CHARACTERISATION OF HUMAN HOLOCARBOXYLASE SYNTHETASE

by

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Abstract

Biotin (also known as vitamin H or vitamin B7) is an essential micronutrient that is utilised by all living organisms. A key enzyme in the lifecycle of biotin is biotin protein ligase (BPL). BPL catalyses the attachment of biotin onto the biotin-requiring enzymes, a family of enzymes that play important roles in essential pathways such as fatty acid synthesis, gluconeogenesis and amino acid metabolism. Whilst there is a catalytic core conserved among the BPLs of all species, the N-terminal region of the molecule varies considerably in length and function. Mammalian BPL, commonly referred to as holocarboxylase synthetase, contains a large N-terminal extension not present in prokaryotes. The function and significance of this N-terminal extension is not understood. The aim of this project was to examine the function of the N-terminal portion of human holocarboxylase synthetase (referred to as HCS).

To date, no crystal structure for HCS has been reported. Insights into the enzyme have come from mutagenesis and proteolytic mapping of eukaryotic BPLs. For example, yeast BPL has a large N-terminal extension analogous to HCS. Previous work in our laboratory has investigated yeast BPL by mapping the domain structure using limited proteolysis. It has been shown that the N-terminus plays a critical role in catalysis as its removal renders the enzyme inactive (Polyak *et al.*, 1999). A similar approach has been applied to map the domain boundaries in HCS. Work by others in our laboratory showed the N-terminal extension of HCS consisted of up to three domains: Met^{1}-Ala^{80}, Ala^{80}-Glu^{151} and Lys^{160}-Asp^{311}, joined by two protease sensitive linker regions between Glu^{151} to Lys^{160}, and Lys^{312} to Glu^{336}.

In this study four polyclonal antibodies, raised against peptide epitopes from different regions of HCS, were affinity purified for characterisation. All antibodies detected multiple

isoforms of HCS by Western Blot analysis upon extracts of human embryonic kidney cells. Mass spectrometry determined that these isoforms varied at their N-terminus.

To investigate if the HCS isoforms are subjected to differential localisation into subcellular compartments, three approaches were attempted: immunocytochemistry, overexpression of GFP-tagged HCS and subcellular fractionation followed by Western Blotting. All methods showed all isoforms of HCS localised primarily in the cytosolic fraction, implying that processing of the protein alone is not responsible for determining HCS localisation.

A novel approach to examine potential protein binding partners, based on the work of Choi-Ree *et al*. (2004), using an engineered HCS variant was attempted. Whilst no novel binding partners were identified, studies with this HCS variant revealed intramolecular interactions occurring between the N- and C-terminal portions of the enzyme, which was confirmed by Yeast Two Hybrid Assay.

Multiple Carboxylase Deficiency (MCD) is a disease caused by a defect in biotin metabolism. The more severe neonatal form of the disease is caused by reduced HCS activity, which subsequently affects biotinylation of all the biotin-dependent enzymes.
To examine the critical role of the N-terminus of HCS in biotin metabolism, two fibroblast cell lines isolated from MCD patients who responded poorly to biotin therapy were investigated. Genotyping revealed that the patients were homozygous for the L216R mutation in the N-terminal extension. The patients' fibroblasts had a reduced proliferation rate compared to wildtype cells, and when grown in biotin-deficient media, were less able to respond to the re-addition of biotin. Whilst the HCS mRNA transcript was readily detected in MCD cell lines, protein and enzyme activity could not be detected, implying mutation of this single residue does have drastic effects on protein stability and function. Overexpression studies revealed that wildtype and mutant proteins localise to the same cellular compartments but enzyme activity was severely compromised for HCS-L216R

and could not be elevated by additional biotin. Furthermore, the turn-over rate for the mutant protein was double that of wildtype HCS. These results help to provide a molecular explanation for the incomplete biotin responsiveness of the MCD condition in patients with the HCS-L216R mutation, and imply that this region of the enzyme is critical for HCS function.

Finally, HCS has been implicated in other cellular processes, such as histone modification. Biotinylation is a new class of histone modification which has been reported in the literature. The most common method of analysis of histone biotinylation is the use of streptavidin as a probe, exploiting the strong non-covalent interaction of avidin for biotin. However studies presented in the thesis show that streptavidin should not be used for analysis of histone biotinylation, as it binds to histones independently of biotin. In a biotin-depletion cell culture system, the amount of streptavidin-reactive material in histone extracts, as examined by Western Blotting, was not affected by biotin availability. This was despite the biotin-depletion treatment reducing cell viability and biotinylation of biotin-dependent carboxylases. Blocking biotin binding sites on streptavidin with free biotin prior to Western Blot analysis did not affect histone binding. In contrast, the biotin-containing protein Pyruvate Carboxylase, was not detected by streptavidin that was pre-incubated with biotin. Finally, cells grown in biotin deficient media supplemented with ^{14}C labelled biotin failed to incorporate biotin onto histone proteins, whereas biotin incorporation onto biotin-dependent carboxylases was readily detected. These results suggest that histone biotinylation may be an artefactual observation, and that streptavidin is not an appropriate tool to measure the dynamics of biotin transfer on and off histone proteins. Analysis with methods which do not rely on secondary detection systems, such as mass spectrometry, will provide the most powerful evidence for histone biotinylation occurring as a physiologically relevant modification.

Statement of Originality

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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Signed

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Lisa Marie Bailey

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List of Abbreviations

aa	amino acid
Ab	antibody
ACC	acetyl CoA carboxylase
AD	activation domain
Amp	ampicillin
AP	alkaline phosphatase
APS	ammonium persulphate
ATP	adenosine triphosphate
BirA	biotin inducible repressor A
BCA	bicinchoninic acid
BCCP	biotin carboxyl carrier protein
BLAST	basic local alignment search tool
BME	beta-mercaptoethanol
bp	base pair
BPL	biotin protein ligase
BSA	bovine serum albumin
C-	carboxyl-
Cα	central carbon atom
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
cHCS	C-terminal amino acids 315-726 of HCS
C-HCS	C-terminal antibody to holocarboxylase synthetase
DAPI	4’,6-diamidino-2-phenylindole
Daxx	Death domain associated protein
DBD	DNA binding domain
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco’s modified eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxynucleotide triphosphate
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol-O,O’-bis-[2-amino-ethyl]-N,N,N’,N’,-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
Eu	europium
FCS	fetal calf serum
G418	geneticin

GFP	green fluorescent protein
hr	hour
HCS	human holocarboxylase synthetase
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
3-HIA	3-hydroxyisovaleric acid
hPC107	107 amino acids encoding the biotin domain of human pyruvate carboxylase
HRP	horseradish peroxidase
IP	immunoprecipitation
K_a	affinity constant
K_M	Michaelis constant
kb	kilobase pair
kDa	kilodalton
LB	Luria broth
LC-ESI-MS	Liquid chromatography electrospray ionisation tandem mass spectrometry
LplA	lipoyl ligase
m	metre
M	molar
μ	micron
mA	milliampere
MAb	monoclonal antibody
Min	minute, minutes
MCC	methylcrotonyl-CoA carboxylase
MCD	multiple carboxylase deficiency
MOPS	3-morpholinopropanesulfonic acid
MS	mass spectrometry
MW	molecular weight
MWCO	molecular weight cut-off
n	nano
N-	amino-
N1	n-terminal antibody 1 to holocarboxylase synthetase
N2	n-terminal antibody 2 to holocarboxylase synthetase
N3	n-terminal antibody 3 to holocarboxylase synthetase
nHCS	N-terminal amino acids 1-314 of holocarboxylase synthetase
NHS-	N-succinimidyl ester
NLS	nuclear localisation sequence
NMR	nuclear magnetic resonance
$OD_{x\ nm}$	optical density at x nm wavelength
p	pico
PAb	polyclonal antibody
PCC	propionyl-CoA carboxylase
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline and 0.1% (v/v) Tween-20

PBMC	peripheral blood mononuclear cells
PC	pyruvate carboxylase
PCR	polymerase chain reaction
PDB	protein data bank
PFA	paraformaldehyde
PhBPL	*P.horikoshii* BPL
PKG	protein kinase G
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinyl difluoride
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
s	second
SEM	standard error of the mean
TBS	tris buffered saline
TBS-T	tris buffered saline and 0.1% (v/v) Tween-20
TEMED	N,N.N,N'N'-tetramethylethylene-diamine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
Tween-20	polyoxyethylene-sorbitan monolaurate
U	units (active)
UTR	untranslated region
V_{max}	maximum velocity
UV	ultra violet
WB	Western blot
WT	wild type
yBPL	Yeast (*S.cerevisiae*) biotin protein ligase
yPC104	104 amino acids encoding the biotin domain of yeast pyruvate carboxylase