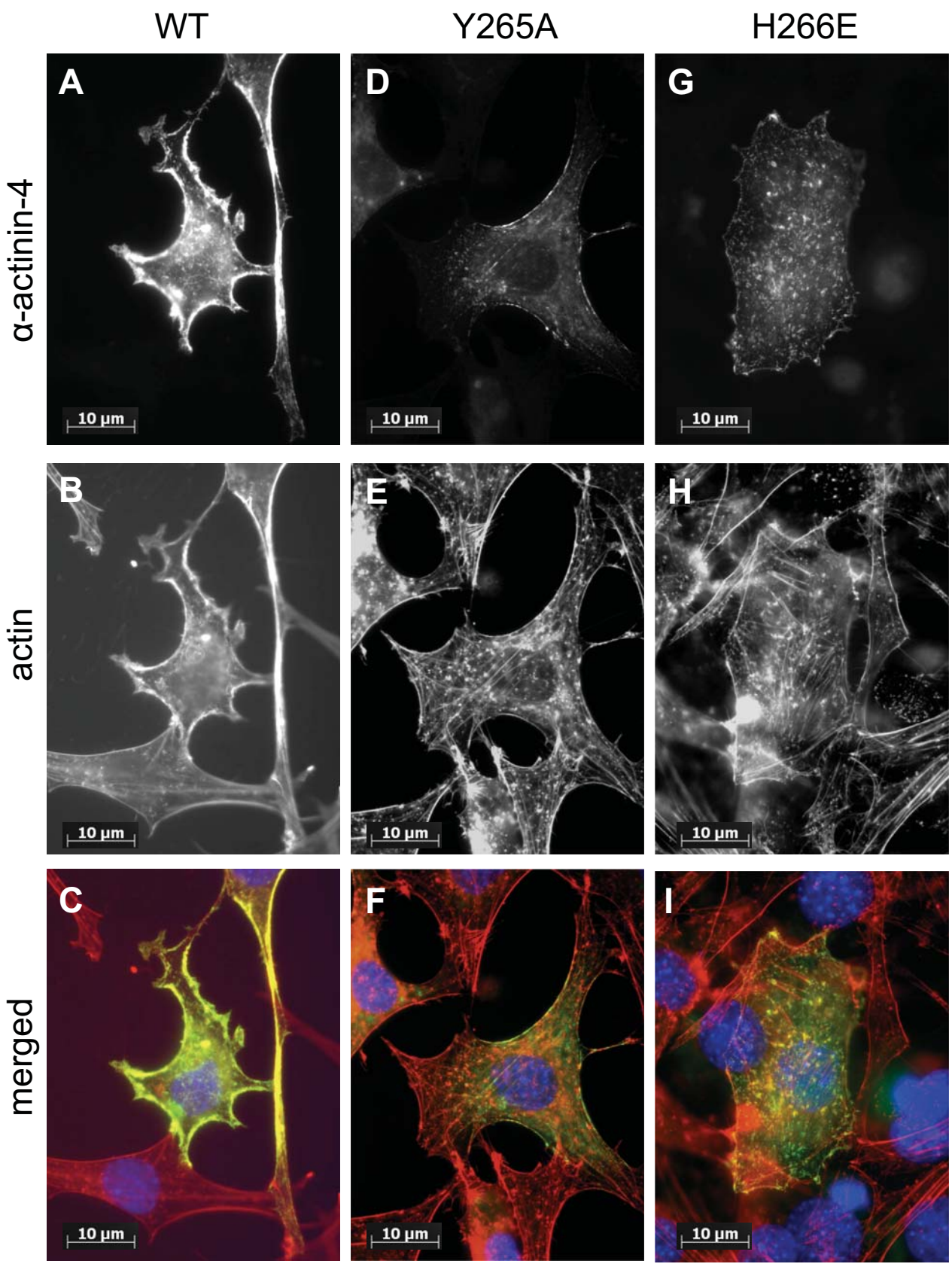
6.4 Discussion

6.4.1 Subcellular Localisation of α-actinin-4

As described in section 6.3.1, α-actinin-4 was co-localised with focal adhesions as well as actin stress fibres in the resting cells. Once the cells were stimulated by EGF, actin filaments were rearranged and α-actinin-4 was redistributed from focal adhesions and actin stress fibres to actin filaments in the cytoplasm. α-actinin-4 was then co-localised with actin filaments on membrane ruffles and cell membrane-associated cortical actin cytoskeleton after EGF stimulation for 30 minutes. These results were similar to that of α-actinin-1 reported by Greenwood *et al.* (Greenwood *et al.*, 2000).

As described in chapter 5, the phosphoinositide binding site was mapped on a region between the N-terminal and the C-terminal α-helices of α-actinin-4 CH2 domain. In order to investigate the biological roles of this phosphoinositide binding site, EGFP-Y265A and -H266E mutants of α-actinin-4 were transiently over-expressed in NIH3T3 cells. As described in section 6.3, Y265A and H266E mutants of α-actinin-4 were co-localised with actin stress fibres but only rarely co-localised with focal adhesions in resting cells. Moreover, both Y265A and H266E mutants of α-actinin-4 were co-localised with actin in the cytoplasm rather than concentrated along the cell membrane after EGF stimulation for

Figure 6.6 Induction the redistribution of α-actinin-4 mutants by EGF in NIH3T3 cells. NIH3T3 cells were transiently transfected with plasmids that express EGFP fusion proteins of α-actinin-4 wildtype (A, B and C), Y265A (D, E and F) and H266E (G, H and I) mutant using Lipofectamine 2000 on sterile 0.2% gelatin-coated coverslips in 6-well trays. After 48 hours, cells were washed and incubated with serum-free medium for 24 hours. Cells were then treated with 100 ng/mL human recombinant EGF for 30 minutes, fixed with 4% paraformaldehyde and stained with TRITC-conjugated phalloidin for F-actin and with DAPI for nucleus. The coverslips were inverted onto microscope slides and inspected with a fluorescence microscopy. The images of each EGFP-α-actinin-4 wildtype (A), Y265A (D) and H266E (G) mutant and actin stress fibres (B, E and H) were merged in (C), (F) and (I) (α-actinin-4 in green, actin in red and nucleus in blue). Bars, 10 μM. The images shown are representative of the major fluorescence patterns observed for each clone.


WT
Y265A
H266E
α-actinin-4
actin
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10 µm

30 minutes. These results suggested that PIP2 assists the co-localisation of α-actinin-4 with focal adhesions.

CHAPTER 7

Final Discussion

7.1 Role of α-actinin-4

α-actinin-4 is associated with focal adhesions by direct interaction with integrin β1 subunit and vinculin and is thought to be essential for stabilising cell morphology and adhesion. The α-actinin-4 deficient mice have been generated using homologous recombination in ES cells (Kos *et al.*, 2003) and show a severe kidney failure phenotype, including proteinuria and glomerulosclerosis, and typically death by several months of age (Kos *et al.*, 2003). It is therefore clear that α-actinin-4 is required for normal kidney function. Podocyte cells were generated from $Actn4^{+/+}$ and $Actn4^{-/-}$ mice to investigate the roles of α-actinin-4 in normal kidney function. Using immunofluorescence analysis, α-actinin-4 was co-localised with focal adhesions and actin stress fibres in podocytes derived from wildtype mice (Yao *et al.*, 2004). Compared with podocytes derived from $Actn4^{+/+}$, podocytes derived from $Actn4^{-/-}$ showed a loss of adherence to the glomerular basement membrane components, such as collagen IV and laminin 10 or 11 (Dandapani *et al.*, 2007). These results suggested that α-actinin-4 is required for normal podocyte adhesion. Three α-actinin-4 mutants, K255E, T259I and S262P have been identified from three families with an autosomal dominant from of FSGS (Kaplan *et al.*, 2001). FSGS (focal segmental glomerulosclerosis) is a common renal lesion characterised by regions of sclerosis in the renal glomeruli from a wide range of primary disorders, such as diabetes, HIV infection and hypertension (Ichikawa and Fogo, 1996; Somlo and Mundel, 2000). In order to investigate the biological function of these α-actinin-4 mutants, an α-actinin-4 K255E mutant mouse was generated by knock-in technology (Yao *et al.*, 2004; Michaud *et al.*, 2003). The phenotype of the $Actn4^{K255E/K255E}$ mutant mice also showed severe kidney failure, but heterozygous mice, $Actn4^{K255E/+}$ showed no obvious ill effect. Podocyte cells derived from K255E mutant mice have been generated to investigate the function of α-actinin-4 K255E mutant. The results showed that α-actinin-4 K255E is aggregated in the cytoplasm and degraded rapidly through the ubiquitin porteasome pathway (Yao *et al.*, 2004). It is assumed that α-actinin-4 K255E could not perform its normal function in the cell. Therefore, the role of α-actinin-4 is clearly demonstrated to be required for kidney function and involved in focal adhesions for stabilising cell adhesion.

Non-muscle isoforms, α-actinin-1 and -4, are expressed ubiquitously and co-localised with

actin in focal adhesions and actin stress fibres. However, the biological function of these two non-muscle isoform α-actinin is not the same because α-actinin-1 does not compensate for the role of α-actinin-4 in FSGS. α-actinin-1 is localised in focal adhesions by interacting with integrin β1 subunit and vinculin and is phosphorylated by focal adhesion kinase (FAK) in the integrin-mediated signaling pathway (Izaguirre *et al.*, 2001; Craig *et al.*, 2007). Although α-actinin-4 is localised in focal adhesions, the phosphorylation of α-actinin-4 by FAK or others focal adhesion associated kinase or phosphatase have not been reported. The biological role of α-actinin-4 in the focal adhesion-associated signaling pathway has been investigated using siRNA knock down analysis in mammalian cells, including human SW620, HT-29, and Caco-2 colon cancer cell lines (Craig *et al.*, 2007). The results indicated that diminishing the expression of α-actinin-4 would not influence the focal adhesion-associated signaling pathway (Craig *et al.*, 2007). Tec kinase has been reported to specifically interact with the spectrin-like repeat 3 domain of α-actinin-4 *in vitro* using yeast two-hybrid analysis and a GST pull down assay (Merkel, 2002). Phosphorylation of α-actinin-4 by Tec kinase has not been investigated. Therefore, there is no evidence that α-actinin-4 might be involved in the focal adhesion-associated signaling pathway.

Both α-actinin-1 and -4 were reported to be involved in tumorigenicity of cells (Gluck *et al.*, 1993; Gluck and Ben-Ze'ev, 1994; Nikolopoulos *et al.*, 2000). Transformed or immortalised cells express low levels of α-actinin-1 or -4 and restoration of α-actinin-1 or -4 expression suppresses the tumorigenicity of these cells *in vitro* and *in vivo*. Therefore, non-muscle α-actinin isoforms are believed to act as tumor suppressors. The expression level of α-actinin-4 might influence the cell growth rate in prostate cancer (Hara *et al.*, 2007). Based on the biochemical evidence, the CH domains and four spectrin-like repeat domains are though to be involved in inhibition of cell proliferation (Hara *et al.*, 2007). According to the subcellular localisation of α-actinin-4, α-actinin-4 is localised in focal adhesions and stress fibres in normal cells but localised in the nucleus and diffusely dispersed in the cytoplasm in cancer cells (Honda *et al.*, 1998; Bolshakova *et al.*, 2007). These observations implied that α-actinin-4 might be involved in transcriptional activity in cancer cells. α-actinin-4 might be directly or indirectly associated in transcription factors or transcription elements (Poch *et al.*, 2004; Babakov *et al.*, 2004; Goffart *et al.*, 2006; Chakraborty *et al.*, 2006). However, these reports did not focus on a specific factor or element. α-actinin-4 K122N mutant and natural

spliced variant, have been reported to be associated in non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), respectively. α-actinin-4 K122N mutant shows high tumorigenicity *in vitro* and *in vivo* (Menez *et al.*, 2004) and might be a potential target in chemotherapy for NSCLC (Mami-Chouaib *et al.*, 2002). In this PhD project, I have investigated the F-actin binding activity of the natural spliced variant of α-actinin-4, its regulation by Ca^{2+} and phosphoinositides (PIP2 and PIP3) and its subcellular localisation (data not shown). However, there are no significant differences between those of wildtype and the natural spliced variant of α-actinin-4. Therefore, how the natural variant α-actinin-4 is involved in SCLC is unclear.

The decreased expression level of α-actinin-4 results in a highly tumorigenic phenotype. However, increased expression levels of α-actinin-4 may result in a high metastatic potential (Honda *et al.*, 1998; 2005; Yamamoto *et al.*, 2007; Fu *et al.*, 2007). E-cadherin and β-catenin play roles as an invasion suppressor in cancer cells (Hirohashi, 1998). The increased expression level of α-actinin-4 might compete with E-cadherin to bind β-catenin, resulting in promoting the invasiveness and metastatic potential in cancer cells (Hayashida *et al.*, 2005). Therefore, α-actinin-4 is believed to be involved in cancer tumorgenicity and metastasis.

7.2 Role of Phosphoinositides in α-actinin-4

The phosphoinositide binding site has been identified from chicken skeletal muscle α-actinin and is mapped on a region, residues 177-193, adjacent to actin binding site-3 on the CH2 domain (Fukami *et al.*, 1996). The critical residues for potential charged interactions with the phosphate group of the phosphoinositide are residues Lys 181, His 189 and Lys 193. These binding residues have been confirmed in human α-actinin-1 by generating point mutation mutants using site-direct mutagenesis. The results showed that these residues are involved in the interaction with phosphoinositides by charged interaction (Fraley *et al.*, 2003). However, in this thesis, the phosphoinositide binding site identified in α-actinin-4 is not located at the same region as for chicken skeletal muscle α-actinin. NMR-based ligand titration analysis showed that a docking site between the first α-helix and last α-helix is involved in the interaction with phosphoinositides and the critical

residues are Glu 167, Gly 168, Leu 169, Leu 170, Trp 172, Tyr 265 and His 266. It is presumed that His 266 directly interacts with the phosphate group of phosphoinositides by a charged interaction and residues Glu 167, Gly 168, Leu 169, Leu 170, Trp 172 and Tyr 265 bind the acyl chains of phosphoinositides by hydrophobic interaction. Moreover, based on the crystal structure of the CH domains (2X CH) of α-actinin-4 K255E mutant (Lee *et al.*, 2008), residues His 66, Arg 65 and Lys 66 within the CH1 domain are proposed to be specifically involved in the binding to PIP3 by charged interaction between these positively charged residues and the phosphate group at position 3 of the inositol (referred to Figure 5.22). The residues identified from α-actinin-1 (Lys 181, His 189 and Lys 193) did not show any binding effect on α-actinin-4. Therefore, the phosphoinositide binding site on α-actinin-1 and α-actinin-4 appear to be different.

According to results described in chapter 6, residues Tyr 265 and His 266 on the CH2 domain might assist the localisation of α-actinin-4 at focal adhesions in un-stimulated NIH3T3 cells. α-actinin-4 is thought to be a membrane-associated protein (Burn *et al.*, 1985). Biochemical evidence showed that α-actinin-4 directly interacts with negatively charged phospholipids, but does not bind to membranes composed of neutral lipids (Fritz *et al.*, 1993; Niggli and Gimona, 1993; Han *et al.*, 1997). Therefore, I predicted that α-actinin-4 may directly interact with cell membrane (Figure 7.1). Both PIP2 and PIP3 interact with α-actinin-4 at the same binding site and with equal binding affinity (the Kd values 109 μM for PIP2 and 127 μM for PIP3) (described in chapter 5). However, in the un-stimulated cells, the concentration of PIP2 and PIP3 at the inner leaflet of the plasma membrane is approximately 5 mM and 5 μM, respectively. Therefore, PIP2 would be the critical phosphoinositide for interacting with α-actinin-4 in un-stimulated cells. According to the *in vitro* F-actin binding analysis, both PIP2 and PIP3 inhibit the F-actin binding activity of α-actinin-4 by competition for the actin binding site-3 on the CH2 domain with F-actin, with PIP3 being more effective (Fraley *et al.*, 2003; chapter 5 in this thesis). The phosphate group at the position 3 of PIP3 inositol head group is proposed to interact with a region within the CH1 domain (referred to Figure 5.22). Therefore, the conformational change of the CH domains (2X CH) (switched between open and closed conformation) is proposed to be regulated by binding to PIP3, resulting in influence of their F-actin binding ability. Indeed, PIP2 appears to stabilise the CH2 domain of α-actinin, whereas PIP3

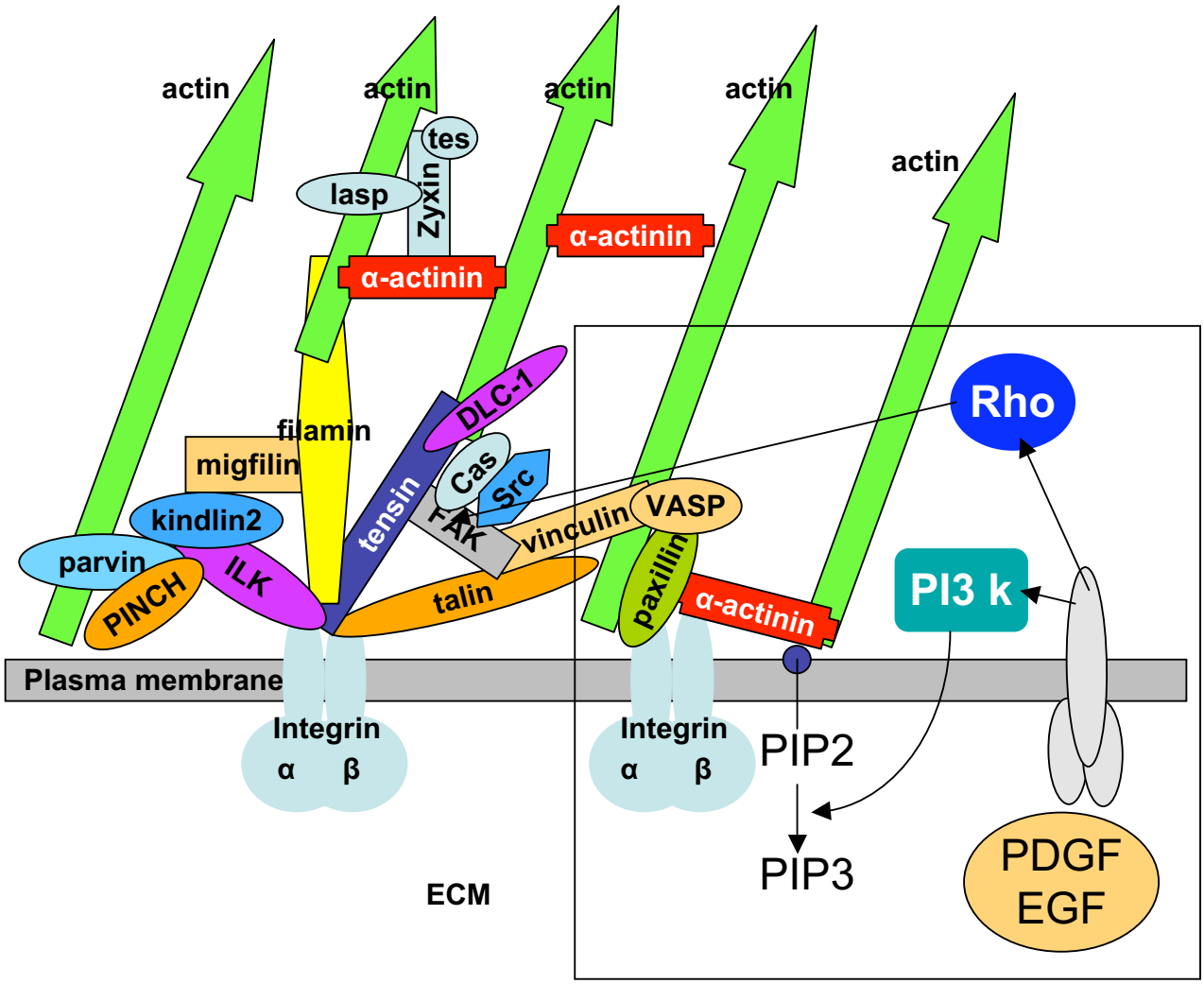

Figure 7.1 Regulation of α-actinin-4 reorganisation. In resting cells, α-actinin-4 directly interacts with integrin β subunit and PIP2 on the cell membrane to maintain cell shape and adhesion. Once cells are stimulated by PDGF or EGF, PI3 kinase would be activated to convert PIP2 to PIP3. PIP3 is proposed to compete α-actinin-4 to F-actin interaction and direct or indirect dissociate α-actinin-4 from integrin β subunit, resulting in re-distribution of α-actinin-4 from focal adhesions to the cytoplasm.

increases the flexibility of the CH2 domain (Corgan *et al.*, 2004). In addition, interactions with PIP2 or PIP3 differently affect the structure of the CH2 domain (Full *et al.*, 2007). Therefore, PIP2 may be a critical phosphoinositide for assistance of α-actinin-4 binding to cell membranes, whereas PIP3 may be responsible for regulating the localisation of α-actinin-4 on the cell membrane.

As described in chapter 6, in EGF-stimulated NIH3T3 cells, α-actinin-4 underwent a redistribution from focal adhesion to cytoplasm and cell membrane. Similar to that of α-actinin-4, the redistribution of non-muscle α-actinin induced by PDGF has been reported in rat embryonic fibroblast (REF) cells (Greenwood *et al.*, 2000). The PDGF induced α-actinin redistribution could be eliminated by PI3 kinase inhibitors, wortmannin or LY294002. These results suggested that PI3 kinase may be involved in the redistribution of α-actinin. When PI3 kinase is activated by PDGF or EGF, the concentration of PIP2 would be decreased from 5 mM to 3.5 mM, whereas that of PIP3 would be increased from 5 μM to 200 μM. In addition, the redistribution of α-actinin can be induced in PIP3 treated REF cells (Greenwood *et al.*, 2000). These results suggested that PIP3 is sufficient to induce the redistribution of α-actinin-4. As described above, PIP3 increases the flexibility of the CH2 domain of α-actinin-4 (Corgan *et al.*, 2004). Therefore, α-actinin-4 dissociates from the cell membrane when PI3 kinase is activated by PDGF or EGF. α-actinin-4 is involved in focal adhesions by directly interacting with integrin β subunit in un-stimulated cells. α-actinin-4 should be dissociated from integrin β subunit when cells were treated with PDGF or EGF. PIP3 might not interrupt the interaction between α-actinin-4 and integrin β subunit because PIP3 and integrin β subunit bind different regions on α-actinin-4. PIP3 binds the CH2 domain on α-actinin-4, wheras, integrin β subunit binds a region between spectrin-like repeat domain 1 and 2 on α-actinin-4 (Kelly and Taylor, 2005). Therefore, it is assumed that other proteins might be involved in this dissociation mechanism.

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APPENDIX

APPENDIX I: Chemical shift list of α-actinin-4 CH2 domain

Residues	Name	^{15}N	^{13}CO	^{13}Cα	^{13}Cβ	^{13}Cγ	HN	Hα	Hβ	Hγ	other
-2	Gly										
-1	Ser										
162	Glu		177.229	57.652	30.084	35.579		4.372	2.028	2.303	
									2.121	2.342	
163	Thr	115.083	175.017	62.905	69.726	21.496	8.223	4.325	4.247	1.235	
164	Ser										
165	Ala		179.107	54.111	18.755			4.231	1.416		
166	Lys	120.155	177.539	59.496	33.335	29.858	8.17	4.013	1.896	1.492	Cδ 25.181
											Cε 42.294
											Hδ2 1.602, Hδ1 1.761
											Hε 3.063
167	Glu	119.219	179.113	59.329	29.874	36.957	8.329	4.109	2.118	2.386	
168	Gly	107.83	176.372	46.973			8.387	3.943			
								4.006			
169	Leu	124.293	179.563	57.81	42.117	27.284	8.119	4.248	1.486	1.725	Cδ2 23.533, Cδ1 26.056
									1.946		Hδ2 0.506, Hδ1 0.668
170	Leu	122.427	178.598	59.175	41.294	26.641	8.759	3.97	1.6	1.239	Cδ2 22.89, Cδ1 25.391
									1.93		Hδ2 0.092, Hδ1 0.608
171	Leu	119.384	179.385	58.183	42.125	27.038	8.078	4.282	1.822	1.766	Cδ 24.519
											Hδ2 0.999, Hδ1 1.02
172	Trp	120.681	178.13	63.05	28.619		8.235	4.308	3.513		Nε1 131.241
									3.853		Hδ1 7.659
											Hε1 10.727
											Hε3 8.183
											Hζ2 7.538
											Hζ3 7.219
											Hη2 7.136
173	Cys	115.858	178.881	64.723	28.139		8.817	3.796	2.629		
									3.675		
174	Gln	121.776	178.16	59.885	27.903	34.38	8.832	3.669	1.884	2.462	Nε2 108.363
									2.479	2.54	Hε21 6.643, Hε22 7.393
175	Arg	119.616	180.628	59.568	30.45	27.885	8.605	4.085	1.874	1.681	Cδ 43.765
									1.979	1.876	Hδ 3.25
176	Lys	115.611	176.849	56.273	32.46	22.969	8.251	3.961	1.26	0.219	Cδ 27.279
									1.439	0.855	Cε 42.067
											Hδ2 1.157, Hδ1 1.416

											Hε2 2.799, Hε1 3.041
177	Thr	101.781	174.895	61.173	69.916	20.929	7.329	4.603	4.337	0.918	
178	Ala	127.769	175.601	56.379	16.648		7.136	4.236	1.488		
179	Pro		175.764	64.854	31.382	27.616		4.261	0.889	1.484	Cδ 51.395
									2.011	1.709	Hδ2 3.21, Hδ1 3.498
180	Tyr	118.191	175.896	57.521	37.309		7.544	4.21	2.684		Hδ 6.514
									2.877		Hε 6.279
181	Lys	122.713	176.703	57.929	31.964	24.93	8.542	4.187	1.805	1.509	Cδ 28.651
									1.928		Cε 42.215
											Hδ 1.667
											Hε 2.982
182	Asn	114.159	173.282	51.937	37.164		8.072	4.63	2.813		Nδ2 110.976
									3.136		Hδ21 6.676, Hδ22 7.716
183	Val	118.844	174.197	61.236	34.962	22.898	7.498	3.878	1.925	0.676	
										0.699	
184	Asn	123.698	174.181	52.568	39.048		8.74	4.594	2.601		Nδ2 110.129
									2.681		Hδ21 6.68, Hδ22 7.446
185	Val	124.898	175.203	64.538	31.257	21.325	9.088	3.292	1.597	0.11	
						22.221				0.654	
186	Gln	125.164	173.885	54.866	32.288	32.853	8.147	4.219	1.45	1.968	Nε2 113.011
									2.332	2.167	Hε21 6.761, Hε22 7.599
187	Asn	112.714	173.691	52.071	39.686		7.541	4.251	2.041		Nδ2 118.735
									2.975		Hδ21 6.134, Hδ22 8.015
188	Phe	116.571	171.87	58.955	38.879		9.337	5.176	2.699		Hδ 6.996
									3.776		Hε 7.421
											Hζ 6.983
189	His	112.608	176.889	57.122	31.91		8.534	5.04	3.044		Hδ2 7.153
									3.105		Hε1 7.829
190	Ile		178.515	66.562	39.15	γ1 28.286		3.934	2.248	γ12 1.743	Cδ 14.297
						γ2 19.07				γ11 1.94	Hδ 1.132
										γ2 1.361	
191	Ser	117.33	173.728	61.959	64.305		9.417	4.377	3.491		
									3.645		
192	Trp	119.805	177.118	57.558	29.538		8.255	4.224	2.926		Nε1 128.971
									3.415		Hδ1 6.971
											Hε1 10.131
											Hε3 5.672
											Hζ2 7.251
											Hζ3 6.06

											Hη2 6.95
193	Lys	120.179	175.197	59.95	34.168	25.92	7.393	3.977	1.912	1.741	Cε 41.753
									2.081		Hδ 1.814
											Hε2 3.242, Hε13.388
194	Asp	111.105	177.169	53.785	40.66		7.868	4.769	2.575		
									3.368		
195	Gly	103.065	175.666	47.451			7.507	4.072			
								4.134			
196	Leu	124.814	178.708	59.297	39.838	27.273	8.903	3.933	1.51	1.255	Cδ2 23.413, Cδ1 25.917
									1.88		Hδ2 0.362, Hδ1 0.677
197	Ala	121.28	179.3	56.401	17.342		8.858	3.634	0.293		
198	Phe	113.73	177.992	63.107	41.055		8.307	4.476	2.981		Hδ 7.399
									3.295		Hε 7.215
											Hζ 7.276
199	Asn	114.523	176.839	58.37	41.545		7.291	4.354	2.446		Nδ2 114.621
									2.922		Hδ21 7.419, Hδ22 7.748
200	Ala	124.487	177.991	55.569	17.63		9.251	3.045	0.53		
201	Leu	115.292	179.079	58.062	43.669	27.277	8.064	3.722	1.783	1.987	Cδ2 24.85, Cδ1 25.558
									2.064		Hδ2 1.011, Hδ1 1.163
202	Ile	115.961	177.189	66.091	39.186	γ1 29.211	7.343	3.959	2.039	γ12 1.009	Cδ 18.457
						γ2 14.473				γ11 2.408	Hδ 1.09
										γ2 1.214	
203	His	122.631	175.47	61.184	31.577		8.957	3.712	2.97		Hδ2 8.174
											Hε1 7.709
204	Arg	116.258	176.366	56.495	29.437	26.75	8.459	3.927	1.394	0.771	Cδ 42.383
									2.113	1.631	Hδ2 3.118, Hδ1 3.403
205	His	113.598	175.151	59.062	31.332		7.005	4.313	1.835		Hδ2 6.938
									2.899		Hε1 7.094
206	Arg	119.617	172.478	51.513	30.2	25.808	8.712	4.876	1.725	1.454	Cδ 41.081
										1.751	Hδ 3.456
207	Pro		177.723	65.266	31.027	27.259		4.104	1.581	1.862	Cδ 50.212
									1.774	1.922	Hδ2 3.108, Hδ1 3.678
208	Glu	118.244	177.596	57.605	27.74	35.533	9.881	4.299	2.027	2.458	
209	Leu	118.297	176.547	55.336	43.354	26.781	7.912	4.239	1.461	1.423	Cδ2 20.716, Cδ1 25.511
									1.851		Hδ2 0.193, Hδ1 0.62
210	Ile	115.821	175.008	57.45	42.181	γ1 26.042	7.111	4.565	2.087	γ12 1.499	Cδ 16.408
						γ2 10.935				γ11 1.685	Hδ 1.139
										γ2 0.997	
211	Glu	128.94	176.93	54.388	27.771	36.549	8.997	4.748	1,998	2.195	

										2.333	
212	Tyr	124.049	177.096	63.282	38.893		7.451	3.368	2.155		Hδ 6.548
									2.386		Hε 6.668
213	Asp	114.42	176.592	56.088	40.409		8.592	4.29	2.652		
									2.731		
214	Lys	116.339	177.121	56.346	33.498	25.469	7.246	4.217	1.747	1.361	Cε 42.346
									1.933		Hδ 1.432
											Hε 2.958
215	Leu	119.889	177.435	55.036	40.965	26.143	7.158	4.255	0.932	1.486	Cδ2 26.569, Cδ1 20.944
									1.167		Hδ2 0.488, Hδ1 0.668
216	Arg	122.683	177.587	54.639	33.14	27.17	8.984	4.576	1.82	1.593	Cδ 43.607
									1.983	1.708	Hδ 3.274
217	Lys		175.251	59.429	33.016	24.789		3.674	1.814	1.46	Cδ 29.072
											Cε 42.182
											Hδ 1.675
											Hε 3.022
218	Asp	112.331	174.585	53.574	40.148		7.957	4.543	2.674		
									2.885		
219	Asp	118.085		51.591	41.297		7.23	5.231	2.319		
									3.128		
220	Pro		178.604	65.708	33.32			4.384	2.143		Cδ 50.929
									2.38		Hδ2 3.494, Hδ1 3.866
221	Val	118.981	177.99	68.073	31.637	21.57	8.332	3.444	2.128	0.917	
						24.15				1.048	
222	Thr	116.28	177.115	65.774	68.098	22.049	7.007	3.896	3.943	1.187	
223	Asn	120.452	178.747	56.594	38.326		7.707	4.174	2.327		Nδ2 108.246
									3.325		Hδ21 7.116, Hδ22 7.44
224	Leu	123.203	175.887	58.349	42.874	28.006	8.933	3.483	0.841	1.676	Cδ2 27.433, Cδ1 24.929
									1.812		Hδ2 1.081, Hδ1 1.303
225	Asn	113.739	177.971	56.613	38.08		8.07	4.353	2.81		Nδ 114.085
											Hδ21 7.375, Hδ22 7.418
226	Asn	117.808	176.553	58.01	40.411		8.404	4.463	2.883		Nδ 114.091
									3.014		Hδ21 7.078, Hδ22 8.029
227	Ala	119.032	179.175	55.734	18.394		7.581	3.961	1.513		
228	Phe	115.119	180.169	56.583	37.262		8.57	4.94	3.298		Hδ 7.27
									3.471		Hε 6.428
											Hζ 6.643
229	Glu	120.926	178.879	59.688	30.026	36.218	8.786	4.329	2.269	2.474	
									2.372		

230	Val	120.968	178.789	67.173	32.42	23.988 24.126	8.538	3.993	2.342	1,216 1.373	
231	Ala	121.16	179.643	55.423	18.838		8.887	4.049	1.727		
232	Glu	119.578	178.49	59.982	31.015	34.622	7.834	4.187	1.988 2.2	2.385	
233	Lys	118.114	178.619	59.214	33.802	25.31	8.492	3.954	1.393 1.78	0.807 1.241	Cδ 29.232 Cε 42.226 Hδ 1.555 Hε 2.892
234	Tyr	112.057	176.925	59.584	40.319		8.632	4.811	2.914 3.137		Hδ 7.492 Hε 6.961
235	Leu	115.963	175.391	53.681	42.315	27.456	7.162	4.777	1.454 2.148	1.646	Cδ 27.08 Hδ 0.946
236	Asp	116.242	174.636	55.706	39.901		7.59	4.346	2.58 3.013		
237	Ile	120.33	173.391	58.786	39.49	γ1 26.512 γ2 17.227	7.647	4.348	1.227	γ12 0.434 γ11 1.354 γ2 0.569	Cδ 13.046 Hδ -0.175
238	Pro		176.389	62.609	32.87	27.156		4.365	1.747 2.264	1.9 1.971	Cδ 51.453 Hδ2 3.674, Hδ1 3.956
239	Lys	120.43	177.445	57.625	32.454	26.809	8.349	4.246	1.643	1.355 1.488	Cδ 29.983 Cε 41.692 Hδ 1.767 Hε 3.044
240	Met	121.031	174.511	56.563	36.011	32.448	6.942	4.309	1.602 1.869	2.433 2.482	
241	Leu	117.738	174.363	52.653	43.874		7.182	4.32	0.722 1.018		Cδ2 27.323, Cδ1 22.841 Hδ2 0.618, Hδ1 0.642
242	Asp	121.481	176.649	52.917	43.708		8.615	4.839	2.487 2.844		
243	Ala	127.873	178.968	56.364	20.614		9.665	4.067	1.552		
244	Glu	115.255	178.478	60.144	29.356	37.18	8.622	3.915	2.08	2.305 2.377	
245	Asp	118.293	178.18	57.152	41.721		7.784	4.469	2.876		
246	Ile	118.98	176.953	64	38.028	γ1 29.211 γ2 19.21	7.753	3.866	2.304	γ12 1.498 γ11 1.844 γ2 1.151	Cδ 13.584 Hδ 1.121
247	Val	114.392	177.763	65.657	32.49	21.775 22.89	7.895	3.874	2.096	1.01 1.069	

248	Asn	116.417	175.3	54.19	39.343		7.96	4.822	2.951		Nδ 113.547 Hδ21 7.034, Hδ22 7.752
249	Thr	115.094	173.736	61.421	69.819	21.145	7.407	4.45	4.076	1.148	
250	Ala	129.096	178.596	55.435	18.542		8.681	4.113	1.467		
251	Arg	116.864	172.266	53.053	31.9	26.953	8.088	4.778	1.607	1.379	Cδ 43.514
									1.803	1.468	Hδ 2.888
252	Pro		173.957	62.911	33.558	27.742		4.416	1.921	1.942	Cδ 51.117
									2.496	2.213	Hδ2 3.527, Hδ1 3.858
253	Asp	119.413	176.151	54.963	41.676		8.453	4.45	2.72		
									2.935		
254	Glu	127.875	178.134	57.796	31.343	36.805	8.354	4.858	2.122	2.376	
									2.236	2.423	
255	Lys	120.834	179.557	59.768	31.833	25.515	8.164	4.009	1.959	1.4	Cδ 29.266
										1.494	Cε 41.768
											Hδ 1.72
											Hε 2.998
256	Ala	124.377	180.271	55.142	18.439		7.736	4.079	1.418		
257	Ile	117.983	177.818	65.972	36.584	γ1 30.467	8.052	3.668	2.382	γ12 1.224	Cδ 17.966
						γ2 12.932				γ11 1.86	Hδ 0.314
										γ2 1.002	
258	Met	118.508	178.381	60.416	32.998		8.701	3.047	1.64	1.487	
									1.775	1.72	
259	Thr	116.859	176.161	67.334	68.423	21.29	8.037	3.727	4.265	1.198	
260	Tyr	124.965	178.359	61.677	39.511		7.995	4.485	2.979		Hδ 6.996
									3.106		Hε 6.603
261	Val	118.963	179.085	67.521	31.454	24.362	9.143	3.54	1.845	0.358	
						23.024				1.081	
262	Ser	116.602	176.06	62.799	62.681		8.568	4.003	3.913		
									4.07		
263	Ser	118.239	177.24	62.842	63.152		7.723	4.333	3.699		
									3.982		
264	Phe	123.735	176.387	61.39	38.272		7.822	3.8	3.08		Hδ 7.275
											Hε 6.87
											Hζ 7.162
265	Tyr	119.966	177.849	61.255	37.788		7.791	3.254	1.984		Hδ 5.45
									2.784		Hε 6.244
266	His	116.818	177.726	59.609	29.956		8.008	4.052	3.063		Hδ2 7.107
									3.137		Hε1 7.99
267	Ala	121.375	179.694	54.83	18.191		7.59	3.997	0.97		

268	Phe	113.95	177.09	55.778	39.229		7.754	5.048	2.679		Hδ 7.094
									3.055		Hε 7.199
											Hζ 7.141
269	Ser	116.293	175.387	60.194	63.193		7.781	4.289	3.435		
									3.566		
270	Gly	110.228	173.811	45.647			8.272	3.781			
								3.946			
271	Ala	123.864	176.988	52.573	19.714		7.921	4.429	1.49		
272	Gln	125.128	180.949	57.738	30.266	32.195	8.073	4.197	1.989	2.375	Nε2 112.498
									2.186		Hε21 6.859, Hε22 7.533

APPENDIX II: Restraints list for α-actinin-4 CH2 domain

The amino acid numbers are full length α-actinin-4 numbers

Intraresidue NOES: 465
Sequential NOES: 275
Short range NOES: 132
Medium range NOES: 55
Long range NOES: 227
Total NOES: 1154

GLU 162
assign (segid " A" and resid 162 and name HB2) (segid " A" and resid 162 and name HA) 2.469 0.762 0.762 weight 1.000 ! spec=Expt_1, no=2518, id=1864, vol=4.043755e+05
assign (segid " A" and resid 162 and name HB2) (segid " A" and resid 163 and name HN) 2.945 1.084 1.084 weight 1.000 ! spec=Expt_1, no=3159, id=2297, vol=1.403062e+05

THR 163
assign (segid " A" and resid 163 and name HG1) (segid " A" and resid 163 and name HB) 1.955 0.478 0.478 weight 1.000 ! spec=Expt_1, no=464, id=416, vol=1.637246e+06
assign (segid " A" and resid 163 and name HG1) (segid " A" and resid 163 and name HA) 1.880 0.442 0.442 weight 1.000 ! spec=Expt_3, no=406, id=2931, vol=1.037311e+07

ALA 165
assign (segid " A" and resid 165 and name HA) (segid " A" and resid 165 and name HB2) 1.746 0.381 0.381 weight 1.000 ! spec=Expt_1, no=318, id=288, vol=3.234840e+06

LYS 166
assign (segid " A" and resid 166 and name HN) (segid " A" and resid 165 and name HB2) 2.095 0.549 0.549 weight 1.000 ! spec=Expt_1, no=693, id=594, vol=1.082463e+06
assign (segid " A" and resid 166 and name HN) (segid " A" and resid 165 and name HA) 2.284 0.652 0.652 weight 1.000 ! spec=Expt_1, no=695, id=595, vol=6.451825e+05
assign (segid " A" and resid 166 and name HN) (segid " A" and resid 166 and name HB2) 1.984 0.492 0.492 weight 1.000 ! spec=Expt_1, no=692, id=593, vol=1.500353e+06
assign (segid " A" and resid 166 and name HA) (segid " A" and resid 166 and name HE2) 2.319 0.672 0.672 weight 1.000 ! spec=Expt_3, no=607, id=3091, vol=2.953105e+06
assign (segid " A" and resid 166 and name HN) (segid " A" and resid 166 and name HE2) 2.499 0.780 0.780 weight 1.000 ! spec=Expt_3, no=4045, id=4057, vol=1.884347e+06
assign (segid " A" and resid 166 and name HN) (segid " A" and resid 166 and name HA) 2.653 0.879 0.879 weight 1.000 ! spec=Expt_4, no=168, id=4374, vol=8.726104e+06
assign (segid " A" and resid 166 and name HN) (segid " A" and resid 167 and name HN) 2.413 0.728 0.728 weight 1.000 ! spec=Expt_1, no=697, id=597, vol=4.631864e+05
assign (segid " A" and resid 166 and name HB1) (segid " A" and resid 169 and name HD13) 2.074 0.538 0.538 weight 1.000 ! spec=Expt_3, no=1594, id=3669, vol=5.767924e+06

GLU 167
assign (segid " A" and resid 167 and name HN) (segid " A" and resid 165 and name HB2) 2.578 0.831 0.831 weight 1.000 ! spec=Expt_1, no=718, id=613, vol=3.117806e+05
assign (segid " A" and resid 167 and name HN) (segid " A" and resid 166 and name HA) 2.412 0.727 0.727 weight 1.000 ! spec=Expt_1, no=714, id=610, vol=4.647902e+05
assign (segid " A" and resid 167 and name HN) (segid " A" and resid 167 and name HA) 2.207 0.609 0.609 weight 1.000 ! spec=Expt_1, no=713, id=609, vol=7.914891e+05
assign (segid " A" and resid 167 and name HN) (segid " A" and resid 167 and name HB2) 2.080 0.541 0.541 weight 1.000 ! spec=Expt_1, no=715, id=611, vol=1.131687e+06
assign (segid " A" and resid 167 and name HA) (segid " A" and resid 167 and name HB1) 2.035 0.518 0.518 weight 1.000 ! spec=Expt_1, no=1699, id=1350, vol=1.287867e+06
assign (segid " A" and resid 167 and name HA) (segid " A" and resid 170 and name HB1) 2.125 0.565 0.565 weight 1.000 ! spec=Expt_3, no=593, id=3080, vol=4.976461e+06
assign (segid " A" and resid 167 and name HA) (segid " A" and resid 170 and name HD21) 2.288 0.654 0.654 weight 1.000 ! spec=Expt_3, no=1370, id=3516, vol=3.196659e+06
assign (segid " A" and resid 167 and name HB1) (segid " A" and resid 170 and name HD21) 2.477 0.767 0.767 weight 1.000 ! spec=Expt_3, no=1599, id=3673, vol=1.984763e+06

GLY 168
assign (segid " A" and resid 168 and name HN) (segid " A" and resid 167 and name HN) 2.062 0.531 0.531 weight 1.000 ! spec=Expt_1, no=7, id=6, vol=1.192063e+06

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assign (segid "   A" and resid    168 and name   HN) (segid "   A" and resid    167 and name  HB2) 2.344 0.687 0.687 weight 1.000 ! spec=Expt_1, no=724, id=618, vol=5.521306e+05
assign (segid "   A" and resid    168 and name   HN) (segid "   A" and resid    167 and name   HA) 2.760 0.952 0.952 weight 1.000 ! spec=Expt_4, no=172, id=4377, vol=6.881260e+06
assign (segid "   A" and resid    168 and name   HN) (segid "   A" and resid    169 and name   HN) 2.228 0.621 0.621 weight 1.000 ! spec=Expt_1, no=8, id=7, vol=7.476413e+05

LEU 169
assign (segid "   A" and resid   169 and name   HN) (segid "   A" and resid    168 and name  HA1) 2.273 0.646 0.646 weight 1.000 ! spec=Expt_1, no=735, id=623, vol=6.634830e+05
assign (segid "   A" and resid   169 and name   HN) (segid "   A" and resid   169 and name   HA) 2.287 0.654 0.654 weight 1.000 ! spec=Expt_1, no=738, id=626, vol=6.388711e+05
assign (segid "   A" and resid   169 and name  HB1) (segid "   A" and resid   169 and name  HB2) 1.684 0.354 0.354 weight 1.000 ! spec=Expt_1, no=1872, id=1479, vol=4.013201e+06
assign (segid "   A" and resid   169 and name   HA) (segid "   A" and resid   169 and name HD21) 2.067 0.534 0.534 weight 1.000 ! spec=Expt_3, no=2030, id=3823, vol=5.873209e+06

LEU 170
assign (segid "   A" and resid   170 and name   HN) (segid "   A" and resid    167 and name   HA) 2.769 0.959 0.959 weight 1.000 ! spec=Expt_4, no=945, id=4944, vol=6.736957e+06
assign (segid "   A" and resid   170 and name   HN) (segid "   A" and resid   169 and name   HN) 2.184 0.596 0.596 weight 1.000 ! spec=Expt_1, no=6, id=5, vol=8.444529e+05
assign (segid "   A" and resid   170 and name   HN) (segid "   A" and resid   169 and name  HB1) 2.436 0.742 0.742 weight 1.000 ! spec=Expt_1, no=742, id=628, vol=4.379542e+05
assign (segid "   A" and resid   170 and name   HN) (segid "   A" and resid   169 and name   HA) 2.706 0.915 0.915 weight 1.000 ! spec=Expt_4, no=176, id=4381, vol=7.742369e+06
assign (segid "   A" and resid   170 and name  HB2) (segid "   A" and resid   170 and name   HG) 2.426 0.736 0.736 weight 1.000 ! spec=Expt_1, no=1932, id=1531, vol=4.483779e+05
assign (segid "   A" and resid   170 and name HD21) (segid "   A" and resid   170 and name  HB1) 2.477 0.767 0.767 weight 1.000 ! spec=Expt_1, no=1954, id=1546, vol=3.966644e+05
assign (segid "   A" and resid   170 and name HD21) (segid "   A" and resid   170 and name   HG) 1.701 0.362 0.362 weight 1.000 ! spec=Expt_1, no=2156, id=1701, vol=3.774287e+06
assign (segid "   A" and resid   170 and name  HB1) (segid "   A" and resid   170 and name   HG) 2.376 0.706 0.706 weight 1.000 ! spec=Expt_1, no=2210, id=1746, vol=5.081125e+05
assign (segid "   A" and resid   170 and name   HN) (segid "   A" and resid   170 and name   HA) 2.259 0.638 0.638 weight 1.000 ! spec=Expt_1, no=3486, id=2582, vol=6.887311e+05
assign (segid "   A" and resid   170 and name HD13) (segid "   A" and resid   170 and name HD21) 1.632 0.333 0.333 weight 1.000 ! spec=Expt_3, no=472, id=2988, vol=2.423749e+07
assign (segid "   A" and resid   170 and name HD13) (segid "   A" and resid   170 and name   HA) 1.894 0.448 0.448 weight 1.000 ! spec=Expt_3, no=1389, id=3527, vol=9.940494e+06
assign (segid "   A" and resid   170 and name  HB1) (segid "   A" and resid   170 and name  HB2) 1.785 0.398 0.398 weight 1.000 ! spec=Expt_3, no=1442, id=3560, vol=1.418996e+07
assign (segid "   A" and resid   170 and name HD21) (segid "   A" and resid   170 and name  HB2) 1.910 0.456 0.456 weight 1.000 ! spec=Expt_3, no=1499, id=3604, vol=9.455587e+06
assign (segid "   A" and resid   170 and name   HA) (segid "   A" and resid   170 and name  HB2) 2.212 0.612 0.612 weight 1.000 ! spec=Expt_3, no=1693, id=3740, vol=3.911995e+06
assign (segid "   A" and resid   170 and name   HG) (segid "   A" and resid   171 and name   HN) 2.836 1.005 1.005 weight 1.000 ! spec=Expt_4, no=656, id=4762, vol=5.839936e+06
assign (segid "   A" and resid   170 and name   HN) (segid "   A" and resid   172 and name   HN) 3.206 1.285 1.285 weight 1.000 ! spec=Expt_4, no=1617, id=5191, vol=2.799578e+06
assign (segid "   A" and resid   170 and name HD13) (segid "   A" and resid   174 and name  HG2) 2.472 0.764 0.764 weight 1.000 ! spec=Expt_1, no=1958, id=1550, vol=4.011889e+05
assign (segid "   A" and resid   170 and name   HN) (segid "   A" and resid   188 and name   HZ) 2.327 0.677 0.677 weight 1.000 ! spec=Expt_1, no=746, id=632, vol=5.761223e+05
assign (segid "   A" and resid   170 and name   HN) (segid "   A" and resid   188 and name  HE1) 2.402 0.721 0.721 weight 1.000 ! spec=Expt_1, no=747, id=633, vol=4.763896e+05
assign (segid "   A" and resid   170 and name  HB2) (segid "   A" and resid   188 and name  HE1) 2.037 0.519 0.519 weight 1.000 ! spec=Expt_3, no=1022, id=3358, vol=6.420516e+06

LEU 171
assign (segid "   A" and resid   171 and name   HN) (segid "   A" and resid   170 and name   HN) 2.123 0.563 0.563 weight 1.000 ! spec=Expt_1, no=4, id=3, vol=1.000828e+06
assign (segid "   A" and resid   171 and name   HN) (segid "   A" and resid   170 and name  HB1) 2.099 0.551 0.551 weight 1.000 ! spec=Expt_1, no=755, id=638, vol=1.070169e+06
assign (segid "   A" and resid   171 and name   HN) (segid "   A" and resid   171 and name  HB1) 1.810 0.410 0.410 weight 1.000 ! spec=Expt_1, no=754, id=637, vol=2.598596e+06
assign (segid "   A" and resid   171 and name   HN) (segid "   A" and resid   171 and name   HA) 2.164 0.585 0.585 weight 1.000 ! spec=Expt_1, no=760, id=641, vol=8.914528e+05
assign (segid "   A" and resid   171 and name   HA) (segid "   A" and resid   171 and name   HG) 2.074 0.538 0.538 weight 1.000 ! spec=Expt_3, no=478, id=2993, vol=5.755764e+06
assign (segid "   A" and resid   171 and name   HA) (segid "   A" and resid   171 and name HD13) 1.776 0.394 0.394 weight 1.000 ! spec=Expt_3, no=479, id=2994, vol=1.463658e+07
assign (segid "   A" and resid   171 and name   HN) (segid "   A" and resid   171 and name HD13) 2.413 0.728 0.728 weight 1.000 ! spec=Expt_4, no=652, id=4758, vol=1.539498e+07
assign (segid "   A" and resid   171 and name   HN) (segid "   A" and resid   172 and name   HN) 2.081 0.541 0.541 weight 1.000 ! spec=Expt_1, no=3, id=2, vol=1.126674e+06

TRP 172
assign (segid "   A" and resid   172 and name  HE3) (segid "   A" and resid   169 and name HD21) 2.044 0.522 0.522 weight 1.000 ! spec=Expt_1, no=698, id=598, vol=1.255543e+06
assign (segid "   A" and resid   172 and name   HN) (segid "   A" and resid   169 and name HD21) 2.994 1.120 1.120 weight 1.000 ! spec=Expt_4, no=954, id=4945, vol=4.221103e+06
assign (segid "   A" and resid   172 and name   HN) (segid "   A" and resid   171 and name  HB1) 2.481 0.769 0.769 weight 1.000 ! spec=Expt_1, no=3142, id=2281, vol=3.923126e+05
assign (segid "   A" and resid   172 and name  HE1) (segid "   A" and resid   172 and name  HD1) 2.248 0.632 0.632 weight 1.000 ! spec=Expt_1, no=630, id=553, vol=7.094256e+05
assign (segid "   A" and resid   172 and name  HE1) (segid "   A" and resid   172 and name  HZ2) 2.714 0.921 0.921 weight 1.000 ! spec=Expt_1, no=631, id=554, vol=2.288981e+05
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assign (segid " A" and resid 172 and name HN) (segid " A" and resid 172 and name HB1) 2.259 0.638 0.638 weight 1.000 ! spec=Expt_1, no=3469, id=2569, vol=6.884718e+05
assign (segid " A" and resid 172 and name HE3) (segid " A" and resid 172 and name HB1) 2.327 0.677 0.677 weight 1.000 ! spec=Expt_1, no=3471, id=2571, vol=5.765881e+05
assign (segid " A" and resid 172 and name HE3) (segid " A" and resid 172 and name HZ3) 1.928 0.464 0.464 weight 1.000 ! spec=Expt_3, no=730, id=3196, vol=8.942308e+06
assign (segid " A" and resid 172 and name HH2) (segid " A" and resid 172 and name HZ2) 1.937 0.469 0.469 weight 1.000 ! spec=Expt_3, no=746, id=3204, vol=8.674074e+06
assign (segid " A" and resid 172 and name HD1) (segid " A" and resid 172 and name HB2) 2.149 0.577 0.577 weight 1.000 ! spec=Expt_3, no=892, id=3270, vol=4.661233e+06
assign (segid " A" and resid 172 and name HA) (segid " A" and resid 172 and name HB2) 2.197 0.603 0.603 weight 1.000 ! spec=Expt_3, no=1190, id=3452, vol=4.084117e+06
assign (segid " A" and resid 172 and name HA) (segid " A" and resid 172 and name HB1) 2.423 0.734 0.734 weight 1.000 ! spec=Expt_3, no=5157, id=4175, vol=2.264197e+06
assign (segid " A" and resid 172 and name HN) (segid " A" and resid 173 and name HN) 2.166 0.586 0.586 weight 1.000 ! spec=Expt_1, no=768, id=646, vol=8.863178e+05
assign (segid " A" and resid 172 and name HE3) (segid " A" and resid 173 and name HN) 2.198 0.604 0.604 weight 1.000 ! spec=Expt_1, no=778, id=655, vol=8.119833e+05
assign (segid " A" and resid 172 and name HE3) (segid " A" and resid 173 and name HA) 2.287 0.654 0.654 weight 1.000 ! spec=Expt_3, no=763, id=3218, vol=3.204729e+06
assign (segid " A" and resid 172 and name HE3) (segid " A" and resid 201 and name HD21) 2.379 0.708 0.708 weight 1.000 ! spec=Expt_3, no=754, id=3210, vol=2.527391e+06
assign (segid " A" and resid 172 and name HZ3) (segid " A" and resid 201 and name HD21) 2.065 0.533 0.533 weight 1.000 ! spec=Expt_3, no=1038, id=3367, vol=5.913893e+06
assign (segid " A" and resid 172 and name HH2) (segid " A" and resid 265 and name HA) 2.352 0.691 0.691 weight 1.000 ! spec=Expt_1, no=2306, id=1770, vol=5.411004e+05
assign (segid " A" and resid 172 and name HZ2) (segid " A" and resid 265 and name HA) 2.330 0.679 0.679 weight 1.000 ! spec=Expt_3, no=938, id=3302, vol=2.867161e+06
assign (segid " A" and resid 172 and name HZ2) (segid " A" and resid 268 and name HD1) 2.080 0.541 0.541 weight 1.000 ! spec=Expt_3, no=2050, id=3836, vol=5.665329e+06
assign (segid " A" and resid 172 and name HZ2) (segid " A" and resid 268 and name HB1) 2.376 0.705 0.705 weight 1.000 ! spec=Expt_3, no=2626, id=3974, vol=2.551783e+06

CYS 173
assign (segid " A" and resid 173 and name HN) (segid " A" and resid 169 and name HD21) 2.707 0.916 0.916 weight 1.000 ! spec=Expt_4, no=959, id=4947, vol=7.728326e+06
assign (segid " A" and resid 173 and name HN) (segid " A" and resid 170 and name HA) 2.469 0.762 0.762 weight 1.000 ! spec=Expt_1, no=776, id=653, vol=4.038841e+05
assign (segid " A" and resid 173 and name HB2) (segid " A" and resid 170 and name HD13) 2.317 0.671 0.671 weight 1.000 ! spec=Expt_3, no=1719, id=3763, vol=2.963909e+06
assign (segid " A" and resid 173 and name HN) (segid " A" and resid 172 and name HB1) 2.281 0.651 0.651 weight 1.000 ! spec=Expt_1, no=3455, id=2555, vol=6.493529e+05
assign (segid " A" and resid 173 and name HB1) (segid " A" and resid 173 and name HB2) 1.967 0.484 0.484 weight 1.000 ! spec=Expt_1, no=1723, id=1368, vol=1.579639e+06
assign (segid " A" and resid 173 and name HN) (segid " A" and resid 173 and name HA) 2.318 0.672 0.672 weight 1.000 ! spec=Expt_4, no=182, id=4383, vol=1.958840e+07
assign (segid " A" and resid 173 and name HN) (segid " A" and resid 173 and name HB2) 2.205 0.607 0.607 weight 1.000 ! spec=Expt_4, no=384, id=4537, vol=2.647819e+07
assign (segid " A" and resid 173 and name HB1) (segid " A" and resid 185 and name HG21) 2.274 0.647 0.647 weight 1.000 ! spec=Expt_3, no=1651, id=3708, vol=3.315012e+06
assign (segid " A" and resid 173 and name HB1) (segid " A" and resid 192 and name HH2) 2.564 0.822 0.822 weight 1.000 ! spec=Expt_1, no=3298, id=2414, vol=3.220562e+05

GLN 174
assign (segid " A" and resid 174 and name HE21) (segid " A" and resid 170 and name HD21) 2.643 0.873 0.873 weight 1.000 ! spec=Expt_1, no=2101, id=1661, vol=2.686720e+05
assign (segid " A" and resid 174 and name HE22) (segid " A" and resid 170 and name HG) 2.286 0.653 0.653 weight 1.000 ! spec=Expt_3, no=1057, id=3382, vol=3.213190e+06
assign (segid " A" and resid 174 and name HG1) (segid " A" and resid 170 and name HD13) 2.368 0.701 0.701 weight 1.000 ! spec=Expt_3, no=1396, id=3532, vol=2.602815e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 170 and name HA) 2.947 1.086 1.086 weight 1.000 ! spec=Expt_4, no=963, id=4951, vol=4.636510e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 170 and name HG) 3.144 1.235 1.235 weight 1.000 ! spec=Expt_4, no=967, id=4953, vol=3.147720e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 170 and name HD13) 2.943 1.082 1.082 weight 1.000 ! spec=Expt_4, no=970, id=4956, vol=4.680634e+06
assign (segid " A" and resid 174 and name HE22) (segid " A" and resid 171 and name HD13) 2.098 0.550 0.550 weight 1.000 ! spec=Expt_3, no=1061, id=3384, vol=5.381566e+06
assign (segid " A" and resid 174 and name HE21) (segid " A" and resid 171 and name HD13) 2.258 0.637 0.637 weight 1.000 ! spec=Expt_3, no=2370, id=3922, vol=3.458791e+06
assign (segid " A" and resid 174 and name HG2) (segid " A" and resid 171 and name HA) 2.416 0.729 0.729 weight 1.000 ! spec=Expt_3, no=5152, id=4171, vol=2.308044e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 171 and name HA) 2.542 0.807 0.807 weight 1.000 ! spec=Expt_4, no=965, id=4952, vol=1.127702e+07
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 172 and name HN) 2.978 1.109 1.109 weight 1.000 ! spec=Expt_4, no=961, id=4949, vol=4.354013e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 173 and name HA) 2.868 1.028 1.028 weight 1.000 ! spec=Expt_4, no=185, id=4385, vol=5.465421e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 173 and name HB2) 2.343 0.686 0.686 weight 1.000 ! spec=Expt_4, no=386, id=4539, vol=1.836498e+07
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 174 and name HB2) 2.002 0.501 0.501 weight 1.000 ! spec=Expt_1, no=783, id=659, vol=1.419362e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 174 and name HG2) 2.179 0.593 0.593 weight 1.000 ! spec=Expt_1, no=784, id=660, vol=8.556038e+05
assign (segid " A" and resid 174 and name HG2) (segid " A" and resid 174 and name HE22) 2.345 0.687 0.687 weight 1.000 ! spec=Expt_1, no=3107, id=2252, vol=5.506110e+05
assign (segid " A" and resid 174 and name HA) (segid " A" and resid 174 and name HG1) 2.154 0.580 0.580 weight 1.000 ! spec=Expt_3, no=29, id=2637, vol=4.596936e+06
assign (segid " A" and resid 174 and name HE22) (segid " A" and resid 174 and name HE21) 2.067 0.534 0.534 weight 1.000 ! spec=Expt_3, no=790, id=3232, vol=5.887694e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 174 and name HA) 2.288 0.654 0.654 weight 1.000 ! spec=Expt_4, no=184, id=4384, vol=2.117985e+07

assign (segid " A" and resid 174 and name HN) (segid " A" and resid 174 and name HE22) 2.986 1.114 1.114 weight 1.000 ! spec=Expt_4, no=962, id=4950, vol=4.290844e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 175 and name HN) 2.142 0.574 0.574 weight 1.000 ! spec=Expt_1, no=779, id=656, vol=9.465566e+05
assign (segid " A" and resid 174 and name HA) (segid " A" and resid 185 and name HG21) 2.502 0.783 0.783 weight 1.000 ! spec=Expt_1, no=2757, id=1985, vol=3.730680e+05
assign (segid " A" and resid 174 and name HG1) (segid " A" and resid 185 and name HG21) 2.249 0.632 0.632 weight 1.000 ! spec=Expt_3, no=1383, id=3524, vol=3.547044e+06
assign (segid " A" and resid 174 and name HN) (segid " A" and resid 185 and name HG21) 2.913 1.060 1.060 weight 1.000 ! spec=Expt_4, no=969, id=4955, vol=4.978726e+06

ARG 175
assign (segid " A" and resid 175 and name HB2) (segid " A" and resid 172 and name HA) 2.224 0.619 0.619 weight 1.000 ! spec=Expt_1, no=2516, id=1862, vol=7.554415e+05
assign (segid " A" and resid 175 and name HN) (segid " A" and resid 174 and name HB2) 2.244 0.629 0.629 weight 1.000 ! spec=Expt_1, no=794, id=664, vol=7.171960e+05
assign (segid " A" and resid 175 and name HN) (segid " A" and resid 174 and name HA) 2.749 0.945 0.945 weight 1.000 ! spec=Expt_4, no=187, id=4387, vol=7.035195e+06
assign (segid " A" and resid 175 and name HA) (segid " A" and resid 175 and name HG1) 1.943 0.472 0.472 weight 1.000 ! spec=Expt_1, no=517, id=463, vol=1.700199e+06
assign (segid " A" and resid 175 and name HD1) (segid " A" and resid 175 and name HN) 2.692 0.906 0.906 weight 1.000 ! spec=Expt_1, no=3447, id=2548, vol=2.402628e+05
assign (segid " A" and resid 175 and name HG2) (segid " A" and resid 175 and name HG1) 1.654 0.342 0.342 weight 1.000 ! spec=Expt_3, no=1441, id=3559, vol=2.241165e+07
assign (segid " A" and resid 175 and name HG2) (segid " A" and resid 175 and name HB1) 1.845 0.426 0.426 weight 1.000 ! spec=Expt_3, no=1612, id=3678, vol=1.161707e+07
assign (segid " A" and resid 175 and name HN) (segid " A" and resid 175 and name HA) 2.334 0.681 0.681 weight 1.000 ! spec=Expt_4, no=186, id=4386, vol=1.882000e+07
assign (segid " A" and resid 175 and name HN) (segid " A" and resid 175 and name HB2) 2.011 0.505 0.505 weight 1.000 ! spec=Expt_4, no=389, id=4542, vol=4.598497e+07
assign (segid " A" and resid 175 and name HN) (segid " A" and resid 176 and name HN) 2.228 0.620 0.620 weight 1.000 ! spec=Expt_1, no=797, id=666, vol=7.485004e+05
assign (segid " A" and resid 175 and name HN) (segid " A" and resid 177 and name HN) 2.930 1.073 1.073 weight 1.000 ! spec=Expt_4, no=972, id=4957, vol=4.807958e+06
assign (segid " A" and resid 175 and name HA) (segid " A" and resid 178 and name HB2) 2.006 0.503 0.503 weight 1.000 ! spec=Expt_1, no=2760, id=1987, vol=1.404988e+06

LYS 176
assign (segid " A" and resid 176 and name HN) (segid " A" and resid 173 and name HA) 2.970 1.103 1.103 weight 1.000 ! spec=Expt_4, no=977, id=4959, vol=4.428835e+06
assign (segid " A" and resid 176 and name HN) (segid " A" and resid 175 and name HB1) 2.282 0.651 0.651 weight 1.000 ! spec=Expt_1, no=804, id=672, vol=6.481087e+05
assign (segid " A" and resid 176 and name HN) (segid " A" and resid 175 and name HA) 2.938 1.079 1.079 weight 1.000 ! spec=Expt_4, no=189, id=4389, vol=4.728949e+06
assign (segid " A" and resid 176 and name HN) (segid " A" and resid 176 and name HG1) 2.317 0.671 0.671 weight 1.000 ! spec=Expt_1, no=800, id=669, vol=5.921080e+05
assign (segid " A" and resid 176 and name HN) (segid " A" and resid 176 and name HD2) 2.195 0.602 0.602 weight 1.000 ! spec=Expt_1, no=803, id=671, vol=8.176187e+05
assign (segid " A" and resid 176 and name HB1) (segid " A" and resid 176 and name HG1) 2.454 0.753 0.753 weight 1.000 ! spec=Expt_1, no=1881, id=1486, vol=4.187973e+05
assign (segid " A" and resid 176 and name HB1) (segid " A" and resid 176 and name HB2) 2.096 0.549 0.549 weight 1.000 ! spec=Expt_3, no=1476, id=3585, vol=5.403453e+06
assign (segid " A" and resid 176 and name HE1) (segid " A" and resid 176 and name HD1) 2.290 0.655 0.655 weight 1.000 ! spec=Expt_3, no=4574, id=4094, vol=3.184232e+06
assign (segid " A" and resid 176 and name HE2) (segid " A" and resid 176 and name HD2) 2.438 0.743 0.743 weight 1.000 ! spec=Expt_3, no=4969, id=4148, vol=2.184932e+06
assign (segid " A" and resid 176 and name HG2) (segid " A" and resid 176 and name HG1) 2.362 0.697 0.697 weight 1.000 ! spec=Expt_3, no=5228, id=4192, vol=2.643027e+06
assign (segid " A" and resid 176 and name HN) (segid " A" and resid 176 and name HA) 2.482 0.770 0.770 weight 1.000 ! spec=Expt_4, no=188, id=4388, vol=1.300999e+07
assign (segid " A" and resid 176 and name HN) (segid " A" and resid 176 and name HB2) 2.325 0.676 0.676 weight 1.000 ! spec=Expt_4, no=665, id=4767, vol=1.925170e+07
assign (segid " A" and resid 176 and name HA) (segid " A" and resid 177 and name HN) 3.052 1.165 1.165 weight 1.000 ! spec=Expt_4, no=983, id=4960, vol=3.758894e+06
assign (segid " A" and resid 176 and name HN) (segid " A" and resid 177 and name HN) 2.314 0.670 0.670 weight 1.000 ! spec=Expt_1, no=811, id=679, vol=5.953613e+05
assign (segid " A" and resid 176 and name HB2) (segid " A" and resid 177 and name HN) 2.814 0.990 0.990 weight 1.000 ! spec=Expt_1, no=3010, id=2167, vol=1.843776e+05
assign (segid " A" and resid 176 and name HN) (segid " A" and resid 178 and name HN) 3.091 1.195 1.195 weight 1.000 ! spec=Expt_4, no=976, id=4958, vol=3.481601e+06
assign (segid " A" and resid 176 and name HB2) (segid " A" and resid 201 and name HA) 2.592 0.840 0.840 weight 1.000 ! spec=Expt_1, no=1987, id=1576, vol=3.020416e+05
assign (segid " A" and resid 176 and name HG2) (segid " A" and resid 201 and name HG) 2.290 0.655 0.655 weight 1.000 ! spec=Expt_3, no=1543, id=3632, vol=3.181650e+06
assign (segid " A" and resid 176 and name HA) (segid " A" and resid 204 and name HG2) 2.374 0.705 0.705 weight 1.000 ! spec=Expt_3, no=4658, id=4110, vol=2.561646e+06

THR 177
assign (segid " A" and resid 177 and name HN) (segid " A" and resid 174 and name HA) 2.754 0.948 0.948 weight 1.000 ! spec=Expt_4, no=984, id=4961, vol=6.971655e+06
assign (segid " A" and resid 177 and name HG1) (segid " A" and resid 177 and name HB) 1.955 0.478 0.478 weight 1.000 ! spec=Expt_3, no=568, id=3062, vol=8.218412e+06
assign (segid " A" and resid 177 and name HG1) (segid " A" and resid 177 and name HA) 2.210 0.610 0.610 weight 1.000 ! spec=Expt_3, no=569, id=3063, vol=3.939067e+06
assign (segid " A" and resid 177 and name HN) (segid " A" and resid 177 and name HA) 2.672 0.893 0.893 weight 1.000 ! spec=Expt_4, no=190, id=4390, vol=8.345302e+06
assign (segid " A" and resid 177 and name HN) (segid " A" and resid 177 and name HB) 2.994 1.120 1.120 weight 1.000 ! spec=Expt_4, no=397, id=4548, vol=4.220649e+06
assign (segid " A" and resid 177 and name HN) (segid " A" and resid 178 and name HN) 2.039 0.520 0.520 weight 1.000 ! spec=Expt_1, no=813, id=680, vol=1.272895e+06

assign (segid " A" and resid 177 and name HG1) (segid " A" and resid 180 and name HD1) 2.670 0.891 0.891 weight 1.000 ! spec=Expt_1, no=2996, id=2156, vol=2.525248e+05
assign (segid " A" and resid 177 and name HG1) (segid " A" and resid 200 and name HB2) 1.902 0.452 0.452 weight 1.000 ! spec=Expt_1, no=1803, id=1434, vol=1.932331e+06
assign (segid " A" and resid 177 and name   HB) (segid " A" and resid 201 and name HD13) 2.496 0.779 0.779 weight 1.000 ! spec=Expt_3, no=5136, id=4165, vol=1.897715e+06

ALA178
assign (segid " A" and resid 178 and name   HN) (segid " A" and resid 174 and name   HA) 3.321 1.378 1.378 weight 1.000 ! spec=Expt_4, no=990, id=4964, vol=2.266595e+06
assign (segid " A" and resid 178 and name   HN) (segid " A" and resid 175 and name   HA) 2.807 0.985 0.985 weight 1.000 ! spec=Expt_4, no=988, id=4963, vol=6.213707e+06
assign (segid " A" and resid 178 and name   HN) (segid " A" and resid 177 and name   HA) 2.826 0.998 0.998 weight 1.000 ! spec=Expt_4, no=398, id=4549, vol=5.965576e+06
assign (segid " A" and resid 178 and name   HN) (segid " A" and resid 178 and name HB2) 1.766 0.390 0.390 weight 1.000 ! spec=Expt_1, no=815, id=682, vol=3.015753e+06
assign (segid " A" and resid 178 and name   HN) (segid " A" and resid 178 and name   HA) 2.199 0.604 0.604 weight 1.000 ! spec=Expt_4, no=399, id=4550, vol=2.691218e+07
assign (segid " A" and resid 178 and name   HN) (segid " A" and resid 179 and name HD1) 2.356 0.694 0.694 weight 1.000 ! spec=Expt_3, no=2714, id=3987, vol=2.678978e+06
assign (segid " A" and resid 178 and name   HN) (segid " A" and resid 180 and name HD1) 3.045 1.159 1.159 weight 1.000 ! spec=Expt_4, no=987, id=4962, vol=3.809152e+06

PRO 179
assign (segid " A" and resid 179 and name HB2) (segid " A" and resid 179 and name HB1) 1.750 0.383 0.383 weight 1.000 ! spec=Expt_1, no=1760, id=1399, vol=3.189487e+06
assign (segid " A" and resid 179 and name HG2) (segid " A" and resid 179 and name HB1) 2.555 0.816 0.816 weight 1.000 ! spec=Expt_1, no=2199, id=1738, vol=3.292756e+05
assign (segid " A" and resid 179 and name HD1) (segid " A" and resid 179 and name HG1) 2.435 0.741 0.741 weight 1.000 ! spec=Expt_1, no=3499, id=2594, vol=4.387438e+05
assign (segid " A" and resid 179 and name   HA) (segid " A" and resid 179 and name HB1) 1.995 0.498 0.498 weight 1.000 ! spec=Expt_3, no=1350, id=3504, vol=7.271564e+06
assign (segid " A" and resid 179 and name HD1) (segid " A" and resid 180 and name HE1) 2.615 0.855 0.855 weight 1.000 ! spec=Expt_1, no=2074, id=1639, vol=2.863975e+05

TYR 180
assign (segid " A" and resid 180 and name HD1) (segid " A" and resid 177 and name   HA) 2.074 0.538 0.538 weight 1.000 ! spec=Expt_1, no=1548, id=1249, vol=1.149291e+06
assign (segid " A" and resid 180 and name HE1) (segid " A" and resid 177 and name   HA) 2.431 0.739 0.739 weight 1.000 ! spec=Expt_3, no=3211, id=4052, vol=2.222345e+06
assign (segid " A" and resid 180 and name   HN) (segid " A" and resid 179 and name HG2) 2.592 0.839 0.839 weight 1.000 ! spec=Expt_1, no=822, id=688, vol=3.021253e+05
assign (segid " A" and resid 180 and name   HN) (segid " A" and resid 179 and name HD1) 2.535 0.804 0.804 weight 1.000 ! spec=Expt_1, no=825, id=690, vol=3.444861e+05
assign (segid " A" and resid 180 and name HD1) (segid " A" and resid 179 and name HD1) 2.315 0.670 0.670 weight 1.000 ! spec=Expt_1, no=1531, id=1236, vol=5.950722e+05
assign (segid " A" and resid 180 and name   HA) (segid " A" and resid 179 and name HB2) 3.079 1.185 1.185 weight 1.000 ! spec=Expt_1, no=2578, id=1897, vol=1.074472e+05
assign (segid " A" and resid 180 and name HD1) (segid " A" and resid 179 and name HG2) 2.408 0.725 0.725 weight 1.000 ! spec=Expt_3, no=999, id=3340, vol=2.351488e+06
assign (segid " A" and resid 180 and name HE1) (segid " A" and resid 179 and name HG2) 2.354 0.693 0.693 weight 1.000 ! spec=Expt_3, no=1000, id=3341, vol=2.692826e+06
assign (segid " A" and resid 180 and name   HN) (segid " A" and resid 180 and name HB2) 2.219 0.615 0.615 weight 1.000 ! spec=Expt_1, no=546, id=486, vol=7.675826e+05
assign (segid " A" and resid 180 and name HD1) (segid " A" and resid 180 and name HE1) 1.752 0.384 0.384 weight 1.000 ! spec=Expt_1, no=579, id=515, vol=3.168809e+06
assign (segid " A" and resid 180 and name   HN) (segid " A" and resid 180 and name HD1) 2.007 0.504 0.504 weight 1.000 ! spec=Expt_1, no=1433, id=1160, vol=1.399678e+06
assign (segid " A" and resid 180 and name HD1) (segid " A" and resid 180 and name   HA) 2.122 0.563 0.563 weight 1.000 ! spec=Expt_3, no=700, id=3172, vol=5.027309e+06
assign (segid " A" and resid 180 and name   HA) (segid " A" and resid 180 and name HB1) 2.129 0.567 0.567 weight 1.000 ! spec=Expt_3, no=1215, id=3469, vol=4.926880e+06
assign (segid " A" and resid 180 and name HD1) (segid " A" and resid 180 and name HB2) 2.250 0.633 0.633 weight 1.000 ! spec=Expt_3, no=2807, id=4006, vol=3.538573e+06
assign (segid " A" and resid 180 and name HD1) (segid " A" and resid 180 and name HB1) 2.231 0.622 0.622 weight 1.000 ! spec=Expt_3, no=2808, id=4007, vol=3.724116e+06
assign (segid " A" and resid 180 and name   HN) (segid " A" and resid 180 and name   HA) 2.253 0.635 0.635 weight 1.000 ! spec=Expt_4, no=191, id=4391, vol=2.320986e+07
assign (segid " A" and resid 180 and name HD1) (segid " A" and resid 183 and name HG12) 2.121 0.562 0.562 weight 1.000 ! spec=Expt_3, no=1065, id=3387, vol=5.038253e+06
assign (segid " A" and resid 180 and name HB2) (segid " A" and resid 183 and name HG12) 2.115 0.559 0.559 weight 1.000 ! spec=Expt_3, no=1375, id=3520, vol=5.120432e+06
assign (segid " A" and resid 180 and name HE1) (segid " A" and resid 200 and name   HA) 2.786 0.970 0.970 weight 1.000 ! spec=Expt_1, no=2649, id=1935, vol=1.957479e+05
assign (segid " A" and resid 180 and name HD1) (segid " A" and resid 200 and name HB2) 2.069 0.535 0.535 weight 1.000 ! spec=Expt_3, no=1087, id=3402, vol=5.854255e+06
assign (segid " A" and resid 180 and name HE1) (segid " A" and resid 200 and name HB2) 2.191 0.600 0.600 weight 1.000 ! spec=Expt_3, no=1088, id=3403, vol=4.145402e+06
assign (segid " A" and resid 180 and name HE1) (segid " A" and resid 204 and name HD1) 2.295 0.659 0.659 weight 1.000 ! spec=Expt_3, no=880, id=3262, vol=3.136665e+06
assign (segid " A" and resid 180 and name HE1) (segid " A" and resid 204 and name HB1) 2.292 0.657 0.657 weight 1.000 ! spec=Expt_3, no=1004, id=3345, vol=3.165180e+06

LYS 181
assign (segid " A" and resid 181 and name   HN) (segid " A" and resid 180 and name   HA) 1.927 0.464 0.464 weight 1.000 ! spec=Expt_1, no=841, id=701, vol=1.789920e+06
assign (segid " A" and resid 181 and name   HN) (segid " A" and resid 181 and name HB2) 2.078 0.540 0.540 weight 1.000 ! spec=Expt_1, no=833, id=696, vol=1.136923e+06

assign (segid " A" and resid 181 and name HB1) (segid " A" and resid 181 and name HB2) 1.582 0.313 0.313 weight 1.000 ! spec=Expt_1, no=1884, id=1489, vol=5.828322e+06
assign (segid " A" and resid 181 and name HA) (segid " A" and resid 181 and name HB1) 1.919 0.460 0.460 weight 1.000 ! spec=Expt_3, no=1228, id=3477, vol=9.196197e+06
assign (segid " A" and resid 181 and name HA) (segid " A" and resid 181 and name HD1) 1.967 0.484 0.484 weight 1.000 ! spec=Expt_3, no=1231, id=3479, vol=7.910447e+06
assign (segid " A" and resid 181 and name HN) (segid " A" and resid 181 and name HG2) 2.494 0.777 0.777 weight 1.000 ! spec=Expt_4, no=674, id=4774, vol=1.264319e+07
assign (segid " A" and resid 181 and name HN) (segid " A" and resid 182 and name HN) 2.427 0.736 0.736 weight 1.000 ! spec=Expt_1, no=835, id=698, vol=4.480226e+05
assign (segid " A" and resid 181 and name HB2) (segid " A" and resid 182 and name HN) 2.516 0.791 0.791 weight 1.000 ! spec=Expt_4, no=407, id=4558, vol=1.197656e+07

ASN 182
assign (segid " A" and resid 182 and name HD22) (segid " A" and resid 182 and name HB2) 2.369 0.702 0.702 weight 1.000 ! spec=Expt_1, no=1346, id=1091, vol=5.177101e+05
assign (segid " A" and resid 182 and name HN) (segid " A" and resid 182 and name HA) 2.547 0.811 0.811 weight 1.000 ! spec=Expt_4, no=204, id=4399, vol=1.114485e+07
assign (segid " A" and resid 182 and name HN) (segid " A" and resid 183 and name HN) 2.205 0.608 0.608 weight 1.000 ! spec=Expt_1, no=855, id=712, vol=7.972220e+05
assign (segid " A" and resid 182 and name HN) (segid " A" and resid 183 and name HG12) 2.808 0.986 0.986 weight 1.000 ! spec=Expt_4, no=1003, id=4971, vol=6.195494e+06
assign (segid " A" and resid 182 and name HD22) (segid " A" and resid 196 and name HD21) 2.032 0.516 0.516 weight 1.000 ! spec=Expt_3, no=1064, id=3386, vol=6.525361e+06
assign (segid " A" and resid 182 and name HB2) (segid " A" and resid 196 and name HD21) 2.286 0.653 0.653 weight 1.000 ! spec=Expt_3, no=1397, id=3533, vol=3.212375e+06

VAL 183
assign (segid " A" and resid 183 and name HG12) (segid " A" and resid 180 and name HB1) 2.152 0.579 0.579 weight 1.000 ! spec=Expt_3, no=1377, id=3522, vol=4.615416e+06
assign (segid " A" and resid 183 and name HN) (segid " A" and resid 181 and name HA) 2.549 0.812 0.812 weight 1.000 ! spec=Expt_4, no=1004, id=4972, vol=1.107352e+07
assign (segid " A" and resid 183 and name HN) (segid " A" and resid 182 and name HA) 2.451 0.751 0.751 weight 1.000 ! spec=Expt_4, no=203, id=4398, vol=1.402306e+07
assign (segid " A" and resid 183 and name HN) (segid " A" and resid 183 and name HG12) 2.166 0.587 0.587 weight 1.000 ! spec=Expt_1, no=821, id=687, vol=8.850472e+05
assign (segid " A" and resid 183 and name HN) (segid " A" and resid 183 and name HA) 2.377 0.706 0.706 weight 1.000 ! spec=Expt_1, no=866, id=722, vol=5.078532e+05
assign (segid " A" and resid 183 and name HG21) (segid " A" and resid 183 and name HA) 1.754 0.384 0.384 weight 1.000 ! spec=Expt_3, no=48, id=2651, vol=1.576660e+07
assign (segid " A" and resid 183 and name HN) (segid " A" and resid 184 and name HN) 3.163 1.251 1.251 weight 1.000 ! spec=Expt_4, no=7, id=4245, vol=3.035072e+06
assign (segid " A" and resid 183 and name HG21) (segid " A" and resid 185 and name HG12) 1.762 0.388 0.388 weight 1.000 ! spec=Expt_3, no=1533, id=3625, vol=1.530362e+07

ASN 184
assign (segid " A" and resid 184 and name HN) (segid " A" and resid 183 and name HG21) 2.053 0.527 0.527 weight 1.000 ! spec=Expt_1, no=872, id=728, vol=1.222230e+06
assign (segid " A" and resid 184 and name HN) (segid " A" and resid 183 and name HA) 1.844 0.425 0.425 weight 1.000 ! spec=Expt_1, no=873, id=729, vol=2.331112e+06
assign (segid " A" and resid 184 and name HD22) (segid " A" and resid 184 and name HB1) 2.701 0.912 0.912 weight 1.000 ! spec=Expt_1, no=3112, id=2256, vol=2.356936e+05
assign (segid " A" and resid 184 and name HD21) (segid " A" and resid 184 and name HB2) 2.560 0.819 0.819 weight 1.000 ! spec=Expt_1, no=3292, id=2408, vol=3.248175e+05
assign (segid " A" and resid 184 and name HN) (segid " A" and resid 184 and name HA) 2.528 0.799 0.799 weight 1.000 ! spec=Expt_4, no=200, id=4395, vol=1.164086e+07
assign (segid " A" and resid 184 and name HN) (segid " A" and resid 185 and name HG12) 2.636 0.869 0.869 weight 1.000 ! spec=Expt_1, no=2589, id=1902, vol=2.727631e+05
assign (segid " A" and resid 184 and name HD22) (segid " A" and resid 186 and name HG1) 2.489 0.774 0.774 weight 1.000 ! spec=Expt_3, no=933, id=3297, vol=1.930635e+06

VAL 185
assign (segid " A" and resid 185 and name HN) (segid " A" and resid 183 and name HG21) 2.362 0.697 0.697 weight 1.000 ! spec=Expt_1, no=1569, id=1261, vol=5.269902e+05
assign (segid " A" and resid 185 and name HN) (segid " A" and resid 184 and name HN) 2.606 0.849 0.849 weight 1.000 ! spec=Expt_1, no=2594, id=1906, vol=2.921593e+05
assign (segid " A" and resid 185 and name HN) (segid " A" and resid 185 and name HA) 2.375 0.705 0.705 weight 1.000 ! spec=Expt_1, no=3239, id=2362, vol=5.099399e+05
assign (segid " A" and resid 185 and name HA) (segid " A" and resid 185 and name HB) 2.074 0.538 0.538 weight 1.000 ! spec=Expt_3, no=58, id=2660, vol=5.757736e+06
assign (segid " A" and resid 185 and name HG12) (segid " A" and resid 185 and name HA) 1.946 0.473 0.473 weight 1.000 ! spec=Expt_3, no=536, id=3039, vol=8.451948e+06
assign (segid " A" and resid 185 and name HN) (segid " A" and resid 185 and name HA) 1.972 0.486 0.486 weight 1.000 ! spec=Expt_4, no=202, id=4397, vol=5.171388e+07

GLN 186
assign (segid " A" and resid 186 and name HN) (segid " A" and resid 185 and name HA) 2.133 0.569 0.569 weight 1.000 ! spec=Expt_1, no=702, id=601, vol=9.721470e+05
assign (segid " A" and resid 186 and name HN) (segid " A" and resid 186 and name HA) 2.329 0.678 0.678 weight 1.000 ! spec=Expt_1, no=2598, id=1909, vol=5.733361e+05
assign (segid " A" and resid 186 and name HN) (segid " A" and resid 186 and name HB1) 2.603 0.847 0.847 weight 1.000 ! spec=Expt_1, no=2599, id=1910, vol=2.942783e+05
assign (segid " A" and resid 186 and name HN) (segid " A" and resid 186 and name HG2) 2.257 0.637 0.637 weight 1.000 ! spec=Expt_1, no=3155, id=2293, vol=6.918122e+05
assign (segid " A" and resid 186 and name HA) (segid " A" and resid 186 and name HB2) 2.023 0.511 0.511 weight 1.000 ! spec=Expt_3, no=1745, id=3783, vol=6.697833e+06

assign (segid " A" and resid 186 and name HE21) (segid " A" and resid 186 and name HN) 2.613 0.854 0.854 weight 1.000 ! spec=Expt_3, no=5196, id=4184, vol=1.440660e+06
assign (segid " A" and resid 186 and name HN) (segid " A" and resid 187 and name HN) 2.085 0.543 0.543 weight 1.000 ! spec=Expt_1, no=632, id=555, vol=1.113898e+06

ASN 187
assign (segid " A" and resid 187 and name HN) (segid " A" and resid 186 and name HB1) 2.838 1.007 1.007 weight 1.000 ! spec=Expt_4, no=420, id=4566, vol=5.818264e+06
assign (segid " A" and resid 187 and name HN) (segid " A" and resid 186 and name HG2) 2.925 1.070 1.070 weight 1.000 ! spec=Expt_4, no=692, id=4788, vol=4.849643e+06
assign (segid " A" and resid 187 and name HD21) (segid " A" and resid 187 and name HB2) 2.146 0.576 0.576 weight 1.000 ! spec=Expt_1, no=1371, id=1111, vol=9.364481e+05
assign (segid " A" and resid 187 and name HD21) (segid " A" and resid 187 and name HB1) 2.636 0.869 0.869 weight 1.000 ! spec=Expt_1, no=3166, id=2304, vol=2.726716e+05
assign (segid " A" and resid 187 and name HN) (segid " A" and resid 187 and name HB2) 3.092 1.195 1.195 weight 1.000 ! spec=Expt_4, no=422, id=4568, vol=3.476096e+06
assign (segid " A" and resid 187 and name HN) (segid " A" and resid 188 and name HN) 3.208 1.286 1.286 weight 1.000 ! spec=Expt_4, no=1013, id=4977, vol=2.790725e+06
assign (segid " A" and resid 187 and name HB1) (segid " A" and resid 189 and name HD2) 2.308 0.666 0.666 weight 1.000 ! spec=Expt_3, no=936, id=3300, vol=3.036672e+06
assign (segid " A" and resid 187 and name HD21) (segid " A" and resid 190 and name HG11) 2.753 0.947 0.947 weight 1.000 ! spec=Expt_1, no=3167, id=2305, vol=2.103493e+05

PHE 188
assign (segid " A" and resid 188 and name HE1) (segid " A" and resid 169 and name HB1) 2.280 0.650 0.650 weight 1.000 ! spec=Expt_1, no=1525, id=1231, vol=6.522860e+05
assign (segid " A" and resid 188 and name HZ) (segid " A" and resid 169 and name HB1) 2.139 0.572 0.572 weight 1.000 ! spec=Expt_3, no=937, id=3301, vol=4.792718e+06
assign (segid " A" and resid 188 and name HZ) (segid " A" and resid 169 and name HD21) 1.922 0.462 0.462 weight 1.000 ! spec=Expt_3, no=1070, id=3391, vol=9.088179e+06
assign (segid " A" and resid 188 and name HZ) (segid " A" and resid 169 and name HD13) 2.076 0.538 0.538 weight 1.000 ! spec=Expt_3, no=2522, id=3954, vol=5.737492e+06
assign (segid " A" and resid 188 and name HE1) (segid " A" and resid 170 and name HA) 2.225 0.619 0.619 weight 1.000 ! spec=Expt_1, no=1562, id=1257, vol=7.546026e+05
assign (segid " A" and resid 188 and name HZ) (segid " A" and resid 170 and name HA) 2.019 0.510 0.510 weight 1.000 ! spec=Expt_3, no=968, id=3317, vol=6.762998e+06
assign (segid " A" and resid 188 and name HE1) (segid " A" and resid 170 and name HD13) 2.117 0.560 0.560 weight 1.000 ! spec=Expt_3, no=2069, id=3843, vol=5.098543e+06
assign (segid " A" and resid 188 and name HE1) (segid " A" and resid 173 and name HB2) 2.495 0.778 0.778 weight 1.000 ! spec=Expt_1, no=1544, id=1246, vol=3.789872e+05
assign (segid " A" and resid 188 and name HN) (segid " A" and resid 187 and name HA) 2.169 0.588 0.588 weight 1.000 ! spec=Expt_1, no=876, id=731, vol=8.779905e+05
assign (segid " A" and resid 188 and name HN) (segid " A" and resid 187 and name HB1) 2.207 0.609 0.609 weight 1.000 ! spec=Expt_1, no=877, id=732, vol=7.921595e+05
assign (segid " A" and resid 188 and name HN) (segid " A" and resid 187 and name HB2) 2.423 0.734 0.734 weight 1.000 ! spec=Expt_1, no=2071, id=1636, vol=4.524010e+05
assign (segid " A" and resid 188 and name HN) (segid " A" and resid 188 and name HB1) 2.392 0.715 0.715 weight 1.000 ! spec=Expt_1, no=875, id=730, vol=4.883675e+05
assign (segid " A" and resid 188 and name HN) (segid " A" and resid 188 and name HA) 2.688 0.903 0.903 weight 1.000 ! spec=Expt_1, no=879, id=734, vol=2.427926e+05
assign (segid " A" and resid 188 and name HD1) (segid " A" and resid 188 and name HA) 1.916 0.459 0.459 weight 1.000 ! spec=Expt_3, no=2002, id=3807, vol=9.265259e+06
assign (segid " A" and resid 188 and name HD1) (segid " A" and resid 188 and name HB2) 2.390 0.714 0.714 weight 1.000 ! spec=Expt_3, no=2054, id=3837, vol=2.462204e+06
assign (segid " A" and resid 188 and name HD1) (segid " A" and resid 188 and name HB1) 2.082 0.542 0.542 weight 1.000 ! spec=Expt_3, no=2734, id=3992, vol=5.631742e+06
assign (segid " A" and resid 188 and name HD1) (segid " A" and resid 192 and name HZ2) 2.349 0.689 0.689 weight 1.000 ! spec=Expt_1, no=2706, id=1969, vol=5.453962e+05
assign (segid " A" and resid 188 and name HD1) (segid " A" and resid 254 and name HG2) 2.588 0.837 0.837 weight 1.000 ! spec=Expt_1, no=2707, id=1970, vol=3.045165e+05
assign (segid " A" and resid 188 and name HN) (segid " A" and resid 254 and name HB2) 3.164 1.251 1.251 weight 1.000 ! spec=Expt_4, no=1027, id=4982, vol=3.030570e+06
assign (segid " A" and resid 188 and name HD1) (segid " A" and resid 257 and name HD11) 1.857 0.431 0.431 weight 1.000 ! spec=Expt_3, no=1111, id=3417, vol=1.119491e+07
assign (segid " A" and resid 188 and name HA) (segid " A" and resid 257 and name HD11) 2.349 0.690 0.690 weight 1.000 ! spec=Expt_3, no=1138, id=3436, vol=2.732043e+06
assign (segid " A" and resid 188 and name HD1) (segid " A" and resid 258 and name HG2) 1.980 0.490 0.490 weight 1.000 ! spec=Expt_3, no=992, id=3335, vol=7.618273e+06
assign (segid " A" and resid 188 and name HE1) (segid " A" and resid 258 and name HG1) 2.186 0.597 0.597 weight 1.000 ! spec=Expt_3, no=2144, id=3864, vol=4.202457e+06
assign (segid " A" and resid 188 and name HE1) (segid " A" and resid 261 and name HG21) 1.884 0.444 0.444 weight 1.000 ! spec=Expt_3, no=5321, id=4239, vol=1.024188e+07

HIS 189
assign (segid " A" and resid 189 and name HN) (segid " A" and resid 187 and name HB1) 2.692 0.906 0.906 weight 1.000 ! spec=Expt_1, no=3205, id=2336, vol=2.402691e+05
assign (segid " A" and resid 189 and name HN) (segid " A" and resid 188 and name HN) 2.363 0.698 0.698 weight 1.000 ! spec=Expt_1, no=647, id=568, vol=5.261400e+05
assign (segid " A" and resid 189 and name HD2) (segid " A" and resid 188 and name HN) 2.598 0.844 0.844 weight 1.000 ! spec=Expt_1, no=819, id=686, vol=2.973045e+05
assign (segid " A" and resid 189 and name HN) (segid " A" and resid 188 and name HA) 2.413 0.728 0.728 weight 1.000 ! spec=Expt_1, no=882, id=736, vol=4.633094e+05
assign (segid " A" and resid 189 and name HD2) (segid " A" and resid 189 and name HB1) 2.141 0.573 0.573 weight 1.000 ! spec=Expt_3, no=962, id=3313, vol=4.757674e+06
assign (segid " A" and resid 189 and name HA) (segid " A" and resid 189 and name HB2) 2.324 0.675 0.675 weight 1.000 ! spec=Expt_3, no=5267, id=4214, vol=2.914198e+06
assign (segid " A" and resid 189 and name HN) (segid " A" and resid 189 and name HA) 2.584 0.835 0.835 weight 1.000 ! spec=Expt_4, no=212, id=4405, vol=1.020262e+07
assign (segid " A" and resid 189 and name HN) (segid " A" and resid 189 and name HD2) 3.003 1.128 1.128 weight 1.000 ! spec=Expt_4, no=1030, id=4984, vol=4.140439e+06

assign (segid " A" and resid 189 and name HE1) (segid " A" and resid 190 and name HG12) 2.056 0.528 0.528 weight 1.000 ! spec=Expt_3, no=973, id=3321, vol=6.073552e+06
assign (segid " A" and resid 189 and name HE1) (segid " A" and resid 190 and name HD11) 1.926 0.464 0.464 weight 1.000 ! spec=Expt_3, no=975, id=3322, vol=8.988315e+06
assign (segid " A" and resid 189 and name HE1) (segid " A" and resid 190 and name HG22) 2.322 0.674 0.674 weight 1.000 ! spec=Expt_3, no=2990, id=4021, vol=2.930351e+06
assign (segid " A" and resid 189 and name   HN) (segid " A" and resid 191 and name   HN) 2.467 0.761 0.761 weight 1.000 ! spec=Expt_1, no=885, id=739, vol=4.063176e+05
assign (segid " A" and resid 189 and name   HN) (segid " A" and resid 192 and name HD1) 2.236 0.625 0.625 weight 1.000 ! spec=Expt_1, no=1440, id=1164, vol=7.313355e+05
assign (segid " A" and resid 189 and name   HN) (segid " A" and resid 192 and name HE1) 2.928 1.072 1.072 weight 1.000 ! spec=Expt_4, no=1028, id=4983, vol=4.824875e+06
assign (segid " A" and resid 189 and name HE1) (segid " A" and resid 251 and name HD1) 2.548 0.812 0.812 weight 1.000 ! spec=Expt_3, no=895, id=3272, vol=1.675184e+06
assign (segid " A" and resid 189 and name HB2) (segid " A" and resid 252 and name HD2) 1.943 0.472 0.472 weight 1.000 ! spec=Expt_1, no=2391, id=1801, vol=1.698587e+06
assign (segid " A" and resid 189 and name HD2) (segid " A" and resid 254 and name HG2) 2.703 0.913 0.913 weight 1.000 ! spec=Expt_1, no=1240, id=1015, vol=2.346423e+05
assign (segid " A" and resid 189 and name HD2) (segid " A" and resid 254 and name HB2) 2.237 0.626 0.626 weight 1.000 ! spec=Expt_3, no=932, id=3296, vol=3.656981e+06
assign (segid " A" and resid 189 and name HD2) (segid " A" and resid 254 and name HB1) 2.109 0.556 0.556 weight 1.000 ! spec=Expt_3, no=934, id=3298, vol=5.209593e+06

ILE 190
assign (segid " A" and resid 190 and name HD11) (segid " A" and resid 190 and name HG22) 1.733 0.375 0.375 weight 1.000 ! spec=Expt_1, no=329, id=298, vol=3.380598e+06
assign (segid " A" and resid 190 and name HG22) (segid " A" and resid 190 and name   HB) 1.770 0.391 0.391 weight 1.000 ! spec=Expt_3, no=84, id=2680, vol=1.493850e+07
assign (segid " A" and resid 190 and name HD11) (segid " A" and resid 190 and name   HB) 1.914 0.458 0.458 weight 1.000 ! spec=Expt_3, no=85, id=2681, vol=9.319077e+06
assign (segid " A" and resid 190 and name   HA) (segid " A" and resid 190 and name   HB) 1.980 0.490 0.490 weight 1.000 ! spec=Expt_3, no=87, id=2683, vol=7.601570e+06
assign (segid " A" and resid 190 and name HG22) (segid " A" and resid 190 and name   HA) 1.702 0.362 0.362 weight 1.000 ! spec=Expt_3, no=422, id=2946, vol=1.888115e+07
assign (segid " A" and resid 190 and name HG22) (segid " A" and resid 190 and name HG12) 1.786 0.399 0.399 weight 1.000 ! spec=Expt_3, no=425, id=2949, vol=1.414356e+07
assign (segid " A" and resid 190 and name HD11) (segid " A" and resid 190 and name HG12) 1.750 0.383 0.383 weight 1.000 ! spec=Expt_3, no=1470, id=3580, vol=1.598019e+07
assign (segid " A" and resid 190 and name HG22) (segid " A" and resid 252 and name HD1) 2.565 0.822 0.822 weight 1.000 ! spec=Expt_3, no=4534, id=4087, vol=1.610358e+06

SER 191
assign (segid " A" and resid 191 and name HB2) (segid " A" and resid 185 and name   HA) 2.048 0.524 0.524 weight 1.000 ! spec=Expt_3, no=1211, id=3465, vol=6.225604e+06
assign (segid " A" and resid 191 and name HB1) (segid " A" and resid 185 and name HG12) 2.311 0.668 0.668 weight 1.000 ! spec=Expt_3, no=1400, id=3536, vol=3.008876e+06
assign (segid " A" and resid 191 and name   HN) (segid " A" and resid 187 and name   HN) 3.080 1.185 1.185 weight 1.000 ! spec=Expt_4, no=1038, id=4990, vol=3.563495e+06
assign (segid " A" and resid 191 and name   HN) (segid " A" and resid 190 and name HG11) 2.530 0.800 0.800 weight 1.000 ! spec=Expt_1, no=888, id=740, vol=3.493799e+05
assign (segid " A" and resid 191 and name   HN) (segid " A" and resid 190 and name   HA) 2.787 0.971 0.971 weight 1.000 ! spec=Expt_4, no=214, id=4407, vol=6.488471e+06
assign (segid " A" and resid 191 and name   HN) (segid " A" and resid 190 and name   HB) 2.653 0.880 0.880 weight 1.000 ! spec=Expt_4, no=694, id=4790, vol=8.725368e+06
assign (segid " A" and resid 191 and name   HN) (segid " A" and resid 190 and name HD11) 2.903 1.053 1.053 weight 1.000 ! spec=Expt_4, no=695, id=4791, vol=5.078254e+06
assign (segid " A" and resid 191 and name   HN) (segid " A" and resid 191 and name   HA) 2.288 0.654 0.654 weight 1.000 ! spec=Expt_1, no=2069, id=1635, vol=6.378250e+05
assign (segid " A" and resid 191 and name   HN) (segid " A" and resid 192 and name HD1) 2.615 0.855 0.855 weight 1.000 ! spec=Expt_1, no=894, id=745, vol=2.861431e+05
assign (segid " A" and resid 191 and name HB1) (segid " A" and resid 192 and name   HN) 2.698 0.910 0.910 weight 1.000 ! spec=Expt_4, no=430, id=4576, vol=7.884786e+06
assign (segid " A" and resid 191 and name   HN) (segid " A" and resid 193 and name   HN) 3.046 1.160 1.160 weight 1.000 ! spec=Expt_4, no=1039, id=4991, vol=3.805091e+06

TRP 192
assign (segid " A" and resid 192 and name HZ2) (segid " A" and resid 185 and name   HB) 2.673 0.893 0.893 weight 1.000 ! spec=Expt_1, no=2532, id=1874, vol=2.506462e+05
assign (segid " A" and resid 192 and name HZ2) (segid " A" and resid 185 and name HG21) 1.888 0.445 0.445 weight 1.000 ! spec=Expt_3, no=1069, id=3390, vol=1.013419e+07
assign (segid " A" and resid 192 and name HE1) (segid " A" and resid 188 and name   HA) 2.057 0.529 0.529 weight 1.000 ! spec=Expt_1, no=1406, id=1137, vol=1.206555e+06
assign (segid " A" and resid 192 and name HE1) (segid " A" and resid 188 and name HD1) 2.173 0.590 0.590 weight 1.000 ! spec=Expt_1, no=1575, id=1264, vol=8.697235e+05
assign (segid " A" and resid 192 and name HZ2) (segid " A" and resid 188 and name HE1) 1.998 0.499 0.499 weight 1.000 ! spec=Expt_3, no=820, id=3249, vol=7.208250e+06
assign (segid " A" and resid 192 and name HD1) (segid " A" and resid 188 and name   HA) 1.955 0.478 0.478 weight 1.000 ! spec=Expt_3, no=2003, id=3808, vol=8.211050e+06
assign (segid " A" and resid 192 and name HE1) (segid " A" and resid 188 and name HE1) 2.881 1.037 1.037 weight 1.000 ! spec=Expt_4, no=1514, id=5155, vol=5.319653e+06
assign (segid " A" and resid 192 and name   HN) (segid " A" and resid 191 and name   HN) 2.416 0.729 0.729 weight 1.000 ! spec=Expt_1, no=637, id=558, vol=4.606085e+05
assign (segid " A" and resid 192 and name HD1) (segid " A" and resid 191 and name HB1) 2.312 0.668 0.668 weight 1.000 ! spec=Expt_3, no=921, id=3289, vol=3.000061e+06
assign (segid " A" and resid 192 and name   HN) (segid " A" and resid 191 and name   HA) 2.906 1.056 1.056 weight 1.000 ! spec=Expt_4, no=217, id=4410, vol=5.044325e+06
assign (segid " A" and resid 192 and name HE1) (segid " A" and resid 191 and name HB1) 2.874 1.033 1.033 weight 1.000 ! spec=Expt_4, no=1523, id=5160, vol=5.387843e+06
assign (segid " A" and resid 192 and name   HN) (segid " A" and resid 192 and name HB1) 2.394 0.717 0.717 weight 1.000 ! spec=Expt_1, no=639, id=560, vol=4.857083e+05

assign (segid " A" and resid 192 and name HH2) (segid " A" and resid 192 and name HZ3) 1.933 0.467 0.467 weight 1.000 ! spec=Expt_1, no=676, id=589, vol=1.754106e+06
assign (segid " A" and resid 192 and name HE1) (segid " A" and resid 192 and name HZ2) 2.216 0.614 0.614 weight 1.000 ! spec=Expt_1, no=789, id=662, vol=7.735465e+05
assign (segid " A" and resid 192 and name   HN) (segid " A" and resid 192 and name HD1) 2.085 0.543 0.543 weight 1.000 ! spec=Expt_1, no=1441, id=1165, vol=1.114545e+06
assign (segid " A" and resid 192 and name HE3) (segid " A" and resid 192 and name HB2) 2.236 0.625 0.625 weight 1.000 ! spec=Expt_1, no=1576, id=1265, vol=7.314033e+05
assign (segid " A" and resid 192 and name HE3) (segid " A" and resid 192 and name   HA) 2.555 0.816 0.816 weight 1.000 ! spec=Expt_1, no=2046, id=1621, vol=3.292696e+05
assign (segid " A" and resid 192 and name HZ3) (segid " A" and resid 192 and name HE3) 1.960 0.480 0.480 weight 1.000 ! spec=Expt_3, no=737, id=3199, vol=8.091351e+06
assign (segid " A" and resid 192 and name HZ2) (segid " A" and resid 192 and name HH2) 1.868 0.436 0.436 weight 1.000 ! spec=Expt_3, no=750, id=3206, vol=1.081380e+07
assign (segid " A" and resid 192 and name HD1) (segid " A" and resid 192 and name HB1) 2.200 0.605 0.605 weight 1.000 ! spec=Expt_3, no=2055, id=3838, vol=4.044559e+06
assign (segid " A" and resid 192 and name   HN) (segid " A" and resid 192 and name   HA) 2.659 0.884 0.884 weight 1.000 ! spec=Expt_4, no=216, id=4409, vol=8.599217e+06
assign (segid " A" and resid 192 and name HE1) (segid " A" and resid 192 and name HD1) 2.019 0.510 0.510 weight 1.000 ! spec=Expt_4, no=1517, id=5157, vol=4.481465e+07
assign (segid " A" and resid 192 and name   HN) (segid " A" and resid 193 and name   HN) 2.000 0.500 0.500 weight 1.000 ! spec=Expt_1, no=638, id=559, vol=1.431891e+06
assign (segid " A" and resid 192 and name   HA) (segid " A" and resid 193 and name   HN) 2.683 0.900 0.900 weight 1.000 ! spec=Expt_4, no=219, id=4411, vol=8.144758e+06
assign (segid " A" and resid 192 and name HE3) (segid " A" and resid 197 and name HB2) 2.005 0.503 0.503 weight 1.000 ! spec=Expt_3, no=1095, id=3407, vol=7.057388e+06
assign (segid " A" and resid 192 and name   HA) (segid " A" and resid 197 and name HB2) 2.102 0.552 0.552 weight 1.000 ! spec=Expt_3, no=1390, id=3528, vol=5.323530e+06
assign (segid " A" and resid 192 and name HZ3) (segid " A" and resid 198 and name   HA) 2.121 0.563 0.563 weight 1.000 ! spec=Expt_1, no=1547, id=1248, vol=1.004031e+06
assign (segid " A" and resid 192 and name HH2) (segid " A" and resid 201 and name HD13) 2.149 0.577 0.577 weight 1.000 ! spec=Expt_3, no=2061, id=3840, vol=4.652079e+06
assign (segid " A" and resid 192 and name HH2) (segid " A" and resid 201 and name HD21) 2.049 0.525 0.525 weight 1.000 ! spec=Expt_3, no=2332, id=3915, vol=6.196963e+06
assign (segid " A" and resid 192 and name HZ3) (segid " A" and resid 201 and name HD21) 2.272 0.646 0.646 weight 1.000 ! spec=Expt_3, no=2497, id=3940, vol=3.331072e+06
assign (segid " A" and resid 192 and name HZ3) (segid " A" and resid 201 and name HD13) 2.504 0.784 0.784 weight 1.000 ! spec=Expt_3, no=2499, id=3941, vol=1.860408e+06
assign (segid " A" and resid 192 and name HB1) (segid " A" and resid 257 and name HD11) 2.531 0.801 0.801 weight 1.000 ! spec=Expt_1, no=1767, id=1405, vol=3.478943e+05
assign (segid " A" and resid 192 and name HH2) (segid " A" and resid 261 and name HG12) 2.197 0.603 0.603 weight 1.000 ! spec=Expt_3, no=1015, id=3353, vol=4.078411e+06
assign (segid " A" and resid 192 and name HZ3) (segid " A" and resid 261 and name HG12) 2.448 0.749 0.749 weight 1.000 ! spec=Expt_3, no=1093, id=3406, vol=2.131523e+06
assign (segid " A" and resid 192 and name HE3) (segid " A" and resid 261 and name HG12) 2.528 0.799 0.799 weight 1.000 ! spec=Expt_3, no=4446, id=4072, vol=1.756451e+06

LYS 193
assign (segid " A" and resid 193 and name   HN) (segid " A" and resid 191 and name   HA) 3.068 1.176 1.176 weight 1.000 ! spec=Expt_4, no=1056, id=4997, vol=3.645537e+06
assign (segid " A" and resid 193 and name   HN) (segid " A" and resid 193 and name HB2) 1.995 0.498 0.498 weight 1.000 ! spec=Expt_1, no=907, id=755, vol=1.450582e+06
assign (segid " A" and resid 193 and name HB1) (segid " A" and resid 193 and name HE2) 1.751 0.383 0.383 weight 1.000 ! spec=Expt_3, no=657, id=3136, vol=1.591077e+07
assign (segid " A" and resid 193 and name HB2) (segid " A" and resid 193 and name HE2) 2.492 0.776 0.776 weight 1.000 ! spec=Expt_3, no=5124, id=4163, vol=1.914267e+06
assign (segid " A" and resid 193 and name HD1) (segid " A" and resid 193 and name HE1) 2.436 0.742 0.742 weight 1.000 ! spec=Expt_3, no=5256, id=4207, vol=2.194405e+06
assign (segid " A" and resid 193 and name   HN) (segid " A" and resid 193 and name HG2) 2.336 0.682 0.682 weight 1.000 ! spec=Expt_4, no=699, id=4794, vol=1.870208e+07
assign (segid " A" and resid 193 and name   HN) (segid " A" and resid 193 and name   HA) 2.172 0.590 0.590 weight 1.000 ! spec=Expt_4, no=1631, id=5198, vol=2.890841e+07
assign (segid " A" and resid 193 and name   HN) (segid " A" and resid 194 and name   HN) 2.210 0.611 0.611 weight 1.000 ! spec=Expt_1, no=925, id=765, vol=7.854629e+05
assign (segid " A" and resid 193 and name   HA) (segid " A" and resid 246 and name HG22) 1.709 0.365 0.365 weight 1.000 ! spec=Expt_3, no=1421, id=3546, vol=1.841669e+07
assign (segid " A" and resid 193 and name   HN) (segid " A" and resid 246 and name HG22) 2.786 0.970 0.970 weight 1.000 ! spec=Expt_4, no=1058, id=4998, vol=6.495112e+06
assign (segid " A" and resid 193 and name HE2) (segid " A" and resid 247 and name   HA) 2.228 0.621 0.621 weight 1.000 ! spec=Expt_3, no=1196, id=3456, vol=3.746886e+06

ASP 194
assign (segid " A" and resid 194 and name   HN) (segid " A" and resid 193 and name   HA) 2.718 0.923 0.923 weight 1.000 ! spec=Expt_4, no=221, id=4413, vol=7.541396e+06
assign (segid " A" and resid 194 and name   HN) (segid " A" and resid 193 and name HB2) 2.541 0.807 0.807 weight 1.000 ! spec=Expt_4, no=437, id=4583, vol=1.130333e+07
assign (segid " A" and resid 194 and name   HN) (segid " A" and resid 194 and name HB2) 2.643 0.873 0.873 weight 1.000 ! spec=Expt_1, no=3410, id=2511, vol=2.684635e+05
assign (segid " A" and resid 194 and name   HN) (segid " A" and resid 194 and name   HA) 2.316 0.670 0.670 weight 1.000 ! spec=Expt_4, no=220, id=4412, vol=1.970662e+07
assign (segid " A" and resid 194 and name   HN) (segid " A" and resid 194 and name HB1) 2.346 0.688 0.688 weight 1.000 ! spec=Expt_4, no=439, id=4585, vol=1.823347e+07

GLY 195
assign (segid " A" and resid 195 and name   HN) (segid " A" and resid 194 and name   HA) 2.700 0.911 0.911 weight 1.000 ! spec=Expt_4, no=223, id=4414, vol=7.850615e+06
assign (segid " A" and resid 195 and name   HN) (segid " A" and resid 196 and name   HN) 2.574 0.828 0.828 weight 1.000 ! spec=Expt_1, no=869, id=725, vol=3.146641e+05
assign (segid " A" and resid 195 and name HA2) (segid " A" and resid 224 and name HD21) 2.152 0.579 0.579 weight 1.000 ! spec=Expt_3, no=1413, id=3542, vol=4.618227e+06

assign (segid " A" and resid 195 and name HA1) (segid " A" and resid 224 and name HD21) 2.382 0.709 0.709 weight 1.000 ! spec=Expt_3, no=5237, id=4197, vol=2.511179e+06

LUE 196
assign (segid " A" and resid 196 and name HN) (segid " A" and resid 194 and name HA) 2.697 0.909 0.909 weight 1.000 ! spec=Expt_4, no=1068, id=5000, vol=7.895029e+06
assign (segid " A" and resid 196 and name HN) (segid " A" and resid 196 and name HB2) 2.272 0.645 0.645 weight 1.000 ! spec=Expt_1, no=946, id=780, vol=6.648851e+05
assign (segid " A" and resid 196 and name HN) (segid " A" and resid 196 and name HA) 2.448 0.749 0.749 weight 1.000 ! spec=Expt_1, no=947, id=781, vol=4.250963e+05
assign (segid " A" and resid 196 and name HD13) (segid " A" and resid 196 and name HA) 1.863 0.434 0.434 weight 1.000 ! spec=Expt_3, no=487, id=3001, vol=1.097355e+07
assign (segid " A" and resid 196 and name HD13) (segid " A" and resid 196 and name HD21) 1.605 0.322 0.322 weight 1.000 ! spec=Expt_3, no=490, id=3004, vol=2.680927e+07
assign (segid " A" and resid 196 and name HD21) (segid " A" and resid 196 and name HG) 1.832 0.420 0.420 weight 1.000 ! spec=Expt_3, no=1512, id=3612, vol=1.213100e+07
assign (segid " A" and resid 196 and name HB2) (segid " A" and resid 196 and name HB1) 1.635 0.334 0.334 weight 1.000 ! spec=Expt_3, no=1613, id=3679, vol=2.400588e+07
assign (segid " A" and resid 196 and name HD13) (segid " A" and resid 199 and name HB1) 2.602 0.846 0.846 weight 1.000 ! spec=Expt_1, no=2124, id=1677, vol=2.947375e+05
assign (segid " A" and resid 196 and name HA) (segid " A" and resid 199 and name HN) 2.785 0.970 0.970 weight 1.000 ! spec=Expt_4, no=1086, id=5005, vol=6.509299e+06
assign (segid " A" and resid 196 and name HD13) (segid " A" and resid 215 and name HD13) 1.862 0.434 0.434 weight 1.000 ! spec=Expt_3, no=1525, id=3620, vol=1.099250e+07

ALA 197
assign (segid " A" and resid 197 and name HA) (segid " A" and resid 177 and name HG1) 2.412 0.727 0.727 weight 1.000 ! spec=Expt_3, no=1675, id=3729, vol=2.331830e+06
assign (segid " A" and resid 197 and name HB2) (segid " A" and resid 183 and name HG21) 1.802 0.406 0.406 weight 1.000 ! spec=Expt_3, no=1532, id=3624, vol=1.340757e+07
assign (segid " A" and resid 197 and name HA) (segid " A" and resid 183 and name HG21) 1.895 0.449 0.449 weight 1.000 ! spec=Expt_3, no=1716, id=3761, vol=9.921198e+06
assign (segid " A" and resid 197 and name HB2) (segid " A" and resid 185 and name HG12) 1.845 0.426 0.426 weight 1.000 ! spec=Expt_3, no=1527, id=3622, vol=1.161202e+07
assign (segid " A" and resid 197 and name HN) (segid " A" and resid 196 and name HA) 2.906 1.056 1.056 weight 1.000 ! spec=Expt_4, no=228, id=4417, vol=5.045002e+06
assign (segid " A" and resid 197 and name HN) (segid " A" and resid 197 and name HB2) 2.030 0.515 0.515 weight 1.000 ! spec=Expt_1, no=949, id=782, vol=1.309074e+06
assign (segid " A" and resid 197 and name HN) (segid " A" and resid 197 and name HA) 2.254 0.635 0.635 weight 1.000 ! spec=Expt_1, no=952, id=785, vol=6.975007e+05
assign (segid " A" and resid 197 and name HA) (segid " A" and resid 197 and name HB2) 1.816 0.412 0.412 weight 1.000 ! spec=Expt_3, no=117, id=2706, vol=1.277554e+07
assign (segid " A" and resid 197 and name HA) (segid " A" and resid 200 and name HB2) 1.926 0.464 0.464 weight 1.000 ! spec=Expt_1, no=1604, id=1287, vol=1.793086e+06

PHE 198
assign (segid " A" and resid 198 and name HN) (segid " A" and resid 192 and name HE3) 2.576 0.830 0.830 weight 1.000 ! spec=Expt_1, no=960, id=793, vol=3.130308e+05
assign (segid " A" and resid 198 and name HE1) (segid " A" and resid 192 and name HB2) 2.708 0.916 0.916 weight 1.000 ! spec=Expt_1, no=2409, id=1810, vol=2.322064e+05
assign (segid " A" and resid 198 and name HD1) (segid " A" and resid 192 and name HZ3) 2.148 0.577 0.577 weight 1.000 ! spec=Expt_3, no=776, id=3223, vol=4.670939e+06
assign (segid " A" and resid 198 and name HN) (segid " A" and resid 192 and name HZ3) 3.016 1.137 1.137 weight 1.000 ! spec=Expt_4, no=1075, id=5002, vol=4.036873e+06
assign (segid " A" and resid 198 and name HD1) (segid " A" and resid 195 and name HA2) 2.101 0.552 0.552 weight 1.000 ! spec=Expt_1, no=916, id=762, vol=1.063536e+06
assign (segid " A" and resid 198 and name HN) (segid " A" and resid 197 and name HB2) 2.239 0.627 0.627 weight 1.000 ! spec=Expt_1, no=611, id=542, vol=7.255188e+05
assign (segid " A" and resid 198 and name HN) (segid " A" and resid 197 and name HN) 2.537 0.805 0.805 weight 1.000 ! spec=Expt_1, no=953, id=786, vol=3.430995e+05
assign (segid " A" and resid 198 and name HE1) (segid " A" and resid 198 and name HD1) 1.747 0.382 0.382 weight 1.000 ! spec=Expt_1, no=674, id=588, vol=3.216856e+06
assign (segid " A" and resid 198 and name HN) (segid " A" and resid 198 and name HB2) 2.339 0.684 0.684 weight 1.000 ! spec=Expt_1, no=956, id=789, vol=5.583457e+05
assign (segid " A" and resid 198 and name HN) (segid " A" and resid 198 and name HA) 2.384 0.710 0.710 weight 1.000 ! spec=Expt_1, no=959, id=792, vol=4.985994e+05
assign (segid " A" and resid 198 and name HD1) (segid " A" and resid 198 and name HN) 2.199 0.604 0.604 weight 1.000 ! spec=Expt_1, no=961, id=794, vol=8.096282e+05
assign (segid " A" and resid 198 and name HA) (segid " A" and resid 198 and name HB2) 2.510 0.788 0.788 weight 1.000 ! spec=Expt_1, no=2457, id=1839, vol=3.657361e+05
assign (segid " A" and resid 198 and name HD1) (segid " A" and resid 198 and name HA) 2.047 0.524 0.524 weight 1.000 ! spec=Expt_3, no=706, id=3178, vol=6.242228e+06
assign (segid " A" and resid 198 and name HZ) (segid " A" and resid 198 and name HE1) 2.000 0.500 0.500 weight 1.000 ! spec=Expt_3, no=2077, id=3848, vol=7.169734e+06
assign (segid " A" and resid 198 and name HD1) (segid " A" and resid 198 and name HB2) 2.068 0.535 0.535 weight 1.000 ! spec=Expt_3, no=2647, id=3978, vol=5.860898e+06
assign (segid " A" and resid 198 and name HN) (segid " A" and resid 199 and name HN) 2.262 0.639 0.639 weight 1.000 ! spec=Expt_1, no=596, id=530, vol=6.839154e+05
assign (segid " A" and resid 198 and name HA) (segid " A" and resid 201 and name HB2) 2.472 0.764 0.764 weight 1.000 ! spec=Expt_1, no=2637, id=1930, vol=4.014758e+05
assign (segid " A" and resid 198 and name HD1) (segid " A" and resid 224 and name HD21) 1.776 0.394 0.394 weight 1.000 ! spec=Expt_1, no=905, id=753, vol=2.915632e+06
assign (segid " A" and resid 198 and name HB2) (segid " A" and resid 227 and name HB2) 2.071 0.536 0.536 weight 1.000 ! spec=Expt_1, no=2596, id=1907, vol=1.161053e+06
assign (segid " A" and resid 198 and name HN) (segid " A" and resid 227 and name HB2) 3.010 1.133 1.133 weight 1.000 ! spec=Expt_4, no=1080, id=5004, vol=4.084154e+06
assign (segid " A" and resid 198 and name HZ) (segid " A" and resid 241 and name HD13) 2.318 0.672 0.672 weight 1.000 ! spec=Expt_3, no=1074, id=3394, vol=2.957111e+06
assign (segid " A" and resid 198 and name HZ) (segid " A" and resid 257 and name HG22) 2.401 0.720 0.720 weight 1.000 ! spec=Expt_1, no=1503, id=1214, vol=4.779344e+05

assign (segid " A" and resid 198 and name HE1) (segid " A" and resid 257 and name HD11) 2.096 0.549 0.549 weight 1.000 ! spec=Expt_1, no=2090, id=1652, vol=1.078627e+06
assign (segid " A" and resid 198 and name HE1) (segid " A" and resid 260 and name HD1) 2.023 0.512 0.512 weight 1.000 ! spec=Expt_3, no=801, id=3237, vol=6.687920e+06
assign (segid " A" and resid 198 and name HE1) (segid " A" and resid 261 and name HG12) 2.040 0.520 0.520 weight 1.000 ! spec=Expt_3, no=1043, id=3371, vol=6.355639e+06
assign (segid " A" and resid 198 and name HD1) (segid " A" and resid 261 and name HG12) 1.806 0.408 0.408 weight 1.000 ! spec=Expt_3, no=1102, id=3411, vol=1.322193e+07
assign (segid " A" and resid 198 and name HD1) (segid " A" and resid 264 and name HE1) 2.386 0.712 0.712 weight 1.000 ! spec=Expt_3, no=795, id=3235, vol=2.486268e+06

ASN 199
assign (segid " A" and resid 199 and name HD21) (segid " A" and resid 196 and name   HA) 2.147 0.576 0.576 weight 1.000 ! spec=Expt_1, no=1119, id=918, vol=9.331474e+05
assign (segid " A" and resid 199 and name HB1) (segid " A" and resid 196 and name   HA) 2.746 0.942 0.942 weight 1.000 ! spec=Expt_1, no=2698, id=1963, vol=2.136061e+05
assign (segid " A" and resid 199 and name   HN) (segid " A" and resid 198 and name HB2) 2.428 0.737 0.737 weight 1.000 ! spec=Expt_1, no=968, id=800, vol=4.470272e+05
assign (segid " A" and resid 199 and name   HN) (segid " A" and resid 198 and name HB1) 2.695 0.908 0.908 weight 1.000 ! spec=Expt_1, no=3334, id=2444, vol=2.386438e+05
assign (segid " A" and resid 199 and name   HN) (segid " A" and resid 199 and name   HA) 2.332 0.680 0.680 weight 1.000 ! spec=Expt_1, no=970, id=802, vol=5.683746e+05
assign (segid " A" and resid 199 and name   HN) (segid " A" and resid 199 and name HD21) 2.249 0.632 0.632 weight 1.000 ! spec=Expt_1, no=971, id=803, vol=7.076518e+05
assign (segid " A" and resid 199 and name HD21) (segid " A" and resid 199 and name HB1) 2.085 0.543 0.543 weight 1.000 ! spec=Expt_1, no=3385, id=2489, vol=1.115095e+06
assign (segid " A" and resid 199 and name   HN) (segid " A" and resid 199 and name HB1) 2.227 0.620 0.620 weight 1.000 ! spec=Expt_1, no=967, id=799, vol=7.500690e+05
assign (segid " A" and resid 199 and name   HN) (segid " A" and resid 200 and name   HN) 2.107 0.555 0.555 weight 1.000 ! spec=Expt_1, no=963, id=796, vol=1.045284e+06
assign (segid " A" and resid 199 and name   HA) (segid " A" and resid 202 and name HG22) 2.207 0.609 0.609 weight 1.000 ! spec=Expt_3, no=1699, id=3745, vol=3.972484e+06
assign (segid " A" and resid 199 and name HB2) (segid " A" and resid 210 and name HG22) 2.152 0.579 0.579 weight 1.000 ! spec=Expt_3, no=1620, id=3684, vol=4.614757e+06
assign (segid " A" and resid 199 and name HB2) (segid " A" and resid 215 and name HD13) 2.148 0.577 0.577 weight 1.000 ! spec=Expt_1, no=1761, id=1400, vol=9.316920e+05
assign (segid " A" and resid 199 and name HB1) (segid " A" and resid 215 and name HD13) 2.289 0.655 0.655 weight 1.000 ! spec=Expt_1, no=1965, id=1556, vol=6.362387e+05
assign (segid " A" and resid 199 and name HD22) (segid " A" and resid 223 and name   HA) 2.334 0.681 0.681 weight 1.000 ! spec=Expt_1, no=3528, id=2612, vol=5.662667e+05
assign (segid " A" and resid 199 and name   HN) (segid " A" and resid 227 and name HB2) 2.450 0.751 0.751 weight 1.000 ! spec=Expt_1, no=965, id=797, vol=4.227691e+05
assign (segid " A" and resid 199 and name HD22) (segid " A" and resid 227 and name HB2) 2.041 0.521 0.521 weight 1.000 ! spec=Expt_1, no=1415, id=1145, vol=1.266498e+06

ALA 200
assign (segid " A" and resid 200 and name   HN) (segid " A" and resid 196 and name   HA) 3.090 1.193 1.193 weight 1.000 ! spec=Expt_4, no=1094, id=5009, vol=3.494431e+06
assign (segid " A" and resid 200 and name   HN) (segid " A" and resid 197 and name   HA) 2.495 0.778 0.778 weight 1.000 ! spec=Expt_1, no=975, id=807, vol=3.790337e+05
assign (segid " A" and resid 200 and name   HN) (segid " A" and resid 199 and name HB1) 2.384 0.710 0.710 weight 1.000 ! spec=Expt_1, no=973, id=805, vol=4.990213e+05
assign (segid " A" and resid 200 and name   HN) (segid " A" and resid 199 and name   HA) 2.898 1.050 1.050 weight 1.000 ! spec=Expt_4, no=231, id=4420, vol=5.131750e+06
assign (segid " A" and resid 200 and name   HN) (segid " A" and resid 199 and name HD21) 3.174 1.259 1.259 weight 1.000 ! spec=Expt_4, no=1091, id=5007, vol=2.971386e+06
assign (segid " A" and resid 200 and name   HN) (segid " A" and resid 200 and name HB2) 1.880 0.442 0.442 weight 1.000 ! spec=Expt_1, no=972, id=804, vol=2.070494e+06
assign (segid " A" and resid 200 and name   HN) (segid " A" and resid 200 and name   HA) 2.257 0.637 0.637 weight 1.000 ! spec=Expt_1, no=974, id=806, vol=6.923974e+05
assign (segid " A" and resid 200 and name   HA) (segid " A" and resid 200 and name HB2) 1.819 0.414 0.414 weight 1.000 ! spec=Expt_3, no=131, id=2716, vol=1.266315e+07
assign (segid " A" and resid 200 and name   HN) (segid " A" and resid 212 and name HE1) 2.107 0.555 0.555 weight 1.000 ! spec=Expt_1, no=1428, id=1156, vol=1.047453e+06
assign (segid " A" and resid 200 and name HB2) (segid " A" and resid 212 and name HE1) 2.135 0.570 0.570 weight 1.000 ! spec=Expt_1, no=2151, id=1697, vol=9.662636e+05

LEU 201
assign (segid " A" and resid 201 and name HD21) (segid " A" and resid 173 and name   HA) 2.136 0.571 0.571 weight 1.000 ! spec=Expt_1, no=2753, id=1984, vol=9.623598e+05
assign (segid " A" and resid 201 and name   HN) (segid " A" and resid 198 and name   HA) 2.986 1.115 1.115 weight 1.000 ! spec=Expt_4, no=1099, id=5011, vol=4.287005e+06
assign (segid " A" and resid 201 and name   HN) (segid " A" and resid 200 and name HB2) 2.119 0.561 0.561 weight 1.000 ! spec=Expt_1, no=748, id=634, vol=1.011269e+06
assign (segid " A" and resid 201 and name   HN) (segid " A" and resid 200 and name   HN) 2.174 0.591 0.591 weight 1.000 ! spec=Expt_1, no=978, id=810, vol=8.671559e+05
assign (segid " A" and resid 201 and name HD13) (segid " A" and resid 200 and name HB2) 2.240 0.627 0.627 weight 1.000 ! spec=Expt_3, no=2032, id=3825, vol=3.627613e+06
assign (segid " A" and resid 201 and name   HN) (segid " A" and resid 200 and name   HA) 2.855 1.019 1.019 weight 1.000 ! spec=Expt_4, no=236, id=4425, vol=5.616789e+06
assign (segid " A" and resid 201 and name   HN) (segid " A" and resid 201 and name HB2) 2.042 0.521 0.521 weight 1.000 ! spec=Expt_1, no=979, id=811, vol=1.263127e+06
assign (segid " A" and resid 201 and name   HG) (segid " A" and resid 201 and name   HA) 2.551 0.813 0.813 weight 1.000 ! spec=Expt_1, no=2268, id=1758, vol=3.322529e+05
assign (segid " A" and resid 201 and name   HN) (segid " A" and resid 201 and name HD13) 2.235 0.624 0.624 weight 1.000 ! spec=Expt_1, no=2931, id=2104, vol=7.348347e+05
assign (segid " A" and resid 201 and name HD13) (segid " A" and resid 201 and name   HA) 1.930 0.466 0.466 weight 1.000 ! spec=Expt_3, no=496, id=3009, vol=8.878543e+06
assign (segid " A" and resid 201 and name   HN) (segid " A" and resid 201 and name   HA) 2.360 0.696 0.696 weight 1.000 ! spec=Expt_4, no=235, id=4424, vol=1.759914e+07

assign (segid " A" and resid 201 and name HN) (segid " A" and resid 202 and name HN) 2.195 0.602 0.602 weight 1.000 ! spec=Expt_1, no=853, id=711, vol=8.179918e+05
assign (segid " A" and resid 201 and name HN) (segid " A" and resid 203 and name HN) 3.094 1.197 1.197 weight 1.000 ! spec=Expt_4, no=1545, id=5165, vol=3.461873e+06

ILE 202
assign (segid " A" and resid 202 and name HN) (segid " A" and resid 199 and name HA) 2.860 1.022 1.022 weight 1.000 ! spec=Expt_4, no=1106, id=5012, vol=5.553385e+06
assign (segid " A" and resid 202 and name HN) (segid " A" and resid 201 and name HA) 2.771 0.960 0.960 weight 1.000 ! spec=Expt_4, no=239, id=4427, vol=6.715123e+06
assign (segid " A" and resid 202 and name HN) (segid " A" and resid 202 and name HA) 2.368 0.701 0.701 weight 1.000 ! spec=Expt_1, no=990, id=817, vol=5.196941e+05
assign (segid " A" and resid 202 and name HG12) (segid " A" and resid 202 and name HG11) 2.122 0.563 0.563 weight 1.000 ! spec=Expt_1, no=1754, id=1394, vol=1.002990e+06
assign (segid " A" and resid 202 and name HN) (segid " A" and resid 202 and name HB) 2.122 0.563 0.563 weight 1.000 ! spec=Expt_1, no=2675, id=1953, vol=1.002961e+06
assign (segid " A" and resid 202 and name HN) (segid " A" and resid 202 and name HG22) 2.344 0.687 0.687 weight 1.000 ! spec=Expt_1, no=3022, id=2178, vol=5.511427e+05
assign (segid " A" and resid 202 and name HD11) (segid " A" and resid 202 and name HB) 2.018 0.509 0.509 weight 1.000 ! spec=Expt_3, no=141, id=2726, vol=6.782798e+06
assign (segid " A" and resid 202 and name HG22) (segid " A" and resid 202 and name HB) 2.091 0.547 0.547 weight 1.000 ! spec=Expt_3, no=437, id=2958, vol=5.482652e+06
assign (segid " A" and resid 202 and name HG12) (segid " A" and resid 202 and name HD11) 2.098 0.550 0.550 weight 1.000 ! spec=Expt_3, no=1465, id=3576, vol=5.374968e+06
assign (segid " A" and resid 202 and name HA) (segid " A" and resid 202 and name HD11) 1.927 0.464 0.464 weight 1.000 ! spec=Expt_3, no=1687, id=3736, vol=8.967611e+06
assign (segid " A" and resid 202 and name HG12) (segid " A" and resid 209 and name HD13) 2.498 0.780 0.780 weight 1.000 ! spec=Expt_3, no=2016, id=3815, vol=1.890182e+06
assign (segid " A" and resid 202 and name HD11) (segid " A" and resid 209 and name HD13) 2.491 0.776 0.776 weight 1.000 ! spec=Expt_3, no=2017, id=3816, vol=1.920378e+06
assign (segid " A" and resid 202 and name HG11) (segid " A" and resid 209 and name HD13) 2.464 0.759 0.759 weight 1.000 ! spec=Expt_3, no=2018, id=3817, vol=2.049636e+06
assign (segid " A" and resid 202 and name HD11) (segid " A" and resid 235 and name HD13) 1.752 0.384 0.384 weight 1.000 ! spec=Expt_1, no=1859, id=1473, vol=3.163613e+06

HIS 203
assign (segid " A" and resid 203 and name HE1) (segid " A" and resid 180 and name HE1) 2.177 0.592 0.592 weight 1.000 ! spec=Expt_3, no=771, id=3221, vol=4.313890e+06
assign (segid " A" and resid 203 and name HB2) (segid " A" and resid 200 and name HA) 2.328 0.677 0.677 weight 1.000 ! spec=Expt_1, no=2667, id=1948, vol=5.752418e+05
assign (segid " A" and resid 203 and name HN) (segid " A" and resid 202 and name HN) 2.186 0.597 0.597 weight 1.000 ! spec=Expt_1, no=657, id=576, vol=8.393930e+05
assign (segid " A" and resid 203 and name HN) (segid " A" and resid 202 and name HG12) 2.987 1.116 1.116 weight 1.000 ! spec=Expt_4, no=713, id=4796, vol=4.275144e+06
assign (segid " A" and resid 203 and name HN) (segid " A" and resid 202 and name HG22) 2.982 1.112 1.112 weight 1.000 ! spec=Expt_4, no=716, id=4797, vol=4.322419e+06
assign (segid " A" and resid 203 and name HN) (segid " A" and resid 203 and name HA) 2.514 0.790 0.790 weight 1.000 ! spec=Expt_1, no=653, id=573, vol=3.627433e+05
assign (segid " A" and resid 203 and name HD2) (segid " A" and resid 203 and name HA) 2.339 0.684 0.684 weight 1.000 ! spec=Expt_1, no=656, id=575, vol=5.595159e+05
assign (segid " A" and resid 203 and name HD2) (segid " A" and resid 203 and name HB1) 2.328 0.677 0.677 weight 1.000 ! spec=Expt_3, no=768, id=3220, vol=2.884712e+06
assign (segid " A" and resid 203 and name HN) (segid " A" and resid 203 and name HB1) 2.289 0.655 0.655 weight 1.000 ! spec=Expt_1, no=3233, id=2359, vol=6.366090e+05
assign (segid " A" and resid 203 and name HN) (segid " A" and resid 204 and name HN) 2.348 0.689 0.689 weight 1.000 ! spec=Expt_1, no=660, id=579, vol=5.460628e+05
assign (segid " A" and resid 203 and name HD2) (segid " A" and resid 207 and name HA) 2.125 0.564 0.564 weight 1.000 ! spec=Expt_1, no=1339, id=1089, vol=9.939877e+05
assign (segid " A" and resid 203 and name HA) (segid " A" and resid 207 and name HA) 2.121 0.562 0.562 weight 1.000 ! spec=Expt_1, no=2688, id=1959, vol=1.005519e+06
assign (segid " A" and resid 203 and name HD2) (segid " A" and resid 210 and name HB) 1.982 0.491 0.491 weight 1.000 ! spec=Expt_1, no=1336, id=1086, vol=1.507901e+06
assign (segid " A" and resid 203 and name HD2) (segid " A" and resid 210 and name HG22) 1.983 0.492 0.492 weight 1.000 ! spec=Expt_3, no=756, id=3211, vol=7.533320e+06
assign (segid " A" and resid 203 and name HB1) (segid " A" and resid 210 and name HG22) 2.039 0.520 0.520 weight 1.000 ! spec=Expt_3, no=1431, id=3552, vol=6.386443e+06
assign (segid " A" and resid 203 and name HN) (segid " A" and resid 210 and name HG12) 2.917 1.064 1.064 weight 1.000 ! spec=Expt_4, no=1110, id=5013, vol=4.931567e+06
assign (segid " A" and resid 203 and name HD2) (segid " A" and resid 212 and name HN) 2.374 0.705 0.705 weight 1.000 ! spec=Expt_1, no=710, id=606, vol=5.108939e+05
assign (segid " A" and resid 203 and name HB1) (segid " A" and resid 212 and name HD1) 2.499 0.781 0.781 weight 1.000 ! spec=Expt_3, no=2797, id=4003, vol=1.881572e+06

ARG 204
assign (segid " A" and resid 204 and name HN) (segid " A" and resid 180 and name HE1) 2.837 1.006 1.006 weight 1.000 ! spec=Expt_4, no=1114, id=5014, vol=5.835143e+06
assign (segid " A" and resid 204 and name HN) (segid " A" and resid 203 and name HB2) 2.225 0.619 0.619 weight 1.000 ! spec=Expt_1, no=3260, id=2382, vol=7.544668e+05
assign (segid " A" and resid 204 and name HN) (segid " A" and resid 204 and name HA) 2.214 0.613 0.613 weight 1.000 ! spec=Expt_1, no=1000, id=822, vol=7.777362e+05
assign (segid " A" and resid 204 and name HD2) (segid " A" and resid 204 and name HD1) 2.018 0.509 0.509 weight 1.000 ! spec=Expt_1, no=1728, id=1372, vol=1.353971e+06
assign (segid " A" and resid 204 and name HG1) (segid " A" and resid 204 and name HG2) 2.118 0.561 0.561 weight 1.000 ! spec=Expt_1, no=1808, id=1438, vol=1.014272e+06
assign (segid " A" and resid 204 and name HB1) (segid " A" and resid 204 and name HG2) 2.289 0.655 0.655 weight 1.000 ! spec=Expt_1, no=1824, id=1452, vol=6.357220e+05
assign (segid " A" and resid 204 and name HA) (segid " A" and resid 204 and name HD2) 2.059 0.530 0.530 weight 1.000 ! spec=Expt_3, no=1193, id=3454, vol=6.015430e+06
assign (segid " A" and resid 204 and name HB1) (segid " A" and resid 204 and name HB2) 2.098 0.550 0.550 weight 1.000 ! spec=Expt_3, no=1450, id=3565, vol=5.378557e+06

assign (segid " A" and resid 204 and name HA) (segid " A" and resid 205 and name HN) 2.790 0.973 0.973 weight 1.000 ! spec=Expt_4, no=242, id=4429, vol=6.441112e+06

HIS 205
assign (segid " A" and resid 205 and name HN) (segid " A" and resid 204 and name HN) 2.510 0.787 0.787 weight 1.000 ! spec=Expt_1, no=664, id=583, vol=3.663462e+05
assign (segid " A" and resid 205 and name HN) (segid " A" and resid 204 and name HB2) 2.282 0.651 0.651 weight 1.000 ! spec=Expt_3, no=935, id=3299, vol=3.248364e+06
assign (segid " A" and resid 205 and name HN) (segid " A" and resid 205 and name HA) 2.506 0.785 0.785 weight 1.000 ! spec=Expt_1, no=667, id=584, vol=3.697336e+05
assign (segid " A" and resid 205 and name HB1) (segid " A" and resid 205 and name HB2) 2.020 0.510 0.510 weight 1.000 ! spec=Expt_3, no=1638, id=3698, vol=6.758437e+06
assign (segid " A" and resid 205 and name HN) (segid " A" and resid 206 and name HN) 2.079 0.540 0.540 weight 1.000 ! spec=Expt_1, no=663, id=582, vol=1.133913e+06
assign (segid " A" and resid 205 and name HB2) (segid " A" and resid 268 and name HE1) 2.604 0.848 0.848 weight 1.000 ! spec=Expt_1, no=3316, id=2427, vol=2.932566e+05
assign (segid " A" and resid 205 and name HD2) (segid " A" and resid 268 and name HD1) 2.285 0.652 0.652 weight 1.000 ! spec=Expt_3, no=798, id=3236, vol=3.226550e+06
assign (segid " A" and resid 205 and name HD2) (segid " A" and resid 268 and name HB2) 2.263 0.640 0.640 weight 1.000 ! spec=Expt_3, no=964, id=3314, vol=3.417524e+06

ARG 206
assign (segid " A" and resid 206 and name HN) (segid " A" and resid 203 and name HA) 2.831 1.002 1.002 weight 1.000 ! spec=Expt_4, no=1119, id=5016, vol=5.898491e+06
assign (segid " A" and resid 206 and name HN) (segid " A" and resid 204 and name HN) 2.991 1.118 1.118 weight 1.000 ! spec=Expt_4, no=1546, id=5166, vol=4.246385e+06
assign (segid " A" and resid 206 and name HN) (segid " A" and resid 205 and name HA) 2.862 1.024 1.024 weight 1.000 ! spec=Expt_4, no=244, id=4431, vol=5.528303e+06
assign (segid " A" and resid 206 and name HN) (segid " A" and resid 205 and name HB2) 2.535 0.803 0.803 weight 1.000 ! spec=Expt_4, no=474, id=4616, vol=1.145122e+07
assign (segid " A" and resid 206 and name HN) (segid " A" and resid 206 and name HB1) 2.330 0.679 0.679 weight 1.000 ! spec=Expt_1, no=1007, id=826, vol=5.714677e+05
assign (segid " A" and resid 206 and name HD1) (segid " A" and resid 206 and name HG2) 2.202 0.606 0.606 weight 1.000 ! spec=Expt_3, no=1669, id=3723, vol=4.026061e+06
assign (segid " A" and resid 206 and name HN) (segid " A" and resid 206 and name HA) 2.765 0.956 0.956 weight 1.000 ! spec=Expt_4, no=245, id=4432, vol=6.799606e+06
assign (segid " A" and resid 206 and name HN) (segid " A" and resid 206 and name HG2) 2.659 0.884 0.884 weight 1.000 ! spec=Expt_4, no=721, id=4800, vol=8.597717e+06
assign (segid " A" and resid 206 and name HB1) (segid " A" and resid 209 and name HD21) 2.209 0.610 0.610 weight 1.000 ! spec=Expt_1, no=1835, id=1459, vol=7.884879e+05
assign (segid " A" and resid 206 and name HD1) (segid " A" and resid 209 and name HD21) 2.445 0.747 0.747 weight 1.000 ! spec=Expt_3, no=1401, id=3537, vol=2.147752e+06

PRO 207
assign (segid " A" and resid 207 and name HD1) (segid " A" and resid 206 and name HN) 2.962 1.096 1.096 weight 1.000 ! spec=Expt_4, no=1120, id=5017, vol=4.503088e+06
assign (segid " A" and resid 207 and name HD2) (segid " A" and resid 207 and name HG2) 2.204 0.607 0.607 weight 1.000 ! spec=Expt_3, no=1323, id=3488, vol=4.003899e+06
assign (segid " A" and resid 207 and name HA) (segid " A" and resid 210 and name HB) 2.408 0.725 0.725 weight 1.000 ! spec=Expt_1, no=1702, id=1351, vol=4.688909e+05

GLU 208
assign (segid " A" and resid 208 and name HN) (segid " A" and resid 206 and name HA) 2.951 1.088 1.088 weight 1.000 ! spec=Expt_4, no=1122, id=5018, vol=4.605916e+06
assign (segid " A" and resid 208 and name HN) (segid " A" and resid 207 and name HD2) 2.712 0.919 0.919 weight 1.000 ! spec=Expt_4, no=726, id=4802, vol=7.636761e+06
assign (segid " A" and resid 208 and name HN) (segid " A" and resid 207 and name HG1) 2.282 0.651 0.651 weight 1.000 ! spec=Expt_4, no=857, id=4882, vol=2.150068e+07
assign (segid " A" and resid 208 and name HN) (segid " A" and resid 208 and name HA) 2.200 0.605 0.605 weight 1.000 ! spec=Expt_1, no=1014, id=832, vol=8.065894e+05
assign (segid " A" and resid 208 and name HA) (segid " A" and resid 208 and name HB1) 2.266 0.642 0.642 weight 1.000 ! spec=Expt_1, no=2515, id=1861, vol=6.763880e+05
assign (segid " A" and resid 208 and name HG2) (segid " A" and resid 208 and name HB1) 2.076 0.538 0.538 weight 1.000 ! spec=Expt_3, no=1627, id=3690, vol=5.737976e+06
assign (segid " A" and resid 208 and name HN) (segid " A" and resid 209 and name HN) 2.178 0.593 0.593 weight 1.000 ! spec=Expt_1, no=1008, id=827, vol=8.564591e+05
assign (segid " A" and resid 208 and name HN) (segid " A" and resid 209 and name HG) 2.877 1.034 1.034 weight 1.000 ! spec=Expt_4, no=1124, id=5020, vol=5.364164e+06

LEU 209
assign (segid " A" and resid 209 and name HD21) (segid " A" and resid 208 and name HG2) 2.482 0.770 0.770 weight 1.000 ! spec=Expt_3, no=2015, id=3814, vol=1.962781e+06
assign (segid " A" and resid 209 and name HD13) (segid " A" and resid 209 and name HD21) 1.771 0.392 0.392 weight 1.000 ! spec=Expt_1, no=406, id=365, vol=2.966763e+06
assign (segid " A" and resid 209 and name HN) (segid " A" and resid 209 and name HG) 2.148 0.577 0.577 weight 1.000 ! spec=Expt_1, no=1018, id=836, vol=9.311457e+05
assign (segid " A" and resid 209 and name HN) (segid " A" and resid 209 and name HA) 2.314 0.670 0.670 weight 1.000 ! spec=Expt_1, no=1020, id=837, vol=5.954398e+05
assign (segid " A" and resid 209 and name HB2) (segid " A" and resid 209 and name HN) 2.653 0.880 0.880 weight 1.000 ! spec=Expt_1, no=3133, id=2273, vol=2.623366e+05
assign (segid " A" and resid 209 and name HB2) (segid " A" and resid 209 and name HA) 2.184 0.596 0.596 weight 1.000 ! spec=Expt_3, no=1235, id=3480, vol=4.224363e+06
assign (segid " A" and resid 209 and name HD21) (segid " A" and resid 209 and name HB2) 1.847 0.427 0.427 weight 1.000 ! spec=Expt_3, no=1495, id=3602, vol=1.154302e+07
assign (segid " A" and resid 209 and name HN) (segid " A" and resid 210 and name HN) 2.046 0.523 0.523 weight 1.000 ! spec=Expt_1, no=1022, id=839, vol=1.246878e+06

assign (segid " A" and resid 209 and name HG) (segid " A" and resid 210 and name HN) 2.645 0.875 0.875 weight 1.000 ! spec=Expt_1, no=3018, id=2175, vol=2.670491e+05

ILE 210
assign (segid " A" and resid 210 and name HD11) (segid " A" and resid 199 and name HA) 2.386 0.712 0.712 weight 1.000 ! spec=Expt_3, no=1727, id=3770, vol=2.484117e+06
assign (segid " A" and resid 210 and name HG12) (segid " A" and resid 202 and name HG22) 1.794 0.402 0.402 weight 1.000 ! spec=Expt_3, no=1516, id=3615, vol=1.375617e+07
assign (segid " A" and resid 210 and name HN) (segid " A" and resid 203 and name HD2) 2.647 0.876 0.876 weight 1.000 ! spec=Expt_1, no=1031, id=845, vol=2.659426e+05
assign (segid " A" and resid 210 and name HB) (segid " A" and resid 203 and name HB1) 2.226 0.620 0.620 weight 1.000 ! spec=Expt_3, no=4928, id=4145, vol=3.766699e+06
assign (segid " A" and resid 210 and name HD11) (segid " A" and resid 209 and name HD13) 2.331 0.679 0.679 weight 1.000 ! spec=Expt_3, no=1538, id=3628, vol=2.862305e+06
assign (segid " A" and resid 210 and name HN) (segid " A" and resid 209 and name HA) 2.730 0.932 0.932 weight 1.000 ! spec=Expt_4, no=249, id=4436, vol=7.334454e+06
assign (segid " A" and resid 210 and name HG11) (segid " A" and resid 210 and name HG12) 1.786 0.399 0.399 weight 1.000 ! spec=Expt_1, no=1889, id=1494, vol=2.821236e+06
assign (segid " A" and resid 210 and name HN) (segid " A" and resid 210 and name HG11) 2.481 0.770 0.770 weight 1.000 ! spec=Expt_1, no=2525, id=1868, vol=3.922342e+05
assign (segid " A" and resid 210 and name HN) (segid " A" and resid 210 and name HB) 2.261 0.639 0.639 weight 1.000 ! spec=Expt_1, no=3068, id=2222, vol=6.852464e+05
assign (segid " A" and resid 210 and name HG22) (segid " A" and resid 210 and name HG12) 1.942 0.471 0.471 weight 1.000 ! spec=Expt_3, no=427, id=2951, vol=8.559970e+06
assign (segid " A" and resid 210 and name HD11) (segid " A" and resid 210 and name HA) 2.150 0.578 0.578 weight 1.000 ! spec=Expt_3, no=434, id=2957, vol=4.640275e+06
assign (segid " A" and resid 210 and name HG22) (segid " A" and resid 210 and name HA) 2.155 0.581 0.581 weight 1.000 ! spec=Expt_3, no=1346, id=3500, vol=4.577572e+06
assign (segid " A" and resid 210 and name HN) (segid " A" and resid 210 and name HA) 2.499 0.781 0.781 weight 1.000 ! spec=Expt_4, no=248, id=4435, vol=1.247018e+07
assign (segid " A" and resid 210 and name HN) (segid " A" and resid 210 and name HD11) 2.792 0.974 0.974 weight 1.000 ! spec=Expt_4, no=735, id=4805, vol=6.417287e+06
assign (segid " A" and resid 210 and name HG22) (segid " A" and resid 215 and name HD13) 1.921 0.461 0.461 weight 1.000 ! spec=Expt_3, no=1509, id=3610, vol=9.122040e+06

GLU 211
assign (segid " A" and resid 211 and name HN) (segid " A" and resid 210 and name HA) 2.149 0.577 0.577 weight 1.000 ! spec=Expt_1, no=1032, id=846, vol=9.289654e+05
assign (segid " A" and resid 211 and name HN) (segid " A" and resid 210 and name HG22) 2.302 0.663 0.663 weight 1.000 ! spec=Expt_1, no=1033, id=847, vol=6.142200e+05
assign (segid " A" and resid 211 and name HN) (segid " A" and resid 210 and name HD11) 2.713 0.920 0.920 weight 1.000 ! spec=Expt_1, no=1034, id=848, vol=2.293086e+05
assign (segid " A" and resid 211 and name HN) (segid " A" and resid 211 and name HG1) 2.497 0.780 0.780 weight 1.000 ! spec=Expt_1, no=1036, id=850, vol=3.773176e+05
assign (segid " A" and resid 211 and name HN) (segid " A" and resid 211 and name HB1) 2.308 0.666 0.666 weight 1.000 ! spec=Expt_1, no=2906, id=2083, vol=6.049086e+05
assign (segid " A" and resid 211 and name HG1) (segid " A" and resid 211 and name HG2) 1.703 0.362 0.362 weight 1.000 ! spec=Expt_3, no=1624, id=3688, vol=1.880882e+07
assign (segid " A" and resid 211 and name HN) (segid " A" and resid 211 and name HA) 2.468 0.761 0.761 weight 1.000 ! spec=Expt_4, no=252, id=4438, vol=1.344783e+07

TYR 212
assign (segid " A" and resid 212 and name HE1) (segid " A" and resid 180 and name HD1) 1.914 0.458 0.458 weight 1.000 ! spec=Expt_3, no=2045, id=3832, vol=9.318312e+06
assign (segid " A" and resid 212 and name HE1) (segid " A" and resid 196 and name HA) 2.387 0.712 0.712 weight 1.000 ! spec=Expt_3, no=875, id=3260, vol=2.478067e+06
assign (segid " A" and resid 212 and name HE1) (segid " A" and resid 196 and name HD13) 2.167 0.587 0.587 weight 1.000 ! spec=Expt_3, no=1121, id=3425, vol=4.432422e+06
assign (segid " A" and resid 212 and name HD1) (segid " A" and resid 196 and name HD13) 2.520 0.794 0.794 weight 1.000 ! spec=Expt_3, no=2094, id=3855, vol=1.791216e+06
assign (segid " A" and resid 212 and name HD1) (segid " A" and resid 199 and name HB2) 2.148 0.577 0.577 weight 1.000 ! spec=Expt_1, no=2183, id=1725, vol=9.309560e+05
assign (segid " A" and resid 212 and name HE1) (segid " A" and resid 199 and name HB1) 2.172 0.590 0.590 weight 1.000 ! spec=Expt_3, no=886, id=3265, vol=4.368031e+06
assign (segid " A" and resid 212 and name HD1) (segid " A" and resid 199 and name HB1) 2.282 0.651 0.651 weight 1.000 ! spec=Expt_3, no=2800, id=4004, vol=3.251645e+06
assign (segid " A" and resid 212 and name HE1) (segid " A" and resid 200 and name HA) 2.163 0.585 0.585 weight 1.000 ! spec=Expt_3, no=881, id=3263, vol=4.475105e+06
assign (segid " A" and resid 212 and name HN) (segid " A" and resid 210 and name HG22) 2.292 0.656 0.656 weight 1.000 ! spec=Expt_1, no=1039, id=853, vol=6.318520e+05
assign (segid " A" and resid 212 and name HN) (segid " A" and resid 210 and name HB) 2.903 1.053 1.053 weight 1.000 ! spec=Expt_1, no=1041, id=855, vol=1.530257e+05
assign (segid " A" and resid 212 and name HD1) (segid " A" and resid 210 and name HG22) 1.948 0.474 0.474 weight 1.000 ! spec=Expt_3, no=1098, id=3410, vol=8.399177e+06
assign (segid " A" and resid 212 and name HN) (segid " A" and resid 212 and name HA) 2.133 0.569 0.569 weight 1.000 ! spec=Expt_1, no=573, id=511, vol=9.725657e+05
assign (segid " A" and resid 212 and name HN) (segid " A" and resid 212 and name HB1) 2.646 0.875 0.875 weight 1.000 ! spec=Expt_1, no=3077, id=2228, vol=2.663934e+05
assign (segid " A" and resid 212 and name HN) (segid " A" and resid 212 and name HB2) 2.440 0.744 0.744 weight 1.000 ! spec=Expt_1, no=3280, id=2398, vol=4.333324e+05
assign (segid " A" and resid 212 and name HA) (segid " A" and resid 212 and name HD1) 2.005 0.503 0.503 weight 1.000 ! spec=Expt_3, no=699, id=3171, vol=7.054763e+06
assign (segid " A" and resid 212 and name HD1) (segid " A" and resid 212 and name HB1) 2.035 0.518 0.518 weight 1.000 ! spec=Expt_3, no=2418, id=3929, vol=6.455355e+06
assign (segid " A" and resid 212 and name HD1) (segid " A" and resid 212 and name HB2) 2.020 0.510 0.510 weight 1.000 ! spec=Expt_3, no=2803, id=4005, vol=6.758657e+06
assign (segid " A" and resid 212 and name HN) (segid " A" and resid 213 and name HN) 2.427 0.736 0.736 weight 1.000 ! spec=Expt_1, no=1045, id=858, vol=4.482498e+05
assign (segid " A" and resid 212 and name HN) (segid " A" and resid 215 and name HD13) 2.633 0.867 0.867 weight 1.000 ! spec=Expt_1, no=1038, id=852, vol=2.745636e+05

assign (segid " A" and resid 212 and name HA) (segid " A" and resid 215 and name HD13) 2.066 0.533 0.533 weight 1.000 ! spec=Expt_1, no=1580, id=1269, vol=1.178316e+06
assign (segid " A" and resid 212 and name HA) (segid " A" and resid 215 and name HG) 2.205 0.608 0.608 weight 1.000 ! spec=Expt_1, no=1584, id=1270, vol=7.963833e+05
assign (segid " A" and resid 212 and name HA) (segid " A" and resid 215 and name HN) 2.659 0.884 0.884 weight 1.000 ! spec=Expt_1, no=3332, id=2442, vol=2.587928e+05
assign (segid " A" and resid 212 and name HD1) (segid " A" and resid 215 and name HD13) 1.985 0.492 0.492 weight 1.000 ! spec=Expt_3, no=1092, id=3405, vol=7.509652e+06
assign (segid " A" and resid 212 and name HA) (segid " A" and resid 215 and name HB2) 2.472 0.764 0.764 weight 1.000 ! spec=Expt_3, no=4571, id=4093, vol=2.010004e+06

ASP 213
assign (segid " A" and resid 213 and name HN) (segid " A" and resid 212 and name HB1) 2.681 0.898 0.898 weight 1.000 ! spec=Expt_1, no=3209, id=2339, vol=2.464675e+05
assign (segid " A" and resid 213 and name HN) (segid " A" and resid 212 and name HA) 2.697 0.909 0.909 weight 1.000 ! spec=Expt_4, no=256, id=4441, vol=7.902136e+06
assign (segid " A" and resid 213 and name HN) (segid " A" and resid 213 and name HB2) 2.639 0.870 0.870 weight 1.000 ! spec=Expt_1, no=3242, id=2365, vol=2.711169e+05
assign (segid " A" and resid 213 and name HN) (segid " A" and resid 213 and name HB1) 2.380 0.708 0.708 weight 1.000 ! spec=Expt_1, no=3245, id=2368, vol=5.032793e+05
assign (segid " A" and resid 213 and name HA) (segid " A" and resid 213 and name HB2) 1.966 0.483 0.483 weight 1.000 ! spec=Expt_3, no=1747, id=3785, vol=7.942811e+06
assign (segid " A" and resid 213 and name HA) (segid " A" and resid 213 and name HN) 2.238 0.626 0.626 weight 1.000 ! spec=Expt_4, no=255, id=4440, vol=2.418366e+07

LYS 214
assign (segid " A" and resid 214 and name HN) (segid " A" and resid 213 and name HN) 2.237 0.626 0.626 weight 1.000 ! spec=Expt_1, no=1052, id=862, vol=7.297780e+05
assign (segid " A" and resid 214 and name HN) (segid " A" and resid 213 and name HB1) 2.430 0.738 0.738 weight 1.000 ! spec=Expt_1, no=1058, id=868, vol=4.449457e+05
assign (segid " A" and resid 214 and name HN) (segid " A" and resid 213 and name HA) 2.581 0.833 0.833 weight 1.000 ! spec=Expt_4, no=258, id=4442, vol=1.028249e+07
assign (segid " A" and resid 214 and name HB1) (segid " A" and resid 214 and name HA) 2.236 0.625 0.625 weight 1.000 ! spec=Expt_1, no=2600, id=1911, vol=7.328016e+05
assign (segid " A" and resid 214 and name HA) (segid " A" and resid 214 and name HN) 2.190 0.599 0.599 weight 1.000 ! spec=Expt_1, no=3521, id=2606, vol=8.301481e+05
assign (segid " A" and resid 214 and name HB2) (segid " A" and resid 214 and name HG2) 1.773 0.393 0.393 weight 1.000 ! spec=Expt_3, no=420, id=2944, vol=1.477624e+07
assign (segid " A" and resid 214 and name HN) (segid " A" and resid 214 and name HB1) 2.168 0.587 0.587 weight 1.000 ! spec=Expt_3, no=982, id=3328, vol=4.419880e+06
assign (segid " A" and resid 214 and name HE2) (segid " A" and resid 214 and name HB1) 2.408 0.725 0.725 weight 1.000 ! spec=Expt_3, no=1314, id=3484, vol=2.355171e+06
assign (segid " A" and resid 214 and name HG2) (segid " A" and resid 214 and name HE2) 2.145 0.575 0.575 weight 1.000 ! spec=Expt_3, no=1664, id=3719, vol=4.704844e+06
assign (segid " A" and resid 214 and name HN) (segid " A" and resid 214 and name HG1) 2.434 0.741 0.741 weight 1.000 ! spec=Expt_4, no=741, id=4811, vol=1.460278e+07
assign (segid " A" and resid 214 and name HN) (segid " A" and resid 214 and name HD1) 2.301 0.662 0.662 weight 1.000 ! spec=Expt_4, no=742, id=4812, vol=2.047220e+07
assign (segid " A" and resid 214 and name HN) (segid " A" and resid 215 and name HB2) 2.970 1.103 1.103 weight 1.000 ! spec=Expt_4, no=1147, id=5027, vol=4.428081e+06

LEU 215
assign (segid " A" and resid 215 and name HD13) (segid " A" and resid 196 and name HA) 2.609 0.851 0.851 weight 1.000 ! spec=Expt_1, no=2625, id=1928, vol=2.901389e+05
assign (segid " A" and resid 215 and name HB2) (segid " A" and resid 196 and name HD13) 2.016 0.508 0.508 weight 1.000 ! spec=Expt_3, no=1502, id=3605, vol=6.826472e+06
assign (segid " A" and resid 215 and name HB1) (segid " A" and resid 196 and name HD13) 2.156 0.581 0.581 weight 1.000 ! spec=Expt_3, no=1504, id=3606, vol=4.570432e+06
assign (segid " A" and resid 215 and name HN) (segid " A" and resid 214 and name HN) 2.054 0.528 0.528 weight 1.000 ! spec=Expt_1, no=1053, id=863, vol=1.217296e+06
assign (segid " A" and resid 215 and name HN) (segid " A" and resid 214 and name HG1) 2.789 0.972 0.972 weight 1.000 ! spec=Expt_4, no=743, id=4813, vol=6.460992e+06
assign (segid " A" and resid 215 and name HN) (segid " A" and resid 215 and name HB2) 2.079 0.540 0.540 weight 1.000 ! spec=Expt_1, no=1067, id=875, vol=1.134173e+06
assign (segid " A" and resid 215 and name HN) (segid " A" and resid 215 and name HA) 2.266 0.642 0.642 weight 1.000 ! spec=Expt_1, no=1070, id=878, vol=6.768425e+05
assign (segid " A" and resid 215 and name HA) (segid " A" and resid 215 and name HD21) 1.710 0.365 0.365 weight 1.000 ! spec=Expt_1, no=2004, id=1590, vol=3.666414e+06
assign (segid " A" and resid 215 and name HA) (segid " A" and resid 215 and name HB1) 2.099 0.551 0.551 weight 1.000 ! spec=Expt_3, no=1349, id=3503, vol=5.365484e+06
assign (segid " A" and resid 215 and name HB1) (segid " A" and resid 215 and name HB2) 1.798 0.404 0.404 weight 1.000 ! spec=Expt_3, no=1484, id=3592, vol=1.356980e+07
assign (segid " A" and resid 215 and name HN) (segid " A" and resid 215 and name HG) 2.065 0.533 0.533 weight 1.000 ! spec=Expt_4, no=747, id=4814, vol=3.915119e+07
assign (segid " A" and resid 215 and name HD21) (segid " A" and resid 223 and name HA) 2.026 0.513 0.513 weight 1.000 ! spec=Expt_3, no=1354, id=3507, vol=6.629138e+06

ARG 216
assign (segid " A" and resid 216 and name HN) (segid " A" and resid 196 and name HD13) 3.198 1.279 1.279 weight 1.000 ! spec=Expt_4, no=1156, id=5030, vol=2.840327e+06
assign (segid " A" and resid 216 and name HN) (segid " A" and resid 215 and name HA) 1.883 0.443 0.443 weight 1.000 ! spec=Expt_1, no=1083, id=887, vol=2.052183e+06
assign (segid " A" and resid 216 and name HN) (segid " A" and resid 215 and name HB1) 2.441 0.745 0.745 weight 1.000 ! spec=Expt_1, no=2181, id=1723, vol=4.326700e+05
assign (segid " A" and resid 216 and name HN) (segid " A" and resid 215 and name HD21) 2.405 0.723 0.723 weight 1.000 ! spec=Expt_1, no=2868, id=2053, vol=4.726551e+05
assign (segid " A" and resid 216 and name HN) (segid " A" and resid 216 and name HB2) 2.296 0.659 0.659 weight 1.000 ! spec=Expt_1, no=1078, id=883, vol=6.253960e+05

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assign (segid "   A" and resid   216 and name  HG2) (segid "   A" and resid   216 and name  HG1) 1.663 0.346 0.346 weight 1.000 ! spec=Expt_1, no=1863, id=1475, vol=4.325117e+06
assign (segid "   A" and resid   216 and name  HB2) (segid "   A" and resid   216 and name  HB1) 1.677 0.352 0.352 weight 1.000 ! spec=Expt_1, no=1879, id=1484, vol=4.111617e+06
assign (segid "   A" and resid   216 and name  HG1) (segid "   A" and resid   216 and name   HA) 2.682 0.899 0.899 weight 1.000 ! spec=Expt_1, no=2592, id=1905, vol=2.456711e+05
assign (segid "   A" and resid   216 and name   HA) (segid "   A" and resid   216 and name  HD1) 2.390 0.714 0.714 weight 1.000 ! spec=Expt_3, no=1218, id=3470, vol=2.462480e+06
assign (segid "   A" and resid   216 and name   HN) (segid "   A" and resid   216 and name   HA) 2.651 0.879 0.879 weight 1.000 ! spec=Expt_4, no=263, id=4445, vol=8.753281e+06

LYS 217
assign (segid "   A" and resid   217 and name  HE2) (segid "   A" and resid   217 and name  HD1) 2.148 0.577 0.577 weight 1.000 ! spec=Expt_1, no=1681, id=1338, vol=9.328120e+05

ASP 218
assign (segid "   A" and resid   218 and name   HN) (segid "   A" and resid   216 and name  HB1) 2.356 0.694 0.694 weight 1.000 ! spec=Expt_1, no=1087, id=891, vol=5.356014e+05
assign (segid "   A" and resid   218 and name   HN) (segid "   A" and resid   217 and name   HA) 2.645 0.874 0.874 weight 1.000 ! spec=Expt_4, no=262, id=4444, vol=8.879161e+06
assign (segid "   A" and resid   218 and name   HN) (segid "   A" and resid   218 and name   HA) 2.362 0.698 0.698 weight 1.000 ! spec=Expt_1, no=1098, id=900, vol=5.267517e+05
assign (segid "   A" and resid   218 and name   HN) (segid "   A" and resid   218 and name  HB1) 2.185 0.597 0.597 weight 1.000 ! spec=Expt_1, no=2555, id=1886, vol=8.414221e+05

ASP 219
assign (segid "   A" and resid   219 and name   HN) (segid "   A" and resid   218 and name   HN) 2.132 0.568 0.568 weight 1.000 ! spec=Expt_1, no=1084, id=888, vol=9.748279e+05
assign (segid "   A" and resid   219 and name   HN) (segid "   A" and resid   218 and name   HA) 2.443 0.746 0.746 weight 1.000 ! spec=Expt_4, no=261, id=4443, vol=1.430486e+07
assign (segid "   A" and resid   219 and name   HN) (segid "   A" and resid   219 and name   HA) 2.386 0.712 0.712 weight 1.000 ! spec=Expt_1, no=1062, id=871, vol=4.955756e+05
assign (segid "   A" and resid   219 and name   HN) (segid "   A" and resid   219 and name  HB1) 2.313 0.669 0.669 weight 1.000 ! spec=Expt_4, no=507, id=4644, vol=1.982467e+07
assign (segid "   A" and resid   219 and name   HN) (segid "   A" and resid   220 and name  HD2) 2.453 0.752 0.752 weight 1.000 ! spec=Expt_3, no=907, id=3280, vol=2.105096e+06

PRO 220
assign (segid "   A" and resid   220 and name  HD2) (segid "   A" and resid   218 and name   HA) 2.483 0.770 0.770 weight 1.000 ! spec=Expt_1, no=2559, id=1889, vol=3.909063e+05
assign (segid "   A" and resid   220 and name  HD1) (segid "   A" and resid   219 and name   HA) 2.162 0.584 0.584 weight 1.000 ! spec=Expt_3, no=867, id=3256, vol=4.497220e+06
assign (segid "   A" and resid   220 and name  HD2) (segid "   A" and resid   219 and name   HA) 2.061 0.531 0.531 weight 1.000 ! spec=Expt_3, no=1172, id=3444, vol=5.986584e+06
assign (segid "   A" and resid   220 and name  HB2) (segid "   A" and resid   220 and name   HA) 2.169 0.588 0.588 weight 1.000 ! spec=Expt_1, no=1708, id=1355, vol=8.798528e+05

VAL 221
assign (segid "   A" and resid   221 and name   HN) (segid "   A" and resid   219 and name   HA) 2.880 1.037 1.037 weight 1.000 ! spec=Expt_4, no=1171, id=5034, vol=5.329837e+06
assign (segid "   A" and resid   221 and name   HN) (segid "   A" and resid   220 and name  HB1) 2.258 0.637 0.637 weight 1.000 ! spec=Expt_4, no=511, id=4646, vol=2.293959e+07
assign (segid "   A" and resid   221 and name   HN) (segid "   A" and resid   220 and name   HA) 2.936 1.077 1.077 weight 1.000 ! spec=Expt_4, no=754, id=4820, vol=4.746663e+06
assign (segid "   A" and resid   221 and name   HN) (segid "   A" and resid   220 and name  HD1) 2.300 0.661 0.661 weight 1.000 ! spec=Expt_4, no=755, id=4821, vol=2.051235e+07
assign (segid "   A" and resid   221 and name   HN) (segid "   A" and resid   221 and name HG21) 1.871 0.438 0.438 weight 1.000 ! spec=Expt_1, no=1102, id=904, vol=2.133174e+06
assign (segid "   A" and resid   221 and name   HN) (segid "   A" and resid   221 and name   HA) 2.164 0.585 0.585 weight 1.000 ! spec=Expt_1, no=3444, id=2545, vol=8.916962e+05
assign (segid "   A" and resid   221 and name HG21) (segid "   A" and resid   221 and name   HA) 2.183 0.596 0.596 weight 1.000 ! spec=Expt_3, no=1667, id=3721, vol=4.234872e+06
assign (segid "   A" and resid   221 and name HG12) (segid "   A" and resid   222 and name   HA) 2.463 0.758 0.758 weight 1.000 ! spec=Expt_1, no=2417, id=1815, vol=4.098593e+05
assign (segid "   A" and resid   221 and name   HN) (segid "   A" and resid   222 and name   HN) 2.133 0.568 0.568 weight 1.000 ! spec=Expt_4, no=58, id=4292, vol=3.231418e+07
assign (segid "   A" and resid   221 and name   HA) (segid "   A" and resid   243 and name  HB2) 2.028 0.514 0.514 weight 1.000 ! spec=Expt_3, no=1325, id=3490, vol=6.602877e+06

THR 222
assign (segid "   A" and resid   222 and name   HN) (segid "   A" and resid   221 and name   HB) 2.012 0.506 0.506 weight 1.000 ! spec=Expt_1, no=1111, id=910, vol=1.381333e+06
assign (segid "   A" and resid   222 and name   HN) (segid "   A" and resid   221 and name   HA) 2.634 0.867 0.867 weight 1.000 ! spec=Expt_4, no=269, id=4449, vol=9.094694e+06
assign (segid "   A" and resid   222 and name   HN) (segid "   A" and resid   221 and name HG12) 2.357 0.695 0.695 weight 1.000 ! spec=Expt_4, no=760, id=4826, vol=1.770927e+07
assign (segid "   A" and resid   222 and name  HG1) (segid "   A" and resid   222 and name   HB) 1.869 0.437 0.437 weight 1.000 ! spec=Expt_1, no=166, id=151, vol=2.145560e+06
assign (segid "   A" and resid   222 and name  HG1) (segid "   A" and resid   222 and name   HN) 2.485 0.772 0.772 weight 1.000 ! spec=Expt_1, no=2982, id=2142, vol=3.888401e+05
assign (segid "   A" and resid   222 and name   HN) (segid "   A" and resid   223 and name   HN) 2.111 0.557 0.557 weight 1.000 ! spec=Expt_1, no=1113, id=912, vol=1.033464e+06
assign (segid "   A" and resid   222 and name  HG1) (segid "   A" and resid   223 and name   HA) 2.456 0.754 0.754 weight 1.000 ! spec=Expt_3, no=1728, id=3771, vol=2.091315e+06
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assign (segid " A" and resid 222 and name HA) (segid " A" and resid 225 and name HD21) 2.380 0.708 0.708 weight 1.000 ! spec=Expt_1, no=3519, id=2604, vol=5.036162e+05
assign (segid " A" and resid 222 and name HA) (segid " A" and resid 225 and name HN) 2.734 0.935 0.935 weight 1.000 ! spec=Expt_4, no=1202, id=5041, vol=7.273011e+06

ASN 223
assign (segid " A" and resid 223 and name HD21) (segid " A" and resid 220 and name HA) 2.373 0.704 0.704 weight 1.000 ! spec=Expt_1, no=1418, id=1148, vol=5.128425e+05
assign (segid " A" and resid 223 and name HN) (segid " A" and resid 221 and name HN) 2.935 1.077 1.077 weight 1.000 ! spec=Expt_4, no=915, id=4933, vol=4.751010e+06
assign (segid " A" and resid 223 and name HN) (segid " A" and resid 222 and name HG1) 2.296 0.659 0.659 weight 1.000 ! spec=Expt_1, no=1116, id=915, vol=6.238996e+05
assign (segid " A" and resid 223 and name HN) (segid " A" and resid 223 and name HB1) 2.393 0.716 0.716 weight 1.000 ! spec=Expt_1, no=1118, id=917, vol=4.868532e+05
assign (segid " A" and resid 223 and name HN) (segid " A" and resid 223 and name HA) 2.241 0.628 0.628 weight 1.000 ! spec=Expt_1, no=1121, id=920, vol=7.228280e+05
assign (segid " A" and resid 223 and name HD21) (segid " A" and resid 223 and name HB2) 2.412 0.727 0.727 weight 1.000 ! spec=Expt_1, no=3273, id=2395, vol=4.646141e+05
assign (segid " A" and resid 223 and name HN) (segid " A" and resid 223 and name HD21) 2.631 0.865 0.865 weight 1.000 ! spec=Expt_4, no=1182, id=5035, vol=9.171949e+06
assign (segid " A" and resid 223 and name HN) (segid " A" and resid 224 and name HN) 2.180 0.594 0.594 weight 1.000 ! spec=Expt_1, no=1114, id=913, vol=8.519475e+05
assign (segid " A" and resid 223 and name HA) (segid " A" and resid 226 and name HB1) 2.468 0.761 0.761 weight 1.000 ! spec=Expt_1, no=1726, id=1371, vol=4.051244e+05
assign (segid " A" and resid 223 and name HA) (segid " A" and resid 226 and name HB2) 2.318 0.672 0.672 weight 1.000 ! spec=Expt_3, no=1219, id=3471, vol=2.957270e+06

LEU 224
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 195 and name HA1) 2.714 0.920 0.920 weight 1.000 ! spec=Expt_1, no=2356, id=1786, vol=2.292361e+05
assign (segid " A" and resid 224 and name HA) (segid " A" and resid 195 and name HA2) 2.481 0.769 0.769 weight 1.000 ! spec=Expt_3, no=5147, id=4169, vol=1.968631e+06
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 220 and name HA) 2.946 1.085 1.085 weight 1.000 ! spec=Expt_4, no=1194, id=5038, vol=4.653063e+06
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 222 and name HA) 3.087 1.191 1.191 weight 1.000 ! spec=Expt_4, no=1195, id=5039, vol=3.511152e+06
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 223 and name HB1) 2.431 0.739 0.739 weight 1.000 ! spec=Expt_1, no=1133, id=929, vol=4.435205e+05
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 223 and name HA) 2.571 0.826 0.826 weight 1.000 ! spec=Expt_1, no=1135, id=931, vol=3.169419e+05
assign (segid " A" and resid 224 and name HD21) (segid " A" and resid 224 and name HA) 1.920 0.461 0.461 weight 1.000 ! spec=Expt_1, no=417, id=374, vol=1.825619e+06
assign (segid " A" and resid 224 and name HD13) (segid " A" and resid 224 and name HD21) 1.704 0.363 0.363 weight 1.000 ! spec=Expt_1, no=421, id=378, vol=3.740176e+06
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 224 and name HG) 2.067 0.534 0.534 weight 1.000 ! spec=Expt_1, no=1130, id=926, vol=1.173467e+06
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 224 and name HB2) 2.148 0.577 0.577 weight 1.000 ! spec=Expt_1, no=1131, id=927, vol=9.309429e+05
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 224 and name HA) 2.254 0.635 0.635 weight 1.000 ! spec=Expt_1, no=1134, id=930, vol=6.983278e+05
assign (segid " A" and resid 224 and name HA) (segid " A" and resid 224 and name HB1) 2.593 0.840 0.840 weight 1.000 ! spec=Expt_1, no=1675, id=1333, vol=3.012530e+05
assign (segid " A" and resid 224 and name HD21) (segid " A" and resid 224 and name HB1) 2.409 0.725 0.725 weight 1.000 ! spec=Expt_1, no=1876, id=1482, vol=4.686337e+05
assign (segid " A" and resid 224 and name HG) (segid " A" and resid 224 and name HB2) 2.427 0.736 0.736 weight 1.000 ! spec=Expt_1, no=2171, id=1714, vol=4.482365e+05
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 224 and name HD13) 2.370 0.702 0.702 weight 1.000 ! spec=Expt_1, no=2871, id=2055, vol=5.166297e+05
assign (segid " A" and resid 224 and name HA) (segid " A" and resid 224 and name HG) 2.258 0.638 0.638 weight 1.000 ! spec=Expt_3, no=226, id=2797, vol=3.456759e+06
assign (segid " A" and resid 224 and name HD13) (segid " A" and resid 224 and name HB2) 2.048 0.524 0.524 weight 1.000 ! spec=Expt_3, no=1564, id=3649, vol=6.212659e+06
assign (segid " A" and resid 224 and name HB1) (segid " A" and resid 228 and name HE1) 2.544 0.809 0.809 weight 1.000 ! spec=Expt_1, no=2038, id=1616, vol=3.379342e+05
assign (segid " A" and resid 224 and name HN) (segid " A" and resid 243 and name HB2) 2.679 0.897 0.897 weight 1.000 ! spec=Expt_1, no=2436, id=1825, vol=2.477803e+05

ASN 225
assign (segid " A" and resid 225 and name HD21) (segid " A" and resid 221 and name HG12) 1.979 0.490 0.490 weight 1.000 ! spec=Expt_1, no=2958, id=2124, vol=1.523772e+06
assign (segid " A" and resid 225 and name HN) (segid " A" and resid 224 and name HN) 2.172 0.590 0.590 weight 1.000 ! spec=Expt_1, no=859, id=716, vol=8.707397e+05
assign (segid " A" and resid 225 and name HN) (segid " A" and resid 224 and name HB2) 1.862 0.433 0.433 weight 1.000 ! spec=Expt_1, no=2442, id=1830, vol=2.196619e+06
assign (segid " A" and resid 225 and name HN) (segid " A" and resid 225 and name HA) 2.353 0.692 0.692 weight 1.000 ! spec=Expt_1, no=851, id=710, vol=5.391462e+05
assign (segid " A" and resid 225 and name HN) (segid " A" and resid 225 and name HB1) 2.038 0.519 0.519 weight 1.000 ! spec=Expt_1, no=1145, id=938, vol=1.276246e+06
assign (segid " A" and resid 225 and name HD21) (segid " A" and resid 225 and name HN) 2.410 0.726 0.726 weight 1.000 ! spec=Expt_1, no=2441, id=1829, vol=4.672373e+05
assign (segid " A" and resid 225 and name HD21) (segid " A" and resid 225 and name HB1) 2.196 0.603 0.603 weight 1.000 ! spec=Expt_1, no=3341, id=2450, vol=8.167750e+05
assign (segid " A" and resid 225 and name HB1) (segid " A" and resid 225 and name HA) 2.220 0.616 0.616 weight 1.000 ! spec=Expt_3, no=5154, id=4173, vol=3.836990e+06
assign (segid " A" and resid 225 and name HN) (segid " A" and resid 226 and name HN) 2.072 0.537 0.537 weight 1.000 ! spec=Expt_1, no=1148, id=941, vol=1.156771e+06
assign (segid " A" and resid 225 and name HB1) (segid " A" and resid 226 and name HN) 2.789 0.973 0.973 weight 1.000 ! spec=Expt_1, no=3256, id=2378, vol=1.942801e+05
assign (segid " A" and resid 225 and name HN) (segid " A" and resid 243 and name HB2) 2.419 0.731 0.731 weight 1.000 ! spec=Expt_1, no=1143, id=936, vol=4.571902e+05

ASN 226
assign (segid " A" and resid 226 and name HB2) (segid " A" and resid 199 and name HD22) 2.292 0.657 0.657 weight 1.000 ! spec=Expt_3, no=2633, id=3977, vol=3.163742e+06
assign (segid " A" and resid 226 and name HB2) (segid " A" and resid 215 and name HD21) 2.242 0.628 0.628 weight 1.000 ! spec=Expt_3, no=1660, id=3715, vol=3.613800e+06
assign (segid " A" and resid 226 and name   HN) (segid " A" and resid 222 and name   HA) 2.977 1.108 1.108 weight 1.000 ! spec=Expt_4, no=1211, id=5045, vol=4.366864e+06
assign (segid " A" and resid 226 and name   HN) (segid " A" and resid 223 and name   HA) 2.791 0.974 0.974 weight 1.000 ! spec=Expt_4, no=1210, id=5044, vol=6.432494e+06
assign (segid " A" and resid 226 and name   HN) (segid " A" and resid 225 and name   HA) 2.711 0.919 0.919 weight 1.000 ! spec=Expt_4, no=277, id=4456, vol=7.650621e+06
assign (segid " A" and resid 226 and name HD21) (segid " A" and resid 226 and name HB2) 2.097 0.550 0.550 weight 1.000 ! spec=Expt_1, no=2835, id=2026, vol=1.076458e+06
assign (segid " A" and resid 226 and name   HN) (segid " A" and resid 226 and name   HA) 2.273 0.646 0.646 weight 1.000 ! spec=Expt_4, no=276, id=4455, vol=2.204492e+07
assign (segid " A" and resid 226 and name   HN) (segid " A" and resid 227 and name   HN) 2.157 0.582 0.582 weight 1.000 ! spec=Expt_1, no=1153, id=945, vol=9.076416e+05
assign (segid " A" and resid 226 and name HD21) (segid " A" and resid 230 and name HG21) 2.585 0.835 0.835 weight 1.000 ! spec=Expt_1, no=1368, id=1108, vol=3.070245e+05
assign (segid " A" and resid 226 and name HD22) (segid " A" and resid 230 and name HG21) 2.682 0.899 0.899 weight 1.000 ! spec=Expt_1, no=3019, id=2176, vol=2.458961e+05

ALA 227
assign (segid " A" and resid 227 and name   HN) (segid " A" and resid 199 and name HD22) 2.197 0.604 0.604 weight 1.000 ! spec=Expt_1, no=1162, id=953, vol=8.127848e+05
assign (segid " A" and resid 227 and name   HN) (segid " A" and resid 224 and name   HA) 2.658 0.883 0.883 weight 1.000 ! spec=Expt_1, no=2437, id=1826, vol=2.593951e+05
assign (segid " A" and resid 227 and name   HN) (segid " A" and resid 226 and name HB2) 2.126 0.565 0.565 weight 1.000 ! spec=Expt_1, no=1157, id=949, vol=9.922082e+05
assign (segid " A" and resid 227 and name   HN) (segid " A" and resid 226 and name   HA) 2.716 0.922 0.922 weight 1.000 ! spec=Expt_4, no=279, id=4458, vol=7.569674e+06
assign (segid " A" and resid 227 and name   HN) (segid " A" and resid 226 and name HD21) 2.851 1.016 1.016 weight 1.000 ! spec=Expt_4, no=1214, id=5046, vol=5.664282e+06
assign (segid " A" and resid 227 and name   HN) (segid " A" and resid 227 and name HB2) 1.877 0.440 0.440 weight 1.000 ! spec=Expt_1, no=1155, id=947, vol=2.092885e+06
assign (segid " A" and resid 227 and name   HN) (segid " A" and resid 227 and name   HA) 2.222 0.617 0.617 weight 1.000 ! spec=Expt_1, no=1160, id=952, vol=7.603316e+05
assign (segid " A" and resid 227 and name   HA) (segid " A" and resid 227 and name HB2) 1.750 0.383 0.383 weight 1.000 ! spec=Expt_3, no=233, id=2802, vol=1.597180e+07
assign (segid " A" and resid 227 and name HB2) (segid " A" and resid 228 and name HE1) 2.354 0.693 0.693 weight 1.000 ! spec=Expt_3, no=997, id=3339, vol=2.694773e+06
assign (segid " A" and resid 227 and name   HA) (segid " A" and resid 228 and name   HN) 2.822 0.996 0.996 weight 1.000 ! spec=Expt_4, no=282, id=4460, vol=6.015960e+06
assign (segid " A" and resid 227 and name   HA) (segid " A" and resid 230 and name   HB) 2.208 0.609 0.609 weight 1.000 ! spec=Expt_3, no=1754, id=3791, vol=3.957493e+06

PHE 228
assign (segid " A" and resid 228 and name HE1) (segid " A" and resid 198 and name HD1) 2.404 0.722 0.722 weight 1.000 ! spec=Expt_1, no=1429, id=1157, vol=4.740302e+05
assign (segid " A" and resid 228 and name   HZ) (segid " A" and resid 224 and name HD21) 2.127 0.565 0.565 weight 1.000 ! spec=Expt_1, no=1477, id=1193, vol=9.896883e+05
assign (segid " A" and resid 228 and name HE1) (segid " A" and resid 224 and name HD21) 2.308 0.666 0.666 weight 1.000 ! spec=Expt_3, no=2458, id=3936, vol=3.034755e+06
assign (segid " A" and resid 228 and name HD1) (segid " A" and resid 225 and name   HA) 2.136 0.570 0.570 weight 1.000 ! spec=Expt_3, no=910, id=3283, vol=4.832551e+06
assign (segid " A" and resid 228 and name HB1) (segid " A" and resid 225 and name   HA) 2.042 0.521 0.521 weight 1.000 ! spec=Expt_3, no=1188, id=3451, vol=6.325598e+06
assign (segid " A" and resid 228 and name   HN) (segid " A" and resid 225 and name   HA) 2.557 0.817 0.817 weight 1.000 ! spec=Expt_4, no=1231, id=5053, vol=1.086970e+07
assign (segid " A" and resid 228 and name   HN) (segid " A" and resid 226 and name   HN) 2.860 1.023 1.023 weight 1.000 ! spec=Expt_4, no=1225, id=5051, vol=5.548474e+06
assign (segid " A" and resid 228 and name   HN) (segid " A" and resid 227 and name   HN) 2.109 0.556 0.556 weight 1.000 ! spec=Expt_1, no=597, id=531, vol=1.040013e+06
assign (segid " A" and resid 228 and name HD1) (segid " A" and resid 227 and name HB2) 2.118 0.561 0.561 weight 1.000 ! spec=Expt_3, no=1025, id=3361, vol=5.087668e+06
assign (segid " A" and resid 228 and name   HN) (segid " A" and resid 227 and name HB2) 2.185 0.597 0.597 weight 1.000 ! spec=Expt_4, no=528, id=4662, vol=2.792264e+07
assign (segid " A" and resid 228 and name   HN) (segid " A" and resid 228 and name HB1) 2.268 0.643 0.643 weight 1.000 ! spec=Expt_1, no=601, id=534, vol=6.730737e+05
assign (segid " A" and resid 228 and name   HN) (segid " A" and resid 228 and name HD1) 2.159 0.583 0.583 weight 1.000 ! spec=Expt_1, no=846, id=706, vol=9.033846e+05
assign (segid " A" and resid 228 and name   HZ) (segid " A" and resid 228 and name HE1) 1.788 0.399 0.399 weight 1.000 ! spec=Expt_3, no=783, id=3227, vol=1.406239e+07
assign (segid " A" and resid 228 and name HD1) (segid " A" and resid 228 and name   HA) 2.126 0.565 0.565 weight 1.000 ! spec=Expt_3, no=829, id=3252, vol=4.965689e+06
assign (segid " A" and resid 228 and name HB1) (segid " A" and resid 228 and name HB2) 1.961 0.481 0.481 weight 1.000 ! spec=Expt_3, no=1210, id=3464, vol=8.055478e+06
assign (segid " A" and resid 228 and name HD1) (segid " A" and resid 228 and name HB2) 2.184 0.596 0.596 weight 1.000 ! spec=Expt_3, no=2668, id=3982, vol=4.224046e+06
assign (segid " A" and resid 228 and name HE1) (segid " A" and resid 228 and name HD1) 1.705 0.363 0.363 weight 1.000 ! spec=Expt_3, no=5322, id=4240, vol=1.868406e+07
assign (segid " A" and resid 228 and name   HN) (segid " A" and resid 228 and name   HA) 2.521 0.794 0.794 weight 1.000 ! spec=Expt_4, no=280, id=4459, vol=1.183981e+07
assign (segid " A" and resid 228 and name   HN) (segid " A" and resid 229 and name   HN) 2.051 0.526 0.526 weight 1.000 ! spec=Expt_1, no=1173, id=963, vol=1.230109e+06
assign (segid " A" and resid 228 and name   HZ) (segid " A" and resid 241 and name HB1) 2.441 0.745 0.745 weight 1.000 ! spec=Expt_1, no=1523, id=1229, vol=4.324670e+05
assign (segid " A" and resid 228 and name HE1) (segid " A" and resid 241 and name HB2) 2.498 0.780 0.780 weight 1.000 ! spec=Expt_3, no=1096, id=3408, vol=1.888974e+06

assign (segid " A" and resid 228 and name HE1) (segid " A" and resid 241 and name HD13) 2.179 0.593 0.593 weight 1.000 ! spec=Expt_3, no=2467, id=3938, vol=4.286958e+06
assign (segid " A" and resid 228 and name HE1) (segid " A" and resid 243 and name HA) 2.165 0.586 0.586 weight 1.000 ! spec=Expt_1, no=1537, id=1241, vol=8.890419e+05
assign (segid " A" and resid 228 and name HZ) (segid " A" and resid 243 and name HA) 2.779 0.965 0.965 weight 1.000 ! spec=Expt_1, no=2357, id=1787, vol=1.987819e+05
assign (segid " A" and resid 228 and name HE1) (segid " A" and resid 243 and name HB2) 2.048 0.524 0.524 weight 1.000 ! spec=Expt_3, no=996, id=3338, vol=6.221622e+06
assign (segid " A" and resid 228 and name HD1) (segid " A" and resid 243 and name HB2) 2.271 0.645 0.645 weight 1.000 ! spec=Expt_3, no=1023, id=3359, vol=3.345241e+06
assign (segid " A" and resid 228 and name HZ) (segid " A" and resid 246 and name HD11) 2.066 0.534 0.534 weight 1.000 ! spec=Expt_1, no=1479, id=1194, vol=1.176585e+06
assign (segid " A" and resid 228 and name HE1) (segid " A" and resid 246 and name HD11) 2.127 0.566 0.566 weight 1.000 ! spec=Expt_3, no=1008, id=3348, vol=4.954091e+06

GLU 229
assign (segid " A" and resid 229 and name HG1) (segid " A" and resid 226 and name HA) 2.694 0.907 0.907 weight 1.000 ! spec=Expt_1, no=2404, id=1807, vol=2.396462e+05
assign (segid " A" and resid 229 and name HB1) (segid " A" and resid 226 and name HA) 2.580 0.832 0.832 weight 1.000 ! spec=Expt_1, no=2458, id=1840, vol=3.105838e+05
assign (segid " A" and resid 229 and name HN) (segid " A" and resid 226 and name HA) 3.089 1.192 1.192 weight 1.000 ! spec=Expt_4, no=1236, id=5054, vol=3.501388e+06
assign (segid " A" and resid 229 and name HN) (segid " A" and resid 228 and name HB1) 2.506 0.785 0.785 weight 1.000 ! spec=Expt_1, no=1171, id=961, vol=3.697058e+05
assign (segid " A" and resid 229 and name HN) (segid " A" and resid 228 and name HA) 2.981 1.111 1.111 weight 1.000 ! spec=Expt_4, no=284, id=4462, vol=4.332466e+06
assign (segid " A" and resid 229 and name HN) (segid " A" and resid 229 and name HB1) 2.028 0.514 0.514 weight 1.000 ! spec=Expt_1, no=1168, id=958, vol=1.313769e+06
assign (segid " A" and resid 229 and name HG1) (segid " A" and resid 229 and name HN) 2.259 0.638 0.638 weight 1.000 ! spec=Expt_1, no=3226, id=2353, vol=6.895316e+05
assign (segid " A" and resid 229 and name HB1) (segid " A" and resid 229 and name HB2) 1.807 0.408 0.408 weight 1.000 ! spec=Expt_3, no=1623, id=3687, vol=1.318931e+07
assign (segid " A" and resid 229 and name HN) (segid " A" and resid 229 and name HA) 2.274 0.646 0.646 weight 1.000 ! spec=Expt_4, no=283, id=4461, vol=2.198193e+07
assign (segid " A" and resid 229 and name HN) (segid " A" and resid 230 and name HG21) 2.786 0.971 0.971 weight 1.000 ! spec=Expt_4, no=1240, id=5055, vol=6.492672e+06

VAL 230
assign (segid " A" and resid 230 and name HG12) (segid " A" and resid 209 and name HD13) 2.116 0.560 0.560 weight 1.000 ! spec=Expt_3, no=1537, id=3627, vol=5.103935e+06
assign (segid " A" and resid 230 and name HN) (segid " A" and resid 229 and name HN) 2.066 0.534 0.534 weight 1.000 ! spec=Expt_1, no=1175, id=965, vol=1.176420e+06
assign (segid " A" and resid 230 and name HN) (segid " A" and resid 229 and name HG2) 2.558 0.818 0.818 weight 1.000 ! spec=Expt_1, no=1180, id=970, vol=3.269173e+05
assign (segid " A" and resid 230 and name HN) (segid " A" and resid 229 and name HA) 2.688 0.903 0.903 weight 1.000 ! spec=Expt_4, no=286, id=4464, vol=8.064188e+06
assign (segid " A" and resid 230 and name HN) (segid " A" and resid 230 and name HG21) 1.875 0.439 0.439 weight 1.000 ! spec=Expt_1, no=1177, id=967, vol=2.107107e+06
assign (segid " A" and resid 230 and name HN) (segid " A" and resid 230 and name HB) 1.890 0.447 0.447 weight 1.000 ! spec=Expt_1, no=1179, id=969, vol=2.005865e+06
assign (segid " A" and resid 230 and name HN) (segid " A" and resid 230 and name HA) 2.227 0.620 0.620 weight 1.000 ! spec=Expt_1, no=3488, id=2584, vol=7.509979e+05
assign (segid " A" and resid 230 and name HN) (segid " A" and resid 231 and name HN) 2.143 0.574 0.574 weight 1.000 ! spec=Expt_1, no=1182, id=972, vol=9.455092e+05
assign (segid " A" and resid 230 and name HA) (segid " A" and resid 234 and name HD1) 1.944 0.473 0.473 weight 1.000 ! spec=Expt_1, no=3503, id=2597, vol=1.693922e+06

ALA 231
assign (segid " A" and resid 231 and name HN) (segid " A" and resid 230 and name HB) 2.140 0.572 0.572 weight 1.000 ! spec=Expt_1, no=1187, id=975, vol=9.534447e+05
assign (segid " A" and resid 231 and name HN) (segid " A" and resid 230 and name HG12) 2.276 0.647 0.647 weight 1.000 ! spec=Expt_1, no=2874, id=2057, vol=6.590722e+05
assign (segid " A" and resid 231 and name HA) (segid " A" and resid 231 and name HB2) 1.925 0.463 0.463 weight 1.000 ! spec=Expt_1, no=194, id=179, vol=1.796030e+06
assign (segid " A" and resid 231 and name HN) (segid " A" and resid 231 and name HB2) 1.920 0.461 0.461 weight 1.000 ! spec=Expt_1, no=1186, id=974, vol=1.828798e+06
assign (segid " A" and resid 231 and name HN) (segid " A" and resid 231 and name HA) 2.275 0.647 0.647 weight 1.000 ! spec=Expt_1, no=3485, id=2581, vol=6.599278e+05
assign (segid " A" and resid 231 and name HN) (segid " A" and resid 232 and name HN) 2.256 0.636 0.636 weight 1.000 ! spec=Expt_1, no=1191, id=978, vol=6.939334e+05
assign (segid " A" and resid 231 and name HA) (segid " A" and resid 235 and name HG) 2.265 0.641 0.641 weight 1.000 ! spec=Expt_1, no=2013, id=1597, vol=6.785189e+05
assign (segid " A" and resid 231 and name HA) (segid " A" and resid 235 and name HB1) 2.377 0.706 0.706 weight 1.000 ! spec=Expt_1, no=2475, id=1845, vol=5.073019e+05
assign (segid " A" and resid 231 and name HA) (segid " A" and resid 235 and name HD13) 1.916 0.459 0.459 weight 1.000 ! spec=Expt_3, no=1416, id=3544, vol=9.271201e+06
assign (segid " A" and resid 231 and name HB2) (segid " A" and resid 235 and name HD13) 1.842 0.424 0.424 weight 1.000 ! spec=Expt_3, no=1478, id=3587, vol=1.175169e+07
assign (segid " A" and resid 231 and name HN) (segid " A" and resid 235 and name HD13) 2.923 1.068 1.068 weight 1.000 ! spec=Expt_4, no=1248, id=5056, vol=4.874642e+06
assign (segid " A" and resid 231 and name HB2) (segid " A" and resid 237 and name HB) 1.775 0.394 0.394 weight 1.000 ! spec=Expt_3, no=1472, id=3582, vol=1.465886e+07
assign (segid " A" and resid 231 and name HB2) (segid " A" and resid 237 and name HD11) 2.048 0.524 0.524 weight 1.000 ! spec=Expt_3, no=1540, id=3630, vol=6.211665e+06
assign (segid " A" and resid 231 and name HB2) (segid " A" and resid 237 and name HG22) 2.041 0.521 0.521 weight 1.000 ! spec=Expt_3, no=1593, id=3668, vol=6.348390e+06
assign (segid " A" and resid 231 and name HA) (segid " A" and resid 237 and name HD11) 2.510 0.787 0.787 weight 1.000 ! spec=Expt_3, no=4826, id=4128, vol=1.835775e+06

GLU 232
assign (segid " A" and resid 232 and name HN) (segid " A" and resid 231 and name HB2) 2.061 0.531 0.531 weight 1.000 ! spec=Expt_1, no=1194, id=981, vol=1.194517e+06
assign (segid " A" and resid 232 and name HN) (segid " A" and resid 232 and name HA) 2.349 0.689 0.689 weight 1.000 ! spec=Expt_1, no=1206, id=990, vol=5.454599e+05
assign (segid " A" and resid 232 and name HN) (segid " A" and resid 232 and name HB1) 2.291 0.656 0.656 weight 1.000 ! spec=Expt_1, no=3176, id=2310, vol=6.334976e+05
assign (segid " A" and resid 232 and name HN) (segid " A" and resid 233 and name HN) 2.101 0.552 0.552 weight 1.000 ! spec=Expt_1, no=1209, id=992, vol=1.063373e+06
assign (segid " A" and resid 232 and name HB2) (segid " A" and resid 238 and name HA) 2.487 0.773 0.773 weight 1.000 ! spec=Expt_3, no=5262, id=4211, vol=1.939302e+06

LYS 233
assign (segid " A" and resid 233 and name HN) (segid " A" and resid 232 and name HA) 2.845 1.012 1.012 weight 1.000 ! spec=Expt_4, no=291, id=4467, vol=5.735670e+06
assign (segid " A" and resid 233 and name HN) (segid " A" and resid 232 and name HB1) 2.278 0.649 0.649 weight 1.000 ! spec=Expt_4, no=545, id=4676, vol=2.174349e+07
assign (segid " A" and resid 233 and name HN) (segid " A" and resid 233 and name HG2) 2.426 0.735 0.735 weight 1.000 ! spec=Expt_1, no=1212, id=994, vol=4.494300e+05
assign (segid " A" and resid 233 and name HN) (segid " A" and resid 233 and name HB1) 1.995 0.498 0.498 weight 1.000 ! spec=Expt_1, no=1214, id=996, vol=1.450389e+06
assign (segid " A" and resid 233 and name HE2) (segid " A" and resid 233 and name HD1) 2.068 0.535 0.535 weight 1.000 ! spec=Expt_1, no=1742, id=1382, vol=1.168703e+06
assign (segid " A" and resid 233 and name HB1) (segid " A" and resid 233 and name HB2) 1.820 0.414 0.414 weight 1.000 ! spec=Expt_1, no=1892, id=1497, vol=2.515502e+06
assign (segid " A" and resid 233 and name HG1) (segid " A" and resid 233 and name HB2) 2.116 0.560 0.560 weight 1.000 ! spec=Expt_3, no=1589, id=3664, vol=5.111833e+06
assign (segid " A" and resid 233 and name HB1) (segid " A" and resid 233 and name HD1) 2.099 0.551 0.551 weight 1.000 ! spec=Expt_3, no=1615, id=3681, vol=5.358711e+06
assign (segid " A" and resid 233 and name HN) (segid " A" and resid 233 and name HA) 2.251 0.634 0.634 weight 1.000 ! spec=Expt_4, no=290, id=4466, vol=2.334359e+07
assign (segid " A" and resid 233 and name HB1) (segid " A" and resid 234 and name HD1) 2.001 0.500 0.500 weight 1.000 ! spec=Expt_3, no=2132, id=3860, vol=7.148118e+06
assign (segid " A" and resid 233 and name HN) (segid " A" and resid 234 and name HD1) 2.994 1.120 1.120 weight 1.000 ! spec=Expt_4, no=1258, id=5059, vol=4.221632e+06
assign (segid " A" and resid 233 and name HN) (segid " A" and resid 235 and name HN) 2.998 1.124 1.124 weight 1.000 ! spec=Expt_4, no=1257, id=5058, vol=4.183645e+06

TYR 234
assign (segid " A" and resid 234 and name HB1) (segid " A" and resid 209 and name HD21) 2.269 0.643 0.643 weight 1.000 ! spec=Expt_3, no=1545, id=3634, vol=3.363415e+06
assign (segid " A" and resid 234 and name HD1) (segid " A" and resid 209 and name HD21) 2.297 0.660 0.660 weight 1.000 ! spec=Expt_3, no=2140, id=3862, vol=3.121080e+06
assign (segid " A" and resid 234 and name HD1) (segid " A" and resid 209 and name HD13) 2.276 0.647 0.647 weight 1.000 ! spec=Expt_3, no=2505, id=3943, vol=3.300378e+06
assign (segid " A" and resid 234 and name HD1) (segid " A" and resid 230 and name HG12) 1.873 0.439 0.439 weight 1.000 ! spec=Expt_1, no=1513, id=1223, vol=2.117452e+06
assign (segid " A" and resid 234 and name HE1) (segid " A" and resid 230 and name HA) 2.048 0.524 0.524 weight 1.000 ! spec=Expt_3, no=3161, id=4047, vol=6.210023e+06
assign (segid " A" and resid 234 and name HN) (segid " A" and resid 230 and name HG12) 2.713 0.920 0.920 weight 1.000 ! spec=Expt_4, no=777, id=4837, vol=7.622020e+06
assign (segid " A" and resid 234 and name HN) (segid " A" and resid 230 and name HA) 2.602 0.846 0.846 weight 1.000 ! spec=Expt_4, no=1604, id=5183, vol=9.792943e+06
assign (segid " A" and resid 234 and name HN) (segid " A" and resid 233 and name HN) 2.162 0.584 0.584 weight 1.000 ! spec=Expt_1, no=563, id=502, vol=8.952366e+05
assign (segid " A" and resid 234 and name HD1) (segid " A" and resid 233 and name HB2) 2.382 0.709 0.709 weight 1.000 ! spec=Expt_3, no=979, id=3325, vol=2.512560e+06
assign (segid " A" and resid 234 and name HE1) (segid " A" and resid 233 and name HD1) 2.101 0.552 0.552 weight 1.000 ! spec=Expt_3, no=990, id=3334, vol=5.335492e+06
assign (segid " A" and resid 234 and name HN) (segid " A" and resid 234 and name HB1) 2.215 0.613 0.613 weight 1.000 ! spec=Expt_1, no=1229, id=1007, vol=7.741390e+05
assign (segid " A" and resid 234 and name HN) (segid " A" and resid 234 and name HD1) 2.145 0.575 0.575 weight 1.000 ! spec=Expt_1, no=1439, id=1163, vol=9.393369e+05
assign (segid " A" and resid 234 and name HD1) (segid " A" and resid 234 and name HB2) 2.120 0.562 0.562 weight 1.000 ! spec=Expt_3, no=2627, id=3975, vol=5.053220e+06
assign (segid " A" and resid 234 and name HD1) (segid " A" and resid 234 and name HB1) 2.160 0.583 0.583 weight 1.000 ! spec=Expt_3, no=2628, id=3976, vol=4.519867e+06
assign (segid " A" and resid 234 and name HN) (segid " A" and resid 234 and name HA) 2.663 0.886 0.886 weight 1.000 ! spec=Expt_4, no=292, id=4468, vol=8.525703e+06
assign (segid " A" and resid 234 and name HN) (segid " A" and resid 234 and name HE1) 2.981 1.111 1.111 weight 1.000 ! spec=Expt_4, no=1265, id=5061, vol=4.329342e+06
assign (segid " A" and resid 234 and name HN) (segid " A" and resid 235 and name HN) 2.044 0.522 0.522 weight 1.000 ! spec=Expt_1, no=561, id=500, vol=1.256441e+06
assign (segid " A" and resid 234 and name HB1) (segid " A" and resid 235 and name HD21) 2.433 0.740 0.740 weight 1.000 ! spec=Expt_3, no=2041, id=3830, vol=2.214250e+06

LEU 235
assign (segid " A" and resid 235 and name HD21) (segid " A" and resid 209 and name HD21) 2.019 0.509 0.509 weight 1.000 ! spec=Expt_3, no=1553, id=3641, vol=6.781923e+06
assign (segid " A" and resid 235 and name HN) (segid " A" and resid 231 and name HA) 2.540 0.807 0.807 weight 1.000 ! spec=Expt_1, no=1072, id=879, vol=3.406995e+05
assign (segid " A" and resid 235 and name HN) (segid " A" and resid 234 and name HB1) 2.453 0.752 0.752 weight 1.000 ! spec=Expt_3, no=960, id=3311, vol=2.103030e+06
assign (segid " A" and resid 235 and name HN) (segid " A" and resid 234 and name HA) 2.752 0.947 0.947 weight 1.000 ! spec=Expt_4, no=295, id=4470, vol=6.994183e+06
assign (segid " A" and resid 235 and name HN) (segid " A" and resid 235 and name HG) 2.352 0.692 0.692 weight 1.000 ! spec=Expt_1, no=3051, id=2206, vol=5.402538e+05
assign (segid " A" and resid 235 and name HD13) (segid " A" and resid 235 and name HB1) 1.981 0.490 0.490 weight 1.000 ! spec=Expt_3, no=1460, id=3571, vol=7.593924e+06

assign (segid " A" and resid 235 and name HN) (segid " A" and resid 235 and name HA) 2.752 0.947 0.947 weight 1.000 ! spec=Expt_4, no=294, id=4469, vol=6.994183e+06
assign (segid " A" and resid 235 and name HB1) (segid " A" and resid 237 and name HN) 2.520 0.794 0.794 weight 1.000 ! spec=Expt_1, no=3079, id=2230, vol=3.576850e+05
assign (segid " A" and resid 235 and name HD13) (segid " A" and resid 237 and name HD11) 1.793 0.402 0.402 weight 1.000 ! spec=Expt_3, no=417, id=2941, vol=1.381971e+07

ASP 236
assign (segid " A" and resid 236 and name HN) (segid " A" and resid 235 and name HA) 3.097 1.199 1.199 weight 1.000 ! spec=Expt_4, no=299, id=4471, vol=3.444258e+06
assign (segid " A" and resid 236 and name HN) (segid " A" and resid 236 and name HB1) 2.433 0.740 0.740 weight 1.000 ! spec=Expt_1, no=1246, id=1018, vol=4.410022e+05

ILE 237
assign (segid " A" and resid 237 and name HN) (segid " A" and resid 231 and name HB2) 2.713 0.920 0.920 weight 1.000 ! spec=Expt_1, no=2509, id=1857, vol=2.297440e+05
assign (segid " A" and resid 237 and name HN) (segid " A" and resid 232 and name HA) 2.107 0.555 0.555 weight 1.000 ! spec=Expt_1, no=1251, id=1020, vol=1.046854e+06
assign (segid " A" and resid 237 and name HD11) (segid " A" and resid 235 and name HB1) 2.423 0.734 0.734 weight 1.000 ! spec=Expt_1, no=1955, id=1547, vol=4.521809e+05
assign (segid " A" and resid 237 and name HG11) (segid " A" and resid 235 and name HD13) 2.341 0.685 0.685 weight 1.000 ! spec=Expt_1, no=3490, id=2586, vol=5.556021e+05
assign (segid " A" and resid 237 and name HN) (segid " A" and resid 236 and name HA) 2.163 0.585 0.585 weight 1.000 ! spec=Expt_4, no=300, id=4472, vol=2.966799e+07
assign (segid " A" and resid 237 and name HN) (segid " A" and resid 237 and name HG11) 2.412 0.727 0.727 weight 1.000 ! spec=Expt_1, no=1249, id=1019, vol=4.652615e+05
assign (segid " A" and resid 237 and name HG11) (segid " A" and resid 237 and name HG12) 1.920 0.461 0.461 weight 1.000 ! spec=Expt_1, no=1788, id=1422, vol=1.828153e+06
assign (segid " A" and resid 237 and name HG11) (segid " A" and resid 237 and name HG22) 2.426 0.736 0.736 weight 1.000 ! spec=Expt_1, no=1836, id=1460, vol=4.486842e+05
assign (segid " A" and resid 237 and name HN) (segid " A" and resid 237 and name HB) 2.187 0.598 0.598 weight 1.000 ! spec=Expt_1, no=2500, id=1856, vol=8.366513e+05
assign (segid " A" and resid 237 and name HG22) (segid " A" and resid 237 and name HB) 1.973 0.487 0.487 weight 1.000 ! spec=Expt_3, no=414, id=2938, vol=7.780030e+06
assign (segid " A" and resid 237 and name HA) (segid " A" and resid 237 and name HG22) 2.008 0.504 0.504 weight 1.000 ! spec=Expt_3, no=415, id=2939, vol=7.004070e+06
assign (segid " A" and resid 237 and name HD11) (segid " A" and resid 237 and name HG22) 1.843 0.425 0.425 weight 1.000 ! spec=Expt_3, no=447, id=2967, vol=1.170608e+07
assign (segid " A" and resid 237 and name HD11) (segid " A" and resid 237 and name HB) 2.154 0.580 0.580 weight 1.000 ! spec=Expt_3, no=448, id=2968, vol=4.596389e+06
assign (segid " A" and resid 237 and name HG22) (segid " A" and resid 237 and name HG12) 2.112 0.558 0.558 weight 1.000 ! spec=Expt_3, no=1526, id=3621, vol=5.161578e+06
assign (segid " A" and resid 237 and name HA) (segid " A" and resid 237 and name HG12) 2.404 0.722 0.722 weight 1.000 ! spec=Expt_3, no=5233, id=4195, vol=2.376605e+06
assign (segid " A" and resid 237 and name HN) (segid " A" and resid 238 and name HD2) 2.896 1.049 1.049 weight 1.000 ! spec=Expt_4, no=1607, id=5186, vol=5.148906e+06
assign (segid " A" and resid 237 and name HG22) (segid " A" and resid 260 and name HD1) 2.201 0.606 0.606 weight 1.000 ! spec=Expt_3, no=2037, id=3827, vol=4.030954e+06
assign (segid " A" and resid 237 and name HD11) (segid " A" and resid 263 and name HB2) 2.311 0.667 0.667 weight 1.000 ! spec=Expt_3, no=1385, id=3525, vol=3.013299e+06
assign (segid " A" and resid 237 and name HD11) (segid " A" and resid 263 and name HB1) 2.380 0.708 0.708 weight 1.000 ! spec=Expt_3, no=2007, id=3809, vol=2.520932e+06
assign (segid " A" and resid 237 and name HG22) (segid " A" and resid 264 and name HE1) 2.429 0.737 0.737 weight 1.000 ! spec=Expt_3, no=1083, id=3399, vol=2.234313e+06

PRO 238
assign (segid " A" and resid 238 and name HD1) (segid " A" and resid 238 and name HB1) 2.483 0.770 0.770 weight 1.000 ! spec=Expt_1, no=2633, id=1929, vol=3.909428e+05
assign (segid " A" and resid 238 and name HA) (segid " A" and resid 238 and name HB2) 1.877 0.440 0.440 weight 1.000 ! spec=Expt_3, no=275, id=2837, vol=1.049717e+07
assign (segid " A" and resid 238 and name HG2) (segid " A" and resid 238 and name HA) 2.398 0.719 0.719 weight 1.000 ! spec=Expt_3, no=283, id=2842, vol=2.414132e+06
assign (segid " A" and resid 238 and name HG1) (segid " A" and resid 238 and name HD1) 2.192 0.601 0.601 weight 1.000 ! spec=Expt_3, no=1332, id=3493, vol=4.133476e+06

LYS 239
assign (segid " A" and resid 239 and name HN) (segid " A" and resid 238 and name HB1) 2.495 0.778 0.778 weight 1.000 ! spec=Expt_1, no=717, id=612, vol=3.790412e+05
assign (segid " A" and resid 239 and name HN) (segid " A" and resid 238 and name HA) 1.846 0.426 0.426 weight 1.000 ! spec=Expt_1, no=1260, id=1028, vol=2.310550e+06
assign (segid " A" and resid 239 and name HN) (segid " A" and resid 239 and name HG1) 2.272 0.645 0.645 weight 1.000 ! spec=Expt_1, no=1256, id=1024, vol=6.658807e+05
assign (segid " A" and resid 239 and name HE2) (segid " A" and resid 239 and name HG2) 2.317 0.671 0.671 weight 1.000 ! spec=Expt_3, no=4533, id=4086, vol=2.962728e+06
assign (segid " A" and resid 239 and name HN) (segid " A" and resid 239 and name HA) 2.376 0.705 0.705 weight 1.000 ! spec=Expt_4, no=302, id=4474, vol=1.690754e+07
assign (segid " A" and resid 239 and name HN) (segid " A" and resid 240 and name HN) 3.160 1.248 1.248 weight 1.000 ! spec=Expt_4, no=1291, id=5066, vol=3.050968e+06

MET 240
assign (segid " A" and resid 240 and name HN) (segid " A" and resid 239 and name HA) 1.940 0.470 0.470 weight 1.000 ! spec=Expt_1, no=3500, id=2595, vol=1.718282e+06
assign (segid " A" and resid 240 and name HN) (segid " A" and resid 240 and name HG1) 2.675 0.894 0.894 weight 1.000 ! spec=Expt_1, no=1269, id=1036, vol=2.497974e+05
assign (segid " A" and resid 240 and name HB1) (segid " A" and resid 240 and name HG1) 2.229 0.621 0.621 weight 1.000 ! spec=Expt_1, no=1746, id=1386, vol=7.459487e+05

assign (segid " A" and resid 240 and name HB1) (segid " A" and resid 240 and name HB2) 1.648 0.340 0.340 weight 1.000 ! spec=Expt_3, no=1609, id=3675, vol=2.287423e+07
assign (segid " A" and resid 240 and name HA) (segid " A" and resid 240 and name HG2) 2.074 0.537 0.537 weight 1.000 ! spec=Expt_3, no=1753, id=3790, vol=5.771033e+06
assign (segid " A" and resid 240 and name HN) (segid " A" and resid 240 and name HA) 2.392 0.715 0.715 weight 1.000 ! spec=Expt_4, no=303, id=4475, vol=1.620941e+07
assign (segid " A" and resid 240 and name HN) (segid " A" and resid 240 and name HB1) 2.337 0.682 0.682 weight 1.000 ! spec=Expt_4, no=1635, id=5201, vol=1.867553e+07
assign (segid " A" and resid 240 and name HN) (segid " A" and resid 241 and name HN) 1.950 0.475 0.475 weight 1.000 ! spec=Expt_1, no=1262, id=1030, vol=1.664992e+06
assign (segid " A" and resid 240 and name HN) (segid " A" and resid 260 and name HE1) 2.466 0.760 0.760 weight 1.000 ! spec=Expt_1, no=1273, id=1039, vol=4.073640e+05
assign (segid " A" and resid 240 and name HG1) (segid " A" and resid 260 and name HE1) 2.653 0.880 0.880 weight 1.000 ! spec=Expt_1, no=2083, id=1646, vol=2.622381e+05

LEU 241
assign (segid " A" and resid 241 and name HD13) (segid " A" and resid 198 and name HE1) 2.106 0.554 0.554 weight 1.000 ! spec=Expt_3, no=1075, id=3395, vol=5.256031e+06
assign (segid " A" and resid 241 and name HN) (segid " A" and resid 240 and name HB1) 2.516 0.791 0.791 weight 1.000 ! spec=Expt_1, no=2352, id=1783, vol=3.609132e+05
assign (segid " A" and resid 241 and name HN) (segid " A" and resid 241 and name HB2) 1.969 0.485 0.485 weight 1.000 ! spec=Expt_1, no=1501, id=1212, vol=1.571079e+06
assign (segid " A" and resid 241 and name HA) (segid " A" and resid 241 and name HB1) 2.111 0.557 0.557 weight 1.000 ! spec=Expt_1, no=1627, id=1306, vol=1.034678e+06
assign (segid " A" and resid 241 and name HB1) (segid " A" and resid 241 and name HB2) 1.850 0.428 0.428 weight 1.000 ! spec=Expt_1, no=2169, id=1713, vol=2.280533e+06
assign (segid " A" and resid 241 and name HN) (segid " A" and resid 241 and name HA) 2.410 0.726 0.726 weight 1.000 ! spec=Expt_1, no=3527, id=2611, vol=4.669100e+05
assign (segid " A" and resid 241 and name HA) (segid " A" and resid 241 and name HD21) 1.721 0.370 0.370 weight 1.000 ! spec=Expt_3, no=1353, id=3506, vol=1.766051e+07
assign (segid " A" and resid 241 and name HN) (segid " A" and resid 260 and name HE1) 2.989 1.117 1.117 weight 1.000 ! spec=Expt_4, no=1300, id=5068, vol=4.262032e+06

ASP 242
assign (segid " A" and resid 242 and name HN) (segid " A" and resid 241 and name HB1) 2.278 0.649 0.649 weight 1.000 ! spec=Expt_1, no=1219, id=999, vol=6.548405e+05
assign (segid " A" and resid 242 and name HN) (segid " A" and resid 241 and name HA) 1.885 0.444 0.444 weight 1.000 ! spec=Expt_1, no=3479, id=2577, vol=2.041071e+06
assign (segid " A" and resid 242 and name HN) (segid " A" and resid 242 and name HB2) 2.090 0.546 0.546 weight 1.000 ! spec=Expt_1, no=1228, id=1006, vol=1.097840e+06
assign (segid " A" and resid 242 and name HN) (segid " A" and resid 242 and name HA) 2.547 0.811 0.811 weight 1.000 ! spec=Expt_4, no=306, id=4477, vol=1.114168e+07
assign (segid " A" and resid 242 and name HN) (segid " A" and resid 243 and name HN) 3.078 1.185 1.185 weight 1.000 ! spec=Expt_4, no=95, id=4321, vol=3.571385e+06

ALA 243
assign (segid " A" and resid 243 and name HB2) (segid " A" and resid 221 and name HG12) 1.880 0.442 0.442 weight 1.000 ! spec=Expt_3, no=1574, id=3658, vol=1.040581e+07
assign (segid " A" and resid 243 and name HB2) (segid " A" and resid 224 and name HB2) 2.245 0.630 0.630 weight 1.000 ! spec=Expt_1, no=1815, id=1444, vol=7.143706e+05
assign (segid " A" and resid 243 and name HA) (segid " A" and resid 224 and name HD13) 1.897 0.450 0.450 weight 1.000 ! spec=Expt_3, no=1415, id=3543, vol=9.852239e+06
assign (segid " A" and resid 243 and name HB2) (segid " A" and resid 224 and name HD13) 1.813 0.411 0.411 weight 1.000 ! spec=Expt_3, no=1454, id=3567, vol=1.293114e+07
assign (segid " A" and resid 243 and name HB2) (segid " A" and resid 225 and name HD21) 2.989 1.117 1.117 weight 1.000 ! spec=Expt_4, no=1484, id=5130, vol=4.264231e+06
assign (segid " A" and resid 243 and name HN) (segid " A" and resid 228 and name HE1) 2.306 0.665 0.665 weight 1.000 ! spec=Expt_1, no=1288, id=1051, vol=6.087193e+05
assign (segid " A" and resid 243 and name HN) (segid " A" and resid 228 and name HD1) 3.197 1.278 1.278 weight 1.000 ! spec=Expt_4, no=1306, id=5071, vol=2.845412e+06
assign (segid " A" and resid 243 and name HN) (segid " A" and resid 242 and name HA) 1.958 0.479 0.479 weight 1.000 ! spec=Expt_4, no=308, id=4479, vol=5.387640e+07
assign (segid " A" and resid 243 and name HN) (segid " A" and resid 243 and name HB2) 1.870 0.437 0.437 weight 1.000 ! spec=Expt_1, no=1286, id=1049, vol=2.140145e+06
assign (segid " A" and resid 243 and name HN) (segid " A" and resid 243 and name HA) 2.286 0.653 0.653 weight 1.000 ! spec=Expt_1, no=2064, id=1631, vol=6.409420e+05
assign (segid " A" and resid 243 and name HA) (segid " A" and resid 243 and name HB2) 1.813 0.411 0.411 weight 1.000 ! spec=Expt_3, no=294, id=2850, vol=1.292910e+07
assign (segid " A" and resid 243 and name HA) (segid " A" and resid 246 and name HD11) 2.315 0.670 0.670 weight 1.000 ! spec=Expt_1, no=1977, id=1567, vol=5.948330e+05
assign (segid " A" and resid 243 and name HA) (segid " A" and resid 246 and name HB) 2.177 0.592 0.592 weight 1.000 ! spec=Expt_3, no=1225, id=3474, vol=4.311732e+06

GLU 244
assign (segid " A" and resid 244 and name HA) (segid " A" and resid 221 and name HG21) 2.077 0.539 0.539 weight 1.000 ! spec=Expt_3, no=1691, id=3738, vol=5.705740e+06
assign (segid " A" and resid 244 and name HN) (segid " A" and resid 242 and name HA) 2.783 0.968 0.968 weight 1.000 ! spec=Expt_4, no=1313, id=5073, vol=6.539547e+06
assign (segid " A" and resid 244 and name HN) (segid " A" and resid 243 and name HB2) 2.100 0.551 0.551 weight 1.000 ! spec=Expt_1, no=1222, id=1001, vol=1.067133e+06
assign (segid " A" and resid 244 and name HN) (segid " A" and resid 243 and name HN) 2.333 0.680 0.680 weight 1.000 ! spec=Expt_1, no=1284, id=1048, vol=5.672757e+05
assign (segid " A" and resid 244 and name HG2) (segid " A" and resid 243 and name HB2) 2.314 0.669 0.669 weight 1.000 ! spec=Expt_3, no=5094, id=4154, vol=2.985624e+06
assign (segid " A" and resid 244 and name HN) (segid " A" and resid 243 and name HA) 2.725 0.928 0.928 weight 1.000 ! spec=Expt_4, no=311, id=4482, vol=7.421630e+06
assign (segid " A" and resid 244 and name HN) (segid " A" and resid 244 and name HG2) 2.243 0.629 0.629 weight 1.000 ! spec=Expt_1, no=1226, id=1004, vol=7.189466e+05

assign (segid " A" and resid 244 and name HG1) (segid " A" and resid 244 and name HA) 2.478 0.767 0.767 weight 1.000 ! spec=Expt_1, no=2682, id=1958, vol=3.954792e+05
assign (segid " A" and resid 244 and name HN) (segid " A" and resid 244 and name HA) 2.223 0.618 0.618 weight 1.000 ! spec=Expt_4, no=310, id=4481, vol=2.519920e+07
assign (segid " A" and resid 244 and name HB1) (segid " A" and resid 244 and name HN) 1.947 0.474 0.474 weight 1.000 ! spec=Expt_4, no=578, id=4704, vol=5.586908e+07
assign (segid " A" and resid 244 and name HN) (segid " A" and resid 245 and name HN) 1.949 0.475 0.475 weight 1.000 ! spec=Expt_1, no=1306, id=1061, vol=1.669612e+06

ASP 245
assign (segid " A" and resid 245 and name HN) (segid " A" and resid 242 and name HB1) 2.678 0.897 0.897 weight 1.000 ! spec=Expt_1, no=3359, id=2467, vol=2.480100e+05
assign (segid " A" and resid 245 and name HN) (segid " A" and resid 242 and name HA) 3.123 1.219 1.219 weight 1.000 ! spec=Expt_4, no=1319, id=5074, vol=3.277046e+06
assign (segid " A" and resid 245 and name HN) (segid " A" and resid 244 and name HB1) 2.212 0.612 0.612 weight 1.000 ! spec=Expt_1, no=1307, id=1062, vol=7.805708e+05
assign (segid " A" and resid 245 and name HN) (segid " A" and resid 245 and name HB1) 2.082 0.542 0.542 weight 1.000 ! spec=Expt_1, no=1317, id=1071, vol=1.122727e+06
assign (segid " A" and resid 245 and name HN) (segid " A" and resid 245 and name HA) 2.220 0.616 0.616 weight 1.000 ! spec=Expt_4, no=312, id=4483, vol=2.537606e+07
assign (segid " A" and resid 245 and name HN) (segid " A" and resid 246 and name HG11) 3.171 1.257 1.257 weight 1.000 ! spec=Expt_4, no=1323, id=5076, vol=2.991582e+06

ILE 246
assign (segid " A" and resid 246 and name HB) (segid " A" and resid 224 and name HD13) 1.965 0.483 0.483 weight 1.000 ! spec=Expt_3, no=1464, id=3575, vol=7.975426e+06
assign (segid " A" and resid 246 and name HD11) (segid " A" and resid 241 and name HB1) 2.203 0.606 0.606 weight 1.000 ! spec=Expt_3, no=1490, id=3598, vol=4.018224e+06
assign (segid " A" and resid 246 and name HD11) (segid " A" and resid 241 and name HD13) 1.832 0.420 0.420 weight 1.000 ! spec=Expt_3, no=1562, id=3647, vol=1.213566e+07
assign (segid " A" and resid 246 and name HN) (segid " A" and resid 243 and name HA) 2.741 0.939 0.939 weight 1.000 ! spec=Expt_4, no=1326, id=5077, vol=7.170410e+06
assign (segid " A" and resid 246 and name HN) (segid " A" and resid 244 and name HN) 2.986 1.114 1.114 weight 1.000 ! spec=Expt_4, no=99, id=4325, vol=4.290387e+06
assign (segid " A" and resid 246 and name HN) (segid " A" and resid 245 and name HA) 2.691 0.905 0.905 weight 1.000 ! spec=Expt_4, no=315, id=4485, vol=8.011517e+06
assign (segid " A" and resid 246 and name HB) (segid " A" and resid 246 and name HA) 2.321 0.673 0.673 weight 1.000 ! spec=Expt_1, no=235, id=215, vol=5.859895e+05
assign (segid " A" and resid 246 and name HN) (segid " A" and resid 246 and name HG11) 2.118 0.561 0.561 weight 1.000 ! spec=Expt_1, no=1296, id=1054, vol=1.012672e+06
assign (segid " A" and resid 246 and name HN) (segid " A" and resid 246 and name HB) 1.918 0.460 0.460 weight 1.000 ! spec=Expt_1, no=1299, id=1056, vol=1.839839e+06
assign (segid " A" and resid 246 and name HB) (segid " A" and resid 246 and name HG11) 2.275 0.647 0.647 weight 1.000 ! spec=Expt_1, no=1745, id=1385, vol=6.600342e+05
assign (segid " A" and resid 246 and name HD11) (segid " A" and resid 246 and name HG12) 2.289 0.655 0.655 weight 1.000 ! spec=Expt_1, no=1902, id=1505, vol=6.360702e+05
assign (segid " A" and resid 246 and name HG22) (segid " A" and resid 246 and name HB) 1.885 0.444 0.444 weight 1.000 ! spec=Expt_3, no=306, id=2857, vol=1.021874e+07
assign (segid " A" and resid 246 and name HD11) (segid " A" and resid 246 and name HB) 1.960 0.480 0.480 weight 1.000 ! spec=Expt_3, no=307, id=2858, vol=8.092268e+06
assign (segid " A" and resid 246 and name HG22) (segid " A" and resid 246 and name HG12) 1.926 0.464 0.464 weight 1.000 ! spec=Expt_3, no=439, id=2959, vol=8.996733e+06
assign (segid " A" and resid 246 and name HD11) (segid " A" and resid 246 and name HG11) 1.838 0.422 0.422 weight 1.000 ! spec=Expt_3, no=1469, id=3579, vol=1.188269e+07
assign (segid " A" and resid 246 and name HN) (segid " A" and resid 246 and name HA) 2.252 0.634 0.634 weight 1.000 ! spec=Expt_4, no=314, id=4484, vol=2.331074e+07
assign (segid " A" and resid 246 and name HA) (segid " A" and resid 252 and name HG2) 2.240 0.627 0.627 weight 1.000 ! spec=Expt_1, no=492, id=438, vol=7.238901e+05
assign (segid " A" and resid 246 and name HA) (segid " A" and resid 252 and name HB2) 2.366 0.700 0.700 weight 1.000 ! spec=Expt_1, no=2382, id=1796, vol=5.219863e+05
assign (segid " A" and resid 246 and name HA) (segid " A" and resid 252 and name HA) 2.102 0.553 0.553 weight 1.000 ! spec=Expt_3, no=1185, id=3448, vol=5.312239e+06

VAL 247
assign (segid " A" and resid 247 and name HA) (segid " A" and resid 193 and name HE1) 2.147 0.576 0.576 weight 1.000 ! spec=Expt_3, no=1194, id=3455, vol=4.687277e+06
assign (segid " A" and resid 247 and name HN) (segid " A" and resid 246 and name HB) 2.114 0.559 0.559 weight 1.000 ! spec=Expt_1, no=933, id=772, vol=1.025044e+06
assign (segid " A" and resid 247 and name HN) (segid " A" and resid 246 and name HN) 1.930 0.465 0.465 weight 1.000 ! spec=Expt_1, no=938, id=775, vol=1.772410e+06
assign (segid " A" and resid 247 and name HN) (segid " A" and resid 246 and name HG22) 2.318 0.672 0.672 weight 1.000 ! spec=Expt_1, no=2944, id=2112, vol=5.894437e+05
assign (segid " A" and resid 247 and name HA) (segid " A" and resid 247 and name HB) 1.958 0.479 0.479 weight 1.000 ! spec=Expt_1, no=241, id=221, vol=1.623950e+06
assign (segid " A" and resid 247 and name HB) (segid " A" and resid 247 and name HN) 2.121 0.562 0.562 weight 1.000 ! spec=Expt_4, no=584, id=4709, vol=3.340803e+07
assign (segid " A" and resid 247 and name HN) (segid " A" and resid 248 and name HN) 1.950 0.475 0.475 weight 1.000 ! spec=Expt_1, no=941, id=778, vol=1.663824e+06

ASN 248
assign (segid " A" and resid 248 and name HB1) (segid " A" and resid 245 and name HA) 2.292 0.657 0.657 weight 1.000 ! spec=Expt_1, no=2375, id=1794, vol=6.307820e+05
assign (segid " A" and resid 248 and name HN) (segid " A" and resid 245 and name HA) 2.732 0.933 0.933 weight 1.000 ! spec=Expt_4, no=1338, id=5079, vol=7.307396e+06
assign (segid " A" and resid 248 and name HD22) (segid " A" and resid 248 and name HB1) 2.698 0.910 0.910 weight 1.000 ! spec=Expt_1, no=3310, id=2422, vol=2.371515e+05
assign (segid " A" and resid 248 and name HN) (segid " A" and resid 248 and name HA) 2.302 0.662 0.662 weight 1.000 ! spec=Expt_4, no=318, id=4488, vol=2.042925e+07

assign (segid " A" and resid 248 and name HN) (segid " A" and resid 248 and name HB1) 2.059 0.530 0.530 weight 1.000 ! spec=Expt_4, no=586, id=4710, vol=3.992469e+07

THR 249
assign (segid " A" and resid 249 and name HN) (segid " A" and resid 247 and name HA) 2.680 0.897 0.897 weight 1.000 ! spec=Expt_4, no=1345, id=5080, vol=8.211015e+06
assign (segid " A" and resid 249 and name HN) (segid " A" and resid 248 and name HN) 2.225 0.619 0.619 weight 1.000 ! spec=Expt_1, no=926, id=766, vol=7.550808e+05
assign (segid " A" and resid 249 and name HG1) (segid " A" and resid 249 and name HB) 1.996 0.498 0.498 weight 1.000 ! spec=Expt_1, no=245, id=225, vol=1.445826e+06
assign (segid " A" and resid 249 and name HN) (segid " A" and resid 249 and name HG1) 2.144 0.575 0.575 weight 1.000 ! spec=Expt_1, no=2960, id=2126, vol=9.411764e+05
assign (segid " A" and resid 249 and name HB) (segid " A" and resid 249 and name HA) 1.953 0.477 0.477 weight 1.000 ! spec=Expt_3, no=319, id=2865, vol=8.267877e+06
assign (segid " A" and resid 249 and name HN) (segid " A" and resid 249 and name HA) 2.258 0.637 0.637 weight 1.000 ! spec=Expt_4, no=320, id=4489, vol=2.292089e+07

ALA 250
assign (segid " A" and resid 250 and name HN) (segid " A" and resid 249 and name HA) 2.525 0.797 0.797 weight 1.000 ! spec=Expt_1, no=1320, id=1074, vol=3.533729e+05
assign (segid " A" and resid 250 and name HA) (segid " A" and resid 250 and name HB2) 1.743 0.380 0.380 weight 1.000 ! spec=Expt_1, no=248, id=227, vol=3.269217e+06
assign (segid " A" and resid 250 and name HN) (segid " A" and resid 250 and name HA) 2.664 0.887 0.887 weight 1.000 ! spec=Expt_1, no=2406, id=1808, vol=2.562316e+05
assign (segid " A" and resid 250 and name HN) (segid " A" and resid 250 and name HB2) 2.944 1.083 1.083 weight 1.000 ! spec=Expt_4, no=589, id=4711, vol=4.667191e+06

ARG 251
assign (segid " A" and resid 251 and name HN) (segid " A" and resid 249 and name HA) 2.995 1.121 1.121 weight 1.000 ! spec=Expt_4, no=1350, id=5081, vol=4.212870e+06
assign (segid " A" and resid 251 and name HN) (segid " A" and resid 250 and name HA) 2.695 0.908 0.908 weight 1.000 ! spec=Expt_4, no=317, id=4487, vol=7.931585e+06
assign (segid " A" and resid 251 and name HN) (segid " A" and resid 250 and name HB2) 2.248 0.631 0.631 weight 1.000 ! spec=Expt_4, no=590, id=4712, vol=2.357446e+07
assign (segid " A" and resid 251 and name HN) (segid " A" and resid 251 and name HB2) 2.288 0.654 0.654 weight 1.000 ! spec=Expt_1, no=2732, id=1979, vol=6.376824e+05
assign (segid " A" and resid 251 and name HB1) (segid " A" and resid 251 and name HD1) 2.428 0.737 0.737 weight 1.000 ! spec=Expt_3, no=1322, id=3487, vol=2.237001e+06
assign (segid " A" and resid 251 and name HB1) (segid " A" and resid 251 and name HB2) 1.957 0.479 0.479 weight 1.000 ! spec=Expt_3, no=1610, id=3676, vol=8.162827e+06
assign (segid " A" and resid 251 and name HN) (segid " A" and resid 251 and name HA) 2.344 0.687 0.687 weight 1.000 ! spec=Expt_4, no=324, id=4491, vol=1.830673e+07

PRO 252
assign (segid " A" and resid 252 and name HB1) (segid " A" and resid 189 and name HA) 2.300 0.661 0.661 weight 1.000 ! spec=Expt_3, no=4318, id=4068, vol=3.095294e+06
assign (segid " A" and resid 252 and name HD2) (segid " A" and resid 190 and name HG22) 2.471 0.763 0.763 weight 1.000 ! spec=Expt_3, no=5246, id=4203, vol=2.017091e+06
assign (segid " A" and resid 252 and name HB2) (segid " A" and resid 246 and name HG22) 2.226 0.620 0.620 weight 1.000 ! spec=Expt_3, no=1334, id=3494, vol=3.766295e+06
assign (segid " A" and resid 252 and name HA) (segid " A" and resid 249 and name HG1) 2.021 0.510 0.510 weight 1.000 ! spec=Expt_1, no=2639, id=1931, vol=1.344460e+06
assign (segid " A" and resid 252 and name HA) (segid " A" and resid 252 and name HB2) 2.015 0.508 0.508 weight 1.000 ! spec=Expt_1, no=249, id=228, vol=1.367259e+06
assign (segid " A" and resid 252 and name HG1) (segid " A" and resid 252 and name HD1) 2.430 0.738 0.738 weight 1.000 ! spec=Expt_1, no=2280, id=1762, vol=4.439614e+05
assign (segid " A" and resid 252 and name HA) (segid " A" and resid 252 and name HG2) 2.477 0.767 0.767 weight 1.000 ! spec=Expt_3, no=4488, id=4075, vol=1.984313e+06
assign (segid " A" and resid 252 and name HG1) (segid " A" and resid 252 and name HG2) 1.869 0.437 0.437 weight 1.000 ! spec=Expt_3, no=5098, id=4158, vol=1.076983e+07

ASP 253
assign (segid " A" and resid 253 and name HN) (segid " A" and resid 246 and name HA) 3.039 1.155 1.155 weight 1.000 ! spec=Expt_4, no=817, id=4860, vol=3.855642e+06
assign (segid " A" and resid 253 and name HN) (segid " A" and resid 252 and name HA) 1.838 0.422 0.422 weight 1.000 ! spec=Expt_1, no=1322, id=1075, vol=2.370526e+06
assign (segid " A" and resid 253 and name HN) (segid " A" and resid 252 and name HB2) 2.625 0.861 0.861 weight 1.000 ! spec=Expt_1, no=3196, id=2328, vol=2.799521e+05
assign (segid " A" and resid 253 and name HN) (segid " A" and resid 253 and name HB1) 2.120 0.562 0.562 weight 1.000 ! spec=Expt_1, no=3253, id=2376, vol=1.007905e+06
assign (segid " A" and resid 253 and name HB1) (segid " A" and resid 256 and name HB2) 2.197 0.604 0.604 weight 1.000 ! spec=Expt_3, no=1335, id=3495, vol=4.075459e+06
assign (segid " A" and resid 253 and name HN) (segid " A" and resid 257 and name HG22) 2.859 1.022 1.022 weight 1.000 ! spec=Expt_1, no=2211, id=1747, vol=1.675855e+05

GLU 254
assign (segid " A" and resid 254 and name HN) (segid " A" and resid 253 and name HA) 1.864 0.434 0.434 weight 1.000 ! spec=Expt_4, no=328, id=4493, vol=7.240230e+07
assign (segid " A" and resid 254 and name HN) (segid " A" and resid 254 and name HB1) 2.124 0.564 0.564 weight 1.000 ! spec=Expt_1, no=1330, id=1082, vol=9.967943e+05
assign (segid " A" and resid 254 and name HN) (segid " A" and resid 254 and name HB2) 2.444 0.747 0.747 weight 1.000 ! spec=Expt_1, no=3191, id=2324, vol=4.290365e+05
assign (segid " A" and resid 254 and name HA) (segid " A" and resid 254 and name HB2) 2.424 0.735 0.735 weight 1.000 ! spec=Expt_3, no=340, id=2880, vol=2.260922e+06

assign (segid " A" and resid 254 and name HN) (segid " A" and resid 254 and name HA) 2.447 0.748 0.748 weight 1.000 ! spec=Expt_4, no=327, id=4492, vol=1.416865e+07
assign (segid " A" and resid 254 and name HB1) (segid " A" and resid 255 and name HN) 2.616 0.856 0.856 weight 1.000 ! spec=Expt_4, no=1585, id=5176, vol=9.480624e+06

LYS 255
assign (segid " A" and resid 255 and name HN) (segid " A" and resid 253 and name HA) 2.725 0.928 0.928 weight 1.000 ! spec=Expt_4, no=330, id=4495, vol=7.418636e+06
assign (segid " A" and resid 255 and name HN) (segid " A" and resid 254 and name HN) 2.290 0.655 0.655 weight 1.000 ! spec=Expt_1, no=1329, id=1081, vol=6.349826e+05
assign (segid " A" and resid 255 and name HN) (segid " A" and resid 254 and name HG1) 2.442 0.746 0.746 weight 1.000 ! spec=Expt_1, no=1338, id=1088, vol=4.311515e+05
assign (segid " A" and resid 255 and name HN) (segid " A" and resid 254 and name HA) 2.792 0.975 0.975 weight 1.000 ! spec=Expt_4, no=331, id=4496, vol=6.409601e+06
assign (segid " A" and resid 255 and name HE2) (segid " A" and resid 255 and name HD1) 1.985 0.492 0.492 weight 1.000 ! spec=Expt_1, no=1741, id=1381, vol=1.496564e+06
assign (segid " A" and resid 255 and name HG2) (segid " A" and resid 255 and name HG1) 1.958 0.479 0.479 weight 1.000 ! spec=Expt_1, no=1907, id=1509, vol=1.622941e+06
assign (segid " A" and resid 255 and name HE2) (segid " A" and resid 255 and name HG2) 2.209 0.610 0.610 weight 1.000 ! spec=Expt_3, no=4530, id=4085, vol=3.947935e+06
assign (segid " A" and resid 255 and name HN) (segid " A" and resid 255 and name HA) 2.171 0.589 0.589 weight 1.000 ! spec=Expt_4, no=329, id=4494, vol=2.903141e+07
assign (segid " A" and resid 255 and name HN) (segid " A" and resid 255 and name HB1) 1.890 0.447 0.447 weight 1.000 ! spec=Expt_4, no=601, id=4722, vol=6.660633e+07
assign (segid " A" and resid 255 and name HN) (segid " A" and resid 255 and name HD1) 2.672 0.893 0.893 weight 1.000 ! spec=Expt_4, no=1586, id=5177, vol=8.343044e+06
assign (segid " A" and resid 255 and name HB1) (segid " A" and resid 256 and name HN) 2.201 0.606 0.606 weight 1.000 ! spec=Expt_1, no=3084, id=2235, vol=8.042825e+05
assign (segid " A" and resid 255 and name HA) (segid " A" and resid 258 and name HB2) 2.269 0.643 0.643 weight 1.000 ! spec=Expt_1, no=1716, id=1363, vol=6.712969e+05

ALA 256
assign (segid " A" and resid 256 and name HN) (segid " A" and resid 241 and name HD21) 3.137 1.230 1.230 weight 1.000 ! spec=Expt_4, no=1375, id=5089, vol=3.190040e+06
assign (segid " A" and resid 256 and name HN) (segid " A" and resid 254 and name HN) 3.122 1.218 1.218 weight 1.000 ! spec=Expt_4, no=1364, id=5086, vol=3.282971e+06
assign (segid " A" and resid 256 and name HN) (segid " A" and resid 254 and name HA) 3.130 1.225 1.225 weight 1.000 ! spec=Expt_4, no=1588, id=5178, vol=3.229863e+06
assign (segid " A" and resid 256 and name HN) (segid " A" and resid 255 and name HN) 2.101 0.552 0.552 weight 1.000 ! spec=Expt_4, no=109, id=4332, vol=3.532471e+07
assign (segid " A" and resid 256 and name HA) (segid " A" and resid 256 and name HB2) 1.728 0.373 0.373 weight 1.000 ! spec=Expt_1, no=268, id=244, vol=3.437150e+06
assign (segid " A" and resid 256 and name HB2) (segid " A" and resid 256 and name HN) 1.841 0.424 0.424 weight 1.000 ! spec=Expt_1, no=3031, id=2187, vol=2.350154e+06
assign (segid " A" and resid 256 and name HN) (segid " A" and resid 258 and name HN) 2.974 1.106 1.106 weight 1.000 ! spec=Expt_4, no=1381, id=5092, vol=4.389058e+06

ILE 257
assign (segid " A" and resid 257 and name HD11) (segid " A" and resid 188 and name HB2) 2.420 0.732 0.732 weight 1.000 ! spec=Expt_1, no=1985, id=1575, vol=4.557648e+05
assign (segid " A" and resid 257 and name HG22) (segid " A" and resid 189 and name HA) 2.552 0.814 0.814 weight 1.000 ! spec=Expt_1, no=2603, id=1913, vol=3.314982e+05
assign (segid " A" and resid 257 and name HD11) (segid " A" and resid 192 and name HE1) 2.588 0.837 0.837 weight 1.000 ! spec=Expt_1, no=2118, id=1675, vol=3.044647e+05
assign (segid " A" and resid 257 and name HN) (segid " A" and resid 241 and name HD21) 2.566 0.823 0.823 weight 1.000 ! spec=Expt_1, no=1138, id=933, vol=3.209126e+05
assign (segid " A" and resid 257 and name HB) (segid " A" and resid 241 and name HD13) 2.556 0.816 0.816 weight 1.000 ! spec=Expt_3, no=4697, id=4118, vol=1.646754e+06
assign (segid " A" and resid 257 and name HN) (segid " A" and resid 254 and name HA) 2.702 0.913 0.913 weight 1.000 ! spec=Expt_4, no=1378, id=5090, vol=7.802229e+06
assign (segid " A" and resid 257 and name HN) (segid " A" and resid 256 and name HN) 2.029 0.515 0.515 weight 1.000 ! spec=Expt_1, no=856, id=713, vol=1.310037e+06
assign (segid " A" and resid 257 and name HN) (segid " A" and resid 256 and name HB2) 2.026 0.513 0.513 weight 1.000 ! spec=Expt_1, no=1142, id=935, vol=1.322962e+06
assign (segid " A" and resid 257 and name HN) (segid " A" and resid 256 and name HA) 2.860 1.023 1.023 weight 1.000 ! spec=Expt_4, no=335, id=4498, vol=5.548571e+06
assign (segid " A" and resid 257 and name HD11) (segid " A" and resid 257 and name HB) 1.956 0.478 0.478 weight 1.000 ! spec=Expt_1, no=270, id=246, vol=1.632666e+06
assign (segid " A" and resid 257 and name HG22) (segid " A" and resid 257 and name HB) 1.808 0.408 0.408 weight 1.000 ! spec=Expt_1, no=355, id=322, vol=2.624321e+06
assign (segid " A" and resid 257 and name HN) (segid " A" and resid 257 and name HG22) 2.301 0.662 0.662 weight 1.000 ! spec=Expt_1, no=1352, id=1096, vol=6.173076e+05
assign (segid " A" and resid 257 and name HD11) (segid " A" and resid 257 and name HG11) 2.147 0.576 0.576 weight 1.000 ! spec=Expt_1, no=1782, id=1417, vol=9.330838e+05
assign (segid " A" and resid 257 and name HG11) (segid " A" and resid 257 and name HG12) 1.743 0.380 0.380 weight 1.000 ! spec=Expt_1, no=1839, id=1462, vol=3.261782e+06
assign (segid " A" and resid 257 and name HD11) (segid " A" and resid 257 and name HG12) 2.409 0.725 0.725 weight 1.000 ! spec=Expt_1, no=1925, id=1524, vol=4.681234e+05
assign (segid " A" and resid 257 and name HA) (segid " A" and resid 257 and name HG22) 2.387 0.712 0.712 weight 1.000 ! spec=Expt_1, no=1989, id=1577, vol=4.949304e+05
assign (segid " A" and resid 257 and name HG12) (segid " A" and resid 257 and name HG22) 2.447 0.748 0.748 weight 1.000 ! spec=Expt_1, no=2209, id=1745, vol=4.265959e+05
assign (segid " A" and resid 257 and name HN) (segid " A" and resid 257 and name HA) 2.278 0.649 0.649 weight 1.000 ! spec=Expt_1, no=3461, id=2561, vol=6.542637e+05
assign (segid " A" and resid 257 and name HB) (segid " A" and resid 257 and name HA) 2.370 0.702 0.702 weight 1.000 ! spec=Expt_3, no=346, id=2882, vol=2.589106e+06
assign (segid " A" and resid 257 and name HG11) (segid " A" and resid 257 and name HN) 2.337 0.683 0.683 weight 1.000 ! spec=Expt_4, no=829, id=4867, vol=1.865627e+07
assign (segid " A" and resid 257 and name HN) (segid " A" and resid 258 and name HN) 2.066 0.533 0.533 weight 1.000 ! spec=Expt_1, no=1355, id=1099, vol=1.177731e+06

assign (segid " A" and resid 257 and name HD11) (segid " A" and resid 258 and name HA) 2.386 0.711 0.711 weight 1.000 ! spec=Expt_3, no=1373, id=3519, vol=2.488032e+06
assign (segid " A" and resid 257 and name HA) (segid " A" and resid 258 and name HN) 2.883 1.039 1.039 weight 1.000 ! spec=Expt_4, no=337, id=4500, vol=5.290601e+06
assign (segid " A" and resid 257 and name HG11) (segid " A" and resid 258 and name HN) 2.991 1.119 1.119 weight 1.000 ! spec=Expt_4, no=831, id=4868, vol=4.241010e+06
assign (segid " A" and resid 257 and name HD11) (segid " A" and resid 261 and name HG21) 2.287 0.654 0.654 weight 1.000 ! spec=Expt_3, no=1506, id=3607, vol=3.207879e+06

MET 258
assign (segid " A" and resid 258 and name HG1) (segid " A" and resid 188 and name HD1) 2.079 0.540 0.540 weight 1.000 ! spec=Expt_1, no=3003, id=2162, vol=1.133141e+06
assign (segid " A" and resid 258 and name HG2) (segid " A" and resid 254 and name HG2) 2.415 0.729 0.729 weight 1.000 ! spec=Expt_3, no=5220, id=4189, vol=2.315104e+06
assign (segid " A" and resid 258 and name HN) (segid " A" and resid 254 and name HA) 2.948 1.086 1.086 weight 1.000 ! spec=Expt_4, no=1384, id=5093, vol=4.633723e+06
assign (segid " A" and resid 258 and name HN) (segid " A" and resid 257 and name HG22) 3.109 1.208 1.208 weight 1.000 ! spec=Expt_4, no=1388, id=5094, vol=3.368242e+06
assign (segid " A" and resid 258 and name HN) (segid " A" and resid 258 and name HB2) 2.060 0.531 0.531 weight 1.000 ! spec=Expt_1, no=1357, id=1101, vol=1.197051e+06
assign (segid " A" and resid 258 and name HN) (segid " A" and resid 258 and name HA) 2.156 0.581 0.581 weight 1.000 ! spec=Expt_1, no=1361, id=1104, vol=9.113257e+05
assign (segid " A" and resid 258 and name HN) (segid " A" and resid 258 and name HG2) 2.521 0.794 0.794 weight 1.000 ! spec=Expt_1, no=2912, id=2089, vol=3.566357e+05
assign (segid " A" and resid 258 and name HB1) (segid " A" and resid 258 and name HB2) 1.589 0.315 0.315 weight 1.000 ! spec=Expt_3, no=1477, id=3586, vol=2.854389e+07
assign (segid " A" and resid 258 and name HA) (segid " A" and resid 261 and name HG21) 2.186 0.598 0.598 weight 1.000 ! spec=Expt_3, no=1372, id=3518, vol=4.198152e+06

THR 259
assign (segid " A" and resid 259 and name HN) (segid " A" and resid 258 and name HN) 2.154 0.580 0.580 weight 1.000 ! spec=Expt_1, no=1364, id=1105, vol=9.160110e+05
assign (segid " A" and resid 259 and name HG1) (segid " A" and resid 259 and name HA) 1.811 0.410 0.410 weight 1.000 ! spec=Expt_1, no=470, id=420, vol=2.593356e+06
assign (segid " A" and resid 259 and name HN) (segid " A" and resid 259 and name HG1) 2.319 0.672 0.672 weight 1.000 ! spec=Expt_1, no=1366, id=1106, vol=5.886158e+05
assign (segid " A" and resid 259 and name HN) (segid " A" and resid 259 and name HB) 2.096 0.549 0.549 weight 1.000 ! spec=Expt_1, no=1374, id=1114, vol=1.079460e+06
assign (segid " A" and resid 259 and name HN) (segid " A" and resid 259 and name HA) 2.274 0.646 0.646 weight 1.000 ! spec=Expt_4, no=611, id=4729, vol=2.197767e+07

TYR 260
assign (segid " A" and resid 260 and name HE1) (segid " A" and resid 228 and name HD1) 1.922 0.462 0.462 weight 1.000 ! spec=Expt_3, no=777, id=3224, vol=9.098340e+06
assign (segid " A" and resid 260 and name HE1) (segid " A" and resid 228 and name HE1) 1.911 0.457 0.457 weight 1.000 ! spec=Expt_3, no=784, id=3228, vol=9.408504e+06
assign (segid " A" and resid 260 and name HE1) (segid " A" and resid 231 and name HB2) 2.471 0.763 0.763 weight 1.000 ! spec=Expt_3, no=995, id=3337, vol=2.014863e+06
assign (segid " A" and resid 260 and name HE1) (segid " A" and resid 237 and name HG22) 1.882 0.443 0.443 weight 1.000 ! spec=Expt_3, no=1084, id=3400, vol=1.032562e+07
assign (segid " A" and resid 260 and name HE1) (segid " A" and resid 239 and name HA) 2.382 0.709 0.709 weight 1.000 ! spec=Expt_1, no=1549, id=1250, vol=5.010319e+05
assign (segid " A" and resid 260 and name HD1) (segid " A" and resid 240 and name HG1) 2.333 0.680 0.680 weight 1.000 ! spec=Expt_3, no=926, id=3293, vol=2.844234e+06
assign (segid " A" and resid 260 and name HD1) (segid " A" and resid 241 and name HD13) 1.870 0.437 0.437 weight 1.000 ! spec=Expt_1, no=1464, id=1181, vol=2.137888e+06
assign (segid " A" and resid 260 and name HB1) (segid " A" and resid 241 and name HD13) 2.182 0.595 0.595 weight 1.000 ! spec=Expt_3, no=1546, id=3635, vol=4.253462e+06
assign (segid " A" and resid 260 and name HN) (segid " A" and resid 259 and name HB) 2.263 0.640 0.640 weight 1.000 ! spec=Expt_1, no=1378, id=1117, vol=6.822927e+05
assign (segid " A" and resid 260 and name HN) (segid " A" and resid 259 and name HA) 2.620 0.858 0.858 weight 1.000 ! spec=Expt_4, no=612, id=4730, vol=9.401414e+06
assign (segid " A" and resid 260 and name HD1) (segid " A" and resid 260 and name HA) 1.960 0.480 0.480 weight 1.000 ! spec=Expt_1, no=590, id=524, vol=1.614508e+06
assign (segid " A" and resid 260 and name HB2) (segid " A" and resid 260 and name HA) 2.336 0.682 0.682 weight 1.000 ! spec=Expt_1, no=2276, id=1760, vol=5.632711e+05
assign (segid " A" and resid 260 and name HD1) (segid " A" and resid 260 and name HB1) 2.094 0.548 0.548 weight 1.000 ! spec=Expt_3, no=2731, id=3991, vol=5.439414e+06
assign (segid " A" and resid 260 and name HN) (segid " A" and resid 260 and name HA) 2.406 0.723 0.723 weight 1.000 ! spec=Expt_4, no=341, id=4502, vol=1.567656e+07
assign (segid " A" and resid 260 and name HN) (segid " A" and resid 261 and name HN) 2.175 0.591 0.591 weight 1.000 ! spec=Expt_1, no=591, id=525, vol=8.637419e+05
assign (segid " A" and resid 260 and name HD1) (segid " A" and resid 261 and name HN) 2.620 0.858 0.858 weight 1.000 ! spec=Expt_1, no=1573, id=1263, vol=2.826507e+05
assign (segid " A" and resid 260 and name HD1) (segid " A" and resid 264 and name HE1) 2.322 0.674 0.674 weight 1.000 ! spec=Expt_3, no=811, id=3240, vol=2.925778e+06

VAL 261
assign (segid " A" and resid 261 and name HG21) (segid " A" and resid 169 and name HD13) 1.949 0.475 0.475 weight 1.000 ! spec=Expt_1, no=1789, id=1423, vol=1.669197e+06
assign (segid " A" and resid 261 and name HG21) (segid " A" and resid 169 and name HD21) 1.897 0.450 0.450 weight 1.000 ! spec=Expt_3, no=1491, id=3599, vol=9.827954e+06
assign (segid " A" and resid 261 and name HN) (segid " A" and resid 257 and name HA) 2.798 0.979 0.979 weight 1.000 ! spec=Expt_1, no=2242, id=1752, vol=1.906911e+05
assign (segid " A" and resid 261 and name HG12) (segid " A" and resid 260 and name HD1) 2.341 0.685 0.685 weight 1.000 ! spec=Expt_1, no=2710, id=1971, vol=5.564991e+05
assign (segid " A" and resid 261 and name HN) (segid " A" and resid 260 and name HA) 2.935 1.077 1.077 weight 1.000 ! spec=Expt_4, no=344, id=4505, vol=4.756630e+06

assign (segid " A" and resid 261 and name HG12) (segid " A" and resid 261 and name HA) 1.913 0.458 0.458 weight 1.000 ! spec=Expt_1, no=456, id=410, vol=1.865997e+06
assign (segid " A" and resid 261 and name HG12) (segid " A" and resid 261 and name HG21) 1.738 0.378 0.378 weight 1.000 ! spec=Expt_1, no=460, id=414, vol=3.314988e+06
assign (segid " A" and resid 261 and name HN) (segid " A" and resid 261 and name HA) 2.251 0.633 0.633 weight 1.000 ! spec=Expt_1, no=3240, id=2363, vol=7.032784e+05
assign (segid " A" and resid 261 and name HA) (segid " A" and resid 261 and name HB) 2.290 0.656 0.656 weight 1.000 ! spec=Expt_3, no=363, id=2893, vol=3.178046e+06
assign (segid " A" and resid 261 and name HN) (segid " A" and resid 261 and name HG21) 2.554 0.816 0.816 weight 1.000 ! spec=Expt_3, no=2868, id=4011, vol=1.651088e+06
assign (segid " A" and resid 261 and name HN) (segid " A" and resid 261 and name HG12) 2.239 0.626 0.626 weight 1.000 ! spec=Expt_3, no=2869, id=4012, vol=3.644314e+06

SER 262
assign (segid " A" and resid 262 and name HN) (segid " A" and resid 169 and name HD13) 2.329 0.678 0.678 weight 1.000 ! spec=Expt_1, no=1165, id=956, vol=5.737495e+05
assign (segid " A" and resid 262 and name HA) (segid " A" and resid 169 and name HD21) 1.968 0.484 0.484 weight 1.000 ! spec=Expt_3, no=1362, id=3513, vol=7.902621e+06
assign (segid " A" and resid 262 and name HB2) (segid " A" and resid 169 and name HD13) 2.449 0.749 0.749 weight 1.000 ! spec=Expt_3, no=4716, id=4119, vol=2.128043e+06
assign (segid " A" and resid 262 and name HA) (segid " A" and resid 169 and name HD13) 2.447 0.748 0.748 weight 1.000 ! spec=Expt_3, no=4717, id=4120, vol=2.137740e+06
assign (segid " A" and resid 262 and name HB1) (segid " A" and resid 169 and name HD13) 2.272 0.645 0.645 weight 1.000 ! spec=Expt_3, no=4722, id=4122, vol=3.338319e+06
assign (segid " A" and resid 262 and name HN) (segid " A" and resid 260 and name HN) 2.992 1.119 1.119 weight 1.000 ! spec=Expt_4, no=1400, id=5097, vol=4.233297e+06
assign (segid " A" and resid 262 and name HN) (segid " A" and resid 261 and name HN) 2.153 0.579 0.579 weight 1.000 ! spec=Expt_1, no=1383, id=1120, vol=9.198130e+05
assign (segid " A" and resid 262 and name HN) (segid " A" and resid 261 and name HG21) 2.510 0.788 0.788 weight 1.000 ! spec=Expt_1, no=1384, id=1121, vol=3.659933e+05
assign (segid " A" and resid 262 and name HN) (segid " A" and resid 261 and name HA) 2.794 0.976 0.976 weight 1.000 ! spec=Expt_4, no=346, id=4506, vol=6.391626e+06
assign (segid " A" and resid 262 and name HB1) (segid " A" and resid 262 and name HB2) 2.212 0.612 0.612 weight 1.000 ! spec=Expt_3, no=1207, id=3463, vol=3.913627e+06
assign (segid " A" and resid 262 and name HN) (segid " A" and resid 262 and name HB1) 2.133 0.569 0.569 weight 1.000 ! spec=Expt_4, no=1596, id=5181, vol=3.227500e+07
assign (segid " A" and resid 262 and name HN) (segid " A" and resid 263 and name HN) 2.255 0.636 0.636 weight 1.000 ! spec=Expt_1, no=848, id=707, vol=6.964111e+05
assign (segid " A" and resid 262 and name HA) (segid " A" and resid 265 and name HB2) 2.159 0.583 0.583 weight 1.000 ! spec=Expt_1, no=1724, id=1369, vol=9.036807e+05

SER 263
assign (segid " A" and resid 263 and name HB1) (segid " A" and resid 237 and name HG22) 2.106 0.554 0.554 weight 1.000 ! spec=Expt_3, no=1359, id=3511, vol=5.259765e+06
assign (segid " A" and resid 263 and name HB2) (segid " A" and resid 237 and name HG22) 2.054 0.527 0.527 weight 1.000 ! spec=Expt_3, no=1368, id=3515, vol=6.114068e+06
assign (segid " A" and resid 263 and name HN) (segid " A" and resid 260 and name HA) 2.844 1.011 1.011 weight 1.000 ! spec=Expt_4, no=1413, id=5101, vol=5.738041e+06
assign (segid " A" and resid 263 and name HN) (segid " A" and resid 261 and name HA) 2.998 1.124 1.124 weight 1.000 ! spec=Expt_4, no=1414, id=5102, vol=4.182396e+06
assign (segid " A" and resid 263 and name HN) (segid " A" and resid 263 and name HA) 2.278 0.648 0.648 weight 1.000 ! spec=Expt_1, no=1350, id=1094, vol=6.557599e+05
assign (segid " A" and resid 263 and name HN) (segid " A" and resid 263 and name HB2) 2.143 0.574 0.574 weight 1.000 ! spec=Expt_4, no=624, id=4737, vol=3.142210e+07
assign (segid " A" and resid 263 and name HN) (segid " A" and resid 264 and name HN) 2.135 0.570 0.570 weight 1.000 ! spec=Expt_1, no=1385, id=1122, vol=9.669059e+05
assign (segid " A" and resid 263 and name HN) (segid " A" and resid 264 and name HD1) 2.988 1.116 1.116 weight 1.000 ! spec=Expt_4, no=1411, id=5100, vol=4.272734e+06
assign (segid " A" and resid 263 and name HA) (segid " A" and resid 266 and name HB2) 2.305 0.664 0.664 weight 1.000 ! spec=Expt_3, no=1187, id=3450, vol=3.061772e+06

PHE 264
assign (segid " A" and resid 264 and name HE1) (segid " A" and resid 202 and name HG22) 2.114 0.559 0.559 weight 1.000 ! spec=Expt_3, no=1018, id=3355, vol=5.139208e+06
assign (segid " A" and resid 264 and name HE1) (segid " A" and resid 202 and name HG11) 2.352 0.691 0.691 weight 1.000 ! spec=Expt_3, no=1020, id=3356, vol=2.710964e+06
assign (segid " A" and resid 264 and name HD1) (segid " A" and resid 202 and name HD11) 2.120 0.562 0.562 weight 1.000 ! spec=Expt_3, no=1042, id=3370, vol=5.052255e+06
assign (segid " A" and resid 264 and name HE1) (segid " A" and resid 231 and name HB2) 2.191 0.600 0.600 weight 1.000 ! spec=Expt_3, no=988, id=3332, vol=4.141196e+06
assign (segid " A" and resid 264 and name HZ) (segid " A" and resid 231 and name HB2) 1.919 0.460 0.460 weight 1.000 ! spec=Expt_3, no=2244, id=3893, vol=9.188436e+06
assign (segid " A" and resid 264 and name HE1) (segid " A" and resid 235 and name HD13) 2.165 0.586 0.586 weight 1.000 ! spec=Expt_3, no=1021, id=3357, vol=4.452519e+06
assign (segid " A" and resid 264 and name HD1) (segid " A" and resid 235 and name HD13) 2.159 0.583 0.583 weight 1.000 ! spec=Expt_3, no=1049, id=3376, vol=4.525769e+06
assign (segid " A" and resid 264 and name HZ) (segid " A" and resid 237 and name HG22) 2.675 0.895 0.895 weight 1.000 ! spec=Expt_1, no=1236, id=1012, vol=2.496222e+05
assign (segid " A" and resid 264 and name HA) (segid " A" and resid 237 and name HD11) 2.178 0.593 0.593 weight 1.000 ! spec=Expt_1, no=2048, id=1622, vol=8.575141e+05
assign (segid " A" and resid 264 and name HZ) (segid " A" and resid 237 and name HD11) 2.751 0.946 0.946 weight 1.000 ! spec=Expt_1, no=2801, id=2005, vol=2.110331e+05
assign (segid " A" and resid 264 and name HD1) (segid " A" and resid 237 and name HD11) 2.316 0.670 0.670 weight 1.000 ! spec=Expt_3, no=1122, id=3426, vol=2.973449e+06
assign (segid " A" and resid 264 and name HE1) (segid " A" and resid 237 and name HD11) 2.406 0.724 0.724 weight 1.000 ! spec=Expt_3, no=1123, id=3427, vol=2.362163e+06
assign (segid " A" and resid 264 and name HN) (segid " A" and resid 237 and name HD11) 3.175 1.260 1.260 weight 1.000 ! spec=Expt_4, no=1423, id=5105, vol=2.964950e+06
assign (segid " A" and resid 264 and name HE1) (segid " A" and resid 260 and name HE1) 2.251 0.633 0.633 weight 1.000 ! spec=Expt_3, no=787, id=3229, vol=3.526893e+06

assign (segid " A" and resid 264 and name HZ) (segid " A" and resid 260 and name HE1) 2.173 0.590 0.590 weight 1.000 ! spec=Expt_3, no=817, id=3246, vol=4.356085e+06
assign (segid " A" and resid 264 and name HN) (segid " A" and resid 261 and name HA) 2.459 0.756 0.756 weight 1.000 ! spec=Expt_1, no=1202, id=987, vol=4.143397e+05
assign (segid " A" and resid 264 and name HE1) (segid " A" and resid 261 and name HA) 2.375 0.705 0.705 weight 1.000 ! spec=Expt_3, no=920, id=3288, vol=2.552798e+06
assign (segid " A" and resid 264 and name HD1) (segid " A" and resid 261 and name HA) 1.953 0.477 0.477 weight 1.000 ! spec=Expt_3, no=943, id=3305, vol=8.270168e+06
assign (segid " A" and resid 264 and name HN) (segid " A" and resid 262 and name HN) 3.149 1.239 1.239 weight 1.000 ! spec=Expt_4, no=1564, id=5171, vol=3.118457e+06
assign (segid " A" and resid 264 and name HN) (segid " A" and resid 263 and name HA) 2.851 1.016 1.016 weight 1.000 ! spec=Expt_4, no=350, id=4509, vol=5.658400e+06
assign (segid " A" and resid 264 and name HN) (segid " A" and resid 263 and name HB2) 2.636 0.868 0.868 weight 1.000 ! spec=Expt_4, no=625, id=4738, vol=9.066810e+06
assign (segid " A" and resid 264 and name HN) (segid " A" and resid 264 and name HD1) 2.072 0.537 0.537 weight 1.000 ! spec=Expt_1, no=1389, id=1125, vol=1.154916e+06
assign (segid " A" and resid 264 and name HZ) (segid " A" and resid 264 and name HE1) 2.044 0.522 0.522 weight 1.000 ! spec=Expt_3, no=727, id=3193, vol=6.295020e+06
assign (segid " A" and resid 264 and name HD1) (segid " A" and resid 264 and name HA) 2.356 0.694 0.694 weight 1.000 ! spec=Expt_3, no=908, id=3281, vol=2.682548e+06
assign (segid " A" and resid 264 and name HD1) (segid " A" and resid 264 and name HB1) 2.472 0.764 0.764 weight 1.000 ! spec=Expt_3, no=5301, id=4225, vol=2.008915e+06
assign (segid " A" and resid 264 and name HN) (segid " A" and resid 264 and name HA) 2.346 0.688 0.688 weight 1.000 ! spec=Expt_4, no=349, id=4508, vol=1.823755e+07
assign (segid " A" and resid 264 and name HA) (segid " A" and resid 267 and name HB2) 1.994 0.497 0.497 weight 1.000 ! spec=Expt_1, no=1600, id=1284, vol=1.454259e+06
assign (segid " A" and resid 264 and name HD1) (segid " A" and resid 268 and name HZ) 2.168 0.588 0.588 weight 1.000 ! spec=Expt_3, no=723, id=3189, vol=4.414286e+06

TYR 265
assign (segid " A" and resid 265 and name HB2) (segid " A" and resid 169 and name HD21) 2.105 0.554 0.554 weight 1.000 ! spec=Expt_3, no=1376, id=3521, vol=5.280169e+06
assign (segid " A" and resid 265 and name HB1) (segid " A" and resid 169 and name HD21) 2.041 0.521 0.521 weight 1.000 ! spec=Expt_3, no=1601, id=3674, vol=6.348858e+06
assign (segid " A" and resid 265 and name HN) (segid " A" and resid 169 and name HD21) 2.942 1.082 1.082 weight 1.000 ! spec=Expt_4, no=1429, id=5109, vol=4.686810e+06
assign (segid " A" and resid 265 and name HE1) (segid " A" and resid 172 and name HD1) 2.444 0.747 0.747 weight 1.000 ! spec=Expt_1, no=1432, id=1159, vol=4.290962e+05
assign (segid " A" and resid 265 and name HD1) (segid " A" and resid 172 and name HD1) 2.542 0.807 0.807 weight 1.000 ! spec=Expt_3, no=1999, id=3805, vol=1.701593e+06
assign (segid " A" and resid 265 and name HN) (segid " A" and resid 261 and name HA) 3.148 1.239 1.239 weight 1.000 ! spec=Expt_4, no=1428, id=5108, vol=3.123191e+06
assign (segid " A" and resid 265 and name HD1) (segid " A" and resid 262 and name HA) 2.331 0.679 0.679 weight 1.000 ! spec=Expt_3, no=1149, id=3440, vol=2.857467e+06
assign (segid " A" and resid 265 and name HN) (segid " A" and resid 262 and name HA) 2.467 0.761 0.761 weight 1.000 ! spec=Expt_4, no=1427, id=5107, vol=1.349141e+07
assign (segid " A" and resid 265 and name HN) (segid " A" and resid 265 and name HA) 2.282 0.651 0.651 weight 1.000 ! spec=Expt_1, no=553, id=492, vol=6.482517e+05
assign (segid " A" and resid 265 and name HN) (segid " A" and resid 265 and name HB2) 2.579 0.832 0.832 weight 1.000 ! spec=Expt_1, no=3382, id=2486, vol=3.108859e+05
assign (segid " A" and resid 265 and name HA) (segid " A" and resid 265 and name HB1) 1.847 0.426 0.426 weight 1.000 ! spec=Expt_3, no=383, id=2913, vol=1.156603e+07
assign (segid " A" and resid 265 and name HD1) (segid " A" and resid 265 and name HA) 2.048 0.524 0.524 weight 1.000 ! spec=Expt_3, no=701, id=3173, vol=6.219005e+06
assign (segid " A" and resid 265 and name HD1) (segid " A" and resid 265 and name HB1) 2.119 0.561 0.561 weight 1.000 ! spec=Expt_3, no=2564, id=3967, vol=5.069462e+06
assign (segid " A" and resid 265 and name HN) (segid " A" and resid 266 and name HN) 2.172 0.590 0.590 weight 1.000 ! spec=Expt_1, no=556, id=495, vol=8.718814e+05
assign (segid " A" and resid 265 and name HD1) (segid " A" and resid 266 and name HN) 2.470 0.763 0.763 weight 1.000 ! spec=Expt_1, no=1435, id=1161, vol=4.031519e+05
assign (segid " A" and resid 265 and name HN) (segid " A" and resid 267 and name HN) 2.996 1.122 1.122 weight 1.000 ! spec=Expt_4, no=1424, id=5106, vol=4.199377e+06

HIS 266
assign (segid " A" and resid 266 and name HN) (segid " A" and resid 263 and name HA) 2.560 0.819 0.819 weight 1.000 ! spec=Expt_1, no=1399, id=1133, vol=3.252858e+05
assign (segid " A" and resid 266 and name HD2) (segid " A" and resid 263 and name HA) 2.433 0.740 0.740 weight 1.000 ! spec=Expt_3, no=911, id=3284, vol=2.211431e+06
assign (segid " A" and resid 266 and name HN) (segid " A" and resid 265 and name HB2) 2.290 0.656 0.656 weight 1.000 ! spec=Expt_1, no=1398, id=1132, vol=6.342528e+05
assign (segid " A" and resid 266 and name HE1) (segid " A" and resid 265 and name HE1) 2.642 0.873 0.873 weight 1.000 ! spec=Expt_3, no=1145, id=3439, vol=1.348394e+06
assign (segid " A" and resid 266 and name HN) (segid " A" and resid 266 and name HA) 2.177 0.593 0.593 weight 1.000 ! spec=Expt_1, no=643, id=564, vol=8.588533e+05
assign (segid " A" and resid 266 and name HN) (segid " A" and resid 266 and name HB2) 2.076 0.539 0.539 weight 1.000 ! spec=Expt_1, no=645, id=566, vol=1.143582e+06
assign (segid " A" and resid 266 and name HA) (segid " A" and resid 266 and name HB2) 2.319 0.672 0.672 weight 1.000 ! spec=Expt_1, no=2473, id=1844, vol=5.879256e+05
assign (segid " A" and resid 266 and name HD2) (segid " A" and resid 266 and name HB2) 2.267 0.642 0.642 weight 1.000 ! spec=Expt_3, no=955, id=3307, vol=3.382864e+06
assign (segid " A" and resid 266 and name HN) (segid " A" and resid 266 and name HD2) 2.820 0.994 0.994 weight 1.000 ! spec=Expt_4, no=1431, id=5110, vol=6.047154e+06
assign (segid " A" and resid 266 and name HN) (segid " A" and resid 267 and name HN) 2.134 0.569 0.569 weight 1.000 ! spec=Expt_1, no=646, id=567, vol=9.691905e+05

ALA 267
assign (segid " A" and resid 267 and name HN) (segid " A" and resid 263 and name HA) 3.053 1.165 1.165 weight 1.000 ! spec=Expt_4, no=1438, id=5114, vol=3.756182e+06
assign (segid " A" and resid 267 and name HN) (segid " A" and resid 264 and name HA) 2.604 0.847 0.847 weight 1.000 ! spec=Expt_4, no=1439, id=5115, vol=9.756704e+06

assign (segid " A" and resid 267 and name HN) (segid " A" and resid 266 and name HB2) 2.422 0.733 0.733 weight 1.000 ! spec=Expt_1, no=3352, id=2460, vol=4.538884e+05
assign (segid " A" and resid 267 and name HA) (segid " A" and resid 267 and name HB2) 1.703 0.363 0.363 weight 1.000 ! spec=Expt_1, no=303, id=273, vol=3.747488e+06
assign (segid " A" and resid 267 and name HN) (segid " A" and resid 267 and name HB2) 1.781 0.397 0.397 weight 1.000 ! spec=Expt_1, no=1245, id=1017, vol=2.866185e+06

PHE 268
assign (segid " A" and resid 268 and name HD1) (segid " A" and resid 172 and name HH2) 1.862 0.434 0.434 weight 1.000 ! spec=Expt_3, no=2089, id=3853, vol=1.099207e+07
assign (segid " A" and resid 268 and name HZ) (segid " A" and resid 202 and name HA) 2.258 0.637 0.637 weight 1.000 ! spec=Expt_1, no=2826, id=2020, vol=6.912772e+05
assign (segid " A" and resid 268 and name HE1) (segid " A" and resid 202 and name HA) 2.166 0.586 0.586 weight 1.000 ! spec=Expt_3, no=904, id=3277, vol=4.445149e+06
assign (segid " A" and resid 268 and name HZ) (segid " A" and resid 202 and name HD11) 2.146 0.576 0.576 weight 1.000 ! spec=Expt_3, no=1044, id=3372, vol=4.694118e+06
assign (segid " A" and resid 268 and name HZ) (segid " A" and resid 235 and name HD13) 1.881 0.442 0.442 weight 1.000 ! spec=Expt_3, no=1051, id=3378, vol=1.035527e+07
assign (segid " A" and resid 268 and name HN) (segid " A" and resid 267 and name HN) 2.028 0.514 0.514 weight 1.000 ! spec=Expt_1, no=607, id=540, vol=1.314176e+06
assign (segid " A" and resid 268 and name HN) (segid " A" and resid 267 and name HB2) 2.043 0.522 0.522 weight 1.000 ! spec=Expt_1, no=1293, id=1052, vol=1.257847e+06
assign (segid " A" and resid 268 and name HD1) (segid " A" and resid 267 and name HB2) 1.994 0.497 0.497 weight 1.000 ! spec=Expt_3, no=1052, id=3379, vol=7.305105e+06
assign (segid " A" and resid 268 and name HN) (segid " A" and resid 267 and name HA) 2.746 0.943 0.943 weight 1.000 ! spec=Expt_4, no=357, id=4513, vol=7.083109e+06
assign (segid " A" and resid 268 and name HN) (segid " A" and resid 268 and name HD1) 2.201 0.606 0.606 weight 1.000 ! spec=Expt_1, no=2321, id=1773, vol=8.039502e+05
assign (segid " A" and resid 268 and name HE1) (segid " A" and resid 268 and name HD1) 1.708 0.365 0.365 weight 1.000 ! spec=Expt_3, no=724, id=3190, vol=1.844732e+07
assign (segid " A" and resid 268 and name HD1) (segid " A" and resid 268 and name HA) 1.910 0.456 0.456 weight 1.000 ! spec=Expt_3, no=828, id=3251, vol=9.435532e+06
assign (segid " A" and resid 268 and name HD1) (segid " A" and resid 268 and name HB1) 2.145 0.575 0.575 weight 1.000 ! spec=Expt_3, no=2723, id=3989, vol=4.712456e+06
assign (segid " A" and resid 268 and name HN) (segid " A" and resid 268 and name HA) 2.593 0.840 0.840 weight 1.000 ! spec=Expt_4, no=356, id=4512, vol=9.998482e+06
assign (segid " A" and resid 268 and name HN) (segid " A" and resid 268 and name HB1) 2.425 0.735 0.735 weight 1.000 ! spec=Expt_4, no=636, id=4747, vol=1.495131e+07
assign (segid " A" and resid 268 and name HB1) (segid " A" and resid 269 and name HN) 2.344 0.687 0.687 weight 1.000 ! spec=Expt_1, no=3362, id=2470, vol=5.515807e+05

SER 269
assign (segid " A" and resid 269 and name HN) (segid " A" and resid 172 and name HZ2) 2.845 1.012 1.012 weight 1.000 ! spec=Expt_4, no=1449, id=5117, vol=5.727868e+06
assign (segid " A" and resid 269 and name HN) (segid " A" and resid 268 and name HA) 2.758 0.951 0.951 weight 1.000 ! spec=Expt_4, no=359, id=4515, vol=6.909089e+06
assign (segid " A" and resid 269 and name HN) (segid " A" and resid 268 and name HD1) 2.924 1.069 1.069 weight 1.000 ! spec=Expt_4, no=1451, id=5118, vol=4.865188e+06
assign (segid " A" and resid 269 and name HN) (segid " A" and resid 269 and name HB2) 2.260 0.638 0.638 weight 1.000 ! spec=Expt_4, no=641, id=4751, vol=2.282372e+07
assign (segid " A" and resid 269 and name HN) (segid " A" and resid 270 and name HN) 2.966 1.099 1.099 weight 1.000 ! spec=Expt_4, no=139, id=4354, vol=4.467866e+06

GLY 270
assign (segid " A" and resid 270 and name HN) (segid " A" and resid 269 and name HA) 2.466 0.760 0.760 weight 1.000 ! spec=Expt_1, no=808, id=676, vol=4.069590e+05

ALA 271
assign (segid " A" and resid 271 and name HN) (segid " A" and resid 270 and name HA1) 2.538 0.805 0.805 weight 1.000 ! spec=Expt_1, no=937, id=774, vol=3.421554e+05
assign (segid " A" and resid 271 and name HN) (segid " A" and resid 271 and name HB2) 1.953 0.477 0.477 weight 1.000 ! spec=Expt_1, no=1089, id=892, vol=1.651380e+06
assign (segid " A" and resid 271 and name HN) (segid " A" and resid 271 and name HA) 2.509 0.787 0.787 weight 1.000 ! spec=Expt_1, no=1097, id=899, vol=3.667325e+05
assign (segid " A" and resid 271 and name HA) (segid " A" and resid 271 and name HB2) 1.775 0.394 0.394 weight 1.000 ! spec=Expt_3, no=401, id=2928, vol=1.464528e+07

GLN 272
assign (segid " A" and resid 272 and name HE21) (segid " A" and resid 271 and name HN) 2.291 0.656 0.656 weight 1.000 ! spec=Expt_1, no=928, id=768, vol=6.330243e+05
assign (segid " A" and resid 272 and name HN) (segid " A" and resid 271 and name HA) 2.083 0.542 0.542 weight 1.000 ! spec=Expt_4, no=366, id=4522, vol=3.724162e+07
assign (segid " A" and resid 272 and name HN) (segid " A" and resid 272 and name HB1) 2.105 0.554 0.554 weight 1.000 ! spec=Expt_1, no=756, id=639, vol=1.050975e+06
assign (segid " A" and resid 272 and name HN) (segid " A" and resid 272 and name HG2) 2.044 0.522 0.522 weight 1.000 ! spec=Expt_1, no=1144, id=937, vol=1.255290e+06
assign (segid " A" and resid 272 and name HN) (segid " A" and resid 272 and name HA) 2.634 0.867 0.867 weight 1.000 ! spec=Expt_4, no=365, id=4521, vol=9.100428e+06

APPENDIX III: Hydrogen bond restraints list for α-actinin-4 CH2 domain

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LEU 170  HN    LYS 166  O
LEU 171  HN    GLU 167  O
TRP 172  HN    GLY 168  O
CYS 173  HN    LEU 169  O
GLN 174  HN    LEU 170  O
ARG 175  HN    LEU 171  O
THR 177  HN    CYS 173  O
TRP 192  HE1   ASN 187  O
ALA 197  HN    ASP 194  O
ASN 199  HN    GLY 195  O
ASN 199  HD22  ASN 223  O
ALA 200  HN    LEU 196  O
LEU 201  HN    ALA 197  O
ILE 202  HN    PHE 198  O
HIS 203  HD1   TYR 180  OH
ARG 204  HN    ALA 200  O
HIS 205  HN    LEU 201  O
ILE 210  HN    ARG 206  O
LYS 214  HN    GLU 211  O
LEU 215  HN    GLU 211  O
ASN 223  HN    ASP 219  O
LEU 224  HN    PRO 220  O
ASN 225  HN    VAL 221  O
ASN 226  HN    THR 222  O
ASN 226  HD21 ASN 199  OD1
ALA 227  HN    ASN 223  O
PHE 228  HN    LEU 224  O
GLU 229  HN    ASN 225  O
VAL 230  HN    ASN 226  O
ALA 231  HN    ALA 227  O
GLU 232  HN    PHE 228  O
TYR 234  HN    VAL 230  O
ILE 237  HN    ALA 231  O
VAL 247  HN    ALA 243  O
ASN 248  HN    GLU 244  O
THR 249  HG1   THR 249  O
ALA 256  HN    ASP 253  O
ILE 257  HN    ASP 253  O
MET 258  HN    GLU 254  O
TYR 260  HN    ALA 256  O
VAL 261  HN    ILE 257  O
SER 262  HN    MET 258  O
PHE 264  HN    TYR 260  O
TYR 265  HN    VAL 261  O
HIS 266  HN    SER 262  O
PHE 268  HN    PHE 264  O
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