PURIFICATION AND PROPERTIES OF NAD(P)-GLUTAMATE DEHYDROGENASE FROM PLANT TISSUE

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DOCTOR OF PHILOSOPHY

by

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SUMMARY

(1). NAD(H)-dependent glutamate dehydrogenase (NAD(H)-GDH) was purified from the green marine alga, Ulva australis. SDS-polyacrylamide gel electrophoresis showed the enzyme subunit to have a molecular size of 45 kDa, whilst the molecular size of native enzyme was 184 kDa. The enzyme displayed both NAD(H)- and NADP(H)-dependent activities with NAD(H) being the preferred substrate (i.e. the ratio of the NAD(H)/NADP(H) dependent activities being of the order of 4:1). Unlike higher plant NAD(H)-GDH, the activity of the enzyme isolated from Ulva was not affected by the addition of Ca^{2+} or of the calcium chelater, EGTA. The NAD(H)-GDH had pH optima of 8.0 and 9.2 for the amination and deamination reactions respectively. The apparent Km values for glutamate, 2-oxoglutarate, ammonia (as ammonium sulphate at pH 8.0), NAD and NADH were 14.7, 1.39, 37.0, 0.21 and 0.16 mM respectively.

(2). NADP(H)-dependent glutamate dehydrogenase (NADP(H)-GDH) was purified from the green alga Bryopsis maxima. The enzyme displayed both NADP(H)- and NAD(H)-dependent activities with NADP(H) being the preferred substrate (i.e. the ratio of NADP(H)/NAD(H) dependent activities being of the order of 18:1). SDS-polyacrylamide gel electrophoresis showed the enzyme subunit had a molecular size of 46 kDa. The molecular size of the

native enzyme was 280 kDa. These results suggest that the NADP(H)-GDH of Bryopsis maxima has a hexameric subunit structure.

(3). NAD(H)-glutamate dehydrogenase (NAD(H)-GDH) was purified from turnip storage root (Brassica rapa L.) and its properties examined. The enzyme was found to be associated with the mitochondria and located entirely within the mitochondrial matrix compartment. The enzyme displayed both NAD(H)- and NADP(H)-dependent activities with NAD(H) being the preferred substrate i.e. the ratio of the NAD(H)/NADP(H)-dependent activities were of the order of 11:1. The pH optima for the amination and deamination reactions were 8.0 and 9.5 respectively. The apparent Km values for glutamate, 2-oxoglutarate, ammonia (as ammonium sulphate at pH 8.0), NAD and NADH were 28.6, 2.0, 22.2, 0.25, and 0.09 mM respectively under optimum pH conditions. The Km values for the substrates varied depending upon the assay pH, except for NAD(H) which did not change significantly. NAD(H)-GDH activity was activated by the bivalent cations Ca^{2+}, Mn^{2+} and Zn^{2+}. Calcium was the most effective cation for activation. The deamination reaction was fully activated by 7-8 μM Ca^{2+}, however, 60 μM Ca^{2+} was required to fully activate the enzyme when catalysing the amination reaction. The enzyme was completely inactivated by the addition of EGTA, but activity was fully restored by the addition of excess Ca^{2+}. The

enzyme was purified 346 fold and the molecular size of the enzyme sub-unit was estimated to be 43 kDa by SDS-PAGE.

(4). The interaction between Ca^{2+} and ammonia on the activity of NADH-GDH from turnip was examined. Adding 4 μM Ca^{2+} was sufficient to fully activate the enzyme in the presence of 15 mM $(NH_4)_2SO_4$. Although enzymic activity was apparent without added Ca^{2+}, under low substrate conditions (10 mM $(NH_4)_2SO_4$ and 20 μM NADH), this activity was completely suppressed by adding EGTA (200μM). The activity could be recovered by subsequent additions of Ca^{2+}. These results suggest that small amounts of Ca^{2+} in the assay solution elicit some enzyme activity but added Ca^{2+} was essential for maximal enzyme activity. It is possible that Ca^{2+} concentrations (μM or perhaps nM levels) may regulate the activity of GDH, <u>in situ</u>.

The sensitivity of the enzyme to Ca^{2+} was affected by proteolysis. After thermolysin treatment, lower concentrations of Ca^{2+} were required to fully activate the enzyme or overcome the inhibitory effect of high ammonium, compared to non-treated enzyme; however, the thermolysin-treated enzyme was still sensitive to EGTA.

NADH-GDH activity could also be activated by the addition of high concentrations of NaCl (300 mM) in the absence of added Ca^{2+}. NADH-GDH activity

was inhibited about 30% by p-HMB (200 μM) under low substrate conditions, however, this residual activity was not affected by subsequent additions of EGTA. These results suggest that hydrophobic and -SH groups are important in the regulation of enzyme activity by Ca^{2+}.

(5) The effect of the transaminase inhibitor, aminooxyacetate (AOA), on glutamate oxidation was investigated using mitochondria isolated from both pea leaf and turnip root.

Glutamate oxidation, without added TPP, was inhibited about 45% in pea mitochondria and about 17% in turnip mitochondria by 1 mM AOA. These inhibitions were increased (pea-50%, turnip-30%) if TPP was present in the reaction medium. No inhibition of glutamate oxidation by AOA was observed in the presence of 2 mM malonate.

These results indicate that glutamate is initially oxidized by NAD-GDH in isolated mitochondria. Further conversion of the product, 2-oxoglutarate (2-OG), via the TCA cycle supplies OAA which contributes to glutamate metabolism via asparate aminotransferase (AsAT). Therefore, GDH is essential for glutamate oxidation in isolated mitochondria and the degree of inhibition of glutamate oxidation by AOA is strongly affected by

the relative velocity of TCA cycle conversions subsequent to 2-OG dehydrogenase.

DECLARATION

The investigations described in this thesis were performed in the Botany Department, University of Adelaide from March, 1985 to March, 1989. The following paper was written and published during the period of study:

(1) Purification and properties of NAD-glutamate dehydrogenase from turnip mitochondria, by T. Itagaki, I.B. Dry and J.T. Wiskich, Phytochem. (1988) 27: 3373-3378.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text. I consent to this thesis being made available for photocopying and loan.

TADASHI ITAGAKI

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ABBREVIATIONS

ADP	adenosine diphosphate
AOA	aminooxyacetate
Asp	aspartate
AsAT	aspartate aminotransferase
BSA	bovine serum alubumin
Chl	chlorophyll
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol (β aminoethylether) N, N' tetraacetic acid
GDH	glutamate dehydrogenase
Glu	glutamate
GOGAT	glutamate synthase
GS	glutamine synthetase
GSH	reduced glutathione
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
MDH	malate dehydrogenase
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
OAA	oxaloacetate
2-OG	2-oxoglutarate
2-OG DH	2-oxoglutarate dehydrogenase
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
p-HMB	p-hydroxymercuribenzoate

p-CMB	p-chloromercuribenzoate
p-CMPSA	p-chloromercuriphenylsulfonic acid
PVP	polyvinyl pyrrolidone
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SDH	succinate dehydrogenase
Tes	N-tris (hydroxymethyl)-methyl-2-aminoethanesulphonic acid
TPP	thiamine pyrophosphate
Tris	tris (hydroxymethyl) aminomethane

CHAPTER I

GENERAL INTRODUCTION

1.1 *Function of glutamate dehydrogenase (GDH)*

Ammonia assimilation is a very important aspect of plant metabolism and it has been thought that NAD(H)-glutamate dehydrogenase (GDH), which catalyses the reductive amination of 2-OG to glutamate (see below), must be a key enzyme in this metabolic pathway.

$$\text{2-oxoglutarate} + NH_3 + \text{NADH} + H^+ \overset{GDH}{\rightleftharpoons} \text{glutamate} + NAD^+ + H_2O$$

However, more recent research has suggested that the primary assimilation of inorganic nitrogen in plants occurs mainly via the glutamine synthetase (GS) /glutamate synthase (GOGAT) pathway (see below), because the Km of GS for ammonia is much lower than that of GDH (Lea and Miflin, 1974; Miflin and Lea, 1977). Thus, the discovery of the GS/GOGAT pathway has obscured the physiological function of GDH in plant tissues.

$$\begin{array}{ccc} \text{glutamate} + NH_3 + \text{ATP} & \overset{GS}{\rightleftharpoons} & \text{glutamine} + \text{ADP} + \text{Pi} \\ \uparrow & & \downarrow \\ 2\ \text{glutamate} + Fd_{ox} & \overset{GOGAT}{\longleftarrow} & \text{glutamine} + \text{2-oxoglutarate} + Fd_{red} \end{array}$$

Alternatively, it has been suggested that the main function of GDH <u>in vivo</u> may be the oxidative deamination of glutamate (Miflin and Lea, 1977). Isolated plant mitochondria

can utilize glutamate as an oxidizable substrate, however, it is thought that both aspartate aminotransferase (AsAT) and GDH may be involved. For example, Yamaya and Matsumoto (1985) reported that glutamate oxidation by mitochondria isolated from corn and pea shoots was inhibited over 60% by the transaminase inhibitor, aminooxyacetate (AOA). They concluded that the major route of glutamate oxidation proceeded via transamination rather than direct deamination by GDH. This supported their previous data suggesting that GDH was involved in glutamate formation (Yamaya et al. 1984).

Large amounts of NH_3 are released during the photorespiratory conversion of glycine to serine in the leaf mitochondria of C_3 plants in the light. Many studies have demonstrated that this NH_3 is also reassimilated via the GS/GOGAT system (Miflin and Lea, 1977; Berger et al. 1985; Wallsgrove et al. 1987; Givan et al. 1988), but an involvement of mitochondrial GDH has not been completely eliminated. Yamaya et al. (1984) have suggested that GDH may have an NH_3-assimilation role ancillary to the GS/GOGAT system. Their estimation of the in vivo concentrations of Ca^{2+} and NADH within the mitochondrial matrix suggested that NADH-GDH would be in the activated configuration, favoring NH_3 assimilation. In contrast, a role for GDH in NH_3 reassimilation during mitochondrial glycine metabolism could not be demonstrated by Day and Wiskich (1981).

Neeman et al. (1985), using N^{15} nuclear magnetic resonance techniques detected NH_3 assimilation via both

cytosolic GS and mitochondrial GDH in Nicotiana protoplasts. However, these experiments were performed in the dark with added glycine; conditions under which the chloroplast GS enzyme would be inoperative. Thus, while it is possible to establish conditions which show an involvement of GDH, these conditions are quite different from physiological 'photorespiratory' conditions. This is well demonstrated by studies using mutants lacking chloroplast GS which only survive under non-photorespiratory conditions (Wallsgrove et al. 1987). Thus, one can conclude that GDH does not have a major or significant role in the refixation photorespiratory ammonia.

Srivastava and Singh (1987) have recently reviewed the role and regulation of GDH in plants under various environmental and physiological conditions. They concluded that the enzyme may be important in the assimilation of ammonia under conditions of stress, such as dark starvation, high temperature, salinity, water stress, environmental pollution, senescence and other abnormalities.

1.2 *Species/cellular distribution and co-enzyme specificity*

Glutamate dehydrogenase has been found in many species of higher plant, algae, bacteria, and number of vertebrate and invertebrates of the animal kingdom (Stewart et al. 1980; Srivastava and Singh, 1987).

The GDH enzyme in plant tissues has been found to be located both in mitochondria (Bone 1959; Joy 1973; Ehmke and Hartmann 1976; Lea and Thurman, 1972; Mc Kenzie and Lees, 1981; Prunkard et al. 1986) and in chloroplasts (Lea and Thurman, 1972; Gayler and Morgan, 1976; Rathnam and Edwards, 1976; Lees and Dennis, 1981; Mc Kenzie and Lees, 1981; Prunkard et al. 1986).

Nauen and Hartmann (1980) reported that NAD(H)-GDH is located exclusively in the mitochondrial matrix, however, Yamaya et al. (1984) have suggested that NAD(H)-GDH may, in fact, be loosely associated with the inner mitochondrial membrane. Chloroplastic NADP(H)-GDH could not be released by osmotic shock of isolated chloroplast (Leech and Kirk, 1968; Lea and Thurman, 1972) and required detergent treatment to isolate it from lettuce leaves (Lea and Thurman, 1972) indicating the enzyme is tightly bound to the thylakoid membrane. In contrast, the enzyme in the green alga Caulerpa simpliciuscula was readily released as a soluble enzyme from the chloroplast fraction by gentle treatments such as suspension in low osmotic media (Gayler and Morgan, 1976).

It is possible to isolate both particulate and soluble enzyme forms from extracts of plant tissues (Bone, 1959; Davies and Teixeira, 1975; Chou and Splittstoesser, 1972). However, the two enzymes usually have the same properties and it may be that the soluble form is simply derived from mitochondria (the chloroplastic enzyme being tightly bound to thylakoid membranes) damaged during

isolation (Bone, 1959; Davies and Teixeira, 1975). In contrast, Chou and Splittstoesser (1972) reported on the separation of a soluble and a particulate GDH enzyme from pumpkin cotyledons and suggested that the two enzyme forms were not identical. This was based on their different response to inhibition by ammonium and activation by metal ions. Their results seems to indicate that GDH occurs in at least two cellular compartments in pumpkin cotyledons.

The enzyme distribution pattern is somewhat more complex in algae. The NAD(H)-GDH of Chlorella sorokiniana was shown to be located within the mitochondria whereas NADP(H)-GDH enzyme activity induced by ammonium treatment was found to be chloroplastic (Bascomb and Schmidt, 1987). The NADP(H)-GDH of Euglena gracilis is located in the cytoplasm (Fayyaz-Chaudhary et al. 1985) and, interestingly, this enzyme is completely repressed if the cells are grown on ammonium (Parker et al. 1985).

Both mitochondrial and chloroplastic forms of the enzyme demonstrate co-enzyme specificity, however, the mitochondrial enzyme shows greater activity with NAD(H) while the chloroplastic enzyme prefers NADP(H) as shown in Table 1.1. Experiments using mixed substrates have shown that for both the mitochondrial and chloroplastic enzymes, NADH and NADPH bind to the same active site on the enzyme indicating that each enzyme shows true dual co-enzyme specificity (Stewart et al. 1980). However, the relative activity of the enzyme, with each co-enzyme, changes markedly during enzyme

purification. Crude enzyme extracted from Caulerpa simpliciuscula showed an NADPH/NADH activity ratio of 9:1, which increased to 75:1 after purification (Gayler and Morgan, 1976).

1.3 *The biochemistry of GDH isolated from plant tissues*

1.3.1 *Effects of ions*

The amination reaction of higher plant NADH-GDH has been shown to be activated by certain species of bivalent cations such as Ca^{2+}, Mn^{2+}, Zn^{2+} (Joy, 1973; Garland and Dennis, 1977; Kindt et al. 1980; Furuhashi and Takahashi, 1982; Yamaya et al. 1984), but not by Mg^{2+} (Garland and Dennis, 1977; Joy, 1973). Ca^{2+} was the most effective cation and high concentrations were not deleterious to enzyme activity (Joy, 1973; Furuhashi and Takahashi, 1982; Yamaya et al. 1984). The concentration of Ca^{2+} required for maximal stimulation of enzymic activity depended on the storage conditions, pH and substrate concentrations in the assay medium (Joy, 1973; Garland and Dennis, 1977; Kindt et al. 1980; Ehmke and Hartmann, 1978). On the the other hand, increasing concentrations of chaotropic anions were found to inactivate GDH activity (Pahlich et al. 1978; Kindt et al. 1980).

The metal-chelating agent, EGTA, inhibits NADH-GDH activity, but activity was regained on the addition of an excess amount of a bivalent cation (Ehmke and Hartmann, 1976; Fawole and Boulter, 1977). EGTA-dependent inhibition could

also be partially overcome (56%) by adding 360 mM Na_2SO_4 (Kindt et al. 1980) whereas high concentrations of K_2SO_4 did not restore any activity.

A number of studies have reported that the deamination reaction of GDH is not affected by the concentration of bivalent cations (Garland and Dennis, 1977; Furuhashi and Takahashi, 1982; Yamaya et al. 1984). In contrast, Ehmke and Hartmann (1976) demonstrated a reversal of EGTA inhibition of the deamination reaction with either Ca^{2+} or Mg^{2+} with Lemna minor GDH. Based on further kinetic studies Ehmke and Hartmann (1978) have suggested that the Ca^{2+} concentration governs an equilibrium between a catalytically inactive form (Ca^{2+} free) and an active (Ca^{2+} saturated) form of the enzyme.

Chou and Splittstoesser (1972) reported that the soluble enzyme found in pumpkin cotyledons was activated by metal ions while the particulate form of the enzyme was not. Similarly, the NAD(H)-GDH from safflower seedlings did not respond to treatment by bivalent cations or EGTA (Errel et al. 1973), nor did the NADP(H)-GDH isolated from Bryopsis maxima (Nishizawa et al. 1978).

1.3.2 *Km values*

Km values for the various substrates have been determined for GDH enzymes isolated from a variety of plant tissues (Table 1.2). The affinity for glutamate and ammonia is particularly important with respect to the physiological

function of this enzyme. Although GDH appears to have a slightly better affinity for glutamate than for ammonia, it is also apparent that this affinity for glutamate is not sufficiently high to support a significant involvement of this enzyme in glutamate oxidation *in vivo*.

The Km values shown in Table 1.2 are highly dependent on the assay conditions i.e. co-substrate concentrations and pH (Stone *et al*. 1979; Nagel and Hartmann, 1980; Lees and Dennis, 1981; Davies and Teixeira, 1975; Mc Kenzie *et al*. 1981; Yamaya *et al*. 1984, Furuhashi and Takahashi, 1982; Meredith *et al*. 1979). It is also still unknown as to whether the true substrate for GDH is NH_4^+ or NH_3. Furuhashi and Takahashi (1982) observed a decrease in the apparent Km of tobacco-callus GDH for NH_4Cl when the pH was raised from 7.3 to 9.0. However, when re-calculated in terms of the actual NH_3 concentration, the Km value was found to increase with increasing assay pH. Therefore, they were unable to determine whether the true substrate for tobacco callus GDH was NH_3 or NH_4^+.

It can be seen in Table 1.2 that the NADP(H)-GDH enzymes isolated from algal species *Caulerpa simpliciuscula* (Gayler and Morgan, 1976), *Chlorella sorokiniana* (Tischner, 1984; Bascomb and Schmidt, 1987) and *Euglena gracilis* (Javed and Merrett, 1986) have a high affinity for ammonia suggesting a role *in vivo* in ammonia assimilation. However, Km estimates alone can not be used to determine the enzyme's physiological function. For example, Parker *et al*. (1985)

have shown that Euglena gracilis had high NADP-GDH activity when grown on glutamate as nitrogen source but the activity was completely repressed in cells grown on ammonium. Fayyaz-Chaudhary et al. (1985) have also suggested this cytoplasmic NADP(H)-GDH has a catabolic function in Euglena generating ammonia from glutamate under conditions of nitrogen limitation.

1.3.3 *Molecular weight*

The molecular weights of NAD(P)-GDH from both higher plants and algae have been estimated by a number of different methods including gel electrophoresis, gel filtration and equilibrium sedimentation (Table 1.3).

The molecular weight of NAD(H)-GDH isolated from higher plants was estimated to be of the order of 230-270 kDa (Table 1.3). Determination of subunit molecular weight using SDS-polyacrylamide gel electrophoresis (PAGE) suggests the enzyme to be hexameric in structure with subunit molecular weight of approximately 46 kDa (Table 1.3). The hexameric nature was also confirmed by the appearance of six electrophoretic bands after cross-linking with diimidates (Kindt et al. 1980).

In algae the enzyme appears to be more variable in size. NAD(H)-GDH from Chlorella sorokiniana (Meredith et al. 1978) was found to have a molecular weight of 180 kDa, being composed of four identical subunits with a molecular weight of 45 kDa. Similarly, the NADP(H)-GDH from Euglena gracilis

appears to be a tetramer of 45 kDa sub-units with a total molecular mass of 180 kDa (Javed and Merrett, 1986), whereas the molecular weight of ammonium-inducible NADP(H)-GDH from Chlorella sorokiniana (Gronostajski et al. 1978) was estimated to be 290 kDa for the native enzyme. Recently, Prunkard et al. (1986) suggested that the NADP(H)-GDH of Chlorella sorokiniana is initially synthesized as a larger precursor protein (58.5 kDa) and further work by this group (Bascomb and Schmidt, 1987) has found evidence for two distinct isoenzymes; composed of either the α- (55.5 kDa) or β- (53 kDa) subunits.

1.3.4 *pH optima*

The pH optima of GDH activities from various plant tissues is shown in Table 1.4. In general, the pH optimum for the amination reaction is around 8 and for the deamination reaction around 9. This 1 pH unit difference between the amination and deamination reactions may indicate that pH has a significant role in regulating the direction of the enzyme activity. However, while such large changes in pH may occur in the stroma of chloroplasts, it is doubtful that similar changes would occur in the mitochondrial matrix.

1.3.5 *Isozymes*

GDH isozymes, having different mobilities, have been separated by gel electrophoresis with the highly purified enzymes from pea seeds and lupin nodules showing 7

and 4 bands respectively. However, the enzyme from both pea seeds and lupin nodules showed only one single subunit peptide band after SDS-PAGE, suggesting that isozymes differ in charge, but not in molecular size (Stone et al. 1979; Kindt et al. 1980).

Isozyme patterns have also been recorded for other plant species. For example; a pattern of 2 bands was detected with soybean; 6 bands with groundnut and 7 bands with mungbean, corn (Yue, 1969), pea and broad bean (Thurman, 1965). Multiple enzyme forms have been found to be associated with both tissue and organelle extracts (Mc Kenzie and Lees, 1981; Lea and Thurman, 1972; Nagel and Hartmann, 1980). Isozyme patterns and enzymic activity are also highly influenced by such factors as plant age (Thurman et al. 1965; Yue, 1969; Chou Splittstoesser, 1972), growth conditions such as the light regime (Barash et al. 1976), extracellular concentrations of ammonia (Kanamori et al. 1972; Barash et al. 1975; Laurière and Daussant, 1983), amino acids (Sahulka, 1972), and sugars (Nauen and Hartmann, 1980) and exposure to stress (Laurière et al. 1981; Postius and Jacobi, 1976).

1.3.6 *Heat stability*

NAD(H)-GDH is a relatively heat stable enzyme; the pea root enzyme surviving short periods of treatment at 60°C (Pahlich and Joy, 1970) while GDH from safflower seedlings was incubated for 10 min at 75°C without appreciable loss of activity (Errel et al. 1973). This property has encouraged

the use of a heat treatment step (55-70°C for 5-25 min) in the isolation of GDH from various plant tissues (Meredith et al. 1978; Chou and Splittstoesser, 1972; Garland and Dennis, 1977; Fawole and Boulter, 1977). Recently, Ehmke et al. (1984) have shown that inactivation by prolonged heat treatment of the purified pea seed NAD(H)-GDH was reduced in the presence of NADH, dithioerythritol and Ca^{2+} and that the seven isozymes present were converted into a more stable form during incubation.

In comparison to the relative heat stability of NAD(H)-GDH, the NADP(H)-GDH enzyme is heat sensitive. The activity of NADP(H)-GDH from Chlorella sorokiniana decreased by approximately 50% in 3-4 min at 60°C (Gronostajski et al. 1978) and the enzyme activity of Bryopsis maxima was almost completely inactivated by heating at 50°C for 10 min (Nishizawa et al. 1978).

1.3.7 *Presence of thiol groups*

There have been a number of reports showing that thiol poisons inhibit GDH isolated from both algae and higher plants suggesting the presence of thiol groups essential to enzyme activity. Stability of purified NAD(H)-GDH from pea seeds was markedly improved in the presence of a reducing agent 2-mercaptoethanol. Activity lost in the absence of reducing agents was almost completely restored upon incubation with 2-mercaptoethanol (Kindt et al. 1980). Whilst similar properties have been reported for the GDH isolated

from soybean seed and leaf (chloroplastic enzyme), the enzyme isolated from soybean root (mitochondrial enzyme) was unaffected by 2-mercaptoethanol or glutathione (Mc Kenzie and Lees, 1981). Another exception appears to be the GDH activity isolated from lupin nodules which was not affected by 10 min at 30°C with 0.1 mM p-CMB (Stone et al. 1979). Apart from these few exceptions, the general pattern of results suggests that -SH groups have an important role in NAD(H)-GDH activity. For example, inhibition by -SH poisons and recovery by reducing agents has been reported for the enzymes isolated from pea seedlings (Yamasaki and Suzuki, 1968), pumpkin cotyledons (Chou and Splittstoesser, 1972), safflower (Errel et al. 1973), pea mitochondria (Davies and Teixeira, 1975), and cowpea (Fawole and Boulter, 1977).

NADP(H)-GDH from the alga, Bryopsis maxima, was inhibited strongly by p-CMB (Nishizawa et al. 1978). Furthermore, dithiothreitol stabilised NADP(H)-GDH during preparation from Chlorella sorokiniana, however, its presence (10 mM) during storage at -20°C caused a loss in activity (of both the purified and crude enzyme; Gronostajski et al. 1978). Based on titration data using the Ellman reagent (5,5'-dithiobis (2-nitrobenzoic acid)), Gronostajski et al. (1978) suggested that there are six cysteines per subunit in this enzyme from Chlorella sorokiniana and that all of the cysteine residues exist in the free sulphydryl form.

1.4 *Present study*

The initial aim of this project was to investigate the properties of NAD(P)-GDH in the marine alga (Ulva australis). However, due to frequent and unpredictable shortages of algal material, it became necessary to modify the project to include a more readily available tissue.

As very little is known about the enzymes involved in nitrogen metabolism in storage tissues, I therefore decided to broaden this study to include turnip storage root (Brassica rapa L.). The basic experimental protocol was as follows;

(1). determination of the general characteristics of GDH of the various plant tissues.

(2). comparison of the properties of these enzymes.

(3). investigation of the role of Ca^{2+} in the regulation of GDH activity.

(4). determination of the relative roles of GDH and aminotransferase enzyme in glutamate oxidation by isolated mitochondria.

Table 1.1 Coenzyme specificity of NAD(P)H-GDH enzymes isolated from various plant source.

Source	NADH/NADPH	Reference
Mitochondria		
Vicia fava	10:1	1
Lettuce leaves	7:1	2
Pea roots	17:1	3
Mung bean seedlings		4
Pea seedlings	20:1	5
Lemna minor	12:1	6
Chlorella pyrenoidosa	5:1	7
Chloroplast		
Vicia fava	1:6	8
Lettuce leaves	1:1	2
Pea	6:1	9
Chlorella pyrenoidosa	1:33	7
Caulerpa simpliciuscula	1:9	10
Chlamydomonas reinhardtii	1:1	11

1. Fawole and Boulter, (1977); 2. Lea and Thurman, (1972); 3. Joy, (1973); 4. Bone, (1959); 5. Davies and Teixeira, (1975); 6. Ehmke and Hartmann, (1976); 7. Talley *et al*. (1972); 8. Leech and Kirk, (1968); 9. Tsenova, (1972); 10. Gayler and Morgan; (1976). 11. Fischer and Klein (1988).

Table 1.2 Km values for the substrates of NAD(P)-GDH.

References for Table 1.2:

1. Kindt et al. (1980); 2. Yamazaki and Suzuki, (1969); 3. Davies and Teixeira, (1975); 4. Pahlich and Joy, (1971); 5. Garland and Dennis, (1977); 6. Fawole and Boulter, (1977); 7. King and Wu, (1971); 8 Chou and Splittstoesser, (1972); 9. Barash et al. (1976); 10. Errel and Barash, (1973); 11. Yamaya et al. (1984); 12. Stone et al. (1979); 13. Stewart and Rhodes, (1977); 14. Ehmke and Hartmann, (1976); 15. Nagel and Hartmann, (1980); 16. Lea and Thurman, (1972); 17. Javed and Merrett, (1986); 18. Gronostajski et al. (1978); 19. Bascomb and Schmidt (1987); 20. Tischner, 1984; 21. Meredith et al. (1978); 22. Shatilov and Kretorich, (1977); 23. Nishizawa et al. (1978); 24. Gayler and Morgan, (1976).

Source		Km (mM)					
		Glu	2-OG	NH_4^+ salt	NAD(P)	NAD(P)H	Ref.
Pea seed	NAD	9.3	2.3	52.6	0.23	0.032	1
Pea seedling	NAD	1.6	-	-	0.041	-	2
Pea epicotyl	NAD	3.5	0.62	70	0.590	0.026	3
Pea root	NAD	7.3	3.3	38	0.65	0.86	4
Pea stem	NAD	12.5	5.6	68	0.24	0.92	5
Cowpea	NAD	16.7	1.79	28.6	0.285	0.059	6
Soybean cotyledon	NAD	8.0	1.2	9.4	0.21	0.015	7
Pumpkin cotyledon	NAD	-	-	24	-	0.036	8
	NAD	-	-	30	-	0.022	
Oat leaf	NAD	-	5	55	-	0.2	9
	NAD	-	4.3	44	-	0.11	
Safflower seedling	NAD	8	4	35.4	0.26	0.065	10
Corn shoot	NAD	-	2.22	12.5	-	0.063	11
Lupin nodule	NAD	14.7	9.1	59	0.26	0.133	12
Lemna minor	NAD	2.5	1.5	29	0.18	0.015	13
Lemna minor	NAD	12	3.3	27	0.46	0.11	14
Medicago seed	NAD	51.3	2.6	70.1	0.349	0.721	15
root	NAD	28.4	3.42	83.4	0.722	0.775	
Lettuce leaf	NAD	-	-	12.36	-	0.42	16
	NADP	-	-	5.2	-	0.118	
Euglena gracilis	NADP	-	1.4	0.4	-	0.18	17
Chlorella sorokiniana	NADP	32	12	68.0	0.038	0.13	18
NADP	(α-)	38.2	-	0.02-3.3	0.040	0.10	19
NADP	(β-)	32.3	-	75	0.031	0.14	
	NADP	-	-	50 (μM)	-	-	20
	NAD	60	2	40	0.15	0.15	21
Chlorella pyrenoidosa	NAD	5	1.25	41	0.143	0.034	22
	NADP	11	0.35	9	0.012	0.018	
Bryopsis	NADP	5.2	1.4	23.8	0.04	0.14	23
Caulerpa	NADP	3.8	6.7	0.67	-	-	24

Table 1.3 The molecular size of NAD(H)-GDH.

Source	Total	Subunit	Reference
Pea epicotyl	230 (kDa)		1
Oat leaves	220-230		2
Pumpkin cotyledon	250		3
Vigna unguiculata		51 (kDa)	4
Barely seed		46	5
Lupin nodules	270	45	6
Pea seeds	260	44	7

1. Davis and Teixeira, (1975); 2.Barash *et al*. (1976); 3. Chou and Splittstoesser, (1972); 4. Fawole and Boulter, (1977); 5. Stewart *et al*. (1980; Fentem unpublished); 6. Stone *at al*. (1979); 7. Kindt *et al*. (1980).

Table 1.4 pH optima for various GDH preparations.

Source		Deamination reaction	Amination reaction	Ref.
Chlorella sorokiniana	NAD	9.1	8.0	1
	NADP	9.2	7.2	2
Bryopsis maxima	NADP	8.43	7.65	3
Euglena gracilis	NAD	-	8.0	4
Caulerpa simpliciuscula	NADP	-	7.5-8.5	5
Lemna minor		9.2	8.2	6
Safflower cotyledon		8.9	8.2	7
Pea seedling		10.0	7.5	8
Pea root		8.0	8.0	9
Pea seeds		9.1-9.2	8.1	10
Pea stem		9.2	8.0	11
Pea (chloroplast)		-	7.8-8.4	12
Lettuce (mitochondria)	NAD	-	8.0-8.4	13
(chloroplast)	NADP	-	7.6	
	NAD	-	8.0	
Lupin nodules		8.8	8.2	14
Cowpea seedlings		10.0	8.0	15
Alfalfa roots		9.2	8.0-8.2	16

1. Meredith *et al*. (1978); 2. Gronostajski *at al*. (1978); 3. Nishizawa *at al*. (1978); 4. Javed and Merrett (1986); 5. Gayler and Morgan (1976); 6. Stewart and Rhodes (1977); 7. Errel *et al*. (1973); 8. Yamasaki and Suzuki (1969); 9. Pahlich and Joy (1971); 10. Kindt *et al*. (1980); 11. Garland and Dennis (1977); 12. Tsenova (1972); 13. Lea and Thurman (1972); 14. Stone *et al*. (1979); 15. Fawole and Boulter (1977); 16. Nagel and Hartmann (1980).

CHAPTER II

MATERIALS AND METHODS

2.1 *Materials*

Pea seeds (Pisum sativum cv. Massey Gem, Greenfeast) and turnip roots (Brassica rapa L.) were purchased locally. Ulva australis was collected at St. Kilda and West Lakes in Adelaide, South Australia. Bryopsis maxima was collected at Choshi on the Pacific coast of Japan. Peas were grown in vermiculite in a glasshouse for 10-15 days.

All chemicals were analytical reagent grade and obtained from B.D.H. Ltd. (Poole, U.K.). Protease XIV (Streptomyces griseus), thermolysin (Bacillus thermoproteolyous rokko) and NADP-malic enzyme (chicken liver) were obtained from Sigma Chemical Co.. Trypsin, subtilisin and NADH-MDH (pig heart) were obtained from Boehringer Mannheim Pty. Ltd.. DEAE-Sephacel, Sephadex S-300, Superose 6, PD-10 columns and Percoll were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

2.2 *Isolation of washed mitochondria*

(a) Pea leaf

Chilled leaves (100 g) were disrupted with a Polytron PTA-35 probe for 2-3 sec in 200 ml of ice-cold medium containing 0.3 M sorbitol, 50 mM Tes, 1 mM EDTA, 1 mM $MgCl_2$, 0.19 g cysteine, 1% (w/v) PVP-40 and 0.4% (w/v) BSA, adjusted to pH

7.5. The homogenate was filtered through two layers of Miracloth and centrifuged at 2,000 g for 5 min. The supernatant was centrifuged at 10,000 g for 20 min and the pellets washed by resuspending in 60 ml of 0.3 M sucrose containing 20 mM Tes and 0.1% (w/v) BSA (adjusted to pH 7.5) and recentrifuged at 10,000 g for 20 min. Final resuspension was in 5 ml of wash medium. All isolation procedures were carried out at 1-2°C.

(b) Turnip

Turnip (400 g tissue chilled for 60 min.) was homogenized with a Braun juice extractor into 44 ml of 0.2 M sucrose, 62.5 mM EGTA (pH 7.2), 0.125 M Tes-KOH (pH 7.2), 90 mM Tris, 1.1% (w/v) BSA and 0.34% (w/v) cysteine with the pH adjusted to 7.5. The homogenate was strained through a double layer of Miracloth and the filtrate centrifuged at 1,500 g for 10 min. The supernatant was centrifuged at 12,000 g for 15 min and the mitochondrial pellet resuspended in 2-3 ml of 0.4 M sucrose.

2.3 *Isolation of purified mitochondria*

(a) Pea leaf

After the second centrifugation, 5 ml of the washed mitochondrial fraction was layered onto three linear PVP gradients (35 ml of 4% (w/w) to 0%

(w/w) PVP containing 0.3 M sucrose, 20 mM Tes, and 28% (w/v) Percoll, 0.1% BSA, adjusted to pH 7.0) and centrifuged at 18,000 rpm for 30 min in a Beckman model L-7 55 ultracentrifuge using a SW-28 rotor. The mitochondrial band was removed with a Pasteur pipette and diluted with wash medium. The suspension was centrifuged at 9,000 g for 15 min twice. The final mitochondrial pellet was resuspended in 2-3 ml of wash medium.

(b) *Turnip*

After the second centrifugation, the pellet was resuspended in 9 ml of 0.4 M sucrose and layered onto three discontinuous percoll gradients (15 ml of 50% (w/v), 30 ml of 33% (w/v), 21 ml of 28% (w/v), 36 ml of 15% (w/v) containing 0.3 M sucrose, 10 mM KH_2PO_4 and 0.1% BSA, adjust to pH 7.4) and centrifuged at 18,000 rpm for 40 min in a Beckman model L-7 55 ultracentrifuge, using a SW-28 rotor. The mitochondrial band was removed with a Pasteur pipette and diluted with 0.4 M sucrose and centrifuged at 7,500 g for 15 min twice. The final mitochondrial pellet was resuspended in 2-3 ml of 0.4 M sucrose.

2.4 *Isolation of washed chloroplasts from Bryopsis maxima*

Chloroplast isolation from Bryopsis maxima was performed essentially according to Nakayama et al. (1974). Algal thalli were cut by scissors and squeezed through three layers of gauze. The green juice was dispersed in a medium containing 25 mM Tris-HCl (pH 7.5) and 0.6 M sorbitol at 4°C, and centrifuged at 1,700 g for 10 min. The precipitate obtained was washed once with the same buffer and frozen at -90°C until use.

2.5 *Oxygen uptake*

Oxygen uptake was measured polarographically with a Rank oxygen electrode (Rank Bros., Cambridge, U.K.) connected to a Rikadenki recorder (Rikadenki Kogyo Co., Ltd., Tokyo Japan). For all measurements the temperature of the reaction vessel was maintained at 25°C with a circulating water bath. Oxygen concentration of air-saturated medium was taken as 240 μM.

(a) Pea leaf mitochondria

Pea leaf mitochondria were assayed in 2.0 ml of medium containing 0.3 M sucrose, 10 mM Tes, 10 mM KH_2PO_4, 2 mM $MgCl_2$, and 0.1% (w/v) BSA, adjusted to pH 7.2.

(b) *Turnip mitochondria*

Turnip mitochondria were assayed in 2.0 ml of medium containing 0.25 M sucrose, 10 mM Tes, 10 mM KH_2PO_4, 5 mM $MgCl_2$, adjusted to pH 7.2.

2.6 *Enzyme assays*

Spectrophotomeric measurements were made with an Aminco Dw 2a spectrophotometer (American Instrument Co., Maryland, U.S.A.) or a Philips UV/VIS 88 000 spectrophotometer.

(a) *Glutamate dehydrogenase (GDH)*

NAD(P)(H)-GDH was assayed essentially according to Pahlich and Joy (1971). The standard amination reaction was carried in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate, 0.166 mM NAD(P)H, 0.213 M $(NH_4)_2SO_4$, 0.4 mM $CaCl_2$ and 0.04 % (w/v) Triton X-100. The standard deamination reaction medium contained 0.1 M Tris-HCl (pH 9.5), 100 mM glutamate, 0.4 mM NADP, 0.1 mM $CaCl_2$ and 0.04% (w/v) Triton X-100. All assays were performed at 28°C by measuring the absorption change at 340 nm using 6.22×10^3 M^{-1} cm^{-1} as the extinction co-efficient.

(b) *Fumarase*

Fumarase activity was measured in a coupled assay system with NADP-malic enzyme according to the method of Hatch (1978). The reaction mixture contained 25 mM Hepes-KOH (pH 7.5), 0.04% (w/v) Triton X-100, 5 mM KH_2PO_4, 4 mM $MgCl_2$, 0.4 mM NADP and 0.2 unit of NADP-malic enzyme in a total of 1 ml. The reaction was initiated with 10 mM fumarate and NADPH production was followed at 340 nm using 6.22×10^3 M^{-1} cm^{-1} as the extinction co-efficient.

(c) *Catalase*

Catalase was assayed using the method of Lück (1965). The reaction medium consisted of 0.1 M phosphate buffer (pH 7.0), 0.05% (w/v) H_2O_2 and 0.04% (w/v) Triton X-100. The reaction was initiated by the addition of sample and the decrease in absorbance at 240 nm was measured using 43.6 M^{-1} cm^{-1} as the extinction co-efficient.

(d) *PEP-carboxylase*

PEP-carboxylase was measured in a coupled assay with NADH-MDH. The reaction mixture contained 30 mM Hepes-KOH (pH 8.0), 6 mM $MgCl_2$, 1 mM glucose-6-phosphate, 4 mM DTT, 8 mM $NaHCO_3$, 0.2 mM NADH and 1.6 unit of NADH-MDH (from pig heart; Boehringer Mannheim Pty. Ltd.). The reaction was initiated with 1.6 mM PEP. NADH oxidation was

followed at 340 nm using 6.22×10^3 M^{-1} cm^{-1} as the extinction co-efficient.

(e) *NADH-malate dehydrogenase*

NADH-MDH was measured at 340 nm by following NADH oxidation in 200 mM Tes-KOH (pH 7.5) containing 0.2 mM NADH. The reaction was initiated with 6 mM OAA and measured by the absorption change at 340 nm using 6.22×10^3 M^{-1} cm^{-1} as the extinction co-efficient.

(f) *Aspartate aminotransferase (AsAT)*

AsAT was measured in a coupled assay with NADH-MDH. The reaction mixture contained pea or turnip mitochondria reaction medium (or 200 mM Tes-KOH, pH 7.5), 0.1% (w/v) Triton X-100, 5 µl NADH-MDH (from pig heart; Boehringer Mannheim Pty. Ltd.), 10 mM 2-OG, 0.025 mM NADH, 5 µl Antimycin A (2 mg/ml). The reaction was started with 10 mM aspartate (Asp) and the decrease in absorbance at 340 nm at 25°C was measured using 6.22×10^3 M^{-1} cm^{-1} as the extinction co-efficient.

2.7 *Enzyme extraction*

(a) *Turnip*

Washed turnip mitochondria were incubated with 0.5% Triton X-100 (w/v) at 5°C for 5 min and

centrifuged at 40,000 rpm for 30 min using a Beckman L-7 55 ultracentrifuge with Ti 50 rotor. The supernatant was passed through a Pharmacia PD-10 column equilibrated with 50 mM Tris-HCl (pH 8.0). The eluate was used as the turnip enzyme extract.

(b) Ulva australis

Ulva australis collected at St. Kilda, Adelaide, Australia, was washed with sea water and frozen in liquid nitrogen. The frozen tissue was pulverized and disrupted with a mortar and pestle, suspended in 50 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol, filtered through three layers of miracloth and centrifuged at 9,000 g for 20 min. The extract was brought to 30% ammonium sulphate saturation, centrifuged, and the supernatant adjusted to 75% saturation with this salt. This second protein precipitate was recovered by centrifugation at 9,000 g for 20 min and dissolved in 50 mM Tris-HCl (pH 8.0) containing 5 mM mercaptoethanol and dialysed overnight against this medium. The extract was centrifuged at 20,000 g for 20 min to remove insoluble material. The supernatant was passed through a Pharmacia PD-10 column which was equilibrated with 50 mM Tris-HCl (pH 8.0) to remove small molecular weight materials. This eluate was used as the enzyme extract.

2.8 *Cell fractionation*

For cell-fractionation studies the turnip homogenate prepared for mitochondrial isolation was centrifuged at 15,000 g for 15 min and the pellet resuspended in a small volume of 0.4 M sucrose. The suspension and the original supernatant were incubated with 0.04% (w/v) Triton X-100 for 5 min and passed through a Pharmacia PD-10 column equilibrated with 50 mM Tris-HCl (pH 8.0).

2.9 *Protease treatment*

Enzyme extracts were incubated with various proteolytic enzymes (Protease XIV, thermolysin, trypsin and subtilisin) at 28°C for up to 2 hours. The ratio of mitochondrial protein to protease is indicated in the legend of the Figures and Tables.

2.10 *Heat treatment*

The enzyme extracts in 50 mM Tris-HCl (pH 8.0) were heated in a thermostated water bath at the appropriate temperature for up to 60 min. An initial assay for each treatment was made before commencing heat treatment.

2.11 *Polyacrylamide gel electrophoresis*

(a) SDS-PAGE

SDS-PAGE was performed essentially according to the method of Laemmli (1970). The gel consisted of 5% acrylamide staking gel (pH 6.8) and 12% acrylamide separation gel (pH 8.8) made up in 125 mM and 375 mM Tris-HCl respectively with 0.1% SDS. The weight ratio of acrylamide:N,N'-methylenebisacrylamide was 30:0.8 in both gels. The gels were polymerized using 0.05% N,N,N',N'-tetramethylethylenediamine and 0.1% ammonium persulphate. The reservoir buffer was 25 mM Tris and 192 mM glycine (pH 8.6) containing 0.1% SDS. SDS-PAGE was performed at 5°C.

Samples were incubated in 3% SDS and 5% 2-mercaptoethanol for 30 min at room temperature. Following electrophoretic migration, the gel was stained with silver.

(b) Non-denaturing PAGE

Non-denaturing PAGE was performed as above, but in the absence of SDS using a 3% acrylamide stacking gel and a 6% acrylamide separation gel. Following electrophoretic migration at 4°C, the gel was stained either for protein or for activity.

(c) Re-electrophoresis

Following the non-denaturing PAGE, the active band was excised and incubated in 70 mM Tris-HCl (pH 8.8), 3% SDS, 6% mercaptoethanol, 1.7 M sucrose for 30 min at room temperature, and then overnight in a freezer. The gel was loaded directly onto the stacking gel of SDS-PAGE.

2.12 *Gel staining*

(a) Activity staining

At the completion of electrophoresis, NAD-GDH activity was detected in the gel with a modified tetrazolium method according to Talley et al. (1972) using 100 mM Tris-HCl (pH 9.5), 65 mM glutamate, 0.1 mM phenazine methosulphate, 0.1 mM nitro-blue tetrazolium, 0.4 mM NAD and 0.4 mM $CaCl_2$ at 28°C.

(b) Protein staining

To detect peptides, the gel was stained with Coomassie Brilliant Blue R-250 or silver.

The protein staining using Coomassie Brilliant Blue R-250 was performed by the method of Satoh et al. (1984). The gel was stained with 0.1% Coomassie Brilliant Blue R-250 (w/v) in 50% methanol and 7% acetic acid (w/v) for 15 min, and

destained overnight in 5% methanol (w/v) and 7% acetic acid (w/v).

For silver staining (Morrissey, 1981), the gel was prefixed in 50% methanol, 10% acetic acid for 30 min, followed by 5% methanol, 7% acetic acid for 30 min. After prefixation the gel was fixed in 10% glutaraldehyde for 30 min. The gel was washed for 3 hours with several changes of distilled water then soaked in 5 µg/ml DTT for 30 min. After soaking, the DTT solution was discarded and replaced with 0.1% silver nitrate for 30 min. The gel was rinsed rapidly with a small amount of distilled water and then twice rapidly with a small amount of developer (50 µl of 37% formaldehyde in 100 ml 3% sodium carbonate (w/v)). The gel was kept in the developer solution until the desired level of staining was attained. Staining was stopped by adding sufficient 2.3 M citric acid stock solution to bring the developer solution to neutral pH. The solution was then discarded and the gel was washed several times in distilled water over a 30 min period, and photographed as soon as possible.

2.13 *Hydroxyapatite preparation*

Hydroxyapatite was prepared according to the method of Tiselius (1956) in which 1 l of 0.5 M Na_2HPO_4 and 1 l of 0.5 M $CaCl_2$ was added at a speed of about 2 drops per second

using a peristaltic pump to about 300 ml of distilled water in a 3 l beaker (If stirring was required, the speed was always kept at a minimum speed to maintain homogeneous diffusion). Brushite ($CaHPO_4 2H_2O$), formed at this stage as a white precipitate, was washed four times using 3 l of distilled water each time. Whilst stirring the precipitate in about 2 l of distilled water, 50 ml of freshly made 40% (w/w) NaOH was added, and boiled for 1 hour. The process converts the Brushite into hydroxyapatite. After boiling, the solution was stirred for 5 min and allowed to set. After a relatively large, particulate precipitate had settled, the supernatant was discarded and the fine particles removed by decantation (4 times using 3 l of distilled water each time). Three litres of 0.01 M $NaHPO_4$- NaH_2PO_4 buffer, pH about 6.8) was added to the precipitate and heated to 97°C, stirred for 5 min and allowed to cool and the supernatant discarded. About 3 l of 0.01 M phosphate buffer was added to the precipitate, the suspension was stirred and boiled for 5 min and the supernatant was discarded as above. This procedure was repeated using 0.01 M phosphate buffer for 15 min once and 0.001 M phosphate buffer for 15 min twice. The last product was suspended in 0.001 M phosphate buffer. Prepared hydroxyapatite was kept in 0.001 M phosphate buffer at 5°C until used.

Note; Care was needed in the handling of hydroxyapatite, because it is a fine crystal in an aggregated form, and is very easily broken into fine particles. When preparating it or packing it into a column, the fine

particles should be removed.

2.14 *Enzyme purification*

(a) Turnip

Mitochondria were treated with 0.5% (w/v) Triton X-100 and centrifuged at 30,000 g for 15 min. Trypsin (from bovine pancreas; Boehringer Mannheim Pty. Ltd.) was added to the supernatant and incubated at 28°C for 1 hr followed by a 5 min heat treatment at 60°C (Meredith et al. 1978). Solid ammonium sulphate was then added and the fraction between 30-75% saturation was collected as a partially purified enzyme preparation. This preparation was diluted in a minimum amount of 50 mM Tris-HCl (pH 8.0) and undissolved materials were removed by centrifugation at 96,000 g for 20 min.

The supernatant fraction was loaded onto a Sephacryl S-300 column (2.6 x 70 cm) and eluted at a flow rate of 0.34 ml min^{-1} using an elution medium containing 50 mM Tris-HCl (pH 8.0). The NADH-GDH fractions were then loaded onto a hydroxyapatite column (2.6 x 7 cm; 0.34 ml min^{-1}) and eluted with a 50-300 mM phosphate buffer gradient (pH 7.2) at a flow rate of 0.34 ml min^{-1}. The NADH-GDH fraction was charged onto a DEAE-Sephacel column (2.6 x 8 cm) and eluted with a NaCl gradient (50-300 mM) at a flow rate of 0.34 ml min^{-1}.

(b) *Ulva australis*

The crude enzyme was chromatographed on a Sephacryl S-300 column (column size, 2.6 x 60 cm, flow rate, 0.42 ml min^{-1}). The active fraction was then loaded onto DEAE-Sephacel (2.6 x 11 cm, 0.36 ml min^{-1}) and the enzyme was eluted with a 0.01-0.5 M NaCl gradient. 50 mM Tris-HCl (pH 8.0),was used as the equilibration buffer for all column chromatography.

The active fraction was then further purified by SDS-free intact disk polyacrylamide gel electrophoresis (PAGE). Enzyme activity in the gel was detected by an activity stain.

(c) *Bryopsis maxima*

Frozen isolated chloroplasts were treated (Satoh et al. 1984) with a preparation buffer (50 mM Tris-HCl, 50 mM NaCl, 25 mM $MgCl_2$ 5 mM 2-mercaptoethanol; pH 7.6), disrupted by a Teflon homogenizer and centrifuged at 28,000 g for 30 min. Ammonium sulphate was added to the supernatant and the fraction between 50-75% saturation was collected as a crude enzyme preparation. The crude enzyme was subjected to gel filtration on Sephacryl S-300 (column size, 2.6 x 73 cm, 0.37 ml min^{-1}) and chromatographed on a DEAE-Sepharose CL-6B column (2.6 cm, 0.37 ml min^{-1}; NaCl gradient 0.05-0.5 M,

50 mM Tris-HCl, pH 7.6). The active fractions were subjected to gel filtration on a Superose 6 column attached to a Pharmacia FPLC system for further purification.

2.15 *Molecular weight determination*

The molecular size of the GDH sub-unit polypeptide was determined using a Bio-Rad or a Pharmacia low molecular weight (LMW) protein standards kit with SDS-PAGE.

Proteins of standard molecular weight were purchased from Pharmacia Fine Chemicals. Gel filtrationwas carried out on Sephacryl S-300 and Superose 6 columns attached to a Pharmacia FPLC system with 50 mM Tris-HCl (pH 8.0) elution buffer.

2.16 *Chemical assays*

Protein was measured by the method of Lowry et al. (1951), with $CuSO_4$ in 1% citrate (rather than tartrate) or using the Bio-Rad reagent (Coomassie dye-binding method). BSA (fraction V) was used as a standard. Chlorophyll was estimated from acetone extracts according to the method of Arnon (1949). The protein content of leaf mitochondrial suspensions was corrected for the contribution by broken thylakoids by assuming a thylakoid protein : chlorophyll ratio of 7:1 (Nash and Wiskich, 1982).

CHAPTER III

PROPERTIES OF GLUTAMATE DEHYDROGENASES ISOLATED FROM ULVA AUSTRALIS AND BRYOPSIS MAXIMA

3.1 INTRODUCTION

It is thought that the GS/GOGAT system is the major pathway for ammonia fixation in higher plants (Miflin and Lea, 1977; Lea and Miflin, 1974) and algae (Cullimore and Sims, 1981; Haxen and Lewis, 1981; Everest and Syrett, 1983; Davison and Stewart, 1984). However, it has also been reported that NADP(H)-GDH enzyme from Caulerpa simpliciuscula (Gayler and Morgan, 1976; McKenzie et al. 1979) and Chlorella sorokiniana (Tischner, 1984; Bascomb and Schmidt, 1987) show a high affinity for ammonia suggesting GDH might be functioning in nitrogen assimilation in these cells. Consequently, I decided to investigate the properties of GDH from the green algae, Ulva australis and Bryopsis maxima. General kinetic parameters have been determined for the Ulva enzyme, but because of a shortage of material, only the molecular size and coenzyme specificity of the enzyme from Bryopsis maxima was determined.

3.2 RESULTS

3.2.1 *Properties of purified NAD(H)-GDH*

NAD(H)-GDH from Ulva australis was purified 16-fold after precipitation and DEAE-Sephacel separation (Table 3.1). Further purification was performed by non-denaturing

PAGE and the subunit molecular size was determined by SDS-PAGE.

When subjected to SDS-PAGE, the enzyme subunit was shown to have a molecular size of 45 kDa (Fig. 3.1). The molecular weight of the native enzyme (as determined with Sephacryl S-300) was 184 kDa (Fig. 3.2). These results suggest that the NAD(H)-GDH of Ulva australis is composed of four identical subunits.

The molecular weight of NADP(H)-GDH from Bryopsis maxima was calculated to be 280 kDa as determined by FPLC with a Superose 6 column (Fig. 3.3). SDS-PAGE showed that the enzyme subunit molecular size was 46 kDa (Fig. 3.4) suggesting that the NADP(H)-GDH of Bryopsis maxima has a hexameric structure.

3.2.2 *Effects of bivalent cations and EGTA*

Unlike higher plant NAD(H)-GDH, the enzyme isolated from Ulva was not affected by the addition of Ca^{2+} or by 1 mM EGTA (a calcium chelater) (Table 3.2.). Addition of 0-1 mM Ca^{2+}, Mg^{2+}, Mn^{2+} had no significant effect on the activity.

3.2.3 *pH optima and Km values*

The pH optima for NAD(H)-GDH were 8.0 and 9.2 for the aminating and deaminating reactions respectively (Fig. 3.5).

The concentration of substrates giving half-maximal velocities are shown in Table 3.3. The Km for ammonia (as ammonium sulphate at pH 8.0) was found to be 47.6 mM. On increasing the assay pH from 7 to 9 the Km for ammonia decreased from 57 to 22 mM, whereas that for 2-oxoglutarate increased from 0.45 to 1.67 mM. However, the Km for NADH was relatively constant over the range of pH. The Km for glutamate and NAD was found to be 14.7 and 0.21 respectively.

The purified enzyme from Ulva australis displayed both NAD(H) and NADP(H)-dependent activity, with NAD(H) being the preferred substrate, having a NAD(H):NADP(H)-dependent activity ratio of 4:1. The ratio of the NAD(H):NADP(H)-dependent activities were 1:18 for the enzyme from Bryopsis maxima.

3.2.4 *Heat stability and trypsin sensitivity*

NAD(H)-GDH has been shown to be a heat stable enzyme in the higher plant (Pahlich and Joy, 1970) and algae (Meredith et al. 1978). The sensitivity of the enzyme from Ulva australis to heat treatment was also examined. NADH-GDH from Ulva australis was stable at 50°C for 60 min, however, the activity decreased rapidly at 60°C (Fig. 3.6.) with a half-time of decay (t 1/2) of 10-15 min. If the enzyme was incubated with NADH at 60°C, the enzyme activity was more stable with a half-time of decay of approximately 40 min.

NAD(H)-GDH from turnip root (see Chapter IV) was found to be insensitive to trypsin treatment. Therefore the

sensitivity of NAD(H)-GDH from Ulva australis to proteolytic enzyme treatment was also examined. The enzyme from Ulva australis was also found to be insensitive to trypsin treatment over 2 hours at 28°C (Fig. 3.7). In contrast, malate dehydrogenase from Ulva australis lost almost all activity over the same time period under the same experimental conditions.

3.2.5 *Sensitivity of NADH-GDH from Ulva australis to p-HMB*

It has been reported that GDH is sensitive to -SH group inhibitors such as p-CMB, p-chloromercuri-phenylsulfonic acid (p-CMPSA), p-HMB and phenylmercuric acetate (Yamasaki and Suzuki, 1968; Chou and Splittstoesser, 1972; Errel et al. 1973; Fawole and Boulter, 1977; Nishizawa et al. 1978). The sensitivity of NADH-GDH from Ulva australis to p-HMB is shown in Table 3.4. No inhibition was observed at concentrations between 0 and 50 μM p-HMB. Significant inhibition was obtained with 500 μM p-HMB, however, the degree of the inhibition did not increase at 1 mM p-HMB.

3.3 DISCUSSION

The kinetic parameters and pH optima determined for NAD(H)-GDH from Ulva australis fall within the range determined for higher plant tissues (i.e. Km of 5-70 mM; Stewart et al. 1980). The very high Km for ammonia (37.0 mM) probably means that, NAD(H)-GDH from Ulva australis may not be involved in ammonia fixation, because such high

concentrations of ammonia are unlikely to occur *in situ*. Although the measured Km value is dependent on the composition of the assay medium, it is nevertheless, always, a very high value. It is, therefore, unlikely that some peculiarity of the intracellular environment could result in a dramatic increase in the affinity of the enzyme for ammonia. Further research is needed to substantiate this point.

The intracellular location of NAD(H)-GDH in *Ulva australis* is still unknown. The existence of two distinct GDH enzymes in higher plants is now well documented; a mitochondrial enzyme which is generally referred to as NAD-linked, and a chloroplast enzyme which is generally referred to as NADP-linked (Stewart *et al*. 1980). From this general consideration, NAD(H)-GDH from *Ulva australis* could be supposed to be mitochondrial in location. However, the enzyme displayed both NAD(H)- and NADP(H)- dependent activity with NAD(H) being preferred by an order of 4:1. The purified *Ulva australis* NAD(H)-GDH had a molecular size of 184 kDa and a subunit molecular size of 45 kDa which is similar to that of NAD(H)-GDH isolated from another green alga, *Chlorella sorokiniana* (Meredith *et al*. 1978). The latter enzyme has been shown to be located in the mitochondria (Bascom and Schmidt, 1987).

NADP(H)-GDH was also isolated from the green algae *Bryopsis maxima*, which Nisizawa *et al*. (1978) has reported does not possess NAD(H)-GDH activity. For the experiments

reported here, NADP(H)-GDH was extracted from isolated chloroplasts. While the purity of the chloroplast preparation was not determined, the enzyme can be assumed to be chloroplastic. The enzyme from Bryopsis maxima has a molecular size of 280 kDa and a subunit molecular size of 46 kDa differing from chloroplastic NADP(H)-GDH of Chlorella sorokiniana which has a subunit molecular size of 53 or 55.5 kDa (Bascom and Schmidt, 1987). On the other hand, cytosolic NADP(H)-GDH from Euglena gracilis has a molecular size of 180 kDa and a subunit molecular size of 45 kDa (Fayyaz-Chaudhary et al. 1985). NADP(H)-specific GDH from Caulerpa simpliciuscula has been found to be located in the chloroplast and has a high affinity for both NADPH and ammonia (Gaylar and Morgan, 1976). This NADP(H)-GDH has suitable kinetic properties for the fixation of ammonia. The location of GDH both in Ulva australis and Bryopsis maxima needs to determined to help understand the physiological role of this enzyme in these cells.

NAD(H)-GDH from Ulva australis was found to be heat resistant as reported for the enzyme from Chlorella sorokiniana (Meredith et al. 1978). Addition of NADH improved this property and possibly NADH might stabilize the active site and protect the enzyme from denaturation as in the case of GDH from pea (Ehmke et al. 1983).

NADH-GDH from Ulva australis was inhibited only 40% of activity at the concentration of 500 μM p-HMB, however, the degree of inhibition did not increase at the

concentration of 1 mM. Therefore it is difficult to determine whether this enzyme can be classified as a classical sulfhydryl enzyme, because if the activity was sensitive to -SH inhibitor, almost complete inhibition should be observed at the concentrations tested (Day and Wiskich, 1985; Nash and Wiskich, 1983).

It has been previously shown that higher plant NADH-GDH is activated by Ca^{2+} *in vitro* (Chou and Splittstoesser, 1972; Joy, 1973 and Furuhashi and Takahashi, 1982). Ca^{2+} had no effect on the activity of *Ulva australis* NADH-GDH nor did EGTA cause any inactivation of the *Ulva australis* enzyme. These differences must represent different tertiary/quaternary arrangements between the two sets of enzymes and are probably an evolutionary response to their different growing environments.

As mentioned in the Introduction, further investigations into such parameters as enzyme location, together with physiological studies into the response of intracellular GDH levels to external concentrations of nitrogen substrates were unable to be completed due to the lack of available algal material.

Table 3.1 Purification of NAD(H)-GDH from Ulva australis.

The amination reaction was carried out in a medium containing 0.133 M Tris, 13 mM 2-oxoglutarate, 0.166 mM NADH, and 0.213 M $(NH_4)_2SO_4$.

Purification Step	Total* activity (Unit)	Total protein (mg)	S.A.* (Units/mg protein)	Recovery (%)	Purif. (fold)
5,000 g Sup.	111.0	3740	0.03	100	1
Ammonium Sulphate Ppt. (35-75%)	46.8	845	0.06	42	2
Sephacryl S-300	46.7	366	0.13	42	4
DEAE-Sephacel	17.5	46.4	0.48	16	16

*One unit is defined as the amount of enzyme necessary to oxidize 1 μmol of NADH min^{-1} at 28°C.

Table 3.2 Effect of Ca^{2+} and EGTA on the activity of NADH-GDH isolated from Ulva australis and Brassica rapa.

The amination reaction was carried out in the medium containing 0.133 M Tris, 13 mM 2-oxoglutarate, 0.166 mM NADH, and 0.213 M $(NH_4)_2SO_4$.

Treatment	NADH-GDH activity (%)	
	Ulva	Brassica
Control (No addition)	100*	100**
+ Ca^{2+} (0.4 mM)	83	791
+ EGTA (1 mM)	110	25
+ EGTA (1 mM), Ca^{2+} (0.4 mM)	107	21
+ EGTA (1 mM), Ca^{2+} (5 mM)	-	864

* 0.014 μmol NADH ox. min^{-1} was calculated as 100%.

** 0.016 μmol NADH ox. min^{-1} was calculated as 100%.

Table 3.3 Km values (mM) for NAD(H)-GDH from Ulva australis.

The standard amination reaction was measured in 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH and 0.213 M $(NH_4)_2SO_4$. The standard deamination reaction was assayed in 0.1 M Tris-HCl (pH 9.2), 100 mM glutamate and 0.4 mM NAD.

pH	Km (mM)			
	$(NH_4)_2SO_4$	$[NH_4^+]$ $[NH_3]$	2-OG	NADH
7.0	57.1	56.8, 0.3	0.45	0.071
8.0	47.6	45.1, 2.5	0.98	0.070
9.0	21.7	14.0, 7.7	1.67	0.065

Table 3.4 Sensitivity of NADH-GDH from Ulva australis to p-HMB.

The standard amination reaction was measured in 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH and 0.213 M $(NH_4)_2SO_4$.

p-HMB conc.	Relative NADH-GDH activties (%)*
None	100*
20 μM	100
50 μM	108.2
500 μM	57.4
1 mM	63.9

* 0.061 μmol NADH ox. min^{-1} mg^{-1} protein was calculated as 100%.

Figure 3.1 SDS-PAGE of purified NAD(H)-GDH from Ulva australis.

Lane A: NAD(H)-GDH from Ulva australis and B: molecular size standard.The molecular size standards were phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The gel was stained with silver as described in Materials and Method.

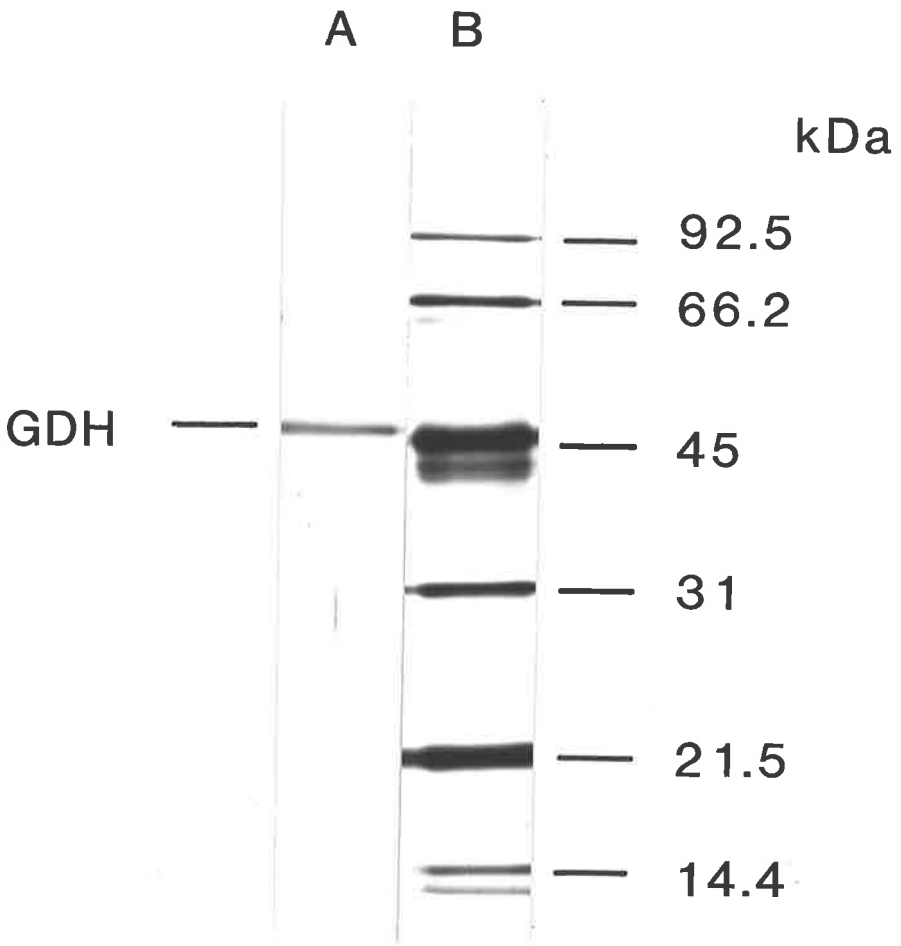

A
B
kDa
92.5
66.2
GDH
45
31
21.5
14.4

Figure 3.2 Molecular size determination of NADH-GDH from *Ulva australis*.

Molecular size was determined by gel filtration through Sephacryl S-300 with 50 mM Tris-HCl (pH 8.0). The marker proteins were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (233 kDa) and aldolase (158 kDa).

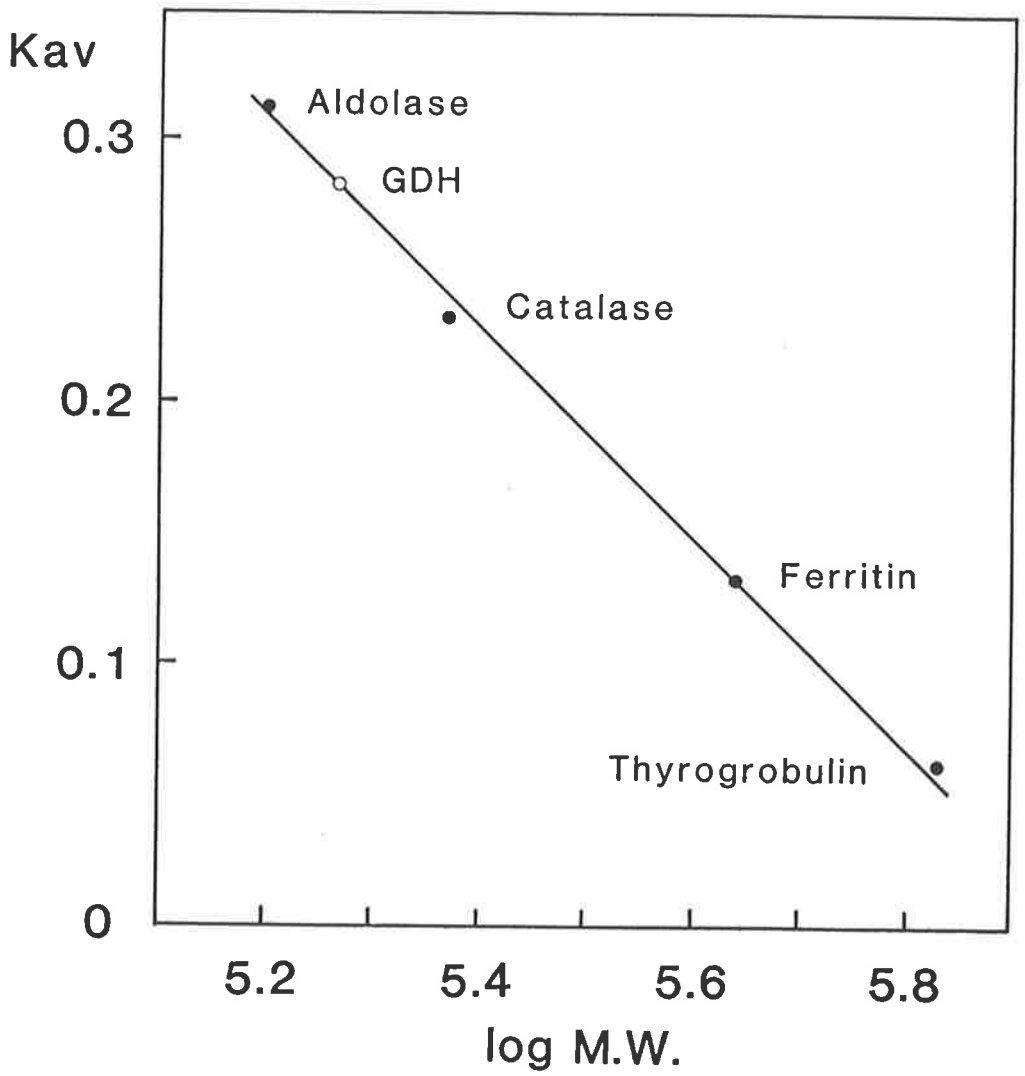

Kav
0.3
0.2
0.1
0
Aldolase
GDH
Catalase
Ferritin
Thyrogrobulin
5.2
5.4
5.6
5.8
log M.W.

Figure 3.3 Molecular size determination of NADP(H)-GDH from Bryopsis maxima.

Molecular size was determined by gel filtration on a Superose 6 column with 50 mM Tris-HCl. The marker proteins were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (233 kDa) and aldolase (158 kDa).

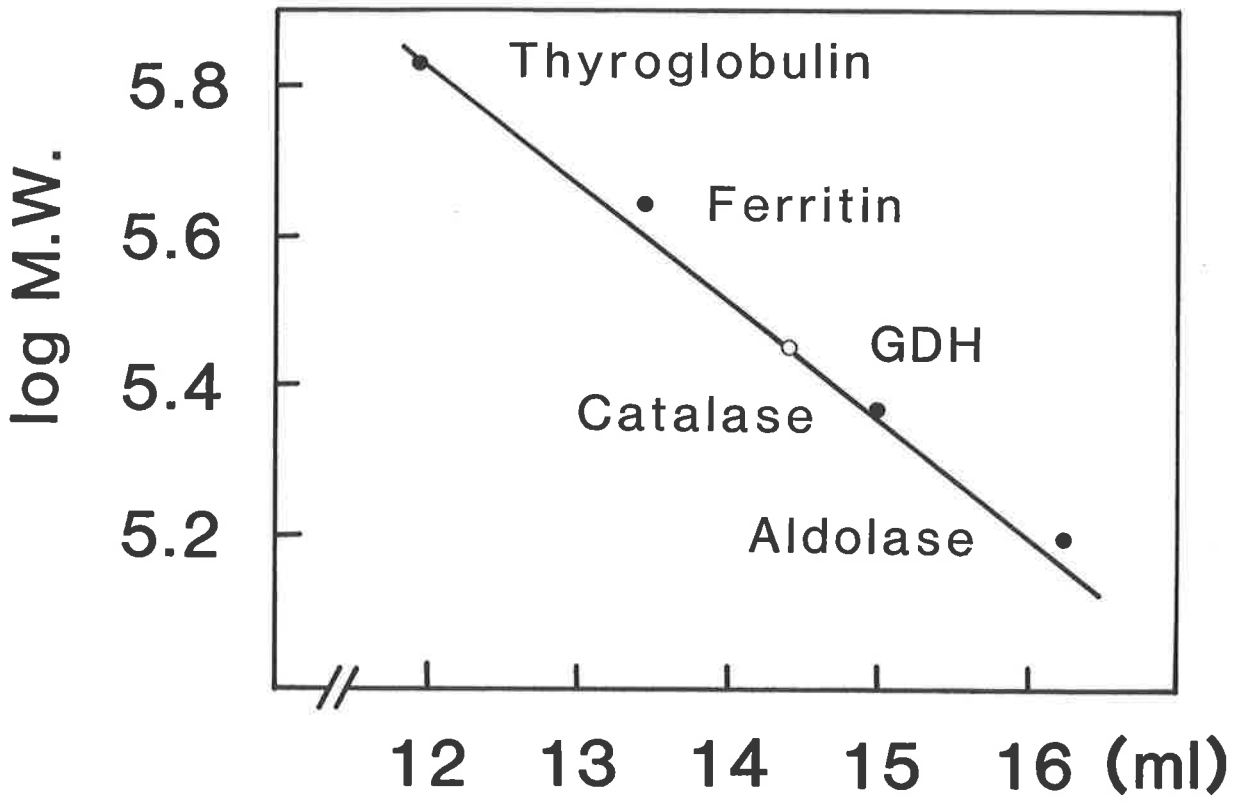

log M.W.
5.8
5.6
5.4
5.2
Thyroglobulin
Ferritin
GDH
Catalase
Aldolase
12
13
14
15
16 (ml)

Figure 3.4 SDS-PAGE of NADP-GDH from Bryopsis maxima.

The molecular size standards and experimental conditions were phosphorylase b (94 kDa), bovin serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The gel was stained by protein with silver as described in Materials and Methods.

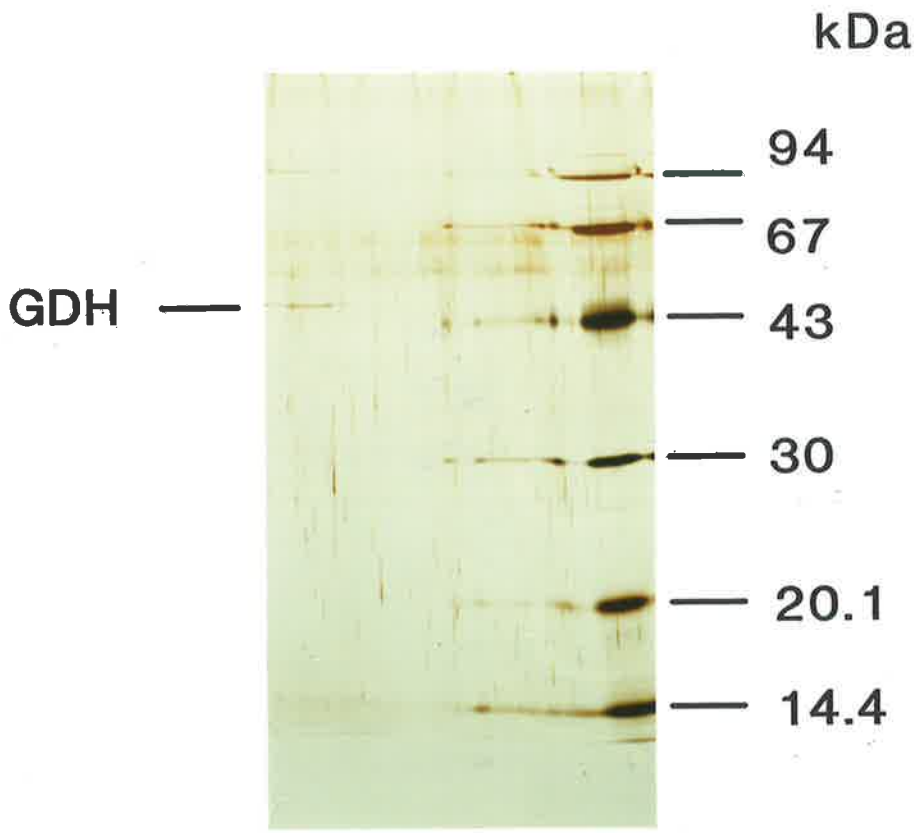

kDa
94
67
GDH
43
30
20.1
14.4

Figure 3.5 pH activity curves for NAD(H)-GDH from Ulva australis.

The amination reaction was carried out in the medium containing 0.133 M Tris, 13 mM 2-oxoglutarate, 0.166 mM NADH, and 0.213 M $(NH_4)_2SO_4$. The deamination reaction was measured in the medium containing 0.1 M Tris, 100 mM glutamate and 0.4 mM NAD. The pH was adjusted by using KOH or HCl.

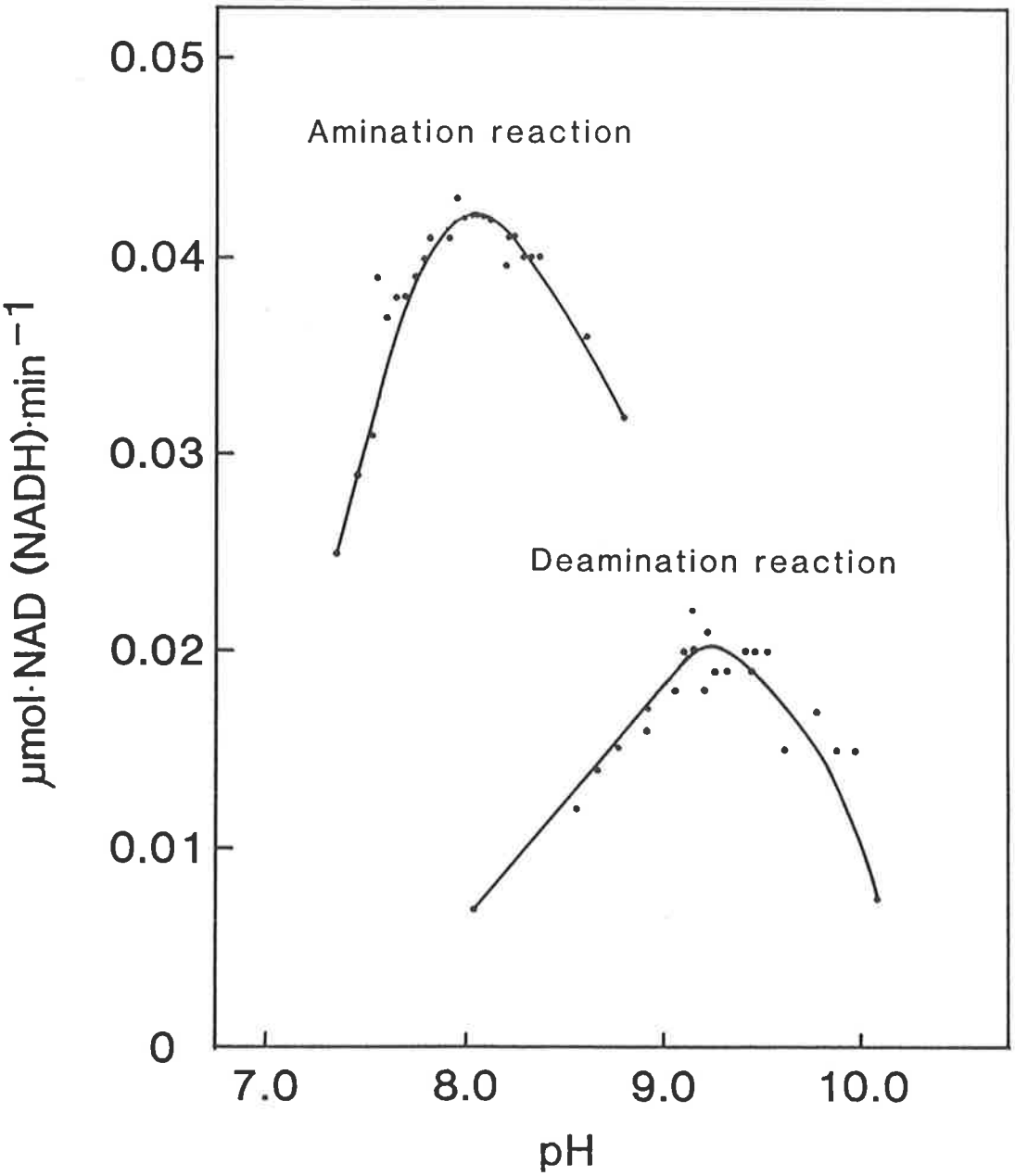

0.05
0.04
0.03
0.02
0.01
0
Amination reaction
Deamination reaction
μmol·NAD (NADH)·min⁻¹
7.0
8.0
9.0
10.0
pH

Figure 3.6 Heat stability of GDH from Ulva australis.

The enzyme was treated at 50°C (●), 60°C (▲) and 60°C with 1 mM NADH (■) for periods up to 1 hour, in 50 mM Tris-HCl (pH 8.0). NADH-GDH was assayed in 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate, 0.166 mM NADH and 0.213 M $(NH_4)_2SO_4$. The control rate (100%) was 0.3 μmol NADH ox. min^{-1} mg^{-1} protein.

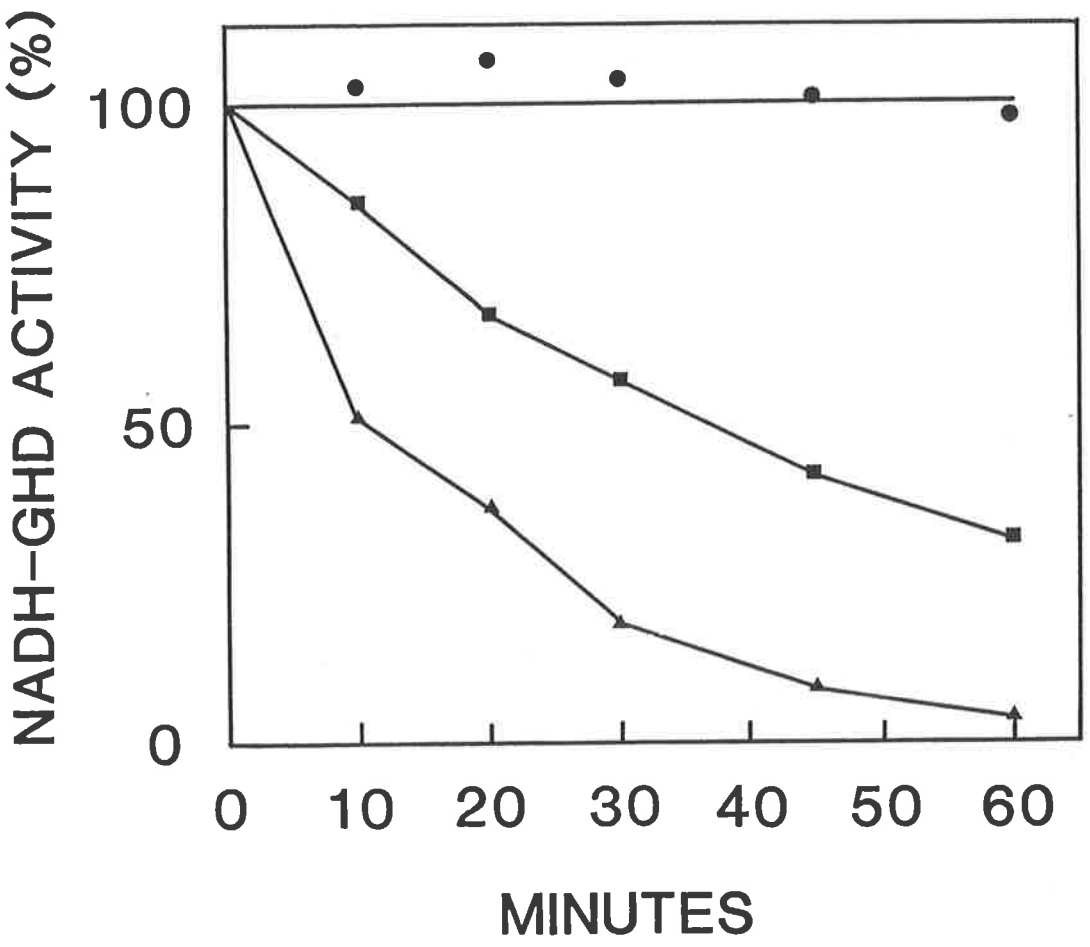

NADH-GHD ACTIVITY (%)
100
50
0
0
10
20
30
40
50
60
MINUTES

Figure 3.7 Comparitive sensitivities of NADH-MDH and NADH-GDH from Ulva australis to trypsin.

The enzymes were incubated in the presence or absence of trypsin (0.2 mg trypsin mg^{-1} protein) in 50 mM Tris-HCl (pH 8.0) at 28°C. Samples were withdrawn at various times. Assays for NADH-GDH was carried in 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate, 0.166 mM NADH and 0.213 M $(NH_4)SO_4$. NADH-MDH was assayed in 0.2 M Tes-KOH (pH 7.5), 0.2 mM NADH and 6 mM OAA.

Symbols:

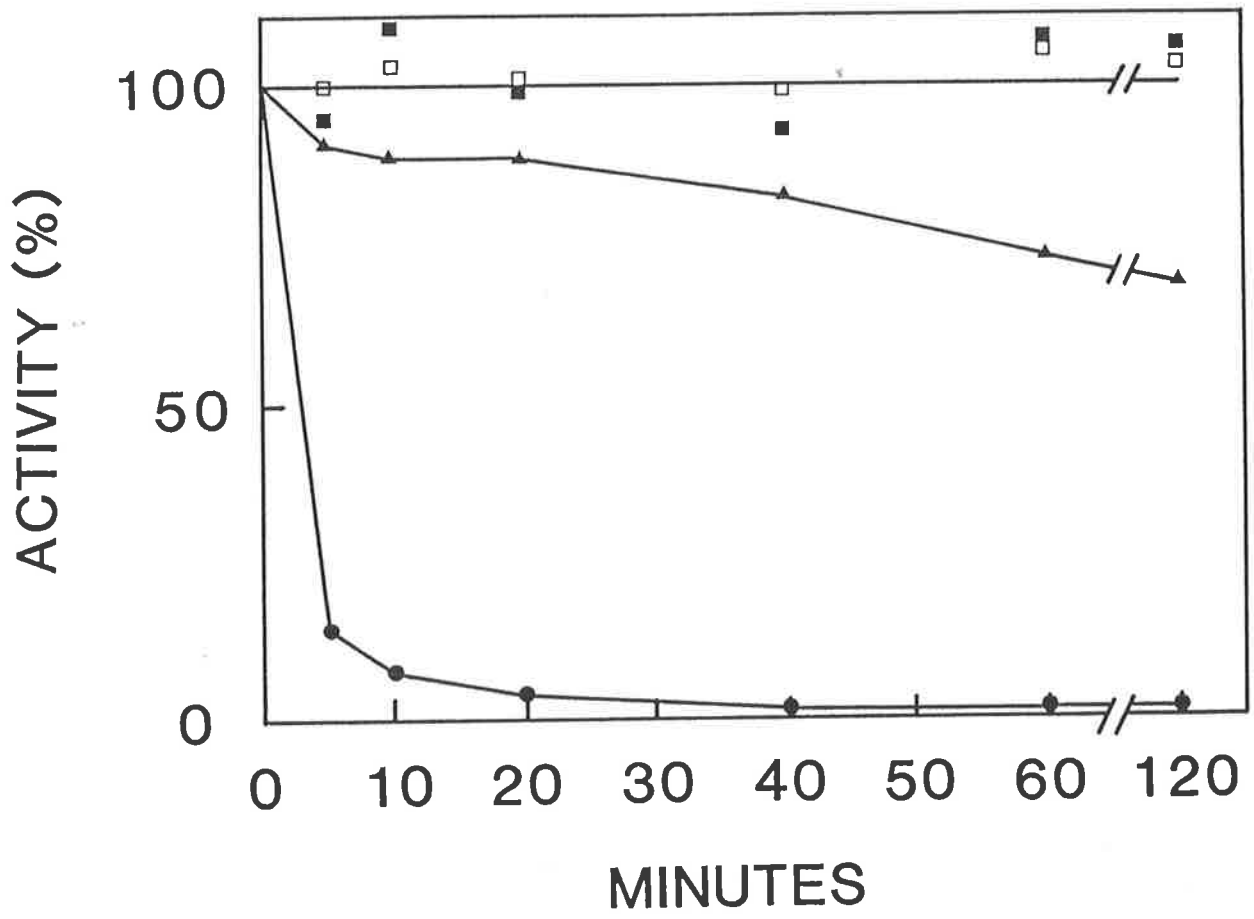
(■); NADH-GDH without trypsin

(□); NADH-GDH with trypsin

(▲); NADH-MDH without trypsin

(●); NADH-MDH with trypsin

The 100% rates were; 3.11 (NADH-MDH without trypsin), 0.07 (NADH-GDH without trypsin) μmol NADH ox. min^{-1} mg^{-1} protein.


100
50
0
ACTIVITY (%)
0
10
20
30
40
50
60
120
MINUTES

CHAPTER IV

PURIFICATION AND PROPERTIES OF NAD(H)-GLUTAMATE DEHYDROGENASE FROM TURNIP MITOCHONDRIA

4.1 INTRODUCTION

There are a number of reports on the properties of NAD(P)-GDH from various plant tissues including leaves (Lea and Thurman, 1972), epicotyls (Davies and Teixeira, 1975), roots (Pahlich and Joy, 1971), seeds (Kindt et al. 1980), cotyledons (Chou and Splittstoesser, 1972) and nodules (Stone et al. 1979). It has been also reported that NAD(H)-GDH activity from higher plant is regulated by bivalent cations (Ehmke and Hartmann, 1976; Ehmke and Hartmann, 1978; Garland and Dennis, 1977; Joy, 1973; Furuhashi and Takahashi, 1982).

It is generally claimed that the GS/GOGAT system is the major pathway for ammonia fixation in higher plants and that GDH does not play a major role in nitrogen assimilation (Miflin and Lea, 1977). However, the function of GDH still remains unclear and little is known about the enzymes involved in nitrogen metabolism in storage tissues. In this chapter, I describe the general characteristics of NAD(H)-GDH isolated and purified from turnip storage root (Brassica rapa L.).

4.2 RESULTS

4.2.1 *Enzyme localization*

The intracellular location of NAD(H)-GDH in turnip storage root was determined by differential centrifugation.

Fumarase, catalase and phosphoenolpyruvate (PEP)-carboxylase were used as marker enzymes for mitochondria, peroxisomes and cytoplasm, respectively. The results given in Table 4.1 show that NADH-GDH activity closely followed the distribution of the mitochondrial enzyme, fumarase. The NADH-GDH activity in the 15,000 g supernatant could not be pelleted at 105,000 g indicating that this activity was not associated with any other cellular membrane fraction (data not shown). The small percentage of activity in the supernatant approximated that of fumarase and so could have been released from broken mitochondria.

Table 4.2 gives a comparison of NADH-GDH activities between intact turnip mitochondria and detergent treated mitochondria. NADH can permeate the outer membrane of plant mitochondria, but cannot cross the inner mitochondrial membrane. Thus, the very low level of GDH activity observed with intact mitochondria compared to that obtained with a detergent treated fraction demonstrates that the enzyme is located almost entirely within the mitochondrial matrix compartment.

4.2.2 Kinetic properties

The general kinetic properties of NAD(H)-GDH were determined using enzyme extracted from isolated mitochondria. The pH optima were 8.0 and 9.5 for the amination and deamination reactions respectively (Fig. 4.1). The apparent Km values for glutamate, 2-oxoglutarate, ammonia (as ammonium

sulphate at pH 8.0), NAD, and NADH under various pH conditions are shown in Table 4.3 and Figs 4.2-4.6. It can be seen that whilst the Km values for glutamate, 2-oxoglutarate or $(NH_4)_2SO_4$ varied depending upon the assay pH, the Km values for NAD(H) did not. The apparent Km for $(NH_4)_2SO_4$ was calculated as 22.2 mM under low substrate conditions (13 mM 2-OG, 20 μM NADH at pH 7.4 - data not shown) which simulates the in vivo environment in the presence of Ca^{2+}. This value was approximately one third of that measured with standard substrate conditions at pH 7.2 (Km=66.7 mM, Table 4.3). The mitochondrial GDH enzyme also displayed some activity with NADP(H), however, activities were of the order of 11 times lower than those observed with NAD(H).

4.2.3 Effect of bivalent cations

NAD(H)-GDH was activated by the addition of $CaCl_2$, $MnCl_2$ and $ZnSO_4$ in both the amination and the deamination direction (Table 4.4), however, there was no effect from 0 to 1 mM of $MgCl_2$, KCl or NaCl. The most effective cation was Ca^{2+} with lower concentrations required to fully activate the enzyme than with Mn^{2+} or Zn^{2+}. The relationship between Ca^{2+} concentration and NAD(H)-GDH activity is shown in Figs. 4.7 and 4.8. The deamination activity of GDH was fully activated in the presence of 4 μM of added Ca^{2+} whereas 60 μM of added Ca^{2+} was required to give maximal enzyme activation in the amination direction. It should be noted that the distilled water used in these experiments did contain a small amount of Ca^{2+}(approx. 3 μM) and this may explain the

significant amount of deamination activity observed in the absence of added Ca^{2+} solution (note complete inhibition of deamination activity by EGTA; Table 4.5). Therefore, maximum activity of the deamination reaction is observed with 7-8 μM Ca^{2+}.

NAD(H)-GDH activity was strongly inhibited by the chelating agent, EGTA. This inhibited enzyme activity could be regained by the addition of Ca^{2+}, Mn^{2+} and, to a lesser extent (for the amination reaction only), of Zn^{2+}. Zn^{2+} was not effective at all in recovering the deaminating activity. The role of calcium in the regulation of GDH activity is considered in more detail in the next chapter.

4.2.4 *Enzyme sensitivity to -SH poison, p-HMB*

The effect of p-HMB was examined to determine whether -SH groups play a significant role in the activity of the GDH enzyme. GDH activity was inhibited approximately 20% at 10-20 μM p-HMB (Table 4.7). The degree of inhibition was only marginally increased at higher p-HMB concentrations. The effect of p-HMB on enzyme activities will be considered in more detail in the next chapter.

4.2.5 *Enzyme stability to heat treatment*

The stability of NADH-GDH to heat treatment is shown in Fig. 4.9. Over 90% of original activity was still maintained after incubation at 65°C for 1 hour. Even after the enzyme was treated at 75°C for 1 hour, over 70% of

the activity remained. If the enzyme was incubated with Ca^{2+} and/or NADH, the enzyme activity became even more stable to heat.

4.2.6 *Properties of purified NAD(H)-GDH*

Mitochondrial GDH was purified 346 fold using the steps outlined in Table 4.6. Initial treatment of the mitochondrial extract included incubation with the proteolytic enzyme, trypsin. GDH activity was found to be insensitive to trypsin treatment in contrast to other mitochondrial enzymes such as NADH-MDH (Fig. 4.10). It can be seen that trypsin treatment markedly reduced the protein concentration (Fig. 4.11-A), but there was no change in the GDH isozyme pattern (Fig. 4.11-B) as judged by the activity stain. It should also be noted that at least 4 isozyme bands were observed (Fig. 4.11-B) as has been reported for the enzyme from lupin nodules (Stone et al. 1979).

Non-denaturing PAGE of the purified enzyme revealed only one protein band which corresponded exactly with a GDH activity band as resolved by activity staining (Fig. 4.12). Only one band was apparent using activity staining in contrast to the four isozyme bands of the mitochondrial extract (Fig 4.11-B). SDS-PAGE of the purified enzyme revealed the protein to consist of subunit with a molecular size of 43 kDa (Fig. 4.13). The apparent molecular size of the native enzyme was estimated to be of the order of 300-310 kDa using either Sephacryl S-300 or Superose 6 columns

(Fig. 4.14). Inclusion of 0.05% Triton X-100 detergent in the elution buffer (50 mM Tris-HCl, pH 8.0) for Sephacryl S-300 had no effect on the estimation of molecular size. It is difficult, therefore, to clearly resolve the exact nature of the protein structure i.e. hexameric, octomeric etc. and may indicate that the enzyme possesses certain characteristics of shape or the presence of non-protein components which complicate the determination of molecular size by gel exclusion.

4.3 DISCUSSION

The results indicate that the NAD(H)-GDH enzyme of turnip storage root is located within the mitochondria as with other plant tissues (Miflin and Lea, 1977; Stewart *et al*. 1980). Nauen and Hartmann (1980) reported that NADH-GDH is located in the mitochondrial matrix. However, recently, Yamaya *et al*. (1984) claimed that NAD(H)-GDH is loosely associated with the inner mitochondrial membrane. Our results show it to be located entirely within the mitochondrial matrix compartment, but do not permit any distinction to be made between the inner surface of the inner membrane and the matrix.

The enzyme displayed both NAD(H)- and NADP(H)-dependent activities with NAD(H) being the preferred substrate. NAD(H)/NADP(H) dependent activity ratios were of the order of 11:1. This is similar to values for mitochondrial GDH isolated from lettuce leaf (Lea and

Thurman, 1972) and Lemna minor (Ehmke and Hartmann, 1976).

The pH optima (Fig. 4.1), Km values (Table 4.3) and the sub-unit molecular size (Fig. 4.13) of turnip root GDH fall within the ranges determined for this enzyme from other plant tissues (Stewart et al. 1980; Kindt et al. 1980; Stone et al. 1979; Meredith et al. 1978). Km values for substrates were variable depending upon the assay condition as in the case of GDH from another plant sources (Stone et al. 1979; Nagel and Hartmann, 1980; Lees and Dennis, 1981; Davies and Teixeira, 1975; Mc Kenzie et al. 1981; Yamaya et al. 1984; Furuhashi and Takahashi, 1982; Meredith et al. 1979). Therefore careful study is required to reveal the real affinity for substrates in vivo.

There have been a number of reports showing that -SH groups have an important role in NAD(H)-GDH (Yamasaki and Suzuki, 1968; Chou and Splittstoesser, 1972; Errel et al. 1973; Fawole and Boulter, 1977). However, the sensitivity of GDH to -SH inhibitor varies markedly depending on the origin of the enzyme. For example 50 μM of p-HMB was sufficient to cause significant inhibition for NAD(H)-GDH from cowpea (Fawole and Boulter, 1977), whereas Stone et al.(1979) could not observe any inhibition with 0.1 mM p-CMB. NAD(H)-GDH from turnip root was inhibited approximately 30% by p-HMB at the concentration of 10 μM (Table 4.7) but the degree of inhibition was not increased at higher concentrations. Similar results were obtained with Ulva australis GDH (see Chapter III) and have also been reported for GDH from pumpkin

cotyledon (Chou and Splittstoesser, 1972). This result indicates that -SH groups might have only a minor role in the enzyme active site compared to other enzymes such as UDP glucose: D-fructose 2-glucosyltransferase (Slack, 1966) and NADH-dehydrogenase (Day and Wiskich, 1975; Nash and Wiskich, 1983) which are completely inhibited by p-CMB in the range 10-50 μM. Further investigations of the effect of p-HMB on GDH activity are discussed in following chapter.

Turnip NADH-GDH was found to be a trypsin-insensitive enzyme. Use of trypsin treatment during the purification procedure achieved a dramatic removal of non-GDH protein (Fig. 4.11). Turnip NADH-GDH was also found to be a heat resistant enzyme and, thus, a heat-treatment procedure was adopted to purify turnip GDH as in the case of GDH from other plant tissues (Meredith et al. 1978; Chou and Splittstoesser, 1972; Garland and Dennis, 1977; Fawole and Boulter, 1977). Addition of Ca^{2+} and/or NADH further improved the heat stability of turnip GDH although the reason for this is uncertain. Ehmke et al. (1984) have suggested that NADH and Ca^{2+} might stabilize the active site of GDH and prevent denaturation. The same interpretation might apply to the present case. Addition of extra Ca^{2+} and/or NADH may allow the use of higher temperatures for longer periods of time to increase the effectiveness of the purification procedure.

NAD(H)-GDH from turnip root was strongly activated by bivalent cations, particularly Ca^{2+} and inactivated by addition of the chelator, EGTA. The amination reaction was

activated by micromolar levels of Ca^{2+} as observed for this enzyme from other sources (Stewart et al. 1980; Ehmke and Hartman, 1976, 1978; Garland and Dennis, 1977; Joy, 1973; Furuhashi and Takahashi, 1982; Yamaya et al. 1984). Furthermore, we have also been able to show for the first time that the deamination activity of this enzyme is also activated by Ca^{2+} (Fig. 4.3).

Yamaya et al. (1984) have suggested that the Ca^{2+} concentration within the matrix of corn mitochondria is of the order of 52 to 56 mM. If this is also true of turnip mitochondria, GDH would be fully activated under all conditions. Thus, Ca^{2+} is unlikely to function in any regulatory capacity. However, if the turnip GDH is bound to the inner mitochondrial membrane (Yamaya et al. 1984), the effective concentration of Ca^{2+} within the immediate environment surrounding the enzyme may be much lower and may therefore play some role in the regulation of intramitochondrial GDH activity.

Of prime importance in considering the metabolic function of this enzyme in turnip root is the apparent poor affinity of the enzyme for the key substrates, ammonia and glutamate. In photosynthetic tissue, large amounts of ammonia are released within the mitochondria by glycine decarboxylation under photorespiratory conditions. It has been suggested that in the presence of these high intramitochondrial ammonia concentrations, mitochondrial GDH may have an ancillary role to the GS/GOGAT system in ammonia

refixation (Yamaya et al. 1984). However, similar high levels of ammonia are unlikely to be present in the mitochondria of storage tissue to support any significant amination activity. Furthermore, while the affinity of the enzyme for glutamate is far superior to ammonia in the pH range found in the matrix of energised mitochondria, the maximal activity of this enzyme in the deamination direction is very much reduced at this pH. Further investigations are required to determine the role of this enzyme in nitrogen metabolism of storage root tissue.

Table 4.1 Distribution of NADH-GDH, fumarase, catalase PEP-carboxylase in various subcellular fractions isolated from turnip root tissue using differential centrifugation.

NADH-GDH was measured in 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)_2SO_4$. 0.4 mM $CaCl_2$ and 0.04% (w/v) Triton X-100. Fumarase activity was measured in a reaction mixture containing 25 mM Hepes-KOH (pH 7.5), 0.04% (w/v) Triton X-100, 5 mM KH_2PO_4, 4 mM $MgCl_2$, 0.4 mM NADP, 0.2 unit of NADP-malic enzyme and 10 mM fumarate. PEP-carboxylase activity was measured in 30 mM Hepes-KOH (pH 8.0), 6 mM $MgCl_2$, 1 mM glucose-6-phosphate, 4 mM DTT, 8 mM $NaHCO_3$, 0.2 mM NADH, 1.6 unit of NADH-MDH and 1.6 mM PEP. Catalase activity was measured in 0.1 M phosphate buffer (pH7.0), 0.05% (w/v) H_2O_2 and 0.04% (w/v) Triton X-100 at 28°C and the decrease in absorbance at 240 nm was measured using 43.6 M^{-1} cm^{-1} as the extinction co-efficient.

	Activity (%)			
	NADH-GDH	Fumarase	Catalase	PEPCK*
Homogenate	100	100	100	100
15,000 g sup.	19	13	75	93
15,000 g pellet	63	67	9	0.3

*PEP-carboxylase

Table 4.2 A comparison of NADH-GDH activities measured using intact mitochondria and detergent treated mitochondria. The NADH-GDH activity was measured in 0.4 M sucrose, 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)_2SO_4$ and 0.4 mM $CaCl_2$ with or without 0.04 % (w/v) Triton X-100.

	NADH-GDH activity*
-Triton X-100	0.02
+Triton X-100	1.71

*μmol NADH ox. min^{-1} mg^{-1} protein

Table 4.3 Influence of pH on the apparent Km values of turnip NAD(H)-GDH.

The standard amination reaction was measured in 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)SO_4$, 0.4 mM $CaCl_2$ and 0.04% (w/v) Triton X-100. The standard deamination reaction was assayed in 0.1 M Tris-HCl (pH 9.5), 100 mM glutamate, 0.4 mM NAD, 0.1 mM $CaCl_2$ and 0.04% (w/v) Triton X-100.

pH	Km (mM)				
	glutamate	2-oxoglutarate	$(NH_4)_2SO_4$	NAD	NADH
7.2	-	1.7	66.7	-	0.08
8.0	13.7	2.0	22.2	0.17	0.09
9.5	28.6	10.0	19.6	0.25	0.07

Table 4.4 Activation of NAD(H)-GDH by bivalent cations.

The amination reaction was assayed in the medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)SO_4$, 0.04% (w/v) Triton X-100. The deamination reaction was measured in the medium contained 0.1 M Tris-HCl (pH 9.5), 100 mM glutamate, 0.4 mM NAD and 0.04% (w/v) Triton X-100. Calcium, Mn^{2+} and Mg^{2+} were added as the chloride salts and Zn^{2+} as the sulphate salt.

Addition	Final conc. (mM)	Relative activities (%)	
		Amination reaction	Deamination reaction
	None	100*	100**
Ca^{2+}	0.1	809	182
	1.0	752	167
Mn^{2+}	0.1	114	103
	1.0	786	114
Zn^{2+}	0.1	94	102
	1.0	197	135
Mg^{2+}	0.1	94	92
	1.0	90	92

* 0.21 μmol NADH ox. min^{-1} mg^{-1} protein was the 100% rate.

** 0.10 μmol NAD red. min^{-1} mg^{-1} protein was the 100% rate.

Table 4.5 Activity recovery experiment.

The amination reaction was assayed in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)SO_4$, 0.04% (w/v) Triton X-100 and EGTA. The deamination reaction was measured in the medium contained 0.1 M Tris-HCl (pH 9.5), 100 mM glutamate, 0.4 mM NAD, 0.04% (w/v) Triton X-100 and EGTA. Calcium and Mg^{2+} were added as the chloride salts, whilst Zn^{2+} was added as the sulphate salt.

Addition		Relative activity (%)					
		Amination reaction			Deamination reaction		
EGTA (mM)	Metal (mM)	Ca^{2+}	Mn^{2+}	Zn^{2+}	Ca^{2+}	Mn^{2+}	Zn^{2+}
0	0	*100	100	100	**100	100	100
1.0	0	25	3	15	0	0	1.6
1.0	0.2	-	-	-	1.5	0	0
1.0	0.5	21	3	13	1.5	0	1.6
1.0	1.0	771	73	26	145	14	1.6
1.0	5.0	864	807	400	153	217	4.5

* 0.18 μmol NADH ox. min^{-1} mg^{-1} protein was the 100% rate.

** 0.41 μmol NAD red. min^{-1} mg^{-1} protein was the 100% rate.

Table 4.6 Purification of turnip NAD(H)-GDH.

Purification Step	Total* activity (Unit)	Total protein (mg)	S.A. (Unit/mg protein)	Recov. (%)	Purif. (fold)
Mitochondria	579	450	1.3	100	1
Partially** purified enzyme	464	46	10.0	80	7.7
Sephacryl S-300	367	3	131	63	123
Hydroxyapatite	267	0.7	381	46	293
DEAE-Sephacel	54	0.12	450	9.4	346

* One unit is defined as the enzyme necessary to oxidize 1 μmol of NADH min^{-1} at 28°C.

** Partially purified refers to the detergent extract after trypsin, heat and ammonium sulphate treatment.

Table 4.7 Sensitivity of turnip NADH-GDH to p-HMB.

The amination reaction was assayed in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)SO_4$ and 0.4 mM $CaCl_2$.

p-HMB conc.	Relative NADH-GDH activties (%)*
None	100*
10 μM	75
20 μM	77
50 μM	81
500 μM	77
1 mM	71

* 11.6 μmol NADH ox. min^{-1} mg^{-1} protein was calculated as 100%.

Figure 4.1 pH optima of NAD(H)-GDH from isolated turnip mitochondria.

The amination reaction was assayed in a medium containing 0.133 M Tris, 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)_2SO_4$, 0.4 mM $CaCl_2$ and 0.04% (w/v) Triton X-100. The deamination reaction was assayed in 0.1 M Tris-HCl, 100 mM glutamate, 0.4 mM NAD, 0.1 mM $CaCl_2$ and 0.04% (w/v) Triton X-100. pH was adjusted using KOH or HCl.

Symbols:

(●); amination reaction

(■); deamination reaction

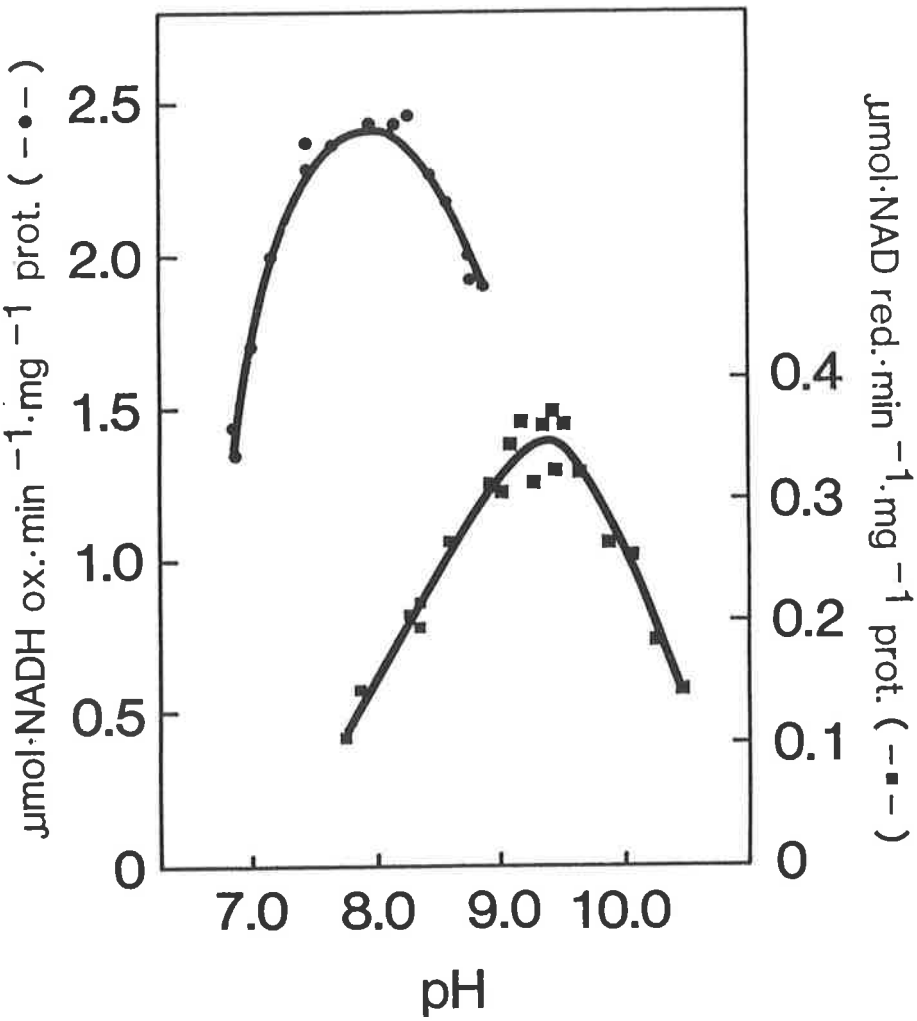

µmol·NADH ox.·min −1·mg −1 prot. (–•–)
µmol·NAD red.·min −1·mg −1 prot. (–▪–)
2.5
2.0
1.5
1.0
0.5
0
0.4
0.3
0.2
0.1
0
7.0
8.0
9.0
10.0
pH

Figure 4.2 Double reciprocal plot of velocity versus $(NH_4)_2SO_4$ concentration.

Assays were carried out in 0.133 M Tris-HCl (pH 7.2, ■ ; pH 8.0, ● ; pH 9.5, ▲), 13 mM 2-OG, 0.166 mM NADH and 0.4 mM $CaCl_2$ at 28°C. Velocities are expressed as μmol NADH ox. min^{-1} mg^{-1} protein.

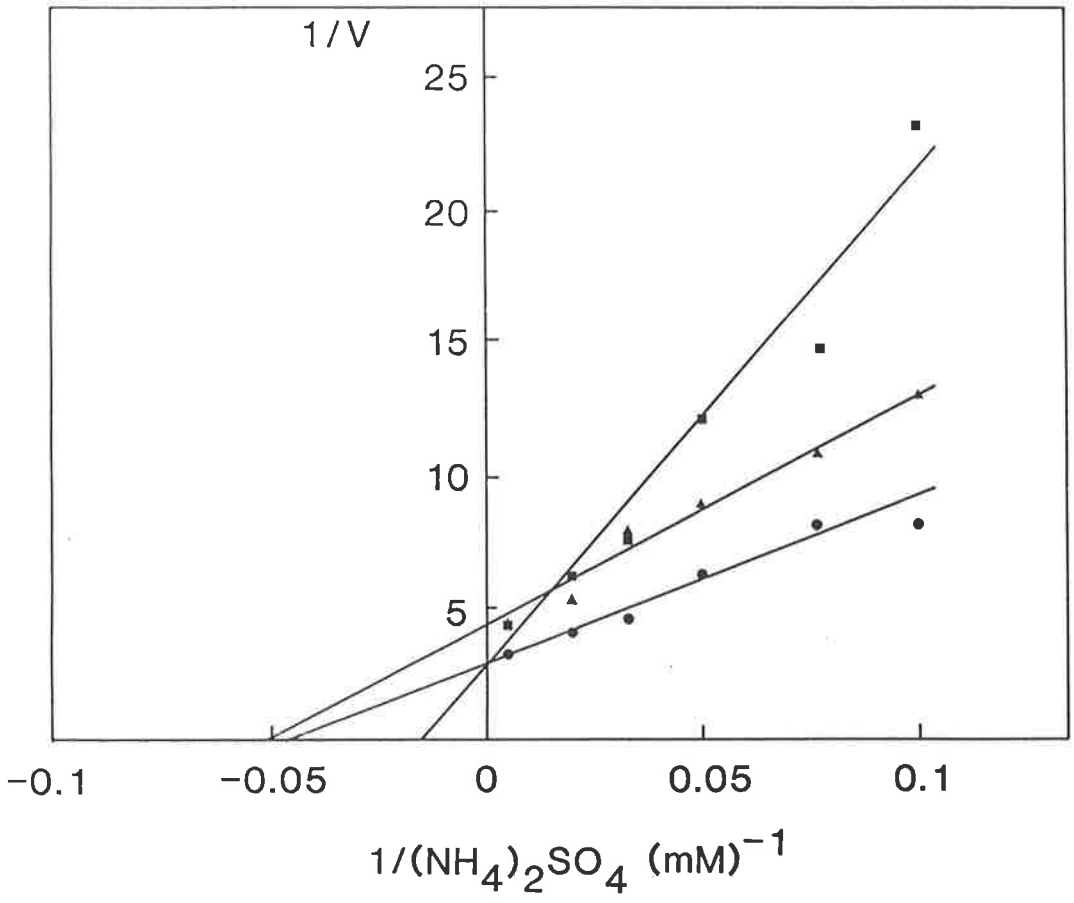

1/V
25
20
15
10
5
-0.1
-0.05
0
0.05
0.1
1/(NH4)2SO4 (mM)-1

Figure 4.3 Double reciprocal plot of velocity versus 2-OG concentration.

Assays were carried out in 0.133 M Tris-HCl (pH 7.2, ■ ; pH 8.0, ● ; pH 9.5, ▲), 213 mM $(NH_4)_2SO_4$, 0.166 mM NADH and 0.4 mM $CaCl_2$ at 28°C. Velocities are expressed as μmol NADH ox. min^{-1} mg^{-1} protein.

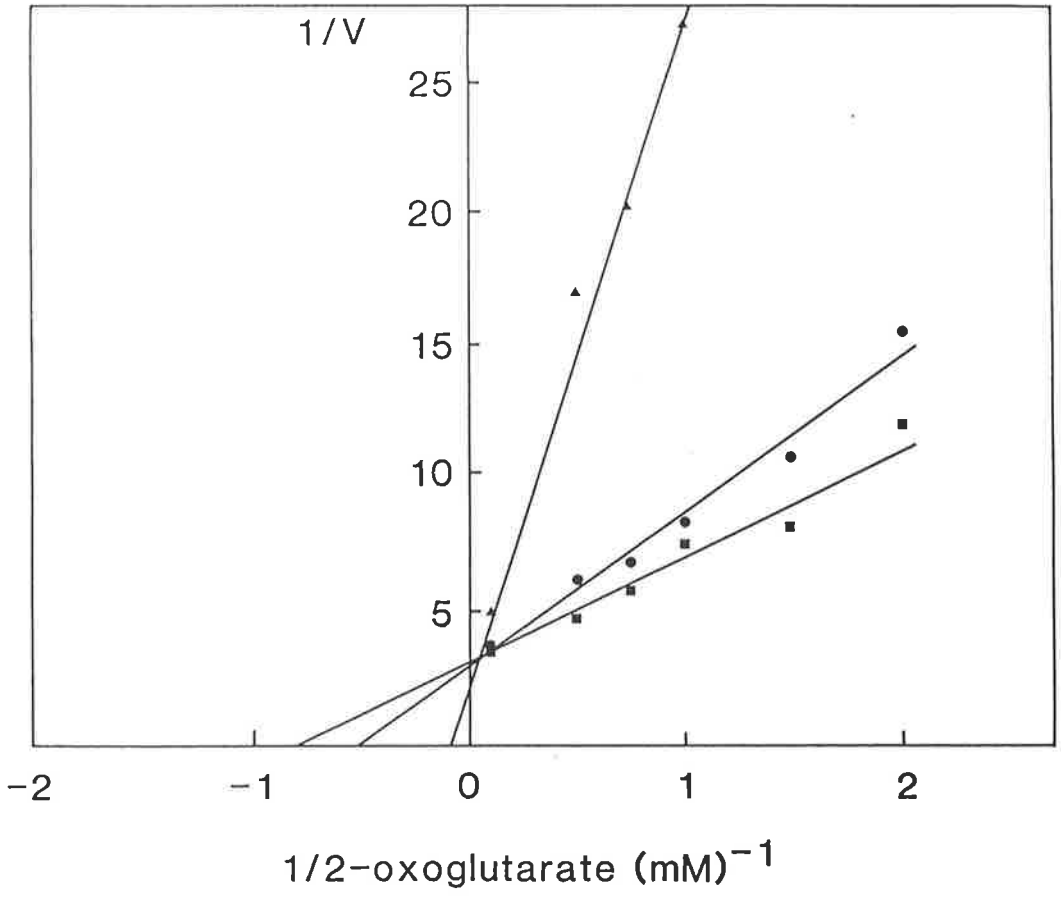

1/V
25
20
15
10
5
−2
−1
0
1
2
1/2-oxoglutarate (mM)$^{-1}$

Figure 4.4 Double reciprocal plot of velocity versus NADH concentration.

Assays were carried out in 0.133 M Tris-HCl (pH 7.2, ■ ; pH 8.0, ● ; pH 9.5, ▲), 13 mM 2-OG, 213 mM $(NH_4)_2SO_4$ and 0.4 mM $CaCl_2$ at 28°C. Velocities are expressed as μmol NADH ox. min^{-1} mg^{-1} protein.

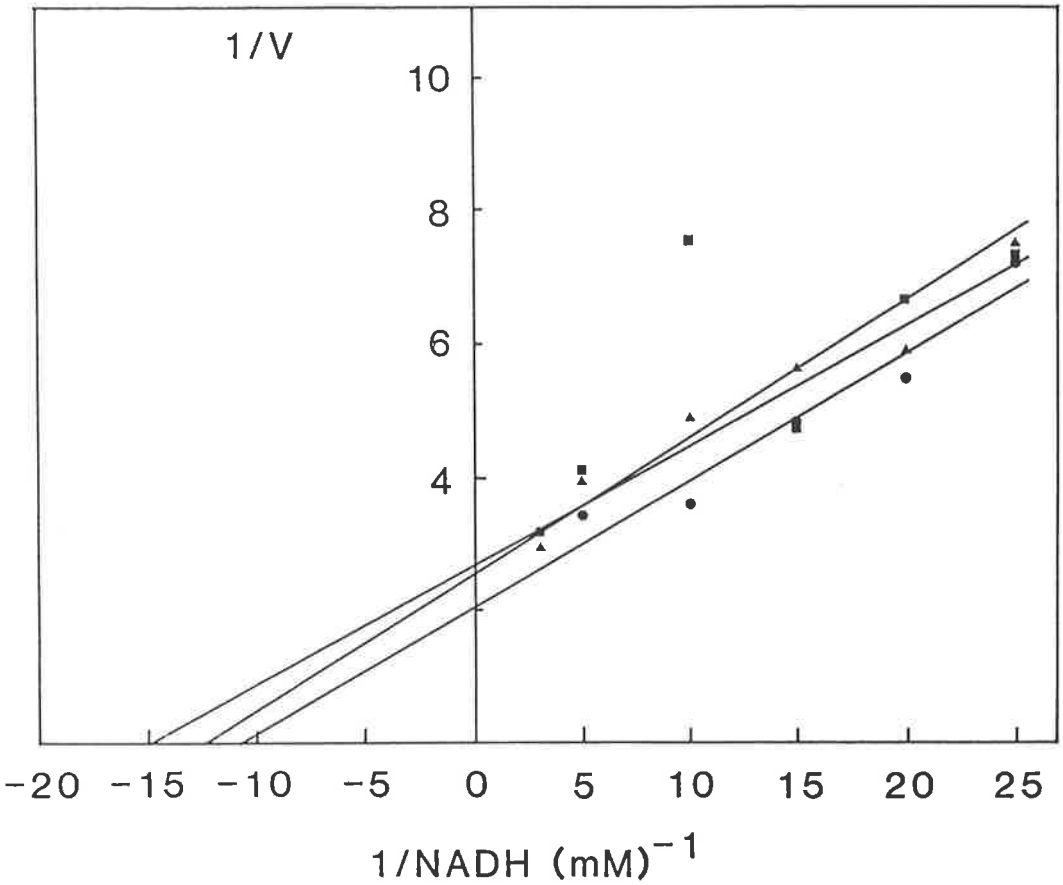

1/V
10
8
6
4
-20
-15
-10
-5
0
5
10
15
20
25
1/NADH (mM)$^{-1}$

Figure 4.5 Double reciplocal plot of velocity versus glutamate concentration.

Assays were carried out in 0.133 M Tris-HCl (pH 8.0, ● ; pH 9.5, ▲), 0.4 mM NAD and 0.4 mM $CaCl_2$ at 28°C. Velocities are expressed as μmol NAD red. min^{-1} mg^{-1} protein.

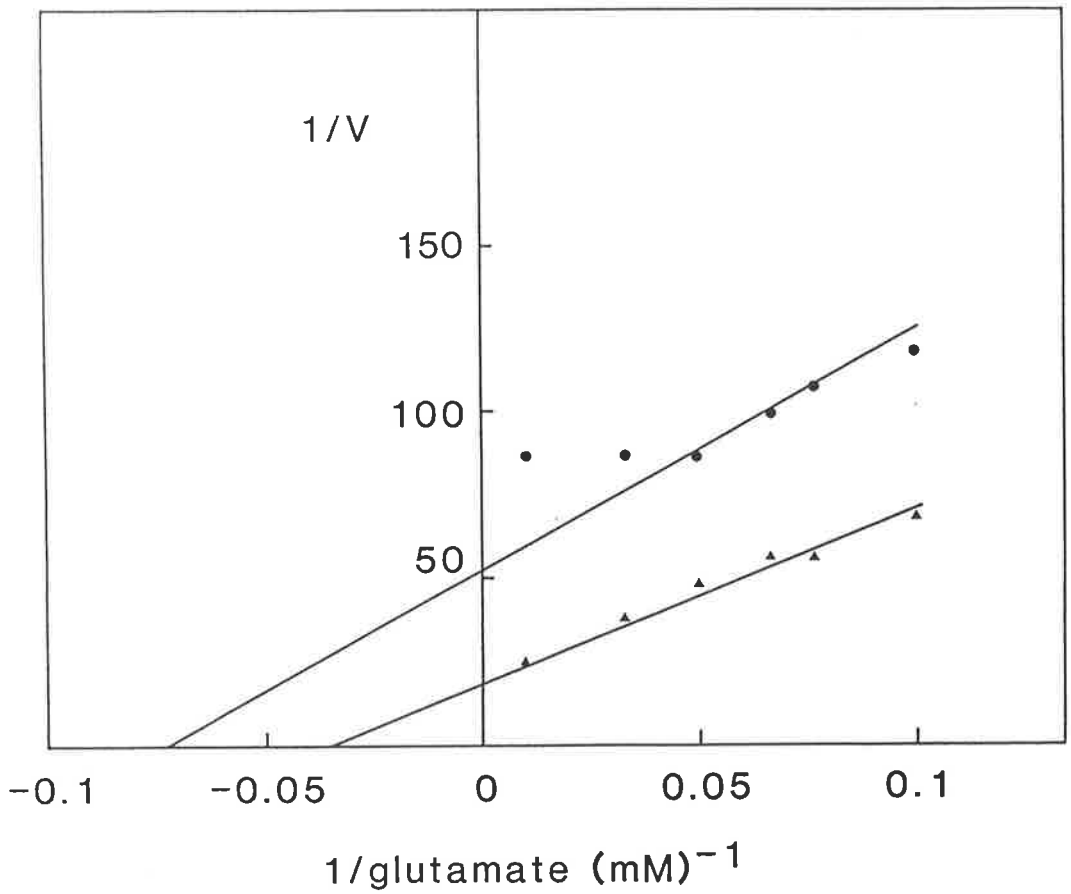

1/V
150
100
50
-0.1
-0.05
0
0.05
0.1
1/glutamate (mM)$^{-1}$

Figure 4.6 Double reciprocal plot of velocity versus NAD concentration.

Assays were carried out in 0.133 M Tris-HCl (pH 8.0, ● ; pH 9.5, ▲), 100 mM glutamate and 0.4 mM $CaCl_2$ at 28°C. Velocities are expressed as μmol NAD red. min^{-1} mg^{-1} protein.

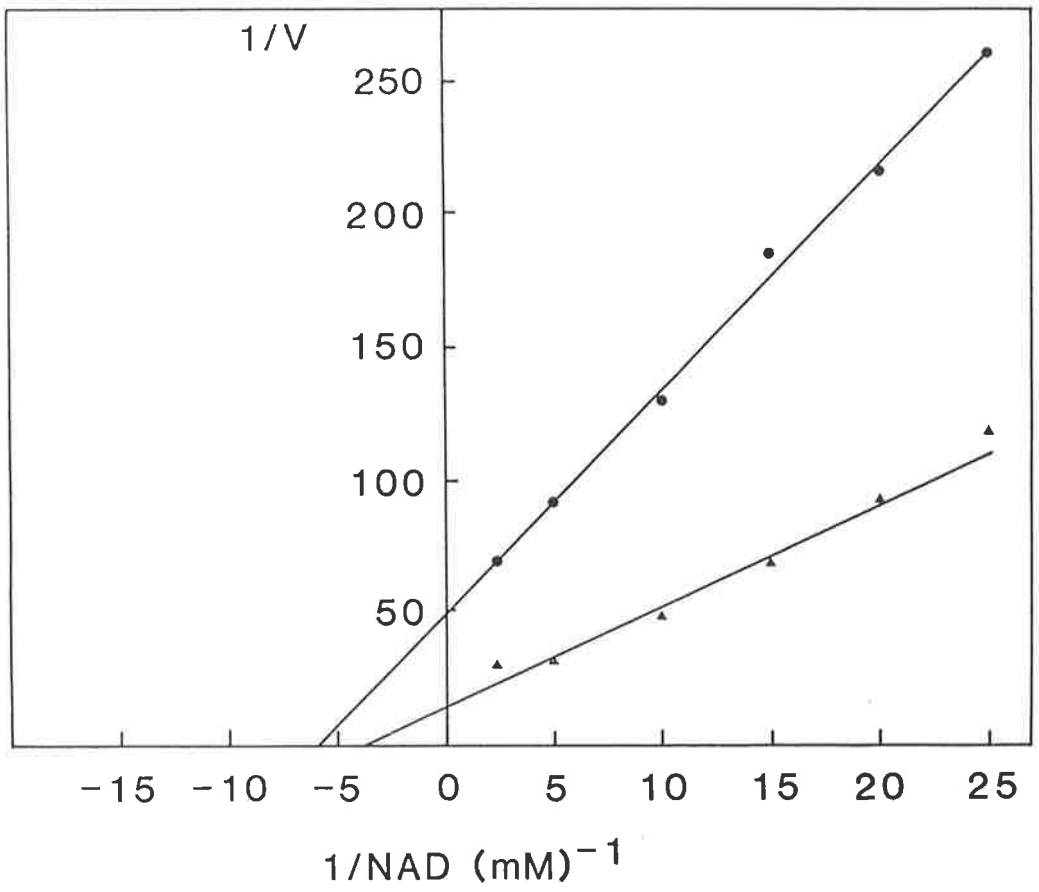

1/V
250
200
150
100
50
-15
-10
-5
0
5
10
15
20
25
1/NAD (mM)$^{-1}$

Figure 4.7 Activation of the amination reaction of NAD(H)-GDH from turnip mitochondria by Ca^{2+}.

Assays were carried out in the medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)SO_4$, 0.04% (w/v) Triton X-100 and $CaCl_2$ which was present at the concentrations as described in Figure.

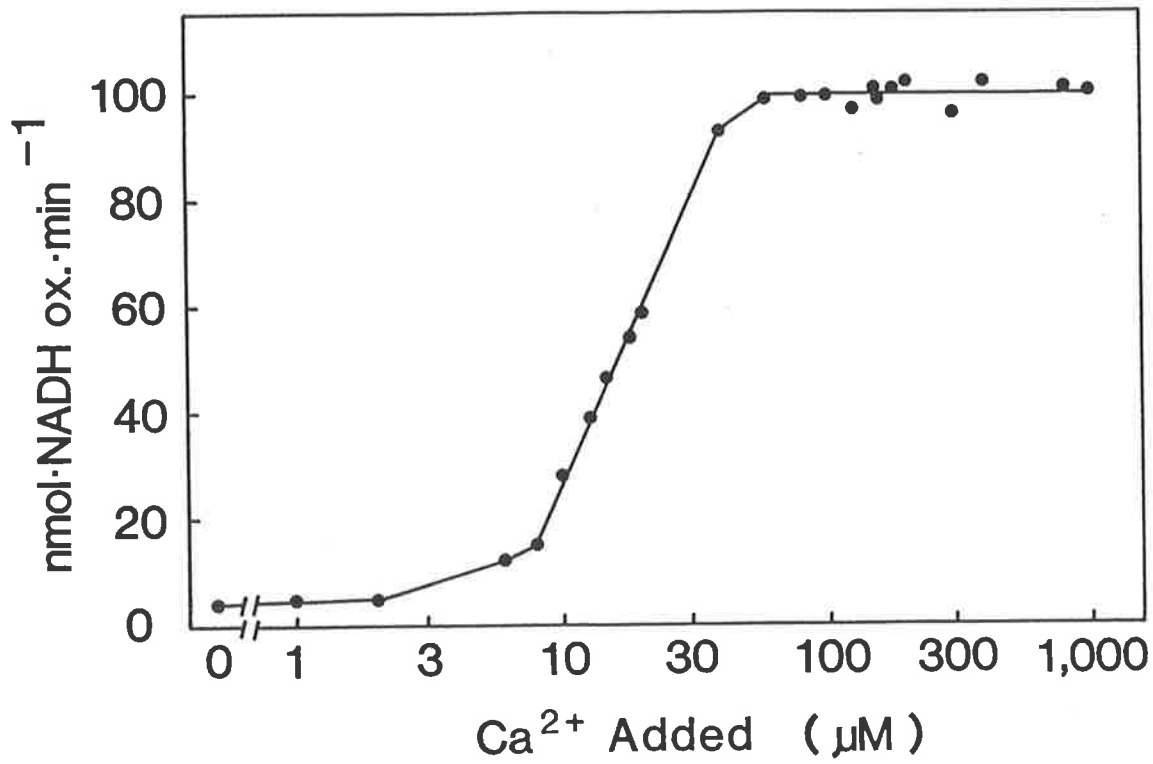

nmol·NADH ox.·min^{-1}
100
80
60
40
20
0
0
1
3
10
30
100
300
1,000
Ca^{2+} Added (μM)

Figure 4.8 Activation of the deamination reaction of NAD-GDH from turnip mitochondria by Ca^{2+}.

Assays were carried out in the medium containing 0.1 M Tris-HCl (pH 9.5), 100 mM glutamate, 0.4 mM NAD, 0.04% (w/v) Triton X-100 and $CaCl_2$ which was present at the concentrations as described in Figure.

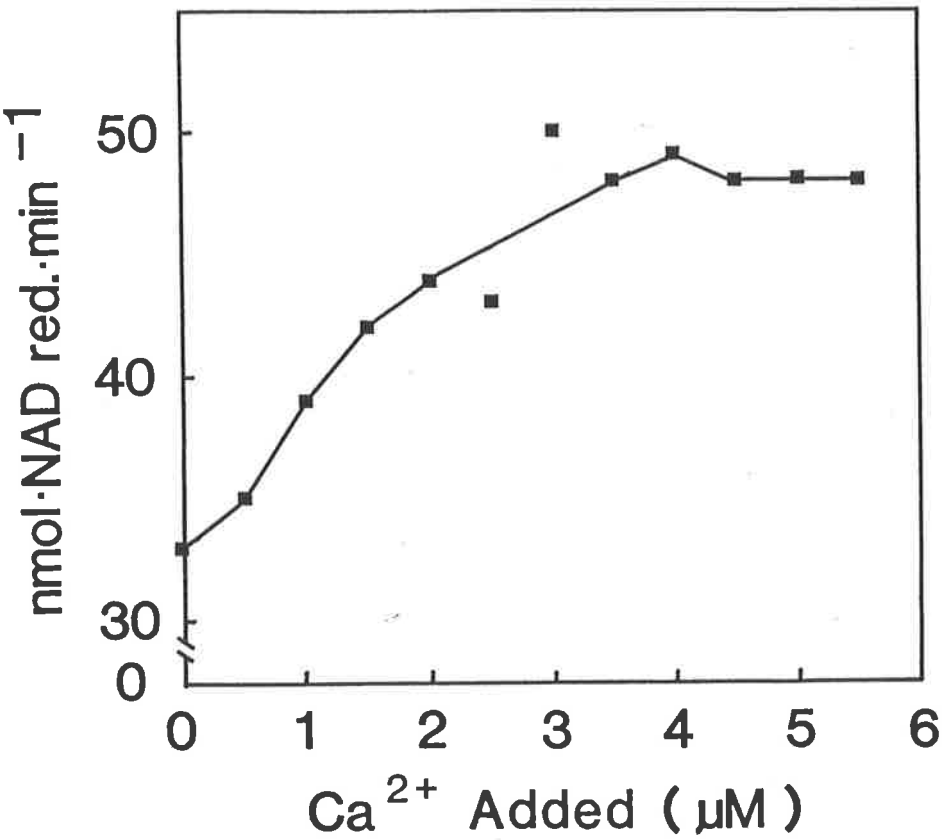

nmol·NAD red.·min $^{-1}$
50
40
30
0
0
1
2
3
4
5
6
Ca $^{2+}$ Added (µM)

Figure 4.9 Heat stability of turnip NADH-GDH.

Assays were carried out in the medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.123 M $(NH_4)SO_4$ and 0.4 mM $CaCl_2$. The heat treatment was performed in 50 mM Tris-HCl (pH 8.0) in a thermostated water bath at the appropriate temperature for up to 60 min. The sample was kept in ice immediately after the treatment. An initial assay for each treatment was made before commencing heat treatment. Samples were incubated at 65°C (○); 75°C (□); 80°C (●); 80°C with 1 mM NADH (▼); 80°C with 1 mM $CaCl_2$ (■); 80°C with 1 mM NADH and 1 mM $CaCl_2$ (▲).

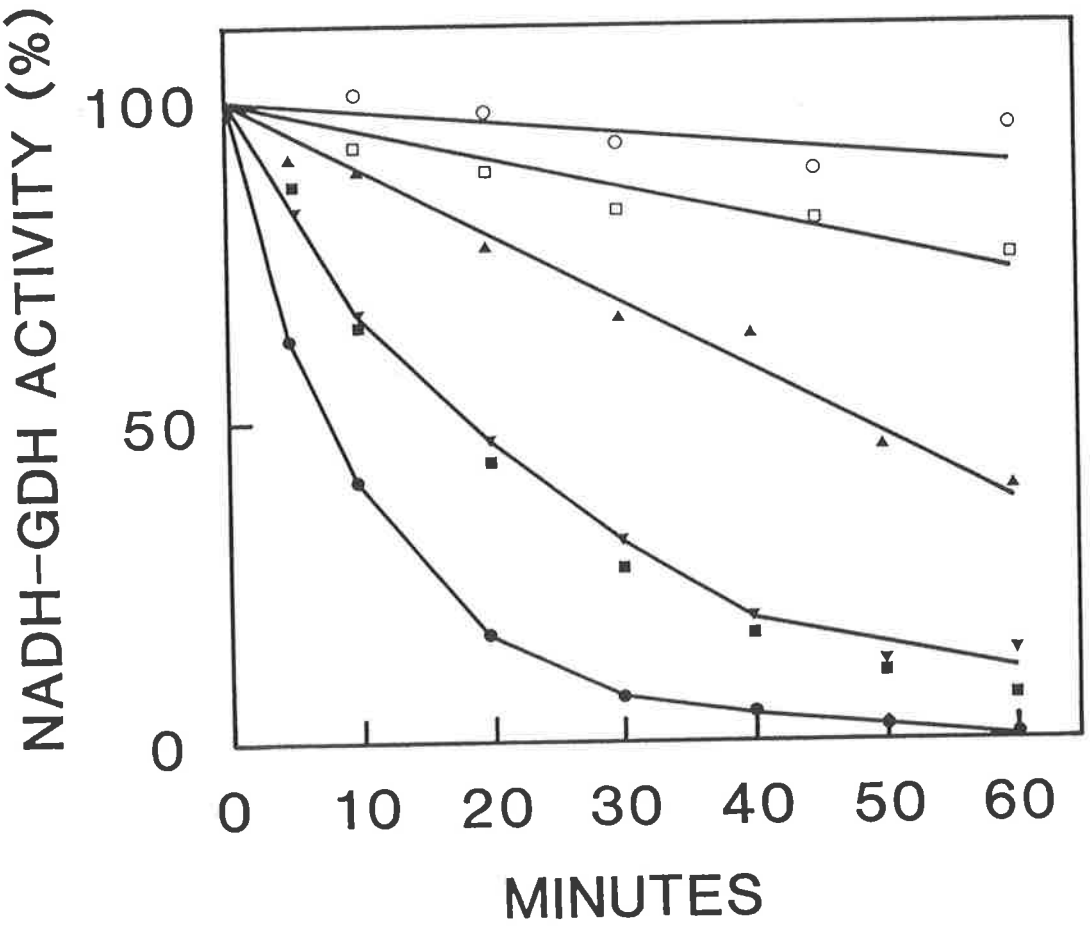

NADH-GDH ACTIVITY (%)
100
50
0
0
10
20
30
40
50
60
MINUTES

Figure 4.10 Comparative effect of Trypsin on NADH-MDH and NADH-GDH from turnip mitochondria.

Mitochondrial extracts were incubated in the presence (open symbols) or absence (closed symbols) of trypsin (0.2 mg trypsin/mg mitochondrial protein) in 50 mM Tris-HCl (pH 8.0) at 28°C. Samples were withdrawn at various times and assayed for NADH-GDH activity in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)_2SO_4$, 0.4 mM $CaCl_2$. NADH-MDH activity was measured in 200 mM Tes-KOH (pH 7.5) and 0.2 mM NADH, with the reaction being initiated with 6 mM OAA.

Symbols:

(△); NADH-GDH activity in the presence of trypsin.

(▲); NADH-GDH activity in the absence of trypsin.

(○); NADH-MDH activity in the presence of trypsin.

(●); NADH-MDH activity in the absence of trypsin.

NADH–MDH ACTIVITY (•,°)
(μmol·NADH ox.·min −1·mg −1 prot.)
NADH–GDH ACTIVITY (▲,△)
(μmol·NADH ox.·min −1·mg −1 prot.)
MINUTES
0
10
20
30
40
50
60
120
0
0.5
1.0
1.5
2.0

Figure 4.11 Polypeptide and GDH isozyme patterns of turnip mitochondrial extract after trypsin treatment as resolved by non-denaturing PAGE.

Mitochondria were extracted with 0.1% Triton X-100 and passed through a PD-10 column equlibrated with 50 mM Tris-HCl (pH 8.0). A. Lane a; mitochondrial extract (0.1 mg protein) at zero time with added trypsin. The gel was stained with Coomassie brilliant blue R-250 and b; extract after incubation with trypsin for 120 min. B. Lane a; mitochondrial extract (0.4 mg protein) without trypsin treatment and b; after trypsin treatment for 120 min. Arrows indicate position of NAD-GDH isozymes. The gel was stained with activity staining using 100 mM Tris-HCl (pH 9.5), 65 mM glutamate, 0.1 mM phenazine methosulphate, 0.1 mM nitro-blue tetrazolium, 0.4 mM NAD and 0.4 mM $CaCl_2$ at 28°C.

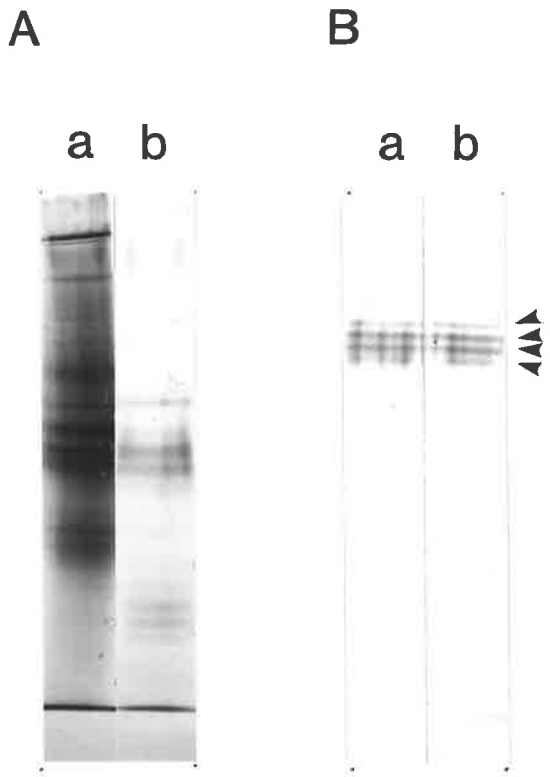
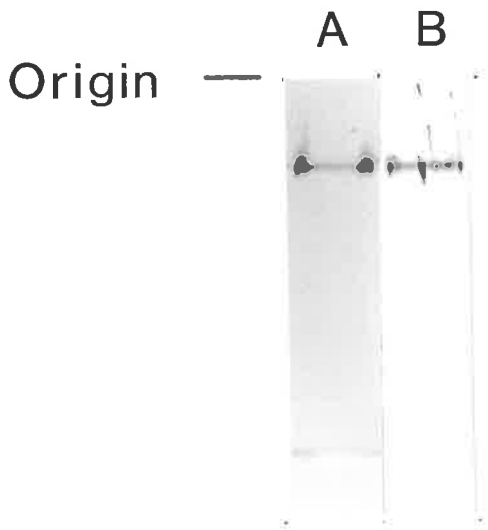

A
B
a
b
a
b

Figure 4.12 Non-denaturing PAGE of purified NAD(H)-GDH from turnip mitochondria.

Lane A; the gel was stained for activity as described in Materials and Methods. Lane B; the gel was stained for protein with silver as described in Materials and Methods.


A
B
Origin

Figures 4.13 SDS-PAGE of purified turnip NAD(H)-GDH.

The molecular mass standards were phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The gel was stained with silver as described in Materials and Method.

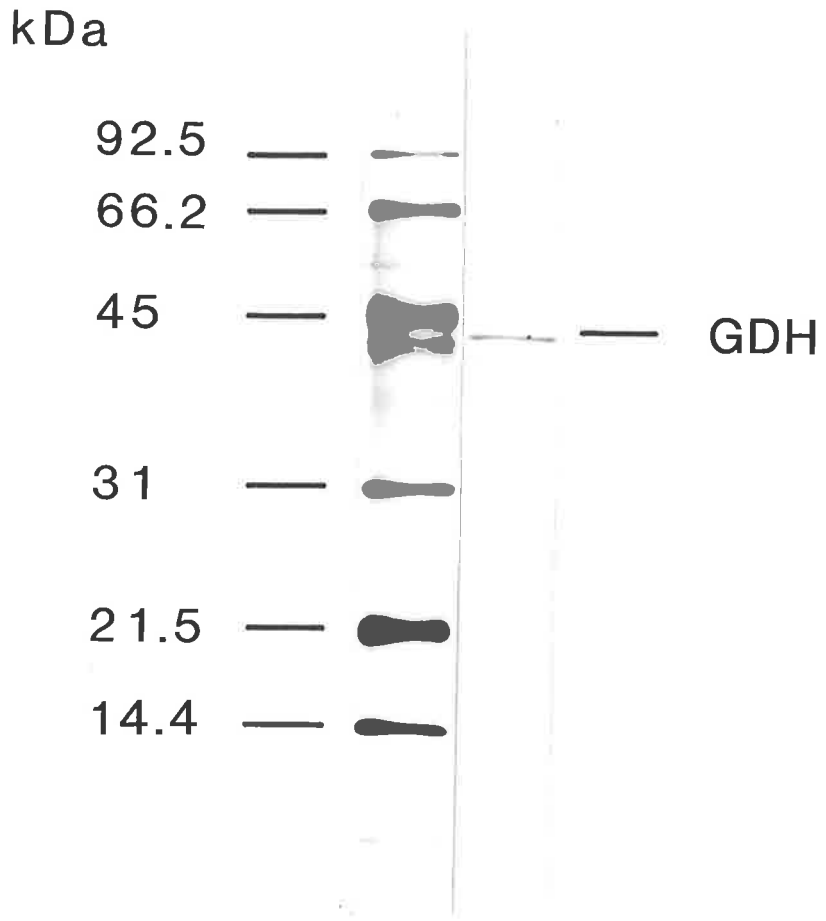
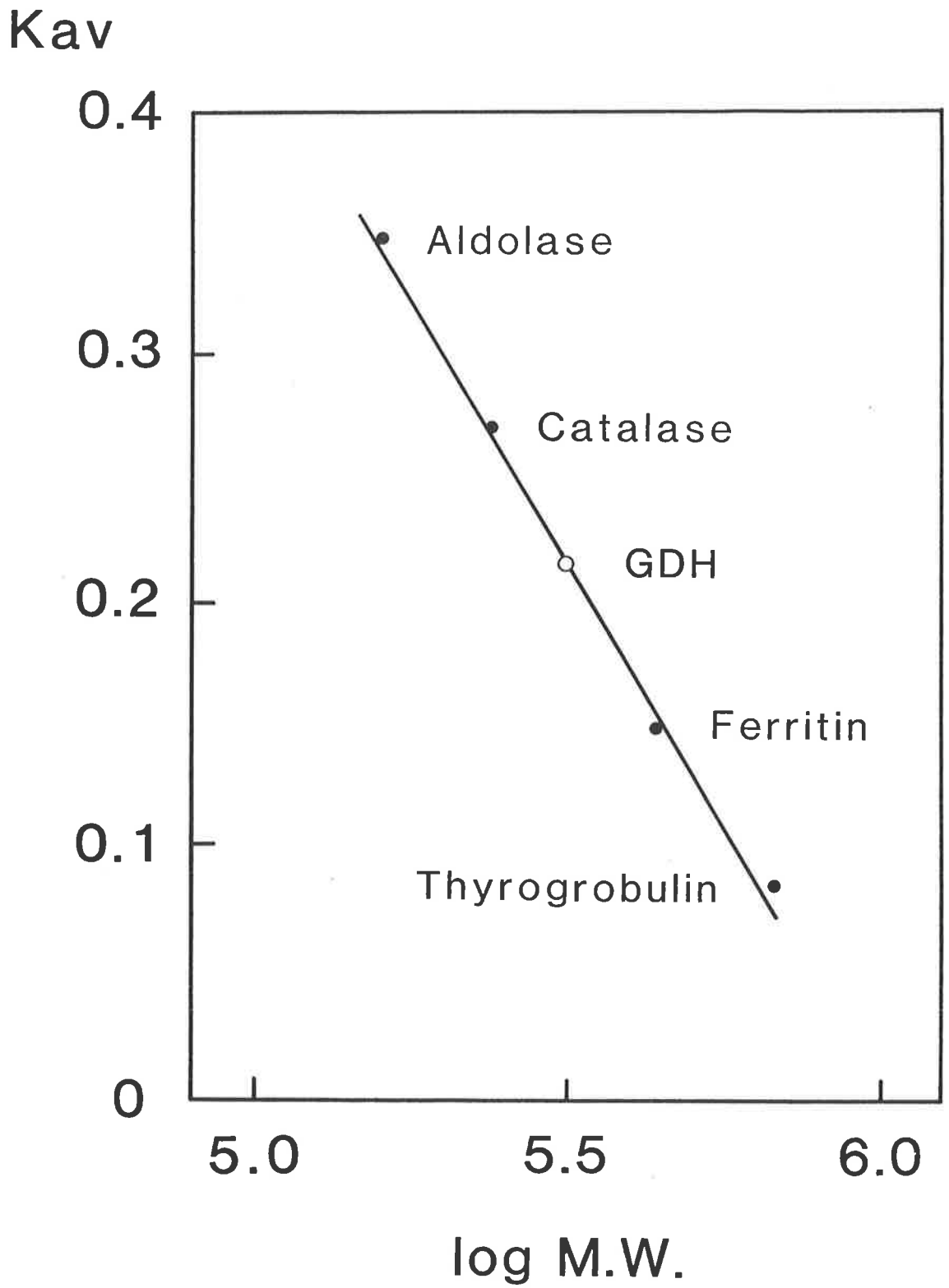

kDa
92.5
66.2
45
31
21.5
14.4
GDH

Figure 4.14 Molecular size determination of turnip NAD(H)-GDH by gel filtration.

The molecular size was determined using Sephacryl S-300 with 50 mM Tris-HCl (pH 8.0). Marker proteins are thyroglobulin (669 kDa), ferritin (440 kDa), catalase (233 kDa) and aldolase (158 kDa).


Kav
0.4
0.3
0.2
0.1
0
Aldolase
Catalase
GDH
Ferritin
Thyrogrobulin
5.0
5.5
6.0
log M.W.

CHAPTER V

EFFECTS OF CALCIUM ON MITOCHONDRIAL NAD(H)-GLUTAMATE DEHYDROGENASE FROM TURNIP (BRASSICA RAPA L.)

5.1 INTRODUCTION

NAD(H)-glutamate dehydrogenase is present in the mitochondria of many plant tissues (Stewart et al. 1980). It is well known that NAD(H)-GDH can be activated by Ca^{2+} (Stewart et al. 1980) and that pronounced substrate (ammonia) inhibition occurs in the absence of Ca^{2+} (Ehmke and Hartmann, 1976, 1978; Furuhashi and Takahashi, 1982. Yamaya et al. 1984). In Chapter IV, it was shown that higher concentrations of Ca^{2+} are required to fully activate the turnip enzyme for amination than for deamination. This effect may suggest that Ca^{2+} has a role in regulating the enzyme activity under various substrate conditions and may also contribute to determining whether the enzyme operates in the amination or deamination direction in vivo. Therefore, a detailed knowledge of the effects of Ca^{2+} on the enzyme activity is required to fully appreciate the potential functions of this enzyme in the cell. Consequently, I decided to analyze the effects of Ca^{2+} on mitochondrial NAD(H)-GDH activity from turnip (Brassica rapa L.).

5.2 RESULTS

5.2.1 *Effects of Ca^{2+} on GDH activity at various ammonia concentrations*

Since ammonia is one of the key substrates in the operation of GDH, a detailed analysis of enzyme activity under various ammonia and calcium concentrations was undertaken. NADH-GDH activity was measured over a range of $(NH_4)_2SO_4$ concentrations in the presence of 10, 20, and 100 μM $CaCl_2$ (Fig. 5.1). Significant substrate inhibition was observed at concentrations above 40 mM $(NH_4)_2SO_4$ in the presence of 10 μM $CaCl_2$ (Fig. 5.1). At 100 mM $(NH_4)_2SO_4$ reduction in the Ca^{2+} concentration from 100 to 20 μM le d to an 80% reduction in GDH activity.

As such high Ca^{2+} and NH_4^+ concentrations are unlikely to be present in vivo, Fig 5.2 shows the effects of Ca^{2+} (2-10 μM) at more 'physiological' $(NH_4)_2SO_4$ concentrations. The enzyme activity was limited not only by $(NH_4)_2SO_4$, but Ca^{2+} concentration. The maximal enzyme activity with 2 μM Ca^{2+} was observed with $(NH_4)_2SO_4$ at the concentration of 10 mM. The higher enzyme activity could be achieved by further addition of Ca^{2+}.

At very low substrate concentrations, NAD(H)-GDH activity was not affected by additions of Ca^{2+} when measured in either the amination or deamination direction. However, both reactions were inhibited by the addition of EGTA, and the activities were virtually completely recovered by the

subsequent addition of Ca^{2+} (Fig. 5.3). The longer time period required to inhibit the deamination reaction with EGTA may indicate the extremely low amount of Ca^{2+} required to activate the enzyme for this reaction under these conditions. Therefore, the distilled water used in these experiments may have contained sufficient Ca^{2+} to activate NAD(H)-GDH activities under these low substrate conditions. The results with EGTA shows that Ca^{2+} is still required to fully express the enzymic activities.

These results indicate that the Ca^{2+} concentration required to fully activate the enzyme strongly depends upon the ammonia concentration and μM, or even of the order of nM, Ca^{2+} concentrations may be able to fully express the enzyme activity *in vivo*.

5.2.2 *Changes in enzyme response to calcium as induced by proteolysis*

It has already been shown in Chapter IV that GDH from both turnip mitochondria and *Ulva australis* was insensitive to treatment by the proteolytic enzyme, trypsin, and that there was no change in the turnip GDH isozyme pattern. This is a rather unique property of the enzyme and it was decided to investigate the response of this enzyme to more general proteolytic treatment. Trypsin is a rather specific proteolytic enzyme and the insensitivity of GDH may implicate a specific feature of the enzyme's primary structure.

Protease XIV, subtilisin and thermolysin were used in these experiments as alternative proteolytic enzymes to trypsin. Protease XIV and subtilisin perform non-specific hydrolysis. Thermolysin hydrolyzes peptide bonds involving the amino groups of hydrophobic amino acid residues in general (Matsubara, 1970).

It can be seen from Fig. 5.4 that Protease XIV did not produce a decrease in maximal Ca^{2+}-stimulated GDH activity over the incubation period. Slight activation was observed by the treatment, however, the enzyme activity itself was totally resistant to proteolytic attack. It is also clear that exposure of GDH to Protease XIV and subtilisin has le d to a change in the Ca^{2+} sensitivity of GDH (Fig. 5.5). The sensitivity towards Ca^{2+} was not altered by the Trypsin treatments. On the other hand, Protease XIV- and subtilisin-treated enzymes showed some activation in the absence of added Ca^{2+}. Subtilisin-treated GDH also showed a differential sensitivity to Ca^{2+} compared to the non-treated enzyme (Fig. 5.5). It should be noted that Protease XIV treated-enzyme preparation contained about 4.5 μM Ca^{2+}, because Protease XIV (from Sigma Chemical Co.) contains approximately 25% calcium acetate. Taking this into account, it is still apparent that Protease XIV-treated GDH showed a different sensitivity to Ca^{2+} compared to the non-treated enzyme.

The relationship between ammonia and Ca^{2+} concentrations was further investigated using Protease XIV

treated enzyme. As has been shown already, only 10% of the maximal activity is observed at 213 mM $(NH_4)_2SO_4$ in the presence of 10μM Ca^{2+} (Fig. 5.1). However, under the same conditions about 80% of activity could be expressed after protease treatment (Fig 5.6). Further, the Protease XIV treated enzyme showed enhanced activity in the absence of added Ca^{2+}. These results indicate that Protease XIV treatment does not damage the active site of GDH, but does effect the Ca^{2+}-sensitive site, or the response to Ca^{2+} binding. Alternatively it may be changing the enzyme structure in such a way to reduce the binding of ammonia at a site which leads to substrate inhibition.

NADH-GDH was also incubated with thermolysin, which had no significant effect for the first 20 min, but after this, enzyme activation was observable, as shown in Fig. 5.7. Enzyme properties were examined after 60 min of thermolysin treatment (Fig. 5.8). No substrate inhibition was observed even under higher concentrations of ammonia. The thermolysin treatment was performed with 5 mM $CaCl_2$ present, because the protease required calcium to express enzyme activity. The thermolysin powder (obtained from Sigma) also contained approximately 20% buffer salt as calcium and sodium acetate. Therefore a minimum of 8 μM, or maximum, of 17 μM Ca^{2+} was already in the assay solution from these sources. Even allowing for this added Ca^{2+}, it is clear that the sensitivity of NADH-GDH to Ca^{2+} was changed by the thermolysin, because the untreated enzyme exhibited only about 70% and 30% of maximum activity with 100 mM and 213 mM

$(NH_4)_2SO_4$ respectively in the presence of 20 μM $CaCl_2$. That is, substrate inhibition could still be observed with the untreated enzyme in the presence of approximately the same concentration of Ca^{2+}.

The non-treated NADH-GDH required added Ca^{2+} for activity (Fig. 5.9-A), and hardly any enzyme activity was observed without the addition of Ca^{2+} (Fig. 5.9-B). This non-treated enzyme required about 60 μM Ca^{2+} for maximal enzyme activity in the presence of 213 mM $(NH_4)_2SO_4$ (Chapter IV and Fig. 5.5). As previously mentioned, the thermolysin treated-enzyme showed maximal activity with 213 mM $(NH_4)_2SO_4$ without added $CaCl_2$ (Fig. 5.9-C), but with 8-17 μM Ca^{2+} which was carried over from the incubation medium. To determine whether the treated enzyme still required added Ca^{2+} for activity, the sensitivity of the thermolysin-treated NADH-GDH to EGTA was also determined (Fig. 5.9-C). The thermolysin-treated enzyme was inactivated by the addition of EGTA, but the activity was fully restored by a subsequent addition of excess Ca^{2+} (Fig. 5.9-C and Fig. 5.9-D). These results indicated that the sensitivity of the enzyme to Ca^{2+} had changed, such that much lower concentrations of Ca^{2+} were sufficient to fully express the enzyme activity after the thermolysin treatment.

5.2.3 *Effect of NaCl*

High salt concentrations have been shown to change the specificity of Ca^{2+}-activated neutral protease (Sakai *et*

al. 1987) and this has been interpreted to mean that the hydrophobic regions of the enzyme have been affected by the salt. Similarly, GDH from turnip has been shown to have its sensitivity to Ca^{2+} altered by thermolysin treatment which supposedly hydrolyses hydrophobic regions. It has also been shown that high concentrations of Na_2SO_4 can replace Ca^{2+} in re-activation of the EGTA-inactivated GDH from pea seeds (Kindt et al. 1980). If the Ca^{2+}-sensitive site of turnip NADH-GDH does involve hydrophobic regions of the protein, then high salt concentrations may be able to substitute for Ca^{2+} in this enzyme.

The effect of NaCl (0 to 500 mM) on the amination reaction of turnip GDH, with saturating $(NH_4)SO_4$, was examined in the presence and absence of added Ca^{2+} (Fig. 5.10). About 300 mM NaCl was required to fully activate the enzyme in the presence of 213 mM $(NH_4)_2SO_4$ and in the absence of any added Ca^{2+}. The NaCl activation could achieve only 60-75% of the maximum activity observed with Ca^{2+} alone. The enzyme activity tended to be reduced at NaCl concentrations above 300 mM, independent of the presence or absence of Ca^{2+}. However, if the enzyme was fully activated with 100 μM $CaCl_2$, additions of NaCl exceeding 75-100 mM tended to inhibit enzyme activity.

The GDH activity was measured with 100, 150, 200 and 300 mM NaCl in the presence of $(NH_4)_2SO_4$ concentrations ranging from 0 to 213 mM (Fig. 5.11). It was found that much higher concentrations of NaCl were required to activate the

enzyme than of Ca^{2+}. However, in all other aspects the effects of NaCl were similar to that of Ca^{2+} in that NaCl could prevent substrate inhibition. These results indicate that the hydrophobic region of the enzyme may be involved in the Ca^{2+}-sensitive site and support the results obtained with thermolysin treatment.

5.2.4 *Effect of p-hydroxymercuribenzoate (p-HMB)*

In Chapter IV, it was shown that the -SH reagent, p-HMB, had only a limited inhibitory effect on NAD(H)-GDH activity. The effect of p-HMB on NADH-GDH from turnip was further investigated as shown in Fig. 5.12. Whereas the enzyme activity was inhibited completely by the addition of 0.5 mM EGTA (Fig. 5.12-C), only approximately 30% inhibition could be observed on the addition of 200 μM p-HMB (Fig. 5.12-B). However, more importantly, the addition of EGTA following an addition of p-HMB did not result in complete inhibition of GDH activity as seen in Fig. 5.12-B. Although p-HMB inhibited only 30% of the enzyme activity, there was a significant effect on the Ca^{2+} requirement of the enzyme as judged by the insensitivity to EGTA. These results indicate that -SH groups in the enzyme may be much more critical for the Ca^{2+} sensitive site than for the active site itself.

5.3 DISCUSSION

The Ca^{2+} concentration required to fully activate NADH-GDH from turnip mitochondria was dependent upon the

substrate concentrations as has been shown for NAD(H)-GDH isolated from Lemna minor (Ehmke and Hartmann, 1976, 1978) and from green tobacco callus (Furuhashi and Takahashi, 1982). Approximately 60 μM Ca^{2+} was required to fully activate the enzyme with 213 mM $(NH_4)_2SO_4$ (Chapter IV). However, enzyme activity can be expressed in the presence of only 2 μM Ca^{2+} at the more 'physiological' $(NH_4)_2SO_4$ concentration of 10 mM. This result indicates the interaction between the Ca^{2+} requirement for maximal activity and the substrate concentration. Thus, intracellular regulation of NAD(H)-GDH activity may be possible with low concentrations of Ca^{2+} (of the order of μM, or even nM).

Turnip NAD(H)-GDH also exhibited activity under low substrate (ammonium and NADH) conditions without any added Ca^{2+} as reported for corn shoot mitochondrial GDH (Yamaya et al. 1984). However, this activity was inhibited by EGTA and the activity could be restored by the subsequent addition of an excess amount of Ca^{2+}. These results indicate that Ca^{2+} is still essential for the enzyme activity to occur under these low substrate conditions.

Chapter IV described results to show that NAD(H)-GDH was insensitive to trypsin treatment. However, Protease XIV-, subtilisin- and thermolysin-treated GDH showed different properties from the non-treated enzyme with respect to its sensitivity to calcium or its capacity to protect the enzyme from substrate inhibition. In particular, the effectiveness of the highly specific proteolytic enzyme,

thermolysin, in eliciting this effect, suggests that the hydrophobic region of the enzyme may be involved in the structure of the Ca^{2+}-binding site. NADH-GDH was activated by a high concentration of NaCl, in the absence of Ca^{2+}, as has been reported for Pea seeds NAD(H)-GDH (Kindt et al. 1980), a result also indicating that hydrophobic regions of the enzyme may be involved in the calcium response. Data from experiments using the -SH inhibitor, p-HMB, suggest that -SH group(s) are also involved in the Ca^{2+}-binding site.

In summary, the results reported in this chapter indicate that Ca^{2+} may have a critical role in regulating GDH activity. Firstly, it is clear that Ca^{2+} is required for catalytic activity of the enzyme as shown by its sensitivity to EGTA even following thermolysin treatment. Secondly, Ca^{2+} appears to protect the enzyme from substrate inhibition by $(NH_4)_2SO_4$. This is particularly relevant when one considers that standard assays of GDH usually involve extremely high concentrations (100-200 mM) of $(NH_4)_2SO_4$ which effectively increase the apparent Ca^{2+}-dependency of the enzyme. There is, of course, a possibility that these two roles are one and the same and that even at very low $(NH_4)_2SO_4$ concentrations (i.e. <1 mM), Ca^{2+} is only required to protect from substrate inhibition. However, enzyme studies at these substrate concentrations are hampered by the extremely low enzyme activity even in the presence of calcium.

In Chapter V, it was shown that smaller amounts of Ca^{2+} are required to fully activate NAD(H)-GDH from turnip

mitochondria in the deamination direction than in the amination direction. Therefore Ca^{2+} may have a role, not only in regulating the degree of expression of the enzyme activity, but also in determining in which direction the enzyme operates. It may also be true that only extremely small amounts of Ca^{2+} might be required for these functions *in vivo*.

Figure 5.1 Effect of Ca^{2+} on the amination reaction of turnip mitochondrial NADH-GDH at various ammonia concentrations.

Assays were carried out in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate and 0.166 mM NADH.

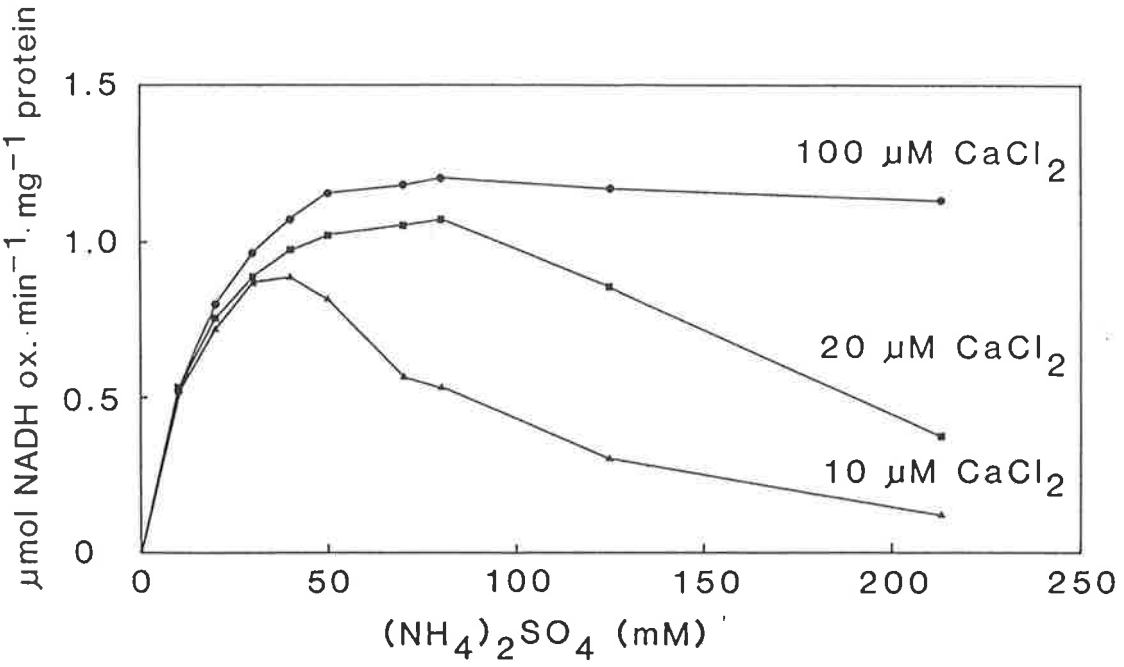

µmol NADH ox. min^{-1}. mg^{-1} protein
1.5
1.0
0.5
0
0
50
100
150
200
250
$(NH_4)_2SO_4$ (mM)
100 µM $CaCl_2$
20 µM $CaCl_2$
10 µM $CaCl_2$

Figure 5.2 Effect of Ca^{2+} on the amination reaction of turnip mitochondrial NADH-GDH at various ammonia concentrations.

Assays were carried out in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate and 0.166 mM NADH.

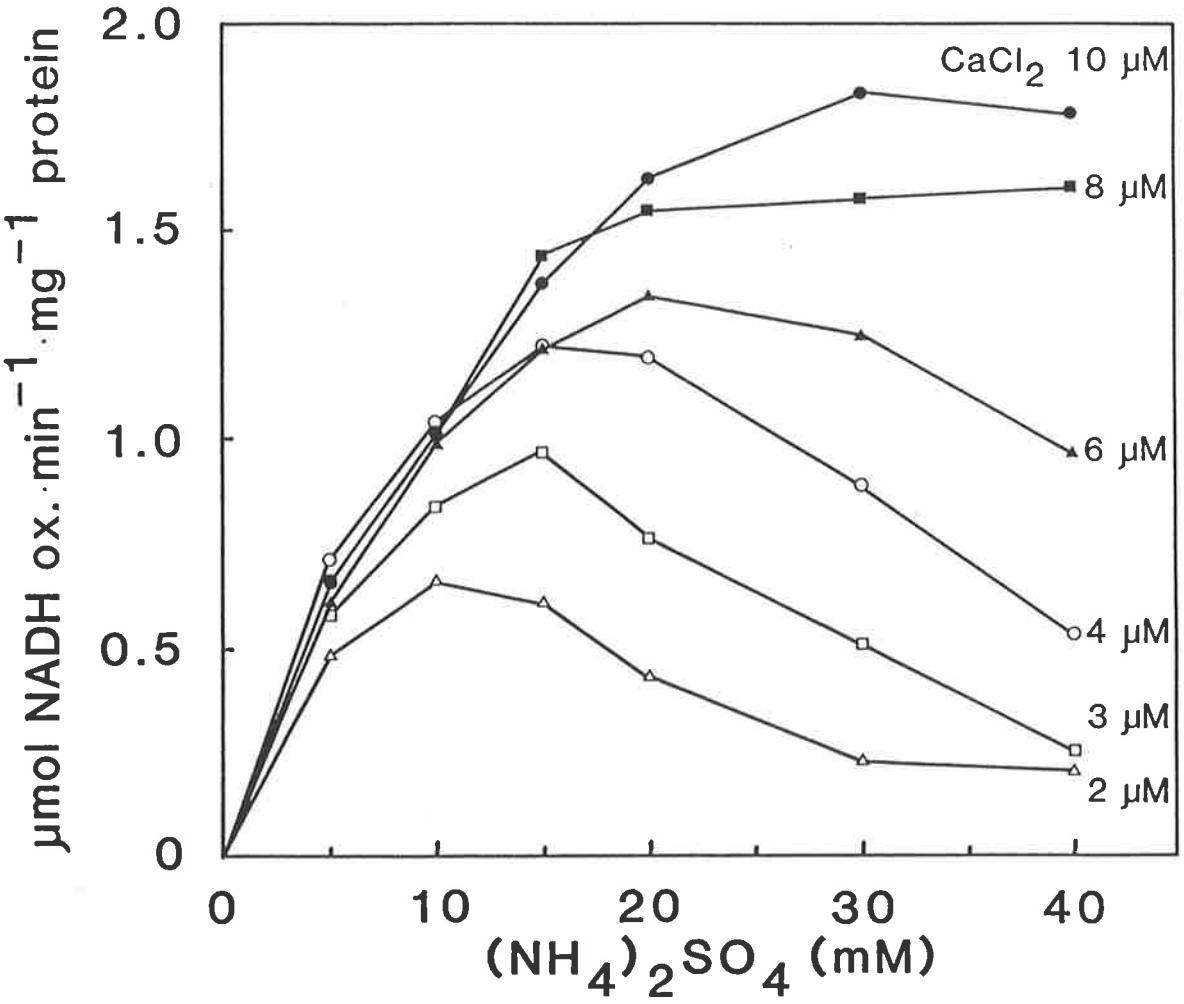

2.0
1.5
1.0
0.5
0
µmol NADH ox. min⁻¹ mg⁻¹ protein
CaCl2 10 µM
8 µM
6 µM
4 µM
3 µM
2 µM
0
10
20
30
40
(NH4)2SO4 (mM)

Figure 5.3 Effect of EGTA and Ca^{2+} on mitochondrial NAD(H)-GDH activity from turnip.

Spectrophotometric assays were carried out in a medium containing 133 mM Tris-HCl (pH 7.4), 13 mM 2-OG, 10 mM $(NH_4)_2SO_4$ and 20 μM NADH for the amination reaction and in 133 mM Tris-HCl (pH 7.4), 10 mM glutamate, 400 μM NAD for the deamination reaction. Rates shown are expressed as μmol NADH ox. min^{-1} mg^{-1} protein.

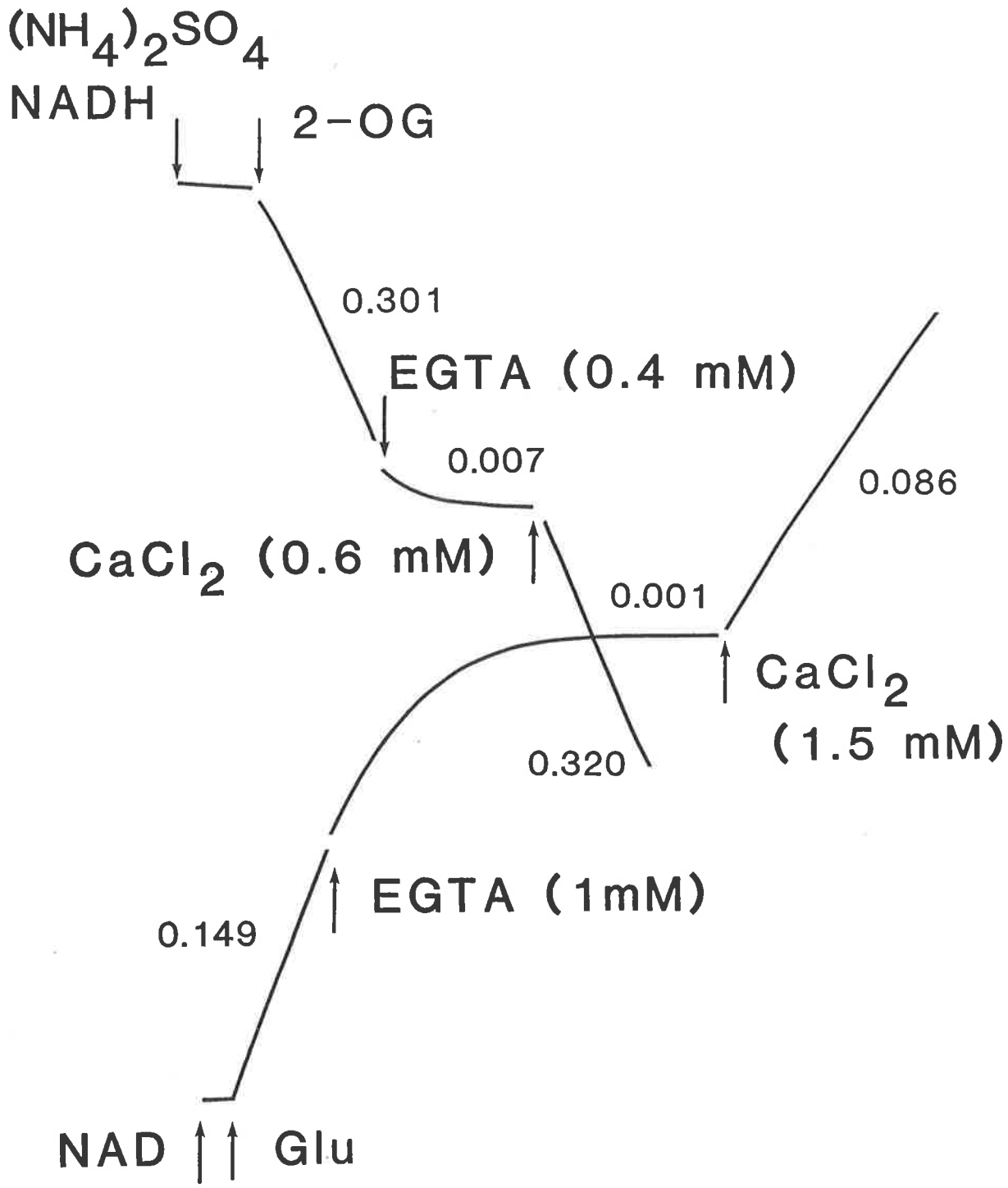

$(NH_4)_2SO_4$
NADH
2-OG
0.301
EGTA (0.4 mM)
0.007
0.086
$CaCl_2$ (0.6 mM)
0.001
$CaCl_2$
(1.5 mM)
0.320
EGTA (1mM)
0.149
NAD
Glu

Figure 5.4 Comparative effects of Protease XIV on NADH-MDH and NADH-GDH from turnip mitochondria.

Mitochondrial extracts were incubated in the Protease XIV (0.2 mg Protease XIV/mg mitochondrial protein) in 50 mM Tris-HCl (pH 8.0) at 28°C. Samples were withdrawn at various times and assayed for NADH-GDH activity in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-OG, 0.166 mM NADH, 0.213 M $(NH_4)_2SO_4$, 0.4 mM $CaCl_2$. NADH-MDH activity was measured in 200 mM Tes-KOH (pH 7.5) and 0.2 mM NADH, with the reaction being initiated with 6 mM OAA.

Symbols:

(●); NADH-GDH activity

(■); NADH-MDH activity

NADH-GDH ACTIVITY (•)
(µmol NADH ox.·min^{-1}·mg^{-1} prot.)
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
MINUTES
40
30
20
10
0
NADH-MDH ACTIVITY (■)
(µmol NADH ox.·min^{-1}·mg^{-1} prot.)

Figure 5.5 Activation of the amination reaction of NAD(H)-GDH from turnip mitochondria by Ca^{2+}.

Mitochondrial extracts were incubated in the presence of the proteolytic enzymes at 28°C in 50 mM Tris-HCl (pH 8.0). Controls without proteolytic enzymes were also incubated at 28°C and 0°C for the same time periods in 50 mM Tris-HCl (pH 8.0). Proteolysis treatment was applied with 0.2 mg of each proteolytic enzymes per mg mitochondrial protein. Assays were carried out in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate, 0.166 mM NADH and 213 mM $(NH_4)_2SO_4$.

Symbols:

(△); Control (28°C)

(●); Control (0°C)

(▲); Trypsin (28°C)

(□); Subtilisin (28°C)

(■); Protease XIV (28°C)

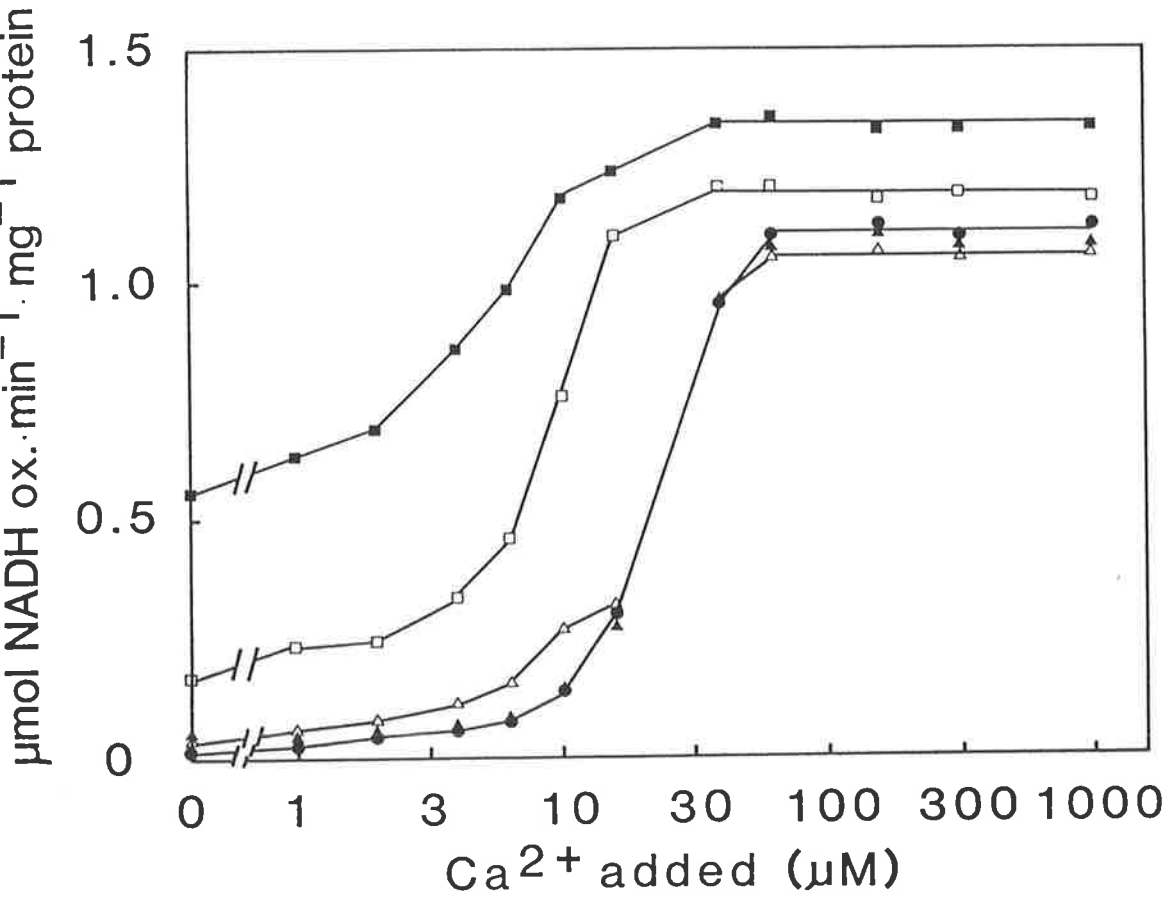

µmol NADH ox. min⁻¹ mg⁻¹ protein
1.5
1.0
0.5
0
0 1 3 10 30 100 300 1000
Ca2+ added (µM)

Figure 5.6 Effect of Ca^{2+} on the amination reaction of mitochondrial NAD(H)-GDH at various ammonia concentrations after Protease XIV treatment.

The Protease XIV treatment (0.2 mg Protease XIV/mg mitochondrial protein) was performed in 50 mM Tris-HCl (pH 8.0) at 28°C for 60 min. Assays were carried out in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate and 0.166 mM NADH.

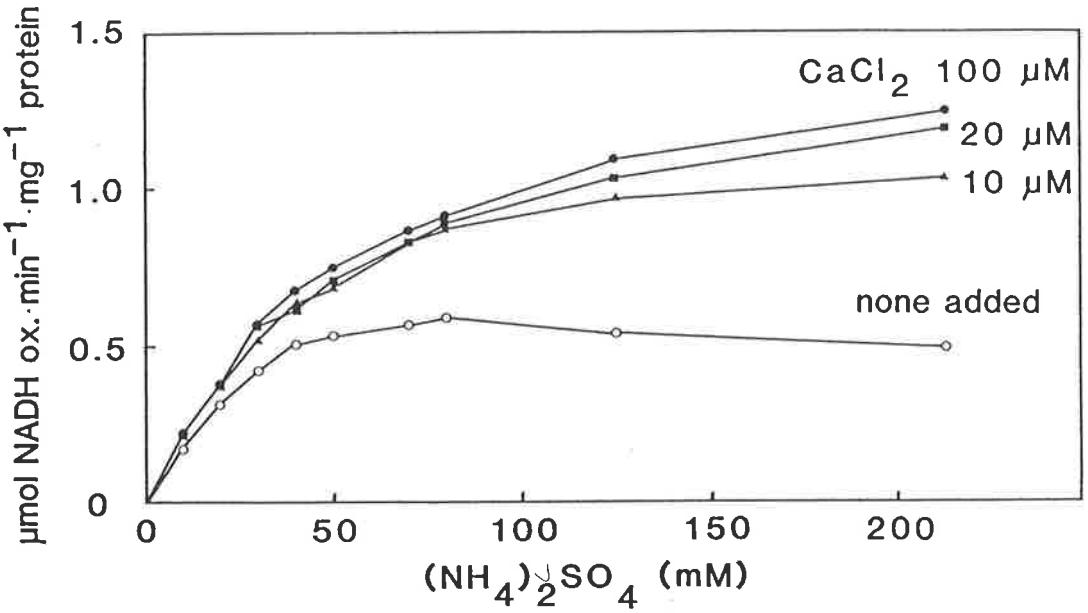

µmol NADH ox. min⁻¹ mg⁻¹ protein
1.5
1.0
0.5
0
0
50
100
150
200
(NH4)2SO4 (mM)
CaCl2 100 µM
20 µM
10 µM
none added

Figure 5.7 Effect of thermolysin on NADH-GDH from Turnip mitochondria.

Mitochondrial extracts were incubated in the presence (●) or absence (■) of thermolysin. Thermolysin was added to the enzyme extract (thermolysin:mitochondrial protein ratio was 1:1) and incubated at 28°C with 50 mM Tris-HCl (pH 8.0) for 60 min in the presence of 5 mM $CaCl_2$ for thermolysin activity. Samples were withdrawn at various times. Assays were carried out in a medium containing 0.133 M Tris-HCl (pH 8.0) 13 mM 2-oxoglutarate, 0.166 mM NADH, 0.213 M $(NH_4)_2SO_4$ and 0.4 mM $CaCl_2$ (Ca^{2+} which is carried over from incubation medium for thermolysin activity (approximately 8-17 μM) was negligible because GDH assays were performed in the presence of 0.4 mM $CaCl_2$).

Symbols:

(●); presence of Thermolysin

(■); absence of Thermolysin

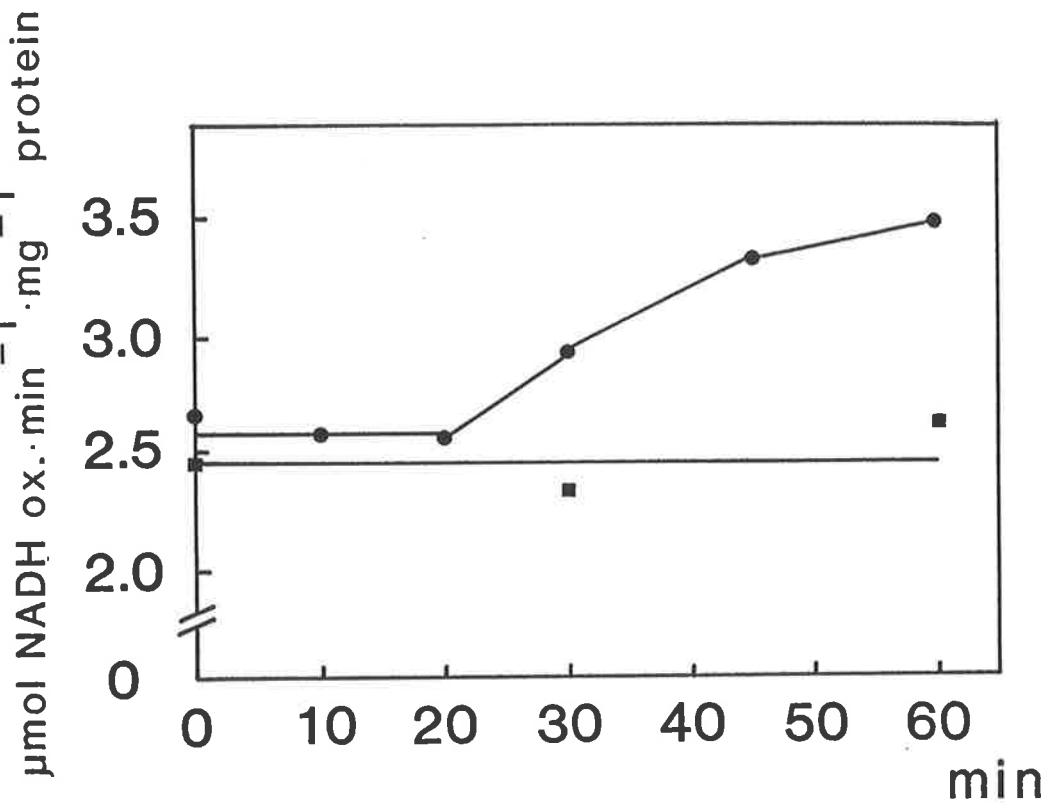

µmol NADH ox.·min⁻¹·mg⁻¹ protein
3.5
3.0
2.5
2.0
0
0
10
20
30
40
50
60
min

Figure 5.8 Substrate saturation curves of NADH-GDH after treatment with thermolysin.

Mitochondrial extracts were incubated in the presence (●) of thermolysin. Thermolysin was added to the enzyme extract (Protein:Thermolysin ratio was 1:1) and incubated at 28°C in the presence of 5 mM $CaCl_2$ for thermolysin activity with 50 mM Tris-HCl (pH 8.0) for 60 min. Assays were carried out in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate, 0.166 mM NADH and $CaCl_2$ and $(NH_4)_2SO_4$ which was present at the concentrations shown in Figure. (As Ca^{2+} was included in the incubation medium for thermolysin activity, approximately 8-17 μM Ca^{2+} was carried over into the assay medium after the incubation).

Symbols:

(●); Thermolysin-treated (maximum 17 μM Ca^{2+})

(▲); control (8 μM Ca^{2+})

(○); control (20 μM Ca^{2+})

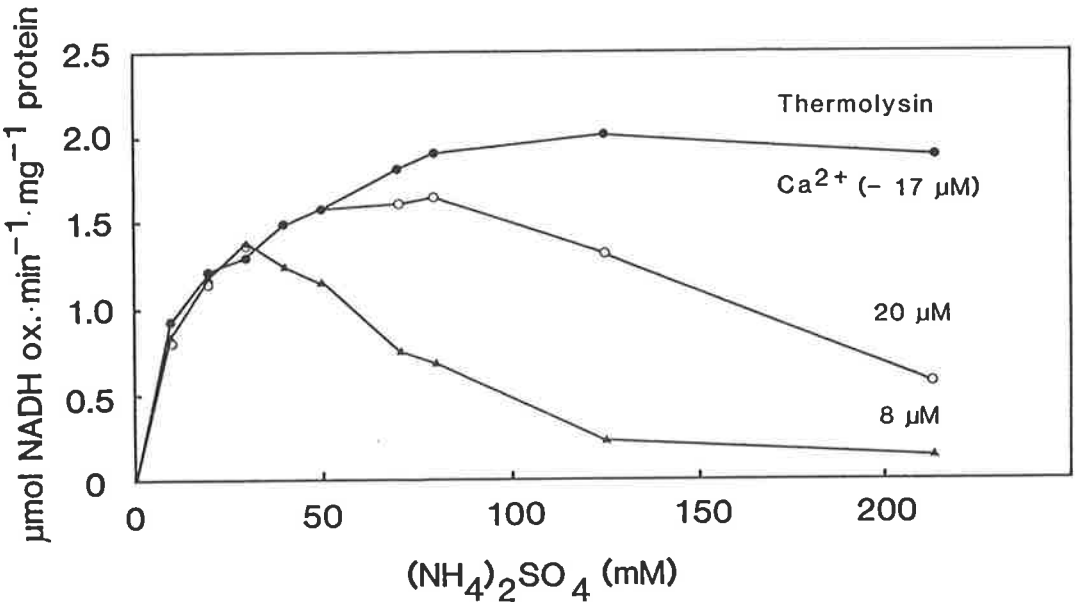

µmol NADH ox. min⁻¹ mg⁻¹ protein
2.5
2.0
1.5
1.0
0.5
0
0
50
100
150
200
(NH4)2SO4 (mM)
Thermolysin
Ca2+ (- 17 µM)
20 µM
8 µM

Figure 5.9 Effect of EGTA and Ca^{2+} on thermolysin treated NADH-GDH from turnip mitochondria.

Assays were carried out in media composed of 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate, 0.166 mM NADH and 0.213 M $(NH_4)_2SO_4$. $CaCl_2$ and EGTA were added at the concentrations indicated. Rates shown are expressed as μmol NADH ox. min^{-1} mg^{-1} protein.

Symbols:

(A); non-treated NADH-GDH activity with Ca^{2+}

(B); non-treated NADH-GDH activity without Ca^{2+}

(C); Thermolysin-treated NADH-GDH activity without addition of Ca^{2+}

(D); Thermolysin-treated NADH-GDH activity with Ca^{2+}

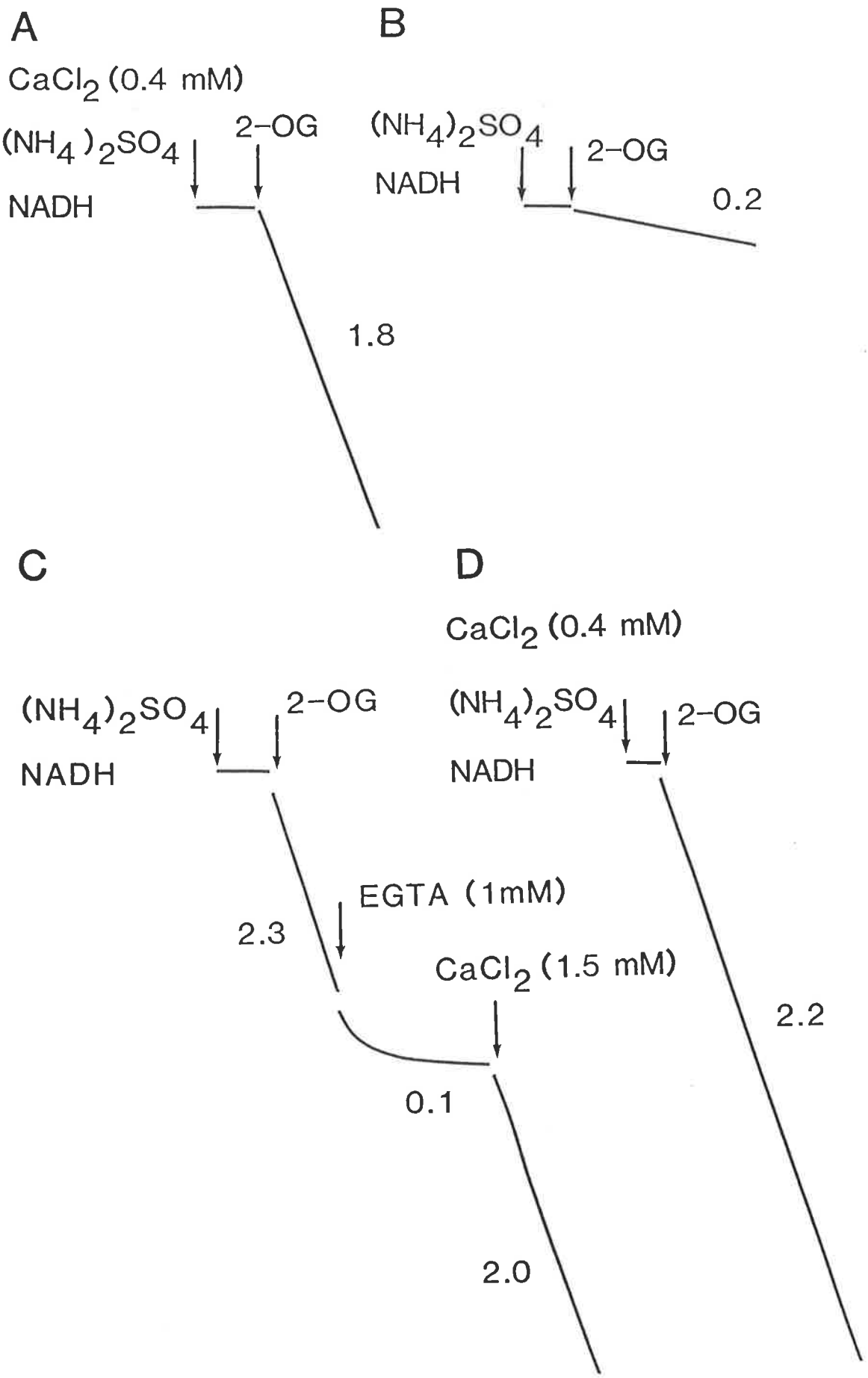

A
$CaCl_2$ (0.4 mM)
$(NH_4)_2SO_4$
2-OG
NADH
1.8
B
$(NH_4)_2SO_4$
2-OG
NADH
0.2
C
$(NH_4)_2SO_4$
2-OG
NADH
2.3
EGTA (1mM)
$CaCl_2$ (1.5 mM)
0.1
2.0
D
$CaCl_2$ (0.4 mM)
$(NH_4)_2SO_4$
2-OG
NADH
2.2

Figure 5.10 Effect of NaCl on NADH-GDH activity with saturating $(NH_4)_2SO_4$.

Assays were carried out in a medium containing 0.133 M Tris-HCl (pH 8.0) 13 mM 2-oxoglutarate, 0.166 mM NADH and 0.213 M $(NH_4)_2SO_4$.

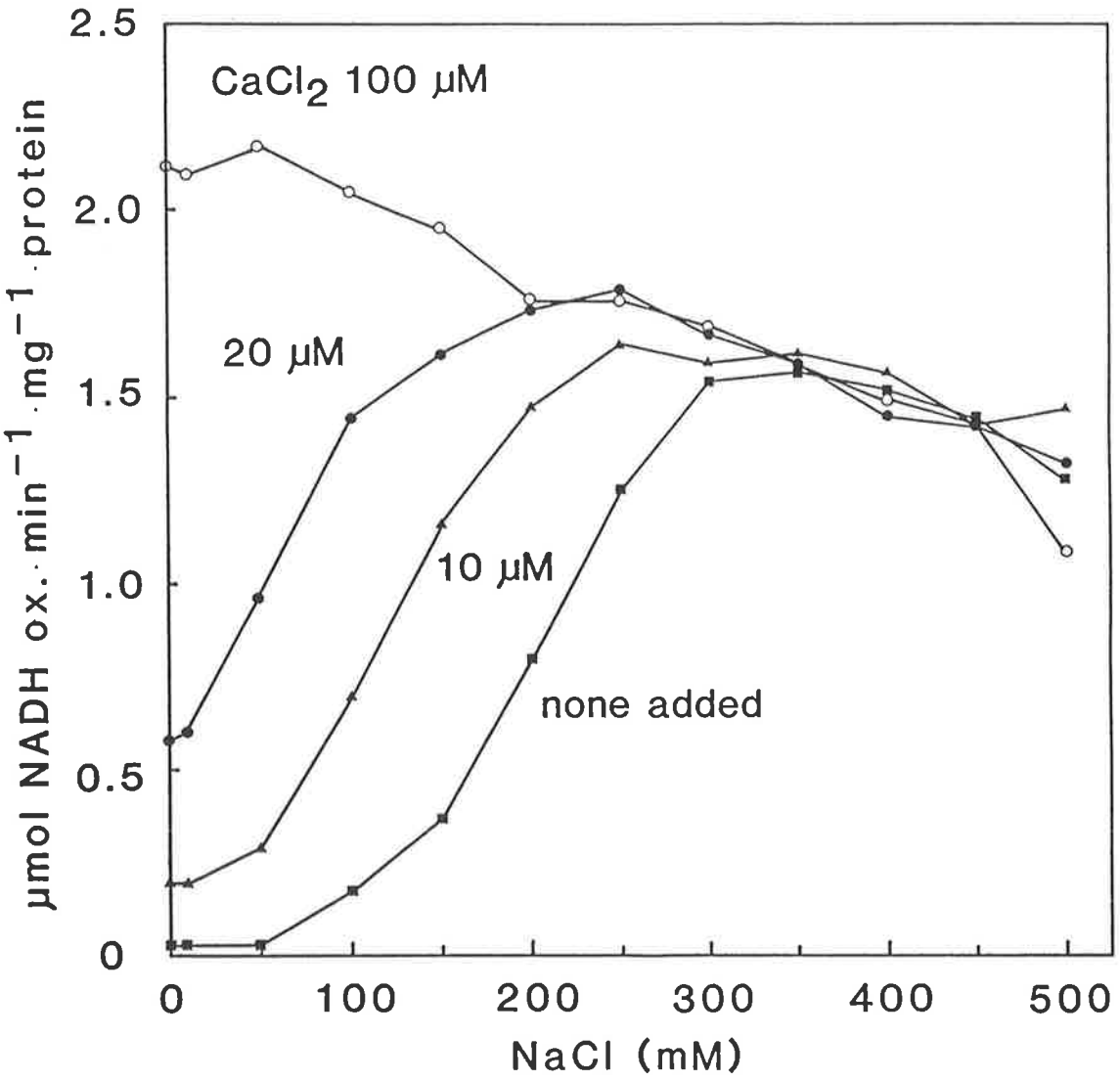

2.5
2.0
1.5
1.0
0.5
0
µmol NADH ox.·min⁻¹·mg⁻¹·protein
CaCl2 100 µM
20 µM
10 µM
none added
0
100
200
300
400
500
NaCl (mM)

Figure 5.11 Effect of NaCl on turnip NADH-GDH activity with varying substrate concentrations.

Assays were carried out in a medium containing 0.133 M Tris-HCl (pH 8.0), 13 mM 2-oxoglutarate and 0.166 mM NADH.

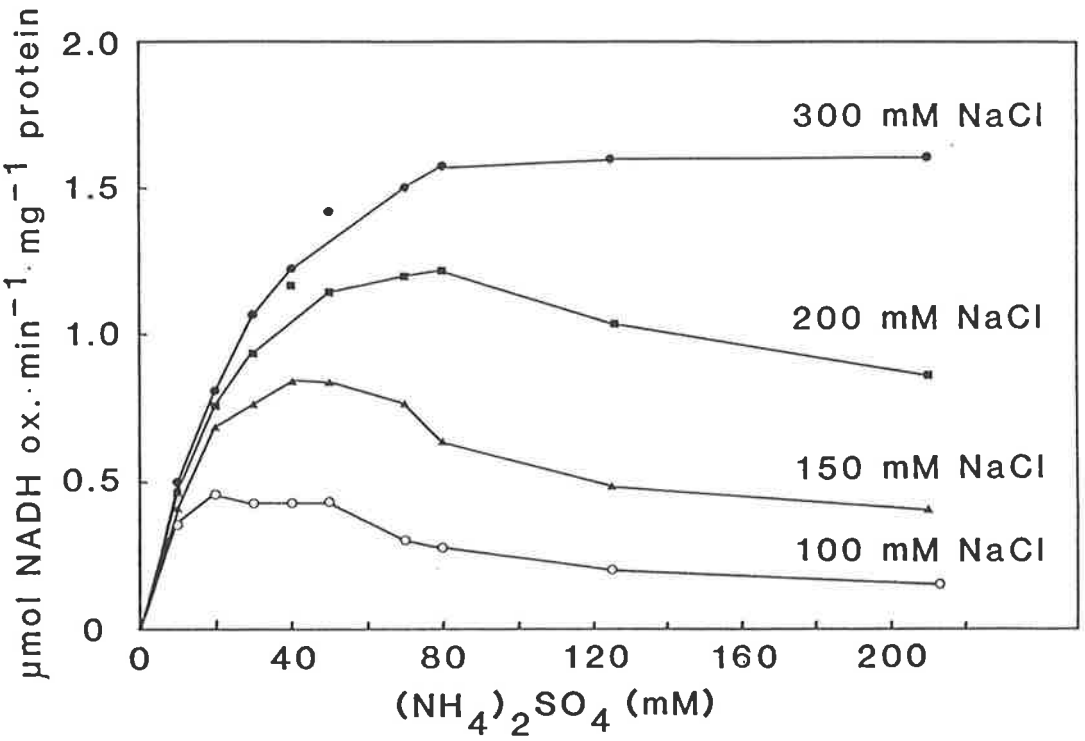
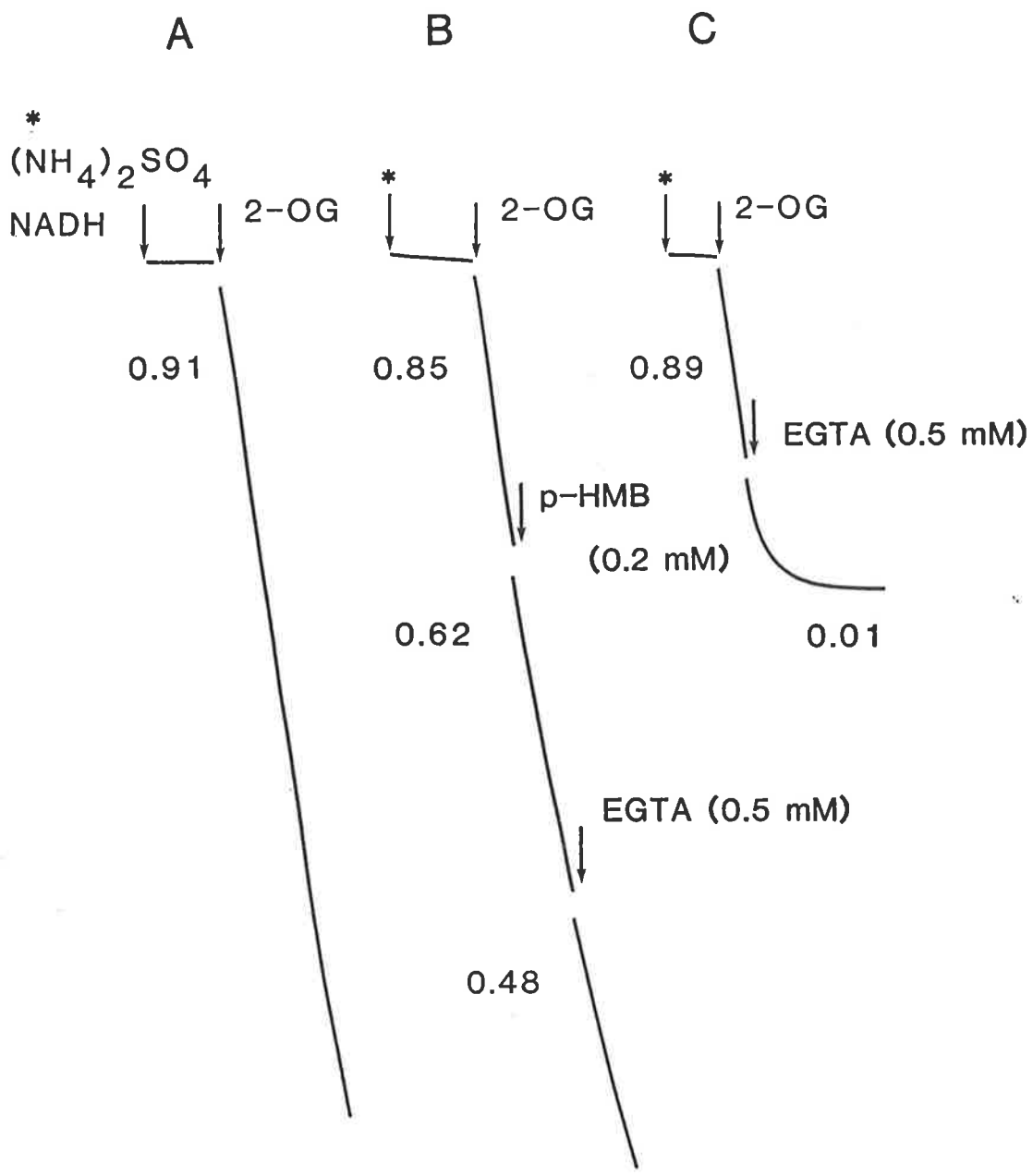

µmol NADH ox. · min⁻¹ · mg⁻¹ protein
2.0
1.5
1.0
0.5
0
0
40
80
120
160
200
(NH4)2SO4 (mM)
300 mM NaCl
200 mM NaCl
150 mM NaCl
100 mM NaCl

Figure 5.12 Effect of p-HMB and EGTA on NADH-GDH activity. Assays were carried out in a medium containing 133 mM Tris-HCl (pH 7.4), 13 mM 2-OG, 10 mM $(NH_4)_2SO_4$, 20μM NADH and 0.2 mM $CaCl_2$. p-HMB and EGTA was added at the concentrations shown in the Figure. Rates shown are expressed as μmol NADH ox. min^{-1} mg^{-1} protein.



(*): $(NH_4)_2SO_4$ and NADH were added.

CHAPTER VI

THE PATHWAY OF GLUTAMATE OXIDATION IN ISOLATED MITOCHONDRIA

6.1 *INTRODUCTION*

It has been suggested that because of its low apparent affinity for ammonia (Km= 5-70 mM; Stewart et al. 1980), the main function of mitochondrial GDH in vivo may be to deaminate glutamate (Miflin and Lea, 1977). Day et al. (1988) has recently postulated that glutamate oxidation via mitochondrial GDH may have a key role in reserve mobilization and amino acid metabolism during seedling growth.

Plant mitochondria are able to utilize glutamate as an oxidisable substrate, but it is thought that both AsAT and GDH may be involved. Recently, Yamaya and Matsumoto (1985) reported that glutamate oxidation by mitochondria isolated from corn and pea shoots was inhibited over 60% by the transaminase inhibitor, AOA. They concluded that the major route of glutamate oxidation proceeded via transamination rather than direct deamination by GDH, supporting their earlier conclusion that GDH functions to synthesize glutamate (Yamaya et al. 1984). However, it would seem unlikely that their isolated mitochondria had sufficient internal OAA to initiate rapid rates of AsAT activity. It seems more likely that GDH is involved in the initiation of glutamate oxidation by plant mitochondria.

Consequently, I decided to study glutamate oxidation, and its interaction with AOA using mitochondria from both photorespiratory (pea leaf) and non-photorespiratory (turnip root) tissues.

6.2 RESULTS

6.2.1 *Effect of aminooxyacetate (AOA) on glutamate oxidation by turnip mitochondria*

The rates of glutamate oxidation by turnip mitochondria in the presence of various substances is shown in Table 6.1. The state 3 rate of glutamate oxidation was inhibited approximately 17% by 1 mM AOA (Table 6.1). TPP, which increases the rate of 2-OG oxidation, also increased the rate of glutamate oxidation (Table 6.1). The addition of AOA, which has no effect on 2-OG oxidation (Table 6.1), inhibited glutamate oxidation by approximately 30% suggesting a greater involvement of AsAT under these conditions (Fig. 6.1). In the presence of malonate, which inhibits succinate dehydrogenase, the rate of glutamate oxidation was markedly reduced, and more importanly, AOA did not cause any inhibition (Fig. 6.2).

6.2.2 *Effects of AOA on glutamate oxidation by pea leaf mitochondria*

Glutamate oxidation was inhibited 45% and 50% by the addition of 1 mM AOA in the presence and absence of TPP respectively (Table 6.1). These values are similar to those

reported by Yamaya and Matsumoto (1985) for both corn shoot and pea leaf mitochondria. The data in Table 6.1 shows that in pea leaf mitochondria, glutamate oxidation was not stimulated by the addition of TPP. This suggests that they were not deficient in TPP and the oxidation of saturating concentrations of 2-OG, showing no effect by TPP, supports this conclusion. The inhibition by AOA was gradual and it took several minutes to achieve a constant inhibited rate (Fig 6.3). Again, glutamate oxidation in the presence of malonate ($\pm$TPP) was not inhibited by AOA (Fig. 6.4).

6.3 DISCUSSION

The results with pea leaf mitochondria were similar to those obtained with turnip except that the latter were deficient in TPP. Hence the addition of TPP stimulated 2-OG oxidation to a greater extent in turnip than in pea leaf mitochondria.

The pathways of oxidation of glutamate present in plant mitochondria are shown in Fig 6.5. It is clear that GDH can operate on the addition of glutamate but that AsAT activity depends on a supply of OAA. Thus, under all circumstances where the supply of OAA was restricted by the inclusion of malonate to inhibit succinate dehydrogenase (SDH), there was no inhibitory effect of AOA (Fig. 6.2 and Fig. 6.4) i.e. glutamate was not being oxidized via AsAT. Turnip mitochondria, being deficient in TPP, showed a slight inhibition with AOA, indicating a slow turnover of 2-OG DH.

The results clearly show that the oxidation of glutamate alone does involve the simultaneous oxidation of 2-OG and subsequent intermediates of the TCA cycle leading to the formation of OAA (Fig 6.5). This, in turn, leads to the involvement of AsAT and so the percent inhibition observed with AOA will depend on the activity of the other TCA cycle enzymes, and a strong inhibitory effect is not sufficient evidence to eliminate the operation of GDH. A complete cycle involving AsAT would consume 3 x O atoms per glutamate whereas, the operation of GDH would consume 4 x O atoms (up to the formation of OAA). The addition of AOA to this system, once established would severely inhibit O_2 uptake i.e. the 3 x O due to AsAT activity would be inhibited as would the 1 x O due to malate dehydrogenase (MDH) (due to its unfavarable equilibrium) resulting from an initial GDH step i.e. the inhibitory effect would be 4/7 or 57%.

These results show that the initiation of glutamate oxidation is dependent on GDH, that other oxidations occur subsequently and that AsAT is not active if the supply of OAA is prevented (e.g. by adding malonate).

The delayed inhibition by AOA in pea leaf mitochondria (with or without TPP) compared to turnip may be explained by the time taken to consume 2-OG that had been generated by AsAT activity prior to the addition of AOA (Fig. 6.1 and 6.3).

These results confirm that the inhibition of glutamate oxidation by AOA is due to the inhibition of AsAT

in pea and turnip mitochondria (Yamaya and Matsumoto, 1985). However, AsAT can not initiate glutamate oxidation but requires a supply of OAA. A detailed analysis of glutamate oxidation by soybean cotyledons mitochondria has recently been published (Day et al. 1988), which supports these conclusions.

Table 6.1 Oxidation of glutamate and 2-oxoglutarate by isolated turnip and pea leaf mitochondria.

O_2 consumption was measured in 2.0 ml of medium containing 0.3 M sucrose, 10 mM Tes, 10 mM KH_2PO_4, 5 mM $MgCl_2$ and 0.1% BSA adjusted to pH 7.2 for pea leaf mitochondria and 0.25 M sucrose, 10 mM Tes, 10 mM KH_2PO_4 and 5 mM $MgCl_2$ adjusted to pH 7.2 for turnip mitochondria. Concentrations used: 10 mM of glutamate (Glu), 2-oxoglutarate (2-OG), malate (Mal), 1 mM AOA, 0.17 mM TPP, 2 mM malonate (Malo.).

Assay conditions	Rate of state 3 respiration (nmol O_2 min^{-1} mg mitochondrial $protein^{-1}$)	
	Pea leaf	Turnip root
Glu	9.8	20.8
Glu +AOA	5.1	17.3
Glu +TPP	9.4	51.2
Glu +TPP +AOA	4.2	35.5
Glu +Malo.	3.9	16.0
Glu +Malo. +AOA	4.2	16.0
Glu +TPP +Malo.	2.2	22.7
Glu +TPP +Malo. +AOA	2.2	23.5
2-OG	32.1	97.1
2-OG +AOA	-	98.1
2-OG +TPP	38.8	293.3
2-OG +AOA + TPP	-	293.3
Mal +Glu	103.8	165.3

Figure 6.1 Glutamate oxidation by turnip mitochondria.

O_2 consumption was measured in 2.0 ml of medium containing 0.25 M Sucrose, 10 mM Tes, 10 mM KH_2PO_4, 5 mM $MgCl_2$, adjusted to pH 7.2. Additions as indicated were 10 mM glutamate, 0.17 mM TPP and 1 mM AOA. Rates shown are expressed as nmol O_2 min^{-1} mg^{-1} protein.

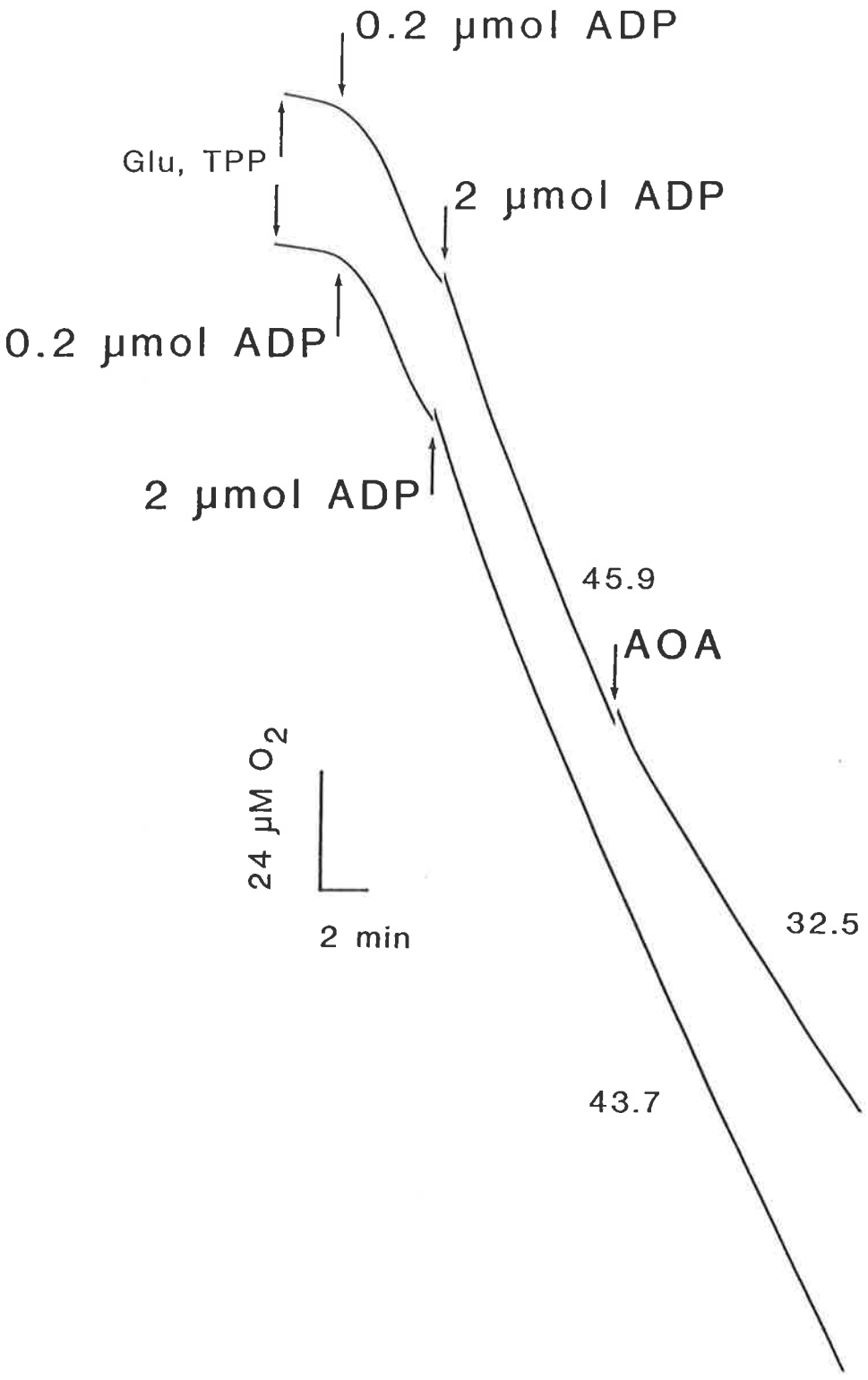

0.2 µmol ADP
Glu, TPP
2 µmol ADP
0.2 µmol ADP
2 µmol ADP
45.9
AOA
24 µM O_2
2 min
32.5
43.7

Figure 6.2 Glutamate oxidation by Turnip mitochondria in the presence of malonate.

O_2 consumption was measured in 2.0 ml of medium containing 0.25 M Sucrose, 10 mM Tes, 10 mM KH_2PO_4, 5 mM $MgCl_2$, adjusted to pH 7.2. Additions as indicated were 10 mM glutamate, 2 mM malonate, 0.17 mM TPP and 1 mM AOA. Rates shown are expressed as nmol O_2 min^{-1} mg^{-1} protein.

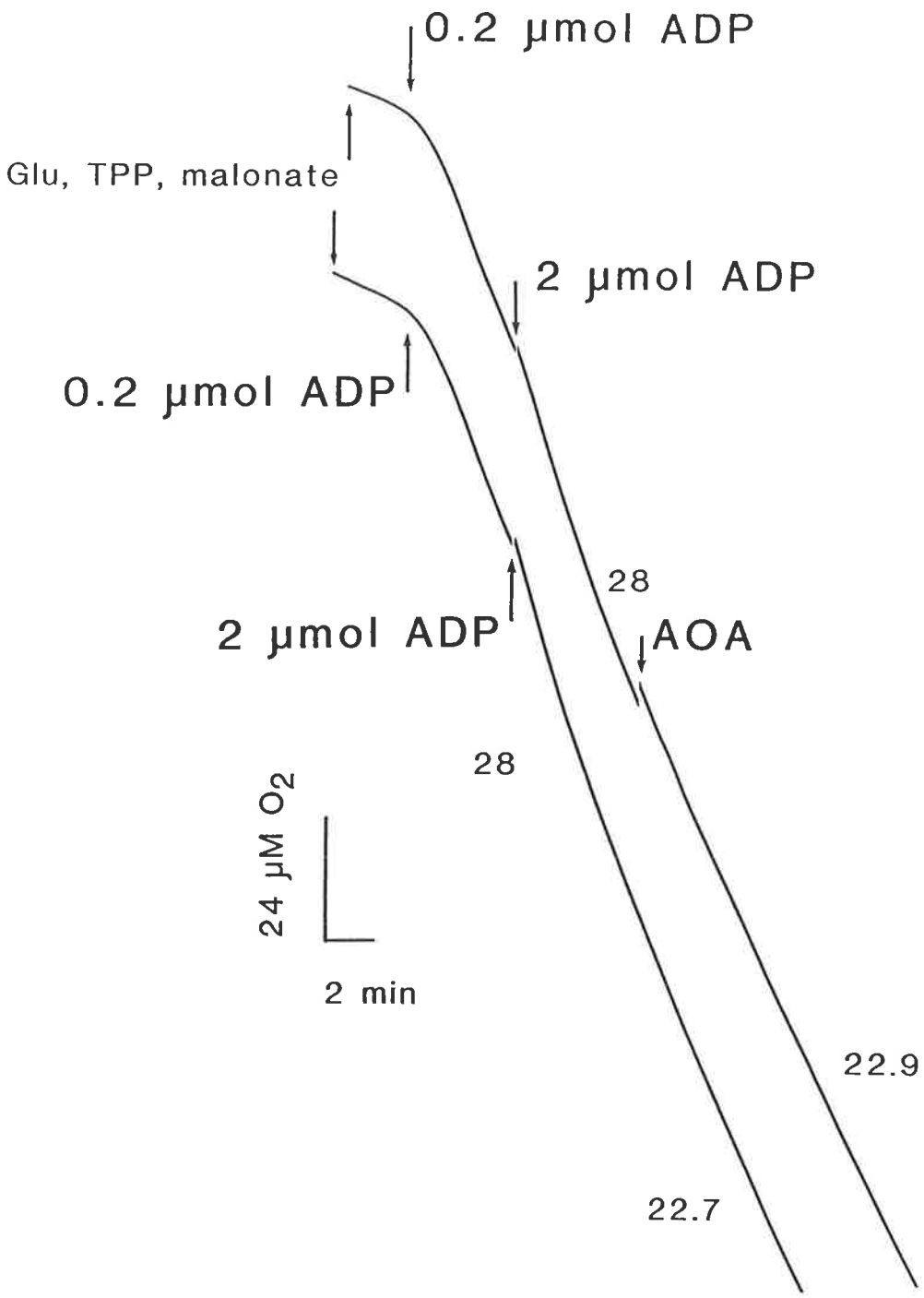

0.2 µmol ADP
Glu, TPP, malonate
2 µmol ADP
0.2 µmol ADP
28
2 µmol ADP
AOA
28
24 µM O2
2 min
22.9
22.7

Figure 6.3 Glutamate oxidation by pea leaf mitochondria.

O_2 consumption was measured in 2.0 ml of medium containing 0.3 M Sucrose, 10 mM Tes, 10 mM KH_2PO_4, 5 mM $MgCl_2$ and 0.1% BSA adjusted to pH 7.2. Additions as indicated were 10 mM glutamate, 0.17 mM TPP and 1 mM AOA. Rates shown are expressed as nmol O_2 min^{-1} mg^{-1} protein.

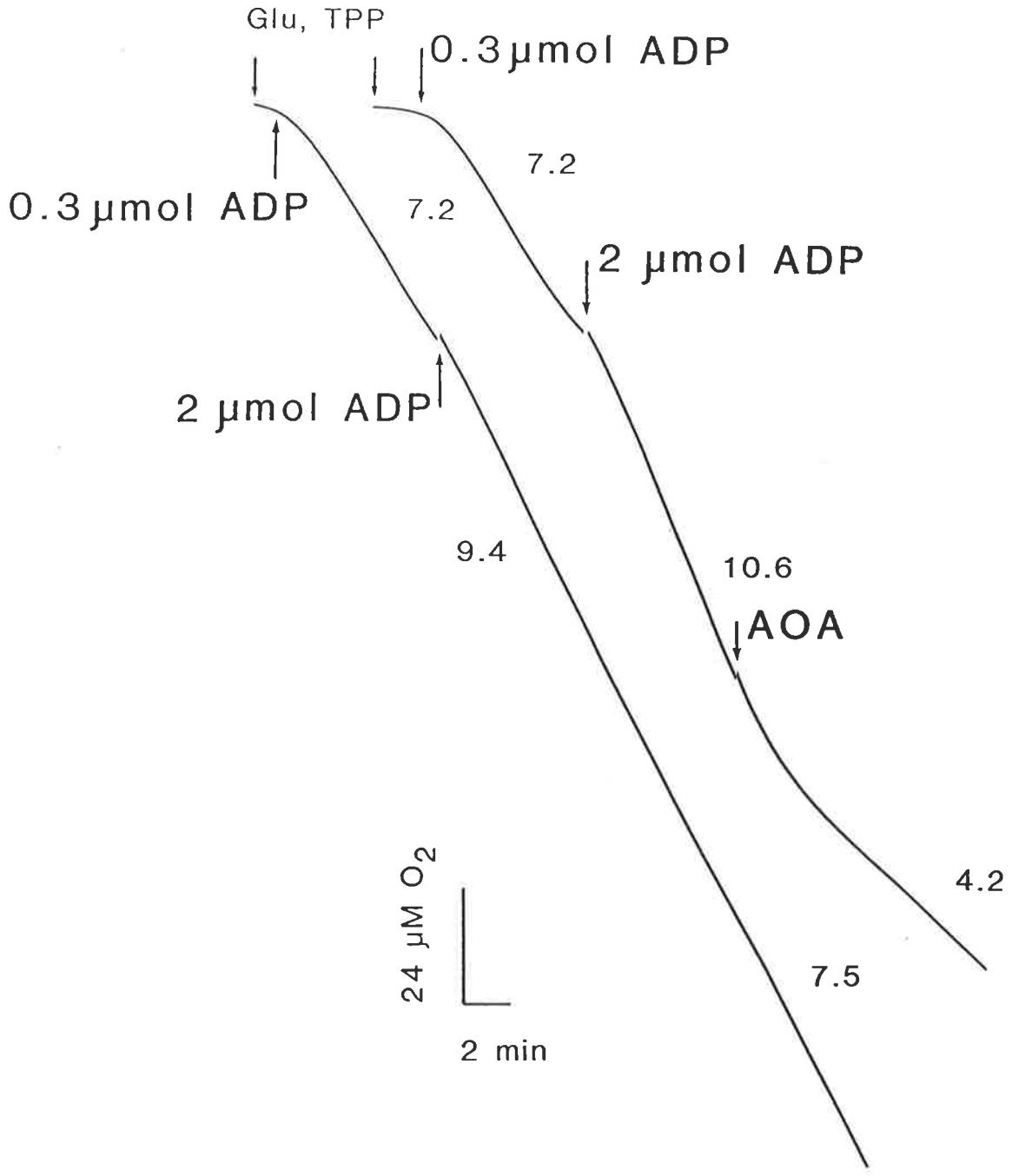

Glu, TPP
0.3µmol ADP
0.3µmol ADP
7.2
7.2
2 µmol ADP
2 µmol ADP
9.4
10.6
AOA
4.2
7.5
24 µM O2
2 min

Figure 6.4 Glutamate oxidation by pea leaf mitochondria in the presence of malonate.

O_2 consumption was measured in 2.0 ml of medium containing 0.3 M Sucrose, 10 mM Tes, 10 mM KH_2PO_4, 5 mM $MgCl_2$ and 0.1% BSA adjusted to pH 7.2. Additions as indicated were 10 mM glutamate, 2 mM malonate, 0.17 mM TPP and 1 mM AOA. Rates shown are expressed as nmol O_2 min^{-1} mg^{-1} protein.

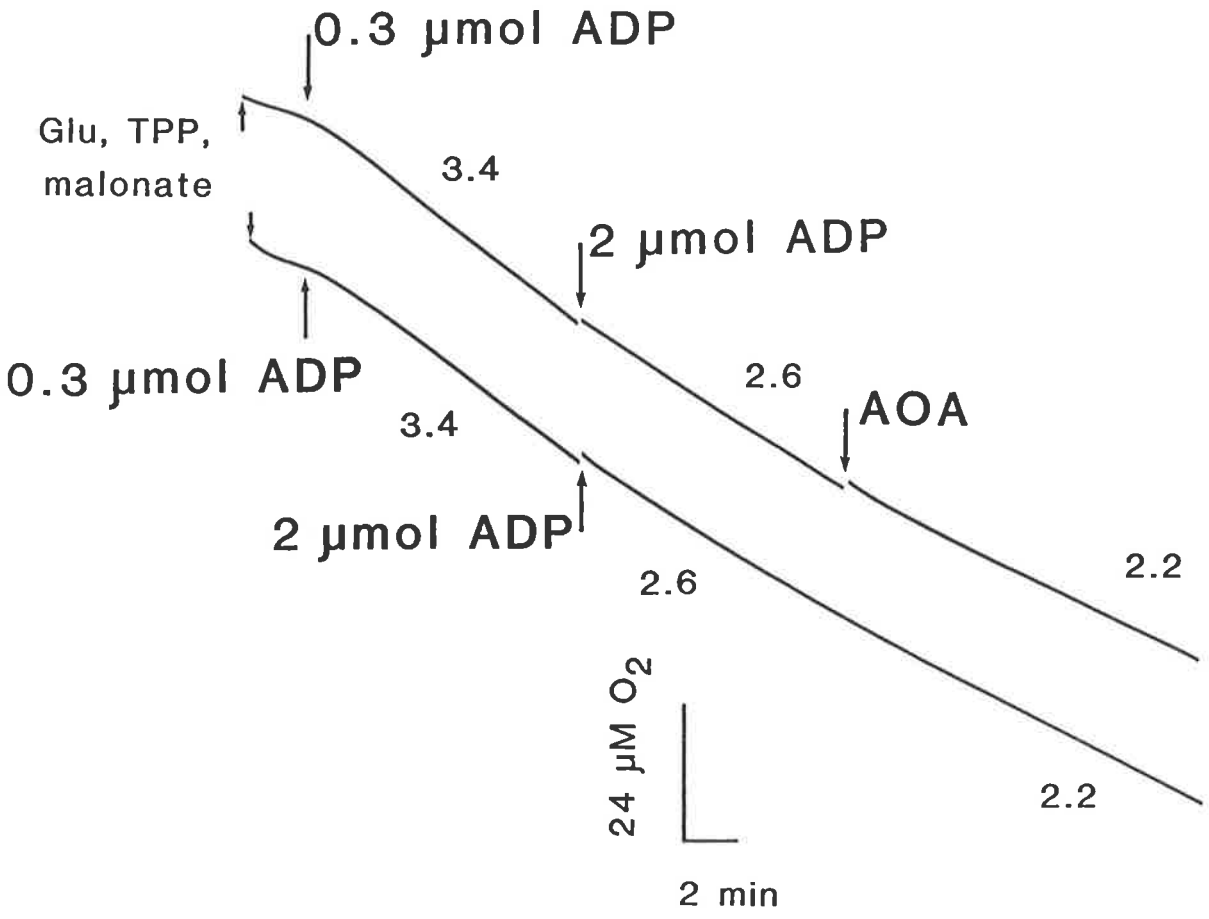

0.3 µmol ADP
Glu, TPP,
malonate
3.4
2 µmol ADP
0.3 µmol ADP
2.6
AOA
3.4
2 µmol ADP
2.2
2.6
24 µM O2
2.2
2 min

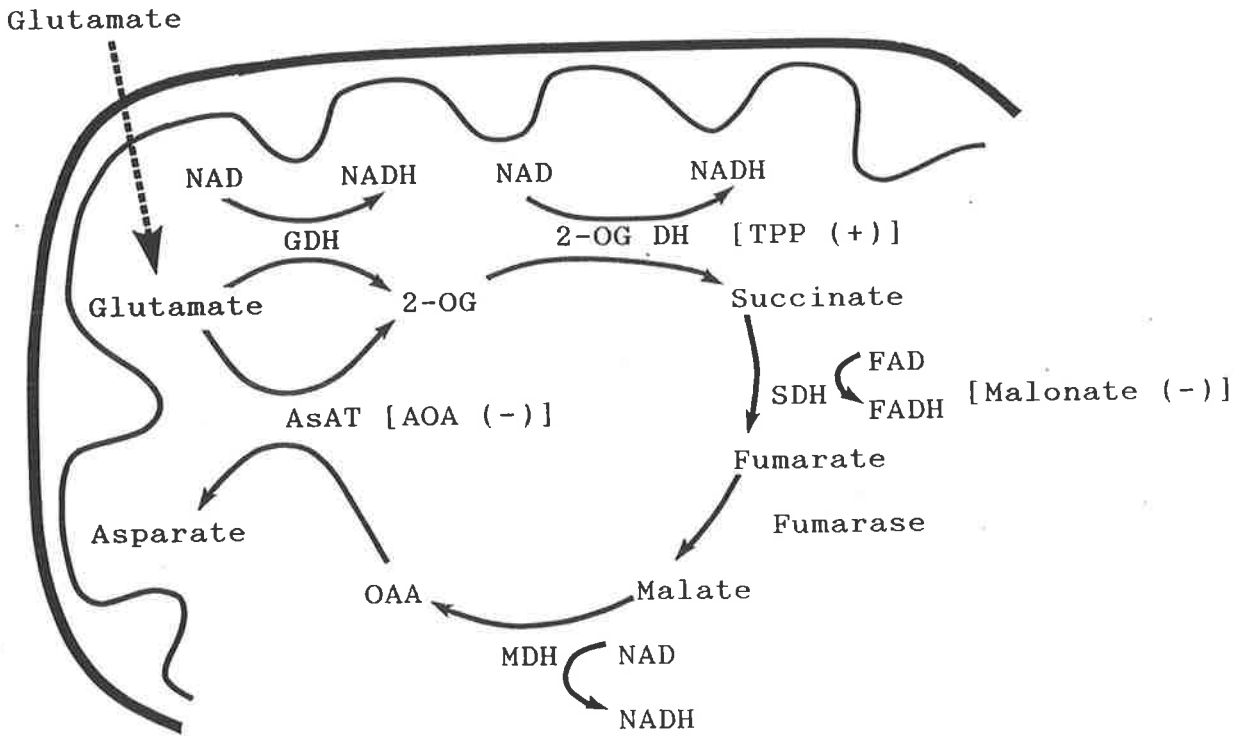
Figure 6.5 Pathways of glutamate oxidation in plant mitochondria.

Abbriviations:

AsAT; aspartate aminotransferase

GDH; glutamate dehydrogenase

SDH; succinate dehydrogenase



CHAPTER VII

GENERAL DISCUSSION

It has been reported that two enzymes, glutamate synthetase (GS) and glutamate dehydrogenase (GDH) could be involved, in parallel, for ammonia fixation. GS would operate in association with glutamate synthase (GOGAT) formulating the so called GS/GOGAT pathway. As GS has a higher affinity for ammonia than GDH, it has been accepted that GS plays the more significant role in ammonia fixation compared to GDH, and that GDH functions in the deamination of glutamate (Miflin and Lea, 1977). However, the function of GDH *in vivo* is still unclear.

Photosynthetic tissues of C_3 plants carry out photorespiration during which ammonia is released from mitochondria as a result of glycine decarboxylation to serine (Keys *et al*. 1978). This ammonia has to be recycled to maintain the operation of the photorespiratory cycle. In general, it has been argued that ammonia released from photorespiration is reassimilated by chloroplastic GS (Givan *et al*. 1988). It has been also suggested that GDH may have an ancillary role to the GS/GOGAT pathway and may contribute to reassimilation of NH_3 produced by photorespiration (Yamaya *et al*. 1984; Yamaya and Oaks, 1987). However, this makes the function of GDH in non-green tissue even more obscure. It has been reported that NADP(H)-GDH from *Chlorella sorokiniana* (Tischner, 1984; Bascomb, 1987) and *Caulerpa simpliciuscula* (Gayler and Morgan, 1976; Mc Kenzie *et al*. 1979) has a

sufficiently high affinity for ammonia to be capable of fixing NH_3. On the contrary, NADP(H)-GDH from Euglena gracilis which also has a high affinity for ammonia (Javed and Merrett, 1986) may in fact have a catabolic function in vivo (Fayyaz-Chaudhary et al., 1985). Therefore, there is a need to clarify the function of GDH in both algae and higher plants.

This study has involved a comparison of the properties of GDH enzymes isolated from the green algae Ulva australis, Bryopsis maxima and the higher plant non-green root tissue of turnip (Brassica rapa L.). The pathways of mitochondrial glutamate oxidation were also examined using green (pea leaf) and non-green (turnip) root tissues.

Comparison of the properties of GDH from Ulva australis, Bryopsis maxima and turnip are shown in Table 7.1. Clearly, the function of these enzymes could not be confidently predicted from Km values. The subunit and total molecular size of turnip and Ulva australis GDH were similar to those already reported for higher plant (Stone et al. 1979; Kindt et al. 1980) and Chlorella sorokiniana GDH (Meredith et al. 1978), respectively. NAD(H)-GDH is located within the mitochondria of turnip tissue, however, the location of NAD(H)-GDH in Ulva australis is unknown. Because of the similarity of molecular size of the enzyme to the mitochondrial NAD(H)-GDH from Chlorella sorokiniana (Meredith et al. 1978; Bascomb and Schmidt, 1987) and because of its similar co-enzyme specificity, NAD(H)-GDH from Ulva australis

is also likely to be mitochondrial in location. The total molecular size and subunit molecular size of NADP(H)-GDH has varied from the different sources. The NADP(H)-GDH extracted from Bryopsis maxia chloroplasts has been assumed to be chloroplastic and its high specificity for NADP supports this speculation (Stewart et al. 1980), but its subunit molecular size of 46 kDa is different from the chloroplastic NADP(H)-GDH of Chlorella sorokiniana (Bascomb and Schmidt, 1987). The subunit molecular weight of NADP(H)-GDH from Bryopsis maxima was similar to the cytosolic GDH from Euglena gracilis (Javed and Merrett, 1986), but the total molecular size of the enzyme was different.

The Km values and pH optima of Ulva australis were similar and fall within the ranges determined for this enzyme from other plant tissues (Stewart et al. 1980). However, a major difference between algal and higher plant GDH was seen in their relative sensitivity towards Ca^{2+} and EGTA. It is not known why these differences have developed and whether they are related to their function in the cell, but it does suggest that they have different ways of regulating their activities. These different properties are, presumably, an evolutionary response to their different growing environments. Therefore, algal GDH would be regulated by some parameter other than bivalent cation as appears to be the case in higher plant. It is important to know the regulatory mechanisms and location of GDH in algae to fully understand the physiological function of this enzyme in these species.

The properties of NAD(H)-GDH from the non-green, root tissue of turnip were shown to be quite similar to the enzyme from other plant sources (Stewart et al. 1980). A disconcerting aspect relating to the function of this enzyme is its affinity for glutamate and ammonia. The Km values for both glutamate and ammonia (as a ammonium sulphate pH 8.0) for turnip enzyme were not sufficiently low to allow firm conclusions to be made about the function of GDH. Furthermore, the Km values for these substrates changed under various assay conditions, and my determinations may not reflect the real in vivo values.

Turnip mitochondrial GDH was activated by calcium in both the amination and deamination direction. The required concentration of calcium to fully activate the enzyme for amination was approximately 10 times higher than for deamination. The calcium concentration required for maximum activity was also dependent on substrate concentration; 60 μM Ca^{2+} was required to fully activate the amination reaction with 213 mM ammonia (as ammonium sulphate at pH 8.0), but only 2 μM calcium was required with 10 mM ammonia. Yamaya et al. (1984) have reported that the calcium level in corn shoot mitochondrial matrix was 52-56 mM. If this value is also valid for the intramitochondrial compartment of turnip mitochondria, then GDH would always be in the fully active form, depending the form of the Ca^{2+} and the enzyme within the mitochondrial matrix. Nauen and Hartmann (1980) have reported that mitochondrial GDH is located in the matrix, whilst, Yamaya et al. (1984) suggest it is located within

mitochondria as a membrane bound enzyme. If Ca^{2+} is bound within mitochondria, its effective concentration is much reduced and if GDH is bound to the membrane, its sensitivity to Ca^{2+} may be quite different to its soluble form, as used in these assays. Turnip GDH was measured under low substrate conditions on the assumption that this was more representative of the _in vivo_ condition. Under these conditions, no added calcium was required to express the enzyme activity, but this activity was still inhibited on the addition of EGTA. Therefore extremely low amounts of calcium (μM or, perhaps, nM) are capable of expressing activity and such low concentrations may have an important role in regulating the activity _in vivo_. At least two possible regulatory systems can be discerned. (1): If the substrate concentration was not a limiting factor, the level of calcium could have a significant role in determining whether the enzyme aminates or deaminates. (2): If low concentrations of substrates only were available, calcium may influence the velocity of the reaction in either direction.

Investigation of the effect of -SH reagents on turnip GDH revealed that only approximately 30% of the activity was inhibited by p-HMB at concentrations as high as a 1.0 mM. Interestingly, however, GDH activity became EGTA-insensitive after p-HMB treatment. These results indicated that sulfhydryl groups in the mitochondrial turnip GDH did not play a major role in the active site itself, but may play a significant role in the calcium sensitive site. Similar observations have been made with bovine liver GDH in

that treatment with organic mercury resulting in changes in enzyme properties leading to the proposal that -SH groups are involved both in catalysis and regulation of GDH activity (Eisenberg et al. 1976).

The sensitivity of turnip GDH to calcium was also changed by the treatment of the enzyme with thermolysin. After the thermolysin treatment, turnip GDH activity required smaller amounts of Ca^{2+}, compared to the non-treated GDH for full activity. It is obvious that the thermolysin-treated enzyme would possess an unusual configuration in terms of primary structure. However, the small amount of Ca^{2+} required to fully activate the thermolysin-treated GDH must be the concentration that represents the truly essential bivalent cation co-factor requirement for enzyme activity. As thermolysin favours the hydrolysis of hydrophobic residues (Matsubara, 1970), this result also implicates a hydrophobic region of turnip GDH in the calcium response. However, it is still unclear what the relationships are among the Ca^{2+} sensitive response, the binding of Ca^{2+} and the hydrophobic site. It is possible that Ca^{2+} may bind at a hydrophobic region and induce a response at the active site. However, Ca^{2+} could interact with a non-hydrophobic region and induce its effects via a hydrophobic region. Whatever the relationship, the Ca^{2+} effect is to 'de-inhibit' the enzyme in the sense that its activity is suppressed before the addition of Ca^{2+}. Thermolysin treatment removes the Ca^{2+} activation by fully activating the enzyme- i.e. Ca^{2+} is no longer required at the high concentrations. If the hydrophobic site was

directly involved with Ca^{2+} binding, an interesting relationship could exist between the mitochondrial membrane and regulation of the enzyme activity, assuming the hydrophobic region to be within the membrane. However, because GDH is quite readily released from broken mitochondria into the surrounding medium it is unlikely to be deeply embedded in the lipid bilayer of the inner membrane.

An analysis of the pathways of glutamate oxidation by isolated mitochondria revealed that both GDH and AsAT may be involved in this process. The results showed that both GDH and AsAT are capable of carrying out glutamate oxidation, however, GDH was absolutely essential to initiate glutamate oxidation and allow for AsAT activity in isolated mitochondria, which are deficient in oxaloacetate.

Some properties of GDH from turnip became apparent in this research, however, further investigations such as careful determination of distribution of this enzyme in the inner mitochondrial compartment need to be undertaken to resolve the function and regulation of this enzyme <u>in vivo</u>.

Table 7.1 Comparison of the properties of GDH from Turnip, Ulva australis and Bryopsis maxima.

	Turnip	Ulva	Bryopsis
Localization	Mito.	?	(Chlp.)
M.W. (Subunit)	43 kDa	45 kDa	46 kDa
M.W. (Total)	300 kDa	180 kDa	280 kDa
Km (Ammonium sulphate)	22.2 mM	37.0 mM	23.8 mM*
Km (Glutamate)	28.6 mM	14.7 mM	5.2 mM*
NAD(H)/NADP(H)	11/1	4/1	18/1
pH opt. (amin., deamin.)	8.0, 9.5	8.0, 9.2	7.65, 8.43*
Trypsin	-	-	?
Calcium	+	-	-
EGTA	+	-	-

*Nishizawa et al. (1978).

Symbols:

(+); sensitive

(-); insensitive

(?); not known

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