# THE PURIFICATION AND STEADY STATE KINETIC CHARACTERISATION OF RAT LIVER MITOCHONDRIAL MALATE DEHYDROGENASE

A thesis presented in partial fulfilment of the
requirements for the degree of
Master of Science in Biochemistry
at Massey University

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1988

# ABSTRACT

Mitochondrial malate dehydrogenase (mMDH) is one of the enzymes in the malate-aspartate shuttle system which facilitates the movement of reducing equivalents across the mitochondrial membrane. The metabolism of ethanol in the cytosol of liver tissue requires large amounts of $NAD^+$ and produces NADH, which must be oxidised back to $NAD^+$ in the mitochondria. The transfer into the mitochondria is carried out by the shuttle system which may thereby contribute significantly to the control of ethanol metabolism under certain circumstances. In the shuttle system mMDH acts in the opposite direction to the cytosolic form of the enzyme. Is this due to differences in the functioning of the two isozymes or to differences in their environments?

In this study a new method for the purification of mMDH from rat liver has been developed. This method was developed to be quick and to cause as little disruption to the protein structure of the enzyme as possible. The scheme involves the partial purification of mitochondria by centrifugation followed by elution, by NADH, off carboxymethyl cellulose chromatography resin. The whole process takes two days, because of the need to dialyse the sample to remove NADH, before any kinetic studies can be carried out. The specific activity of the purified enzyme compares favourably with other published values and the enzyme shows up as a single band on an SDS polyacrylamide electrophoresis gel. The major problem with the enzyme produced was its lack of stability during storage.

The second part of the project was to carry out steady state kinetic experiments with the purified enzyme under conditions that simulate the *in vivo* state. These are 37°C, pH 7.4, and 0.1 M phosphate buffer. The dissociation and Michaelis constants, for other tissues, have been published before but not those for rat liver. Very few of these experiments have been carried out under these near physiological conditions. The kinetic parameters produced are (μM): Ka, 72; $K_{ia}$, 12; $K_b$, 86; $K_{ib}$, 18; $K_p$, 1900; $K_{ip}$, 15000; $K_q$, 170; $K_{iq}$, 1100; where a = NADH, b = oxaloacetate, p = malate, and q = $NAD^+$. Some of these values have extremely high standard errors and so need further refining.

The values produced in this work can be compared to those for cytosolic malate dehydrogenase (Crow *et al.*, 1982) and because the two sets of constants are quite similar it appears that the different modes of action *in vivo* are mostly due to differences in the environment of the two isozymes.

## ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. Kathryn E. Crow and Dr. Michael J. Hardman, for their advice and support throughout the course of this work. I would also like to acknowledge the assistance of the other members of the Department of Chemistry and Biochemistry.

In particular I would like to thank Ms Kathryn Stowell for her encouragement and her invaluble assistance with experimental methods.

Lastly I wish to thank Miss Rachel Page, Miss Jean Garman, and my parents for their helpful advice and assistance with proof reading.

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# ABBREVIATIONS

## GENERAL

| | |
|---|---|
| ADP | adenosine-5'-diphosphate |
| AMP | adenosine-5'-monophosphate |
| ATP | adenosine-5'-triphosphate |
| BSA | bovine serum albumin |
| CM | carboxy methyl |
| Co A | coenzyme A |
| DEAE | diethyl aminoethyl |
| DTT | dithiothreitol |
| EDTA | ethylenediamine tetra acetic acid |
| $H_2O$ | water |
| $H^+$ | hydrogen ion |
| α-KG | alpha-keto glutarate |
| NADH | nicotinamide adenine dinucleotide (reduced form) |
| $NADP^+$ | nicotinamide adenine dinucleotide phosphate (oxidised form) |
| $NAD^+$ | nicotinamide adenine dinucleotide (oxidised form) |
| NBT | nitroblue tetrazolium |
| OAA | oxaloacetate |
| $O_2$ | oxygen gas |
| PMS | phenazine metho-sulphate |
| PMSF | phenylmethylsulphonylfluoride |
| SDS | sodium dodecyl sulphate |
| TCA | tricarboxylic acid |

ENZYMES

| | |
|---|---|
| ADH | alcohol dehydrogenase |
| AlDH | aldehyde dehydrogenase |
| CS | citrate synthase |
| GDH | glutamate dehydrogenase |
| gMDH | glyoxysomal malate dehydrogenase |
| mAAT | mitochondrial aspartate aminotransferase |
| mMDH | mitochondrial malate dehydrogenase |
| sAAT | cytosolic (or soluble) aspartate aminotransferase |
| sMDH | cytosolic (or soluble) malate dehydrogenase |

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# CHAPTER 1

# INTRODUCTION

## 1.1 THE ROLE OF MITOCHONDRIAL MALATE DEHYDROGENASE IN THE METABOLISM OF ETHANOL

Ethanol is oxidised in the cytosol of liver cells by the enzyme alcohol dehydrogenase (ADH). The acetaldehyde produced is further oxidised to acetate by the enzyme aldehyde dehydrogenase (AlDH). Acetate is a normal metabolic product which can be used by the body.

$$\text{Ethanol} + NAD^+ \xleftrightarrow{\text{Alcohol Dehydrogenase}} \text{Acetaldehyde} + NADH + H^+ \quad \text{(Equation 1)}$$

$$\text{Acetaldehyde} + NAD^+ \xrightarrow{\text{Aldehyde Dehydrogenase}} \text{Acetate} + NADH + H^+ \quad \text{(Equation 2)}$$

All the detectable ADH activity is found in the cytosol of the liver cells. AlDH activity is found in both the cytosol and the mitochondrial matrix although it is not clear whether the oxidation of acetaldehyde during ethanol metabolism takes place in the cytosol, the mitochondrial matrix, or in both. As shown in the Equations 1 and 2, the oxidition of ethanol to acetate requires $NAD^+$ and produces NADH. As neither $NAD^+$ and NADH can cross the inner mitochondrial membrane the cell contains two distinct pools of these cofactors, one in the cytosol and the other in the mitochondrial matrix. During the metabolism of ethanol NADH is produced in both the cytosol and the mitochondria, but almost all of the reoxidation to $NAD^+$ is carried out by the electron transport system in the mitochondrial matrix. Because of this, an indirect method is required to reoxidise the cytosolic NADH. The chief process by which this is achieved is the malate-aspartate shuttle (Lardy *et al.*, 1965; Haynes, 1965; Dawson, 1979). Only the reducing equivalent from the cytosolic NADH is transferred to the mitochondrial $NAD^+$ (Equation 3).

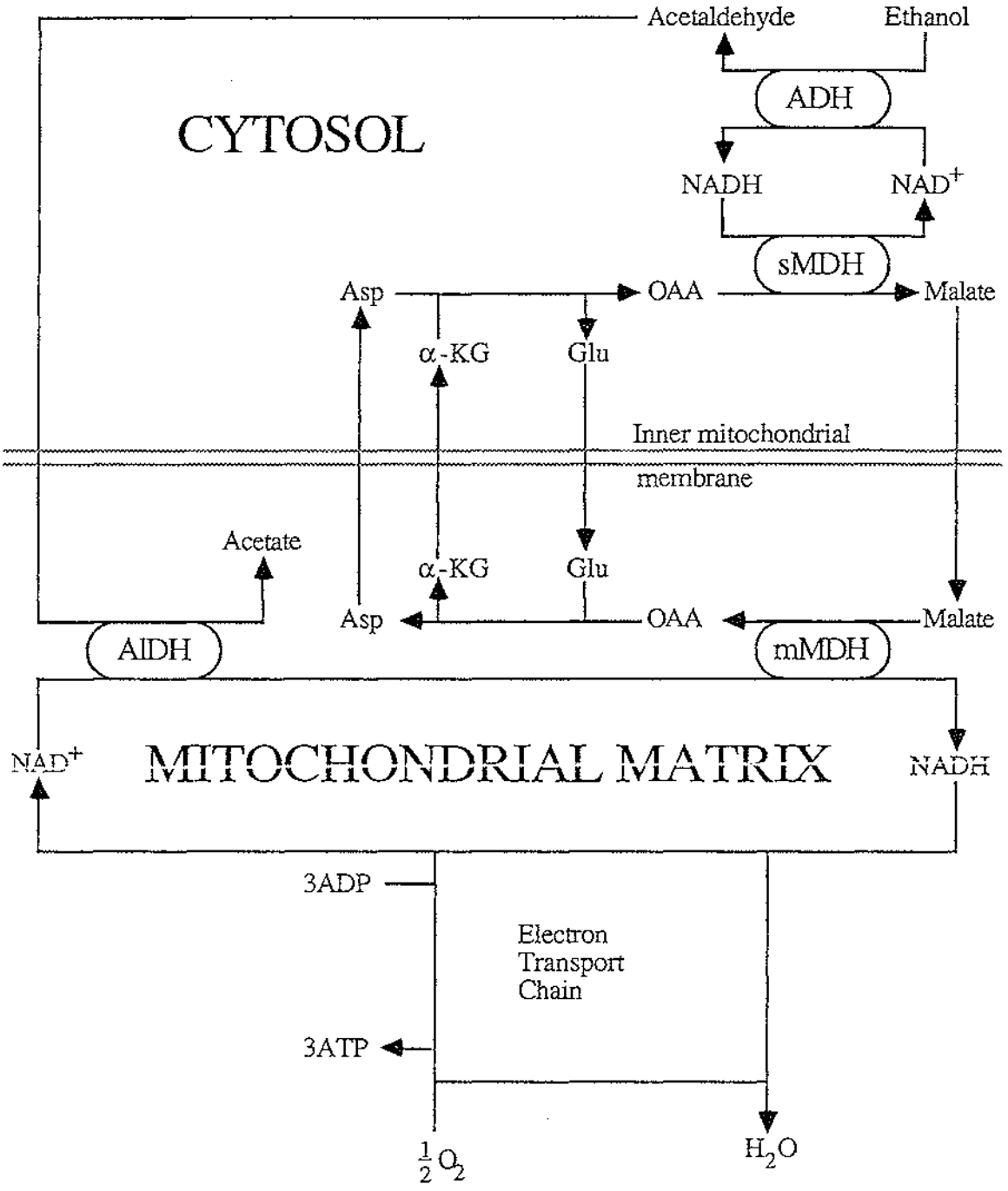
$$NADH_{cyt} + NAD^{+}_{mito} \xrightarrow[\text{Shuttle}]{\text{Malate Aspartate}} NAD^{+}_{cyt} + NADH_{mito} \qquad \text{(Equation 3)}$$

Figure 1 shows the malate-aspartate shuttle system and how it relates to the metabolism of ethanol. NADH in the cytosol is oxidised to $NAD^+$ by cytosolic, or soluble, malate dehydrogenase (sMDH), with the conversion of oxaloacetate to malate. The malate produced can pass across the mitochondrial inner membrane and is then used by mitochondrial malate dehydrogenase (mMDH) to reduce mitochondrial $NAD^+$ to NADH. The NADH can then be reoxidised, by the electron transport system, back to $NAD^+$ with the production of energy in the form of ATP. As oxaloacetate cannot cross the inner mitochondrial membrane the shuttle system is completed by mitochondrial aspartate aminotransferase (mAAT) converting oxaloacetate and glutamate into aspartate and alpha-ketoglutarate, which can both cross the membrane to the cytosol, where they are converted back into glutamate and oxaloacetate by cytosolic aspartate amino-transferase (sAAT).

The oxidation of ethanol is normally controlled by ADH with the $V_{max}$ of the enzyme being the limiting factor (Crow, 1985). The rate at which NADH can be reoxidised to $NAD^+$ may also play some role in the regulation when rates are lower than those set by the $V_{max}$ of ADH (Crow, 1985). It is of interest to know the extent of the control that the malate-aspartate shuttle system, and in particular the cytosolic and mitochondrial malate dehydrogenases, may have on the rate of NADH reoxidation and therefore on the rate of ethanol metabolism in liver tissue. A significant increase in liver mMDH activity, in drinking alcoholics, has been reported (Jenkins and Peters, 1978). The same study also suggested an increased level of sMDH in alcoholics, but this could not be proven to be statistically significant. Steady state kinetic data for these enzymes can be used in computer simulations to show their action under the conditions of ethanol oxidation. This has been done for rat liver and human liver sMDH in previous studies from this laboratory (Crow *et al.*, 1982 and 1983). The present study aims to produce the steady state kinetic constants for rat liver mMDH. Rat liver is used as the source of mMDH because it is much easier to obtain than human liver tissue samples and comparisoms can still be made with the earlier work.

Cytosolic and mitochondrial MDH are enzymes that carry out the same reaction, but normally they must work in opposite directions *in vivo* to allow the shuttle system to carry out its function. One of the main questions we hoped to answer in this study was

whether the kinetic parameters for the two enzymes are different enough to account for their opposite direction of activity, or whether this is due to differences in the environment of the two enzymes.




ADH = Alcohol Dehydrogenase
AlDH = Aldehyde Dehydrogenase
sMDH = cytosolic Malate Dehydrogenase
mMDH = mitochondrial Malate Dehydrogenase

**Figure 1**

Interactions between ethanol metabolism, the malate-aspartate shuttle, and the electron transport chain, showing how the reducing equivalents, produced during ethanol metabolism in the cytosol, are transfered across the inner mitochondrial membrane into the mitochondrial matrix for reoxidation by the electron transport chain.

## 1.2 CHARACTERISATION OF MALATE DEHYDROGENASES

### 1.21 Physical properties

Malate dehydrogenase is a relatively small (molecular weight ≈ 70,000 daltons) globula protein, first discovered independently by Batelli and Stern in 1910 and by Thunberg in 1911. The existence of two distinct isozymes, located in different subcellular fractions, was first shown in 1959 (Delbrück *et al.*, 1959a and 1959b). These were named cytosolic or soluble MDH (sMDH) and mitochondrial MDH (mMDH) after the fractions in which they were found. More recently a third form has been found in the microbodies of several plant tissues (Breidenbach, 1969; Yamazaki and Tolbert, 1969), and this was named glyoxysomal MDH (gMDH). In animal tissues only the first two are found, and most of the discussion here will be about these two. They are very similar in molecular weight with sMDH being about 70,000 daltons and mMDH about 68,000 daltons depending on the species. Rat liver mMDH has a molecular weight of 66,300 daltons (Mann and Vestling, 1969). Both molecules are made up of two identical subunits (Dévényi *et al.*, 1966) each of which binds one molecule of coenzyme, $NAD^+$ or NADH (Kitto and Kaplan, 1966). The three dimensional crystallographic structures of both sMDH and mMDH have been published (Hill *et al.*, 1972; Roderick and Banaszak, 1983). Comparisons between these structures, and the structures of other dehydrogenases, show similarity in overall conformation, quarternary structure, location of the $NAD^+$ binding site, and several catalytically active residues (Roderick and Banaszak, 1983 and 1986), although the amino acid compositions are quite different.

Dissociation of the dimer into two identical monomers was shown to occur at pH 2, in 5 or 7 M guanidine hydrochloride, and in 8 M urea (Mann and Vestling, 1969). Under these conditions the monomers were inactive, but the enzymes could be reactivated by neutralisation of the acid or removal of the denaturant. The amount of activity recovered decreased the longer the enzyme was inactive. Experiments with mMDH, using fluorescent labels, showed concentration dependant dissociation with the $K_D = 2 \times 10^{-7}$ M at pH 8.0 (Shore and Chakrabarti, 1976). Coenzyme binding affected this equilibrium, with NADH causing a shift toward the monomer and $NAD^+$ binding preventing dissociation even at below $10^{-8}$ M. Experiments using gel filtration chromatography and sedimentation velocity ultracentrifugation (Bliele *et al.*, 1977) showed that porcine heart mMDH dissociated at concentrations less than 0.2 μM or when the pH was less than 7.0. Bovine mMDH also dissociated at less than pH 7.0 but

did not exhibit a concentration dependant dissociation and porcine heart sMDH did not dissociate under either of the conditions examined. The presence of either coenzyme caused dimer formation to be favoured. The increase in specific activity and the decrease in protein fluoresence during subunit reassociation both followed first order kinetics (Wood *et al.*, 1981). A model of subunit reassociation was proposed in which the rate limiting step was the cis/trans isomerisation about proline imino bonds during protein refolding. Immobilised protomers have been made that exhibit normal mMDH activity and kinetics (DuVal *et al.*, 1985) and kinetic studies with mMDH molecules that have one subunit inactivated showed normal kinetics (McEvily *et al.*, 1985a). The conclusion from this is that catalytic activity and regulation by effectors is independant of the dimeric structure of the native enzyme. However the intramitochondrial MDH concentration is estimated to be high enough to ensure that mMDH is a dimer *in vivo* (Shore and Chakrabarti, 1976).

Upon electrophoresis of mMDH several catalytically active bands are observed (Kitto *et al.*, 1966). These were suggested to be different conformational forms of the enzyme as they could not be separated on the basis of catalytic activity or amino acid composition (Kitto *et al.*, 1966). Mann and Vestling (1968 and 1970) suggested that the different bands were due to the combinations of two different subunits, but no further evidence has been produced to support this.

One of the major differences between sMDH and mMDH is in the amino acid compositions. mMDH has no tryptophan residues, whereas sMDH has 5 - 15 depending upon the species (Kitto and Kaplan, 1966). sMDH also has a much greater number of charged residues than the mitochondrial form, and in mMDH a high level of asparagine and glutamine residues are found with about 50% amidation of the aspartate and glutamate residues (Jones and Vestling, 1979). The mitochondrial enzyme has no disulphide linkages, but does have a number of free sulphydryl groups, 14 in porcine heart (Thorne and Kaplan, 1963).

A number of amino acid types have been examined as possibilities for active site residues in mMDH. Histidine has been implicated as a catalytically active residue (Banaszak and Bradshaw, 1975). Modification of cysteine residues causes loss of activity because of a thiol group located near the coenzyme binding site which appears to be necessary for enzymatic activity (Gregory *et al.*, 1971). Lysine has also been implicated as involved in enzyme activity (Yost and Harrison, 1971).

The two isozymes have very different isoelectric points, due to the differences in amino

acid composition. On an isoelectric focusing gel each enzyme shows a range of catalytically active bands (Kitto *et al.*, 1966) covering about half a pH unit. The different bands have most recently been explained as being caused by the binding of varying amounts of phospholipid to the mMDH molecule (Jones and Vestling, 1979). The phospholipid covers up charged groups on the protein's surface and therefore affects the isoelectric point.The isoelectric point of mMDH ranges from pH 8.9 - 9.6 and sMDH has an isoelectric point that ranges from pH 5.2 - 5.6. Calculation of the isoelectric point of mMDH using normal pKa values for the ionisable groups and 50% amidation of glutamate and aspartate gives a value in the range of pH 9 - 10 (Jones and Vestling, 1979). The difference between the isoelectric points is of great use when separating the two isozymes as this is the reason for the difference in the binding properties on ion exchange resins (see Chapter 3).

1.22 Kinetic properties of mMDH (also see Chapter 4)

A lot of work has been carried out on the kinetic properties of mMDH from various sources, most commonly porcine heart. This enzyme has a number of interesting kinetic properties, including substrate inhibition by oxaloacetate, substrate activation by malate and allosteric control by a variety of compounds, including citrate. The mechanism of action of mMDH has been described as a compulsory ordered bi-bi system (Raval and Wolfe, 1962). Harada and Wolfe (1968b) proposed a reciprocating compulsory ordered mechanism involving a switch from subunit to subunit, but this was later discounted when immobilised monomers were shown to have normal catalytic activity (DuVal *et al.*, 1985).

Most of the kinetic studies that have been carried out previously have used conditions that could not be described as physiological. As mMDH has an activity optimum at about pH 10 in the direction of malate oxidation and pH 7.4 in the reverse direction most investigators have used pH 8.0 and above for their experimental conditions so as to strike a balance between the two directions. Because of this it is often hard to distinguish between those results which are physiologically relevant and those which may be artifacts of the nonphysiological conditions. Since for the present work, kinetic data was required for the examination of the physiological activity of mMDH, conditions similar to those *in vivo* were used.

## 1.23 mMDH *in vivo*

All the isozymes of MDH are synthesised on ribosomes in the cytoplasm. mMDH and gMDH are both then transported into the appropriate organelle. These two isozymes appear to have a common higher molecular weight precursor (Hock, 1984). Mitochondrial MDH is synthesised with an N-terminal transit peptide which contains a large number of basic residues, although there is little conservation of sequence between species. There are only 12 differences, out of 314 residues, between the mature subunits of rat heart and porcine heart mMDH's (Grant *et al.*, 1987). Purified mature mMDH can also be transfered into the mitochondria whereas sMDH can not (Passerella *et al.*, 1980). It appears that for this process there is a common receptor for mMDH and mAAT (Marra *et al.*, 1985) although only one molecule is transfered at a time.

The location of mMDH in the mitochondria is not completely certain. It is not found in the inner or outer membranes, but there is evidence which indicates that lipids do affect the properties of mMDH in very dilute solutions (Callahan and Kosicki, 1967). Lysolecithin reduces the rate of thermal inactivation of mMDH (Dodd, 1973), which is normally rapidly inactivated at elevated temperatures (Müller and Klein, 1982). It seems probable that mMDH is located in the matrix of the mitochondria but that under certain conditions it is able to interact with the inner membrane.

The low level of free oxaloacetate believed to exist in the mitochondria has led to the proposal that enzymes in the mitochondria interact to allow the formation of localised high substrate concentrations (Srere *et al.*, 1973). Interactions between mMDH and mAAT have been measured using counter-current distribution (Backman and Johansson, 1976). sMDH and sAAT were also shown to interact, but no interaction was found between cytosolic and mitochondrial species. An effective increase in the rate of the reaction of coupled sMDH and sAAT has been shown (Arrio-Dupont *et al.*, 1985). The mitochondrial enzymes probably show a similar enhancement of activity when coupled together. mMDH also interacts with NADH: ubiquinone reductase, complex I of the electron transport chain, which could use the NADH produced by the mMDH (Sumegi and Srere, 1984). A bienzyme complex is also formed between mMDH and citrate synthase (CS). The dissociation constant for this reaction is $10^{-6}$ M (Tompa *et al.*, 1987). Each of these interactions cause one of the products of the mMDH reaction to be removed and therefore assist an otherwise energetically unfavourable reaction. The interactions of mMDH with other enzymes were not investigated in this work as it was first considered necessary to obtain the kinetic parameters of pure mMDH, under physiological conditions.

## 1.3 AIMS OF THIS PROJECT

The first aim of this project was to develop a cheap, gentle, and quick method of purification for mitochondrial malate dehydrogenase from rat liver. The second aim was to use this purified enzyme to carry out steady-state kinetic studies under simulated physiological conditions so that the kinetic constants determined can be compared to data determined for cytosolic malate dehydrogenase and information gained on their differing modes of action.