UNIVERSITY OF SOUTHAMPTON

THE BIOSYNTHESIS OF METHANOL DEHYDROGENASE

by

Ian William Richardson

A thesis submitted to the
University of Southampton
for the degree of
Doctor of Philosophy

Department of Biochemistry

June 1992

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

BIOCHEMISTRY

Doctor of Philosophy

THE BIOSYNTHESIS OF METHANOL DEHYDROGENASE

by Ian William Richardson

This thesis reports studies on the periplasmic quinoprotein methanol dehydrogenase (MDH), principally from the facultative methylotroph *Methylobacterium extorquens*, to investigate which factors are necessary for the formation of active enzyme. The approach used was to utilise mutants of this organism which are known to be impaired in methanol oxidation (Mox mutants).

MoxC and MoxH were shown to produce a MDH protein, although this was not in the typical $\alpha_2\beta_2$ tetrameric configuration as found for the enzyme from wild-type bacteria. Growth on methanol of these mutants could be restored by supplementation of the growth medium with pyrroloquinoline quinone (PQQ; the prosthetic group of MDH). Neither mutant was able to synthesise PQQ; hence the *moxC* and *moxH* genes are involved in PQQ biosynthesis.

The MDH from wild-type *Mb. extorquens* was shown to have two molecules of PQQ per $\alpha_2\beta_2$ tetramer, the PQQ being predominantly in the semiquinone form. MDHs from the MoxA, K and L mutants were identical to the enzyme isolated from wild-type bacteria with respect to molecular size, subunit configuration, isoelectric point, N-terminal amino acid sequence and stability in denaturing conditions (low pH, high urea and high guanidinium chloride) and in the nature and content of the prosthetic group (2 mol PQQ per mol of MDH). They differed in the oxidation state of their PQQ (fully oxidised), absence of the semiquinone form of PQQ in the enzyme, lack of reactivity with the suicide inhibitor cyclopropanol, and in their absorption spectra and circular dichroism spectra which indicated that their PQQ is bound differently from that in normal MDH.

The MDHs from wild-type *Mb. extorquens*, *Methylophilus methylotrophus*, *Paracoccus denitrificans* and *Hyphomicrobium* X all contained a single atom of calcium per $\alpha_2\beta_2$ tetramer. The role of calcium was investigated using the MDH from *Mb. extorquens*. MDH isolated from the MoxA, K and L mutants contained virtually no calcium. Incubation of MDH from the mutants in calcium salts led to irreversible time-dependent reconstitution of full activity concomitant with restoration of a spectrum corresponding to that of fully reduced normal MDH.

It is concluded that Ca^{2+} in MDH is directly or indirectly involved in binding PQQ in the active site. The MoxA, K and L proteins may be involved in maintaining a high Ca^{2+} concentration in the periplasm. It is more likely, however, that they fill a molecular chaperone function, stabilising a configuration of MDH which permits incorporation of low concentrations of Ca^{2+} into the protein.

Acknowledgements

I should like to thank the following people for their valuable contributions, both directly and indirectly, to this study:-

Professor Chris Anthony, my supervisor, for his help, encouragement and tolerance of my idiosyncracies (even if he is sizist).

Professor M. Akhtar, FRS, for the provision of excellent research facilities.

Dr. Mark Carver, my industrial supervisor, for his advice and timely discussions.

SERC and ICI for financial support.

The following for their generous help: Dr. David Lowe, University of Sussex (ESR spectroscopy); Dr. Peter Cary, Portsmouth Polytechnic (CD spectroscopy); Neville Wright, this Department (cyclopropanol).

My fellow 'methylotrophs'; Dr. Tony Long, Dr. Darren Day, Leslie Horsley, Alain Avezoux and Simon Dales, for their valuable discussions. Dave Pearce, Daren Rossiter and Chris Chan for technical assistance.
I owe a special thanks to Dr. Claude 'Clod' Chan and 'Juggling' Jon Cox who, over the past 3½ years, have made my life hell!!! Seriously though, they are good friends, who have helped tremendously, and made considerable contributions to my sanity (or lack of it) through their continual jocularity.
Included in this list are my friends (yes Bob, you aswell) and colleagues inside and outside of the Biochemistry Department.

A very sincere thankyou goes to my family for their love, support (financial and spiritual), encouragement, and for believing in me; without them, I could not have achieved this goal. There are too many to mention them all, but I must acknowledge my mother who has done so much for me over the past 27 years.

Finally, to Jill for her love, willingness to laugh at my feeble jokes and timely presen(ts)ce. More importantly, she has consented to share my life. For you Jill, a very special message; Я ТЕБЯ ЛЮБЛЮ.

To My Family,

Past, Present and Future.

"When you have eliminated the impossible, whatever remains, however improbable, must be the truth"

A. Conan Doyle, 'The Sign of the Four'

CONTENTS

CHAPTER 2 - Materials and Methods

LIST OF FIGURES

LIST OF TABLES

Abbreviations

ADH	Alcohol dehydrogenase
CD	Circular dichroism
Cyt.	Cytochrome
DEAE	Diethylaminoethyl
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediamine-*N,N,N',N'*-tetracetic acid
ESR	Electron spin resonance
GDH	Glucose dehydrogenase
HAP	Hydroxylapatite
Hepes	*N*-(2-Hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid
MDH	Methanol dehydrogenase
MNDH	Methylamine dehydrogenase
Mes	3-(*N*-Morpholino)ethanesulphonic acid
Mops	3-(*N*-Morpholino)propanosulphonic acid
Mox	Methanol oxidation
ORF	Open reading frame
P-6	10DG rapid gel filtration/desalting column
PAGE	Polyacrylamide gel electrophoresis
PES	Phenazine ethosulphate
pI	Isoelectric point
PIP	2,6-dichlorophenolindophenol
PQQ	Pyrroloquinoline quinone
$PQQH^{\bullet}$	The semiquinone (free radical) form of pyrroloquinoline quinone
$PQQH_2$	Pyrroloquinoline quinol
$PQQ\text{-}H_2O$	The hydrated form of pyrroloquinoline quinone
PTS	Phosphotransferase system
SDS	Sodium dodecyl sulphate
Sulpho-NHS	Sulpho-*N*-hydroxysuccinimide
TMBZ	3,3',5,5'-Tetramethylbenzidine
TMPD	*N,N,N'N'*-tetramethyl-*p*-phenylenediamine hydrochloride
Tris	Tris(hydroxymethyl)aminoethane
TPQ	3,4,6-trihydroxyphenylalanyl quinone, 6-hydroxydopa quinone or topaquinone
TTQ	Tryptophan tryptophyl quinone

CHAPTER 1

General Introduction

1.1. Introduction

The work presented in this thesis is primarily concerned with the periplasmic quinoprotein, methanol dehydrogenase (MDH). The components of this enzyme are known but it is not known how these components are assembled into the final active protein and which features are essential for activity.

The aim of this first chapter is to provide an introduction to methylotrophic bacteria, and then describe in detail the current knowledge about MDH, it's prosthetic group (pyrrolo-quinoline quinone; PQQ) and the molecular biology of the methanol oxidation system. An overview of some other quinoproteins is also given.

1.2. Methylotrophs

The first description of bacteria able to oxidise methane and methanol, and to make use of the energy liberated by this process was made around the turn of this century (Sohngen, 1906). These bacteria are now collectively known as methylotrophs and are defined as micro-organisms capable of growth at the expense of reduced carbon compounds (C_1-compounds) that contain one or more carbon atoms but no carbon-carbon bonds (Colby and Zatman, 1972; Zatman, 1981). The principal C_1-compounds used as growth substrates for the study of methylotrophs are methane, methanol and methylamine. Neither bacteria able to oxidise methane nor the methylotrophic yeasts will be discussed here but a full review of these organisms has been published (Anthony, 1982).

Methylotrophic bacteria can be obligate or facultative and are usually strict aerobes. Notable exceptions to this rule are *Paracoccus denitrificans* and *Hyphomicrobium* X. During anaerobic, methylotrophic growth of these organisms, methanol is oxidised by the same dehydrogenase that operates during aerobic growth, the terminal electron acceptor being nitrate instead of oxygen (Anthony, 1982).

1.3. *Methylobacterium extorquens*; the organism used in this study

Methylobacterium extorquens, strain AM1, was previously called *Pseudomonas* AM1 (Green and Bousfield, 1982) and was first isolated as an air-borne contaminant of methylamine liquid cultures (Peel and Quayle, 1961). It is a pink-pigmented facultative methylotroph capable of growth on methanol and methylamine, and some multi-carbon compounds such as succinate. Carbon is assimilated at the level of formaldehyde and carbon dioxide via the serine pathway (Anthony, 1982).

In the years since its isolation, *Mb. extorquens* has served as a major workhorse for methylotrophic research in the study of carbon oxidation and assimilation pathways, and, more recently, in the study of the molecular biology of methanol oxidation (see Section 1.10).

1.4. Methanol oxidation

The general scheme for the oxidation of methanol by methylotrophic bacteria is shown in Fig. 1.1. In the first step, oxidation of methanol to formaldehyde is catalysed by the NAD^+-independent enzyme, methanol dehydrogenase (MDH) and is discussed in detail in the next section. Formaldehyde is then either assimilated into cell material or oxidised further to carbon dioxide.

The physiological electron acceptor for MDH has been shown to be a *c*-type cytochrome called cytochrome c_L which is unique to methylotrophs (Fig. 1.2). Cytochrome c_L passes its electron onto cytochrome c_H which is a typical Class I *c*-type cytochrome. Cytochrome c_H then passes its electron onto a membrane bound cytochrome oxidase, which, in the case of *Mb. extorquens* is of the a/a_3 type (Fukumori *et al.*, 1985a,b). The components of this electron transport chain are discussed in detail in the comprehensive reviews by Anthony (1982, 1986, 1992b) and will not be dealt with further here.

1.5. Methanol dehydrogenase; a quinoprotein

As mentioned in the previous section, in the majority of methylotrophic bacteria the oxidation of methanol is catalysed by the NAD^+-independent enzyme, methanol dehydrogenase (MDH; E.C. 1.1.99.8). MDH was first identified and purified over a quarter of a century ago by Anthony and Zatman (1964a,b) from *Pseudomonas* M27 (now referred to as *Mb. extorquens*; Green and Bousfield, 1982). During characterisation of the MDH, it was discovered that this enzyme was a novel

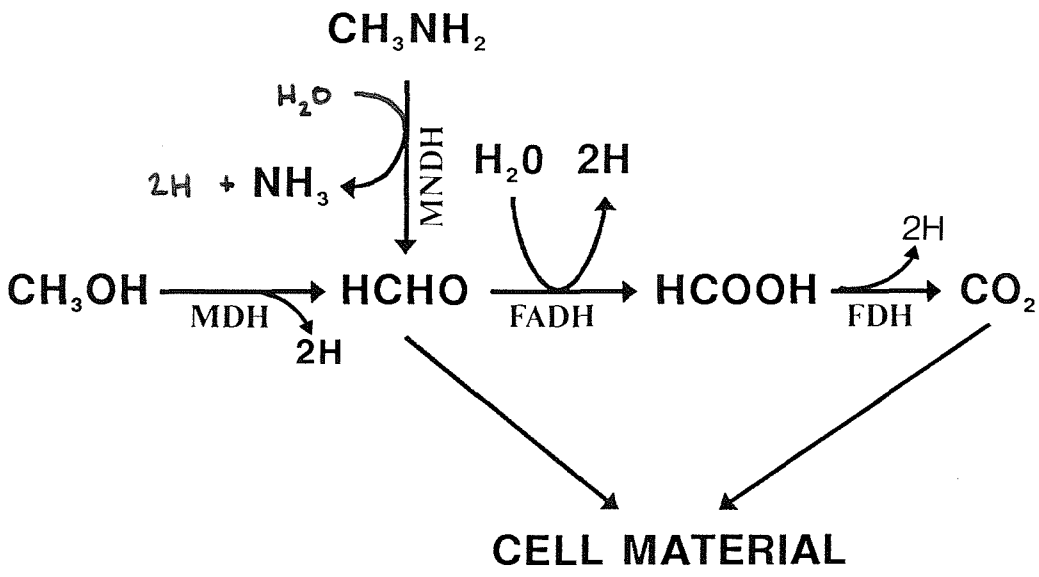


Fig. 1.1. The oxidation of methanol and methylamine by *Methylobacterium extorquens*

The oxidation of both methanol and methylamine proceeds via formaldehyde, which is then further oxidised to formate and carbon dioxide. *Mb. extorquens* assimilates carbon at the level of formaldehyde and carbon dioxide via the serine pathway.
MDH, methanol dehydrogenase; MNDH, methylamine dehydrogenase, FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase.

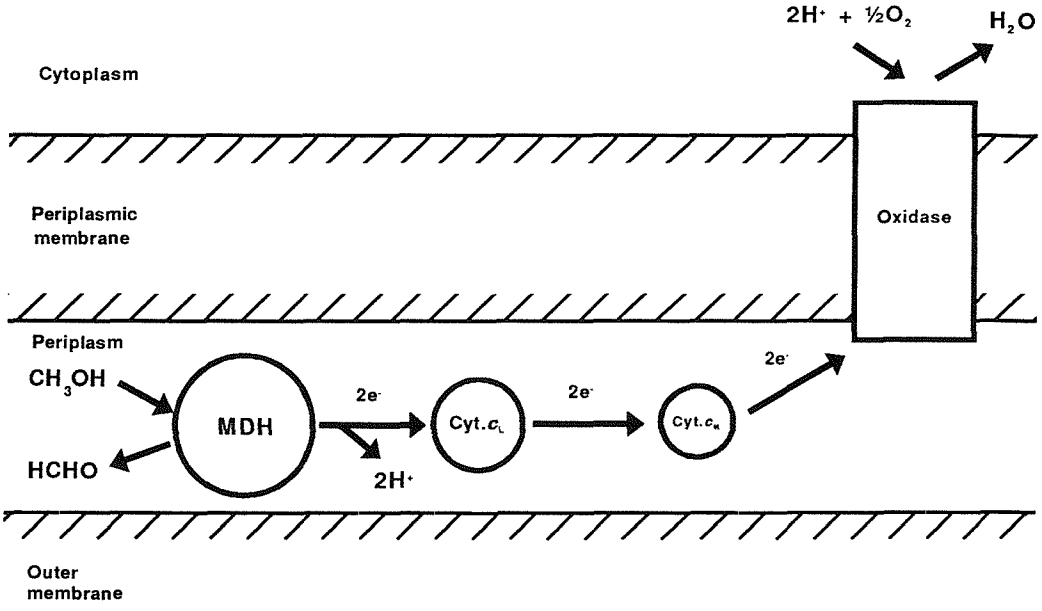


Fig. 1.2. The electron transport chain for methanol oxidation

This is the one-electron transport chain for a typical methylotroph oxidising methanol. The component proteins are soluble and located exclusively in the periplasm with the exception of the terminal oxidase which is an integral membrane protein. MDH, methanol dehydrogenase; cyt. c_L, cytochrome c_L; cyt. c_H, cytochrome c_H.

oxidoreductase containing an unusual prosthetic group (Anthony and Zatman, 1965, 1967a,b). Originally the prosthetic group was thought to be a pteridine derivative (Anthony and Zatman, 1967b), but its identity was subsequently shown to be pyrrolo-quinoline quinone (PQQ; Salisbury *et al.*, 1979; Duine *et al.*, 1978; Westerling *et al.*, 1979). The structure and characteristics of PQQ are discussed in detail in Section 1.6. Enzymes that contain PQQ (or a closely related structure) have been collectively termed quinoproteins (Duine *et al.*, 1980); further examples are mentioned in Section 1.7.

MDH has been purified from a number of different methylotrophs with the enzymes having similar properties (Table 1.1; Anthony, 1986). Although the differences between these MDHs are relatively minor, one feature they all have in common is their absorption spectrum (Fig. 1.3). The key feature of the spectrum is the presence of an absorption maximum at 345-350 nm which is due entirely to the prosthetic group.

During methylotrophic growth, MDH can constitute up to 15% of the total soluble protein with the concentration in the periplasm estimated at 0.5 mM (Beardmore-Gray *et al.*, 1983). The physiological electron acceptor for MDH is cytochrome c_L, but for the purified enzyme, activity can be assayed using an artificial electron acceptor such as phenazine ethosulphate (PES; Bamforth and Quayle, 1978a,b). In this dye-linked assay, re-oxidation of reduced PES is coupled either to the reduction of oxygen (measured in an oxygen electrode) or to the reduction of a second dye, 2,6-dichlorophenol-indophenol (PIP) which is measured spectrophotometrically at 600 nm. An alternative assay where reduced MDH is oxidised by the reduction of cytochrome c_L, which in turn is oxidised by the reduction of a second cytochrome *c* has been developed (Beardmore-Gray *et al.*, 1983; Beardmore-Gray and Anthony, 1984). Although not as convenient for general use as the dye-linked assay, the cytochrome-linked assay has proved invaluable in the study of the protein-protein interactions between MDH and cytochrome c_L (Chan and Anthony, 1991; Cox *et al.*, 1992).

Table 1.1. Properties of some methanol dehydrogenases

This table is taken from Anthony (1986) with the addition of the MDH from *Methylophaga marina* (Janvier and Gasser, 1987) which does not appear to fit in any of the categories below. The division into these groups is somewhat arbitrary and sometimes based on incomplete or preliminary descriptions. Native molecular weights are derived from gel filtration experiments. A 'high' isoelectric point is above 7.0 and based solely on observations during ion-exchange chromatography.

Source of MDH	Molecular weight (kDa)	Subunit molecular weight (kDa)	Isoelectric point
Group A			
Methylobacterium extorquens AM1	120	60	8.8
Pseudomonas M27	120	60	high
Pseudomonas S25	128	62	9.4
Pseudomonas 2941	128	62	7.38
Hyphomicrobium X	120	60	high
Methylophilus methylotrophus	115	62	high
Methylomonas J	135	60	9.3
Group B			
Methylobacterium organophilum	135	62	high
Pseudomonas C	128	60	
Group C			
Paracoccus denitrificans	151	76	3.7
Strain S50	158	76	3.82
Group D			
Methylomonas methanica	60	60	high
Methylomonas sporium	60	60	high
Group E			
Rhodopseudomonas acidophila	116	63	9.35
'Group F'			
Methylophaga marina	145	65	6.4

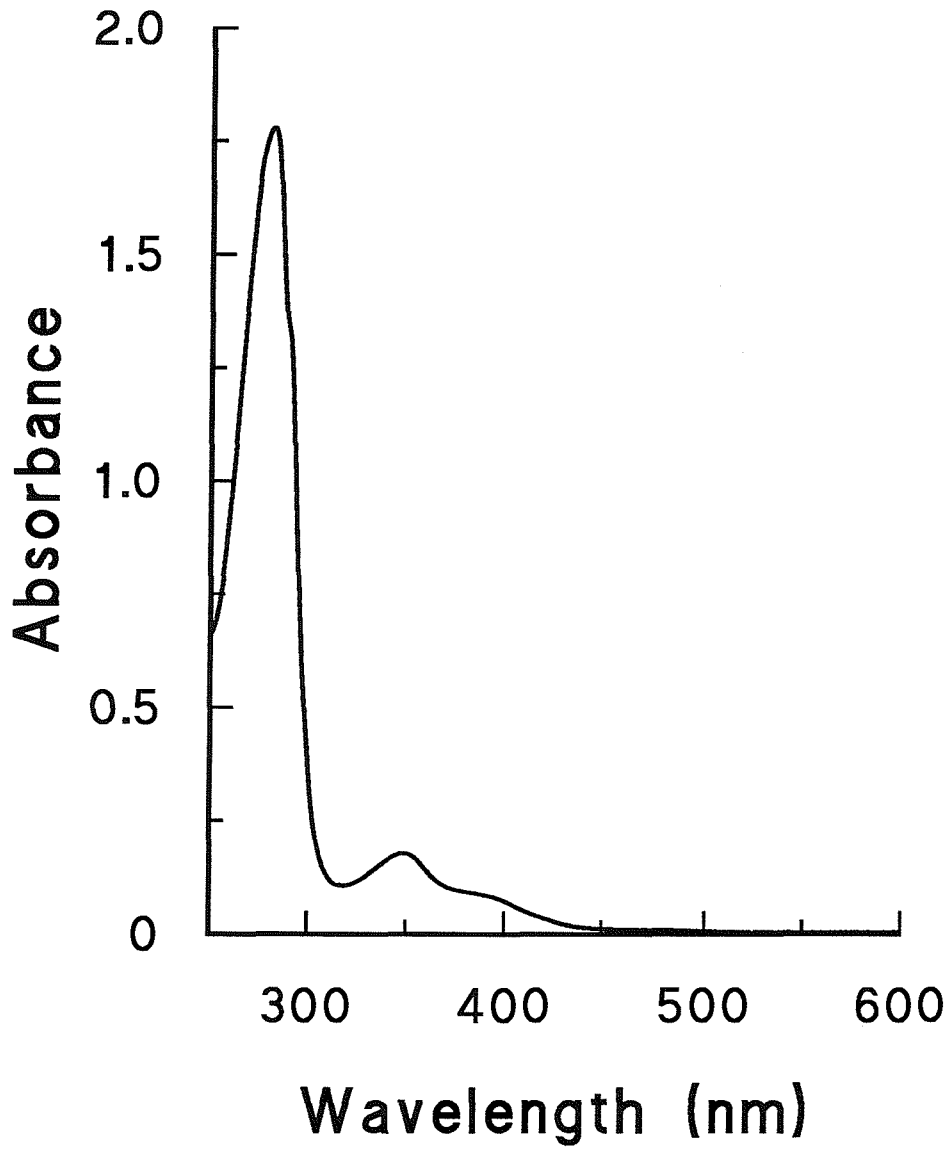


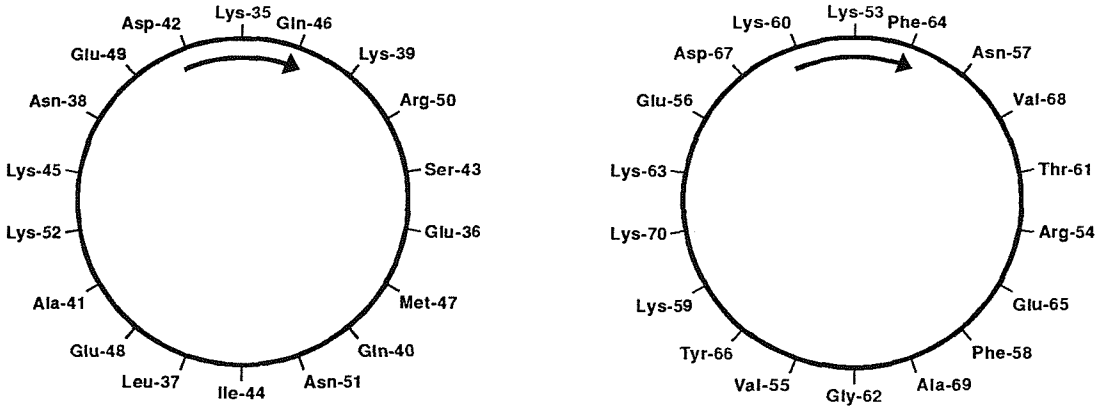
Fig. 1.3. The absorption spectrum of MDH

Methanol dehydrogenase (MDH) was purified from *Mb. extorquens* and the spectrum of a 1 $mg.ml^{-1}$ solution in 100 mM potassium phosphate (pH 7.0) recorded. The peak at 280 nm (and the shoulder at 290 nm) is due to aromatic amino acid residues; the peak at 350 nm (and the shoulder at 400 nm) is due entirely to the prosthetic group, pyrrolo-quinoline quinone (PQQ). The 280 nm : 350 nm ratio is typically 10 : 1 for pure MDH.

1.5.a. Subunit structure of MDH

As can be seen from Table 1.1., MDH has a native molecular weight of between 112 and 158 kDa depending on the source of the enzyme. In the majority of cases, MDH was believed to consist of 2 identical subunits. When pure MDH was run on SDS-PAGE, however, a small protein of about 8 kDa that migrated close to dye-front had often been observed, and it was suggested that this may be a second, smaller subunit of MDH (Elliot and Anthony, 1988). A similarly sized protein was observed when a DNA fragment from *Mb. extorquens* containing the MDH and cytochrome c_L structural genes was expressed in *E. coli* (Anderson and Lidstrom, 1988). Nunn *et al.* (1989) demonstrated that this small protein present in purified MDH is in fact a second subunit of the enzyme. *N*-terminal sequencing of the protein, separated from the larger subunit by gel filtration in the presence of SDS or guanidinium chloride, revealed an identical sequence to that deduced from the DNA fragment containing the MDH and cytochrome c_L structural genes. Furthermore, the gel filtration experiment showed the subunits to be in a 1:1 ratio. Thus the overall structure of MDH was shown to be an $\alpha_2\beta_2$ tetramer.

The β-subunit from *Mb. extorquens* is highly basic and secondary structure prediction indicated the presence of 2 α-helices at the *C*-terminus (Fig. 1.4.a). A helical wheel representation of these regions showed the helices to be amphipathic with one face rich in positively charged lysyl and arginyl residues (Fig. 1.4.b). This feature led to the proposal that the β-subunit was involved in the binding of cytochrome c_L during electron transport (Nunn *et al.*, 1989). However, recent studies have shown this not to be the case. Firstly, the amino acid sequence of the MDH β-subunit from different methylotrophs has been determined (Fig. 1.5). The lysyl residues seen in the β-subunit from *Mb. extorquens* and proposed to be involved in binding are not conserved in the other sequences which suggests that these residues are not involved in the interaction with cytochrome c_L. Secondly, 'zero-length' cross-linking studies (Grabarek and Gergely, 1990) showed that in *Mb. extorquens*, *Acetobacter methanolicus* and *Methylophilus methylotrophus*, cytochrome c_L cross-linked only with the α-subunit of MDH (Chan and Anthony, 1991; Cox *et al.*, 1992). These results clearly showed that the β-subunit is not involved in interaction with cytochrome c_L and its precise role remains unknown.




```
          10        20        30        40        50        60        70
          |         |         |         |         |         |         |
YDGTKCKAAGNCWEPKPGFPEKIAGSKYDPKHDPKELNKQADSIKQMEERNKKFRENFKKTGKFEYDVAKISAN
>>>>------>>>>>*>*>**XXXXXX>>>*>>**XXXXXXXXXXXXXXXXXXXXXXXXXXXX**XXXXXXXXXXX
```

Fig. 1.4. Predicted secondary structure of the β-subunit of MDH from *Methylobacterium extorquens*

This semi-graphical representation of the secondary structure as predicted by the method of Garnier *et al.* (1978) is taken from Nunn *et al.* (1989). X, helical conformation; >, turn conformation; —, extended conformation (β-strand); *, random coil conformation. The potential helical region is presented as two helical wheels in order to demonstrate the amphipathic potential of this region and to illustrate the asymmetrical distribution of the lysyl residues.

```
                                                +    +       +        ++       ++   +        +
     !!!   !!   ! !!!   !   !     ! !!!!   !! !!   !        !   !    !          !     !!
(1)   YDGTKCKAAGNCWEPKPGFPEKIAGSKYDPKHDPKELNKQADSIKQMEERNKKRVENFKKTGKFEYDVAKISAN
(2)   YDGQTCKEAGNCWEAKPGYPEKIAGSKYDPKHDPVELNKQEQAIKAMDERNAARIANAKSSGNFVFDVKK
(3)   YDGANCKAPGTCWEPKPDYPAKVEGSKYDPQHDPAELSKQGESLAVMDARYEWRVWNMKKTGKFEYDVKKIDGYGDETKAPPAD
(4)  AYDGTHCKKPGVCWEPQPGYPEQLVGVKYDPHFDPAELGVQxESxxxMNARNEARTAYFILTGTWEEDVNQIPK
```

Fig. 1.5. Sequence comparison of the MDH β-subunits from different methylotrophs

The sequences shown here were deduced either from the DNA sequence of the gene (1 and 3) or by direct protein sequencing (2 and 4) and are taken from the following sources:

(1) *Methylobacterium extorquens*; Nunn *et al.* (1989)

(2) *Methylophilus methylotrophus*; Cox (1992)

(3) *Paracoccus denitrificans*; van Spanning *et al.* (1991)

(4) *Acetobacter methanolicus*; Chan and Anthony (1991)

(!) indicates conserved residues, (+) indicates the lysyl residues originally proposed to be involved in interactions with cytochrome c_L (Nunn *et al.*, 1989) and x in a sequence indicates an unidentified residue.

1.5.b. Metal content of MDH

In the initial description of MDH, spectrographic analysis showed no appreciable amount of any metal; only copper, iron, nickel, silver, zinc, tin, aluminium, lead, sodium and magnesium were detectable and these were present at less than 0.1 p.p.m. ($\equiv$ 0.1 $\mu g.litre^{-1}$) (Anthony and Zatman, 1967a). By contrast, other quinoproteins have been shown to require metal ions. For example, *E. coli* synthesizes an apo-form of membrane-bound glucose dehydrogenase (GDH) that can only be reconstituted if PQQ and magnesium ions are present (Ameyama *et al.*, 1985a). Similarly, *Pseudomonas testosteroni* synthesizes a soluble apo-alcohol dehydrogenase (ADH) quinohaemoprotein that requires PQQ and calcium ions for reconstitution (Groen *et al.*, 1986). The role of the metal ions in these quinoproteins is discussed further in Section 1.6.d. Very recently, atomic absorption spectroscopy of the MDH from a newly described methylotroph, *Methylobacillus glycogenes*, revealed the presence of a single calcium ion per $\alpha_2\beta_2$ tetramer (Adachi *et al.*, 1990b). This unusual stoichiometry and the role of calcium in MDH is discussed in detail in Chapter 6.

1.5.c. Substrate specificity of MDH

The specificity of MDH is extensive but well defined in that only primary alcohols are oxidised. Whether or not an alcohol is oxidised is determined by its steric configuration (a second substituent on the C-2 atom appears to prevent binding) rather than the electron-displacing effects of the substituent groups (Anthony and Zatman, 1965; Sperl *et al.*, 1974; Duine and Frank, 1980a; Anthony, 1986). Using MDH from *Mb. extorquens* M27, Anthony and Zatman (1965) showed that the general formula for an oxidizable substrate is **R.CH_2OH** where **R** can be **H-**, **OH-**, **R'.CH_2-** or **R'.R''.C=CH-**. Oxidation of these substrates is achieved at rates of at least 30% of that for methanol, the best substrate. Typically the K_m for methanol is 10 - 20 µM. Although most MDHs have very similar specificities, some are unusual. The most notable exception to the rule is the MDH from *Rhodopseudomonas acidophila* which can oxidise secondary as well as primary alcohols (Sahm *et al.*, 1976; Bamforth and Quayle, 1978a, 1979).

A common feature of MDHs is their ability to oxidise the product of methanol oxidation, formaldehyde (in the hydrated gem-diol form) (Ladner and Zatman, 1969; Heptinstall and Quayle, 1969). Formaldehyde has a similar affinity for MDH and is oxidised at a similar rate to methanol, but oxidation of formaldehyde is energetically

wasteful and should be prevented to allow it to enter the bacteria for cell synthesis (Anthony, 1982). Therefore the affinity of MDH for formaldehyde must be governed. This is achieved by a protein that appears to be ubiquitous in methylotrophs called the modifier protein (see Section 1.5.g).

In the dye-linked assay, MDH can reduce the electron acceptor in the absence of added methanol even after extensive dialysis of the enzyme (Anthony and Zatman, 1964b; Goldberg, 1976; Bamforth and Quayle, 1978b; Duine *et al.*, 1978). It was originally thought that methanol was very tightly bound to MDH. However, Ghosh and Quayle (1981) showed that each MDH molecule had 2 molecules of bound methanol and a further 90 molecules of an unidentified endogenous reductant, whose nature remains unknown.

1.5.d. Activators of MDH

The activity of aerobically-prepared MDH, when assayed with PES and PIP, has an absolute requirement for ammonium salts which can be substituted by methylamine but not di-amines, tri-amines or long-chain alkylamines. At lower pH values, a higher concentration of the salt is required suggesting that the free-base is the active species (Anthony and Zatman, 1964b). By contrast, MDH purified from *Hyphomicrobium* X under anaerobic conditions appeared not to be ammonia-dependent, suggesting that ammonia was not important as an activator for MDH in the cell. Exposure of the anaerobically-prepared MDH to air resulted in the enzyme becoming ammonia-dependent (Duine *et al.*, 1979). Similar results were sometimes seen with anaerobic preparations of MDH from *Mb. extorquens* and *Mp. methylotrophus*, but more often than not, anaerobically-prepared cell-free extracts showed typical ammonia-dependence in the dye-linked assay (Beardmore-Gray and Anthony, 1984). The role of ammonia in the mechanism of MDH is discussed in Section 1.8.b.

When cytochrome c_L is used as the electron acceptor at pH 7.0. ammonia usually has little or no effect, supporting the theory that it is not important in the cell (Beardmore-Gray *et al.*, 1983; Elliot and Anthony , 1988). Work with *Hyphomicrobium* X has revealed the presence of an oxygen-labile 'factor X' which could replace ammonia as an activator in the dye-linked assay but was not itself an ammonium salt. Furthermore, 'factor x' stimulated the methanol oxidation rate at pH 7.0 using purified MDH and oxidised cytochrome c_L (Dijkstra *et al.*, 1988).

1.5.e. Inhibitors of MDH

EDTA, *p*-nitrophenylhydrazine and high phosphate concentrations have all been shown to inhibit methanol oxidation by whole cells but do not inhibit MDH when assayed with PES and PIP (Anthony and Zatman, 1964a,b; Anthony, 1975). In the dye-linked assay, PES is a potent, irreversible inhibitor of MDH, inactivating the enzyme within a few seconds although substrates and/or competitive inhibitors (such as cyanide and hydroxylamine) can protect the MDH (Anthony, 1975). Although not effective for all MDHs, the inhibitors usually compete for the substrate binding site, as is the case for the MDHs from *Hyphomicrobium* X (Duine and Frank, 1980a) and *Mp. methylotrophus* (Beardmore-Gray *et al.*, 1983). MDH from *Rhodopseudomonas acidophila*, however, differs in that inhibitors compete for the activator binding site (Bamforth and Quayle, 1978b).

The manner in which EDTA inhibits methanol oxidation is not clear. Anthony and Zatman (1964a) showed that whole cell methanol oxidation by *Mb. extorquens* M27 was completely inhibited by 0.1 mM EDTA; this inhibition was not significantly relieved by various metal ions. It was suggested that the inhibition by EDTA was not due to chelation of any essential cation. Using *Mp. methylotrophus*, it was also shown that EDTA inhibited whole cell methanol oxidation (I_{50} = 32 μM), and this was partially reversible (but not completely) by the addition of divalent cations. By contrast, MDH activity measured by the dye-linked assay was only inhibited 30% by 300 μM EDTA. This suggested that inhibition of whole cell methanol oxidation by EDTA was due to chelation of a magnesium ion that formed a bridge between MDH and the cytoplasmic membrane (Carver and Jones, 1984; Carver *et al.*, 1984). However, this is probably not the case since experiments using purified MDH and cytochrome c_L suggest that polyvalent anions (such as EDTA) somehow affect the interaction between these proteins (Beardmore-Gray *et al.*, 1983; Beardmore-Gray and Anthony, 1984; Cox *et al.*, 1992).

Other inhibitors of MDH include the cyclopropane derived 'suicide substrates' such as cyclopropanol and cyclopropanone ethyl hemiketal. These compounds, which inhibit MDH by formation of an irreversible adduct with the prosthetic group, have been very useful in the study of the reaction mechanism of MDH (Section 1.8; Mincey *et al.*, 1981; Parkes and Abeles, 1984; Dijkstra *et al.*, 1988; Frank *et al.*, 1988; Frank *et al.*, 1989a,b).

1.5.f. Location of MDH

After disruption of cell paste by sonication or passage through a French pressure cell, MDH activity is located almost entirely in the soluble fraction. Up to 50% of the MDH activity has also been observed in the membrane fraction from some methylotrophs, suggesting that the enzyme may be loosely associated with the membrane, although this is probably an artefact due to the method of preparation employed (Anthony, 1986).

A few Gram -ve methylotrophs, such as *Pa. denitrificans*, can be treated with EDTA and lysozyme, in the presence of an osmotic stabiliser (sucrose or mannitol), to yield spheroplasts and a periplasmic fraction. Analysis of this fraction revealed the presence of not only MDH but also all soluble cytochromes *c*; neither of these proteins were found in the cytoplasmic fraction obtained from the spheroplasts (Alefounder and Ferguson, 1981; Jones *et al.*, 1982; Burton *et al.*, 1983; Long and Anthony, 1991). This result suggested that either MDH was freely diffusible in the periplasm or loosely associated with the outer face of the cytoplasmic membrane. An alternative approach, by radio-labelling with [^{14}C]-isothionylacetamide, also indicated that MDH and the soluble cytochromes *c* were located entirely in the periplasm (Kasprzak and Steenkamp, 1983; Quilter and Jones, 1984).

1.5.g. Regulation of MDH activity

It has already been mentioned that formaldehyde, in addition to being the product from the oxidation of methanol, is also a very good substrate for MDH, although oxidation of formaldehyde to formate by MDH is energetically wasteful. Furthermore, formaldehyde is a potentially lethal metabolite capable of numerous adverse reactions in the cell. Clearly there is a need for the activity of MDH to be regulated in this respect. This is most probably achieved by a modifier (M-) protein that has been identified in many methylotrophs (Bolbot and Anthony, 1980; Ford *et al.*, 1985). Partial purification of the M-protein from *Mb. extorquens* and *Mp. methylotrophus* suggested that it was a homo-dimer of subunit molecular weight 67 kDa (Page and Anthony, 1986). However, a further investigation with these organisms and *Pa. denitrificans* showed that the 67 kDa protein was cytoplasmic and that the real M-protein was multimeric (most probably a trimer) of subunit molecular weight 45 kDa which was located in the periplasm along with MDH (Long and Anthony, 1991). The action of M-protein is two-fold. Firstly, it decreases the affinity of MDH for

formaldehyde and reduces the maximum oxidation rate (Page and Anthony, 1986; Long and Anthony, 1991). A second, fortuitous action of M-protein was that it increased the affinity of MDH for some alcohols (such as butane-1,3-diol) which are otherwise poor substrates (Bolbot and Anthony, 1980; Ford *et al.*, 1985). Interestingly, the maximal *in vitro* effect of M-protein was observed when MDH was present in a 5-fold excess. Analysis of the periplasmic fraction showed that M-protein was present at a much lower level than this *in vivo* (Long and Anthony, 1991). Hence, it is possible that M-protein may play a more complex role in MDH activity regulation than simply affecting formaldehyde oxidation. These studies were further complicated by the presence of an unidentified, non-protein factor that mimicked M-protein activity in the normal assay (Long and Anthony, 1991).

1.6. The prosthetic group of MDH

The absorption spectrum of MDH as isolated (Fig. 1.3) has a distinctive maximum between 345 and 350 nm. This peak is due entirely to the prosthetic group of MDH, which was first isolated from the enzyme and characterised by Anthony and Zatman (1967a,b). Although MDH has only typical protein fluorescence (due to aromatic residues), treatment of the enzyme by boiling or by exposure to high or low pH led to inactivation concomitant with the appearance of the green fluorescent prosthetic group (Anthony and Zatman, 1967b). The spectral characteristics of this compound were consistent with those of pteridines and the authors proposed that the prosthetic group of MDH was a pteridine derivative. However, as shall be seen in the next section, the prosthetic group is now known to be a tricyclic compound called pyrrolo-quinoline quinone (PQQ).

Excellent reviews of PQQ, its chemistry, structure and function in quinoproteins have been published (Anthony, 1982, 1986, 1988; Duine *et al.*, 1987; Duine and Jongejan, 1989). The following sections summarize some of the more salient points.

1.6.a. The structure of PQQ

The proposal that the prosthetic group of MDH was a pteridine derivative stood for some 15 years, until two groups working independently elucidated the correct structure. Using esr, nmr and mass spectroscopy, Duine, Frank and their colleagues investigated the prosthetic group of the MDH from *Hyphomicrobium* X. They showed the compound to be multicyclic with 2 uncoupled aromatic protons, 2 nitrogen atoms,

an inner ring ortho-quinone and 2 or more carboxylic acid groups (Duine *et al.*, 1978; Westerling *et al.*, 1979; de Beer *et al.*, 1979; Duine and Frank, 1980b). At the same time, Forrest, Kennard and their colleagues crystallised an acetonyl derivative of the putative prosthetic group of the MDH from *Pseudomonas* TP1. X-ray analysis of this derivative revealed a heterocyclic quinone (Fig. 1.6.a; Salisbury *et al.*, 1979). Shortly afterwards the first, complete structure was published (Fig. 1.6.b; Duine *et al.*, 1980). Originally, Forrest proposed the trivial name methoxatin for the prosthetic group. However, it is now generally referred to by the semi-systematic name pyrrolo-quinoline quinone. This name has the advantage that the abbreviated form, PQQ, allows indication of the various catalytically important redox states (PQQ, $PQQH^{\bullet}$ and $PQQH_2$; see Section 1.6.c).

ESR spectroscopy of MDH from *Hyphomicrobium* X revealed the presence of the free-radical semiquinone form of PQQ ($PQQH^{\bullet}$) (Duine *et al.*, 1978). Extraction of the prosthetic group from the enzyme at low pH followed by reverse phase HPLC analysis gave two peaks in the UV elution profile (Duine *et al.*, 1981). Spectral analysis of these peaks identified them as the quinone (PQQ; which eluted first) and the quinol ($PQQH_2$). Subsequent integration of the peak areas revealed the two forms to be in a 1:1 ratio. Oxidation of the extract by PIP prior to loading onto the column produced only the quinone form and integration of the single peak area showed that 2 molecules of PQQ were present per MDH molecule. It was concluded that the $PQQH^{\bullet}$ present in the isolated enzyme, upon extraction, disproportionated into equimolar amounts of PQQ and $PQQH_2$.

1.6.b. Physical properties of PQQ

As a solid PQQ is a brick red compound of molecular weight 331 Da. It dissolves readily in water to give a green fluorescent solution which has characteristic absorption and fluorescence spectra (Figs. 1.7, 1.8). Examination of the spectra reveals an interesting fact. For most fluorescent compounds, the excitation spectrum is identical to the absorption spectrum; clearly for PQQ they are not. Furthermore, these spectral characteristics are highly dependent on temperature, pH and nature of the solvent (Dekker *et al.*, 1982; Ameyama *et al.*, 1984). The reason for the difference in spectra of PQQ is the fact that in aqueous solutions, PQQ is partially hydrated at the C-5 carbonyl group. Hence the absorption spectrum is a sum of the contributions from

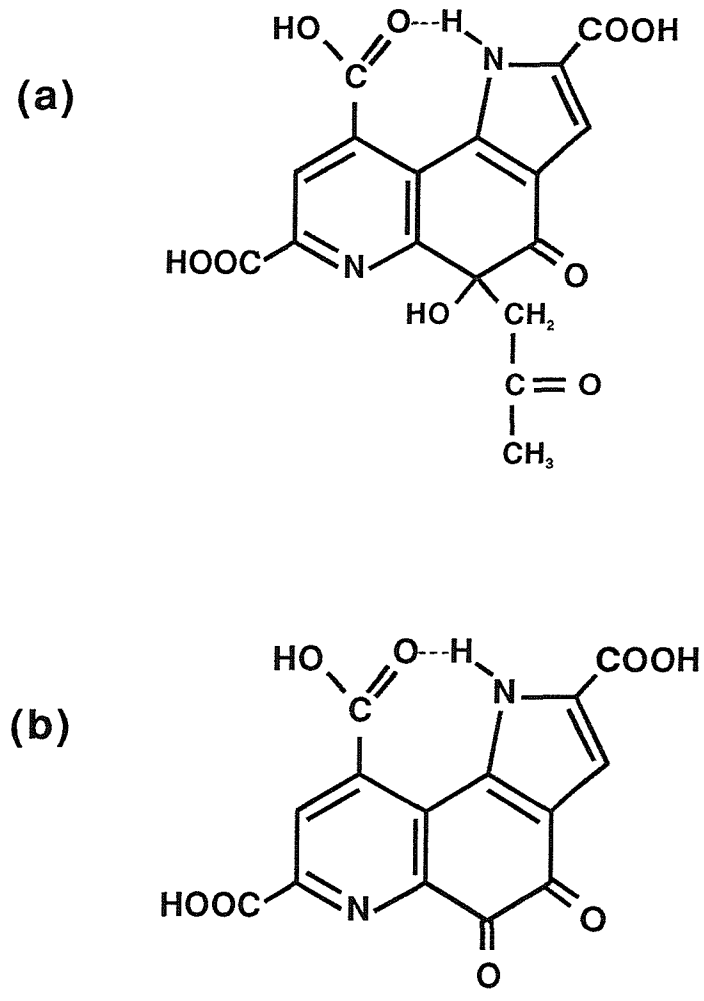


Fig. 1.6. The structure of PQQ

(a) Structure of the prosthetic group of MDH from *Pseudomonas* TP1 (Salisbury *et al.*, 1979). This structure was determined by X-ray diffraction analysis of a crystalline acetonyl derivative of the presumed prosthetic group extracted from whole cells.
(b) Complete structure of pyrrolo-quinoline quinone (PQQ). This structure was deduced from nmr, esr and endor measurements of the prosthetic group extracted from the MDH from *Hyphomicrobium* X (Duine *et al.*, 1980), and from the X-ray structure shown in (a). The full systematic name for PQQ is 2,7,9-tricarboxy-1H-pyrrolo-[2,3-*f*] quinoline-4,5-dione.

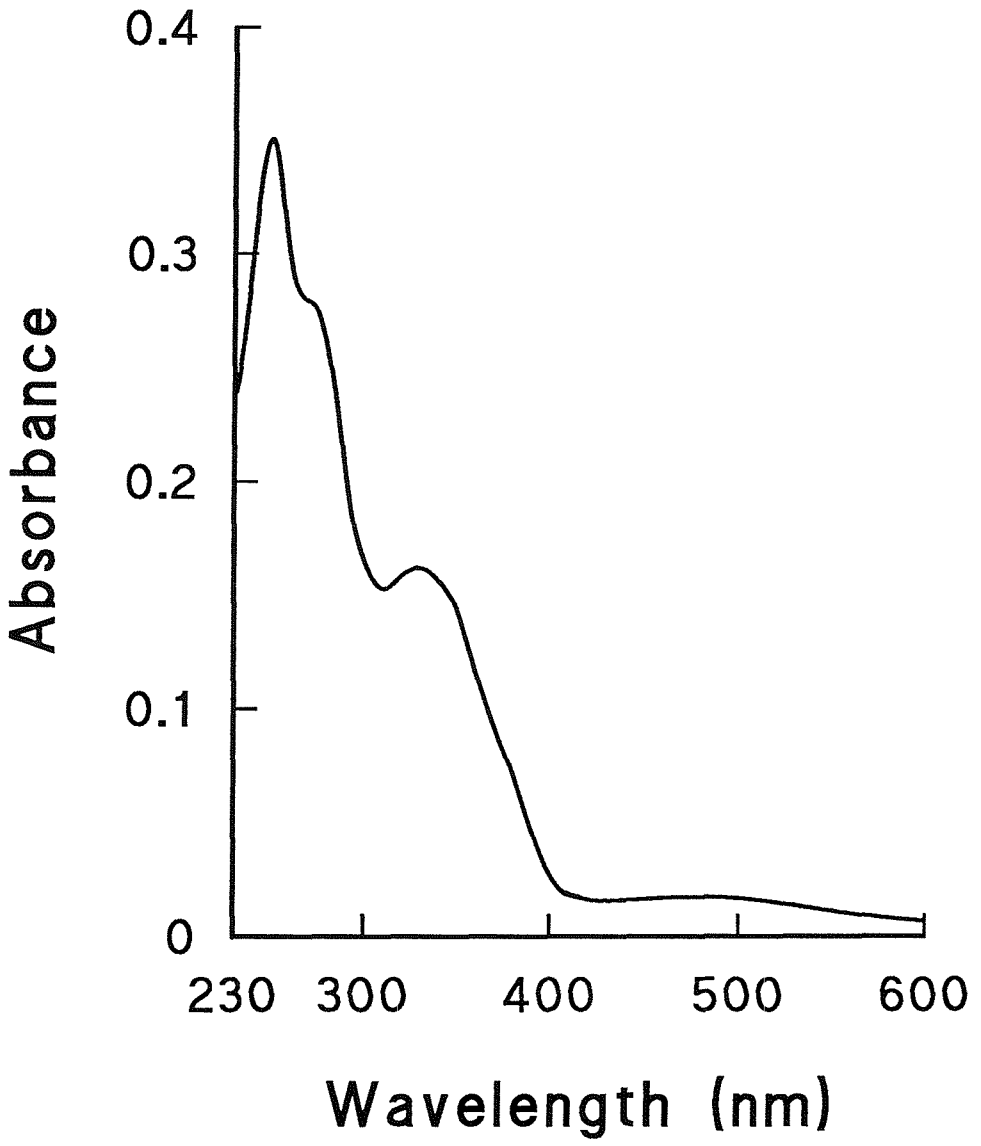


Fig. 1.7. Absorption spectrum of PQQ

The absorption spectrum of a 250 μM solution of PQQ (Fluka) in 20 mM potassium phosphate buffer (pH 7.0). This spectrum is a sum of the contributions of the oxidised form (PQQ) and the hydrated form PQQ-H_2O.

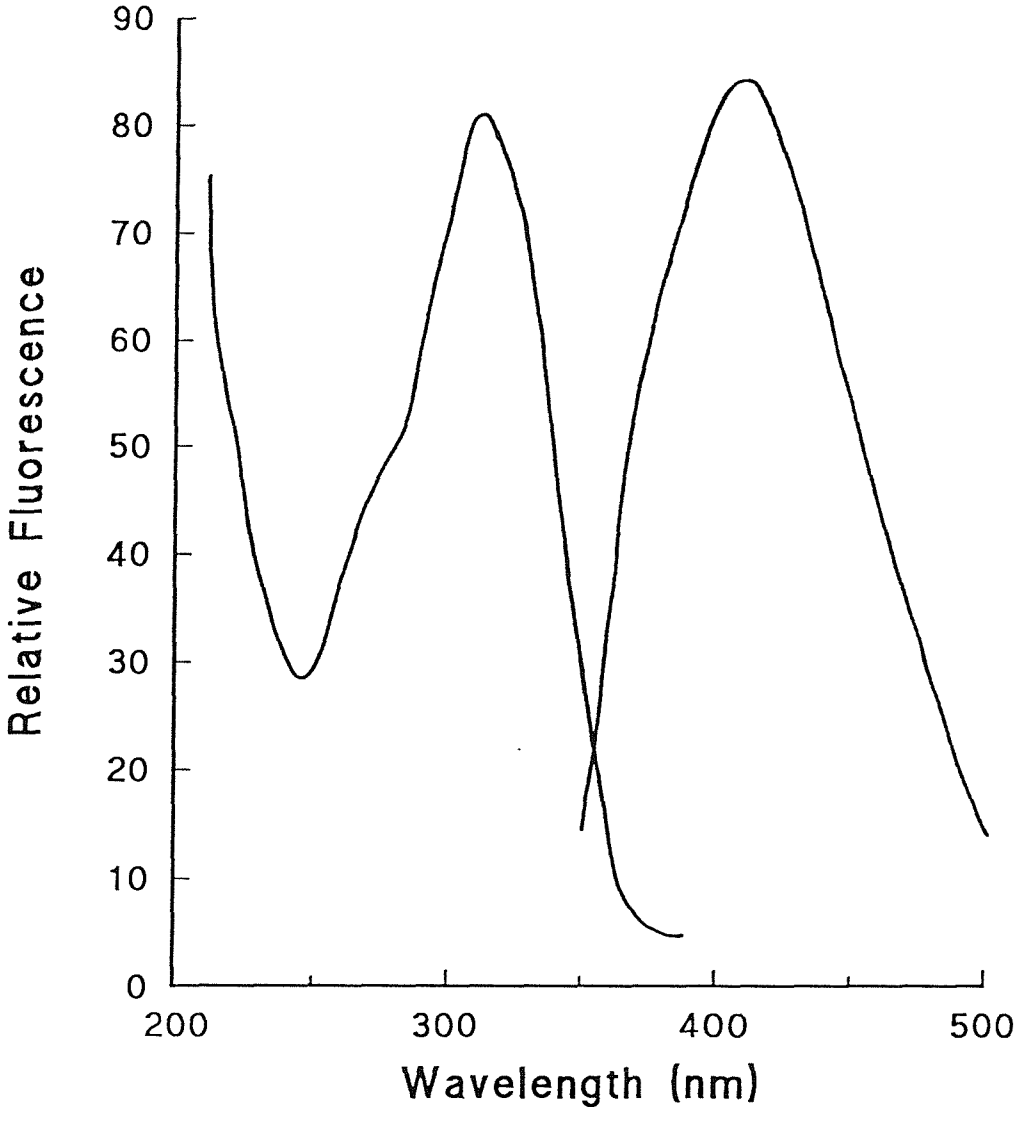


Fig. 1.8. Fluorescence spectra of PQQ

The fluorescence spectra of the same solution of PQQ used in Fig. 1.7. The excitation spectrum was recorded at 470 nm emission; the emission spectrum recorded at 365 nm excitation. Both spectra were recorded using an expansion factor of 0.1 (see Chapter 2). These spectra are due solely to the hydrated form (PQQ-H_2O); The oxidised, quinone form (PQQ) is non-fluorescent.

the non-hydrated form (PQQ) and the hydrated form (PQQ-H_2O); as the temperature decreases so the degree of hydration increases (Dekker *et al.*, 1982). By contrast, only the hydrated form is fluorescent; the fluorescence spectrum is therefore is due solely to PQQ-H_2O. Divalent cations such as Ca^{2+} have also been shown to affect the absorption spectrum of PQQ causing a red shift of the maximum at 330 nm; in the case of calcium the new maximum is at 345 nm (Geiger and Görisch, 1989). This is interesting since MDH has an absorption maximum at 345 nm. It is therefore possible that this peak in the MDH spectrum appears as a result of PQQ binding to calcium that is present in the enzyme. It should be noted, however, that the PQQ is almost certainly bound directly to the protein as well as possibly bound to calcium. That PQQ can bind metal ions is not surprising since the molecule has three carboxylic acid groups. Such groups are important in the chelating action of chelators such as EDTA.

PQQ is a very reactive molecule capable of undergoing many addition reactions, principally at the C-5 carbonyl group which is amenable to attack by nucleophilic agents (Fig. 1.9). The majority of these reactions are reversible (a notable exception is the adduct formed with cyclopropyl derivatives; see Section 1.8.d), and generally have distinctive spectral characteristics and/or retention times on HPLC columns which allow identification (Dekker *et al.*, 1982). Methylation of the carboxylic acid groups is also possible using dimethyl sulphate to give the trimethyl ester (Duine *et al.*, 1980).

1.6.c. The redox forms of PQQ

The redox behaviour of PQQ is reflected solely in the *o*-quinone grouping, which can exist in three biologically important forms; the fully oxidised or quinone form (PQQ), the semiquinone form ($PQQH^{\bullet}$) and the fully reduced or quinol from ($PQQH_2$) (Fig. 1.10). All of these forms have been implicated in the reaction mechanism of MDH (see Section 1.8). The mid-point redox potential of the PQQ/$PQQH_2$ couple is +90 mV at pH 7.0 and +419 mV at pH 2.0 indicating that PQQ is a $2e^-/2H^+$ redox carrier (Duine *et al.*, 1981).

1.6.d. Attachment of PQQ to quinoproteins

In the case of MDH, even though the prosthetic group is non-covalently bound, PQQ can only be released by quite harsh treatment of the enzyme such as boiling or exposure to low or high pH (Anthony and Zatman, 1967b), or by extraction with

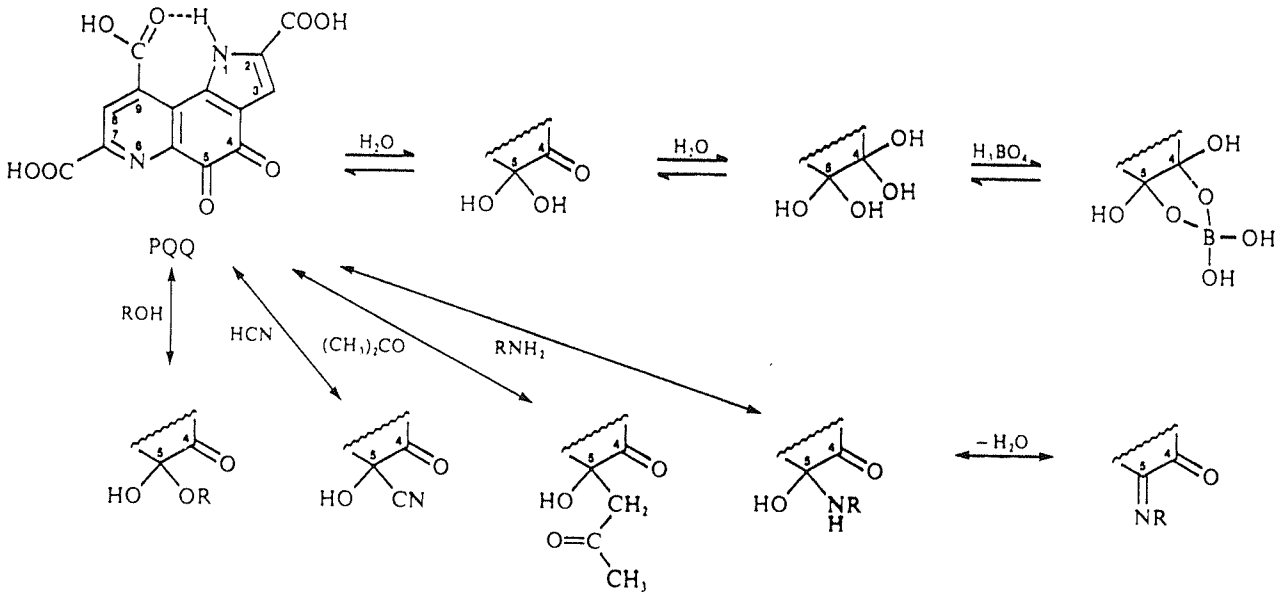


Fig. 1.9. Adducts of PQQ

This figure is taken from the review by Anthony (1986). These adducts and their significance are discussed extensively by Dekker *et al.* (1982). The production of these adducts is reversible in aqueous solution.

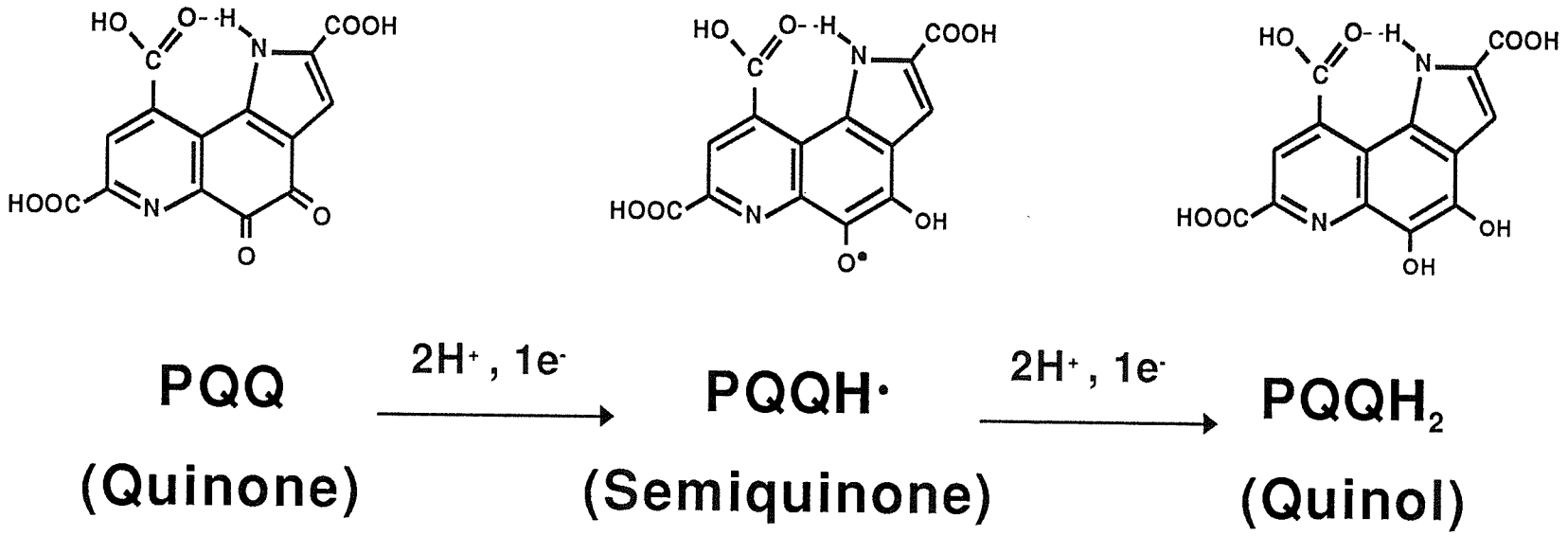


Fig. 1.10. The redox forms of PQQ

The quinone, semiquinone and quinol forms of PQQ are all involved in the reaction cycle of MDH. The semiquinone form is a free radical and is the species normally present in isolated MDH; its presence is detectable by esr spectroscopy. In MDH, only the quinone form is able to react with substrate.

methanol (Duine *et al.*, 1978). In all of these treatments, release of PQQ is concomitant with denaturation of the protein; usually it has not readily possible to release PQQ or separate MDH subunits without denaturation. Apparent removal of PQQ without denaturation has been observed using the monomeric MDH from *Methylomonas methanica* (Patel *et al.*, 1978). Their method relied on extensive dialysis against 3M potassium bromide at pH 4.5 and only gave a 50% yield of apo-MDH. It was not possible to reconstitute holo-MDH by the addition of PQQ. By contrast, Davidson *et al.* (1985) apparently did successfully reconstitute holo-MDH from the apo-form of the enzyme (prepared as described above) from bacterium W3A1 by the addition of a 10-fold excess of PQQ (obtained by heat denaturation of MDH). However, only the absorption spectrum was shown as evidence of reconstitution; the authors stated that to compare activities of the reconstituted and native enzyme was invalid because activity of the native MDH itself was labile. It should be noted that MDH is usually very stable (Anthony and Zatman, 1967a,b) and these comments suggest that the authors failed to reconstitute active MDH. The general failure to reconstitute active MDH was probably due to ignorance about the β-subunit and, as shall be demonstrated in Chapter 6, the fact that the presence of calcium is essential for active MDH.

By contrast with MDH, some quinoproteins that contain non-covalently bound PQQ have been successfully reconstituted. Examples include the membrane-bound glucose dehydrogenase (GDH) from *E. coli* (Ameyama *et al.*, 1985a) and the soluble quinohaemoprotein alcohol dehydrogenase (ADH) from *Pseudomonas testosteroni* (Groen *et al.*, 1986). These enzymes are peculiar in that the organism synthesises the enzyme in the apo-form; neither bacteria are able to synthesise PQQ and rely on scavenging the prosthetic group from their environment in order to make active enzyme. GDHs and ADHs from other organisms that can synthesise PQQ (and hence active enzymes) also have been reconstituted following generation of the apo-forms by treatment with chelating agents (Mutzel and Görisch, 1991) or by heat treatment (Geiger and Görisch, 1989). In all cases reconstitution was possible with the addition of both PQQ and divalent metal ions; addition of either alone had no effect. It was concluded that an enzyme-metal-PQQ complex was necessary for activity; this supports an earlier theory that metal ions may serve physically to bind PQQ to the enzyme (Ameyama *et al.* 1985b).

Studies using synthetic analogues of PQQ to reconstitute apo-quinoproteins

have been used to investigate which structural elements of the prosthetic group are important for binding. One analogue in which the C-9 carboxylic acid group had been omitted gave no appreciable activity in reconstitution of apo-GDH from *E. coli* (Shinagawa *et al.*, 1986). This suggested that this particular moiety was directly involved in the binding of PQQ to the enzyme. Similar studies using the apo-ADH from *Ps. testosteroni* have suggested that rotation of the C-9 carboxylic acid group out of the plane of the molecule may affect activity by altering the electrophilic nature of the C-5 carbonyl group (Jongejan *et al.*, 1989).

A *g*-value calculated from the esr spectrum of MDH suggested that the *o*-quinone moiety was not involved in binding (de Beer *et al.*, 1980) and ENDOR spectroscopy has suggested that PQQ may lie in a hydrophobic pocket in MDH (Duine *et al.*, 1984).

Major advances have been made in the cloning and sequencing of many of the quinoprotein dehydrogenases which has facilitated primary sequence analysis (Table 1.2; Anthony, 1992a). Such analysis led to independent identification of a putative PQQ-binding site in ADH, GDH and MDH (Cleton-Jansen *et al.*, 1990; Inoue *et al.*, 1990; Anthony, 1992a). Fig. 1.11 shows this 65 amino acid region and quite clearly illustrates the high level of conservation between the primary sequences of these distinct enzymes. Although some of the 65 residues will probably bind PQQ directly, the majority are likely to confer secondary structural features and possibly substrate-binding regions (Anthony, 1992a).

The precise nature of the attachment of PQQ to MDH and other quinoproteins will probably only be clarified once the 3-dimensional structure of these enzymes is known. It will then be possible to see how accurate the above proposals were.

1.6.e. Detection and determination of PQQ

In view of its spectral characteristics in solution, PQQ is quite readily identifiable. Determination of the concentration of a solution is possible using the absorption maximum at 249 nm (ε = 18400 $M^{-1}.cm^{-1}$; Duine *et al.*, 1980) although this coefficient depends on temperature, pH and nature of the solvent. To overcome this problem it is more practicable to use coefficients calculated from three isosbestic points in the spectrum ; 233 nm (ε = 14397 $M^{-1}.cm^{-1}$), 259 nm (ε = 16037 $M^{-1}.cm^{-1}$) and 322 nm (ε = 8963 $M^{-1}.cm^{-1}$) (Dekker *et al.*, 1982).

Table 1.2. Quinoproteins whose primary sequences are available

This table is taken from the review by Anthony (1992a). MDH, methanol dehydrogenase (soluble); ADH, alcohol dehydrogenase (membrane bound); GDH-A, glucose dehydrogenase (membrane bound); GDH-B, glucose dehydrogenase (soluble). The molecular weights for the β-subunits from *Acetobacter methanolicus* and *Methylophilus methylotrophus* are estimated from SDS-PAGE since the DNA sequences for these proteins are not known. All other molecular weights are calculated from amino acid sequences deduced from the relevant genes.

Quinoprotein	No. of amino acids	Mol. wt	Location	Signal peptide	References
MDH					
α-subunit					
Mb. extorquens	599	65,795	Periplasm	27	Anderson *et al.* (1990)
Mb. organophilum	599	66,996	Periplasm	27	Machlin and Hanson (1988)
Pa. denitrificans	599	66,845	Periplasm	32	Harms *et al.* (1987)
β-subunit					
Mb. extorquens	74	8,463	Periplasm	22	Nunn and Anthony (1988a)
Pa. denitrificans	83	9,445	Periplasm	20	van Spanning *et al.* (1991)
Ac. methanolicus	76	9,000	Periplasm	?	Chan and Anthony (1991)
Mp. methylotrophus	70	8,000	Periplasm	?	Cox (1992)
ADH					
Ac. aceti	707	78,028	Periplasm	35	Inoue *et al.* (1989, 1990)
GDH					
GDH-A					
An. calcoaceticus	801	86,956	Membrane	0	Cleton-Jansen *et al.* (1988)
E. coli	796	86,993	Membrane	0	Cleton-Jansen *et al.* (1990)
GDH-B					
An. calcoaceticus	454	50,237	Periplasm	24	Cleton-Jansen *et al.* (1989)

```
MDH     M.ext   477- GGTMATAGDL VFYGTLDGY L-KARDSDTG DL-LWKFKIP SGAIGYPMT YTHKGTQYVA IYYGVGG
MDH     M.org   477- GGTLATAGDL VFYGTLDGY L-KARDSDTG DL-LWKFKIP SGAIGYPMT YTHKGTQYVA IYYGVGG
MDH     P.den   476- GGTMATAGGL TFYVTLDGF I-KARDSDTG EL-LWKFKLP SGVIGHPMT YKHDGRQYVA IMYGVGG
ADH     A.ace   486- GGILATGGDL LFQGLANGE F-HAYDATNG SD-LYKFDAQ SGIIAPPMT YSVNGKQYVA VEVGWGG
                    **·**·*·* *   ·* · ·* *···* ·  *·**· · **·*· *** *· ·* **** ·  * ** -Identity in MDHs and ADH
                    !! ··!·!·· !   ··   ·! · ··! ·  !···  · ·!  · !!! !· ·! !!!· ·  !  ! -Identity in MDHs, ADH & GDHA
GDHA    E.col   713- GGPISTAGNV LFIAATADN YLRAYNMSNG EK-LWQGRLP AGGQATPMT YEVNGKQYVV ISAGGHG

GDHA    A.cal   719- GGSISTAGNV MFVGATQDN YLRAFNVTNG KK-LWEARLP AGGQATPMT YEINGKQYVV IMAGGHG
                    * · *·· ·   ·  ··*·  ·    ··*  · *   ** · ·* **   *·***·* ·   ·     -Identity in all GDHs
GDHB    A.cal   137- GLPSSKDHQS GRLVIGPDQ KIYYTIGDQG RNQLAYLFLP NQAQHTPTQ QELNGKDYHT YMGKVLR
                    *  ··· ·       ·        · *   *    · ·    *   · ·* ·*  ·       -Identity in all proteins
```

Fig. 1.11. The putative PQQ binding region on quinoproteins

This figure is taken from the review by Anthony (1992a). The residue numbers refer to the mature proteins with signal peptides excluded. The methanol dehydrogenase (MDH) α-subunits are from *Methylobacterium extorquens*, *Methylobacterium organophilum* and *Paracoccus denitrificans*. The glucose dehydrogenases (GDH-A and GDH-B) are from *Acinetobacter calcoaceticus* and *E. coli*, and the alcohol dehydrogenase (ADH) is from *Acetobacter aceti*. (!) indicates identity in all quinoproteins except for the periplasmic GDH-B from *Ac. calcoaceticus*. (*) indicates identity; (.) indicates a conserved residue. It should be noted that although there is considerable identity between all the GDHs (15 amino acids out of 65), only six of these are the same as the 19 amino acids that are identical in all the other quinoproteins.

It has already been mentioned that PQQ can be analyzed using reverse phase HPLC. Monitoring of the eluting molecules is possible by both absorption (UV) and fluorescence detection; the latter being far more sensitive and selective where complex samples such as cell-free extracts are being examined (Duine *et al.*, 1983). Confirmation that a peak is due to PQQ is achieved by treatment of the original sample with butyraldehyde which converts PQQ into a stable adduct with a retention time different from that of authentic PQQ (van der Meer *et al.*, 1990b). The sensitivity and selectivity of this technique can be further enhanced by reduction of the sample by sodium borohydride, followed by oxidation with sodium periodate. This treatment results in a strongly fluorescing (but as yet unidentified) adduct with a detection limit of about 80 nM (Duine *et al.*, 1983).

An alternative means of determining PQQ is by the reconstitution of apo-quinoproteins. The use of these biological assays is preferential to HPLC for 2 reasons. Firstly, the sample does not generally have to undergo any chemical pretreatment and, secondly, a much higher degree of sensitivity is achievable. For example, using the soluble GDH from *Acinetobacter calcoaceticus*, as little as 0.02 nM PQQ can be accurately detected (van der Meer *et al.*, 1990b). Selectivity of these biological assays is implied by the very fact that enzymes that rely on PQQ for activity are being employed. It should, of course, be borne in mind that some PQQ analogues are active in these reconstitution experiments.

Another method for specifically detecting PQQ and quinoproteins is the so-called redox cycling assay developed by Gallop and his colleagues (Paz *et al.*, 1989; Killgore *et al.*, 1989; Gallop *et al.*, 1989). This assay is based on the ability of PQQ to oxidatively decarboxylate certain amino acids resulting in $PQQH_2$ formation. Re-oxidation to the quinone is achieved using a tetrazolium dye (acting as an electron acceptor) and this can be followed colorimetrically. The method has been criticised for its poor selectivity for PQQ; several other compounds that may be present in biological samples (for example riboflavin) mimic the behaviour of PQQ (van der Meer, 1990a). Gallop, however, insists that his method is valid (Paz *et al.*, 1990a,b) and the argument still rages (Duine, 1990; Gallop, 1990; van der Meer *et al.*, 1990c).

1.7. Methylamine dehydrogenase and other quinoproteins

Methylamine dehydrogenase (MNDH; E.C. 1.4.99.3) was first isolated and characterised shortly after the first description of MDH (Eady and Large, 1968, 1971).

Subsequently MNDH has been identified in numerous bacteria (for a thorough review see Anthony, 1982).

MNDH is similar to MDH in that it is a soluble $\alpha_2\beta_2$ tetrameric enzyme located exclusively in the periplasm (Burton *et al.*, 1983; Lawton and Anthony, 1985). By contrast with MDH, however, the prosthetic group in MNDH is covalently bound. In the original description of MNDH, Eady and Large (1971) suggested that the prosthetic group was a pyridoxal derivative; a proposal based on reaction of the enzyme with carbonyl reagents. A later study using esr spectroscopy showed that the prosthetic group of MNDH was similar to PQQ in that it too was a quinone (de Beer *et al.*, 1980). Absorption and ENDOR spectroscopy, however, suggested that if the prosthetic group of MNDH was PQQ, it was either bound completely differently or was a modified form of PQQ (de Beer *et al.*, 1980; Kenney and McIntire, 1983; Hartmann and Klinman, 1988). Extraction of the prosthetic group was only possible by very harsh chemical treatment such as acid hydrolysis (Ameyama *et al.*, 1984a) and always resulted in a PQQ derivative and never authentic PQQ.

By this time, the structure of PQQ was known and other enzymes, especially from eukaryotic sources, where the prosthetic group was thought to be covalently bound PQQ, were being investigated with the result that a large number of papers appeared claiming the presence of PQQ in the enzyme under study (for a review, see Duine *et al.*, 1987, 1990).

Amino acid analysis of the light (β) subunit of MNDH from *Mb. extorquens* AM1 revealed 2 unidentifiable residues that were proposed to bind PQQ (Ishii *et al.*, 1983). Mass spectral analysis suggested that the prosthetic group was PQQ without the C-1, C-7 and C-9 carboxylic acid groups, and that it was attached to the protein by a cysteine thio-ether and a serine ether linkage (McIntire and Stults, 1986). That the prosthetic group was not PQQ as found in MDH was confirmed recently following elucidation of the 3-dimensional structure of MNDH from *Thiobacillus versutus* (Vellieux *et al.*, 1989). When trying to fit structures into the electron density map, neither normal PQQ nor PQQ without the carboxylic acid groups could be accommodated. The fact that no primary sequence of any MNDH was known at the time did not help the structure solution and led to two proposals for the nature of the prosthetic group (Fig. 1.12; Vellieux *et al.*, 1989; Vellieux and Hol, 1990). The identity of the prosthetic group in MNDH has now been resolved following, firstly, the cloning and sequencing of the gene encoding the light subunit of MNDH from *Mb.*

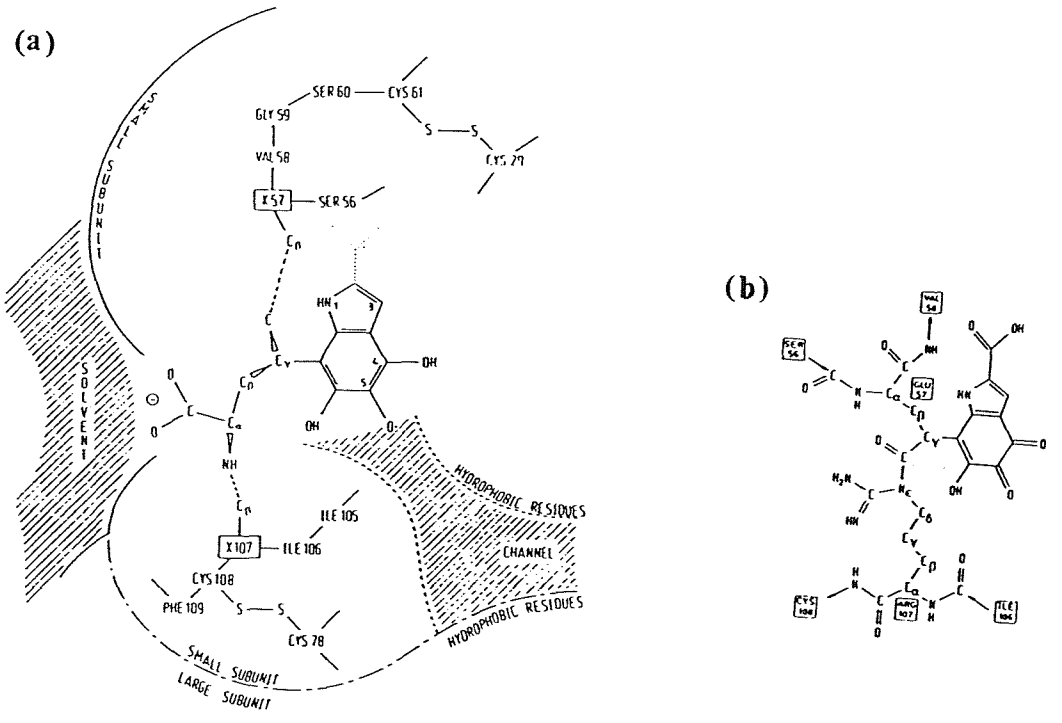


Fig. 1.12. Proposed structures of the prosthetic group of MNDH

(a) 'Pro-PQQ'; the structure proposed by Vellieux *et al.* (1989) following X-ray diffraction analysis of the MNDH from *Thiobacillus versutus*. It was not possible to accurately determine the linkages to the prosthetic group; these are indicated by dotted lines. The primary sequence of this enzyme was not known and it was not possible to identify the amino acids that were responsible for binding the prosthetic group.
(b) 'TGA-pro-PQQ'; the structure proposed by Vellieux and Hol (1990) following a re-evaluation of the electron density map described used in the proposal above. These structures were deduced from X-ray diffraction analysis of MNDH from *Thiobacillus versutus*

extorquens (Chistoserdov *et al.*, 1990) and , secondly, nmr and mass spectral data from the same enzyme (McIntire *et al.*, 1991). These studies showed that the two previously unidentified residues were both tryptophan and that these residues were linked and modified to form the prosthetic group (Fig. 1.13.a).

Some of the other enzymes previously thought to contain covalently bound PQQ have been re-investigated with quite surprising results. Mild treatment of bovine serum amine oxidase (BSAO) with hydrazine, followed by thermolysin hydrolysis yielded a peptide with the hydrazone of 6-hydroxydopaquinone (Janes *et al.*, 1990), and not the hydrazone of PQQ as claimed earlier by Duine's group (Lobenstein-Verbeek *et al.*, 1984). This suggested that in BSAO, the prosthetic group was 6-hydroxydopaquinone (Fig. 1.13.b). Three-dimensional structural analysis of galactose oxidase (Ito *et al.*, 1990) has revealed a third form of prosthetic group, where a tyrosyl residue formed a thio-ether bond with a cystyl residue (Fig. 1.13.c). Similar studies with dopamine β-hydroxylase (Robertson *et al.*, 1989), soybean lipoxygenase-1 (Veldink *et al.*, 1990) and dopa decarboxylase (De Biase *et al.*, 1991), have all shown that these enzymes do not, as originally thought, contain PQQ, but a novel prosthetic group composed of modified aromatic acid residues.

Although these enzymes all have structurally very different prosthetic groups, it is proposed that they still be called quinoproteins in view of the quinonoid nature of the prosthetic group (Duine, 1991). Galactose oxidase is also proposed to be a quinoprotein even though the prosthetic group clearly is not a quinone; Duine has extended his definition of quinoproteins to include those enzymes which have a free radical form of tyrosine or tryptophan.

1.8. The mechanism of MDH

1.8.a. The catalytic cycle

Although MDH and the structure of its prosthetic group have been known for some time, insight into the enzyme mechanism had to wait for some 20 years after the initial description. This was primarily because MDH is isolated in a partially reduced form (called MDH_{sem}) and addition of substrate had no observable effect on the enzyme's absorption spectrum. This is because MDH_{sem} has 2 molecules of $PQQH^{\bullet}$ which are unable to accept the 2 electrons from methanol (Duine *et al.*, 1981). Additionally, MDH was rapidly inactivated when incubated with artificial electron

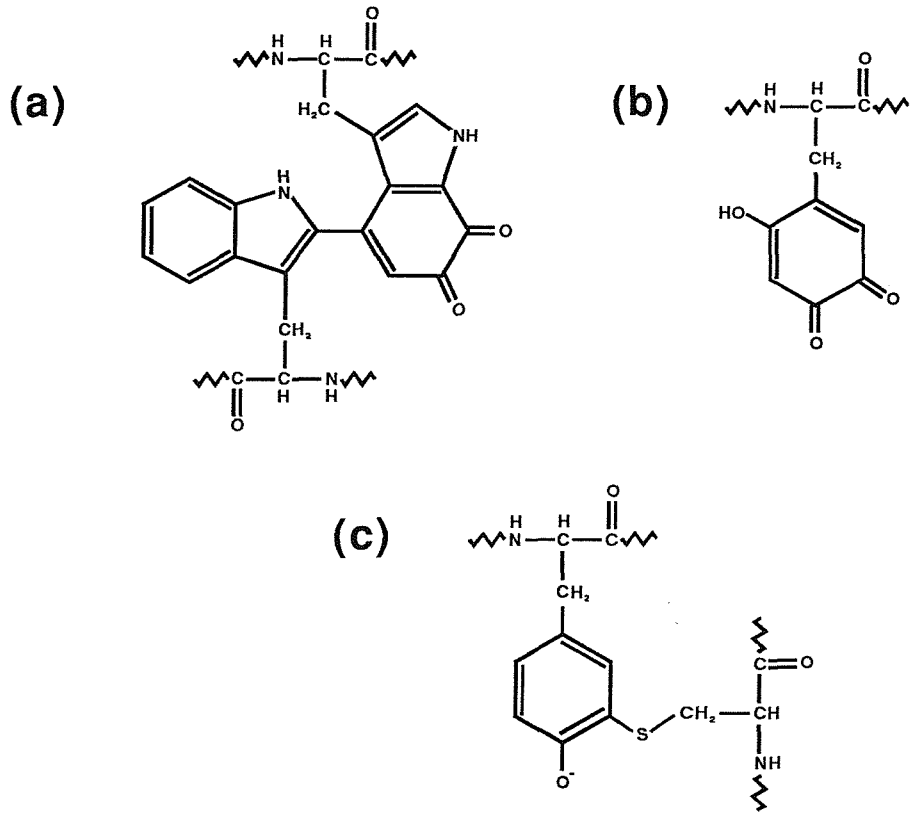


Fig. 1.13. Structures of the covalently-bound prosthetic groups in quinoproteins previously thought to contain PQQ

(a) Tryptophan tryptophyl quinone (TTQ); the prosthetic group of methylamine dehydrogenase (MNDH) (McIntire *et al.*, 1991). TTQ is composed of two tryptophan residues both present in the light subunit of this enzyme. It is not yet known how the two sidechains are modified and linked to form the final structure.

(b) 6-hydroxydopa quinone or topaquinone (TPQ); the prosthetic group of bovine serum amine oxidase (BSAO) (Janes *et al.*, 1990). TPQ is formed from the modification of a tyrosyl residue again by an unknown pathway.

(c) 'Tyr-S'; the prosthetic group of galactose oxidase (GAO) (Ito *et al.*, 1991). The name 'tyr-S' was coined by Thomson (1991) to describe the prosthetic group in GAO which is composed of one tyrosyl residue and one cystyl residue linked by a thio-ether bond.

Both BSAO and GAO contain a copper ion as cofactor in addition to the modified aromatic amino acid residue; no metal ions have been detected in MNDH.

acceptors (Anthony and Zatman, 1967a,b; Duine and Frank, 1980a). A major step forward was taken when it was discovered that cyanide (a competitive inhibitor) prevented the inactivation of MDH and facilitated the isolation of a fully oxidised enzyme form (MDH_{ox}) that contained PQQ (quinone). Addition of substrate to MDH_{ox} resulted in the fully reduced enzyme form (MDH_{red}) containing $PQQH_2$ (Duine and Frank, 1980a). All of the different enzyme forms have distinct absorption spectra (Fig. 1.14). Additionally, it was found that direct reduction of MDH_{sem} to MDH_{red} was possible using the single-electron donor methylviologen, which indicated the intermediate status of MDH_{sem} (de Beer *et al.*, 1983). Studies on the kinetics of the catalytic cycle have been extended using stopped-flow spectrophotometry and has led to the reaction cycle presented in Fig. 1.15 (Frank *et al.*, 1988). The high pH optimum (pH 9.0) of the reaction is related to the pH dependency of the oxidation, by the electron acceptor, of MDH_{red} and MDH_{sem} (reactions 1 and 4). The rate limiting step is the conversion of MDH_{ox}.S (a putative enzyme-substrate complex) to MDH_{red} plus product (P) (reaction 3), and this is the only step that requires ammonia as activator. Using deuterated methanol, the transient MDH_{ox}.S has been observed and, using fluorescence measurements, its decomposition to MDH_{red} and formaldehyde detected (Frank *et al.*, 1989a).

1.8.b. The role of ammonia

Activation by ammonia is confined to the oxidation of substrate in the catalytic cycle. How ammonia is involved is not clear although it has been seen to react with the C-5 carbonyl group of PQQ (Dekker *et al.*, 1982).

Forrest *et al.* (1980) proposed that amines can form an iminoquinone with a covalent carbon-nitrogen bond at the C-4 position of PQQ. The substrate then binds to the same position, hydrogen is transferred to PQQ, and the product is released. However, this scheme is not consistent with the observation that reactivity of isolated PQQ resides in the C-5 position. Neither does the scheme take into account the fact that ammonia or amines are not always essential for activity. Furthermore, the iminoquinone structure is assumed to be active in catalysis. However, the spectrum of MDH does not change upon addition of ammonia and it is possible that ammonia reacts with another part of the enzyme, or that normally an amine group on MDH can replace the ammonia (Frank *et al.*, 1989b).

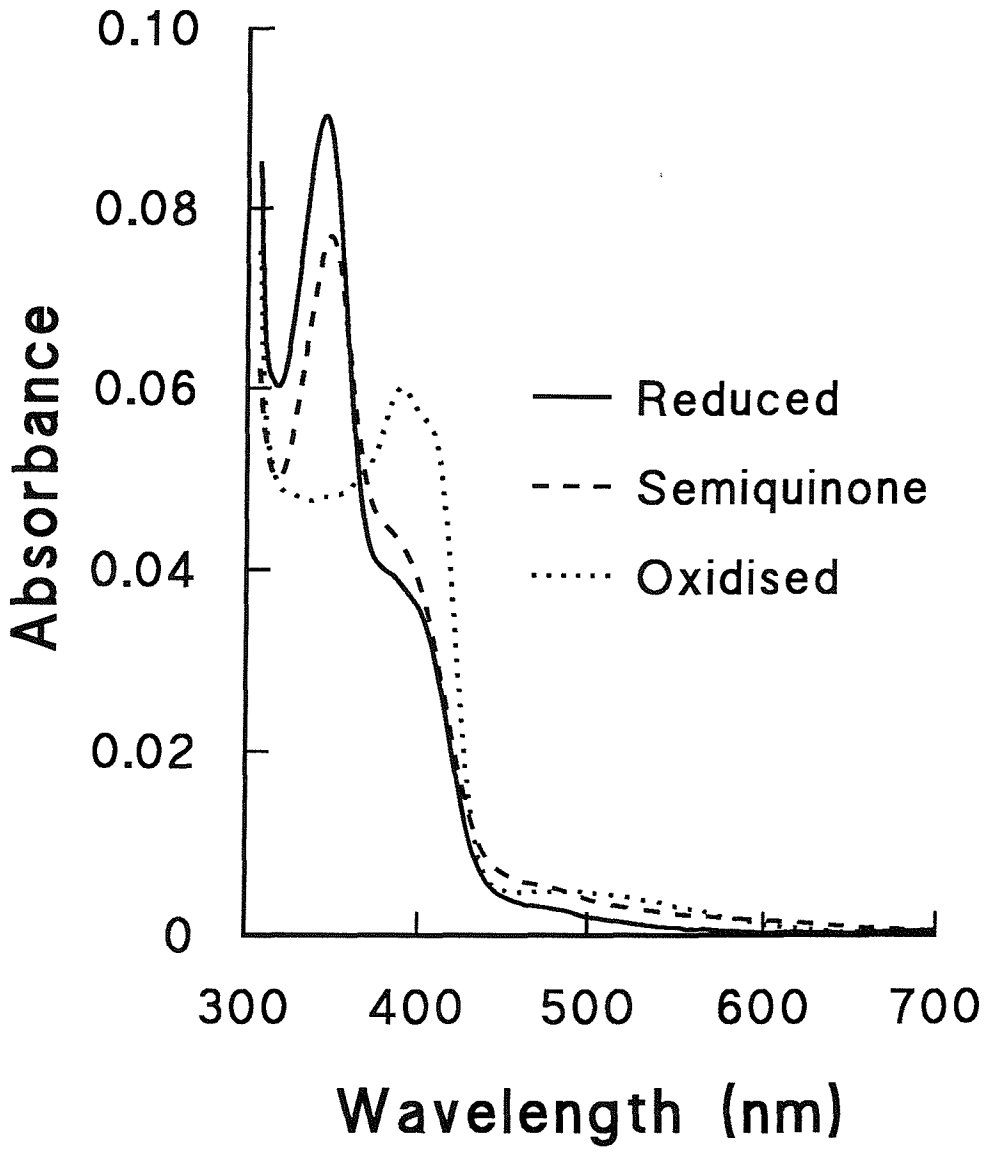


Fig. 1.14. Absorption spectra of the redox forms of MDH

Spectra of MDH (1 mg.ml^{-1}) were recorded in 100 mM potassium phosphate buffer (pH 7.0). ——, fully-reduced MDH of *M. extorquens*; - - - -, semiquinone form of MDH of *M. extorquens*; ·····, fully-oxidised MDH of *Hyphomicrobium* X (taken from Frank *et al.*, 1989).

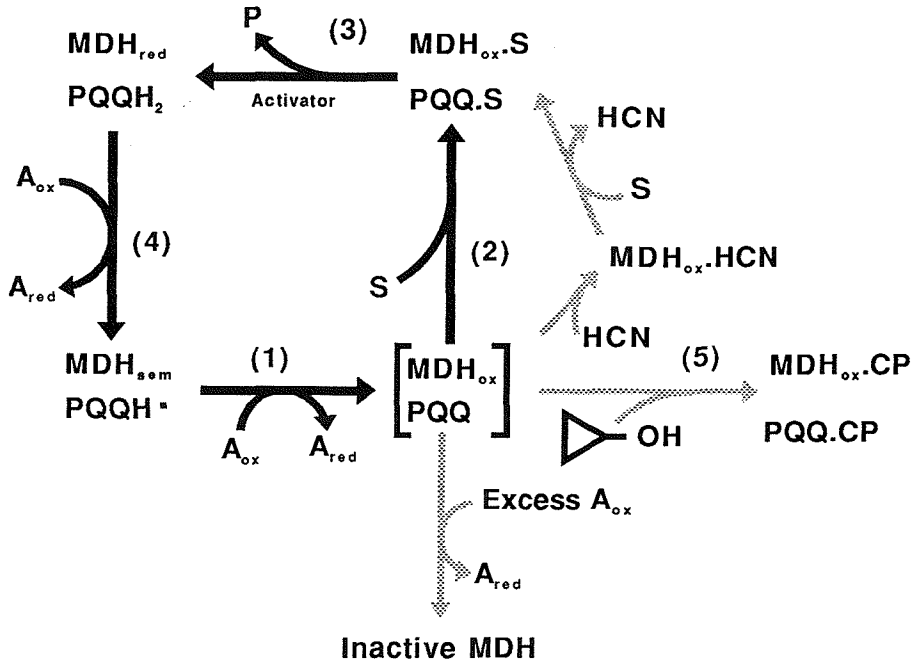


Fig. 1.15. The reaction cycle of MDH

This reaction cycle is taken from the review by Anthony (1992c) and is based on the work of Frank and his colleagues (Frank *et al.*, 1988; Frank *et al.*, 1989a,b). The normal reaction cycle is outlined by dark arrows. The starting point is the semiquinone form of the enzyme (MDH_{sem}) as it is usually isolated. Reactions 1, 2 and 4 are reversible; reaction 3 is not. A is the one-electron acceptor, which can be either PES, PMS, Wurster's blue or cytochrome c_L. MDH_{red}, fully reduced MDH; MDH_{ox}, fully oxidised MDH; MDH_{sem}, semiquinone form of MDH. The reaction with cyclopropanol is described in Section 1.8.d. Possible mechanisms for the reactions catalysed in steps 2 and 3 are given in Figs. 1.16 and 1.18.

1.8.c. The reaction mechanism

The cycle presented in Fig. 1.15 suggests that MDH oxidises the substrate, and releases the product, prior to reaction with an electron acceptor. The two types of mechanism mentioned here are consistent with this assumption.

In the first example (Fig. 1.16), reducing equivalents are transferred directly from substrate to the prosthetic group generating $PQQH_2$ and product. In this mechanism there is an acid-base catalysed hydride transfer to the C-5 carbonyl group of PQQ; a process analogous to the mechanism of typical alcohol:NAD^+ oxidoreductases (Akhtar and Wilton, 1973). The second type of mechanism involves formation of a PQQ-substrate complex at the active site prior to generation of $PQQH_2$. However, the only direct evidence for the existence of such a complex relates to the spectral changes observed on reaction of MDH with methanol, cyanide or cyclopropanol (see next Section) in the presence of an electron acceptor which converts the enzyme to MDH_{ox} (Frank *et al.*, 1988, 1989a).

1.8.d. The reaction of MDH with cyclopropanol

Treatment of MDH with cyclopropanol has provided the most important piece of evidence towards the existence of $MDH_{ox}.S$. The first use of cyclopropanol as an inhibitor for MDH was recorded by Mincey *et al.* (1981). Unfortunately, their conclusions about the action of this compound, coupled to misinterpreted esr spectroscopy data, led the authors to propose an erroneous reaction mechanism in which PQQ could accept 3 electrons. Frank, Duine and their colleagues repeated this work to arrive at the following conclusions and the scheme presented in Fig. 1.15. The inhibition by cyclopropanol derivatives is by way of an irreversible reaction with the C-5 carbonyl group of PQQ at the active site of MDH (Dijkstra *et al.*, 1984). This compound was isolated and identified as a ring-closed form of the C-5 3-propanal adduct (Frank *et al.*, 1988, 1989a). In order for this reaction to proceed, the 3-carbon ring of cyclopropanol must be opened. The most direct way for this to happen is by way of deprotonation by a base which may be present at the active site. It has been suggested that the reaction consists of a concerted proton abstraction followed by re-arrangement of the cyclopropoxy anion to a ring-opened carbanion, which then attacks the electrophilic C-5 carbonyl group of PQQ (Fig. 1.17; Frank *et al.*, 1988; 1989a,b). Such behaviour is unusual in that an 'unbreakable' carbon-carbon bond is formed instead of a carbon-oxygen bond which would be expected with a typical alcohol as

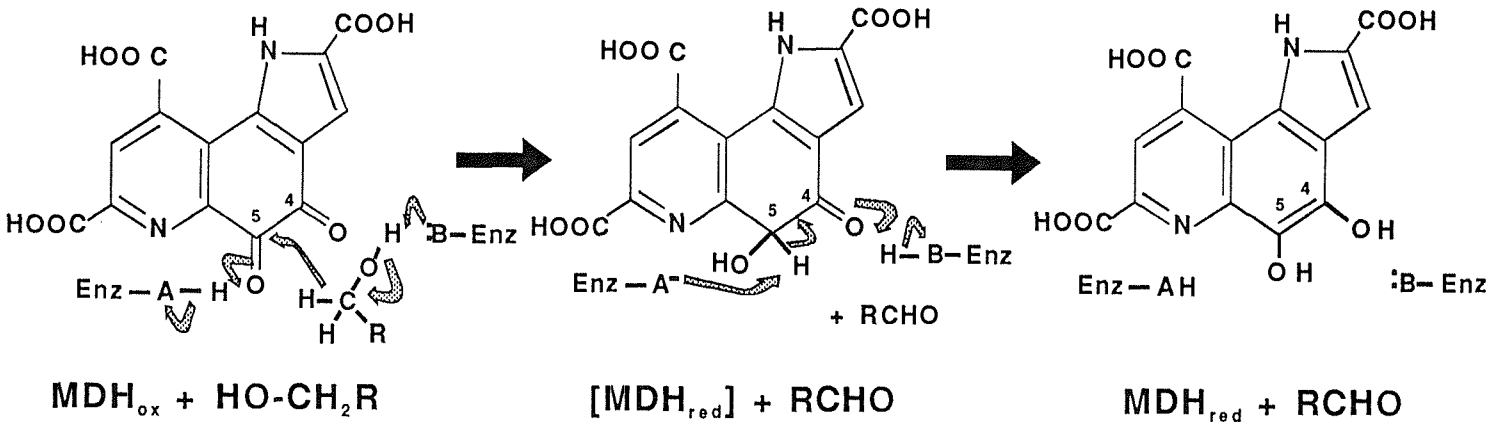


Fig. 1.16. A straightforward mechanism for the reaction of PQQ with substrate

This figure is taken from Anthony (1992c). The mechanism is analogous to that of the typical NAD^+-linked dehydrogenases and involves acid/base-catalysed hydride transfer to the C-5 carbonyl of PQQ.

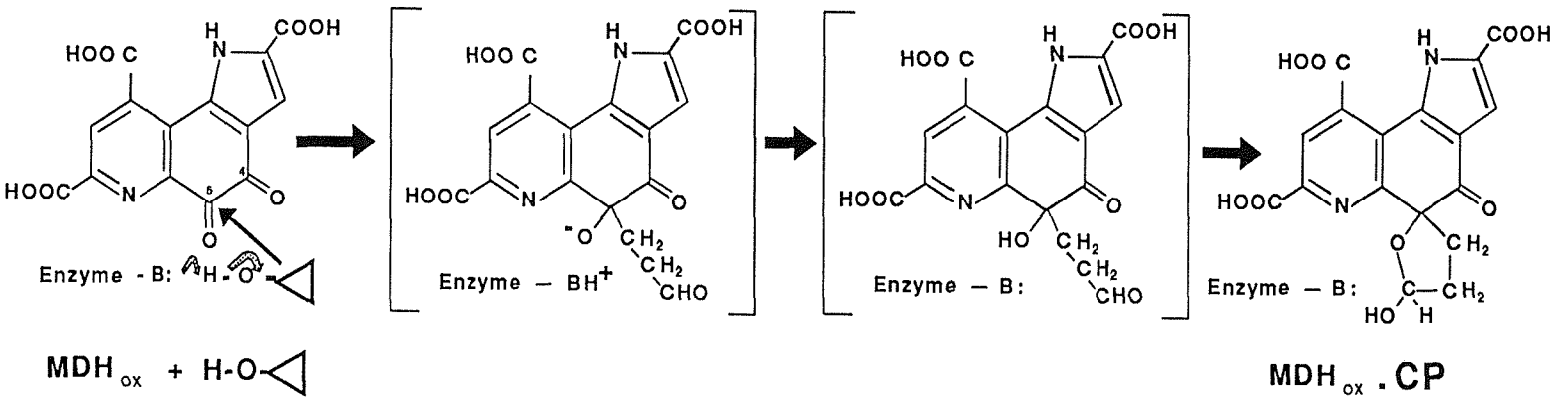


Fig. 1.17. A possible mechanism for the reaction of PQQ with cyclopropanol

This figure is taken from Anthony (1992c) and is based on the work of Frank and his colleagues (Dijkstra *et al.*, 1984; Frank *et al.*, 1989a,b). Identification of the product of the reaction demonstrated the necessity for a base (-B:) at the active site. It is this demonstration that is the basis of the other mechanisms described for alcohol oxidation in Figs. 1.16 and 1.18.

substrate. In this respect, the straightforward mechanism presented in Fig. 1.16 might be more appropriate for the cyclopropanol reaction since it also requires a base but does not require formation of different types of bond with the 2 types of substrate. Fig. 1.18 is taken from the review by Anthony (1992c) and illustrates proposed mechanisms for the reaction of MDH with typical substrates. All of these proposals involve direct proton plus hydride transfer, or reaction of the substrate with the C-5 carbonyl group of PQQ. It should be noted, however, that the scarce evidence available at present does not preclude more unusual reactions such as PQQ acting as a *p*-quinone or a mechanism in which PQQ exists as a ring-opened form on the enzyme and only closes upon extraction (Duine *et al.*, 1987).

1.9. The biosynthesis of PQQ

Shortly after elucidation of the structure of PQQ, a number of routes for its complete chemical synthesis were published (Corey and Tramontano, 1981; Gainer and Weinreb, 1981, 1982; Hendrickson and de Vries, 1982, 1985; Mackenzie *et al.*, 1986; Buechi *et al.*, 1985). The synthesis developed by Corey and Tramontano has been exploited in the commercial production of PQQ by a number of companies.

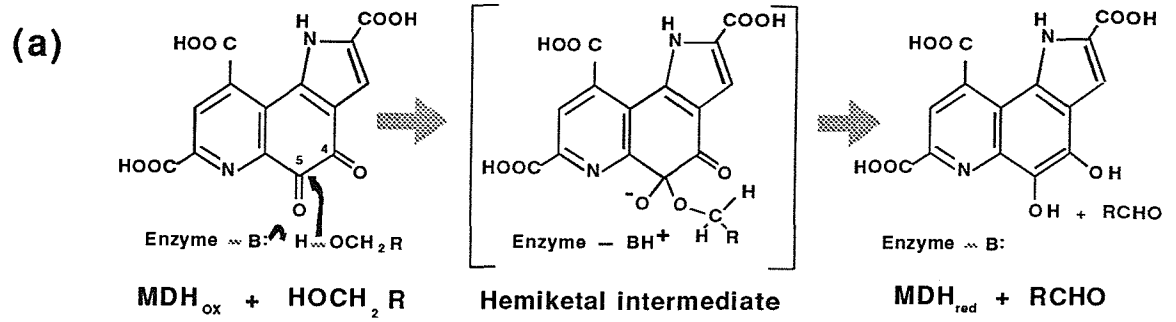
It was several years after these syntheses were developed before insight into the biosynthetic origin of PQQ was gained. The following sections summarise what is presently known about the biosynthetic precursors and the molecular biology of PQQ biosynthesis.

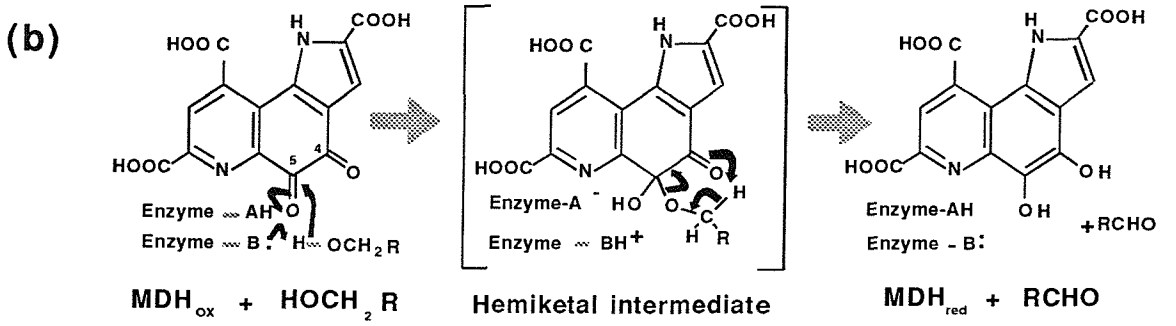
1.9.a. The precursors of PQQ biosynthesis

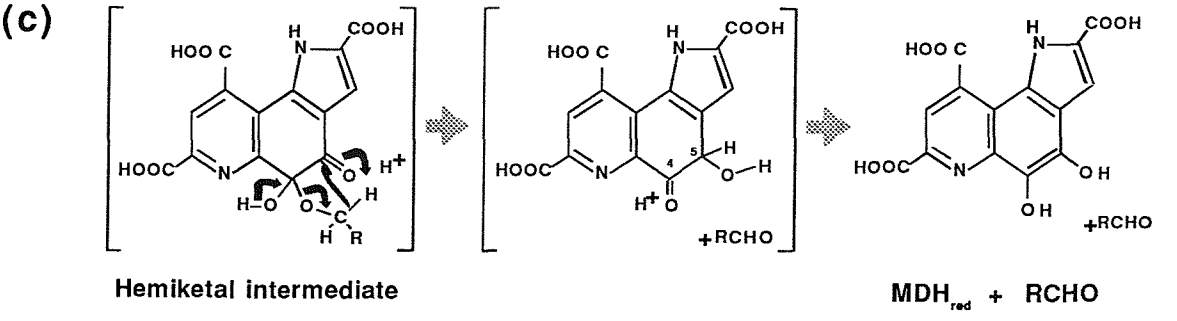
Two groups working independently came to the same conclusions that PQQ was composed of fused amino acid residues.

Unkefer and his colleagues used ^{13}C labelling studies in *Mb. extorquens* initially to assign the complete ^{13}C-nmr spectrum of PQQ. They then used [1-^{13}C]- and [2-^{13}C]-ethanol as the sole carbon source and examined the pattern of labelling in isolated amino acids and PQQ. The pattern seen in two amino acids, tyrosine and glutamate, was identical to two distinct patterns seen in the isolated [^{13}C]-PQQ. It was concluded that tyrosine and glutamate were the precursors of PQQ (Houck *et al.*, 1988). The incorporation of these two amino acids into the final structure of PQQ is shown in Fig. 1.19.a. In an extension of this study, Houck *et al.* (1989, 1991) proposed a biosynthetic

Fig. 1.18. Other possible mechanisms for the reaction of PQQ with substrate


(a)
HOO C
COOH
HOOC
Enzyme ~ B:
H ~ OCH2R
Enzyme – BH+
OH
+ RCHO
Enzyme ~ B:
MDHox + HOCH2R
Hemiketal intermediate
MDHred + RCHO


(b)
HOO C
COOH
HOOC
Enzyme ~ AH
Enzyme ~ B:
H ~ OCH2R
Enzyme-A
Enzyme ~ BH+
Enzyme-AH
Enzyme - B:
+RCHO
MDHox + HOCH2R
Hemiketal intermediate
MDHred + RCHO


(c)
HOO C
COOH
HOOC
+RCHO
Hemiketal intermediate
MDHred + RCHO

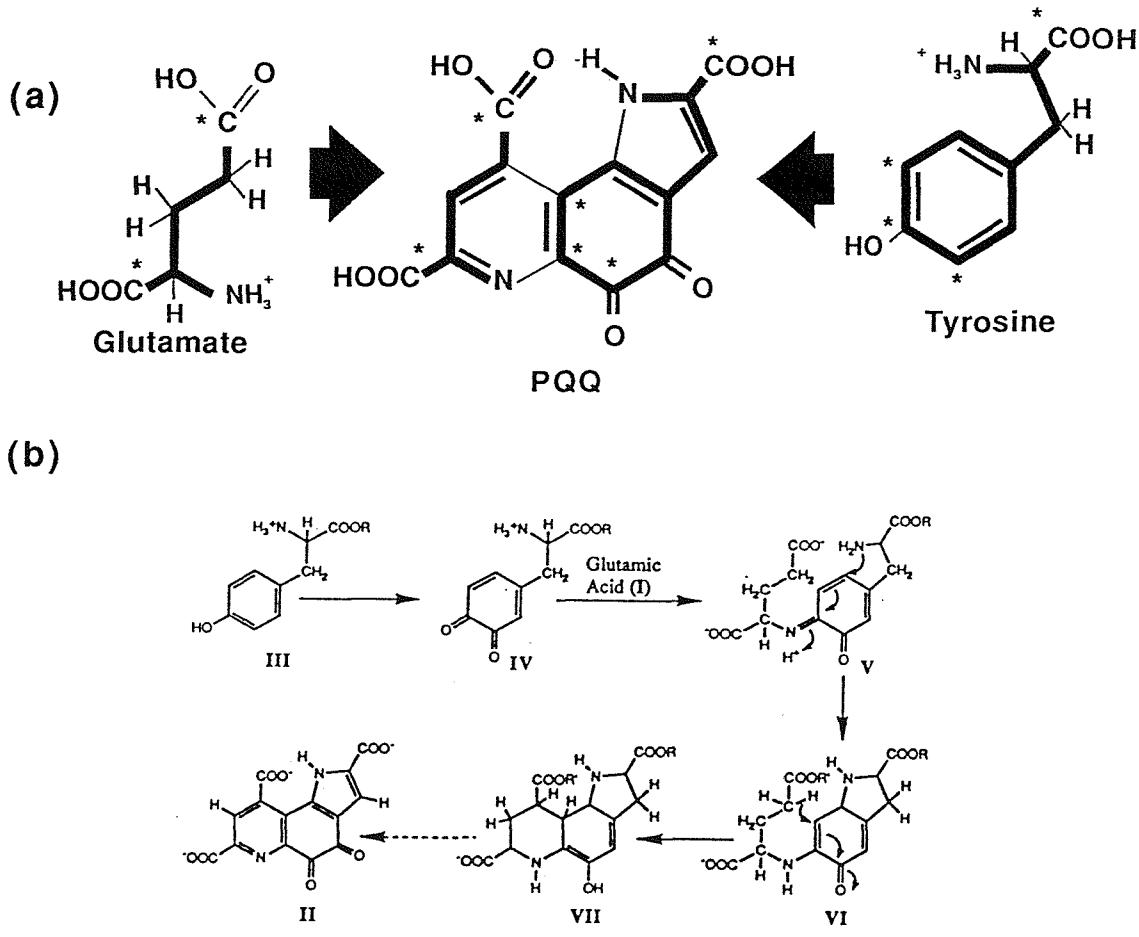


Fig. 1.19. The precursors of, and a possible pathway for, the biosynthesis of PQQ

(a) The incorporation of tyrosine and glutamate into the final structure of PQQ. This figure is taken from Houck *et al.* (1988). The precursors of PQQ biosynthesis were identified using incorporation of [1-^{13}C]- and [2-^{13}]-ethanol. (*) represents the primary sites in glutamate, PQQ and tyrosine labelled by [1-^{13}C]-ethanol.

(b) This speculative pathway, taken from Houck *et al.* (1991), was proposed following incorporation studies using [^{13}C]-tyrosine and [^{15}N,^{13}C]-tyrosine. These experiments showed that all of the carbons, and probably both nitrogens, of the precursors were present in PQQ. There is no evidence for any of the intermediates, although the reactions are primarily oxidative since production of PQQ from tyrosine and glutamate involves the loss of 12 electrons.

pathway for PQQ based on further labelling studies with [^{13}C]-tyrosine and [^{15}N, ^{13}C]-tyrosine (Fig. 1.19.b).

Van Kleef and Duine (1988a) also showed that tyrosine was a precursor of PQQ. Cultures of *Hyphomicrobium* X already growing on [^{13}C]-methanol were supplemented with unlabelled amino acids. ^{1}H- and ^{13}C-nmr analysis of PQQ purified from the culture medium revealed that the complete carbon skeleton of L-tyrosine was incorporated into the final structure of PQQ. The other constituent of PQQ was not identified.

The pathway presented in Fig. 1.19.b involves tyrosine as the initial intermediate which is then oxidised to dopaquinone by, presumably, a tyrosinase-like enzyme. However, no tyrosinase activity was found in cell-free extracts of *Mb. extorquens* (Houck *et al.*, 1991). A further problem is that none of the proposed intermediates have been found in PQQ deficient strains of *Ac. calcoaceticus* (van Kleef and Duine, 1988b).

1.9.b. The molecular biology of PQQ biosynthesis

The first breakthrough in this area came with the characterisation of mutants of *Ac. calcoaceticus* which were deficient in PQQ biosynthesis (Goosen *et al.*, 1987). This organism uses PQQ for the synthesis of active GDH. In a subsequent study, a 5 kb DNA fragment, which complemented the four classes of mutant, was cloned and sequenced (Goosen *et al.*, 1989). Within this fragment, 3 open reading frames (ORFs), which correspond to genes, were identified that could encode polypeptides of molecular weights 10.8 kDa (gene II), 29.7 kDa (gene I) and 43.6 kDa (gene III). *In vitro* transcription/translation of the fragment revealed the presence of 29 and 44 kDa proteins which corresponded to the products from genes I and III. No protein corresponding to the gene II was observed, probably because the SDS-PAGE system used was run for too long. A peculiarity about the fragment was the presence of a fourth ORF (gene IV) which could only encode a polypeptide of 24 amino acids. This 'gene' was shown to be essential for PQQ biosynthesis by *in vivo* expression studies. Expression of a plasmid containing a truncated fragment lacking gene IV did not result in PQQ biosynthesis by *E. coli* and *Ac. lwoffi* (two bacteria normally unable to synthesise PQQ); expression of a plasmid containing the whole fragment did, however, result in PQQ biosynthesis by these bacteria. A further ORF (gene V) was also identified in the fragment but did not appear essential for PQQ biosynthesis. The order

of the ORFs in the fragment was gene I-V-IV-II-III and none of the genes had an identifiable signal sequence indicating that the gene products were probably cytoplasmic. It was speculated that the gene IV product of 24 amino acids may be involved in PQQ transport although this is unlikely since the sequence does not have the hydrophobic character necessary for passage through a membrane. Another suggestion was that the polypeptide may serve as a 'template' for PQQ biosynthesis; both tyrosine and glutamate were present in the sequence. A similar theory had been proposed by van Kleef and Duine (1988a, 1989) following their fruitless search for PQQ biosynthesis intermediates.

Further advances have been made by Gasser and his colleagues using the methylotroph *Methylobacterium organophilum* DSM 760. By contrast with *Ac. calcoaceticus*, this organism uses the PQQ for active MDH. Again PQQ mutants were isolated and characterised resulting in the identification of 6 genes (called *pqqA* to *F*) proposed to be involved in PQQ biosynthesis (Biville *et al.*, 1988; Mazodier *et al.*, 1988; Biville *et al.*, 1989). The genes *pqqA* to *D* were found to be clustered, but not co-transcribed, in a 3.9 kb fragment, and were suggested to be analogous to genes I, II, III and IV described above. Although no sequence data were available, the suggestion is feasible in view of the mapped locations of the genes. By contrast with the genes from *Ac. calcoaceticus*, attempts to express the *Mb. organophilum* genes in *E. coli* or *Ac. lwoffi* failed. The reason, it was suggested, was due to the different G/C ratios that exist between the parent and host organisms. However, an attempt to express the genes in *Ps. testosteroni*, an organism with a very similar G/C ratio to *Mb. organophilum*, also failed (Biville *et al.*, 1989). It should be noted that an attempt to express all 6 genes in one organism was not made because one plasmid with all of the genes was not isolated. Of the other genes, *pqqE* was closely linked to *pqqD*, whereas *pqqF* was located some 19 kb from *pqqE*. Other studies by this group using *E. coli* have produced some very interesting results. It has always been assumed that *E. coli* cannot synthesise PQQ because it makes only the apo-form of GDH. Normally this is no problem because *E. coli* usually utilises glucose by way of the phosphotransferase system (PTS) of glucose transport and concomitant phosphorylation. PTS mutants obviously cannot utilise glucose. However, some spontaneous mutants of a PTS^- strain were able to utilise glucose (Turlin *et al.*, 1991; Biville *et al.*, 1991). Knowing that the PTS system was still impaired, the only way for these mutants to be able to grow on glucose was if GDH was being made in the holo-form. Therefore PQQ was being

synthesised by the mutants. Furthermore, a cloned fragment of wild-type *E. coli* DNA was able to complement the PQQE and PQQF mutants of *Mb. organophilum* (Biville *et al.*, 1991). It was concluded that *E. coli* possesses all of the necessary genetic information for PQQ biosynthesis but this is only expressed under conditions where the PTS system is not operating.

A third organism has been investigated with respect to PQQ biosynthesis genes using the similar approach of PQQ^{-} mutant isolation. *Klebsiella pneumoniae*, like *Ac. calcoaceticus* synthesises both GDH and PQQ. By contrast with *Ac. calcoaceticus*, however, *K. pneumoniae* is similar to *E. coli* in having the PTS system for glucose utilisation. A 6.9 kb fragment was cloned and expressed in *E. coli* resulting in high levels of PQQ biosynthesis in this organism (Meulenberg *et al.*, 1990). Sequencing of this fragment revealed the presence of 6 ORFs, labelled *pqqA* to *F*, which could encode polypeptides of 2.8 kDa, 33.5 kDa, 29.0 kDa, 10.4 kDa, 42.9 kDa and 88.4 kDa. Proteins corresponding in molecular weight to the gene products of *pqqB*, *C*, *E* and *F* were detected in maxicell experiments and there was a short polypeptide (encoded by *pqqA*) which corresponded to the gene IV product of *Ac. calcoaceticus*. The genes *pqqA* to *E* also had similar sequences to genes IV, V, I, II and III from *Ac. calcoaceticus*. The 84 kDa protein, encoded by *pqqF* was essential for PQQ biosynthesis but did not correspond to any previously identified PQQ gene product. It did, however, resemble proteases from various sources and it was suggested that this protein may cleave the final PQQ molecule from the short template polypeptide (Meulenberg *et al.*, 1990; Meulenberg, 1991).

The final piece of evidence relating to PQQ biosynthesis genes has come from PQQ supplementation experiments with **m**ethanol **ox**idation (Mox) mutants of *Mb. extorquens* (Lidstrom, 1990; Lidstrom and Stirling, 1990; Lidstrom and Tsygankov, 1991). Some of the mutants were able to grow on methanol-media agar plates if a filter disk saturated with PQQ was placed on the agar surface. Seven classes of mutant were able to be supplemented in this fashion suggesting that at least seven genes are involved in either PQQ biosynthesis or transport; there was no way of distinguishing between these different phenotypes.

In summary, it would appear that at least 6 genes are required for PQQ biosynthesis, one of which may encode a precursor polypeptide from which is excised the complete PQQ molecule; a process possibly achieved by a protease encoded by one of the other genes.

1.10. The molecular biology of methanol oxidation

This is also an area of extensive research and a number of very thorough reviews have been published (de Vries, 1986; de Vries *et al.*, 1990; Goodwin, 1990; Lidstrom, 1990; Lidstrom and Stirling, 1990; Lidstrom and Tsygankov, 1991; Harms and van Spanning, 1991). This section serves to summarise the progress that has been made with *Mb. extorquens*.

Although mutants impaired in C_1 metabolism have been known for some time (Heptinstall and Quayle, 1970; Dunstan *et al.*, 1972a,b; Dunstan and Anthony, 1973, Tatra and Goodwin, 1983), perhaps the most significant advance was made by Nunn and Lidstrom (1986a,b). These authors isolated ten classes of Mox mutant which were identified by complementation with a library of genomic DNA (Nunn and Lidstrom, 1986a). The mutants were isolated by plating UV-exposed or nitrous acid treated wild-type cells onto nutrient agar plates containing methanol and allyl alcohol. Bacteria with an unimpaired methanol oxidation system (induced by methanol) were able to oxidise the allyl alcohol into acrolein, a very toxic metabolite. Therefore the method was specific for the isolation of Mox mutants. All of the mutant strains were able to utilise methylamine which is metabolised, like methanol, via formaldehyde, indicating that all mutations were restricted to the oxidation of methanol. The ten classes of mutant, labelled MoxA1, A2, A3 and B to H, were characterised with respect to MDH and cytochrome c_L content, whole cell methanol oxidation and dye-linked MDH activity. Definite functions for only two of the relevant genes were ascribed; *moxF* and *moxG* which were co-transcribed and found to encode MDH and cytochrome c_L (Nunn and Lidstrom, 1986b). A later study by Anderson and Lidstrom (1988) showed that two other genes were co-transcribed with *moxF* and *G* to form a four gene operon. These other genes were labelled *moxJ* and *I* and the order of transcription was *moxFJGI*. Using a coupled *in vivo* T7 RNA polymerase/promoter gene expression system this region was seen to encode four proteins of molecular weights 60 kDa (*moxF*), 30 kDa (*moxJ*), 20 kDa (*moxG*) and 12 kDa (*moxI*). The gene product of *moxI* was weakly cross-reactive with anti-MDH antibodies and it was suggested that this protein might be a component of a MDH enzyme complex. Sequencing of the *moxG* and *I* genes (Nunn and Anthony, 1988a,b; Nunn *et al.*, 1989) demonstrated that *moxI* encoded a second subunit of MDH (see Section 1.5.a). The entire *moxFJGI* region has now been

sequenced (Nunn and Anthony, 1988a,b; Nunn *et al.*, 1989; Anderson *et al.*, 1990), and has shown that each gene starts with a region coding for a signal sequence of between 22 and 27 amino acids indicating that the final destination for each gene product is the periplasm. Recently a MoxJ mutant was isolated following insertion of a Tn5 element into the *moxJ* gene (Lee *et al.*, 1991). This mutant lacked both the α-subunit of MDH and cytochrome c_L and it was suggested that the *moxJ* gene product may be involved in regulation. However, it is also possible that the lack of MDH and cytochrome c_L was due to a polar effect by the insertion on the expression of the *moxF* and *G* genes as reported by Nunn and Lidstrom (1986a).

Definite functions for the other genes could not be ascribed. Originally the *moxD* gene product was proposed to be involved in transport of proteins. This is because the mutant lacked MDH and cytochrome c_L but contained high levels of a 23 kDa cytochrome *c*. This cytochrome was thought to be a precursor of cytochrome c_L with the signal sequence still attached (Nunn and Lidstrom, 1986b). However, it has now been demonstrated that the 23 kDa cytochrome *c* (now called cytochrome c_{553}) is distinct from cytochrome c_L and is present at very low levels in wild-type bacteria (Day *et al.*, 1990). The authors suggested that cytochrome c_{553} is induced to high levels in the MoxD mutant in order to replace a function normally carried out by cytochrome c_L, but one not specifically involved in methanol oxidation. A cluster of three other genes were mapped about 2 kb from *moxI* (see Fig. 1.21). These genes, originally labelled *moxA1*, *A2* and *A3* but now referred to as *moxA*, *K* and *L* (Lidstrom, 1990), were not co-transcribed and had virtually identical phenotypes (Nunn and Lidstrom, 1986b). The mutants all produced MDH to near wild-type levels but the pure protein was inactive and had an altered absorption spectrum (Fig. 1.20). It was proposed that, due to the shifts in the PQQ region of the spectrum, the three genes were responsible either for PQQ biosynthesis/modification or for the processing of PQQ and apo-MDH to form active holo-enzyme. The first proposal was discounted because methylamine dehydrogenase (thought at the time to contain covalently bound PQQ) was fully active in all three mutants. However, it is now known that the prosthetic group of MNDH is not any form of PQQ, and so the PQQ biosynthesis/modification role for *moxA*, *K* and *L* remains. The remaining genes (*moxB*, *C*, *E* and *H*) were originally assigned regulatory functions, although it is now proposed that *moxC* and *H* are involved in either PQQ biosynthesis or transport (Table 1.3).

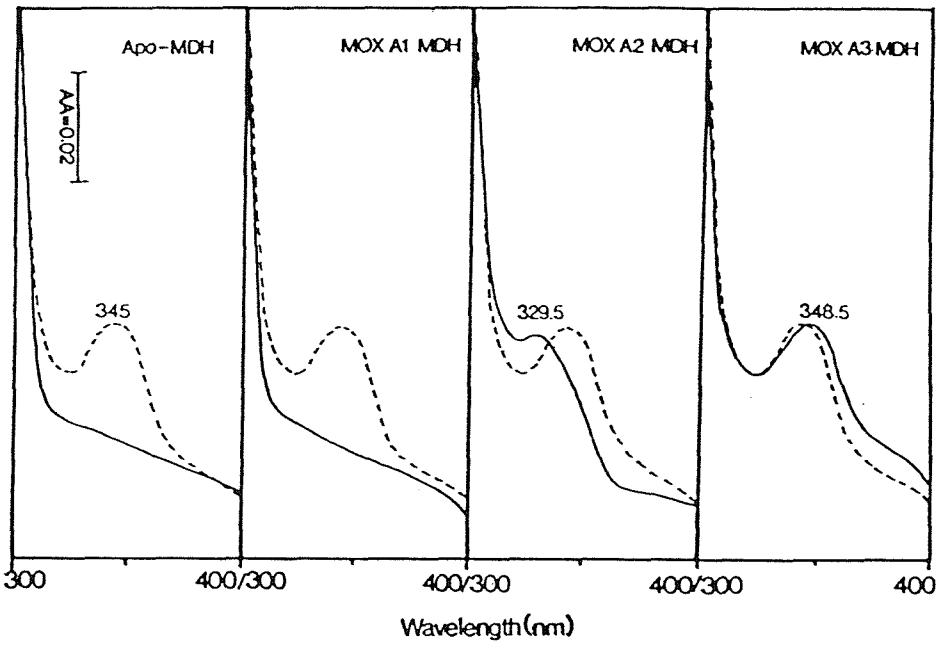


Fig. 1.20. Absorption spectra of the MDHs from the MoxA, K and L mutant strains of *Methylobacterium extorquens*

This figure is taken from Nunn and Lidstrom (1986b). The spectra of MDH (500 µg/ml) were recorded in 150 mM potassium phosphate buffer (pH 7.0). Apo-MDH was prepared using the method of Patel *et al.* (1978). The dashed line in each spectrum is the absorption spectrum of MDH from wild-type bacteria. MoxA1, A2 and A3 are now referred to as MoxA, K and L (Lidstrom, 1990).

Recently, cross-complementation of Mox mutants of *Mb. extorquens* and *Mb. organophilum* XX has resulted in the identification of ten more genes, bringing the total number of genes involved in methanol oxidation to 22 (Lidstrom, 1990; Lidstrom and Stirling, 1990; Lidstrom and Tsygankov, 1991). All of these genes and their possible functions are shown in Table 1.3. The genomic organisation of the genes (Fig. 1.21) and a model of their functions in methanol oxidation (Fig. 1.22) are also shown.

1.11. Regulation of the genes involved in methanol oxidation

The regulation of MDH and other enzymes involved in C_1 metabolism has been studied previously in a number of bacteria following transfer of cells grown on a non-C_1 compound (usually succinate) to medium containing methanol or methylamine (Dunstan *et al.*, 1972b; O'Connor and Hanson, 1977; McNerney and O'Connor, 1980; Weaver and Lidstrom, 1985; de Vries *et al.*, 1988; Ameyama *et al.*, 1988; Dawson *et al.*, 1990). Evidence has been presented that MDH is de-repressed when the cells are growing slowly (Roitsch and Stolp, 1986). However, growth rate alone could not be the sole determinant of MDH synthesis; *Mb. extorquens* and *Mb. organophilum* both grew at similar rates with methanol or succinate as sole carbon source, yet MDH activity increased markedly when cells were transferred from succinate to methanol medium (O'Connor and Hanson, 1977; McNerney and O'Connor, 1980). This effect was unlikely to be due to catabolite repression during growth on succinate; when cells were grown on medium containing both succinate and methanol, there was no evidence of diauxic growth and the activity of MDH was very similar to that from cells grown only on methanol (O'Connor, 1981). In general it appeared that a substantial level of MDH activity was attained following a lag of about 12 hours following the change in substrate. It has been suggested that the addition of PQQ to the growth medium of various organisms results in the decrease in this lag period, although it is not clear whether PQQ is directly affecting MDH regulation (Ameyama *et al.*, 1988).

Thus it would appear that methanol was responsible for the induction of MDH, and this theory is supported by the evidence obtained from an investigation into the regulation of methanol and methylamine dehydrogenases from *Mp. methylotrophus* (Dawson *et al.*, 1990). In these experiments, MDH was present in cells grown solely on methylamine but the specific activity was low compared with that in bacteria grown solely on methanol, suggesting that methanol was the inducer. It has been suggested, however, that in *Pa. denitrificans* the methanol first has to be metabolised to

Table 1.3. Proposed *mox* gene functions in *Methylobacterium extorquens*

This table is taken from Lidstrom and Tsygankov (1991) and is based on the work of Nunn and Lidstrom (1986a,b); Anderson and Lidstrom (1988); Machlin *et al.* (1988); Nunn and Anthony (1988a,b); Bastien *et al.* (1989); Biville *et al.* (1989); Nunn *et al.* (1989); Anderson *et al.* (1990); Day *et al.* (1990); Lidstrom (1990).

Gene designation	Encoded protein (kDa)	Proposed function
moxF	60	MDH α-subunit
moxJ	30	?
moxG	19	Cytochrome c_L
moxI	10	MDH β-subunit
moxA		PQQ/apo-MDH assembly or modification
moxK		PQQ/apo-MDH assembly or modification
moxL	19	PQQ/apo-MDH assembly or modification
moxB		Regulation
moxP		PQQ biosynthesis or transport
moxV		PQQ biosynthesis or transport
moxT		PQQ biosynthesis or transport
moxU		PQQ biosynthesis or transport
moxH		PQQ biosynthesis or transport
moxC		PQQ biosynthesis or transport
moxO		PQQ biosynthesis or transport
moxM		?
moxN		?
moxD		?
moxQ		?
moxE		?
moxR		?
moxS		?

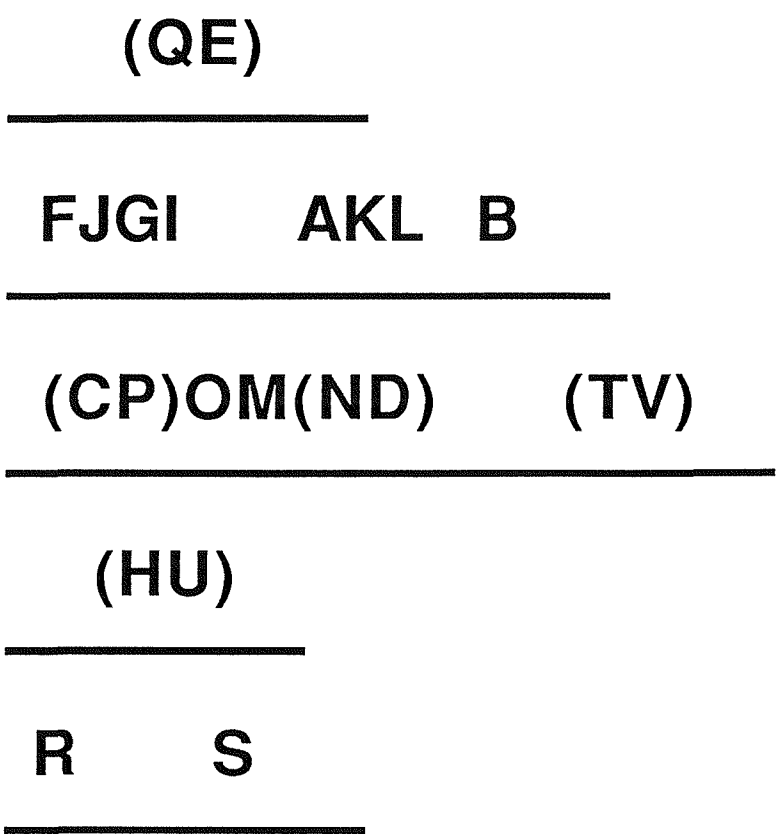


Fig. 1.21. Genomic organisation of the genes proposed to be involved in methanol oxidation

This figure is taken from Lidstrom and Tsygankov (1991) and is based on the work of Nunn and Lidstrom (1986a,b); Anderson and Lidstrom (1988); Machlin *et al.* (1988); Nunn and Anthony (1988a,b); Bastien *et al.* (1989); Biville *et al.* (1989); Nunn *et al.* (1989); Anderson *et al.* (1990); Day *et al.* (1990); Lidstrom (1990).
Parentheses indicate that the order of the enclosed genes is not known. The arrangement of the DNA fragments relative to each other is also unknown.

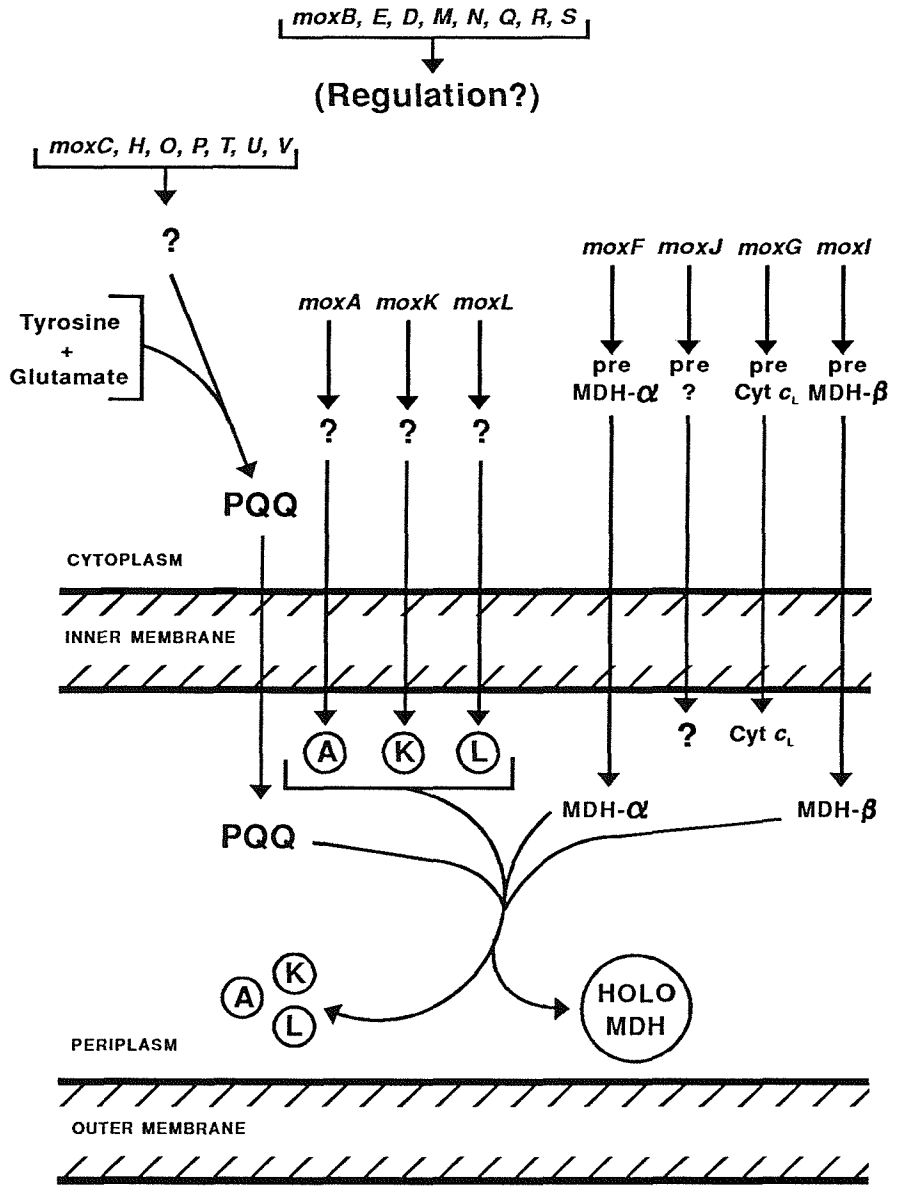


Fig. 1.22. A model for the proposed functions of methanol oxidation genes

This figure is based on the work of Nunn and Lidstrom (1986a,b); Anderson and Lidstrom (1988); Machlin *et al.* (1988); Nunn and Anthony (1988a,b); Bastien *et al.* (1989); Biville *et al.* (1989); Nunn *et al.* (1989); Anderson *et al.* (1990); Day *et al.* (1990); Lidstrom (1990). The function of MoxJ is unknown but it may be involved in some form of regulation in the periplasm (Lee *et al.*, 1991). There is no evidence that the *moxA, K* and *L* gene products are periplasmic, but this location is necessary if they are involved in PQQ-apo-MDH processing.

formaldehyde before any induction could occur (de Vries *et al.*, 1988). This is because the addition of methanol to cultures growing on a multi-carbon compound did not result in expression of MDH indicating that, in this organism at least, catabolite repression does occur. Such product-induction mechanisms have been previously observed in bacteria and are said to give more specificity to the induction process (Hayashi and Lin, 1965; Stanier and Ornston, 1973). In the case of methylotrophs, product-induction by formaldehyde could result in over expression of MDH and hence a high level of product. This situation is further complicated by the presence of the M-protein which regulates the activity of MDH by altering the enzymes affinity for formaldehyde and also the rate of reaction. Formaldehyde is very toxic to cells in high concentration and its use to induce a system that could possibly make more than could normally be coped with would create a hazard. Therefore the cells must have some other means of regulating MDH production besides product- induction. In the obligate methylotroph, *Mp. methylotrophus*, it was suggested that the methanol oxidase system as a whole was regulated by dissolved oxygen tension and/or growth rate; MDH activity was lower in cells grown with a methanol excess than when the methanol was exhausted (Greenwood and Jones, 1986; Jones *et al.*, 1987; Dawson *et al.*, 1990). The repression observed when cells were grown with excess methanol was unlikely to be due to methanol itself, but rather due to the accumulation of a product of methanol oxidation. In *Pa. denitrificans,* however, where the expression of MDH is under tight control by catabolite repression, tentative proposals that another regulating factor, which affects MDH synthesis and activity, may be PQQ biosynthesis, PQQ-apo-MDH processing or disproportionate expression of MDH subunits have been put forward, but without any evidence (Harms and van Spanning, 1991). In summary, it appears that the autotrophic methylotrophs differ from the facultative and obligate methylotrophs in their regulation of methanol oxidation although it is possible that they all rely on a product of methanol metabolism to act as an inducer of the methanol oxidase system.

1.11. The aims of the present work

From the above, it is evident that a large number of genes are involved, directly or indirectly, with the oxidation of methanol. Some of these genes are proposed to be involved in PQQ biosynthesis and/or transport. The induction of MDH is known to occur in response to C_1 compounds even in the presence of other growth substrates. The actual inducer of the system may be methanol itself but is more likely to be its

oxidative product, formaldehyde. It is not known whether the induction of MDH is co-regulated with the synthesis of PQQ.

Although the structural genes for MDH have been identified and sequenced from a number of methylotrophs, it is not known how the various components are brought together and assembled into active enzyme. Mutations in three separate, but closely linked, genes, *moxA*, *K* and *L*, all result in very similar phenotypes. Principally the MoxA, K and L mutants all produce wild-type levels of MDH but the protein is inactive in the dye-linked assay. The present work was initiated to answer the following questions:

(1) Are MoxC and MoxH PQQ^- mutants, and what is the nature of the MDH produced by these strains?

(2) Is the MDH synthesised by the MoxA, K and L mutants in the correct $\alpha_2\beta_2$ tetrameric configuration?

(3) If both α and β subunits are present, is there any difference in the arrangement of the subunits and the binding of the prosthetic group?

(4) Is the prosthetic group contained in the MDHs from these mutant strains authentic PQQ, or is it modified in some way?

(5) If the prosthetic group is authentic PQQ, then is it present in the correct stoichiometry of two molecules per molecule of MDH?

CHAPTER 2

Materials and Methods

2.1. Chemicals

All chemicals were of analytical grade and were obtained from either BDH Ltd., Poole, Dorset, or from Sigma Chemical Company Ltd., Poole, Dorset, except for those listed below:

Aldrich Chemical Company Ltd., Gillingham, Dorset, U.K.
Cyclobutanol
Cyclopropanemethanol
Cyclopropyl methyl ketone
1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (EDC)
Trifluoracetic anhydride
3,3',5,5'-tetramethylbenzidine

Bio-Rad Laboratories Ltd., Hemel Hempstead, U.K.
Bio-Gel HT Hydroxylapatite
Econo-Pac 10DG desalting column (P-6 column)

Difco Laboratories, Detroit, Michigan, U.S.A.
Bacto-Agar
Nutrient Agar
Nutrient Broth
Yeast extract

National Diagnostics, New Jersey, U.S.A.
Protogel (30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide)

Pharmacia LKB, Milton Keynes, U.K.
DEAE-Sepharose fast flow
Mono-Q HR 5/5 prepacked anion exchange column
Mono-S HR 5/5 prepacked cation exchange column

Sephacryl S-200

S-Sepharose fast flow

Superose-12 HR 10/30 prepacked gel filtration column

Pierce Europe B.V., The Netherlands.

Sulpho-*N*-hydroxysuccinimide (sulpho-NHS)

Serotec, Oxford, U.K.

Sheep anti-rabbit IgG,A,M-Horseradish peroxidase conjugate

2.2. Organisms

Methylobacterium extorquens AM1 wild-type (NCIMB 9133) and methanol oxidation mutants were gifts from Dr. D.N. Nunn (formerly of this department). The strains referred to in this work as wild-type, MoxA and MoxL are rifampicin resistant derivatives of the wild-type (NCIMB 9133), mutant PG1 (Tatra and Goodwin, 1983) and mutant M15A (Heptinstall and Quayle, 1970) respectively. Details of how these derivatives and the other mutant strains were obtained is described by Nunn and Lidstrom (1986a).

Escherichia coli (ATCC 10798) for the preparation of membrane bound glucose dehydrogenase used in a biological PQQ assay was obtained from the NCIMB (No. 12805).

Pseudomonas testosteroni (ATCC 15667) for the preparation of soluble alcohol dehydrogenase used in an alternative biological PQQ assay was a kind gift from Dr. F. Gasser (Pasteur Institute, France).

Paracoccus denitrificans (NCIMB 8944 Oxford strain) was obtained from Dr. A.R. Long, formerly of this department.

2.3. Maintenance of organisms

All *Mb. extorquens* strains were maintained on nutrient agar plates kept at 4°C or, for long-term storage, in nutrient broth with 50% glycerol (v/v) kept at -20°C.

E. coli (ATCC 10798) and *Ps. testosteroni* (ATCC 15667) were maintained on L-broth solidified with 1.5% (w/v) bacto-agar (Difco) kept at 4°C or, for long term storage, in L-broth with 50% glycerol (v/v) kept at -20°C.

Pa. denitrificans (NCIMB 8944 Oxf) was maintained on nutrient agar plates containing 0.2% (w/v) succinate and kept 4°C.

2.4. Growth and harvesting of cells

All *Mb. extorquens* strains were grown on the minimal salts medium of Meiberg and Harder (1978). This contained the following (per litre): $(NH_4)_2SO_4$, 3.0 g; $NaH_2PO_4.2H_2O$, 0.57 g; K_2HPO_4, 1.53 g; $MgSO_4.7H_2O$, 0.2 g and 0.5 ml of a trace element solution which contained the following ($mg.l^{-1}$): $CaCl_2.2H_2O$, 5300; $FeSO_4.7H_2O$, 2000; $MnSO_4.3H_2O$, 200; $ZnSO_4.7H_2O$, 200; $CuSO_4.5H_2O$, 40; $CoCl_2.6H_2O$, 40; Na_2MoO_4, 40; H_3BO_3, 30; 1M HCl, 10 ml. The final pH of this medium was pH 7.0.

The basic medium was sterilised by autoclaving at 121°C for 20 min for volumes up to 1 litre. Larger volumes (15 litres for use in fermenters) were sterilised initially by autoclaving as above followed by filtration into a sterilised vessel through an autoclavable cartridge with an absolute pore size of 0.22 µm. Wild-type cells were grown using 0.5% (v/v) methanol as carbon source which was filter sterilised and added to the medium after autoclaving. Mutant strains were grown using 0.4% (w/v) methylamine hydrochloride (added before autoclaving) as carbon source with 0.5% (v/v) methanol present to act as an inducer of the methanol-oxidising system.

All cultures were grown aerobically at 30°C in 15 litre batches using a 20 litre vessel in 1:1000 series fermenter (L.H. Fermentation, Stoke Poges, Bucks.). Smaller cultures acting as seed cultures for the fermenter were grown in baffled flasks at 30°C on an orbital shaker (L.H. Fermentation).

Cells were harvested at late log-phase using a "Sharples" continuous flow centrifuge (Sharples centrifuges, Camberley, Surrey) pre-cooled to 4°C. Cells were washed once with 20 mM Tris-HCl buffer (pH 8.0) and collected by centrifugation (6,000 g) for 10 min. Washed cells were frozen in liquid nitrogen and stored at -20°C.

For supplementation experiments, various amounts of a filter-sterilised aqueous solution of PQQ were added to standard minimal salts medium containing 0.5% (v/v) methanol as carbon source.

E. coli (ATCC 10798) was grown on the medium described by Geiger and Görisch (1987). This medium contained (per litre): D-glucose, 5 g; citrate, 2 g; K_2HPO_4, 10 g; $NaNH_4HPO_4$, 3.5 g. Cultures were grown overnight at 30°C on an orbital shaker and harvested by centrifugation (6,000 g) for 10 min. Cells were washed

once with distilled water and stored at -20°C. *E. coli* strains used in complementation analysis were grown on L-broth solidified with 1.5% (w/v) Bacto-agar (Difco).

Ps. testosteroni (ATCC 15667) was grown on the medium described by Groen *et al.* (1986). This medium contained (per litre): $K_2HPO_4.3H_2O$, 15.4 g; KH_2PO_4, 4.52 g; $MgCl_2.6H_2O$, 0.5 g; $(NH_4)_2SO_4$, 3 g; $CaCl_2$, 15 mg; $FeSO_4.7H_2O$, 15 mg and 1 ml of a trace element solution containing ($mg.l^{-1}$): $ZnSO_4.7H_2O$, 70; H_3BO_3, 10; $MnSO_4.4H_2O$, 10; $NaMoO_4$, 10; $CuSO_4.5H_2O$, 7.8. The final pH of this medium was pH 7.0. Filter sterilised ethanol (0.3% (v/v) final concentration) was used as carbon source and added after the medium was autoclaved at 121°C for 20 min. Cells were harvested by centrifugation (6,000 g) for 10 min, washed once with 20 mM potassium phosphate buffer (pH 7.0) and stored at -70°C.

Pa. denitrificans (NCIMB 8944 Oxf) was grown on the medium described by Kornberg and Morris (1965). This medium contained (per litre): K_2HPO_4, 6 g; KH_2PO_4, 4 g; NH_4Cl, 1.6 g; $NaHCO_3$, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.04 g; Fe^{2+}EDTA, 0.12 g; Hoagland's trace elements solution, 0.1 ml; Yeast extract (Difco), 0.1 g. Filter sterilised methanol (0.5% v/v) was added after autoclaving. The final pH of this medium was pH 7.0. Starter cultures (10 ml) were grown on nutrient broth supplemented with 0.2% (w/v) succinate before transfer into the methanol minimal medium as a 10% inoculum. Cells were harvested by centrifugation (6,000 g) for 10 min, washed once with 10 mM Hepes buffer (pH 7.3) containing 150 mM NaCl, resuspended in the same buffer and used for the preparation of spheroplasts (Section 2.9).

When plates were required, all minimal salts media were solidified by the addition of 1.5% (w/v) Bacto-agar (Difco).

2.5. Complementation of mutant strains

In order to check the authenticity of the mutant strains, complementation using a tri-parental mating method was used essentially as described by Nunn and Lidstrom (1986a). The *E. coli* strain carrying the mobilising plasmid pRK2013 was a gift from Dr. D.N. Nunn. *E. coli* clones carrying specific fragments of *Mb. extorquens* DNA were a gift from Dr. P.M. Goodwin (Wellcome Trust, London). Table 2.1 shows the complementing abilities of these clones. Fresh cultures of all the bacteria were used in the following manner. Onto a nutrient agar (Difco) plate was placed a spot of each of *Mb. extorquens* mutant strain, *E. coli* (pRK2013) and an *E. coli* clone (each mutant

was tested with each clone) in an approximate 5:1:1 ratio. These were mixed thoroughly and the plate incubated overnight at 30°C. After incubation, samples of the mating mixture were streaked onto minimal salts medium agar plates containing either 0.5% (v/v) methanol or 0.2% (w/v) succinate together with rifampicin (20 $\mu g.ml^{-1}$) and tetracycline (40 $\mu g.ml^{-1}$), and then incubated at 30°C for 5 to 7 days. The antibiotics were added to inhibit the growth of *E. coli* and any un-complemented *M. extorquens* cells. All mutant strains of *Mb. extorquens* should grow on succinate but should only grow on methanol when successfully complemented.

2.6. Preparation of cell-free extracts of *Methylobacterium extorquens*

Frozen cells were thawed and resuspended in 20 mM Tris-HCl (pH 8.0) to a density of 1 g(wet weight).ml^{-1} and disrupted by sonication for 10 cycles at full power (30 sec on, 30 sec cooling) using a MSE Soniprep 150 in a salt/ice bath. Whole cells and debris were removed by centrifugation (10,000 g) for 10 min at 4°C. Crude extract was further centrifuged (130,000 g) for 2 hours at 4°C to fractionate membranes and soluble proteins. Supernatant (containing soluble proteins) was used directly for the purification of methanol dehydrogenase. The membrane fraction was resuspended in 20 mM Tris-HCl buffer (pH 8.0), frozen in liquid nitrogen and stored at -20°C.

2.7. Spheroplasting of *Paracoccus denitrificans*

The preparation of 'Type II' spheroplasts was carried out essentially as described by Alefounder and Ferguson (1981). The method described below is for 1 litre of original culture; larger volumes of culture were treated using the same proportion of materials.

Aerobic, methanol-grown cells were harvested at late log phase and resuspended in 250 ml of 10 mM Hepes buffer (pH 7.3) containing 150 mM NaCl. The cells were pelleted by centrifugation (10,000 g for 10 min) and resuspended in 50 ml of 200 mM Hepes buffer (pH 7.3) containing 500 mM sucrose, 0.5 mM Na_2EDTA and 25 mM methanol at room temperature. Lysozyme (50 mg) dissolved in 0.5 ml distilled water was added followed by 50 ml distilled water containing 25 mM methanol to create a mild osmotic shock. The suspension was then shaken gently for 40 min at 30°C. After incubation the spheroplasts were harvested by centrifugation (5,000 g for 10 min) leaving a supernatant comprising the periplasmic fraction. The pelleted spheroplasts were then either lysed by resuspension in 50 ml 20 mM Hepes

Table 2.1. Complementing abilities of the clones used in the complementation of the mutant strains of *Methylobacterium extorquens*

Clone label	***mox* gene(s) complemented**
pVK102	none (negative control)
Clone bank	all (positive control)
pLH1	*moxA*
A1-2-A3-B	*moxA*, *K*, *L* and *B*
A1-A2-A3	*moxA*, *K* and *L*
A3-B	*moxL* and *B*
C-D	*moxC* and *D*
C	*moxC*
D	*moxD*
E	*moxE*
F-G	*moxF*, *J*, *G*, and *I*
H1	*moxH*
H2	*moxH*

buffer (pH 7.3) to yield the cytoplasmic fraction or very gently resuspended in growth medium containing 0.5% (v/v) methanol and 500 mM sucrose (to maintain osmotic balance and hence prevent lysis). Intact spheroplasts were then used to follow transport of MDH across the cytoplasmic membrane.

2.8. Purification of MDH

MDH was purified using a modification of the method described by Day and Anthony (1990a). The soluble protein fraction from 195 g (wet weight) of cells in 20 mM Tris-HCl buffer (pH 8.0) was applied at a flow rate of 2 $ml.min^{-1}$ to a column (24 x 140 mm) of DEAE-Sepharose (Pharmacia) previously equilibrated in the same Tris-HCl buffer. MDH does not bind to this column and was washed off with the Tris-HCl buffer. Non-binding fractions were pooled, dialysed against 20 mM potassium phosphate buffer (pH 7.0) and then applied to a column (24 x 100 mm) of hydroxylapatite (HAP) (Bio-Rad bio-gel HT) equilibrated with the same phosphate buffer. MDH was eluted at about 250 mM phosphate using a linear gradient of 20-300 mM phosphate (pH 7.0) in a total volume of 600 ml. Fractions containing MDH were pooled, dialysed against 10 mM Mes buffer (pH 5.5) and loaded onto a column (24 x 95 mm) of S-Sepharose (Pharmacia) equilibrated with the same Mes buffer. MDH was eluted at about 100 mM NaCl using a linear gradient of 0-250 mM NaCl in the Mes buffer in a total volume of 500 ml. MDH fractions were pooled, concentrated using a YM-30 membrane (Amicon) to a volume of about 10 ml and loaded onto a column (50 x 800 mm) of Sephacryl S-200 (Pharmacia) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM NaCl using an upward flow rate of 10 $ml.h^{-1}$. The pure MDH obtained after the last step was concentrated using a YM-30 membrane to about 20 $mg.ml^{-1}$, frozen in liquid nitrogen and stored at -20°C.

2.9. Purification of cytochrome c_L

Cytochrome c_L was purified as described by Day and Anthony (1990b). The cytochrome c_L in the above soluble protein fraction remained bound to the DEAE-Sepharose column and was eluted at about 100 mM NaCl using a linear gradient of 0-250 mM NaCl in 20 mM Tris-HCl buffer (pH 8.0) in a total volume of 500 ml. The cytochrome fractions were pooled, concentrated using a PM-10 membrane (Amicon) to about 5 ml and loaded onto a column (50 x 800 mm) of Sephadex G-75 (Pharmacia) equilibrated in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl

using an upward flow rate of 10 ml.h^{-1}. Cytochrome fractions were pooled and concentrated as above and the buffer exchanged into 20 mM Mops buffer (pH 7.0) using a prepacked 10DG desalting column (P-6 column; Bio-Rad). Solid ammonium sulphate (enzyme grade) was then added to a final concentration of 1.7 M; any precipitate that formed was removed by centrifugation and discarded. The cytochrome was then applied to a 1 ml FPLC phenyl superose column (Pharmacia) equilibrated with 20 mM Mops buffer (pH 7.0) containing 1.7 M ammonium sulphate using a flow rate of 0.5 ml.min^{-1}. The ammonium sulphate concentration was decreased stepwise with the cytochrome eluting at 45 mM ammonium sulphate. Any remaining ammonium sulphate in the cytochrome was removed using a P-6 column equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The cytochrome was then loaded onto an 8 ml FPLC Mono-Q column (Pharmacia) equilibrated with the same Tris-HCl buffer. Pure cytochrome c_L was eluted at 100 mM NaCl using a linear gradient of 0-300 mM in the same Tris-HCl buffer in a total volume of 30 ml, frozen in liquid nitrogen and stored at -20°C.

2.10. Purification of methylamine dehydrogenase

Methylamine dehydrogenase (MNDH) was purified using essentially the same method as for methanol dehydrogenase (Section 2.8), but without using the HAP column. MNDH remained bound to the DEAE-Sepharose column and was eluted with 90 mM NaCl in a linear gradient of 0-250 mM NaCl in 20 mM Tris-HCl buffer (pH 8.0). Active fractions were pooled, dialysed against 10 mM Mes buffer (pH 5.5) and then loaded onto the S-Sepharose column previously equilibrated in the same Mes buffer. MNDH was eluted with about 150 mM NaCl in a linear gradient of 0-250 mM NaCl in the same MES buffer. Active fractions were pooled, concentrated using a YM-30 membrane to about 10 ml and loaded onto a column (50 x 800 mm) of Sephacryl S-200 (Pharmacia) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM NaCl using an upward flow rate of 10 ml.h^{-1}. The pure MNDH from this step was concentrated to about 15 mg.ml^{-1}, frozen in liquid nitrogen and stored at -20°C.

2.11. Measurement of MDH

2.11.a. Dye-linked assay system

Methanol dehydrogenase was assayed using the dye-linked assay system based on that of Anthony and Zatman (1967a,b) and a modification of Bamforth and Quayle (1978a,b). Activity was measured at pH 9.0 in 100 mM Tris-HCl buffer containing 15 mM NH_4Cl, 5 mM methanol, 87 µM dichlorophenolindophenol (PIP) and enzyme in a volume of 1 ml at room temperature; the reaction was started by addition of phenazine ethosulphate (PES; 0.5 mM final concentration). The reduction rate was followed by the decrease in absorbance at 600 nm of PIP ($\mathscr{E}$= 21,500 $M^{-1}.cm^{-1}$).

2.11.b. Cytochrome *c*-linked assay system

This method was based on that described by Day and Anthony (1990a) in which the reduction of horse heart cytochrome *c* was measured in the presence of a small amount of purified cytochrome c_L. The reaction mixture contained, in a 0.5 ml volume in a spectrophotometer cuvette (1 cm light path), 12 mM Mops buffer (pH 7.0), 5 mM NH_4Cl, 5 mM methanol, 2 µM cytochrome c_L and 50 µM oxidised horse heart cytochrome *c*. Reactions were started by addition of MDH and the rate of reduction of cytochrome *c* was measured at 550 nm ($\mathscr{E}$= 19,500 $M^{-1}.cm^{-1}$).

2.12. Measurement of methylamine dehydrogenase

MNDH was assayed using the method described by Eady and Large (1969) with phenazine methosulphate (PMS) replaced by PES. Activity was measured at pH 7.5 in 66.7 mM potassium phosphate buffer containing 0.3 mM PES, 0.05 mM PIP and enzyme in a volume of 1 ml at room temperature; the reaction was started by the addition of methylamine hydrochloride to a final concentration of 6.7 mM. The reduction rate was followed by the decrease in absorbance at 600 nm.

Active fractions eluting from columns during the purification of MNDH were detected using a similar micro-titre plate adaptation to that described for MDH (Section 2.14.a).

2.13. Production of anti-MDH antiserum

Holo-MDH from wild-type cells was purified as described in Section 2.8. Antibody was raised by immunising a New Zealand White rabbit with pure MDH

(400 µg) emulsified in 1 ml of Freunds complete adjuvant. A second, booster injection of MDH (400 µg) emulsified in 1 ml Freunds incomplete adjuvant was administered 2 weeks later. Blood was collected from the ear and whole serum was prepared. This serum was serially diluted to find the highest concentration that did not give appreciable background staining when used to probe a Western blot of a crude extract of wild-type cells. The antiserum raised against MDH from *Mb. extorquens* wild-type cells was cross-reactive with the MDH from certain mutant strains of *Mb. extorquens* but did not cross-react with MDH from *Pa. denitrificans*, *Acetobacter methanolicus* strain MB58 or *Methylophilus methylotrophus* strain AS1.

2.14. Detection of MDH

2.14.a. Microtitre dye-linked assay

The Bamforth and Quayle (1978a,b) adaptation of the MDH dye-linked assay (Section 2.11.a) was used with the following modification. Sample (10 µl) was placed in a well of a microtitre plate followed by the addition of 200 µl of the complete assay reagent mixture. Reduction of the PIP, resulting in a colour change from green to yellow, indicated active MDH was present in the fraction.

2.14.b. 'Dot-blotting'

This method was used for the detection of inactive MDH as found in certain mutant strains of *Mb. extorquens*. Antiserum was used at a 500 fold dilution. Proteins samples (5 µl) were applied to a nitrocellulose filter in as small a dot as possible. The filter was shaken gently overnight with blocking mixture (20 mM Tris-HCl pH 7.4 containing 150 mM NaCl, 5% (w/v) low fat dried skimmed milk and 0.02% (w/v) sodium azide) to block any free binding sites on the nitrocellulose. The blocking mixture was then poured off and the primary antibody (rabbit anti-MDH Ig raised against holo-MDH from wild-type cells) in blocking mixture with 0.05% (v/v) Tween-20 added and shaken gently for 6 h. The primary antibody was poured off and the nitrocellulose washed three times with 50 ml TBS (20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl) plus 0.05% (v/v) Tween-20. The secondary antibody (sheep anti-rabbit IgG,A,M conjugated to horseradish peroxidase; Serotec) in blocking mixture plus 0.05% (v/v) Tween-20 was added and the nitrocellulose shaken gently for 2 h. The secondary antibody was poured away and the nitrocellulose was

washed three times with TBS plus 0.05% (v/v) Tween-20 and then washed three times with TBS. The nitrocellulose was then incubated in TBS for 15 min. After this incubation, substrate (10 mg of 4-chloro-1-naphthol dissolved in 2 ml methanol mixed with 20 ml TBS plus 50 µl hydrogen peroxide) was added and the blot developed.

2.14.c. Polyacrylamide gel electrophoresis

The discontinuous Tris-HCl/glycine system of Laemmli (1970) was used routinely. Gels (12% or 15%), with a 3% stacking gel were cast using the mini-protean II system from Bio-Rad laboratories and were run at 20 mA constant current for about 1 h. Proteins were stained with Coomassie brilliant blue R250, as described by Weber and Osborn (1975). Gels were stained for haem proteins with 3,3',5,5'-tetramethyl-benzidine (TMBZ) by the method of Thomas *et al.* (1976). Dalton Mark VII standard proteins (Sigma) were used as molecular weight markers.

Non-denaturing PAGE was performed using the high pH (pH 8.9) discontinuous system described by Goldenberg (1989). A 5.0% running gel was used with a 2.5% stacking gel. Proteins were stained with Coomassie brilliant blue (see above). Alternatively, gels were stained using a method to detect activity (Kohler and Schwartz, 1982); Gels were equilibrated in 20 mM potassium phosphate buffer (pH 7.0) containing nitroblue tetrazolium (1.5 $mg.ml^{-1}$) for 15 min. and then incubated in the same phosphate buffer containing PES (1.5 $mg.ml^{-1}$) and 10 mM methanol. Active bands were visible after about 5 min as blue-black precipitates of formazan against a clear yellow background.

2.15. Western blotting

Proteins for immunoblotting were separated by SDS-PAGE using the mini-gel system described in section 2.14.c. After electrophoresis, the proteins were transferred onto nitrocellulose (0.2 µm, Schleicher and Schnell, Dassel, W. Germany) using the mini Trans-Blot system (Bio-Rad). Gels and nitrocellulose were presoaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol; pH 8.3) for 15 min prior to blotting. After assembly of the transfer cassette, blotting was carried out at 100 V, 250 mA for 30 min.

After electroblotting the nitrocellulose was treated exactly as described in section 2.14.b.

2.16. Protein estimation

Protein concentrations were estimated using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985) adapted for microtitre plates (Redinbaugh and Turley, 1986) and read on a Dynatech MR580 micro-ELISA auto reader . Bovine serum albumin fraction V (Sigma) was used as standard in the range 0-1.5 $mg.ml^{-1}$.

2.17. Spectrophotometric measurements

2.17.a. Absorption spectroscopy

Absorption spectra were recorded using either a SLM-AMINCO DW-2000 UV-VIS spectrophotometer or a Shimadzu UV-3000 dual wavelength/double beam spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Unless otherwise indicated, all spectra were recorded at a scan speed of 100 $nm.min^{-1}$ with a 2 nm spectral band width and a 10 mm light path.

Low temperature spectra were recorded on a SLM-AMINCO DW-2000 UV-VIS spectrophotometer using the manufacturer's purpose-built accessory. Samples were stabilised by the addition of 40% sucrose (w/v) prior to freezing with liquid nitrogen. In all cases, the light path was 2 mm and spectra were recorded at a scan rate of 1 $nm.sec^{-1}$ using a spectral band width of 2 nm.

2.17.b. Fluorescence spectroscopy

Fluorescence spectra were recorded on Perkin-Elmer LS-3B fluorescence spectrophotometer at a scan speed of 120 $nm.min^{-1}$ with fixed excitation and emission slits of 10 nm nominal bandpass. The sensitivity of this machine can be adjusted manually by changing the expansion factor in the range 0.001 to 999.9, the latter value being the most sensitive. The actual expansion factors used for fluorescence measurements or recording spectra are given in the appropriate figure legends.

2.17.c. Electron spin resonance spectroscopy

Electron spin resonance (ESR) spectra were recorded on a Bruker ESP 300 *X* band ESR spectrometer with the help of Dr. David Lowe at The University of Sussex. Spectra were recorded at 120°K using the following parameters: time constant, 81.92 ms; modulation amplitude, 4.166 G; receiver gain, 2×10^4 s; microwave power, 2×10^{-2} mW; centre field, 3340 G; sweep width, 50 G; microwave frequency,

9.37 GHz.

The instrument was calibrated using a diphenyl picryl hydrazyl standard (G value = 2.034). Concentration was estimated by comparison of the double integral of the signal to the double integral of the signal from a 0.18 µM Cu^{2+}-EDTA standard.

2.17.d. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a JASCO model 20 dual function circular dichroism/optical rotation spectrophotometer with the help of Dr. Peter Cary at Portsmouth Polytechnic. Spectra were recorded in 5 mM potassium phosphate buffer (pH 7.0) at a scan rate of 2 nm/min with a spectra band width of 1 nm and a 2 mm path length.

2.18. Determination of PQQ

PQQ was determined using two reconstitutable quinoproteins that are synthesised by the bacteria concerned in the apo-form.

2.18.a. Membrane bound apo-glucose dehydrogenase from *E. coli*

This system was used as described by Geiger and Görisch (1987). An overnight culture of *E. coli* ATCC 10798 was used for the preparation of the membrane fraction. Cells were resuspended in 5 mM potassium phosphate buffer (pH 7.0) containing DNase and RNase (10 $\mu g.ml^{-1}$) and passed twice through a French pressure cell at 20,000 psi. Whole cells and debris were removed by centrifugation (10,000 g) for 10 min. The supernatant from this step was further centrifuged (110,000 g) for 60 min to fractionate membranes. The membrane fraction (containing apo-glucose dehydrogenase) was washed with Analar water, resuspended in Analar water and stored at -70°C. Membranes were used at a concentration of 1 $mg.ml^{-1}$ protein and reconstitution was for 10 minutes at room temperature in a mixture containing 25 mM potassium phosphate buffer (pH 6.5) and 1.25 mM $MgSO_4$. PQQ for the standard curve was a gift from Fluka Chemicals and was used in the range 0-25 $ng.ml^{-1}$ (in the reconstitution mixture). Reconstitution mixture (0.1 ml) was used in an assay containing (in a total volume of 1 ml) 16.7 mM Tris-HCl buffer (pH 8.75), 0.67 mM PES, 0.1 mM PIP and 4 mM sodium azide. The reaction was started by the addition of 33 µmol D-glucose and activity was measured as a decrease in absorbance at 600 nm.

2.18.b. Apo-alcohol dehydrogenase from *Pseudomonas testosteroni*

The use of this system was first described by Groen *et al.* (1986). A later method, adopted here, using an abbreviated purification protocol is described by Groen and Duine (1990) and van der Meer *et al.* (1990b).

In this system, crude extract was prepared from 25 g of ethanol-grown cells suspended in 20 ml of 100 mM Tris-HCl buffer (pH 7.5) containing 3 mM $CaCl_2$ (buffer A) by 2 passes through a French pressure cell at 20,000 psi. Centrifugation (100,000 g) for 2 hours resulted in about 30 ml of pink supernatant free from debris and membranes. The supernatant was then diluted 10 fold with buffer A and applied to a CM-cellulose column (50 x 100 mm) equilibrated with the same buffer. The column was washed with two column volumes of buffer A and then a linear gradient of 0-300 mM NaCl in buffer A in a total volume of 400 ml was applied. The enzyme eluted with approximately 150 mM NaCl and active fractions were pooled and dialysed overnight against 20 mM Mes buffer (pH 5.5) (buffer B). Dialysate was concentrated over a YM30 membrane (Amicon) and then applied to a mono-S column (Pharmacia) equilibrated with buffer B. Pure enzyme was eluted with 250 mM NaCl in a linear gradient of 0-500 mM NaCl in buffer B in a total volume of 25 ml, dialysed against 20 mM Tris-HCl buffer (pH 7.5) containing 3 mM $CaCl_2$, concentrated and stored in small aliquots at -20°C.

For the determination of PQQ, 20 µl of a 1 µM solution of ADH was mixed with either a PQQ standard or unknown sample to a volume of 200 µl and left to reconstitute for 10 min at 20°C. After incubation, 725 µl of buffer A and 50 µl of 2 mM Wurster's Blue were added followed by 25 µl of 5 mM butan-1-ol to start the reaction. Reduction of Wurster's Blue was followed by the decrease in absorbance at 600 nm. This assay is very sensitive allowing the detection of 1 nM PQQ in a sample. In addition, the reconstitution response of ADH is linear over several decades.

2.19. Separation of the components of MDH by gel filtration in the presence of SDS

Complete dissociation of MDH was carried out as described by Nunn *et al.* (1989). Pure enzyme was concentrated to about 60 µl containing about 2 mg protein using a Centricon-30 microconcentrator (Amicon). This was incubated at 80°C for 30 min in the presence of 2.0% (w/v) SDS followed by gel filtration on a Superose-12 column equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl

and 0.1% (w/v) SDS at a flow rate of 0.3 ml.min^{-1}. Elution of the components was monitored by absorbance at 280 nm.

2.20. Dissociation of MDH

2.20.a. Dissociation using low pH

The dissociation of MDH using low pH was carried out as described by Anthony and Zatman (1967b). Purified enzyme (3 mg) was dissolved in 3 ml of deionized water and the pH carefully lowered to a value between 2.0 and 6.0 using 0.01 M HCl. After incubation at room temperature for 40 min the pH was raised to 7.5 by the addition of 450 µl of 500 mM potassium phosphate buffer (pH 7.5) and the volume made up to 4.5 ml. Total fluorescence measurements using 3.0 ml of this solution were made at two wavelength pairs: 282 nm excitation/340 nm emission (to detect fluorescence due to tryptophan; Schmid, 1989) and 365 nm excitation/470 nm emission (to detect fluorescence due to PQQ; Anthony and Zatman, 1967b). For the enzyme from wild-type bacteria, a sample was removed and used in the standard dye-linked assay. Measurement of the activity of the MDHs from the mutant strains was not possible.

To measure dissociation of MDH at low pH values as a function of time, the pH of a purified enzyme solution (2 mg in 40 ml of deionized water) was lowered to pH 2.6 or pH 3.1 as rapidly as possible using 0.01 M HCl. At suitable time intervals 2 ml samples were removed and mixed with potassium phosphate buffer (pH 7.5; final concentration 50 mM) and made up to 3.0 ml with deionized water. Fluorescence and dye-linked activity measurements were made as described above.

2.20.b. Dissociation using the chaotropic agents urea and guanidinium chloride

The method was based on a general protocol described by Jaenicke and Rudolph (1989). Pure MDH (50 µg) in 100 mM potassium phosphate buffer (pH 7.5) was incubated in the presence of various concentrations of urea (0-8 M) or guanidinium chloride (0-6 M) at 25°C for 30 min. The final volume for each of the mixtures was 500 µl. When using MDH from wild-type cells, 100 µl of the incubation mixture was removed for use in the dye-linked assay following incubation. The remainder was diluted 10 fold by the addition of 3.6 ml of the above phosphate buffer and used for fluorescence measurements. When using MDH from the mutant strains,

the activity assay was not performed and the samples were diluted 10 fold following incubation by the addition of 4.5 ml of 100 mM potassium phosphate buffer (pH 7.5). Total fluorescence measurements were made at two wavelength pairs: 282 nm excitation/340 nm emission (to detect fluorescence due to tryptophan; Schmid, 1989) and 365 nm excitation/470 nm emission (to detect fluorescence due to PQQ; Anthony and Zatman, 1967b). In order to detect PQQ fluorescence effectively, the sensitivity of the fluorometer was increased 100 fold for the second wavelength pair. In addition to the fluorescence measurements at these wavelength pairs, emission spectra at 282 nm excitation and 365 nm excitation were recorded.

To measure the dissociation of MDH by chaotropic agents as a function of time, purified MDH (4 mg) in a final volume of 40 ml of potassium phosphate buffer (pH 7.5) was incubated with denaturant at 20°C. Urea was used at final concentrations of 5 and 7 M, guanidinium chloride was used at final concentrations of 2 and 3 M; in all cases the denaturant was the final reagent to be added. At regular intervals 3.0 ml of the solution was removed and used for fluorescence and activity measurements.

2.21. Extraction of PQQ for analysis by reverse phase HPLC

The extraction method used was essentially that described by Duine *et al.* (1981), van der Meer *et al.* (1990b). MDH (25 µl of a 10 $mg.ml^{-1}$ solution) in 20 mM potassium phosphate buffer (pH 7.0) was mixed with an equal volume of 1 M KH_2PO_4 acidified to pH 1.0 with HCl. To this was added 100 µl of methanol and the sample was centrifuged (12,000 g) for 5 min to remove precipitated protein. The supernatant was filtered using a 0.22 µm Ultrafree-MC filter unit (Millipore) and 100 µl loaded onto a Novapak C_{18} column (Waters). The column was run isocratically using methanol/water/85% H_3PO_4 (30/69.6/0.4 % v/v/v) as the solvent with a flow rate of 1 $ml.min^{-1}$. Eluent was monitored by absorbance detection at 313 nm (Applied Biosystems model 1783A UV detector) and fluorescence detection (Applied Biosystems model 980; excitation at 365 nm, total emission at over 418 nm).

Identification of the peaks was achieved by performing identical runs with authentic PQQ and $PQQH_2$. $PQQH_2$ was obtained by the reduction of PQQ with phenylhydrazine as described by Duine *et al.* (1981). To quantify the amount of PQQ present in the samples, dilutions of a known concentration of PQQ dissolved in the above phosphate buffer were treated as above. The peak due to the quinone form (in both UV and fluorescence traces) for each dilution was integrated to establish a

standard curve of peak area against PQQ concentration (Fig. 2.1). The peak due to quinone in the samples was also integrated and the amount of PQQ estimated by comparison to the standard curve. It was not possible to directly quantify the amount of quinol present since the prepared $PQQH_2$ had some remaining unreduced PQQ; instead the total amount of PQQ was determined by oxidising the extract (so that all PQQ was in the quinone form) and then calculating the percentage of quinol present in the enzyme sample. Oxidation of $PQQH_2$ to PQQ was achieved by adding 5 µl of 2.6 mM PIP (an excess) to the enzyme extract before loading onto the column.

2.22. Preparation of Wurster's Blue

Wurster's Blue (a one electron-accepting free radical) was prepared from *N,N,N',N'*-tetramethyl-*p*-phenylenediamine hydrochloride (TMPD) as described by Michaelis and Granick (1944). TMPD (1 g) was dissolved in 17.5 ml distilled water. To this was added 28.75 ml of methanol and 12.5 g of sodium perchlorate. The solution was cooled in a salt/ice bath and 34 ml of 0.125 N aqueous bromine solution was added slowly with gentle stirring. The solution turned dark blue rapidly and, after addition of the bromine solution, was filtered using fluted Whatman No. 1 filter paper. The precipitate was washed with about 6 x 20 ml volumes of ice-cold methanol, followed by 3 x 50 ml volumes of dry ether. This protocol yielded about 0.9 g of metallic brown crystals which were stored at room temperature in a foil-covered bottle. The calculated molecular weight of this compound is 263.7 using the formula $(CH_3)_2NC_6H_4N(CH_3)_2ClO_4$. Using extinction coefficients of 12,700 $mM^{-1}.cm^{-1}$ (612 nm), 1,770 $mM^{-1}.cm^{-1}$ (646 nm) and 1,072 $M^{-1}.cm^{-1}$ (652 nm; Frank *et al.*, 1988), the estimated molecular weight was 262.3.

2.23. Oxidation of MDH

Oxidation of MDH was attempted using the method of Duine and Frank (1980a). This method relies on the presence of KCN to protect the enzyme whilst oxidation is achieved by the electron acceptor PES/PIP. Enzyme (10 nmol) in a total volume of 1 ml of 100 mM tetrasodium pyrophosphate buffer (pH 9.0) or 100 mM sodium borate buffer (pH 9.0) containing 50 mM NH_4Cl, 10 mM KCN and 100 µM PIP was mixed with PES at a final concentration of 1 mM. The mixture was passed down a P-6 desalting column (Bio-Rad) equilibrated in the pyrophosphate or

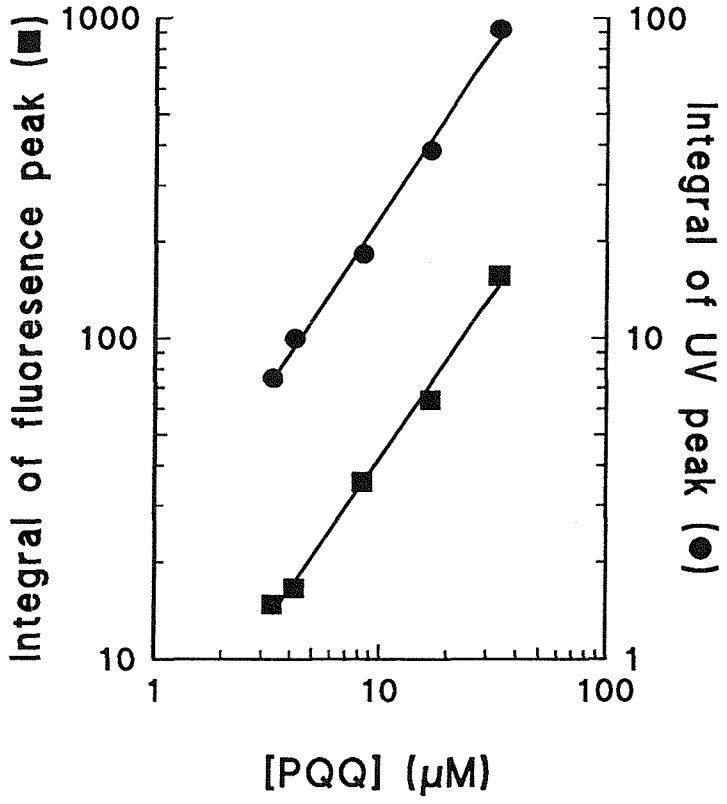


Fig. 2.1. Standard curve for the determination of PQQ using reverse phase HPLC

PQQ solutions of known concentration were mixed with 1 M KH_2PO_4 and methanol as described in Section 2.21. After centrifugation and filtration, 100 µl of each standard PQQ solution was loaded onto a Novapak C_{18} column and the eluent monitored by UV and fluorescence detectors. The area of the peak due to the quinone form of PQQ in both UV and fluorescence elution profiles was integrated and used to establish this standard curve.

borate buffer, to remove low molecular weight compounds, and an absorption spectrum recorded.

Another method, described by Dijkstra *et al.* (1984), was also used. Purified MDH (10 nmol) in 1 ml of 100 mM tetrasodium pyrophosphate buffer (pH 9.0) or 100mM sodium borate buffer (pH 9.0) containing 20 mM NH_4Cl (activator) was mixed with 5 µl of 200 mM KCN (pH 9.0). After the addition of Wurster's Blue (equimolar with the active site concentration of MDH), endogenous substrate and substrate present in the buffer was oxidised by adding 0.1 µl aliquots of 100 mM $K_3Fe(CN)_6$ until the blue colour persisted. Typically 0.5 µl of ferricyanide was sufficient to restore the blue colour. After oxidation, the solution was passed down a P-6 column equilibrated in the pyrophosphate or borate buffer and an absorption spectrum recorded.

2.24. Preparation of cyclopropanol

I am indebted to Neville Wright for his considerable help, fortitude and time in the hazardous preparation of cyclopropanol.

Cyclopropyl acetate was prepared from cyclopropyl methyl ketone using essentially the method described by Emmons and Lucas (1955). Due to the unavailability of 90% (v/v) hydrogen peroxide, a greater proportion of 60% (v/v) hydrogen peroxide was used in order to keep the ratio of peroxide to trifluoracetic anhydride constant. Unfortunately, this resulted in the presence of a greater proportion of water in the reaction mixture, the consequence of which was a lower yield of the cyclopropyl acetate.

Cyclopropanol was prepared from cyclopropyl acetate by the enzymic hydrolysis of the acetate group using porcine liver esterase (Sigma) as described by Jongejan and Duine (1987). Again a much lower product yield was obtained than that quoted by the authors, the reason for which is unclear.

After a number of attempts, approximately 200 mg of cyclopropanol was obtained which proved sufficient for all subsequent experiments with this compound. It was stored with desiccant at -20°C.

2.25. Reaction of MDH with cyclopropanol

MDH was reacted with cyclopropanol using the method described by Dijkstra *et al.* (1984). MDH (10 µM) in a total volume of 1 ml of 100 mM tetrasodium pyrophosphate buffer (pH 9.0) or 100 mM sodium borate buffer (pH 9.0) containing

20 mM NH_4Cl, 10 mM KCN and 20 µM Wurster's blue was oxidised by the addition of 0.2 µl aliquots of 100 mM $K_3Fe(CN)_6$ until the blue colour persisted. At this time, either 20 µM (equimolar) or 200 µM (10-fold excess) cyclopropanol was added. The mixtures were then left for 1 h at room temperature to react, adding more ferricyanide if necessary to keep the MDH oxidised and then low molecular weight compounds were removed by passage through a P-6 column (Bio-Rad) equilibrated with the appropriate buffer. Two other cyclic compounds, cyclobutanol and cyclopropanemethanol, were also used to treat MDH in an identical fashion.

2.26. Cross-linking of MDH with cytochrome c_L

The two stage sulpho-NHS-enhanced cross-linking with carbodiimide (EDC; Grabarek and Gergely, 1990) was used as described by Chan and Anthony (1991) and Cox *et al.* (1992).

Cytochrome c_L (230 µM) in 20 mM Mes buffer (pH 6.0) was activated by the addition of 5 mM sulpho-NHS and 2 mM EDC (final concentrations). After 30 min, excess reagents were removed by gel filtration on a P-6 column (Bio-Rad) equilibrated with 5 mM Hepes buffer (pH 7.5) and an equimolar concentration of unmodified MDH was added. After a further 90 min the reaction was terminated by addition of hydroxylamine to a final concentration of 15 mM. After cross-linking, samples of the reaction mixture were loaded onto both a 13% SDS-polyacrylamide gel and a 5% non-denaturing polyacrylamide gel. The SDS-gel was stained for haem proteins whilst the non-denaturing gel was stained for haem proteins and total protein complement.

2.27. Preparation of MDH in calcium-free buffer

Calcium-free Hepes or Mops buffer (10 mM, pH 7.0) was prepared by dissolving the buffer salt in MilliQ water, adjusting the pH to 7.0 with NaOH and then passage through a column (16 x 150 mm) containing Chelex-100 chelating resin (Bio-Rad) in the sodium form.

To remove any free calcium present in the enzyme solution, pure MDH was passed down a P-6 gel filtration column (Bio-Rad) equilibrated with 40 ml of this calcium-free buffer.

2.28. Estimation of calcium in MDH

The amount of calcium in MDH was determined using a Perkin-Elmer 280 atomic absorption spectrophotometer calibrated with standard calcium chloride solutions in the range 0 to 10 $\mu g.ml^{-1}$ (0 to 250 µM). The standard curve for the calcium determination is shown in Fig. 2.2. The calcium chloride stock solution (100 $\mu g.ml^{-1}$) was prepared by dissolving calcium carbonate in HCl and then making up to volume with MilliQ water. Three different concentrations of MDH were used to get an accurate measurement of calcium in the enzyme. Additionally, three solutions of MDH containing different amounts of added calcium were tested to confirm that MDH does not affect the standard assay system.

2.29. Estimation of free thiols, *N*-terminal sequencing and amino acid composition analysis

The assay of free thiols was carried out using Ellman's reagent as described by Riddles *et al.* (1983). MDH was assayed in 25 mM Hepes buffer pH 7.0.

N-terminal sequencing of MDH was performed using an Applied Biosystems model 407A 'gas phase' (pulsed liquid) protein sequencer coupled to a model 120 phenylthiohydantoin derivative analyzer.

Amino acid composition of MDH was determined using an Applied Biosystems model 420AH phenylthiocarbamoyl derivatiser coupled to a model 130A analyzer.

The *N*-terminal sequencing and the amino acid analyses were performed by Lawrence Hunt in the Protein Sequencing Unit of the Institute of Biomolecular Sciences, University of Southampton.

2.30. Transformation of *E. coli* strain HB101

The transformation of *E. coli* strain HB101 was carried out using a modification of the method described by Maniatis *et al.* (1982); to render the cells competent, the cell suspension in ice-cold 50 mM $CaCl_2$ was left for 20 min instead of 12 h (Dr. A. Worrall, personal communication).

The plasmid (pND5) used for the transformation was a kind gift from Dr. R. Lathigra, Hammersmith Hospital, London. This plasmid is a pBR322 derivative carrying the *E. coli groEL* gene cloned into the ampicillin resistance gene.

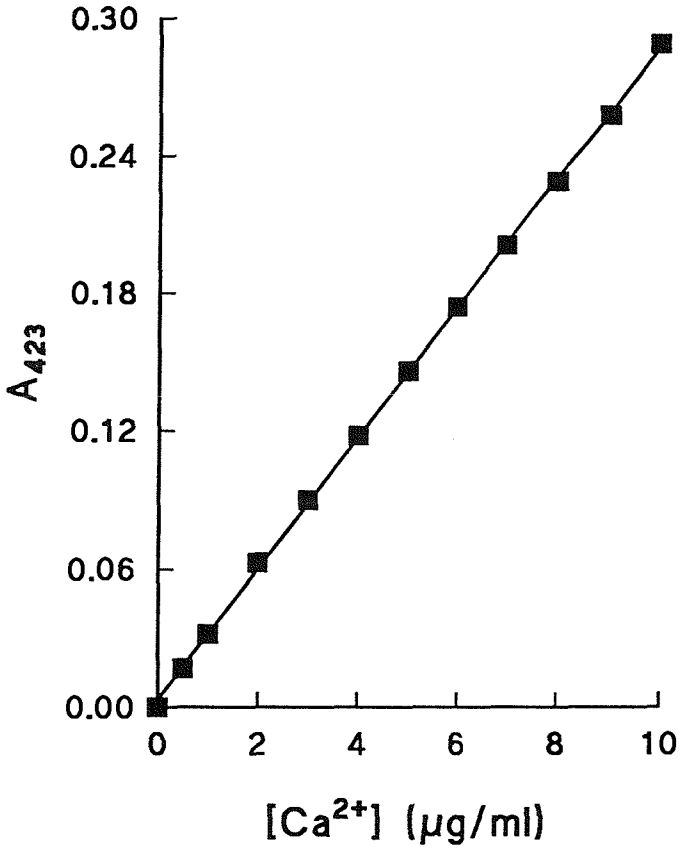


Fig. 2.2. Standard curve for the determination of calcium content in MDH by atomic absorption spectrophotometry

The stock solution of calcium chloride was prepared by dissolving calcium carbonate in dilute HCl and making up to volume with MilliQ water. Dilutions of this stock solution were then made with MilliQ water to cover the range 0 - 10 $\mu g.ml^{-1}$.

CHAPTER 3

Studies on the MoxC and MoxH mutant strains of *Methylobacterium extorquens*

3.1. Introduction

Both MoxC and MoxH mutants have previously been reported to produce MDH and cytochrome c_L, but the amounts of these proteins were much lower than that found in the wild-type strain. Furthermore, the purified MDH from these mutants was inactive in the dye-linked assay and had an altered absorption spectrum resembling that seen for the MoxK (formerly MoxA2) mutant. It was suggested that the *moxC* and *moxH* genes have a regulatory function, controlling not only expression of MDH and cytochrome c_L, but also the expression of the *moxK* gene product. The *moxC* and *H* gene products may also regulate the *moxL* gene, since this gene is immediately downstream of *moxK* (Nunn and Lidstrom, 1986b). Subsequently, however, it has been suggested that the *moxC* and *moxH* genes, along with 5 other genes, are involved in either PQQ biosynthesis or PQQ transport to the periplasm (Section 1.10; Lidstrom, 1990). This suggestion was based on the postive growth response of MoxC and MoxH to PQQ when used to saturate a filter disc placed on a methanol-medium agar plate inoculated with a number of the isolated Mox mutants. It was not possible to differentiate between the lack of PQQ biosynthesis or transport.

This chapter describes experiments carried out using wild-type, MoxC and MoxH strains, firstly, to further characterise the MDH reported to be present in thse mutants when grown on methylamine in the presence of methanol; secondly, to confirm the PQQ^- phenotype of the MoxC and MoxH mutants held in this laboratory; lastly, to study the induction of MDH in the MoxC and MoxH mutants.

3.2. Purification of MDH from MoxC and MoxH mutants grown on methylamine in the presence of methanol

MoxC and MoxH mutant strains were grown on mineral salts medium containing 0.4% (w/v) methylamine and 0.5% (v/v) methanol to late log phase, harvested and cell free extracts prepared as described in Chapter 2. In view of the reported low level of MDH present in these strains (Nunn and Lidstrom, 1986b), about

100 g (wet weight) of cell paste was used for each mutant. Detection of the inactive MDH was initially performed by SDS-PAGE gels stained with Coomassie blue.

In both MoxC and MoxH strains, a protein corresponding in molecular weight to the α-subunit of MDH was detected in the non-binding fractions from the DEAE-Sepharose column. Since MDH does not normally bind to this column, it was assumed this protein was MDH. Fractions containing the putative MDH were pooled, concentrated and dialysed against the phosphate buffer used to equilibrate the next column in the purification procedure (hydroxylapatite; HAP). Following loading of the sample, this column was treated as described in Section 2.8. Unfortunately, no protein corresponding to the α-subunit of MDH were detected in fractions eluting from the HAP column with the linear gradient. Even when the column was washed with 1 M potassium phosphate buffer (pH 7.0) no protein corresponding to the α-subunit was detected. The non-binding fraction from this column was also checked for the presence of MDH but the result was negative.

It is difficult to explain these observations. One possibility is that the MDH might have bound irreversibly to the HAP column; this is unlikely unless the protein was not in the usual $\alpha_2\beta_2$ conformation and hence had different binding characteristics. Alternatively, MDH was present but eluted from the HAP column too diluted to allow detection with Coomassie blue staining. A further possibility is that there was no MDH present in the cells in the first place. To test the first and third possibilities, crude cell extracts of the MoxC and MoxH mutants grown on methylamine in the presence of methanol were investigated using SDS and non-denaturing PAGE, and Western blotting; the results from these experiments are shown in the next section.

The second possibility that the eluting MDH was too dilute to allow detection by Coomassie blue stained SDS gels was tested using two modifications to the purification procedure. Firstly, the DEAE-sepharose and HAP columns were connected in series; both columns were equilibrated initially with the normal 20 mM Tris-HCl buffer (pH 8.0) used for the DEAE-Sepharose column. After loading the cell free extract, the columns were washed with the same Tris-HCl buffer. When all non-binding fractions had washed off from the DEAE-Sepharose onto the hydroxylapatite, the columns were disconnected and then treated separately as described in Section 2.10. A trial run using a cell free extract from wild-type cells showed that, in this case, the MDH bound perfectly well to the HAP column under these conditions, with no apparent detrimental effect on the subsequently purified protein; this abbreviated procedure was often used to decrease the time taken in

purification of MDH from wild-type, MoxA, MoxK and MoxL strains by about 2 days. The second modification used was in the means of detection of MDH; fractions were 'dot-blotted' onto nitrocellulose and then probed with anti-MDH antibodies (using this method it is possible to detect very low levels of the target protein).

Using these modifications, it was still not possible to detect any MDH protein eluting from the hydroxylapatite column. This suggests that one of the other two possibilities was correct. This was further investigated as described below.

3.3. Detection of MDH in MoxC and MoxH mutants

The previous section indicated either that MoxC and MoxH contained no MDH or that the MDH present was not in the normal $\alpha_2\beta_2$ tetrameric configuration. This section describes experiments carried out to investigate this problem.

Fresh cultures (1 litre) of wild-type and mutant strains were grown on methylamine in the presence of methanol. Under these conditions, some strains of *Mb. extorquens* are known to produce MDH (wild-type, MoxA, MoxK, MoxL and MoxG) whereas others are known not to produce MDH (MoxB, MoxD, MoxE and MoxF) (Nunn and Lidstrom, 1986b); hence these strains were used as positive and negative controls. From the 1 litre cultures, about 2-3 g (wet weight) of cells were obtained. These cells were washed with about 50 ml of 25 mM Hepes buffer (pH 7.0) and resuspended in 5 ml of the same buffer. Samples of the cell suspensions (2 ml) were disrupted by sonication and debris removed by centrifugation. Crude extracts were assayed for protein content, and a constant amount of total protein loaded per lane onto SDS-PAGE and non-denaturing PAGE gels. The gels were electrophoresed, proteins transferred onto nitrocellulose using Western blotting and the blots probed with antibodies raised against holo-MDH. The Western blot of the SDS-PAGE gel (Fig. 3.1.a) clearly shows the presence of a protein corresponding to the α-subunit of MDH in crude extracts of both MoxC and MoxH mutants, as well as in the crude extracts of the strains known to produce MDH. Unfortunately, the antibodies used do not cross-react with the ß-subunit even though they were raised against holo-MDH; the reason for this is not known. The Western blot of the non-denaturing PAGE gel gave an intriguing result (Fig. 3.1.b). In the lanes where MDH was known to be present, two cross-reactive bands were detected; the upper band having the same mobility as native MDH when similar gels were run and stained either with Coomassie blue (Fig. 4.1.b). In the lanes for the MoxC and MoxH mutants, however, there was only

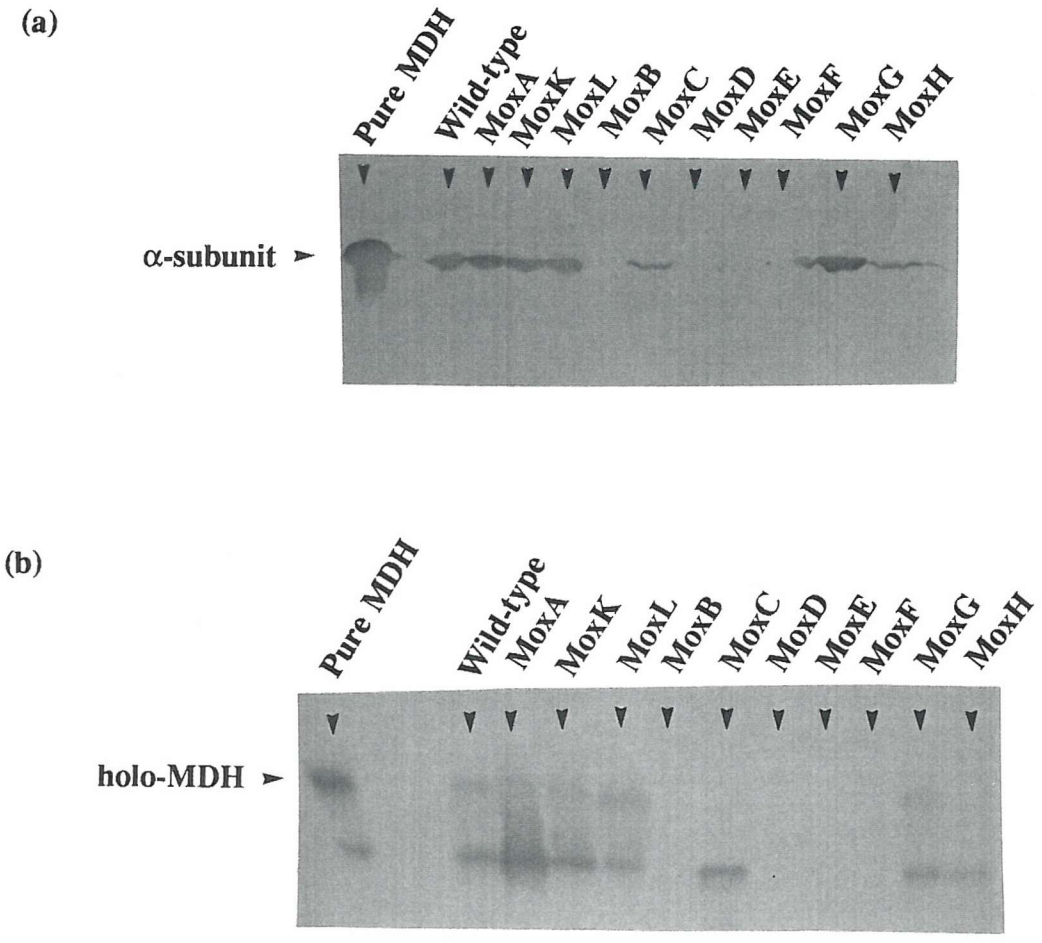


Fig. 3.1. Western blots of SDS-PAGE and non-denaturing PAGE gels of crude extracts of wild-type and Mox mutant strains of *Methylobacterium extorquens*

Crude extracts of all strains were prepared as described in Section 2.6, and 50 µg total protein loaded per lane of a 13% SDS-PAGE gel and a 5% non-denaturing PAGE gel. These gels were electrophoresed as described in Section 2.14.c, and proteins transferred to nitrocellulose filters by Western blotting as described in Section 2.15. The blots were then probed with antibodies raised against holo-MDH purified from wild-type bacteria, and developed as described in Section 2.14.b.

(a) 13% SDS-PAGE

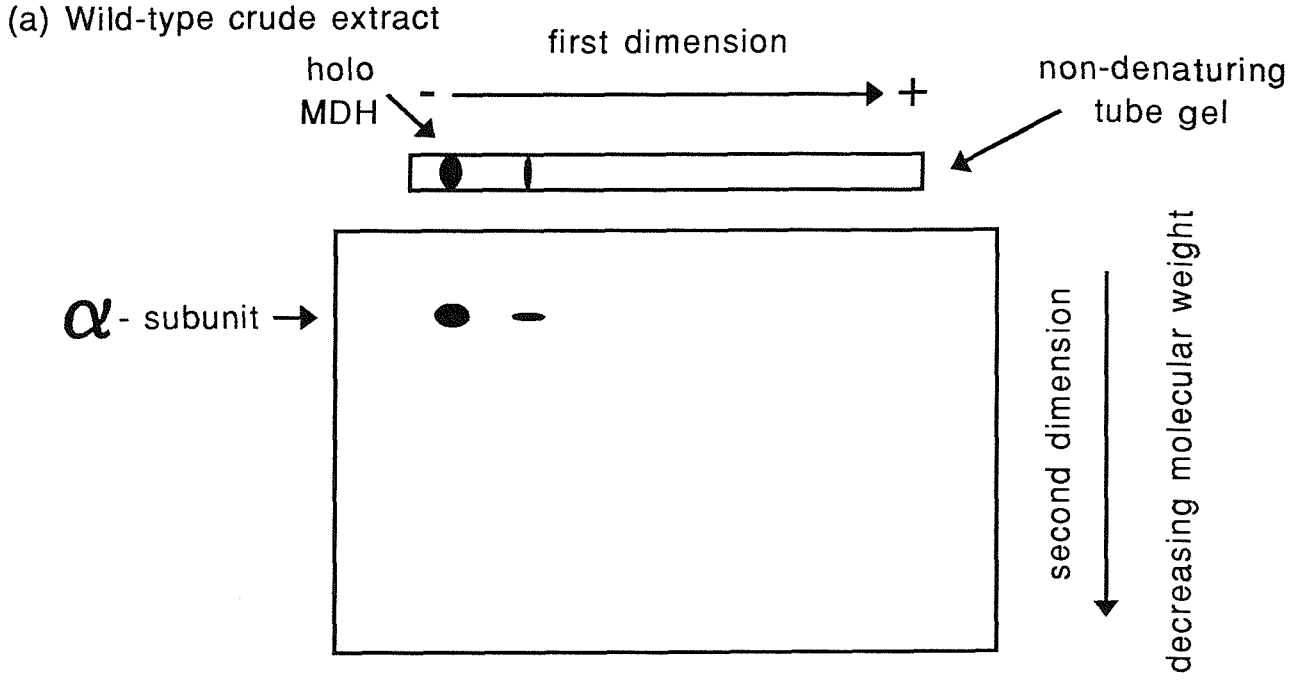
(b) 5% non-denaturing PAGE

one cross-reactive band which had the same mobility as the lower band seen in the positive control lanes. Interestingly, the lane containing MDH purified from wild-type bacteria also showed the presence of the two cross-reactive bands, although the lower band was very feint. This shows that the cross-reactive band observed in the lanes for the MoxC and MoxH mutants is a component of MDH and not a contaminant present in the crude extracts. Since non-denaturing PAGE separates proteins on the basis of charge and size (SDS-PAGE separates proteins on the basis of size alone), it was not possible to get a direct estimation of the molecular weight of the two bands and hence identify their component(s). In an attempt to overcome this problem, 2-dimensional gel electrophoresis was performed using the Bio-Rad mini-Protean II 2-D module. In 2-D PAGE, the first dimension is normally run using iso-electric focussing, which separates proteins on the basis of charge, and the second dimension is typical SDS-PAGE. In this experiment, however, the first dimension was run using tube gels prepared for the non-denaturing procedure descibed in Section 2.16.c. This was done in order to maintain the same relative positions of the two cross-reactive bands. Following electrophoresis the gel was incubated in SDS-PAGE loading buffer for 30 min at room temperature and then laid along a very short stacking gel of a 13% SDS polyacrylamide gel. This second dimension was then electrophoresed as normal (20 mA for 1 h; Section 2.16.c). Following electrophoresis, the gel was Western blotted and probed with anti-holo-MDH antibodies (to detect the α-subunit). Two cross-reactive spots were detected in the blot of the 2-D PAGE gel probed with these antibodies. These spots corresponded to the two cross-reactive bands observed in the blot of the non-denaturing PAGE gel, and both had the same mobility as the α-subunit of MDH in SDS-PAGE (Fig. 3.2). A second gel was run, blotted and probed with antibodies raised specifically against the β-subunit (a gift from A. Avezoux of this department). However, the control of the crude extract of wild-type bacteria showed no reactivity, indicating that the antibody was too weak to use with crude extracts; a preliminary experiment with MDH purified from wild-type bacteria showed that the antibodies were able to detect the β-subunit.In the blots of the 2-D PAGE gel probed with anti-MDH-β antibodies, however, no cross-reactive spots were detected, in any of the gels run using crude extracts of wild-type bacteria, and the MoxC and MoxH mutants.

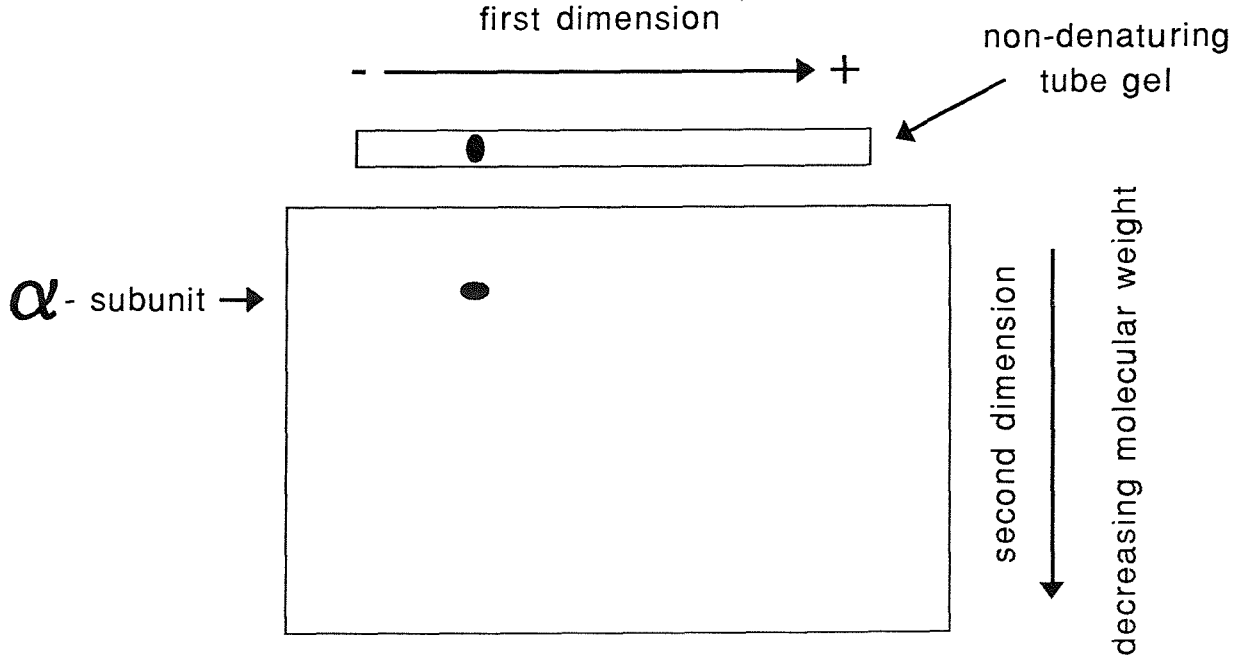
These results show that both MoxC and MoxH synthesise at least the α-subunit of MDH as indicated by the Western blots probed with anti-holo-MDH antibodies. Furthermore, the MDH produced by these mutants is not in the typical $\alpha_2\beta_2$ tetrameric

Fig. 3.2. Diagrams of the Western blots of 2-D PAGE gels run using crude extracts of wild-type bacteria and the MoxC mutant

(a) Wild-type crude extract



(b) MoxC crude extract



configuration of holo-MDH, indicated by the Western blotting of non-denaturing PAGE gels. This suggests that the MDH produced by MoxC and MoxH is a precursor form of the holo-enzyme which definitely contains the α-subunit. This is supported by the fact that the MDH from MoxC and MoxH could not be purified; it remained tightly bound to the HAP column. It was not possible to detect the β-subunit in crude extracts of wild-type bacteria or the Mox mutants, hence it cannot be stated whether this precursor form is an unusual $\alpha_2\beta_2$ tetramer, an α_2 dimer, an αβ dimer or just an α monomer. By adopting a different purification protocol, it should be possible to isolate the MDH produced by MoxC and MoxH and hence characterise this protein fully.

3.4. Supplementation of Mox mutant strains with PQQ

This experiment was performed for two reasons. Firstly, to test whether the mutant strains of *Mb. extorquens* held in this laboratory respond to PQQ supplementation like those in Lidstrom's laboratory. A second aim was to find a concentration of PQQ that supports full growth of these mutants in liquid culture on methanol.

Wild-type *Mb. extorquens* and all of the Mox mutant strains were inoculated, using 5 colonies from freshly grown cultures on nutrient agar plates, into 10 ml volumes of methanol-medium containing various concentrations of PQQ. The cultures were then incubated, without shaking, at 30°C for 1 day, followed by incubation with shaking (150 rpm) at 30°C for 2 days, after which time, growth was measured by absorbance at 650nm.

Of the mutant strains, only MoxC and MoxH were able to grow on methanol with PQQ supplementation. Full growth of these strains was achieved with 1 μM PQQ, and was not markedly different from that observed for the wild-type strain (Fig. 3.3). Higher concentrations of PQQ caused some inhibition of growth. Subsequent inoculation of cultures grown on methanol in the presence of PQQ onto methanol-agar plates (with no added PQQ) did not result in growth except, as expected, in the case of the wild-type strain.

3.5. Detection of PQQ in MoxC and MoxH

The fact that MoxC and MoxH can be supplemented by PQQ suggests that they are impaired either in PQQ biosynthesis or in PQQ transport. In order to determine which of these phenotypes is correct, heat-treated crude extracts of

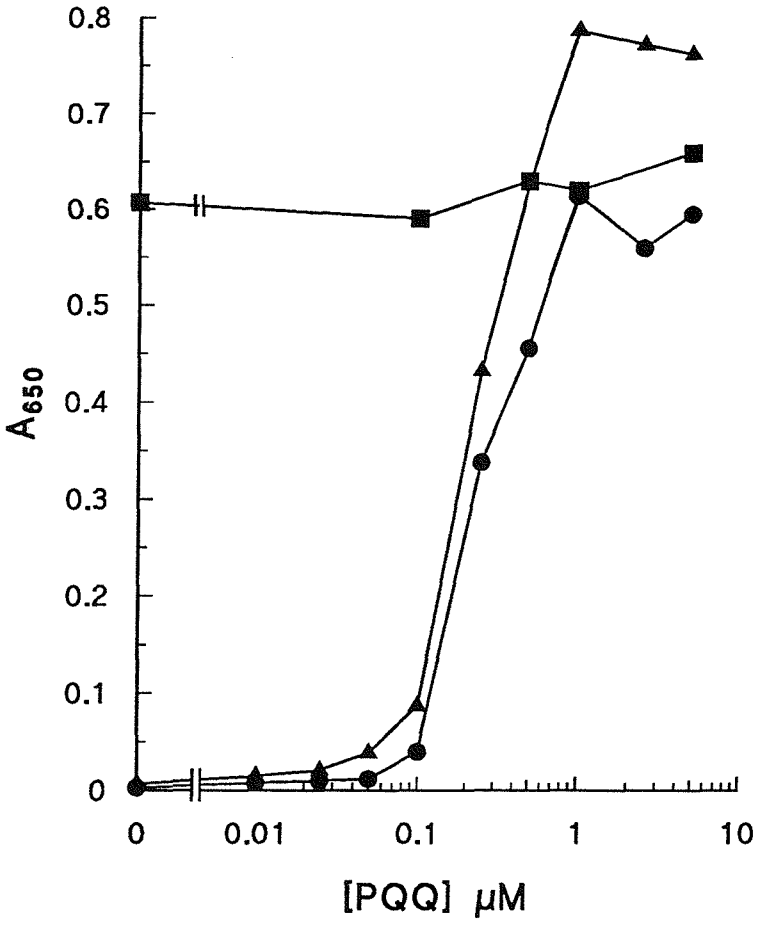


Fig. 3.3. Supplementation of MoxC and MoxH with PQQ

After autoclaving, standard methanol-medium was supplemented with various concentrations of PQQ, added from a filter-sterilised stock. The final volume of the culture medium was 10 ml in each case. These cultures were inoculated using 5 colonies from freshly-grown strains on nutrient agar plates. Cultures were incubated at 30°C without shaking for 1 day followed by incubation at 30°C with shaking for 2 days. After incubation, growth was measured by absorbance at 650 nm. (■), wild-type; (▲), MoxC; (●), MoxH.

methylamine/methanol-grown cultures were used in a biological PQQ assay.

Samples (1 ml) of the crude extracts of wild-type bacteria and MoxC and MoxH mutants were boiled for 10 min, and denatured protein was removed by centrifugation (12,000 g for 10 min). Supernatants were then used directly, with dilutions where necessary, for the reconstitution of apo-alcohol dehydrogenase from *Pseudomonas testosteroni* as described in Section 2.20.b. Only the supernatant prepared from the crude extract of wild-type bacteria contained any material that could reconstitute activity. Supernatants prepared from the crude extracts of MoxC and MoxH were not able to reconstitute activity, even when used undiluted.

It is concluded, therefore, that both MoxC and MoxH have mutations which affect the biosynthesis of PQQ. Of course it is possible that they may *also* be impaired in PQQ transport, but there is no means, at present, of investigating this possibility.

3.6. Discussion

The failure to isolate MDH from methanol/methylamine-grown cells of either MoxC or MoxH suggests that both strains do not, as previously thought (Nunn and Lidstrom, 1986b), synthesise holo-MDH under these growth conditions. However, a protein of molecular weight 60 kDa (judged from SDS-PAGE), and cross-reactive to anti-MDH antibodies, was detected in crude extracts and in fractions eluting from the DEAE column. This suggests that at least the α-subunit of MDH was synthesised by MoxC and MoxH, although the level of protein was lower than that seen for holo-MDH in wild-type bacteria. Non-denaturing PAGE showed that the MDH produced by the MoxC and MoxH mutants was not in the normal $\alpha_2\beta_2$ tetrameric conformation, as found in wild-type bacteria. Western blots revealed the presence of the α-subunit but it was not possible to detect the β-subunit in MoxC, MoxH or wild-type bacteria in these experiments. Therefore, it is not possible to state what conformation the MDH protein from the MoxC and MoxH mutants is in; it could be an atypical $\alpha_2\beta_2$, α_2, αβ or even monomeric α. Another possibility is that the protein is in an $\alpha_2\beta_2$ conformation but without the PQQ; the absence of PQQ affecting the pI of the protein and hence its characteristics for binding to columns. The pI was unlikely to be markedly affected since the protein was detected eluting from the DEAE column. Examination of the individual subunit amino acid sequences using PC/Gene (Genofit, Switzerland) predicts that the α-subunit has a pI of 5.66 and the β-subunit has a pI of 9.77. Using isoelectric focussing, MDH from *Mb. extorquens* has been shown to have a pI of 8.8 (O'Keeffe,

1980). In order to reach this final pI, the β-subunit must have a substantial influence even though it is considerably smaller than the α-subunit. It is possible that PQQ could affect the pI of MDH although, due to its three carboxylic acid groups, PQQ is acidic and will probably lower the pI if present. Therefore, the *absence* of PQQ would result in an *increased* pI for apo-MDH which means that the protein would not bind to the DEAE column. Whether or not the absence of PQQ could significantly affect the binding properties of MDH is not clear, although a precedent for the apo- and holo-forms of a protein having different binding characteristics exists in cytochrome c_{550} from *Paracoccus denitrificans*. The apo- and holo-forms of this redox protein, which differ only in the absence or presence of haem, can be separated by ion-exchange chromatography (Page and Ferguson, 1989, 1990). In the original descripiton of MoxC and MoxH (Nunn and Lidstrom, 1986a,b), MDH purified from these mutants had altered absorption spectra in the region where the prosthetic group absorbs. This suggests that the MDH did contain PQQ, or a modified form of PQQ and, hence, that they were able to, at least partially, synthesise the prosthetic group. The fact that MoxC and MoxH are now known to be mutants impaired in PQQ biosynthesis suggests that the prosthetic group in the MDHs from MoxC and MoxH was derived from contamination. This is supported by the suggestion that PQQ is a common contaminant of glassware, buffers etc, and requires careful treatment to ensure its removal (van Kleef *et al.*, 1987). The fact that a protein cross-reactive to anti-MDH antibodies was present in MoxC and MoxH grown in the presence of methanol, suggests that the *moxFJGI* region was being expressed. This suggests that the synthesis of MDH can occur in the absence of PQQ biosynthesis. This is consistent with an earlier suggestion that there is no coordination between the biosynthesis of PQQ and quinoproteins (van Schie *et al.*, 1984).

The supplementation experiments confirmed the finding of Lidstrom (1991) that both MoxC and MoxH mutants are capable of growth on methanol provided that PQQ is added to the growth medium. Thus these mutants are impaired in either PQQ biosynthesis or transport. The fact that PQQ is able to supplement these mutants suggests that PQQ is free to diffuse across the outer membrane into the periplasm; the reverse direction has already been indicated by the fact that some (if not all) methylotrophs can accumulate PQQ in the growth medium as the cells enter stationary phase (Ameyama *et al.*, 1986). It is thought that the appearance of this extracellular PQQ is due to MDH turnover rather than an excess of PQQ being synthesised

(Ameyama *et al.*, 1988). This would suggest that once transported into the periplasm, PQQ is unable to return to the cytoplasm for re-processing; that is, there does not appear to be any specific PQQ uptake system. The supplementation of MoxC and MoxH by PQQ is reversible; there is no reversion in the mutations since MoxC and MoxH mutants return to their normal methanol$^-$ phenotype once the supply of PQQ is removed. Crude extracts of both MoxC and MoxH were seen not to contain PQQ. This suggests that these mutants are impaired in PQQ biosynthesis. The earlier observation that MDH synthesis can occur in the absence of PQQ suggests that the components of MDH (α-subunit, β-susunit and PQQ) are transported separately into the periplasm, where they are assembled to form holo-MDH.

Clearly, until the biosynthetic pathway for PQQ biosynthesis is elucidated, and extensive genetic analysis performed, the precise nature of the mutations in MoxC and MoxH, and how these mutations affect MDH synthesis, will remain unknown.

CHAPTER 4

Characterisation of the methanol dehydrogenases from wild-type *Methylobacterium extorquens* and MoxA, K and L mutant strains

4.1 Introduction

Originally, methanol dehydrogenase (MDH) from *Methylobacterium extorquens* was thought to be dimeric enzyme of subunit molecular weight 60 kDa, although it is now known to be an $\alpha_2\beta_2$ tetramer (α, 66 Kda; β, 8.5 Kda) with two molecules of pyrrolo-quinoline quinone (PQQ) per tetramer. The genes that encode the two subunits lie in an operon along with two other genes, one of which encodes cytochrome c_L. Analysis of the primary sequences showed that each gene has a region that encodes a signal peptide, indicating that the final destination of each protein is the periplasm and that each precursor is translocated separately (Anderson *et al.*, 1990). Once in the periplasm, the signal peptides are cleaved (presumably by a membrane-bound signal peptidase), and the two subunits assembled, along with PQQ, to form holo-MDH. It is not known, however, if PQQ is transported separately or in association with one or other of the subunits. Radiolabelling studies with Bacterium W3A1 have suggested that there is a co-translational processing and export of the individual apo-protein subunits, and that PQQ can be added to apo-MDH to form holo-enzyme (Davidson *et al.*, 1985). Although this reconstitution was based on the absorption spectrum (no measure of activity was reported), the results suggest that the individual components of MDH are transported separately. If the components are transported separately, it is not known how and it what order they are assembled, and whether or not they can self-assemble or require one or more mediating factors. It is the aim of the remainder of this thesis to answer some or all of these questions.

The approach used was to take advantage of the MoxA, K and L mutants of *Methylobacterium extorquens*. These mutants were introduced in Chapter 1, but their preliminary characterisation is repeated here. They were able to grow on succinate or methylamine, and produced normal levels of cytochrome c_L and (presumably) methylamine dehydrogenase (MNDH). They could not, however, utilise methanol because, although wild-type levels of MDH protein were produced, it was inactive in the dye linked assay. However, the existence of the β-subunit was not known at the

time and so it was not possible to state whether or not this subunit was present in the MDHs produced by these mutants. Additionally, the MDHs from MoxA, K and L had absorption spectra that differed between the mutants and were markedly different from the characteristic spectrum seen for the enzyme from wild-type bacteria (see Fig. 1.3). Consequently, it was concluded that the mutations affected either PQQ biosynthesis or apo-MDH-PQQ processing (Nunn and Lidstrom, 1986b).

This chapter describes the complete characterisation of the MDHs from the MoxA, K and L mutants. In order to qualify this characterisation in terms of any defects in the proteins, MDH from wild-type *Mb. extorquens* was used as a control. That the mutations were restricted to MDH was confirmed by purifying cytochrome c_L and MNDH from the mutants, and showing them to be indistinguishable from those purified from wild-type bacteria.

The MDHs in this chapter are referred to as 'wild-type MDH' and 'mutant MDHs'; this is purely for convenience, and does not imply that there any structural mutations in the genes for these enzymes as would be the case if site-directed mutagenised proteins were being studied.

4.2. Purification of methanol dehydrogenase

In order to ensure that any differences that were observed between the MDHs from the different sources were due solely to MDH and not to any contaminant, it was necessary to purify the proteins to as near 100% homogeneity as possible. Many different purification protocols have been adopted for the MDHs from various bacteria, and depend primarily on the pI and stability of the protein. The method used in this work is based on a three-step protocol described by Day and Anthony (1990a). The second step in this method involves the use of hydroxylapatite (HAP). HAP is crystalline calcium phosphate and, because of its non-uniform particle size, it sometimes causes problems with column packing and sample loading. In the course of this study, it was found that due to irregularly packed columns, reproducible resolution was not always attainable and, even after the third step (cation-exchange chromatography), a significant levels of contaminating proteins remained. To counter this problem, sphaeroidal HAP (BDH) was tried. This is a more mechanically stable form (due to inclusion of HAP crystals into inert porous beads) and, hence, has better

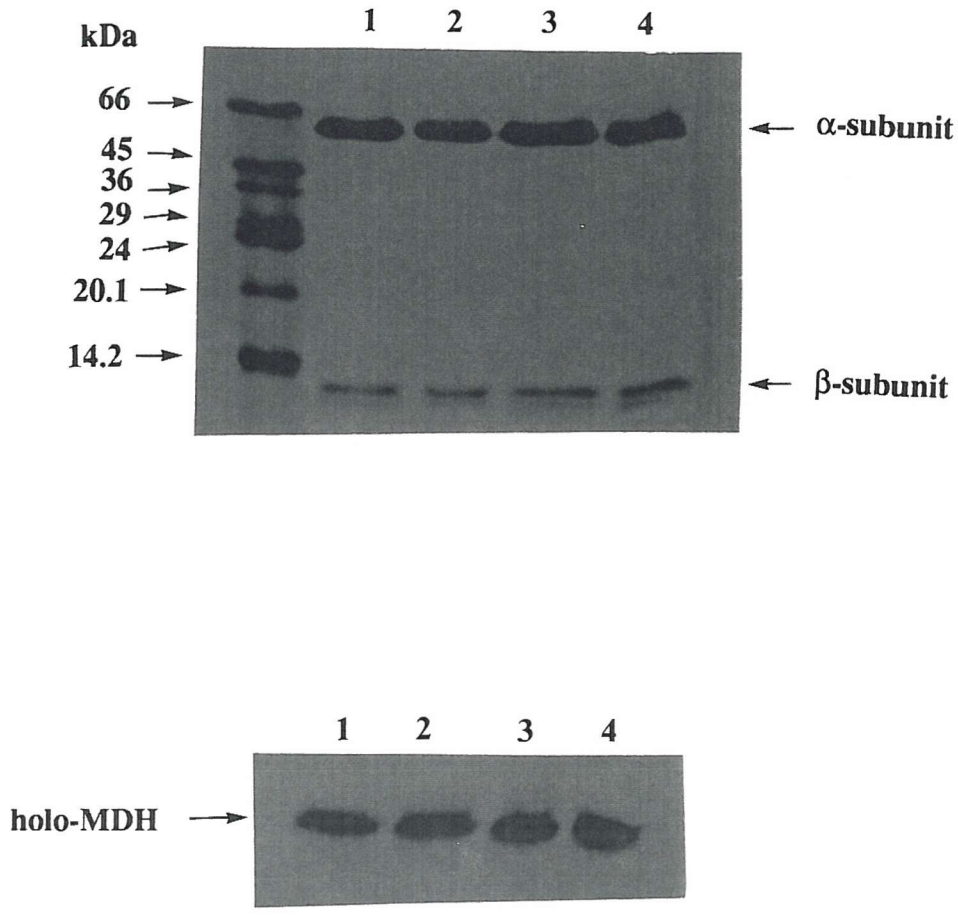


Fig. 4.1. PAGE analysis of wild-type, MoxA, MoxK and MoxL MDHs

(a) SDS-PAGE (13%) prepared and run as described in Section 2.14.c. Each MDH (10 µg) was loaded and, after electrophoresis, the gel was stained with Coomassie Blue.

(b) Non-denaturing PAGE (5%) prepared and run as described in Section 2.14.c. Each MDH (10 µg) was loaded and, after electrophoresis, the gel was stained with Coomassie Blue.

Track 1: Wild-type MDH

Track 2: MoxA MDH

Track 3: MoxK MDH

Track 4: MoxL MDH

packing and flow characteristics. However, proteins are less strongly bound to this form of HAP and, in this study, it was found that MDH often co-eluted with proteins that would otherwise elute separately with the conventional material. I therefore decided to return to the conventional material and found that the most reliable source of HAP was Bio-Rad Bio-Gel HT. The original problem of contaminating proteins remained, however, and so I incorporated a fourth step, gel-filtration, into the purification protocol. The MDH after this step was judged (by SDS-PAGE) to be in excess of 99% homogeneous, and the protocol was adopted routinely.

4.3. Dye-linked and cytochrome *c*-linked activity of the MDHs

The specific activity of wild-type MDH, measured in both assay systems, varied considerably from batch to batch. This variation was apparently related to the length of time taken in purification and the period of storage of the cells; in both cases, the longer the time, the lower the specific activity of the pure protein. The range of specific activity measured in the dye-linked assay was 400-1200 nmol dye reduced.min^{-1}.mg $protein^{-1}$; that measured in the cytochrome *c*-linked assay was 50-95 nmol cytochrome reduced.min^{-1}.mg $protein^{-1}$. Typical values were 600 nmol dye reduced.min^{-1}.mg protein and 80 nmol cytochrome reduced.min^{-1}.mg $protein^{-1}$; these are similar to those in the literature.

MDH from the mutants was previously reported to be inactive in the dye-linked assay due to the actual level of activity being below the detectable limit (Nunn and Lidstrom, 1986b). However, in this study, detectable levels of activity in both assay systems were measured, but only when relatively large amounts of protein was used. In all cases, the activity was ammonia-dependent and exhibited a typical endogenous rate in the absence of methanol. Just as with wild-type MDH, the specific activities of the mutant enzymes varied from batch to batch, but was always less than 5% of a typical wild-type value (Table 4.1).

4.4. Polyacrylamide gel electrophoresis

Coomassie-blue stained SDS-PAGE of the wild-type and mutant MDHs (Fig. 4.1.a) clearly shows that each enzyme consists of both α- and β-subunits and that these subunits are the expected molecular weight (α, 60 Kda; β, 8.5 Kda). Gel scanning confirmed that the subunits were in a 1:1 ratio for the wild-type and mutant enzymes.

Table 4.1. Specific activities of wild-type, MoxA, MoxK and MoxL MDHs

Specific activities for the dye-linked and cytochrome *c*-linked assays were measured as described in Section 2.11. The terminal electron acceptor for the dye-linked assay is PIP, that for the cytochrome *c*-linked assay is horse-heart cytochrome *c*. Specific activities are expressed in nmol electron acceptor reduced. min^{-1}. mg $protein^{-1}$. The values given are typical results although there was considerable batch to batch variation (see text for details).

	Specific activity			
Source of MDH	Dye-linked assay	Percentage of wild-type	Cytochrome *c*-linked assay	Percentage of wild-type
Wild-type	516	100	90.0	100
MoxA	12	2	0.2	<1
MoxK	3	<1	0.1	<1
MoxL	5	1	0.4	<1

Non-denaturing PAGE gels stained with Coomassie-blue (Fig. 4.1.b) showed that each MDH had the same native electrophoretic mobility. This result suggests that the overall structure of the MDHs from the mutants is the same as the wild-type enzyme. With an identical gel stained with nitroblue tetrazolium to detect activity, only the wild-type lane gave a detectable band, confirming that the mutant MDHs are inactive.

4.5. Detection of free-thiols, *N*-terminal sequencing and amino acid composition

The PAGE experiments showed that the MDHs contained both α- and β-subunits, and were all of the same native molecular weight. However, it is possible that there are some minor post-translational modifications of the proteins that could affect activity but would not necessarily be detected by PAGE. Examples of post-translational modification that may be relevant in this instance are the removal of the signal peptides and the formation of disulphide bridges. At least part of the signal peptide for each subunit of the mutant MDHs is removed since there is no obvious difference in molecular weight, but it is possible that cleavage occurs at the wrong site resulting in a slight but structurally significant difference. A consensus sequence for the *mox* genes from *Mb. extorquens* has been identified as Ala-Xxx-Ala-CUT (Anderson *et al.*, 1990), and so *N*-terminal sequencing of the native MDHs should reveal any additions or deletions. However, such sequencing of the mutant MDHs showed that the subunits in each protein were identical to those of the wild-type enzyme (α, NDKLV; β, YDGTK) thereby proving, that in all three mutants, the signal peptidase functions correctly.

Amino acid analysis of the four MDHs (Table 4.2) showed them to be identical within the limits of the method, suggesting that there is no major sequence modification to any of the proteins. This is in keeping with the knowledge that the *moxA*, *K* and *L* genes are distinct from the MDH structural genes (*moxF* and *moxI*).

Disulphide bridges confer stability to proteins and hence are often structurally important features. Whether or not MDH has any disulphide bridges has not been published, but they are probable for two reasons. Firstly, the enzyme is periplasmic and may exist in a relatively harsh environment; therefore stability is paramount. Secondly, the gene sequence shows that holo-MDH has 12 cystyl residues, 4 in each α-subunit, 2 in each β-subunit, allowing the possible formation of 6 disulphide bridges; any bridges that do exist must be intramolecular since the subunits can be separated by

Table 4.2. Amino acid composition of wild-type, MoxA, MoxK and MoxL MDHs

Compositions were determined as described in Section 2.29. This method does not detect cystyl or tryptophyl residues. The predicted values are taken from Nunn *et al.* (1989) and Anderson *et al.* (1990). The high values for Gly and Glu/Gln are most probably due to contamination of the buffers used in the analysis.

	Composition				
Amino acid	Predicted from gene sequence	Wild-type	MoxA	MoxK	MoxL
Ala	104	103	106	97	97
Arg	38	42	43	45	43
Asp/Asn	188	182	183	186	180
Glu/Gln	88	101	103	107	115
Gly	160	197	182	187	200
His	22	27	26	28	27
Ile	48	44	43	42	42
Leu	94	74	77	78	76
Lys	120	120	125	123	123
Met	34	22	22	23	22
Phe	46	45	43	42	40
Pro	74	77	81	73	75
Ser	46	41	42	43	40
Thr	86	85	77	88	83
Tyr	68	62	63	64	63
Val	78	69	70	68	69

detergent in the absence of any reducing agent (Nunn *et al.*, 1989). Disulphide bridges obviously affect the folding of the proteins but not their native molecular weight. The existence of disulphide bridges was determined by assaying the proteins for free-thiols with Ellman's Reagent. No free-thiols could be detected in any of the MDHs both in the native conformation and when unfolded by urea or SDS. This suggests that, in each of the MDHs, all of the cystyl residues are involved in disulphide bridges. It is not possible, however, to state whether the mutant MDHs contain the same bridges as the wild-type enzyme.

4.6. Cross-linking of the wild-type and mutant MDHs with cytochrome c_L

One possible reason for the methanol$^-$ phenotype of MoxA, K and L is that, although MDH of the correct molecular weight is synthesised, the protein may not be folded properly and so cannot interact with the physiological electron acceptor cytochrome c_L. This was tested using 2-stage sulpho-NHS enhanced EDC cross-linking as described in Section 2.26. In these experiments, cytochrome c_L from wild-type bacteria was used, although cytochrome c_L from any of the mutants could have been used since these cytochromes have been shown to cross-link perfectly well with both wild-type MDH and horse-heart cytochrome *c* (Dr. C. Chan, J. Cox, personal communication).

After incubation of the four MDHs with activated cytochrome c_L, cross-linked proteins were analyzed by SDS-PAGE and non-denaturing PAGE. The haem-stained SDS-gel shows a number of bands, the identity of which are indicated (Fig. 4.2.a). Of these bands, perhaps the most significant is that corresponding to the cross-linked MDH α-subunit/cytochrome c_L product, which confirms the finding of Chan and Anthony (1991), and Cox *et al.* (1992) in that this is the physiologically important interaction. The non-denaturing gel stained with Coomassie Blue clearly shows the presence of only three proteins in each of the lanes (Fig. 4.2.b). These proteins were identified as MDH, cytochrome c_L and the MDH/cytochrome c_L cross-linked product. These results clearly show that the mutant MDHs can interact with cytochrome c_L and, hence, that there is no major conformational difference between the wild-type and mutant MDHs in the vicinity of the cytochrome c_L binding site.

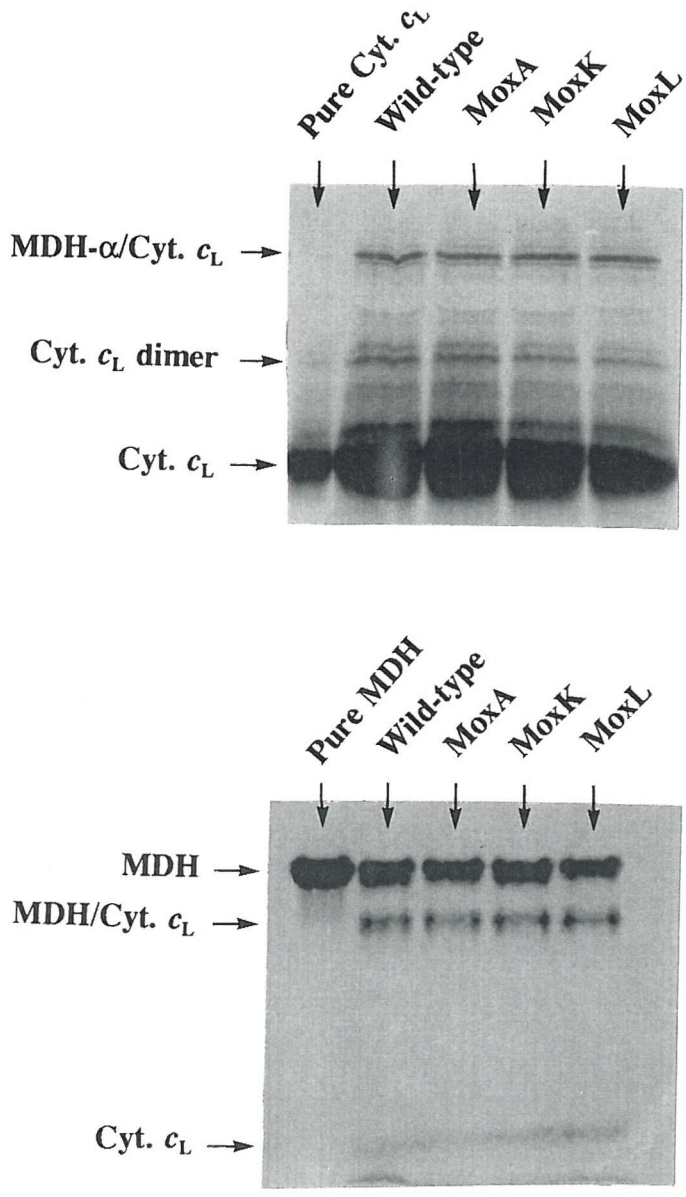


Fig. 4.2. Two-stage EDC/Sulpho-NHS cross-linking of MDH with cytochrome c_L

Cytochrome c_L was activated with EDC (5 mM) and Sulpho-NHS (2 mM) for 30 min in 20 mM Mes buffer (pH 6.0). Activated protein was exchanged into 5 mM Hepes buffer (pH 7.0)using a P-6 column. After buffer exchange, cross-linking was initiated by the introduction of non-activated MDH. After 60 min, the reaction was terminated and samples (10 µg total protein) loaded onto a 13% SDS-PAGE gel and a 5% non-denaturing PAGE gel. After electrophoresis, the SDS-PAGE gel was stained for haem proteins, whereas the non-denaturing PAGE gel was stained with Coomassie blue.

(a) 13% SDS-PAGE gel stained for haem proteins

(b) 5% non-denaturing PAGE gel stained with Coomassie blue

4.7. Dissociation of MDH

The previous sections have shown that the mutant MDHs all contain both α- and β-subunits apparently in the correct stoichiometry and, that with respect to primary structure, they are all identical to the wild-type enzyme. Similarly, they can all interact normally with the physiological electron acceptor cytochrome c_L, yet electron transfer does not occur. The following sections investigate the stability of the MDHs in a variety of denaturing conditions to indicate any gross differences in secondary and tertiary structure that may affect the activity.

4.7.a. Gel filtration of MDH in the presence of SDS

This technique was first used to identify the β-subunit of MDH and determine the subunit stoichiometry (Nunn *et al.*, 1989). Fig. 4.3 shows the elution profile for the MDH from the MoxA mutant. Under identical running conditions, the MDHs from the other strains, including wild-type, gave very similar profiles; the elution volumes for the components of the MDHs are shown in Table 4.3. Integration of the peaks due to the α- and β-subunits showed that, for each MDH, the subunits were present in 1:1 ratio confirming that the MDHs are $\alpha_2\beta_2$ tetramers. The elution profiles also showed that each MDH contained a third component, the prosthetic group. The characterisation of this component in the mutant MDHs is described in the next chapter.

4.7.b. Dissociation of MDH using low pH values

In an early description of MDH (Anthony and Zatman, 1967b), low pH values were used to examine the stability of MDH in a different strain of *Mb. extorquens*. I decided to use identical experiments to investigate the mutant MDHs. The dissociations of the MDHs as a function of pH are shown in Fig. 4.4, and time courses at two pH values shown in Fig. 4.5. These results show that the wild-type MDH is slightly more stable than that observed by Anthony and Zatman (1967b). The mutant MDHs had identical dissociation curves to the wild-type enzyme suggesting that they all have very similar stabilities in this system. Additionally, the time courses were virtually indistinguishable suggesting that the rates of dissociation were very similar. Furthermore, the rates of protein dissociation and 'prosthetic group' release were alike suggesting that these two processes are concomitant.

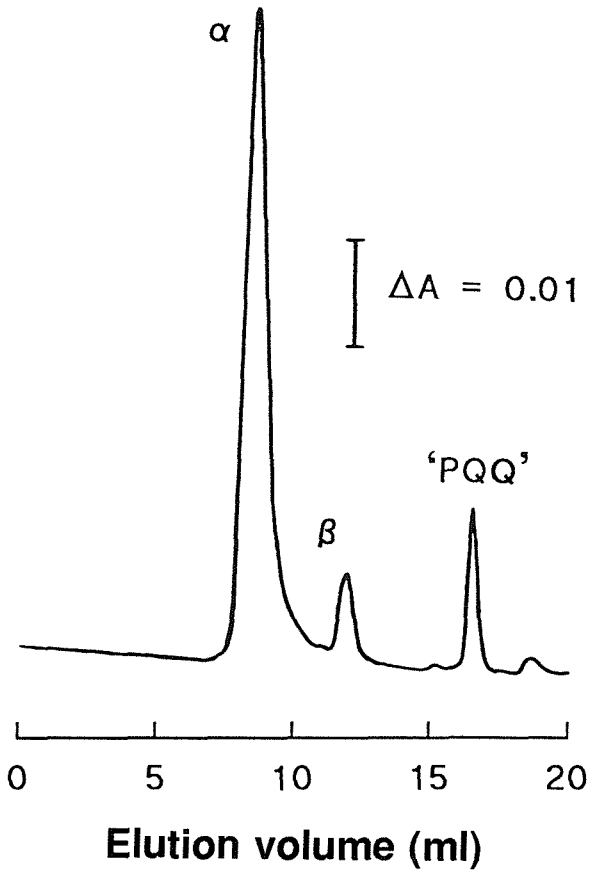


Fig.4.3. Dissociation of MoxA MDH using gel filtration in the presence of SDS

Pure MDH (2 mg) was incubated at 80°C for 30 min in 60 µl of 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 2% SDS. The sample was then loaded onto a Superose-12 column (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 0.2% SDS, and gel filtration performed at a flow rate of 0.3 $ml.min^{-1}$. Peaks were detected by absorbance at 280 nm. Very similar profiles were obtained for the MDHs from wild-type bacteria and the MoxK and L mutant strains.

Table 4.3. Elution volumes for the SDS/gel filtration dissociation components of the wild-type, MoxA, MoxK and MoxL MDHs

MDHs from the different strains were dissociated by gel filtration in the presence of SDS as described in Section 2.19. The prosthetic group of wild-type MDH is known to be PQQ; the characterisation of the prosthetic group from the mutant MDHs is described in Chapter 5. Elution volumes are expressed in ml.

	MDH component		
Source of MDH	α-subunit	β-subunit	Prosthetic group
Wild-type	8.8	12.7	17.6
MoxA	8.8	12.3	17.0
MoxK	8.7	12.4	17.3
MoxL	8.6	12.3	17.1

Fig. 4.4. Dissociation of MDH as a function of pH

Fig. 4.4. Dissociation of MDH as a function of pH

MDHs were dissociated as described in Section 2.20.a. Purified enzyme (3 mg) was dissolved in 3 ml of deionized water and the pH carefully lowered to a value between 2.0 and 6.0 using 0.01 M HCl. After incubation at room temperature for 40 min the pH was raised to 7.5 by the addition of 450 µl of 500 mM potassium phosphate buffer (pH 7.5) and the volume made up to 4.5 ml. Total fluorescence measurements using 3.0 ml of this solution were made at two wavelength pairs: 282 nm excitation/340 nm emission (to detect fluorescence due to tryptophan) and 365 nm excitation/470 nm emission (to detect fluorescence due to PQQ). For the enzyme from wild-type bacteria, a sample was removed and used in the standard dye-linked assay. Measurement of the activity of the MDHs from the mutant strains was not possible.

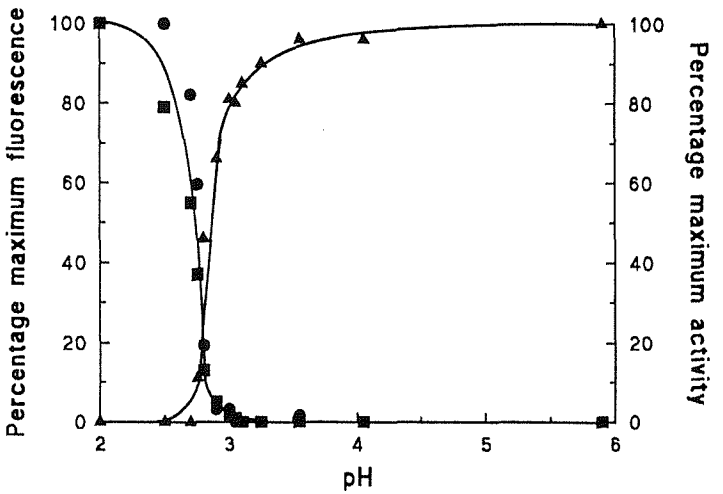
(a), Wild-type MDH; (b), MoxA MDH; (c), MoxK MDH; (d), MoxL MDH.

(■), fluorescence due to tryptophan residues measured at 280 nm excitation/340 nm emission (factor = 3).

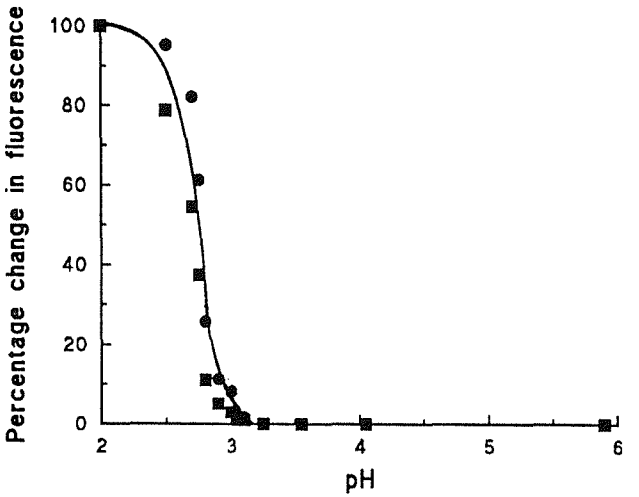
(●), fluorescence due to prosthetic group measured at 365 nm excitation/470 nm emission (factor = 300).

(▲), activity measured in the dye-linked assay.

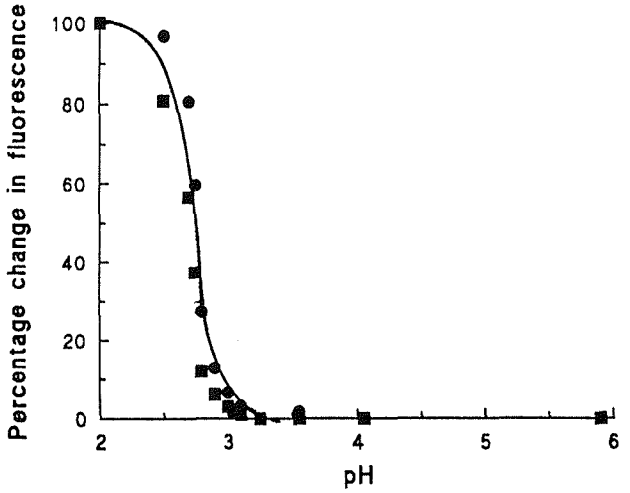
(a) Wild-type



(b) MoxA



(c) MoxK



(d) MoxL

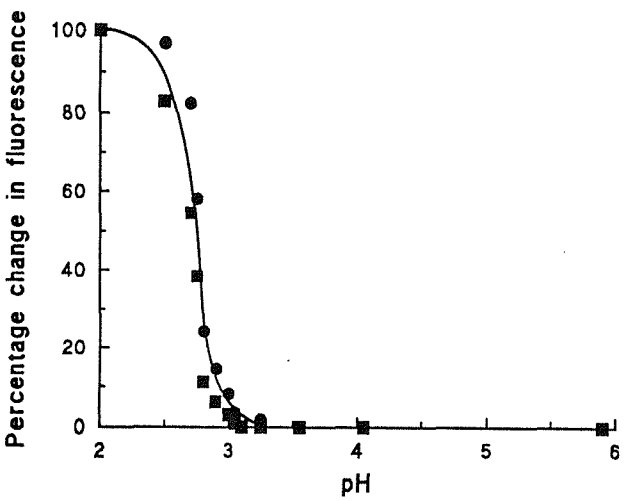


Fig. 4.5. Dissociation of MDH at low pH as a function of time

Fig. 4.5. Dissociation of MDH at low pH as a function of time

MDHs were dissociated as described in Section 2.20.a. To measure dissociation of MDH at low pH values as a function of time, the pH of a purified enzyme solution (2 mg in 40 ml of deionized water) was lowered to pH 2.6 or pH 3.1 as rapidly as possible using 0.01 M HCl. At suitable time intervals 2 ml samples were removed and mixed with potassium phosphate buffer (pH 7.5; final concentration 50 mM) and made up to 3.0 ml with deionized water. Fluorescence and dye-linked activity measurements were made as described in Fig. 4.4.

(a), Wild-type MDH; (b), MoxA MDH; (c), MoxK MDH; (d), MoxL MDH.

(**solid**), dissociation at pH 2.6.

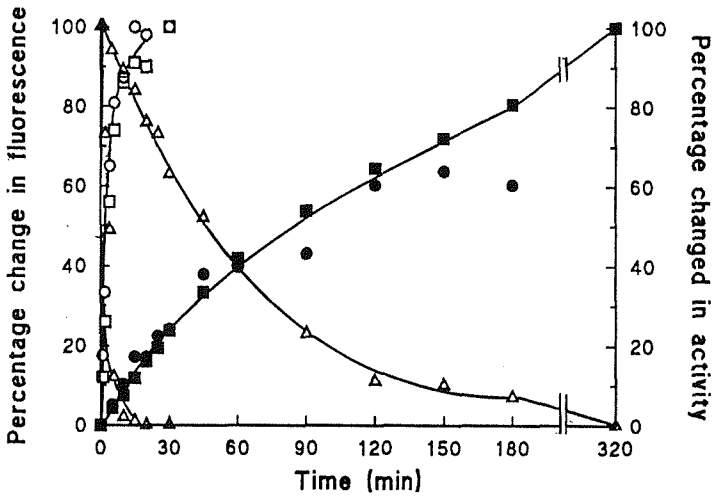
(**open**), dissociation at pH 3.1.

(■/□), fluorescence due to tryptophan residues measured at 280 nm excitation/340 nm emission (factor = 3).

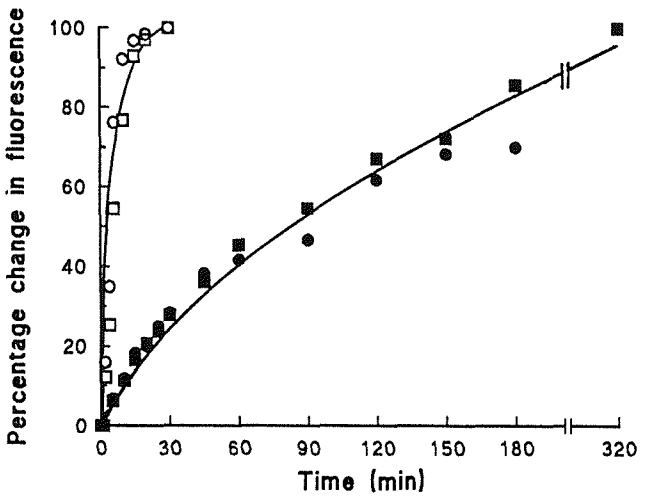
(●/○), fluorescence due to prosthetic group measured at 365 nm excitation/470 nm emission (factor = 300).

(▲/△), activity measured in the dye-linked assay.

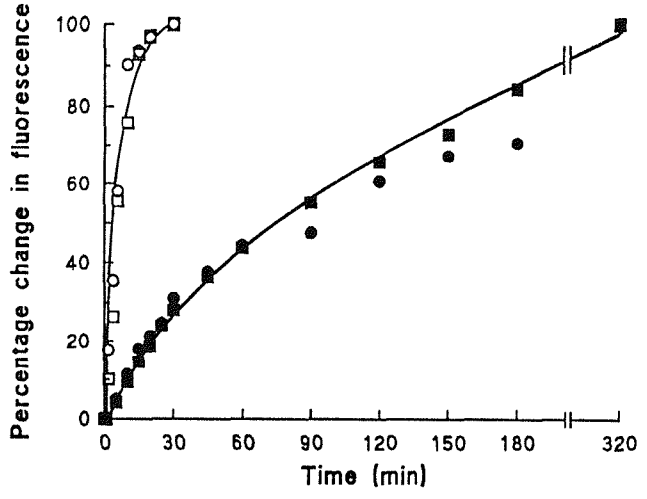
(b) Wild-type



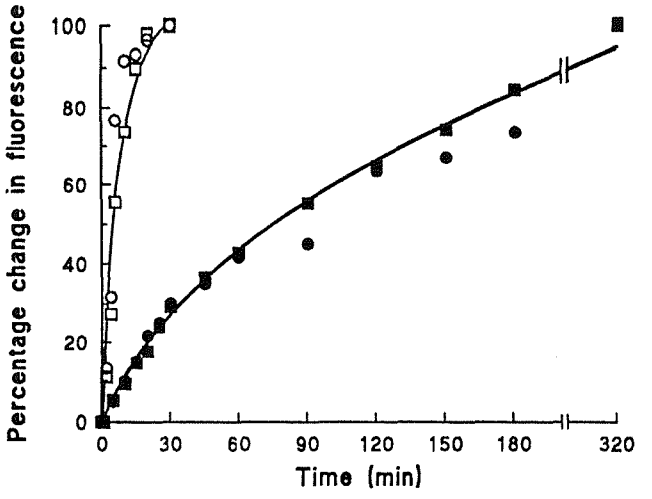
(b) MoxA



(c) MoxK



(d) MoxL



4.7.c. Dissociation of MDH using guanidinium chloride

Guanidinium chloride and urea (next section) are well known chaotropic agents, widely used in experiments to determine the folding characteristics of proteins (Creighton, 1984). It was originally thought that these agents denature proteins simply by their ability to form hydrogen bonds and hence disrupt those that already exist between the protein and water molecules, thereby destabilising the folded structure. This feature is almost certainly important but is not the sole cause of their effectiveness. One theory described by Creighton (1984) suggests that denaturants act indirectly by diminishing hydrophobic interactions between amino acid residues in a uniform, predictable manner, but they may also act directly with the folded and unfolded states producing a wide range of effects, dependent on the local geometry of the interacting groups in the protein. In addition, recently it has been suggested that chaotropic agents can increase the surface tension of water and hence increase the solvation of solutes by direct interaction (Breslow and Guo, 1990).

The experiments described here, and in the next section, were performed for a number of reasons. Firstly to further investigate the stability of the MDHs by using chaotropic agents. Secondly, to destabilise the protein in a controlled fashion, so that either the prosthetic group could be left attached to one or other of the subunits, or the prosthetic group could be removed without complete denaturation of the protein. It would then be possible to determine what features of MDH and/or the prosthetic group are important in the assembly of MDH, in a manner analogous to the well-characterised quinoproteins that have been used in reconstitution studies (see Section 1.6.e).

Although it is generally assumed that when tryptophyl residues move from a hydrophobic environment to a hydrophilic one, the fluorescence intensity decreases and the emission spectrum peak shifts from about 330 nm to 340 nm. However, it is quite normal in protein unfolding experiments to see either an increase in tryptophan fluorescence together with a 'red-shift' (Jaenicke and Rudolph, 1989). With the unfolding of MDH, using both guanidinium chloride and urea, an increase in tryptophan fluorescence and the typical spectral shift were observed.

The dissociation of the MDHs as a function of guanidinium chloride concentration clearly showed very similar unfolding profiles for each of the proteins (Fig. 4.6), supporting the theory proposed in the above section that the mutant MDHs have a similar folded state to that of the wild-type enzyme. Furthermore, time courses at two guanidinium chloride concentrations also showed that release of the 'prosthetic

Fig. 4.6. Dissociation of MDH as a function of guanidinium chloride concentration

Fig. 4.6. Dissociation of MDH as a function of guanidinium chloride concentration

MDHs were dissociated as described in Section 2.20.b. Pure MDH (50 µg) in 100 mM potassium phosphate buffer (pH 7.5) was incubated in the presence of various concentrations of guanidinium chloride (0-6 M) at 25°C for 30 min. The final volume for each of the mixtures was 500 µl. When using MDH from wild-type cells, 100 µl of the incubation mixture was removed for use in the dye-linked assay following incubation. The remainder was diluted 10 fold by the addition of 3.6 ml of the above phosphate buffer and used for fluorescence measurements. When using MDH from the mutant strains, the activity assay was not performed and the samples were diluted 10 fold following incubation by the addition of 4.5 ml of 100 mM potassium phosphate buffer (pH 7.5). Fluorescence measurements were made as described in Fig. 4.4. In order to detect PQQ fluorescence effectively, the sensitivity of the fluorometer was increased 100 fold for the second wavelength pair.

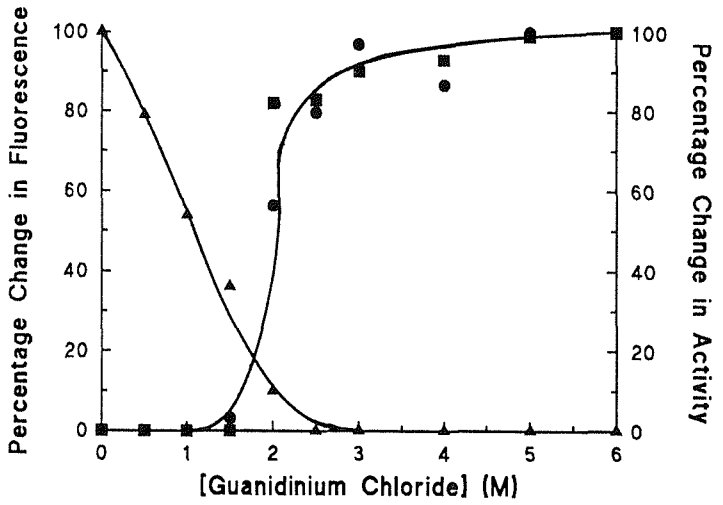
(a), Wild-type MDH; (b), MoxA MDH; (c), MoxK MDH; (d), MoxL MDH.

(■), fluorescence due to tryptophan residues measured at 280 nm excitation/340 nm emission (factor = 3).

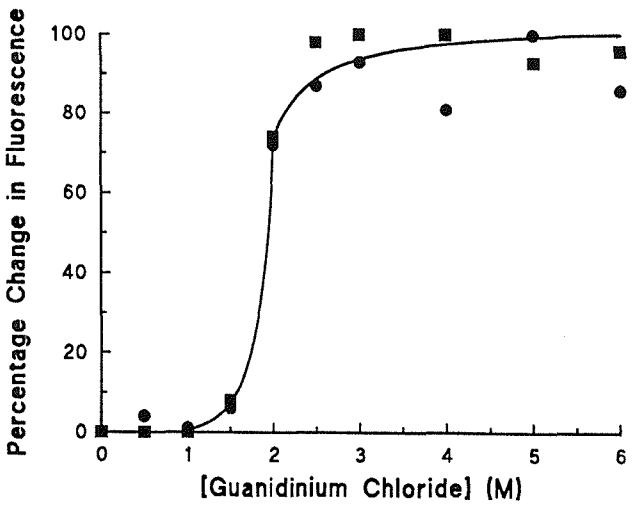
(●), fluorescence due to prosthetic group measured at 365 nm excitation/470 nm emission (factor = 300).

(▲), activity measured in the dye-linked assay.

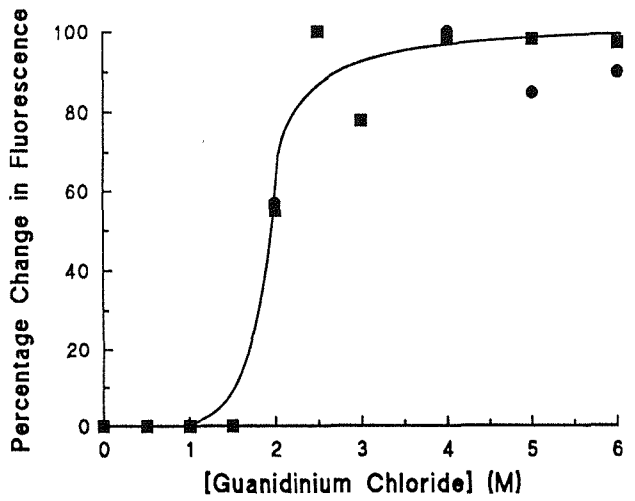
(a) Wild-type



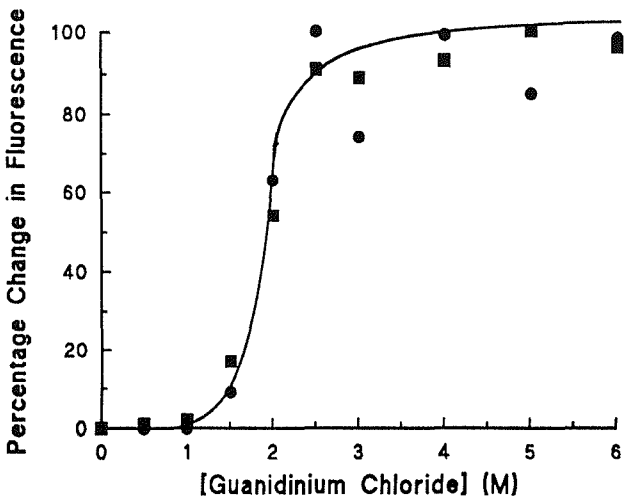
(b) MoxA



(c) MoxK



(d) MoxL



group' occurred at a rate very similar to that of protein unfolding (Fig. 4.7). This suggests that, with guanidinium chloride at least, it is not possible to separate the subunits with the 'prosthetic group' still attached, or to remove prosthetic group without complete denaturation of the protein.

4.7.d. Dissociation of MDH using urea

Guanidinium chloride is a very effective denaturant, and the results presented in Fig. 4.6 showed that complete denaturation of MDH occurred in 30 min with about 2.5 M guanidinium chloride. By contrast, urea is generally much less effective, and so the above experiments were repeated using this milder denaturant.

The dissociations of the MDHs as a function of urea concentration are shown in Fig. 4.8 and time courses at 2 urea concentrations shown in Fig. 4.9. These results clearly indicate that complete denaturation only occurred at 8 M urea. That the values obtained at this concentration were maximal was confirmed by incubating identical samples of the MDHs with 8 M urea overnight at 25°C. Just as with guanidinium chloride, the MDHs all gave very similar dissociation profiles and virtually identical rates of dissociation. This further is consistent with the observation that MDH cannot be dissociated into anything other than its three components;
α-subunit, β-subunit and prosthetic group.

With all of the dissociation protocols employed in this work, inactivation of the wild-type MDH occurred at a slightly faster rate than either detectable protein unfolding or detectable release of prosthetic group.

4.8. Is the assembly of MDH mediated or can the enzyme self-assemble?

In the denaturation experiments with guanidinium chloride, MDH was incubated with 4-6 M guanidinium chloride, and after incubation for some time, the sample was diluted 10-fold with buffer to give sufficient volume for the fluorescence measurements (a minimum of 3 ml was required), thus bringing the final guanidinium chloride concentrations to 0.4-0.6 M. Since very little protein unfolding was observed with initial concentrations (ie before dilution) of 1 M or less, the dilution effectively removed the denaturing effect of guanidinium chloride. During this dissociation with guanidinium chloride, an unexpected observation was made. The fluorescence due to protein unfolding in the diluted samples, as expected had increased. However, when these diluted samples were left for a few hours and more measurements made, the

Fig. 4.7. Dissociation of MDH by guanidinium chloride as a function of time

Fig. 4.7. Dissociation of MDH by guanidinium chloride as a function of time

MDHs were dissociated as described in Section 2.20.b. To measure the dissociation of MDH by guanidinium chloride as a function of time, purified MDH (4 mg) in a final volume of 40 ml of potassium phosphate buffer (pH 7.5) was incubated with denaturant at 25°C. Guanidinium chloride was used at final concentrations of 2 and 3 M and was the final reagent to be added. At regular intervals, 3.0 ml of the solution was removed and used for fluorescence and activity measurements as described in Fig. 4.4.

(a), Wild-type MDH; (b), MoxA MDH; (c), MoxK MDH; (d), MoxL MDH.

(solid), dissociation at 2 M guanidinium chloride.

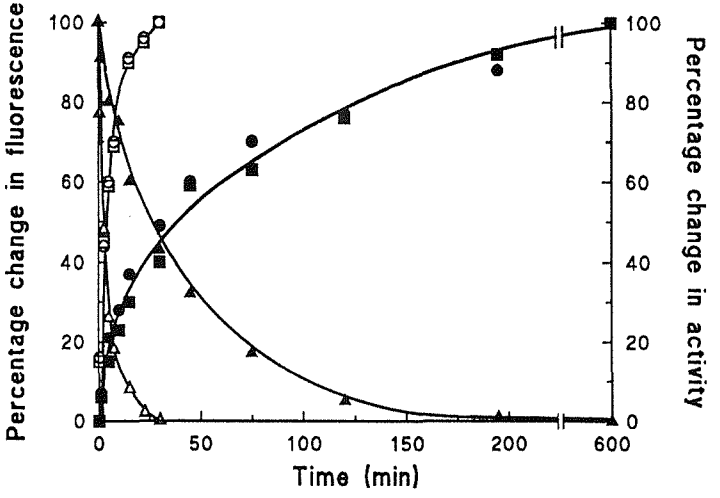
(open), dissociation at 3 M guanidinium chloride.

(■/□), fluorescence due to tryptophan residues measured at 280 nm excitation/340 nm emission (factor = 3).

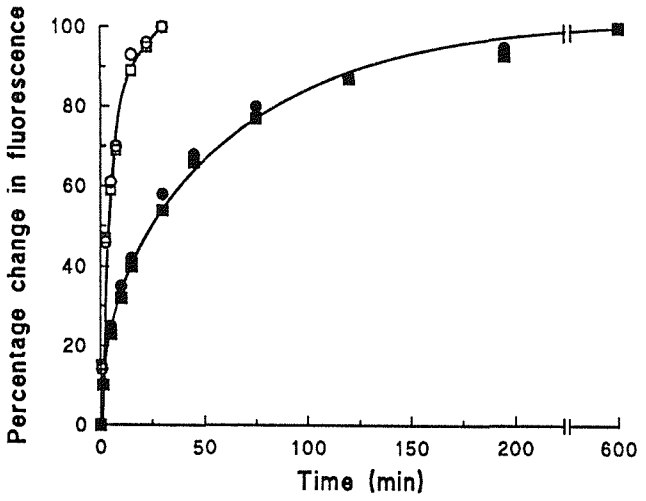
(●/○), fluorescence due to prosthetic group measured at 365 nm excitation/470 nm emission (factor = 300).

(▲/△), activity measured in the dye-linked assay.

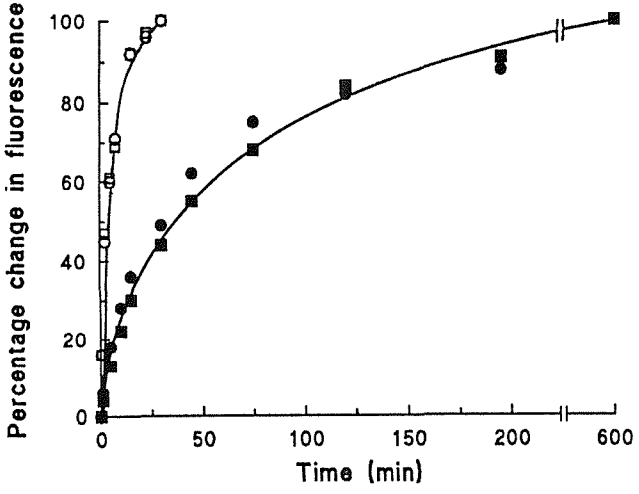
(a) Wild-type



(b) MoxA



(c) MoxK



(d) MoxL

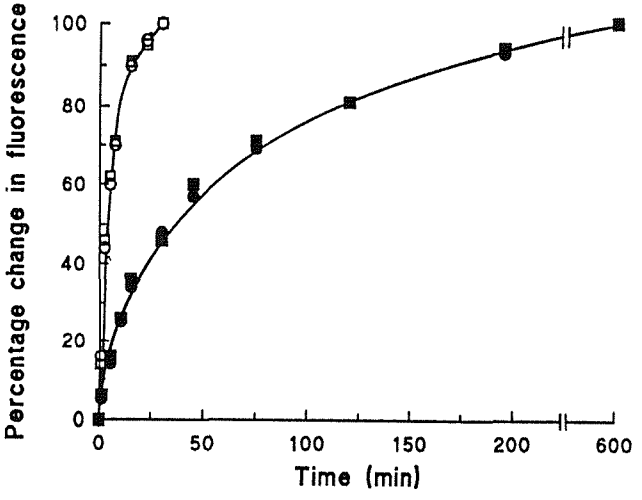


Fig. 4.8. Dissociation of MDH as a function of urea concentration

Fig. 4.8. Dissociation of MDH as a function of urea concentration

MDHs were dissociated as described in Section 2.20.b. Pure MDH (50 µg) in 100 mM potassium phosphate buffer (pH 7.5) was incubated in the presence of various concentrations of urea (0-8 M) at 25°C for 30 min. The final volume for each of the mixtures was 500 µl. When using MDH from wild-type cells, 100 µl of the incubation mixture was removed for use in the dye-linked assay following incubation. The remainder was diluted 10 fold by the addition of 3.6 ml of the above phosphate buffer and used for fluorescence measurements. When using MDH from the mutant strains, the activity assay was not performed and the samples were diluted 10 fold following incubation by the addition of 4.5 ml of 100 mM potassium phosphate buffer (pH 7.5). Fluorescence measurements were made as described in Fig. 4.4. In order to detect PQQ fluorescence effectively, the sensitivity of the fluorometer was increased 100 fold for the second wavelength pair.

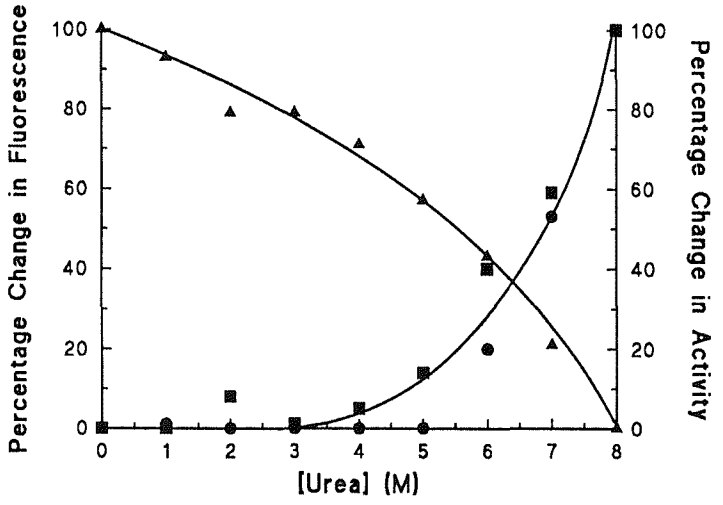
(a) Wild-type MDH; (b), MoxA MDH; (c), MoxK MDH; (d), MoxL MDH.

(■), fluorescence due to tryptophan residues measured at 280 nm excitation/340 nm emission (factor = 3).

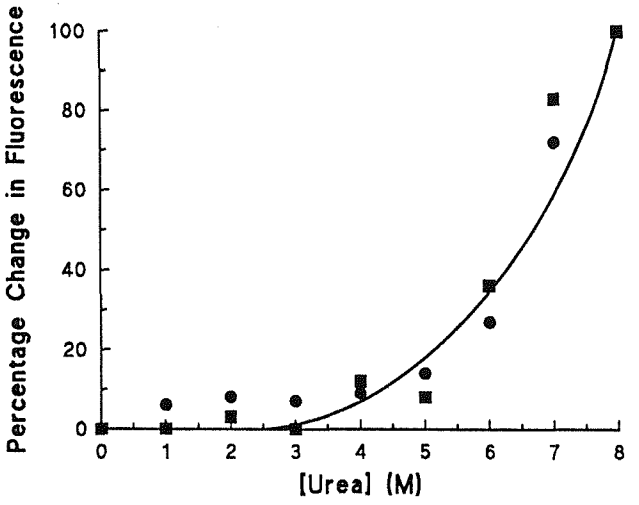
(●), fluorescence due to prosthetic group measured at 365 nm excitation/470 nm emission (factor = 300).

(▲), activity measured in the dye-linked assay.

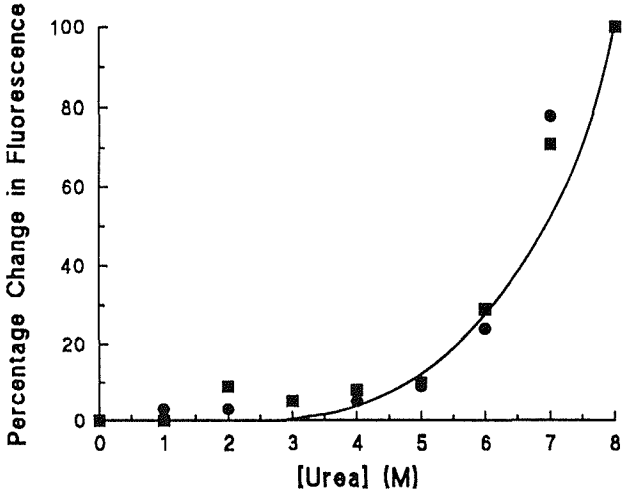
(a) Wild-type



(b) MoxA



(c) MoxK



(d) MoxL

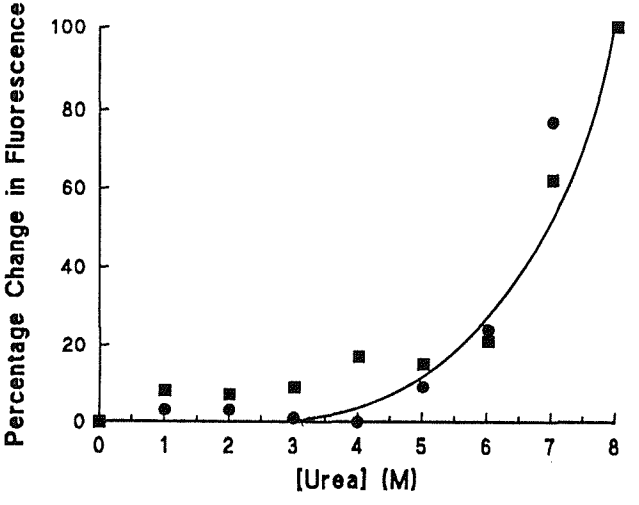


Fig. 4.9. Dissociation of MDH by urea as a function of time

Fig. 4.9. Dissociation of MDH by urea as a function of time

MDHs were dissociated as described in Section 2.20.b. To measure the dissociation of MDH by urea as a function of time, purified MDH (4 mg) in a final volume of 40 ml of potassium phosphate buffer (pH 7.5) was incubated with denaturant at 25°C. Urea was used at final concentrations of 5 and 7 M and was the final reagent to be added. At regular intervals, 3.0 ml of the solution was removed and used for fluorescence and activity measurements as described in Fig. 4.4.

(a), Wild-type MDH; (b), MoxA MDH; (c), MoxK MDH; (d), MoxL MDH.

(solid), dissociation at 5 M urea.

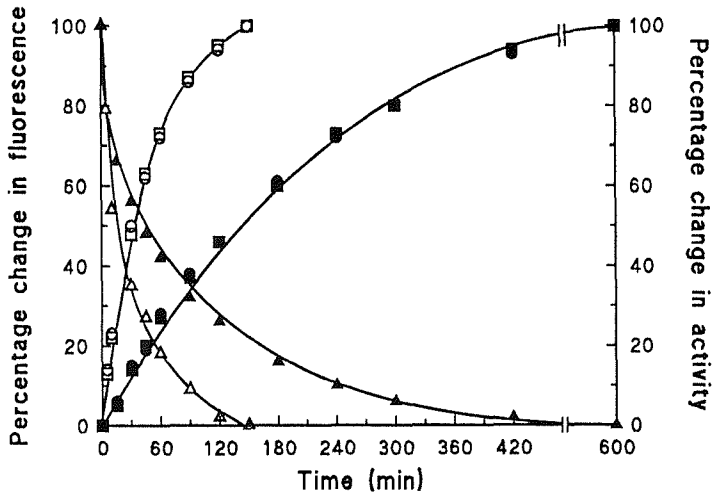
(open), dissociation at 7 M urea.

(■/□), fluorescence due to tryptophan residues measured at 280 nm excitation/340 nm emission (factor = 3).

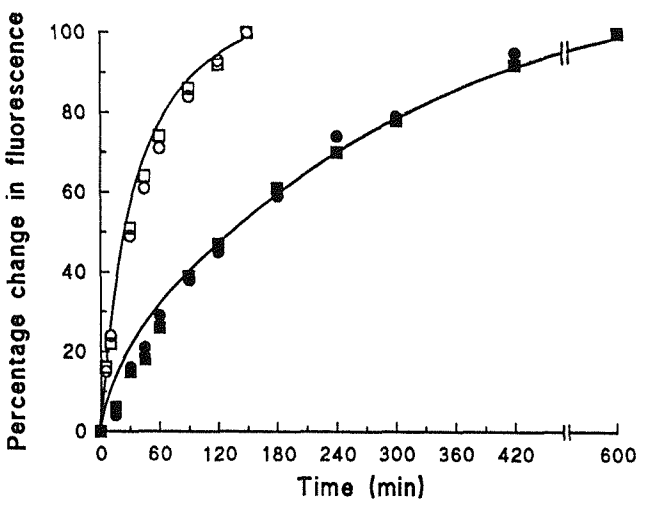
(●/○), fluorescence due to prosthetic group measured at 365 nm excitation/470 nm emission (factor = 300).

(▲/△), activity measured in the dye-linked assay.

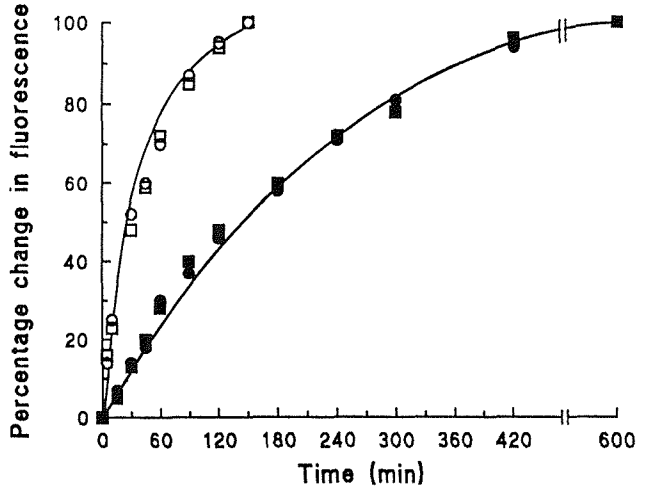
(a) Wild-type



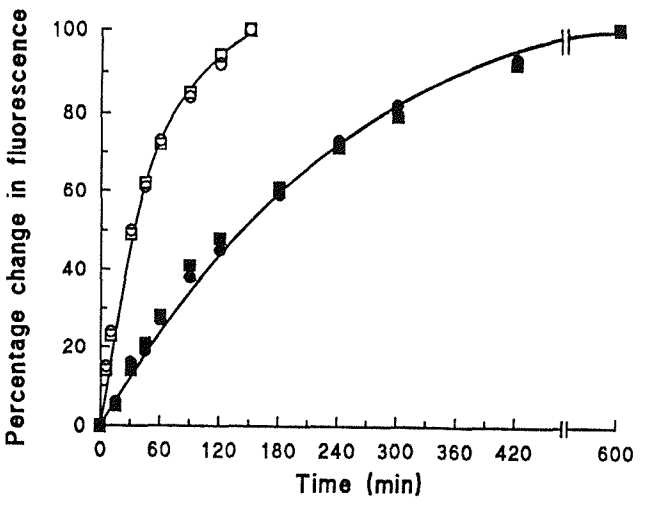
(b) MoxA



(c) MoxK



(d) MoxL



fluorescence was seen to have decreased in intensity to a value lower than that seen for the folded enzyme (in the control experiment), whereas the fluorescence due to the prosthetic group had remained constant. This feature was seen for all MDHs, and was not restricted to any one source, indicating that the observation was not an artefact. Knowing that an increase in protein fluorescence accompanies, in the case of MDH, protein unfolding, it was concluded that the subsequent decrease in protein fluorescence was due to refolding of the MDH. That the fluorescence due to the prosthetic group remained at a constant level suggests that it was not spontaneously incorporated into the folding enzyme.

In order to determine whether MDH was refolding to a native state, a number of attempts were made to isolate the protein after denaturation, dilution and further incubation. In all of these experiments, PQQ was included at a concentration of 10 mol PQQ/mol MDH. The major problem with this type of experiment is that the protein concentration needs to be quite low (< 100 $\mu g.ml^{-1}$) to avoid incorrect aggregation/association, and this is especially true for multimeric proteins (Jaenicke and Rudolph, 1989). Using wild-type MDH, several variations were attempted in order to isolate the refolded enzyme. These included varying the following; the initial concentration of guanidinium chloride (3-6 M), the length of incubation time for unfolding (30 min - 2 h), the extent of dilution (10-, 20- or 30-fold), the length of incubation following dilution (12, 18 or 24 h). The concentration of diluted MDH was kept constant in all of these experiments at 50 $\mu g.ml^{-1}$. After incubation of the diluted samples, they were concentrated using a centricon-30 micro-concentrator (Amicon), and the buffer exchanged to 10 mM potassium phosphate buffer (pH 7.0) using a P-6 column (Bio-Rad). Unfortunately, a stable protein could not be isolated. It is not clear why a stable protein could not be obtained, but is most likely to be due to the treatment employed after dilution of the unfolded MDH. This treatment was necessary to remove all traces of guanidinium chloride and to give a sufficiently high protein concentration so that the sample could be analyzed by PAGE and absorption spectrophotometry.

An alternative approach to test the theory of MDH self-assembly was tried using spheroplasts of *Paracoccus denitrificans* grown on methanol. It was hoped that the intact spheroplasts would be stable enough to continue synthesising and exporting the MDH subunits. Spheroplasts were prepared as described in Section 2.7 and separate samples resuspended in either fresh growth medium or 20 mM Hepes buffer

(pH 7.0); both suspensions were stabilised by the presence of 500 mM sucrose, and PQQ was added to a final concentration of 1 μM. At regular intervals, samples of the suspensions were removed and analyzed by SDS-PAGE and assayed for dye-linked activity. In neither suspension was there a measurable increase in MDH activity or was there detectable level of α- or β-subunit in the SDS-PAGE gels. This failure was probably due to the spheroplasts being too 'shocked' by the treatment in preparation such that all protein synthesis had stopped. Of course it is possible that MDH cannot self-assemble and requires some mediating factor, such as a molecular chaperone, which could be encoded by the *moxA*, *K* and/or *L* genes, to produce active enzyme.

4.8.a. The molecular chaperone concept

Molecular chaperone is a relatively recent term used to describe proteins that mediate the correct folding of other proteins, including oligomerisation into polymers, but are not themselves components of the final structures; for thorough reviews see Ellis and Hemmingsen (1989), Ellis (1990, 1991). One of the earliest examples of a chaperone (although the term had not been coined at the time) were the so-called scaffold-proteins that are essential for the correct assembly of T-even bacteriophage heads (see King *et al.*, 1980 for a thorough review). Once the icosahedral head has formed, the scaffold-proteins are released to allow packaging of DNA and addition of the tail and plate regions. The main progress in the understanding of chaperone action has been made using ribulose bisphosphate carboxylase-oxygenase (rubisco), which is a 16-mer consisting of 8 large and 8 small subunits, assembled by two chaperones called GroEL and GroES (Gatenby and Ellis, 1990). GroEL and GroES, sometimes referred to as chaperonin (cpn-) 60 and cpn-10, have been found in virtually all systems studied, in both eukaryotes and prokaryotes, indicating that they are ubiquitous (Zeilstra-Ryalls *et al.*, 1991). GroEL functions by forming a large complex, GroEL as a 14-mer, with a newly translated protein, and holds it in a stable conformation. This complex then binds GroES, as a 7-mer, and the correctly folded protein is released with the hydrolysis of ATP (Gatenby and Ellis, 1990). The manner in which chaperones, in general, work is to bind aliphatic residues in the nascent polypeptide, accommodating them in an environment equivalent to the interior of a folded protein (Flynn *et al.*, 1991), either in a helical conformation (Landry and Gierasch, 1991) or in the so-called 'molten globule' conformation (Martin *et al.*, 1991).

I decided to test for the presence of a chaperone function in the Mox mutants

of *Mb. extorquens* using two approaches. Firstly, a crude extract of the MoxF mutant (which does not synthesise MDH, but has unaltered *moxA*, *K* and *L* genes) was mixed with crude extracts of the MoxA, K and L mutants (which do have MDH). A similar experiment was performed using unfolded wild-type MDH added to the MoxF mutant crude extract. In both of these experiments, an excess of PQQ was added and the presence of native MDH tested by measuring dye-linked activity of the mixtures. However, no activity could be detected, even after prolonged incubation. This suggests that there is some other factor necessary for MDH assembly that was not present in the crude extract mixtures. In view of the fact that GroEL is one of the best characterised chaperones, the second approach used was to investigate crude extracts of Mox mutants for the presence of GroEL by Western blotting. Antibodies raised against GroEL were gifts from Professor J. Ellis, University of Warwick (*E. coli* GroEL) and Dr. R. Lathigra, Hammersmith Hospital (*Mycobacterium leprae* GroEL). Fig. 4.10 shows the Western blot of all Mox mutant crude extracts probed with the *My. leprae* GroEL antibodies; an identical staining pattern was observed with the *E. coli* GroEL antibodies. This blot clearly shows the presence of a cross-reactive band corresponding to GroEL in all of the mutants tested. This band was not due to MDH, even though GroEL has roughly the same mobility in this system as the α-subunit of MDH, since some of the mutants do not synthesise the enzyme (Nunn and Lidstrom, 1986b). A similarly sized band was observed in the original phenotypic characterisation of the Mox mutants, and was referred to as the 'common antigenic protein' (CA protein) present in all Gram -ve bacteria (Jensen *et al.*, 1985; Nunn and Lidstrom, 1986b). It is possible that this CA protein was GroEL, although there is no conclusive evidence for this assumption. Although GroEL is a soluble protein, analysis of any of the known gene sequences reveals the absence of a signal peptide sequence, indicating that the protein is cytoplasmic and, hence unlikely to be involved in MDH assembly. There is very little knowledge about specific periplasmic chaperones and only two have described that are known not to be located in the cytoplasm. These are the pilus assembly protein, PapD, which is periplasmic but so far only identified in *E. coli* (Holmgren and Branden, 1989, Hultgren *et al.*, 1991), and the binding protein, BiP, which is a member of the heat shock protein 70 class of chaperones and found in the lumen of endoplasmic reticulum (Pelham, 1989). In view of the lack of knowledge about periplasmic chaperones, the possibility that one or all of *moxA*, *K* and *L* encodes such a protein cannot be ruled out, and this is discussed further in Chapter 6.

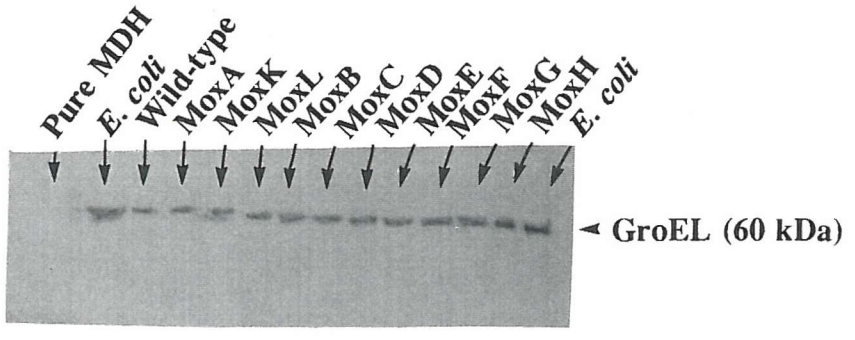


Fig. 4.10. Western blot of crude extracts of Mox mutants of *Methylobacterium extorquens* probed with antibodies raised against GroEL from *Mycobacterium leprae*

Wild-type and Mox mutants of *Mb. extorquens* were grown in liquid culture on methanol and methylamine. The *E. coli* sample is the crude extract of the HB101 strain transformed with pND5 (a plasmid that encodes GroEL; Section 2.31). This organism was grown on L-broth, cells harvested and resuspended in 10 mM Hepes buffer (pH 7.0), and the suspension heat-shocked at 42°C for 10 min to induce synthesis of GroEL. Crude extracts of all organisms were prepared as described in Section 2.6, and 50 µg of each loaded onto a 13% SDS-PAGE gel. Electrophoresis (Section 2.14.c) and Western blotting (Section 2.15) were performed exactly as described in the relevant sections. The lanes are labelled according to the sample.

4.9. Absorption spectra of MDH

The absorption spectra of the MDHs from MoxA, K and L have previously been shown to be altered in the region attributed to the prosthetic group (Fig. 1.20). These spectra led the authors to propose that the *moxA*, *K* and *L* genes were involved in either PQQ biosynthesis/modification or apo-MDH-PQQ processing (Nunn and Lidstrom, 1986b). The published spectra only showed the region between 300 and 400 nm, omitting both the important shoulder at 400 nm that is also due to the prosthetic group, and the peak (due to aromatic residues) at 280 nm. This peak is useful as a rough measure of the purity and activity of the protein; for pure MDH, the 345 nm peak height is typically 10% of that for the 280 nm peak (Duine *et al.*, 1978) and, as the enzyme ages and becomes inactive, this value decrease to about 5% of the 280 nm peak height. Analysis of the absorption spectra can also give information about the redox state of the protein (Frank *et al.*, 1988).

Fig. 4.11 shows the absorption spectra of the MDHs from wild-type bacteria and the MoxA, K and L mutants. It should be noted here that the spectra of the mutant MDHs shown in Fig 4.11 are examples; just as with specific activity, the spectra varied from batch to batch, although there was a general trend in that the peak due to the prosthetic group was always between 350 nm and 360 nm (that is 5-15 nm higher than that seen in wild-type MDH). These spectra are clearly different from those obtained by Nunn and Lidstrom (1986b), and are different from published spectra of the known redox states of MDH from *Hyphomicrobium* X (Frank *et al.*, 1988). The spectra of the mutant MDHs, however, do have several important features. Firstly, all of the spectra have a typical 400 nm shoulder. Secondly, the presence of a peak between 300 nm and 400 nm in the spectrum of the MoxA MDH confirms the presence of a prosthetic group in this protein; Nunn and Lidstrom (1986b) claimed that the enzyme from the MoxA strain was the apo-form of MDH due to the absence of a peak in this region. Thirdly, the absorbance at 345 nm for the mutant MDHs was between 5% and 6% of that at 280 nm; this is in keeping with the inactive character of these proteins. The final feature of the spectra is the presence in the mutant MDHs of a broad, low absorption band at 520 nm, which is absent from the spectrum of the wild-type enzyme. This absorption band gives the mutant MDHs a red colour; wild-type MDH is normally olive green. However, red wild-type MDH, which also had the 520 nm peak, has been observed previously in this laboratory after the protein had become inactive following prolonged storage at -20°C (Day, 1990). The differences

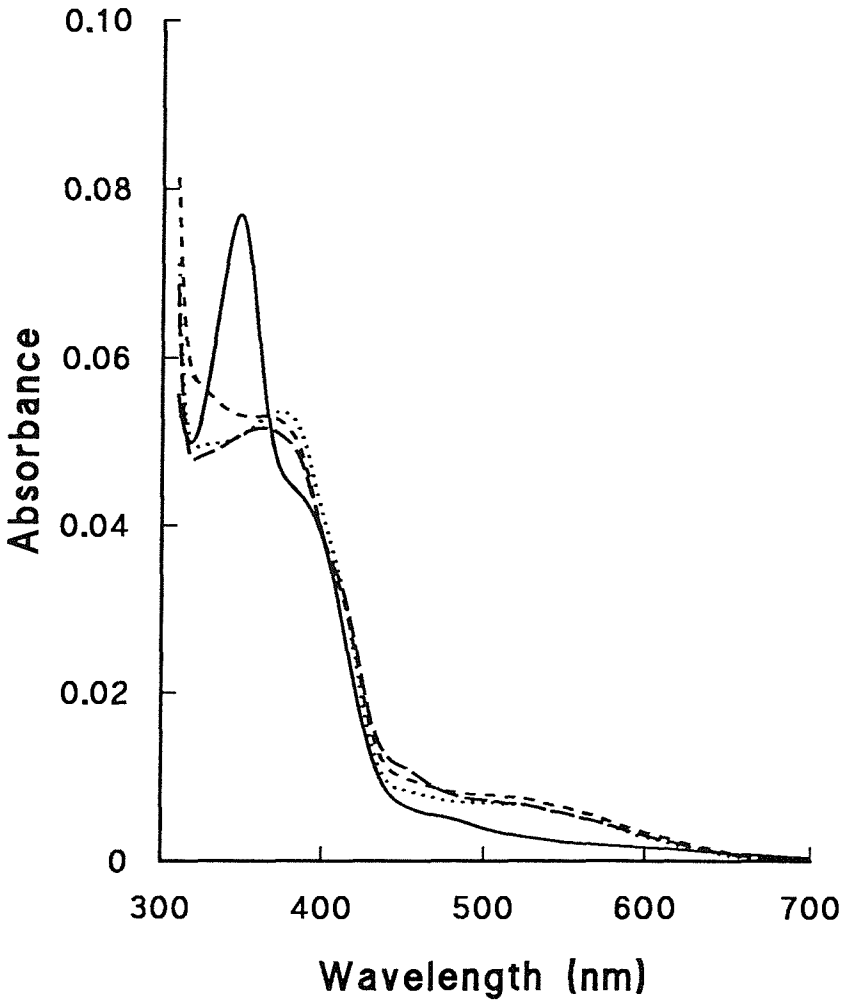


Fig. 4.11. Absorption spectra of wild-type, MoxA, MoxK and MoxL MDHs

MDHs were purified as described in Section 2.8 and spectra recorded of the enzymes (1 $mg.ml^{-1}$) in 100 mM potassium phosphate buffer (pH 7.0) using the SLM-Aminco DW2000 UV/Vis spectrophotometer as described in Section 2.17.a. The spectra of the MDHs from the MoxA, K and L mutants shown here are examples; there was significant batch-to-batch variation. ———, wild-type MDH; – – –, MoxA MDH; - - - -, MoxK MDH; ·····, MoxL MDH.

between the absorption spectra of the mutant MDHs published by Nunn and Lidstrom and those observed in this work were not due to reversion; complementation of the mutants showed that I was working with the correct strains.

4.9.a. The effect of methanol and buffer pH on absorption spectra

MDH has been shown previously to be isolated in a partially reduced form that contains the semiquinone form of PQQ (Duine *et al.*, 1981). Addition of either activator (ammonia) or substrate (methanol) does not affect the spectrum (Anthony and Zatman, 1967a; Duine and Frank, 1980a; Anthony, 1992c), neither does the pH of the buffer (Dekker *et al.*, 1982). This is because the free-radical form of PQQ is unable to directly accept reducing equivalents from the substrate. By contrast, the spectral characteristics of free PQQ do change in the presence of methanol and are dependent on the pH, temperature and nature of the buffer (Dekker *et al.*, 1982). The different absorption spectra in the mutant MDHs might indicate that the prosthetic group may be in a different redox state. If it is in the oxidised state, then the prosthetic group should be able to react directly with substrate, assuming that it is functionally analogous to PQQ if it is not authentic PQQ. An alternative theory is that the prosthetic group in the mutant MDHs is bound in a different configuration than that in the wild-type enzyme. In this case, the spectral characteristics of the mutant enzymes may be affected by buffer pH. The addition of methanol (5 mM) had no noticeable effect on the absorption spectra of the mutant MDHs. By contrast, the pH of the buffer had a marked effect on the spectra of the mutant MDHs. When transferred from a neutral pH buffer into 100 mM tetrasodium pyrophosphate buffer (pH 9.0) or 100 mM sodium borate buffer (pH 9.0), the absorption band at 520 nm was more prominent and the peak between 350 nm and 360 nm was altered (Fig. 4.12). Additionally, with one batch of MoxL MDH, the absorption spectrum was seen to change when the protein was transferred from 20 mM potassium phosphate buffer (pH 7.0) to 20 mM Mes buffer (pH 5.5). In this case, the 520 nm peak disappeared and the peak due to the prosthetic group was shifted to about 385 nm (Fig. 4.13). However, this effect was only seen with the one batch of protein from MoxL, and was not seen for any other batches of protein from this or the other two mutants. In all cases, when the MDHs were returned to potassium phosphate or Hepes buffers at pH 7.0, the original spectrum was restored. With the wild-type MDH, there was no effect on the spectrum when the protein was transferred to buffer at pH 5.5 or pH 9.0.

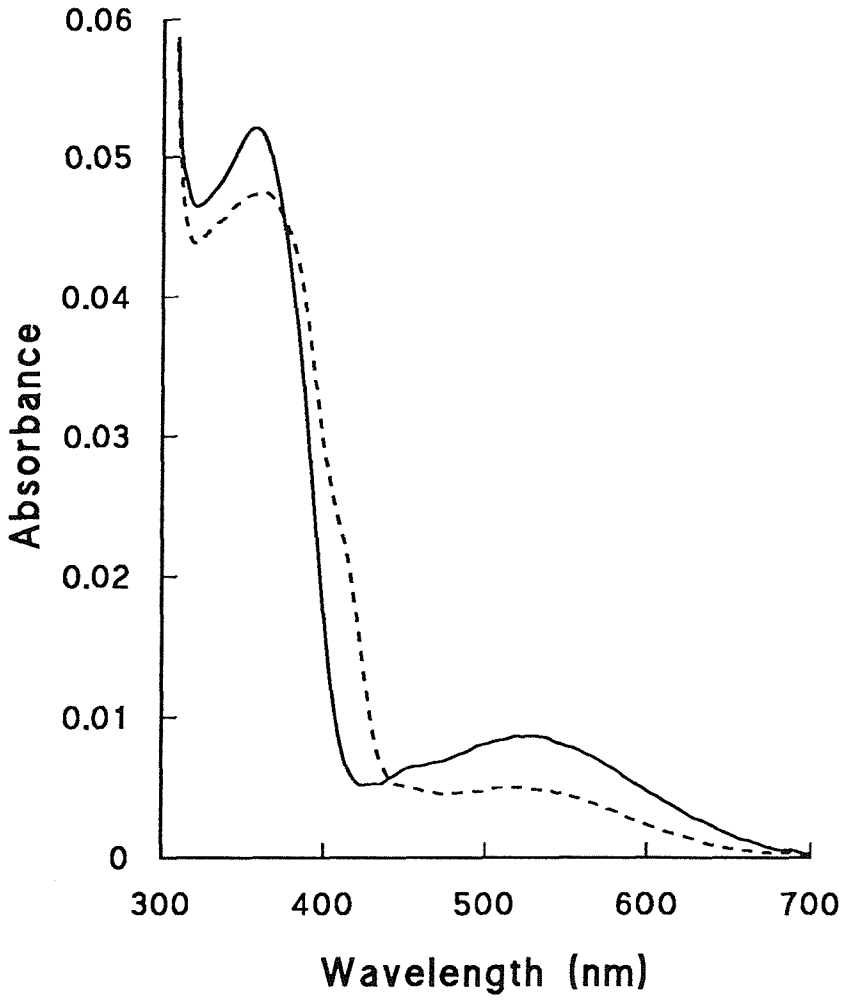


Fig. 4.12. Absorption spectra of MoxA MDH in potassium phosphate and sodium borate buffers

MDH from the MoxA mutant was purified as described in Section 2.8 and transferred into 100 mM potassium phosphate buffer (pH 7.0) or 100 mM sodium borate buffer (pH 9.0) by rapid gel filtration on P-6 columns equilibrated with the same buffers. Spectra of the MDHs (1 $mg.ml^{-1}$) were recorded on the SLM-Aminco DW2000 UV/Vis spectrophotometer as described in Section 2.17.a. Very similar spectra were recorded for the MDHs from the MoxK and MoxL mutant strains. ——, MoxA MDH in sodium borate buffer; ----, MoxA MDH in potassium phosphate buffer.

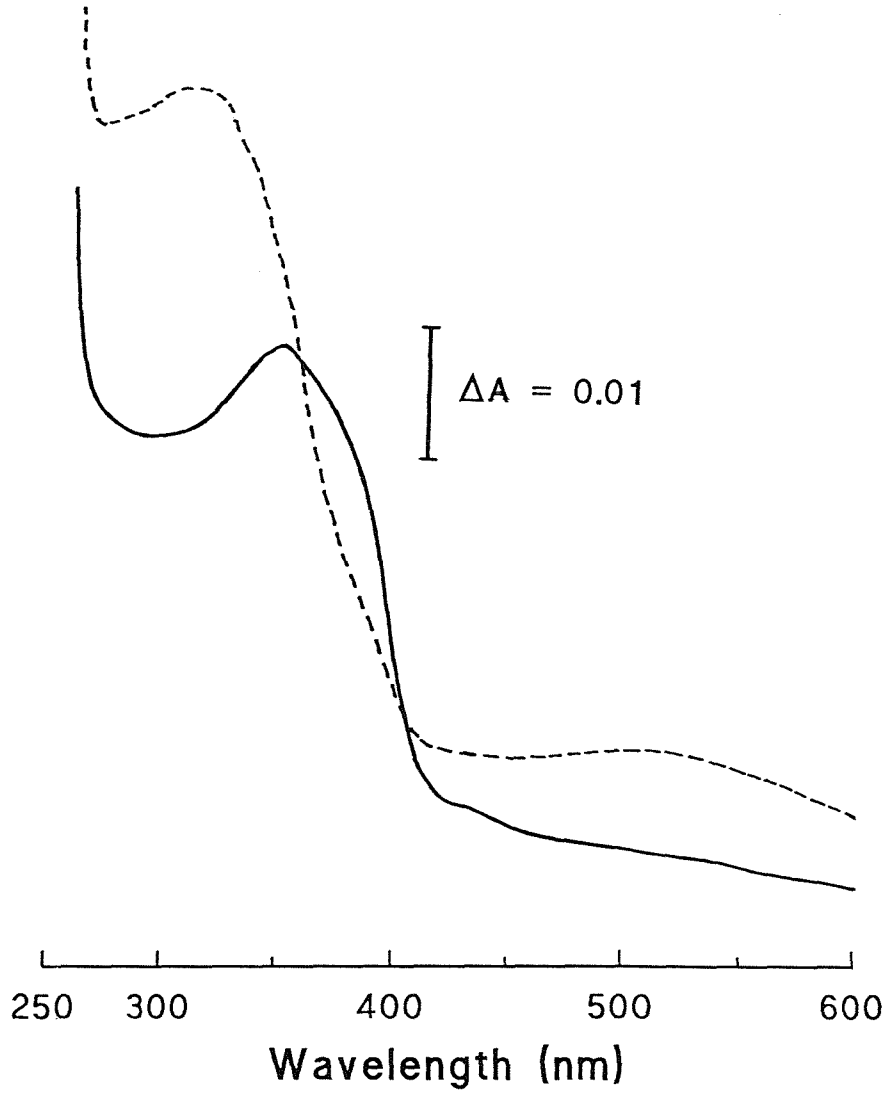


Fig. 4.13. Absorption spectra of MoxL MDH in potassium phosphate and Mes buffers

MDH from the MoxL mutant was purified as described in Section 2.8 and transferred into 100 mM potassium phosphate buffer (pH 7.0) or 20 mM Mes buffer (pH 5.5) by rapid gel filtration on P-6 columns equilibrated with the same buffers. Spectra of the MDH samples were recorded on the Shimadzu UV-3000 spectrophotometer at a concentration of 1 $mg.ml^{-1}$ as described in Section 2.17.a. ——, MoxL MDH in Mes buffer; ----, MoxL MDH in potassium phosphate buffer.

These results suggest that the prosthetic group in the mutant MDHs can be influenced by pH and possibly by the nature of the buffer salt. Fig. 4.14 shows the absorption spectrum of PQQ in a borate buffer at pH 9.0. Comparison of this spectrum with that of a mutant MDH in the same borate buffer shows that both spectra have an absorption maximum at about 350 nm. Knowing that water and some buffer salts (notably borate) readily form adducts with free PQQ and affect the spectral characteristics (Dekker *et al.*, 1982), it is possible that the prosthetic group in the mutant MDHs is bound in such a way to allow interaction with these compounds, and hence give rise to the observed spectral changes.

4.10. Fluorescence spectra of MDH

Wild-type MDH has only its typical protein fluorescence, due to aromatic amino acid residues (most notably tryptophan), and has no fluorescence that can be attributed to PQQ (Fig. 4.15; Anthony and Zatman, 1967b). This observation is in keeping with the proposal that PQQ lies in a hydrophobic environment (de Beer *et al.*, 1983) and the knowledge that PQQ is only fluorescent when hydrated (Dekker *et al.*, 1982). The above section has suggested that the absorption spectrum of the mutant MDHs can be affected by the buffer. Hence it is possible, again assuming that the prosthetic group in the mutant MDHs is authentic PQQ, that these proteins may display a fluorescence signal other than that due to aromatic residues. If the prosthetic group is modified PQQ, it may also be aromatic and display a characteristic fluorescence. However, when the fluorescence spectra of the mutant MDHs were recorded, they were identical to that seen for the wild-type enzyme and no fluorescence besides that due to the aromatic amino acids could be detected, even when the samples were examined using the excitation and emission maxima of PQQ. This result suggests two possibilities. Firstly, that the prosthetic group in the mutant MDHs is not authentic PQQ and is not fluorescent or, secondly, that the prosthetic group is authentic PQQ, but is present in an environment that does not permit hydration, and hence give rise to PQQ fluorescence. Thus the changes observed in the absorption spectra may be because the prosthetic group in the mutant MDHs is normal PQQ but present in a different environment.

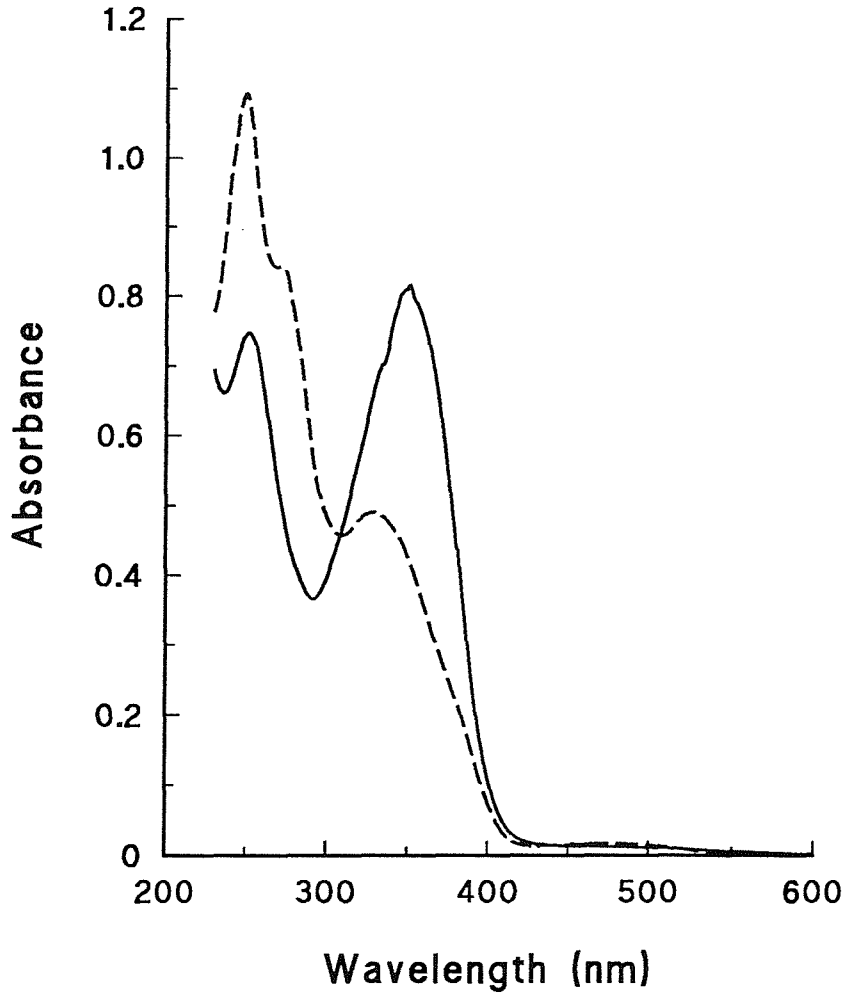


Fig. 4.14. Absorption spectra of PQQ in potassium phosphate and sodium borate buffers

PQQ (50 µM) was dissolved in 100 mM potassium phosphate buffer (pH 7.0) and in 100 mM sodium borate buffer (pH 9.0), and spectra recorded on the SLM-Aminco DW2000 UV/Vis spectrophotometer as described in Section 2.17.a. The spectrum of PQQ in sodium borate buffer shown here is identical to that previously published (Dekker *et al.*, 1982). ——, PQQ in sodium borate buffer; ----, PQQ in potassium phosphate buffer.

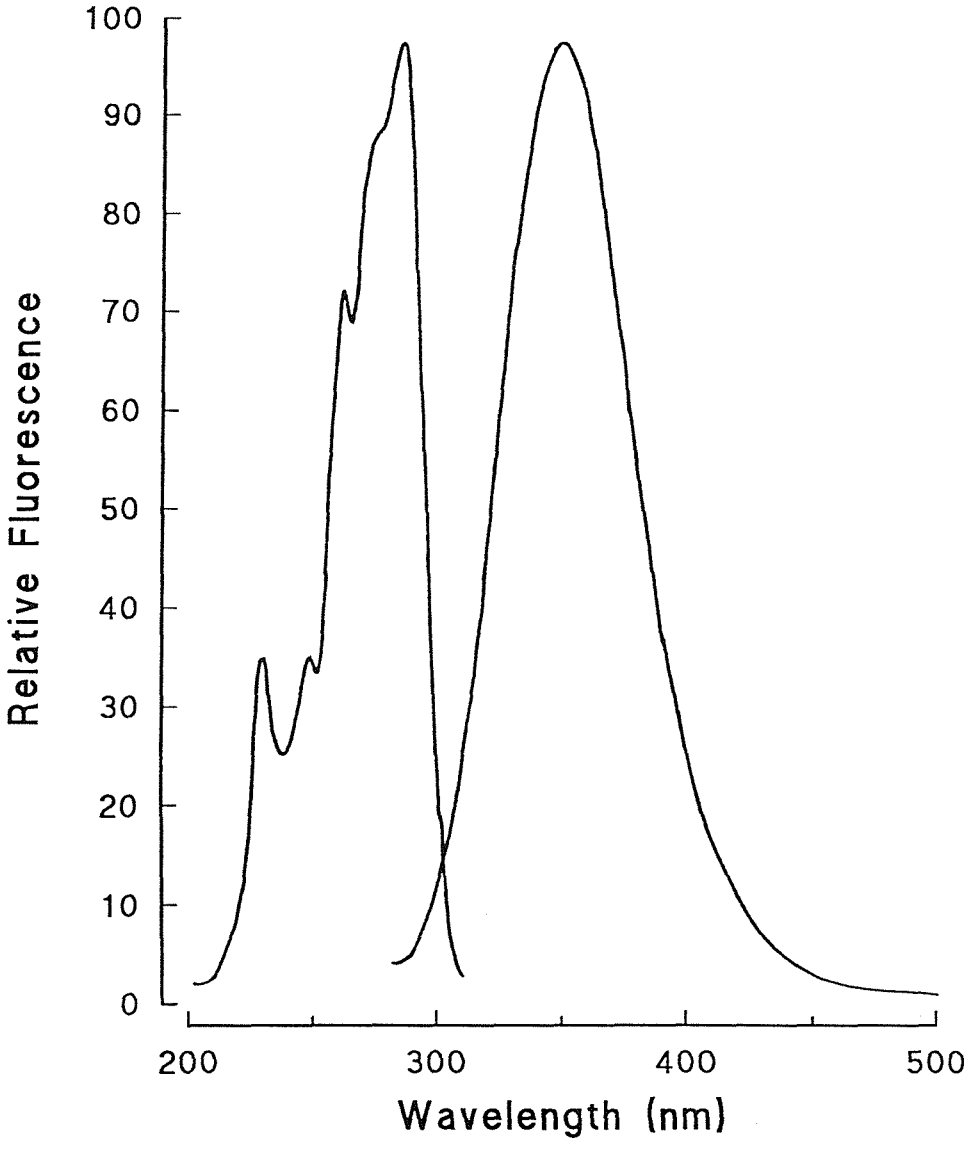


Fig. 4.15. Fluorescence spectra of wild-type MDH

MDH was purified as described in Section 2.8. Fluorescence spectra of a 30 $\mu g.ml^{-1}$ solution were recorded using an expansion factor of 1. The excitation spectrum was recorded at 330 nm emission; the emission spectrum was recorded at 280 nm excitation. Virtually identical spectra were recorded for the MDHs from the MoxA, K and L mutant strains.

4.11. Oxidation of MDH and reaction with cyclopropanol

The oxidised form of MDH is the only one which can react with substrate. This is because this form of MDH contains the fully oxidised quinone form of PQQ, which is the only redox species able to accept the two hydrogens (as reducing equivalents) from methanol. The isolation of the oxidised form of MDH from *Hyphomicrobium* X has been reported by Duine and Frank (1980a), and was seen to have a characteristic absorption spectrum (Fig. 1.14). By contrast, an investigation by Beardmore-Gray (1984) using the MDH from *Methylophilus methylotrophus* did not yield the oxidised form; instead, following the same procedure as Duine and Frank, the spectrum of the treated enzyme appeared identical to that before treatment. I repeated these experiments with the MDHs from *Mb. extorquens* in order to investigate the redox species of the wild-type enzyme and to test whether the redox state of the prosthetic group in the mutant MDHs could be affected.

A number of attempts were made to oxidise wild-type MDH using the method of Duine and Frank (1980a), and subsequently a modification of this method described by Dijkstra *et al.* (1984). Unfortunately, both of these methods failed to yield the oxidised form of MDH. Varying the buffer (sodium borate or tetrasodium pyrophosphate at pH 9.0 and potassium phosphate at pH 7.0), and changing the mediating dyes (PES/PIP or Wurster's blue) also failed. Although the spectra of the treated MDH at first appeared to be the same as that of the untreated enzyme, closer investigation of the spectra showed that there was a small but significant difference (Fig. 4.16); the spectrum of the treated MDH was very similar to that published for the fully reduced form of MDH, MDH_{red} (Section 1.8; Frank *et al.*, 1988). This result suggests that the MDH had been oxidised by the artificial electron acceptor, but had then been rapidly reduced even though methanol had not been added. This is not surprising since both methanol and formaldehyde are common contaminants of most biological buffers, and Wurster's blue readily degrades at pH 9.0 releasing formaldehyde which can also act as a substrate for MDH (Dr. J. Frank, personal communication). By contrast with wild-type MDH, treatment of the mutant enzymes did not result in any noticeable change in spectrum, suggesting that the redox state of these proteins cannot be altered by treatment with artificial electron acceptors.

The generation of oxidised MDH is important in the treatment of the enzyme with the 'suicide substrate' cyclopropanol. This compound, which is a primary alcohol, inhibits MDH by forming an irreversible adduct with the C-5 carbonyl group of PQQ,

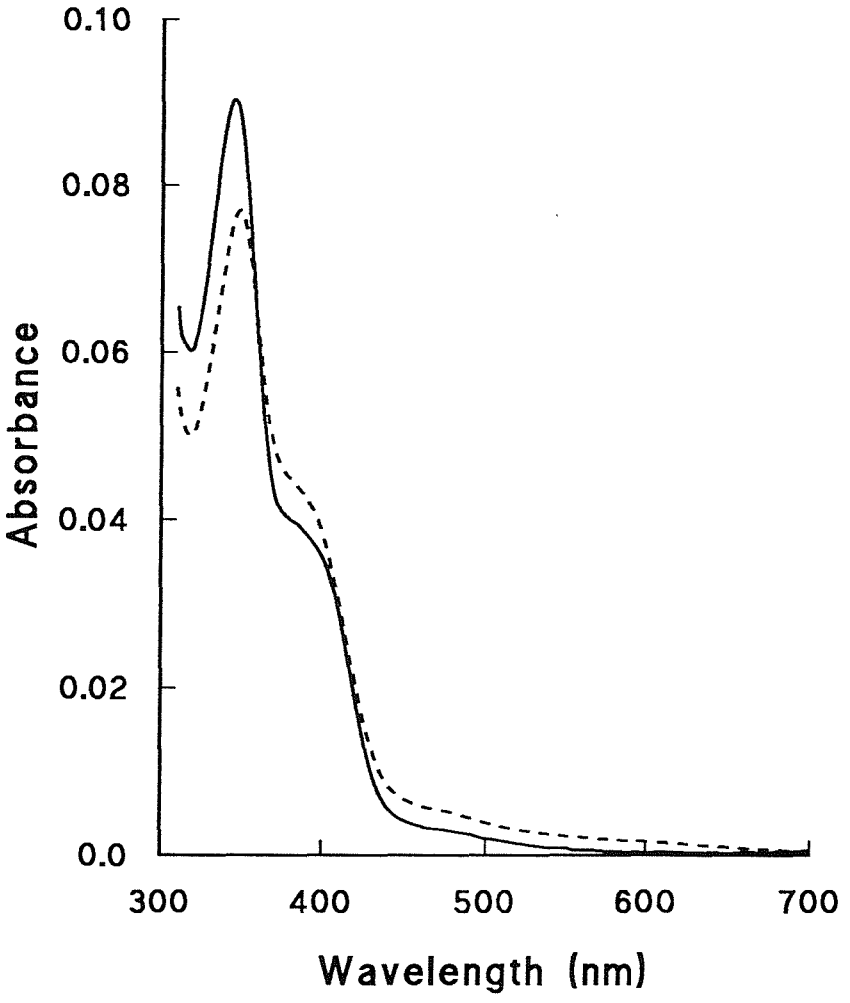


Fig. 4.16. Absorption spectra of wild-type MDH before and after oxidation of the enzyme with artificial electron acceptor

The oxidation of wild-type MDH was attempted as described in Section 2.23. Enzyme was transferred into 100 mM tetrasodium pyrophosphate buffer (pH 9.0) containing 20 mM NH_4Cl using a P-6 column equilibrated in the same buffer. After buffer exchange, the MDH (10 µM) was mixed with KCN (10 mM) and Wurster's Blue (20 µM). To oxidise endogenous substrate and substrate present in the buffer, 0.1 µl aliquots of 100 mM $K_3Fe(CN)_6$ were added until the blue colour persisted. After oxidation, the mixture was passed down a P-6 column equilibrated in 100 mM potassium phosphate buffer (pH 7.0) and an absorption spectrum recorded. ——, MDH after attempted oxidation; ----, MDH before attempted oxidation.

but only when the enzyme, and hence PQQ, has been oxidised (see Section 1.8.d). As expected, unoxidised wild-type MDH did not react with cyclopropanol. After oxidation, however, treatment of wild-type MDH with cyclopropanol, in either sodium borate or tetrasodium pyrophosphate buffer, resulted in an inactive enzyme with a spectrum very similar to that published previously for the cyclopropanol-treated and oxidised form of MDH from *Hyphomicrobium* X (Fig. 4.17; Duine and Frank, 1980a; Dijkstra *et al.*, 1984; Frank *et al.*, 1988). The inhibition of *Mb. extorquens* MDH differed from that of the *Hyphomicrobium* X enzyme, in that the rate of inhibition was quicker and gave 100% inhibition (Fig. 4.18; Dijkstra *et al.*, 1984). Treatment of the mutant MDHs, however, did not result in the enzymes having altered spectra, whether the MDHs were oxidised or not. This suggests that the prosthetic group in the mutant MDHs is unable to react with substrate, either because it cannot be oxidised, or because it exists in a different arrangement so that cyclopropanol cannot be acted on by the base at the active site or, after reaction with the base, the ring-opened form of cyclopropanol cannot attack the prosthetic group.

4.12. Circular dichroism spectroscopy

Previous sections have shown that there are no gross conformational differences between the MDHs from wild-type bacteria and the MoxA, K and L mutant strains, and the differences that were observed between these proteins appear to be related to the prosthetic group. One possibility is that the prosthetic group in the mutant MDHs is bound differently from that in the wild-type enzyme, due to a small but significant change in the secondary structure. The experiments using denaturing agents were too crude to be able to detect any subtle changes that may exist, and so it was decided to specifically investigate the secondary structure using circular dichroism (CD) spectrophotometry. CD is a powerful technique in the study of proteins because it can reveal important information about conformation; the relative amounts of α-helix, β-sheets (parallel and anti-parallel) and β-turns can be determined with very reasonable accuracy (see Brahms and Brahms, 1980; Provencher and Glöckner, 1981; Compton and Johnson, 1986). CD relies on the presence of chiral carbon atoms, and values, in units of molar elipticity ($deg.cm^2.decimole^{-1}$), are a measure of the difference between left- and right-rotated light at the given wavelength.

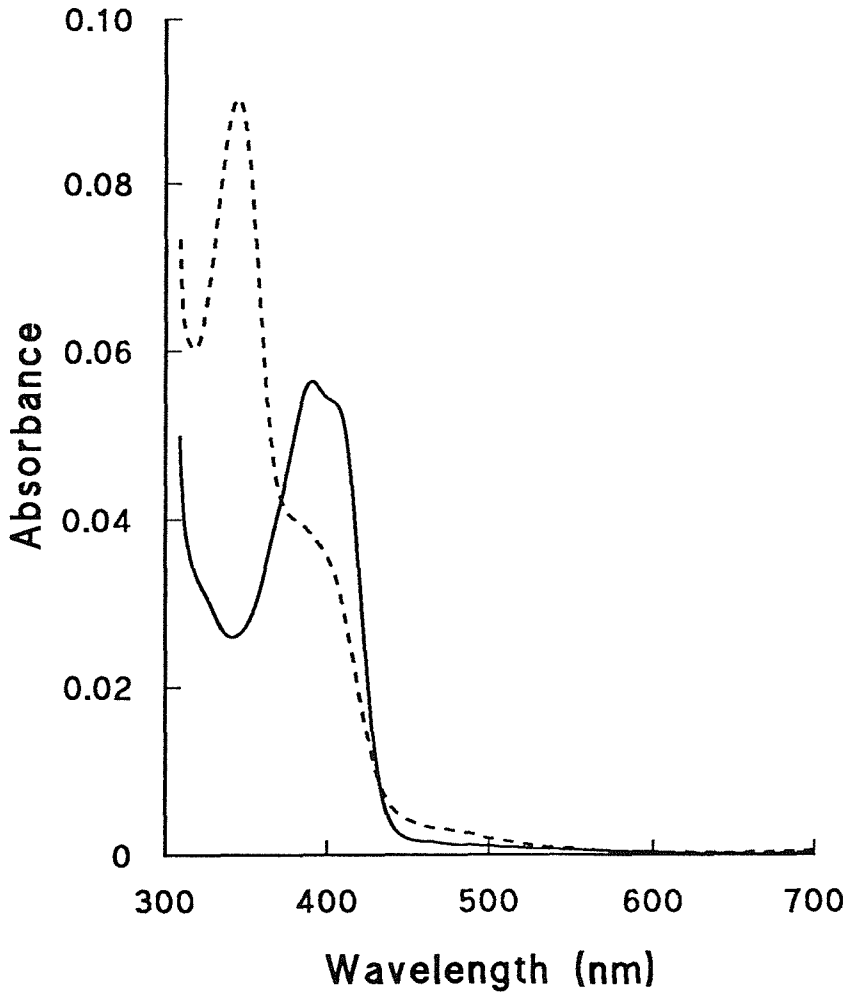


Fig. 4.17. Absorption spectrum of wild-type MDH after treatment with cyclopropanol

MDH was reacted with cyclopropanol using the method described by Dijkstra *et al.* (1984). MDH (10 µM) in a total volume of 1 ml of 100 mM sodium borate (pH 9.0) containing 20 mM NH_4Cl, 10 mM KCN and 20 µM Wurster's blue was oxidised by the addition of 0.2 µl aliquots of 100 mM $K_3Fe(CN)_6$ until the blue colour persisted. At this time, 200 µM (10-fold excess) cyclopropanol was added. The mixture was then left for 1 h at room temperature to react, adding more ferricyanide if necessary to keep the MDH oxidised. Low molecular weight compounds were removed by passage through a P-6 column (Bio-Rad) equilibrated with 100 mM potassium phosphate buffer (pH 7.0), and an absorption spectrum recorded. ——, MDH treated with cyclopropanol; ----, MDH as isolated.

Fig. 4.18. The inhibition of wild-type MDH by cyclopropanol in sodium borate and tetrasodium pyrophosphate buffers, both at pH 9.0

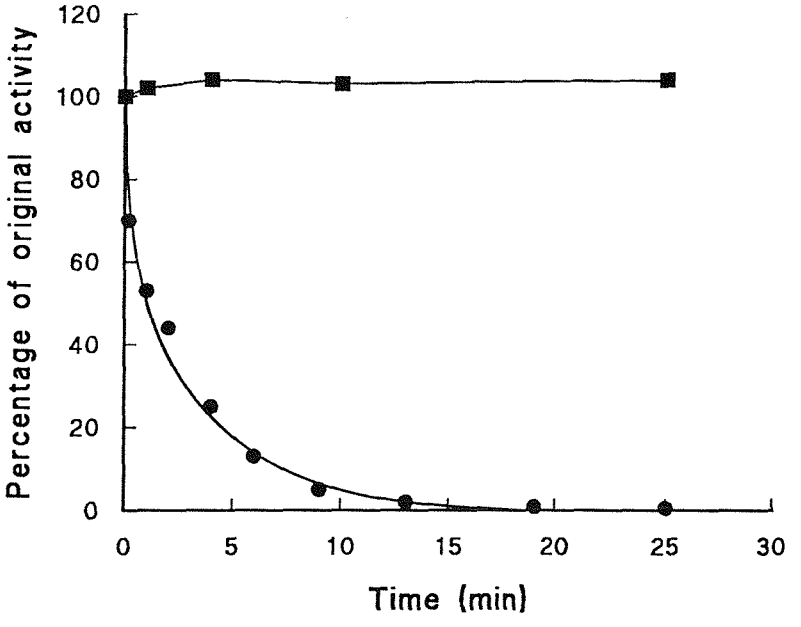
Fig. 4.18. The inhibition of wild-type MDH by cyclopropanol in sodium borate and tetrasodium pyrophosphate buffers, both at pH 9.0

MDH was reacted with cyclopropanol using the method described by Dijkstra *et al.* (1984). MDH (10 µM) in a total volume of 1 ml of 100 mM sodium borate buffer (pH 9.0), or 100 mM tetrasodium pyrophosphate buffer (pH 9.0), both containing 20 mM NH_4Cl, 10 mM KCN and 20 µM Wurster's blue was oxidised by the addition of 0.2 µl aliquots of 100 mM $K_3Fe(CN)_6$ until the blue colour persisted. At this time, 20 µM cyclopropanol was added. The mixture incubated at 20°C. At regular intervals, samples (25 µl) were removed and used to measure activity in the standard dye-linked assay. Control experiments were performed by preparation of identical reaction mixtures; after addition of $K_3Fe(CN)_6$, water was added instead of cyclopropanol.

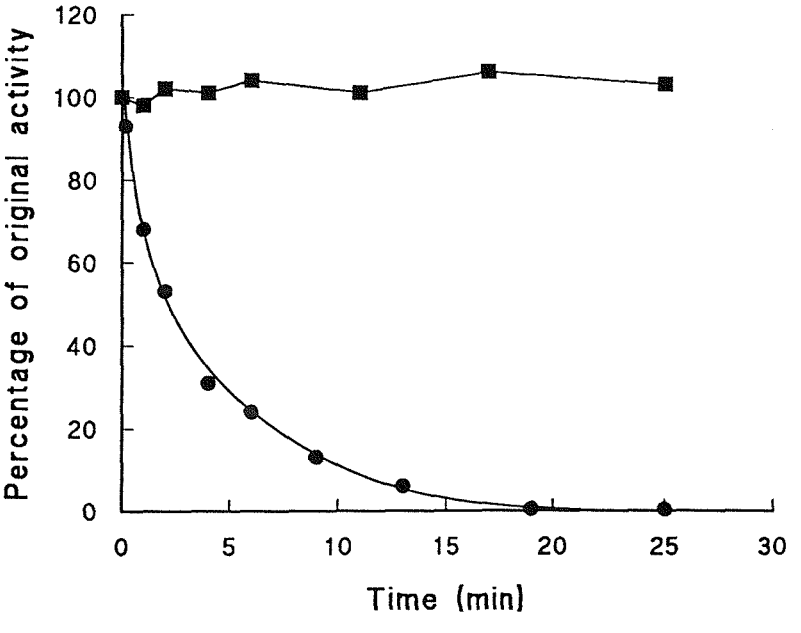
(a) Reaction in sodium borate buffer
(●), cyclopropanol-treated MDH; (■), control

(b) Reaction in tetrasodium pyrophosphate buffer
(●), cyclopropanol-treated MDH; (■), control

(a)


Percentage of original activity
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (min)

(b)


Percentage of original activity
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (min)

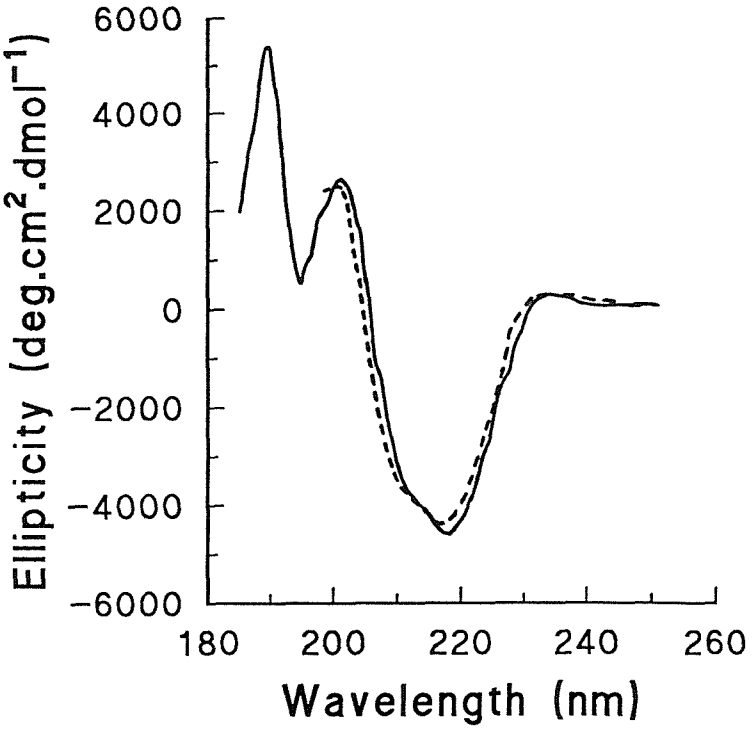
The CD spectra of the MDHs from wild-type and the MoxK mutant are shown in Fig. 4.19; it was not possible to scan the mutant enzymes beyond 190 nm due to poor signal to noise ratios. Similar spectra to that of MoxK MDH were observed for the MDHs from the MoxA and MoxL mutants. The spectra of the MDHs are very similar in the region between 185 nm and 250 nm and show that MDH is predominantly a β-protein (Sears and Beychok, 1973). Signals in this region are due to the peptide backbone and any variations would indicate a significant difference in secondary structure of the protein. It can be concluded, therefore, that, in terms of secondary structure, there are no gross differences between the MDHs from wild-type bacteria and the MoxA, K and L mutants. The spectra do, however, reveal a difference in the near-UV region, between 250 nm and 350 nm. The spectrum of wild-type MDH shows two negative ellipticity maxima at 290 nm and 300 nm which are diminished considerably in the spectra of the mutant MDHs. Additionally, the spectra of the mutant MDHs show a negative ellipticity maximum at about 270 nm which is virtually absent from the spectrum of wild-type MDH. Variations in this region are attributable to differences in the folded state of aromatic rings, notably from tryptophyl residues (Sears and Beychok, 1973) and, in this case, probably represents a small disorder in the tertiary structure of the mutant MDHs. These differences are unlikely to be due to PQQ since, although this molecule is aromatic, it has no chiral carbon atoms and does not give a CD signal. Therefore, the prosthetic group in the mutant MDHs is either PQQ modified so that a chiral carbon atom is present or, more likely, that the prosthetic group binding site involves aromatic rings and this environment is altered in the mutant MDHs.

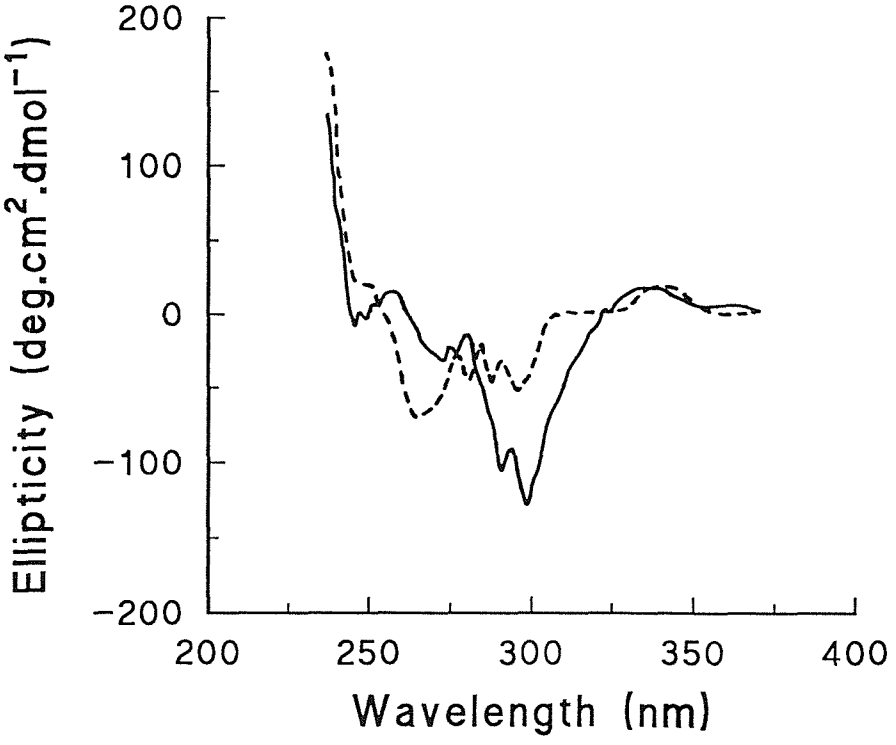
Using the database matrix of Compton and Johnson (1986), it was calculated that wild-type MDH contained approximately 20% parallel β-sheet, 30% anti-parallel β-sheet, 10% β-turn and 5-10% α-helix; the remaining 30-35% being random coil. Secondary structure prediction from the primary sequence of the β-subunit by the method of Garnier (1978), showed that this subunit is about 60% α-helical. Hence, the helical regions in the β-subunit may account for the majority of the helical regions in MDH predicted by the CD spectrum. This suggests that the α-subunit of MDH is mainly composed of β-sheet. The three-dimensional structure of another periplasmic, $\alpha_2\beta_2$ tetrameric enzyme, methylamine dehydrogenase (MNDH), has been elucidated (Vellieux *et al.*, 1990), and shows that the large subunit of this enzyme is predominantly anti-parallel β-sheet arranged into 7 'W'-shaped motifs. In view of the

Fig. 4.19. Circular dichroism spectra of wild-type and MoxK MDHs

Fig. 4.19. Circular dichroism spectra of wild-type and MoxK MDHs

Circular dichroism (CD) spectra were recorded on a JASCO model 20 dual function circular dichroism/optical rotation spectrophotometer as described in Section 2.17.d. Spectra were recorded in 5 mM potassium phosphate buffer (pH 7.0) using a scan rate of 2 $nm.min^{-1}$ with a spectral band width of 1 nm and a 2 mm path length. Very similar spectra to that for MoxK were seen for the MoxA and MoxL mutants. ——, wild-type MDH; ----, MoxK MDH.


6000
4000
2000
0
−2000
−4000
−6000
Ellipticity (deg.cm^2.dmol^{-1})
180
200
220
240
260
Wavelength (nm)


200
100
0
−100
−200
Ellipticity (deg.cm^2.dmol^{-1})
200
250
300
350
400
Wavelength (nm)

similarities between MNDH and MDH, it is possible that the α-subunit of MDH is arranged in similar motifs. Preliminary crystallographic data from the MDH from *Mp. methylotrophus* confirms that this is the case with the α-subunit of MDH arranged into 8 'W'-shaped anti-parallel β-sheets (Dr. F.S. Mathews, personal communication to Prof. C. Anthony).

4.13. Discussion

The results from the experiments presented in this chapter have been discussed at the end of the relevant sections; this final section serves to summarise these results and, where possible, draw definite conclusions.

The MDHs from the MoxA, K and L mutant strains of *Methylobacterium extorquens* are all produced to wild-type levels and contain both α-and β-subunits in a typical $\alpha_2\beta_2$ tetrameric conformation. These MDHs were seen to have typical ammonia- and methanol-dependent activity in both assay systems employed, but the specific activities were generally less than 5% of that obtained for the wild-type enzyme. Structurally, the mutant MDHs are very similar to the wild-type enzyme; they have the same mobility in non-denaturing PAGE, the same *N*-terminal sequences, very similar amino acid compositions, free-thiols were not detected in either the native or unfolded states, and they have very similar stabilities in a variety of denaturing conditions. These results suggest that there are no gross conformational or structural differences between the mutant and wild-type MDHs that could account for the marked inactivity of the mutant enzymes. The mutant MDHs could also cross-link with cytochrome c_L in an identical fashion to that seen for the wild-type enzyme, suggesting that if there was any conformational difference, it was not in the cytochrome c_L binding domain.

Absorption spectra of the MDHs suggested that each enzyme contains a prosthetic group, a result confirmed by gel filtration in the presence of SDS. The characterisation of the prosthetic group present in each of the mutant MDHs is described in the next chapter. Clearly, the inactivity is not due to the lack of a prosthetic group, but may be due to it not being normal PQQ. The absorption spectra of the mutant enzymes do not conform to any of those for the known redox states of MDH. These spectra could not be altered by treatment with artificial electron acceptors nor could the mutant MDHs react with cyclopropanol, suggesting that there is some fundamental difference between the prosthetic group in the mutant MDHs and that in

the wild-type enzyme. Also, the absorption spectra were dependent on the pH of the buffer suggesting that the environment of the prosthetic group in the mutant MDHs could be influenced by the solvent. Fluorescence spectra showed that any change of the prosthetic group was not due to hydration, since no other signal besides that due to aromatic amino acids was seen, and this supports a theory that the prosthetic group lies in a hydrophobic pocket on MDH (de Beer *et al.*, 1983). Circular dichroism spectra revealed a difference between wild-type and mutant MDHs in the region due to aromatic amino acids, suggesting that there is a small conformational difference and that aromatic amino acid residues may be involved in binding the prosthetic group.

In summary, this chapter has investigated the MDHs from wild-type bacteria and the MoxA, K and L strains in order to identify any differences that may exist between these proteins that could account for the lack of activity seen for the mutant proteins. All of the results suggest that this lack of activity is not due to any gross structural differences in the proteins, but is more likely to be due to differences either in the prosthetic group itself, or in the manner in which the prosthetic group is bound. These problems are addressed in the following chapter.

CHAPTER 5

Characterisation of the prosthetic group in the MDHs from wild-type *Methylobacterium extorquens* and the MoxA, K and L mutant strains

5.1 Introduction

The previous chapter investigated the structure of the MDHs produced by the MoxA, K and L mutant strains of *Methylobacterium extorquens*. The results suggested that, in terms of primary and quaternary structure, there were no differences between these MDHs and that from wild-type bacteria. The lack of activity in the mutant MDHs was not due to the absence of a prosthetic group; absorption spectra and dissociation experiments clearly demonstrated the presence of a prosthetic group. That the mutant MDHs are inactive may be due, however, to the presence of an altered prosthetic group (ie different from PQQ) that is incapable of reacting with substrate. Alternatively, the lack of activity may be due to an altered binding of the prosthetic group in the mutant enzymes.

This chapter describes experiments performed in order to fully characterise the prosthetic group present in the mutant MDHs.

5.2. Isolation and characterisation of the prosthetic group fraction after gel filtration of MDH in the presence of SDS

The prosthetic group fraction of each MDH from the gel filtration experiment (Section 4.7.a) was analyzed by absorption and fluorescence spectrophotometry.

The absorption spectra of the prosthetic group fractions from each of the MDHs were very similar (a typical example is shown in Fig. 5.1). Similarly, the fluorescence spectra were virtually identical (Fig. 5.2). Furthermore, these spectra were indistinguishable from those obtained for chemically synthesised PQQ (Fluka) dissolved in the same dissociation buffer used for the gel filtration. These results strongly suggest that the prosthetic group present in the mutant MDHs is the same as that in the wild-type enzyme, and that all of the prosthetic groups are normal PQQ.

The spectra shown in Figs. 5.1 and 5.2 were recorded within 30 min of elution of the fractions from the Superose-12 column. When the samples were left for a few hours and further spectra recorded, a noticeable change in the absorption spectra was

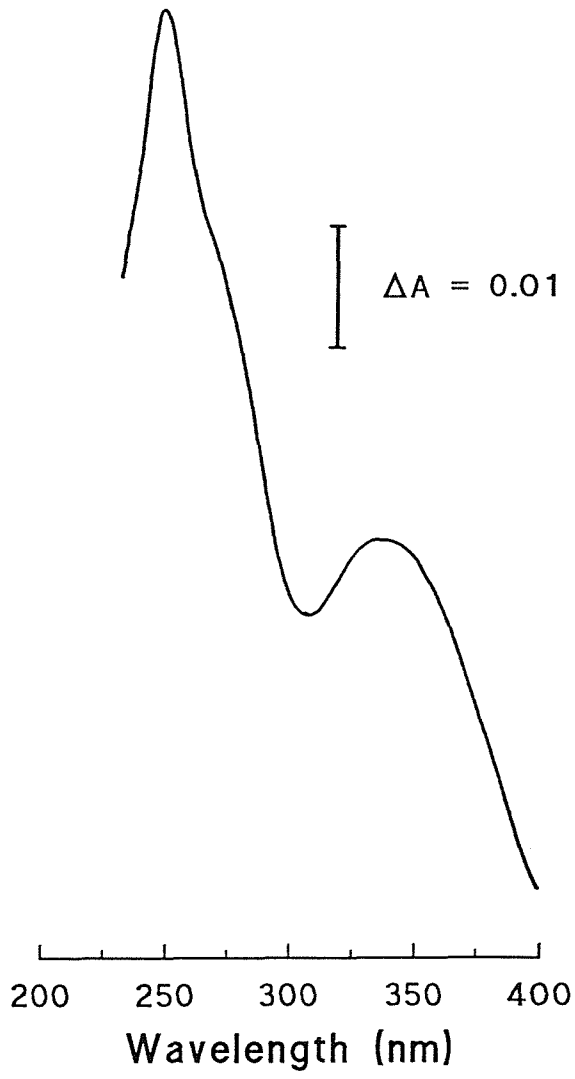


Fig. 5.1. Absorption spectrum of the prosthetic group from MoxA MDH

The prosthetic group from MoxA MDH was obtained from the gel filtration of this MDH in the presence of SDS as described in Section 2.19. The spectrum was recorded on the Shimadzu UV3000 UV/Vis spectrophotometer as described in Section 2.17.a. Virtually identical spectra were recorded for the prosthetic group fractions from wild-type, MoxK and MoxL MDHs. The spectrum shown here is very similar to that obtained for normal PQQ dissolved in the same buffer used for the dissociation of MDH; it is also very similar to that obtained for PQQ in, for example, potassium phosphate buffer (pH 7.0) (Fig. 1.7).

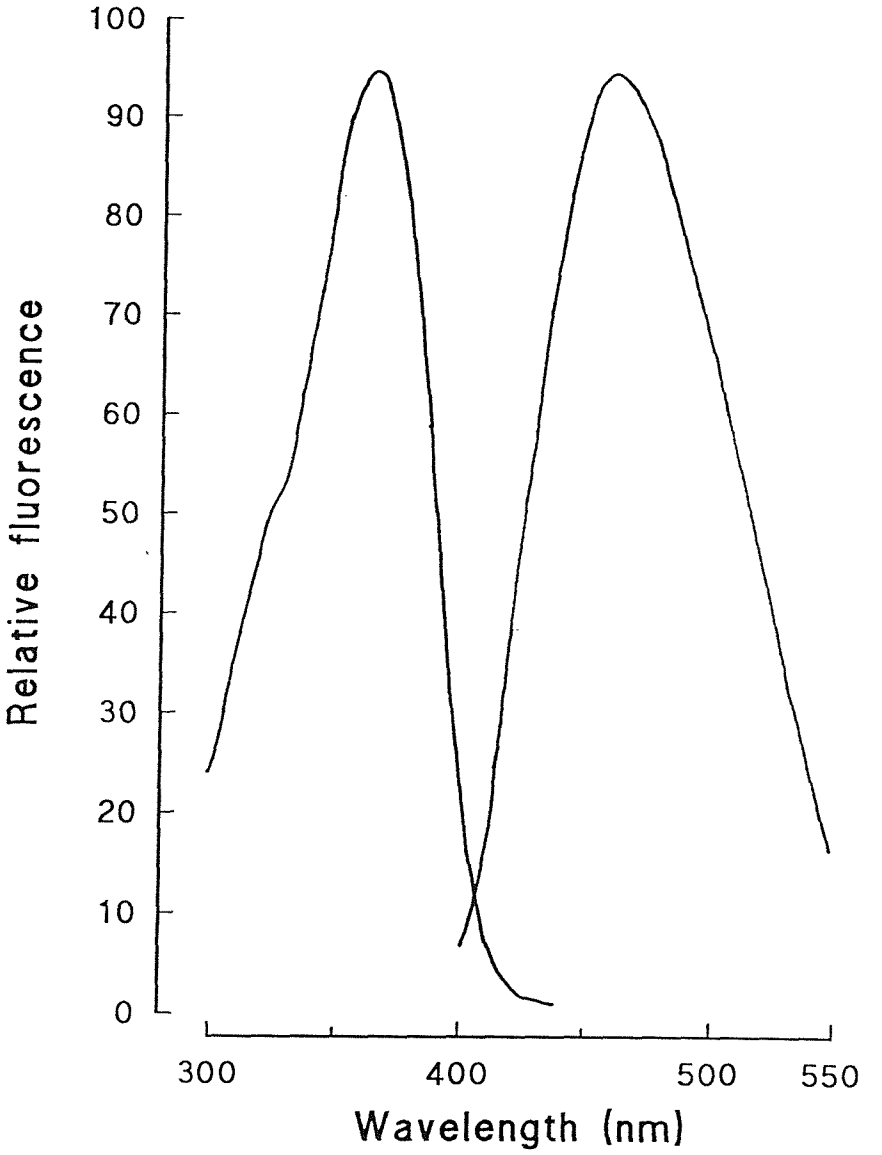


Fig. 5.2. Fluorescence spectra of the prosthetic group from MoxK MDH

The prosthetic group from MoxK MDH was obtained from the gel filtration of this MDH in the presence of SDS as described in Section 2.19. The spectra were recorded as described in Section 2.17.b. Virtually identical spectra were recorded for the prosthetic group fractions from wild-type, MoxA and MoxL MDHs. The spectra shown here are very similar to those obtained for normal PQQ dissolved in the same buffer used for the dissociation of MDH; they are also very similar to those obtained for PQQ in, for example, potassium phosphate buffer (pH 7.0) (Fig. 1.8).

observed, in which the typical shoulder at 270 nm was absent and the peak at 330 nm had red-shifted to about 350 nm (Fig. 5.3). This change was observed for all of the samples, including chemically synthesised PQQ. By contrast, there was no detectable change in the fluorescence spectra. In view of the high reactivity of PQQ in solution, it is probable that an adduct had been formed. The altered spectrum shown in Fig. 5.3 resembles that observed for the 5-imino-quinone derivative of PQQ, formed when PQQ is dissolved in 2 M ammonium chloride at pH 9.0 (Fig. 5.4; Dekker *et al.*, 1982). The fluorescence spectra of the 5-imino-quinone adduct are very similar to those observed for PQQ dissolved in a more usual buffer, such as potassium phosphate, at pH 7.0 (Dekker *et al.*, 1982). This suggests that the prosthetic group fractions had formed the 5-imino-quinone adduct with some component of the dissociation buffer. Of the compounds present in the dissociation buffer (Tris, NaCl and SDS), only Tris has an amine group capable of forming this adduct. However, confirmation that this, or any other, adduct had been formed was not performed.

5.3. Reconstitution of quinoproteins with the prosthetic group extracted from wild-type and mutant MDHs

The previous section has suggested that, in terms of spectral characteristics, the prosthetic group present in the mutant MDHs is normal PQQ. The change in absorption spectrum observed upon standing suggests that, if the 5-imino-quinone adduct was formed, the prosthetic groups all have a C-5 carbonyl group. Although this is the most reactive group on PQQ, other groups (notably the C-9 carboxylic acid) have been shown to be essential for activity (Section 1.6.d; Shinagawa *et al.*, 1986). It is, therefore, possible that the prosthetic group from the mutant MDHs are missing, for example, the C-9 carboxylic acid group and hence are not biologically active. This possibility was tested by using the prosthetic groups, extracted from the MDHs by boiling and centrifugation, in two biological PQQ assays that rely on the reconstitution of apo-quinoproteins. The two systems used in this work involved two very different quinoproteins, one a membrane-bound glucose dehydrogenase (GDH) from *E. coli*, the other a soluble alcohol dehydrogenase (ADH) quinohaemoprotein from *Pseudomonas testosteroni*; both systems are fully described in Section 2.18.

Table 5.1 summarises the results obtained using the above biological assays

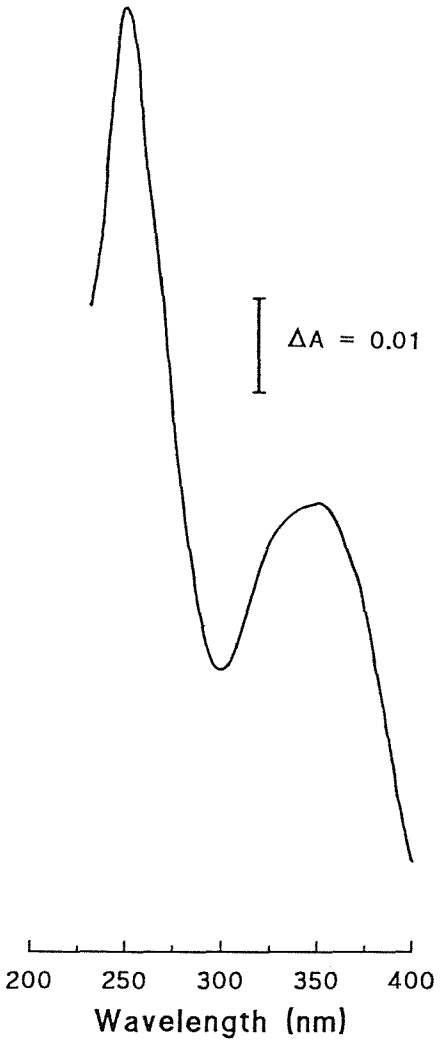
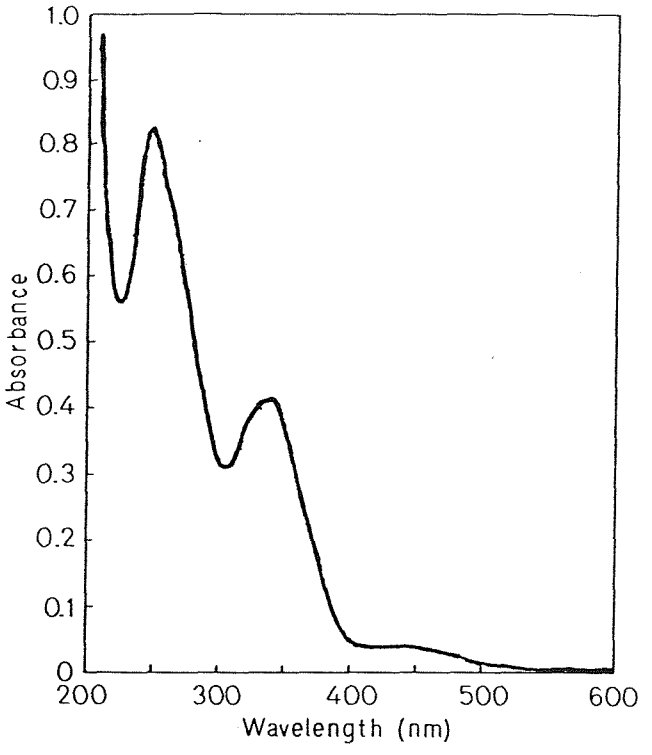


Fig. 5.3. Absorption spectrum of the prosthetic group form MoxA MDH recorded a few hours after the spectrum shown in Fig. 5.1

This spectrum was recorded from the same solution as that used for Fig. 5.1 after it had been left at room temperature for 4 h. Similar changes in spectrum were observed for the prosthetic group fractions from wild-type, MoxK and MoxL MDHs, and for normal PQQ dissolved in the same buffer. The spectra is very similar to that previously published for the 5-imino-quinone adduct of PQQ (Fig. 5.4; Dekker *et al.*, 1982).



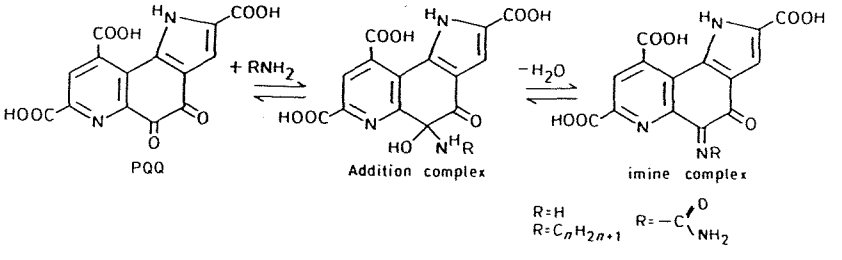


Fig. 5.4. Absorption spectrum of the 5-imino-quinone adduct of PQQ

This figure is taken from Dekker *et al.* (1982). The 5-imino-quinone adduct was obtained by dissolving PQQ in 2 M NH_4Cl (pH 9.0). A possible mechanism for the formation of this adduct is also shown.

Table 5.1. Determination of the amount of prosthetic group present in the MDHs from wild-type *Methylobacterium extorquens* and the MoxA, K and L mutant strains by the reconstitution of active enzyme from the apo-quinoproteins of alcohol dehydrogenase and glucose dehydrogenase

The prosthetic group was released from the MDHs by boiling a solution of protein (1 $mg.ml^{-1}$) for 10 min. Denatured protein was removed by centrifugation (12,000 g for 10 min) and samples of the supernatant used directly in the reconstitution assays as described in Chapter 2. The values shown here are the average of 3 determinations from the same batch of protein. Apo-ADH, apo-alcohol dehydrogenase quinohaemoprotein (soluble) from *Pseudomonas testosteroni*; apo-GDH, apo-glucose dehydrogenase (membrane-bound) from *E. coli*.

	moles PQQ per mole MDH	
Source of MDH	apo-ADH	apo-GDH
Wild-type	2.01 ± 0.18	2.01 ± 0.30
MoxA	1.85 ± 0.26	2.02 ± 0.40
MoxK	1.97 ± 0.20	2.14 ± 0.45
MoxL	1.98 ± 0.21	2.28 ± 0.27

with extracts of wild-type and mutant MDHs. These results clearly show firstly, that all of the MDHs contain normal PQQ, capable of reconstituting both assay systems and, secondly, that the same amount of PQQ (2 mol per mol MDH) is present in each of the MDHs. Thus the prosthetic group present in the mutant MDHs is normal, biologically active PQQ. This conclusion indicates that the reason for the inactivity of the mutant MDHs does not reside in the structure of the prosthetic group.

5.4. Electron spin resonance spectroscopy

Previous sections have shown that the prosthetic group extracted from the mutant MDHs is normal PQQ. This suggests that the difference between the wild-type and mutant enzymes lies not in the structure of the prosthetic group, but rather in the redox state or the binding of the PQQ in the enzyme.

MDH is normally isolated in a partially reduced form that contains the semiquinone form of PQQ (Fig. 1.10; Section 1.8.a; Duine *et al.*, 1981). This semiquinone is a free-radical and consequently gives MDH a characteristic electron spin resonance (ESR) spectrum (Duine *et al.*, 1981). Having established that the prosthetic group in the mutant MDHs is PQQ, the next direction for study was to determine the redox state of the prosthetic group. Absorption spectra of the mutant enzymes did not correspond to any of the redox forms described for MDH (Fig. 4.11), and circular dichroism (CD) spectra revealed a difference in the folding of aromatic acid residues (Fig. 4.19). Hence it is possible that the PQQ in the mutant MDHs is not in the semiquinone form and/or it may be bound differently from that in wild-type MDH.

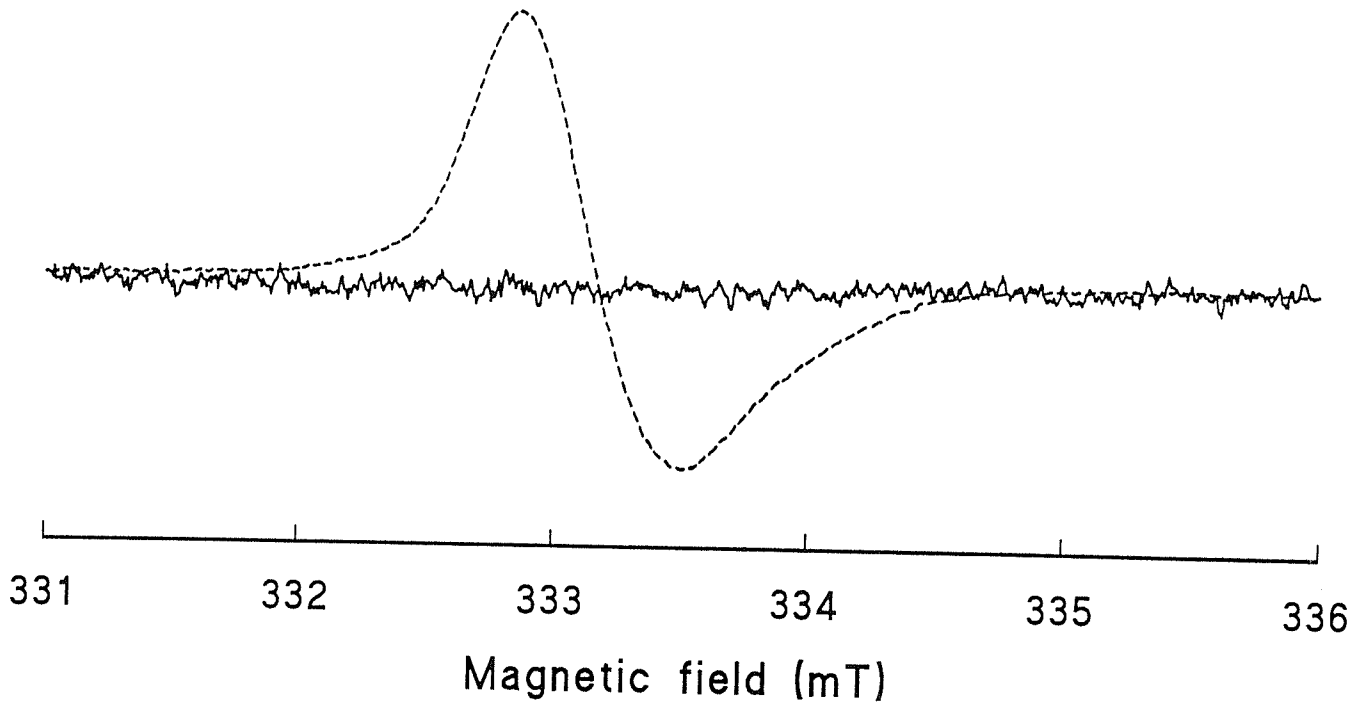
X-band ESR spectra of the mutant MDHs were recorded at 120°K with the help of Dr. David Lowe at the University of Sussex. Fig. 5.5 shows the ESR spectra of the MDHs from wild-type bacteria and the MoxA mutant strain. The spectrum of the mutant MDH clearly shows that this enzyme contains no PQQ in the semiquinone form; the spectrum shown (showing no signal) is the accumulation of 40 scans, whereas that for the wild-type enzyme was a single scan. Identical spectra to that for the MDH from the MoxA mutant were obtained for the MDHs from the MoxK and MoxL mutant strains. The asymmetric peak observed in the spectrum of wild-type MDH is very similar to that published previously for the enzyme from *Hyphomicrobium* X (Westerling *et al.*, 1979). Similarly, the *g*-value (2.0054) and line width (0.60 mT) of the spectrum of wild-type MDH are consistent with those values

Fig. 5.5. Electron spin resonance spectra of wild-type and MoxA MDHs

Fig. 5.5. Electron spin resonance spectra of wild-type and MoxA MDHs

Electron spin resonance (ESR) spectra were recorded as described in Section 2.17.c. Spectra were recorded of the MDHs in 5 mM potassium phosphate buffer (pH 7.0) at 120°K at a concentration of 100 µM. The spectrum of wild-type MDH is the result of a single scan; that for the MoxA MDH is the accumulation of 40 scans. Similar spectra to that for MoxA MDH were recorded for the MoxK and MoxL MDHs. The complete absence of signal in the spectra of the mutant MDHs shows that the prosthetic group in these proteins is not in a free-radical form.

The spectrum of wild-type MDH has a line width of 0.60 mT and a *g*-value of 2.0054; these values are in good agreement with those obtained for the MDH from *Hyphomicrobium* X (Westerling *et al.*, 1979).


331
332
333
334
335
336
Magnetic field (mT)

obtained for the *Hyphomicrobium* enzyme (g-value = 2.0046, line width = 0.69 mT; Westerling *et al.*, 1979).

The concentration of free-radical in wild-type MDH can be calculated. This is achieved by comparison of the double integral of the MDH spectrum to the double integral of the spectrum from a known standard. The standard used in this work was a 0.18 μM solution of Cu^{2+}-EDTA, with the spectrum recorded under identical conditions to MDH. Using the equation presented in Fig. 5.6, the calculated concentration of two separate samples of wild-type MDH were 18.7 μM and 29.7 μM. The concentration of both of these MDHs were estimated to be 100 μM by protein assay. It has been stated by Duine and his colleagues, who have produced the most significant work on ESR spectroscopy of MDH, that MDH from *Hyphomicrobium* X contains PQQ entirely in the semiquinone form (Duine *et al.*, 1981). However, this result was based on HPLC analysis of the extracted prosthetic group; there have been no reports of the concentration of free-radical estimated from ESR spectroscopy for the MDH from this organism. The results presented here show that the two batches of MDH from wild-type *Mb. extorquens* contained only 19% and 30% of PQQ in the semiquinone form. A similar value was found by Mincey *et al.* (1981) who found that only 13% of the MDH from *Methylomonas methanica* contained the free-radical form of PQQ; this finding was used as the basis of the authors erroneous description of the reaction mechanism of MDH in which PQQ could act as a 3-electron acceptor. There is no reason why all of the MDH should contain only the semiquinone form of PQQ; it is unlikely that all of the MDH molecules in the periplasm will be synchronised in their redox state, and so, upon extraction, a mixture of the three known redox species could be expected. In reality, only two of the three species would be observed; the fully oxidised form of MDH would be rapidly reduced by any substrate present in the buffers used for enzyme purification (see Section 4.11).

5.5. HPLC analysis of the PQQ from MDH

It has been shown previously that the semiquinone form of PQQ in wild-type MDH disproportionates, upon extraction at low pH, into equimolar amounts of the quinone (oxidised) and quinol (reduced) forms. These two forms can be separated by reverse phase HPLC (Duine *et al.*, 1981); again low pH is essential for the HPLC analysis since the quinol form is rapidly oxidised at values above pH 4.0 (Duine *et al.*, 1981). The total PQQ determination clearly confirmed the finding of the reconstitution

$$\frac{\left(\frac{(conc\ of\ std\ (\mu M)\ \times\ MDH\ signal\ integral)}{(std\ signal\ integral)}\right)}{\left(\frac{std\ scan\ sweep\ (Gauss)}{MDH\ scan\ sweep\ (Gauss)}\right)^2 \times \sqrt{\left(\frac{std\ scan\ power\ (mW)}{MDH\ scan\ power\ (mW)}\right)}} \ /\ \left(\frac{No\ of\ MDH\ scans}{No\ of\ std\ scans}\right) = MDH\ conc\ (\mu M)$$

Fig. 5.6. The equation used for the estimation of free-radical concentration from electron spin resonance spectroscopy

This equation was provided by Dr. David Lowe from the Nitrogen Fixation Laboratory, University of Sussex

studies in that wild-type MDH contains 2 mol PQQ per mol MDH. The relative amounts of quinone (40%) and quinol (60%) in the wild-type MDH extract were shown to be consistent with the ESR spectroscopy; the PQQ in this enzyme does not appear to be entirely in the semiquinone form (Fig. 5.7.a; Table 5.2). However, assuming that the quinone form of PQQ represents half of the semiquinone content, then the free-radical content should be 80% of the total PQQ content. Clearly this is not the case, the ESR spectroscopy showed that no more than 30% of the PQQ was present in the semiquinone form. However, it should be pointed out that the ESR and HPLC experiments were performed with different batches of wild-type MDH, and this may account for the discrepancy.

The results from the ESR spectroscopy suggested that the PQQ in the mutant MDHs must be in either the oxidised or reduced form. These MDHs were not able to react directly with substrate which suggests that the PQQ was not in the oxidised form (Section 4.8.b), since this is the only form that can react directly with substrate.

The redox state of the PQQ in the mutant MDHs was investigated using a Novapak C_{18} reverse phase HPLC column under isocratic running conditions as described in Section 2.21. Fig. 5.7.b shows a typical elution profile for the MDH from the MoxA mutant strain. Very similar profiles to that for the Mox A mutant MDH were obtained for the MDHs from the MoxK and L mutants. These results clearly show that the extracts from the mutant MDHs contain predominantly oxidised PQQ by contrast with that from the wild-type enzyme which contained the expected mix of quinone and quinol forms. Further extracts from each MDH were prepared and a slight excess of PIP added before loading onto the column. This procedure converts all of the PQQ present into the quinone form, and hence allows quantification of the total PQQ content, and determination of the relative amounts of quinone and quinol present in the original, unoxidised extracts.

The very high proportion of oxidised PQQ present in the mutant MDHs is an intriguing finding. That the PQQ is oxidised suggests that it should react directly with substrate, but it has already been mentioned that this does not happen. This suggests that the PQQ in the mutant MDHs must be bound differently from that in the wild-type enzyme, and that this altered binding accounts for the lack of activity in the mutant MDHs. This conclusion is consistent with the CD spectra which revealed a small secondary structure difference between the mutant and wild-type MDHs. It is also consistent with the demonstration that the mutant MDHs could not react with cyclopropanol, even after oxidation of the enzyme with artificial electron acceptors.

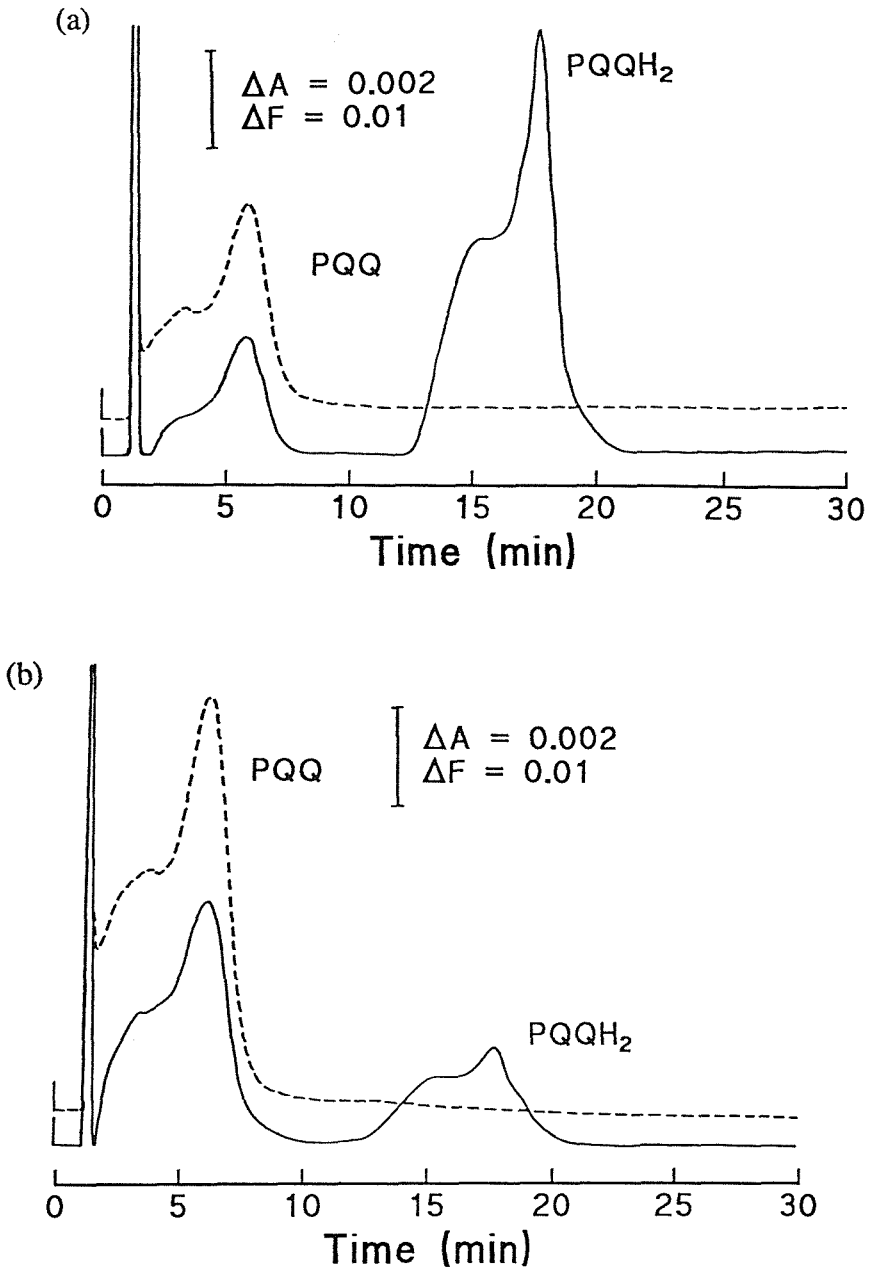


Fig. 5.7. HPLC analysis of the prosthetic groups extracted from wild-type and MoxA MDHs

The prosthetic group was extracted from 250 μg MDH and analysed by HPLC as described in Section 2.21. ——, absorbance at 313 nm; ·····, total fluorescence above 418 nm (excited at 365 nm). Almost identical results to that for the MoxA mutant were obtained using prosthetic group extracted from MDH of MoxK and MoxL mutant strains. (a) MDH from wild-type bacteria; (b) MDH from the MoxA mutant.

Table 5.2. Determination of the total amount of prosthetic group, and the relative amounts of quinone and quinol present in the MDHs from wild-type *Methylobacterium extorquens* and the MoxA, K and L mutant strains by reverse phase HPLC

The prosthetic group was released from MDH by treatment with acid and extracted into methanol as described in Chapter 2. After centrifugation and filtration to remove precipitate, the extract was loaded onto a Novapak C_{18} reverse phase column (Waters), equilibrated with methanol/water/85% H_3PO_4 (30/69.6/0.4 % v/v/v) at a flow rate of 1 $ml.min^{-1}$. Eluent was monitored by absorbance detection at 313 nm and fluorescence detection (excitation at 365 nm; total emission at over 418 nm). After HPLC, the proportions of quinone and quinol were estimated from the integral of the quinone peak in the fluorescence profile of unoxidised exracts; simlar proportions were observed using the absorbance profile. The total amount of PQQ was estimated from the integral of the quinone peak in the fluorescence profile of extracts oxidised with a slight excess of PIP. The values shown here are the average of 2 determinations from the same batch of protein. Different batches of protein were used for the reconstitution assays (Table 5.1) and the HPLC analysis (this Table).

Source of MDH	moles PQQ per mole MDH	percentage quinone	percentage quinol
Wild-type	1.88 ± 0.20	40	60
MoxA	2.04 ± 0.15	84	16
MoxK	2.13 ± 0.10	96	4
MoxL	2.05 ± 0.13	83	17

5.6. Discussion

This chapter has investigated the nature of the prosthetic group present in the MDHs from wild-type *Mb. extorquens* and the MoxA, K and L mutant strains. The results have demonstrated that, in all cases, the prosthetic group is normal PQQ in terms of spectral characteristics and in the ability to reconstitute two different apo-quinoproteins. Furthermore, the PQQ in the mutant MDHs is present in the correct stoichiometry of 2 mol PQQ per mol MDH. These results have showed that the lack of activity in the mutant enzymes is not due either to a structurally altered PQQ or to an incorrect amount of PQQ. Hence the *moxA*, *K* and *L* genes are not involved in PQQ biosynthesis, nor do they affect the amount of PQQ in MDH.

A difference from the wild-type MDH was found in the redox state of the PQQ; the mutant enzymes contain predominantly oxidised PQQ, whereas the wild-type enzyme contains the semiquinone form. The fact that the mutant MDHs could not react with substrate, either directly or after oxidation with artificial electron acceptors, suggests that the PQQ in these enzymes is bound in a conformation different from that in the wild-type MDH. This is supported by the lack of reactivity with cyclopropanol; this depends on the reaction of cyclopropanol with a base on the protein prior to interaction with the PQQ (Section 4.11). Analysis of the primary structure of a number of quinoproteins has revealed the presence of a conserved region proposed to be involved in PQQ binding (Section 1.6.d; Inoue *et al.*, 1990; Cleton-Jansen *et al.*, 1990; Anthony, 1992a). PQQ has three carboxylic acid groups which have low pKa values and hence will be deprotonated at physiological pH (Duine and Frank, 1980b). One of these groups (at C-9) has been demonstrated to be essential for activity in reconstitution studies (Shinagawa *et al.*, 1986). It is therefore possible that the interaction between PQQ and its binding region will be electrostatic in nature, these interactions occurring between the carboxyls on PQQ and positively-charged (lysyl, arginyl and histidyl) residues in the primary protein sequence. The CD spectra (Section 4.12) suggest that aromatic amino acid residues may also be involved. The putative PQQ-binding regions in the different quinoproteins have a number of conserved aromatic amino acids, but there are no conserved positively-charged residues. It must be stressed here that the *moxA*, *K* and *L* genes are distinct from the MDH structural genes (Fig. 1.21), and the results from Chapter 4 have shown that the MDHs from the MoxA, K and L mutants have the same amino acid composition as that from wild-type bacteria. Therefore, it is very unlikely that there are any

differences in the primary sequences of the four MDHs. This suggests either that the interaction is not electrostatic and involves only aromatic amino acids, or that the interaction is electrostatic but occurs between the carboxylic acid groups on PQQ and some other amino acid residues in the PQQ-binding region. There are a number of negatively-charged amino acids that are conserved in this region, but if these residues were involved in the interaction with PQQ, then there must be some other factor that serves as bridge between the prosthetic group and its binding domain. If this is the case, then the lack of activity in the mutant MDHs may be due to this factor being absent or altered.

CHAPTER 6

The role of calcium in methanol dehydrogenase

6.1. Introduction

Chapters 4 and 5 have investigated the structure and the prosthetic group of the MDHs from the MoxA, K and L mutants of *Methylobacterium extorquens* in order to determine the reason(s) why these enzymes are inactive. It was concluded that the prosthetic group was normal PQQ, but this was bound in a slightly different conformation from that in wild-type MDH.

It has already been mentioned previously that the C-9 carboxylic acid group of PQQ is essential for the reconstitution of active glucose dehydrogenase (GDH; Shinagawa *et al.*, 1986; Section 1.6.d). A highly conserved region has been identified in number of different quinoproteins (Fig. 1.11), and specific amino acids in this region may interact directly with PQQ, possibly through the C-9 carboxylic acid group. With some alcohol and glucose dehydrogenases, the presence of certain divalent cations (notably calcium and magnesium) have also been shown to be necessary in the reconstitution of these enzymes (Ameyama *et al.*, 1985; Groen *et al.*, 1986; Adachi *et al.*, 1988; Geiger and Görisch, 1989; Shinagawa *et al.*, 1989; Hommel and Kleber, 1990; Mutzel and Görisch, 1991). These enzymes differ from MDH in that some are produced in the apo-form by the host organism, whereas others, once purified, can be treated to generate the apo-form. In two of the latter examples, the presence of calcium in the pure enzyme has been reported; these are the soluble alcohol dehydrogenase (ADH) from *Pseudomonas aeruginosa* (Mutzel and Görisch, 1991), and the soluble GDH from *Acinetobacter calcoaceticus* (Geiger and Görisch, 1989). Recently, Adachi *et al.* (1991) reported the presence of a single calcium atom in the MDH from a newly described methylotroph, *Methylobacillus glycogenes*. By contrast, Anthony and Zatman (1967a) failed to find any metal ions in the MDH from *Mb. extorquens* strain M27.

This Chapter describes experiments carried out to determine whether the MDHs from wild-type *Mb. extorquens* and the MoxA, K and L mutants contain calcium (or any other metal ion), and discusses a possible role for calcium in quinoproteins.

6.2. The presence of calcium in MDH

The detection of calcium in MDH using atomic absorption spectroscopy was carried out as described in Section 2.28. MDH was also analyzed for the presence of other metal ions by plasma source mass spectroscopy. This analysis was performed by Dr. Trevor Delves of the Trace Element Unit, Southampton General Hospital. The following metal ions were not detectable in MDH using this method (the detection limit was 0.1 $\mu g.l^{-1}$): Mg^{2+}, Al^{3+}, Ni^{2+}, Zn^{2+}, Ba^{2+} and Sr^{2+}.

The MDHs purified from wild-type *Mb. extorquens*, *Methylophilus methylotrophus*, *Paracoccus denitrificans* and *Hyphomicrobium* X were all seen to contain one atom of calcium per MDH tetramer (Table 6.1). These results demonstrate that the MDHs from more characterised methylotrophs have the same stoichiometry of calcium as the MDH from the newly described *Methylobacillus glycogenes* (Adachi *et al.*, 1991). This unusual stoichiometry is discussed in detail in Section 6.4. By contrast with the above MDHs, those from the MoxA, K and L mutant strains of *Mb. extorquens* were shown to contain virtually no calcium (Table 6.1). The observation that the mutant MDHs do not contain calcium whereas it is present in the wild-type enzyme suggests that the absence of this ion is the reason why the mutant MDHs are inactive. Extensive work has been carried out on the reaction mechanism of MDH, and a number of possible mechanisms have been proposed, none of which involves a metal ion (Section 1.8). Hence it is unlikely that the calcium has a role in the reaction mechanism. It is more likely that the calcium serves a structural function, possibly in the binding of PQQ.

6.3. Reconstitution of active enzyme by addition of Ca^{2+} ions to the mutant MDHs

Some quinoproteins have been shown to be reconstituted by the addition of PQQ and certain divalent cations. These enzymes differ from MDH in that a stable apo-form of the protein can be isolated and then used in reconstitution studies. In the thirty years since its discovery, there have been no reports of the reconstitution of active MDH, even when excess PQQ has been added. This failure is probably due either to previous ignorance about the β-subunit in MDH (none of the reconstitutable quinoproteins have more than one type of subunit), or to ignorance about the presence of calcium in MDH. The observation that the MDHs from the MoxA, K and L mutants are identical to the MDH from wild-type bacteria except for the presence of calcium,

Table 6.1. The presence of calcium in MDH from methylotrophic bacteria

Pure MDH was exchanged into calcium-free 10 mM Mops buffer (pH 7.0) by gel filtration. MDH at three dilutions (50, 100 and 150 µM) was then analyzed for calcium using atomic absorption spectroscopy as described in Section 2.28. The detection limit for calcium was 0.1 $\mu g.ml^{-1}$. The values shown here for *Methylobacterium extorquens* are the averages for 3 different amounts of MDH each from two different batches of protein. The values for *Hyphomicrobium* X, *Paracoccus denitrificans* and *Methylophilus methylotrophus* are the average for 3 different amounts of MDH from a single batch of protein from each organism.

Source of MDH	mol Ca^{2+} / mol MDH
Methylobacterium extorquens	
Wild-type	1.04 ± 0.06
MoxA	0.03 ± 0.04
MoxK	0.05 ± 0.07
MoxL	0.01 ± 0.03
Hyphomicrobium X	1.40 ± 0.13
Paracoccus denitrificans	1.12 ± 0.10
Methylophilus methylotrophus	1.05 ± 0.08

gives an opportunity to investigate the reconstitution of active MDH from an apo-form of the enzyme.

Samples of MDH (70 - 700 pmol) in 100 mM Tris-HCl buffer (pH 9.0) were incubated at 20°C with various concentrations of calcium chloride (0 - 10 mM). After 60 min, reconstitution mixtures were used directly to measure activity in the standard dye-linked assay after the addition of methanol, ammonium chloride, PIP and PES (Fig. 6.1). These results demonstrate that full activity in the mutant MDHs can be restored by incubation with 10 mM calcium chloride for 60 min. For time-dependent reconstitution, MDH (3.7 nmol) in 2 ml of 100 mM Tris-HCl buffer (pH 9.0) was incubated with 500 μM calcium chloride. At regular intervals, absorption spectra were recorded and samples (50 μl) removed and assayed in the standard dye-linked assay. Fig. 6.2 shows that full activity was restored after 24 h with this concentration of calcium chloride. The specific activity of wild-type MDH incubated in identical conditions did not vary significantly over the same time period. By contrast with previous reconstitution experiments using apo-MDH, an excess of PQQ was not needed for the reconstitution of the mutant MDHs since they already had the correct stoichiometry of 2 moles of PQQ per MDH tetramer.

During the reconstitution of MoxA MDH, changes in the absorption spectrum were observed; these were the disappearance of the peak at 520 nm and the appearance of a peak at 345 nm (Fig. 6.3). Similar changes were observed for MoxK and MoxL MDHs. The spectra of the MDHs after 24 h incubation were very similar to that of wild-type MDH in the fully reduced state (Fig. 1.14), in which the PQQ is present entirely in the quinol form ($PQQH_2$). The presence of quinol in the reconstituted MoxL MDH was confirmed by analysis of the prosthetic group by reverse phase HPLC as described in Section 2.21 (Fig 6.4). These results are consistent with the observations in Chapter 5 that the PQQ in the mutant MDHs was originally in the oxidised form. After reconstitution of active MDH by incubation in calcium, the oxidised PQQ becomes available for reduction by the endogenous substrate present on the enzyme resulting in the quinol form of the prosthetic group.

The concentration of calcium chloride used in the time-dependent reconstitution experiment is considerably higher than that present in the growth medium where only 20 μM calcium is added. Calcium is a common contaminant of virtually every chemical so that the actual concentration of calcium in the growth medium is usually between 30 and 40 μM. Incubation of the mutant MDHs with this concentration of

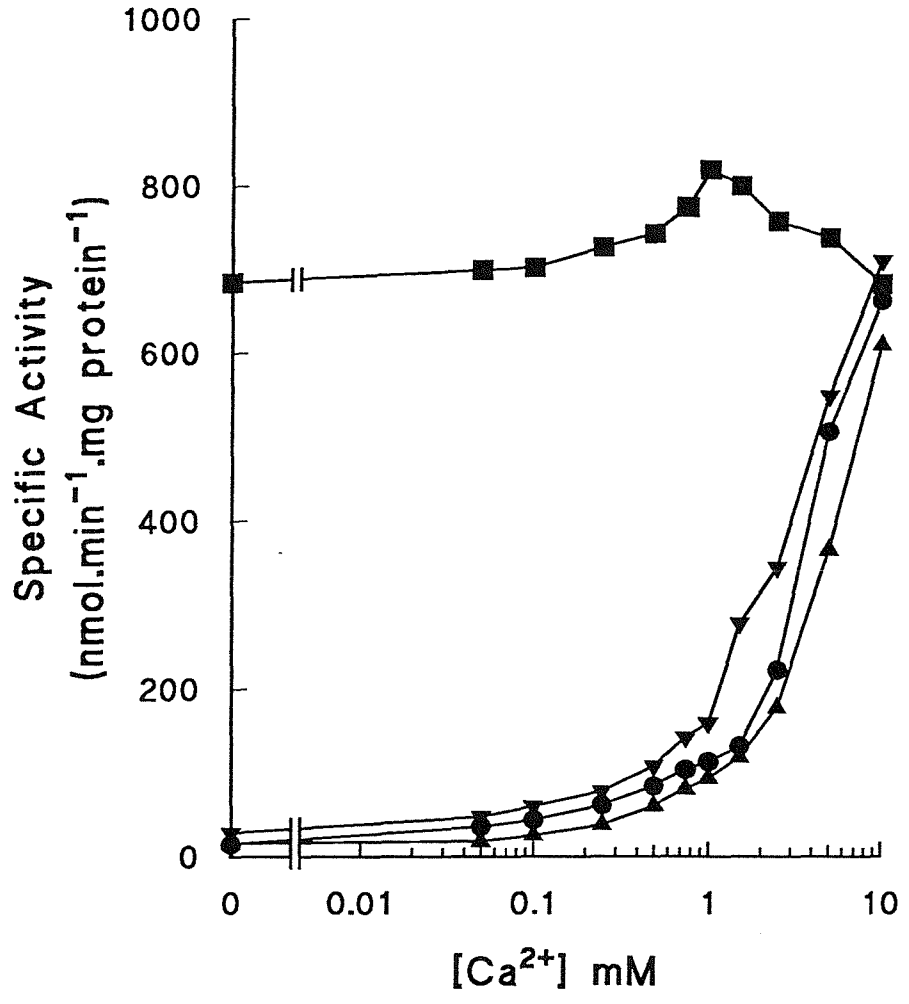


Fig. 6.1. Effect of calcium concentration on reconstitution of active MDH from Ca^{2+}-free MDH purified from MoxA, K and L mutant strains

MDH was incubated at 20°C for 60 min in various concentrations of calcium chloride in 100 mM Tris buffer at pH 9.0. After incubation artificial electron acceptors were added together with substrate (methanol) and activator (ammonium chloride) for determination of enzyme activity as described in methods. The concentration of MDH (wild-type bacteria) was 10 µg/ml. For the MDH from mutants 100 µg/ml was used for lower concentrations of Ca^{2+} (0-500µM) and 10µg/ml for higher concentrations.

■ , wild-type; ▲ , MoxA; ● , MoxK; ▼ , MoxL.

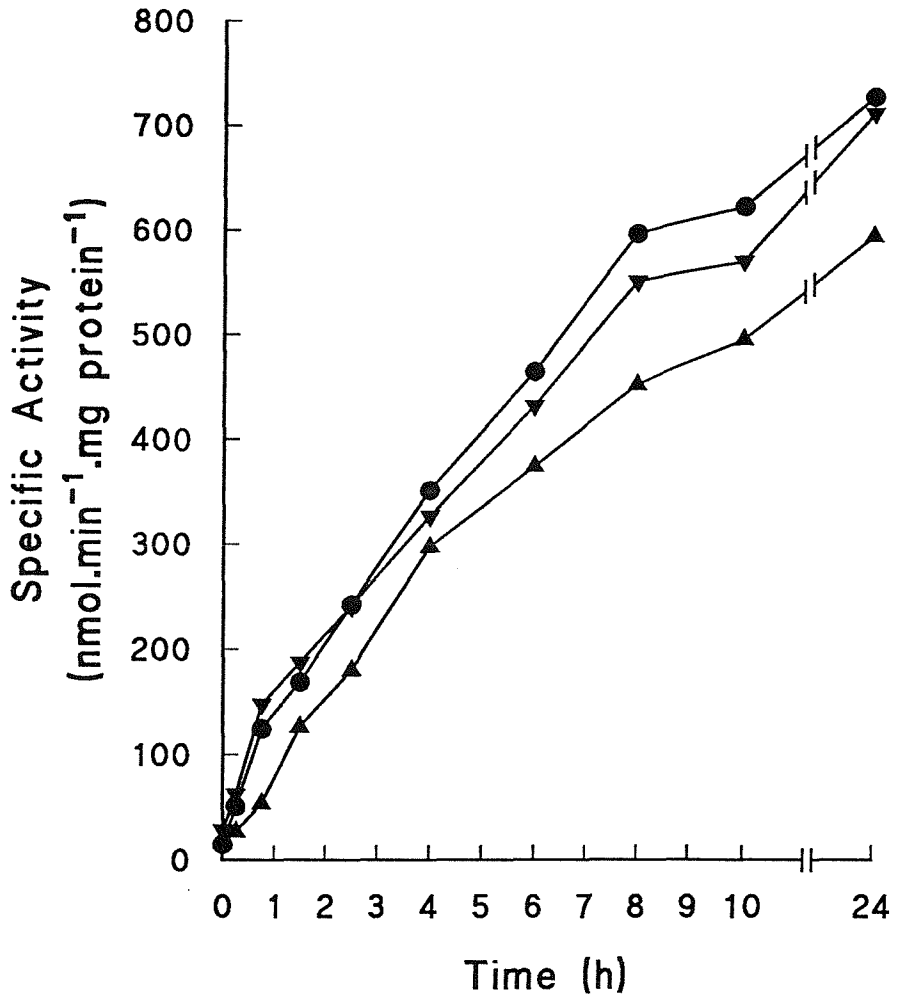


Fig. 6.2. Time-dependent reconstitution of MDH by calcium addition (500 µM)

MDH (100 µg/ml) from the MoxA, K and L mutant strains was incubated with 500 µM calcium chloride in 100 mM Tris buffer (pH 9.0) at 20 °C. Samples (50 µl) were removed from the incubation mixture and assayed in the standard dye-linked assay system.

▲ , MoxA; ● , MoxK; ▼ , MoxL.

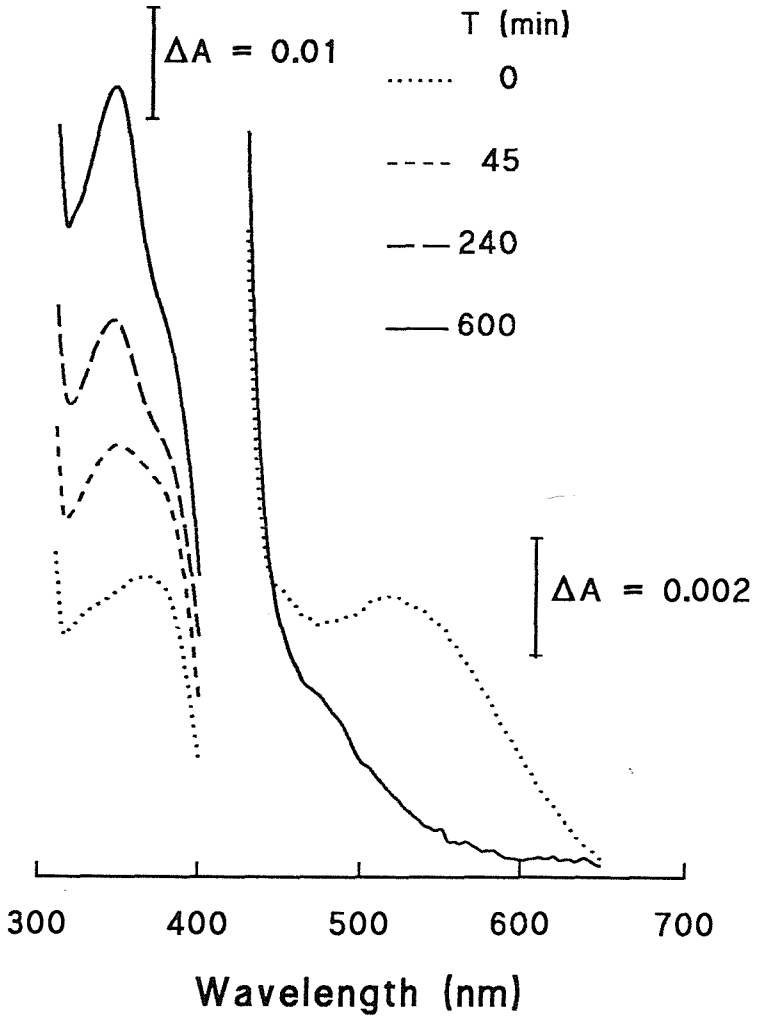


Fig. 6.3. Absorption spectra of MDH from the MoxA mutant strain during reconstitution with calcium ions

The absorption spectra of MDH in the incubation mixtures described in Fig. 6.2 were recorded at the time intervals indicated.

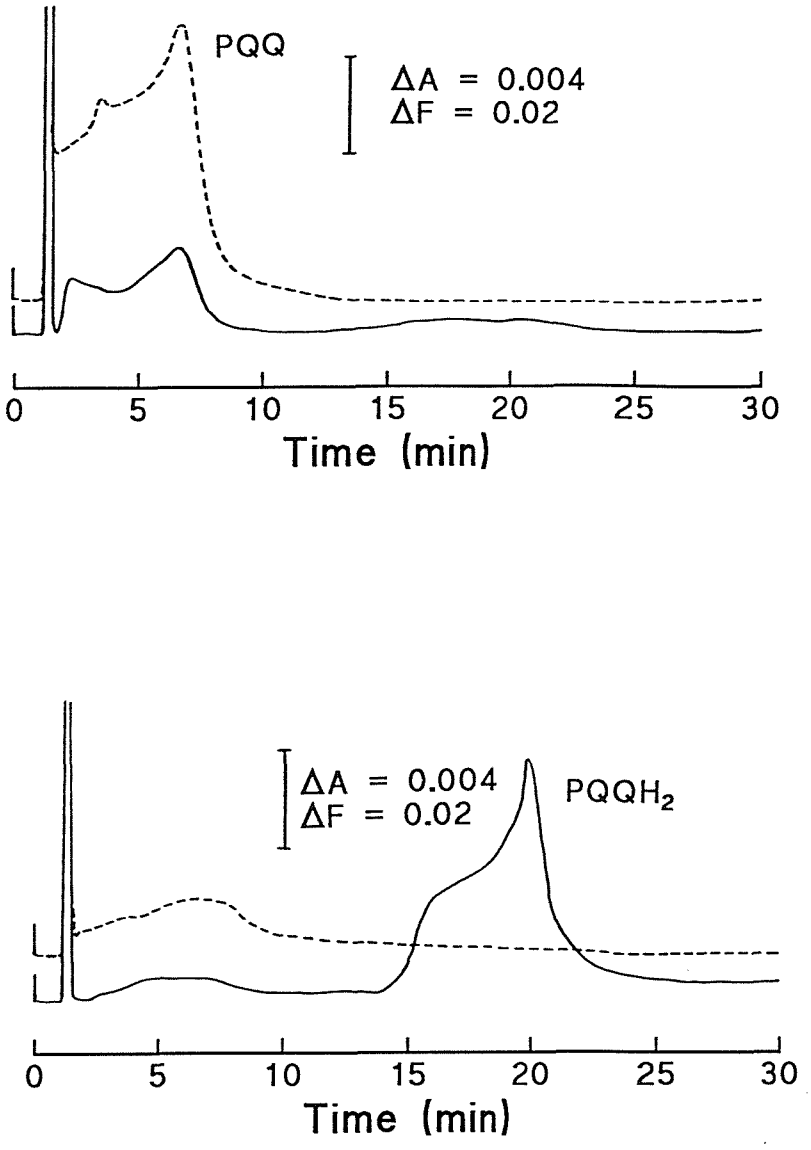


Fig. 6.4. HPLC analysis of prosthetic groups extracted from MDH from the MoxA mutant strain before and after reconstitution by incubation with calcium

The prosthetic group was extracted from 250 µg MDH and analysed by HPLC as described. ——, absorbance at 313 nm; ·····, total fluorescence above 418 nm (excited at 365 nm). Almost identical results to that for the MoxA mutant were obtained using prosthetic group extracted from MDH of MoxK and MoxL mutant strains.

(a) MDH as isolated from the MoxA mutant

(b) The same MDH after reconstitution with calcium.

calcium chloride only resulted in the restoration of 25% of maximum activity after incubation for 4 days; after this time, the proteins began to denature. Furthermore, the rate of reconstitution was very slow; 10% reconstitution after 2 days (Fig. 6.5). Even though this rate was slow, the specific activity achieved after 2 days was considerably higher than that observed for the freshly purified MDHs, which were 'incubated' in the cells at 30°C with about 40 μM calcium for 2 days prior to harvesting. This suggests that the *moxA, K and L* gene products may affect the availability of calcium in the periplasm, or that they are involved in the insertion of calcium into MDH.

Table 6.2 summarises the changes seen after the reconstitution of the mutant MDHs and includes measurements of the enzyme's specific activity in the cytochrome *c*- linked assay system. For these measurements, MDH (1.7 nmol) in 1 ml of 100 mM Tris-HCl buffer (pH 9.0) was incubated for 24 h with 500 μM calcium chloride. After incubation, excess calcium was removed by passage through a P-6 column (Bio-Rad) equilibrated with calcium-free 10 mM Mops buffer (pH 7.0), and activity measured as described in Section 2.11.b. It was necessary to remove the excess calcium prior to assay because activity in this system is very sensitive to high ionic strength (Chan and Anthony, 1991; Cox *et al.*, 1992).

The reconstituted mutant MDHs were able to react with cyclopropanol in oxidising conditions, giving spectra identical to that of cyclopropanol-inactivated MDH from wild-type bacteria shown in Fig. 4.17. This suggests that the addition of calcium to the mutant MDHs has restored the correct conformation of the active site such that the cyclopropanol can be acted upon by the base, and the ring-opened form of cyclopropanol can attack the prosthetic group.

Calcium could not be removed from either wild-type or the reconstituted mutant MDHs, nor were the spectra changed, nor the specific activities diminished by the following procedures: gel filtration, extensive dialysis against buffer containing Chelex-100 (a resin chelator of divalent cations with a high affinity for calcium) (Bio-Rad), or treatment with 5 mM EDTA at pH 7.0 (in calcium-free 10 mM Mops buffer) followed by gel filtration or dialysis against the same buffer (1000 volumes) containing Chelex-100. These results show that once the calcium is present in MDH, it cannot be removed.

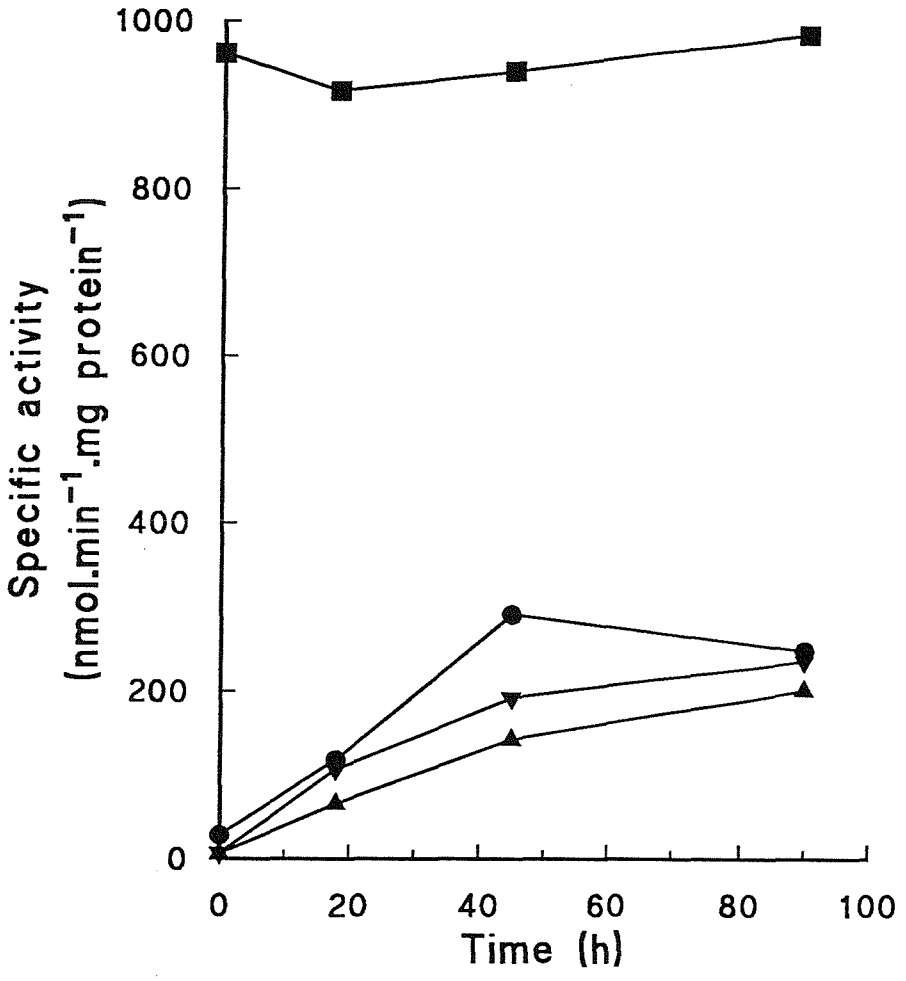


Fig. 6.5. Time-dependent reconstitution of MDH by calcium addition (40 µM)

MDH (100 µg/ml) from wild-type bacteria and the MoxA, K and L mutant strains was incubated with 40 µM calcium chloride in 100 mM Tris buffer (pH 9.0) at 20 °C. Samples (50 µl) were removed from the incubation mixture and assayed in the standard dye-linked assay system.

■, Wild-type; ▲, MoxA; ●, MoxK; ▼, MoxL.

Table 6.2. Reconstitution of active MDH by addition of Ca^{2+} ions to MDH from the MoxA, K and L mutants

MDH (1.5 μM) was exchanged by gel filtration into calcium-free 100 mM Tris-HCl buffer (pH 9.0) and then incubated in the presence or absence of calcium (500 μM) at 20°C for 24 h. After incubation, any excess calcium was removed by gel filtration of MDH in calcium-free 10 mM Mops buffer (pH 7.0) and activities measured in the dye-linked and cytochrome *c*-linked assay systems as described in Section 2.11. Specific activities are in nmol/min/mg.

	Before calcium addition				After calcium addition			
	Absorbance peaks (nm)		Specific activity		Absorbance peaks (nm)		Specific activity	
Source of MDH	345-350	520	Dye	Cyt. *c*	345-350	520	Dye	Cyt. *c*
Wild-type	+	-	686	90.0	+	-	808	102
MoxA	-	+	16	0.2	+	-	712	92
MoxK	-	+	15	0.1	+	-	840	89
MoxL	-	+	28	0.4	+	-	794	95

6.4. Discussion

This Chapter has investigated the MDHs from a number of methylotrophs. The finding of a single atom of calcium in the MDHs from *Methylobacterium extorquens*, *Methylophilus methylotrophus*, *Paracoccus denitrificans* and *Hyphomicrobium* X, adds further examples to the recent report of its presence in the MDH of a newly described methylotroph, *Methylobacillus glycogenes* by Adachi *et al.* (1990b). In all cases there was a single atom of calcium per $\alpha_2\beta_2$ tetramer.

Other quinoproteins have been shown previously to contain calcium; these are the soluble alcohol dehydrogenase from *Pseudomonas aeruginosa* (Mutzel & Görisch, 1991) and the soluble glucose dehydrogenase (GDH-B) from *Acinetobacter calcoaceticus* (Geiger & Gorisch, 1989). By contrast with the methanol dehydrogenases these enzymes can be prepared in the apoenzyme form and the active holo-enzyme reconstituted with PQQ; calcium or strontium were essential for this reconstitution and could not be replaced with magnesium. Similar reconstitution experiments have implicated calcium (not magnesium) in the structure of the quinoprotein alcohol dehydrogenase from membranes of *Gluconobacter suboxydans* (Shinagawa *et al.*, 1989) and in the soluble quinohaemoprotein alcohol dehydrogenase from *Pseudomonas testosteroni* (Groen *et al.*, 1986). Divalent cations have also been shown to be required for reconstitution of active holoenzyme from apoenzyme plus PQQ using the following quinoproteins: membrane glucose dehydrogenase from *Escherichia coli* and *Pseudomonas fluorescens* (Mg^{2+} more effective than Ca^{2+}) (Ameyama *et al.*, 1985b), membrane glycerol dehydrogenase from *Gluconobacter industrius* (Co and Ni were most effective) (Adachi *et al.*, 1988), glycerol dehydrogenase from membranes of *Gluconobacter suboxydans* (Ca^{2+} was most effective) (Adachi *et al.*, 1988) and the membrane-bound quinohaemoprotein aldehyde dehydrogenase from *Acetobacter rancens* (Mg^{2+} and Mn^{2+} were more effective than Ca^{2+}) (Hommel & Kleber, 1990). Insufficient information is available at present to determine the roles of the metal ions in most of these quinoproteins because, although essential for reconstitution, neither the ion present in native holoenzyme, nor the number of ions per subunit has been determined. In MDH from all the bacteria examined there was a single atom of calcium per $\alpha_2\beta_2$ tetramer. By contrast the soluble alcohol dehydrogenase from *Pseudomonas aeruginosa* contained 2 Ca^{2+}/α_2 dimer (Mutzel & Gorisch, 1991) and the soluble glucose dehydrogenase (GDH-B) from *Acinetobacter calcoaceticus* contained 4 Ca^{2+}/α_2 dimer (Geiger & Gorisch, 1989).

This would suggest that the roles of calcium in these three types of soluble quinoproteins might be different. In the case of MDH the presence of a single atom of calcium per $\alpha_2\beta_2$ tetramer suggests that the calcium might be located at the interface between the two $\alpha\beta$ dimers.

The demonstration that the quinoproteins discussed here contain calcium is noteworthy because involvement of calcium as a structural element in enzymes is rather unusual, an important example being DNAase I, in which there are 2 structural calcium ions are essential for stabilization of loop regions within the enzyme (Fig. 6.6), in addition to the calcium ion essential for activity of the enzyme (Oefner & Suck, 1986). The coordination of the 2 calcium-binding sites in DNAase I are quite different. Site 1 has 8 ligands formed with oxygen atoms of main-chain carbonyl groups, side-chain carboxylate groups and 2 water molecules (Fig. 6.6.a). Site 2, however, has only 6 ligands formed with oxygen atoms present in the side-chains of 4 amino acid residues (2 Aspartyl, 1 Glutamyl and 1 Phenylalanyl) and 2 water molecules (Fig. 6.6.b). Further examples of enzymes which have structurally important calcium include bovine galactosyltransferase (O'Keeffe *et al.*, 1980), and phospholipase A2 (Dijkstra *et al.*, 1981, 1983). The presence of calcium in some quinoproteins is likely to be relevant to the one feature that they have in common, their prosthetic group, PQQ. Analysis of the primary amino acid sequences of quinoproteins shows a conserved region that has been suggested to be the domain for binding the prosthetic group (Fig. 6.7; Cleton-Jansen *et al.*, 1990; Inoue *et al.*, 1990; Anthony, 1992a). The calcium might be involved in binding to acidic amino acids (or amides) in this region to stabilize a PQQ-binding conformation, or it may form a bridge between specific amino acid carboxylates and carboxylates of PQQ. Analysis of the putative PQQ-binding domain in MDH reveals the presence of a number of conserved aspartyl residues. The oxygen atoms of the carboxylate groups in these residues have been shown to be important in the function of calcium-binding proteins such as calmodulin. In these proteins, the peptide backbone forms a helix-loop-helix conformation called the EF hand (for a review see Strynadka and James, 1989; Fig. 6.7). Using the PC/Gene program, comparison of the PQQ-binding domain in MDH with a consensus sequence for the EF hand, or any other known calcium-binding motifs, did not result in sequence similarity. This suggests that the calcium-binding domain in MDH does not correspond to any of the known calcium-binding motifs. This suggestion is consistent with the fact that MDH contains only 1 calcium atom per

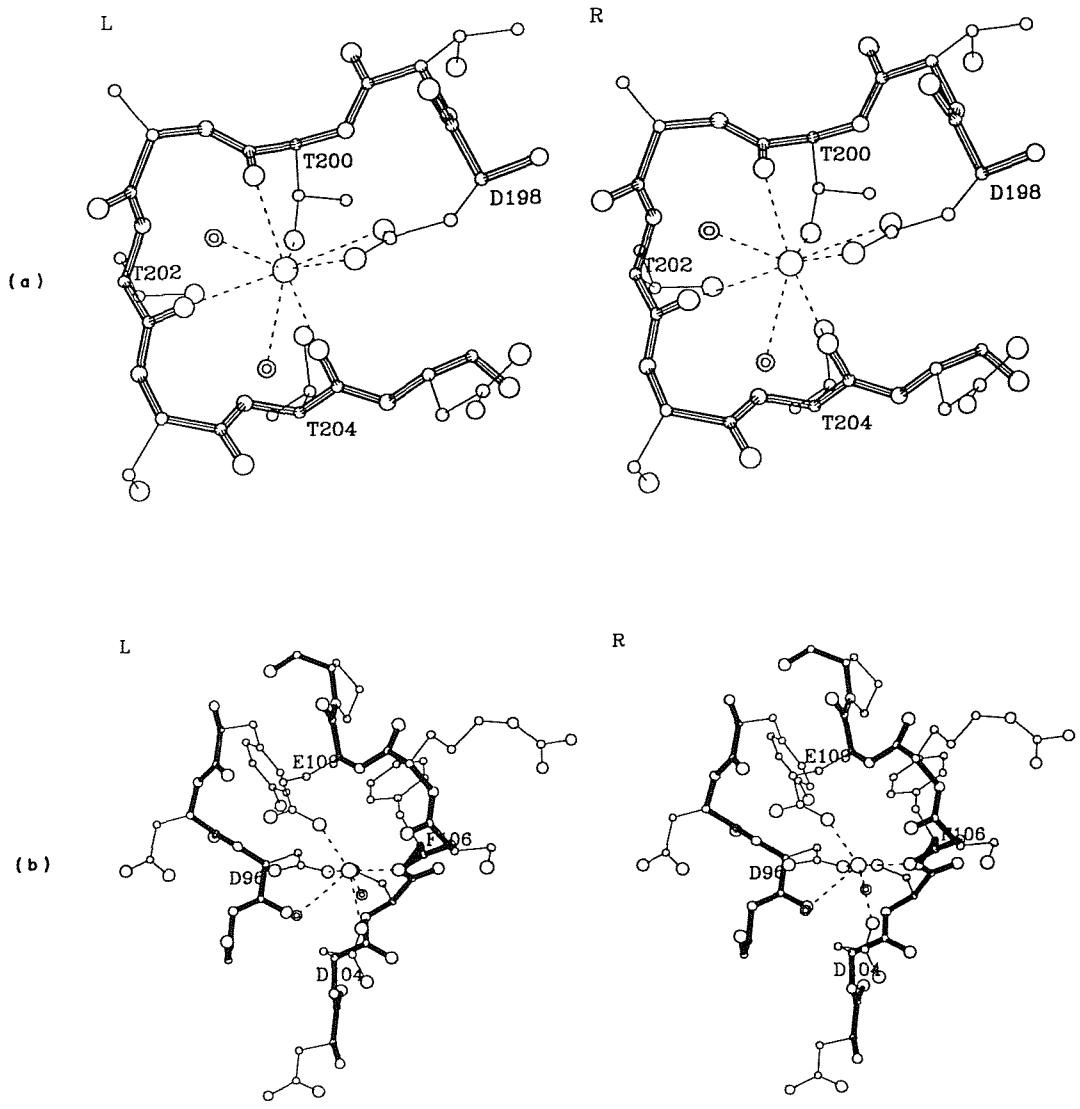


Fig. 6.6. The binding site for the two structural calcium atoms in DNAase I

These figures are taken from Oefner and Suck (1986). Site 1 (a) has 8 ligands formed with the oxygen atoms of main-chain carbonyl groups, side-chain carboxylate groups and 2 water molecules. Site 2 (b) has only 6 ligands formed with oxygen atoms present in the side-chains of 4 amino acids (2 Asp, 1 Glu and 1 Phe) and 2 water molecules. The calcium in both of these sites is involved in the stabilisation of loop regions in the protein. ◎ represents a water molecule.

```
MDH     M.ext   477- GGTMATAGDL VFYGTLDGY L-KARDSDTG DL-LWKFKIP SGAIGYPMT YTHKGTQYVA IYYGVGG
MDH     M.org   477- GGTLATAGDL VFYGTLDGY L-KARDSDTG DL-LWKFKIP SGAIGYPMT YTHKGTQYVA IYYGVGG
MDH     P.den   476- GGTMATAGGL TFYVTLDGF I-KARDSDTG EL-LWKFKLP SGVIGHPMT YKHDGRQYVA IMYGVGG
ADH     A.ace   486- GGILATGGDL LFQGLANGE F-HAYDATNG SD-LYKFDAQ SGIIAPPMT YSVNGKQYVA VEVGWGG
                     **·**·*·*  *    ·*  · ·* *···* ·  *·**· · **·*· *** *· ·* **** ·  * ** -Identity in MDHs and ADH
                     !! ··!·!··  !    ··    ·! · ··! ·  !··· · ·!  · !!! !· ·! !!!· ·  !  ! -Identity in MDHs, ADH & GDHA
GDHA    E.col   713- GGPISTAGNV LFIAATADN YLRAYNMSNG EK-LWQGRLP AGGQATPMT YEVNGKQYVV ISAGGHG

GDHA    A.cal   719- GGSISTAGNV MFVGATQDN YLRAFNVTNG KK-LWEARLP AGGQATPMT YEINGKQYVV IMAGGHG
                     * · *·· ·    ·  ··*·  ·     ··*  · *    ** · ·* **    *·***·* ·   ·     -Identity in all GDHs
GDHB    A.cal   137- GLPSSKDHQS GRLVIGPDQ KIYYTIGDQG RNQLAYLFLP NQAQHTPTQ QELNGKDYHT YMGKVLR
                     *   ··· ·         ·         · *    *     · ·     *    · ·* ·*   ·       -Identity in all proteins
```

Fig. 6.7. The putative PQQ binding region on quinoproteins

This figure is taken from the review by Anthony (1992a) and is the same figure as that shown in Fig. 1.11. The residue numbers refer to the mature proteins with signal peptides excluded. The methanol dehydrogenase (MDH) α-subunits are from *Methylobacterium extorquens*, *Methylobacterium organophilum* and *Paracoccus denitrificans*. The glucose dehydrogenases (GDH-A and GDH-B) are from *Acinetobacter calcoaceticus* and *E. coli*, and the alcohol dehydrogenase (ADH) is from *Acetobacter aceti*. (!) indicates identity in all quinoproteins except for the periplasmic GDH-B from *Ac. calcoaceticus*. (*) indicates identity; (.) indicates a conserved residue. It should be noted that although there is considerable identity between all the GDHs (15 amino acids out of 65), only six of these are the same as the 19 amino acids that are identical in all the other quinoproteins.

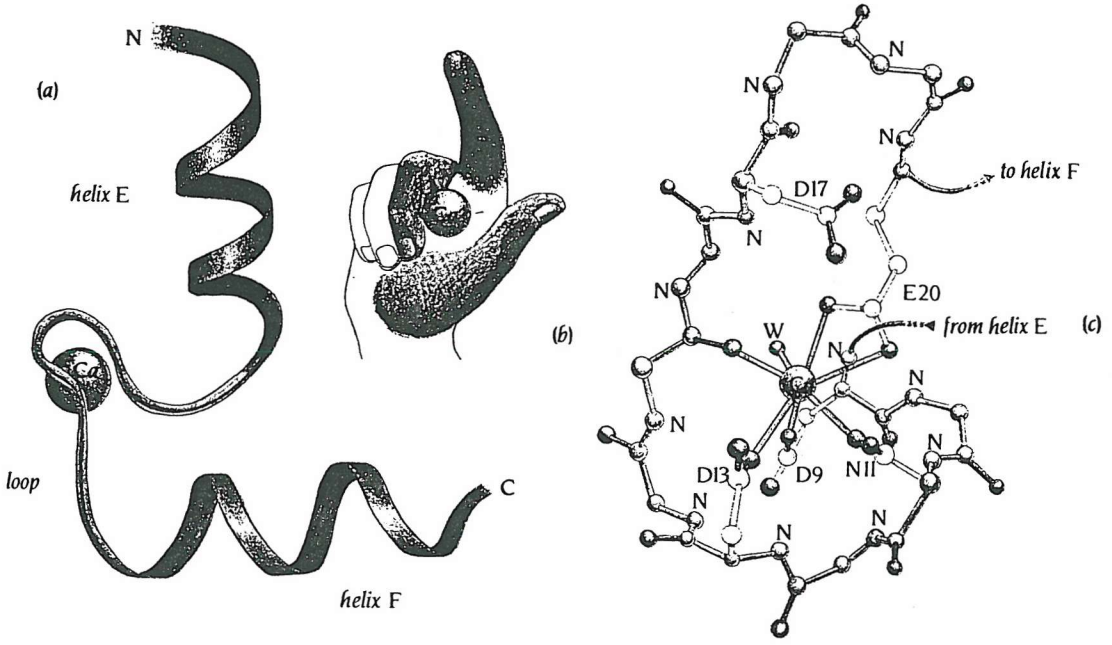


Fig. 6.8. The EF hand region present in troponin-C

This figure is taken from Branden and Tooze (1991). Troponin-C is a calcium binding protein located in muscle. It serves to trigger the muscle contraction by undergoing a conformational change upon binding calcium which initiates ATPase activity in myosin. The term EF hand was coined after identification of a calcium-binding domain in another muscle protein, parvalbumin (Kretsinger and Nockolds, 1973). Two of the helices present in troponin-C (labelled E and F) and the separating loop region are arranged to form a hand-like domain (a). The site binds a single calcium atom through oxygen atoms present in main-chain carbonyl groups and side-chain carboxylate groups. A water molecule is the 7th ligand (b). Similar sites have been identified in many calcium-binding proteins.

$\alpha_2\beta_2$ tetramer, and that this calcium probably lies at the interface between the 2 $\alpha\beta$ dimers and so forms bonds with both dimers. This does not necessarily eliminate the possibility that the calcium is also involved in binding PQQ to the enzyme. The fact that the C-9 carboxylic acid group of PQQ is essential for the reconstitution of active GDH in the presence of Mg^{2+} (Shinagawa *et al.*, 1986), suggests that this metal ion may form a bridge between this group and the protein. It is possible that the calcium in MDH may have a similar role.

Although the putative PQQ-binding domain is seen in the 4 types of quinoprotein that have been analyzed (MDH, alcohol dehydrogenase and 2 types of glucose dehydrogenase), it was noted that there was sufficient difference between these regions for the PQQ binding to be different in the glucose dehydrogenases from that in the MDH or alcohol dehydrogenase. The possibility that divalent cations other than calcium might be involved in the membrane glucose dehydrogenase supports this suggestion. Furthermore, it can be seen that although highly conserved in the MDH sequences, the carboxylates and amides likely to be involved in ligand formation with Ca^{2+} are not so well conserved between the 4 types of quinoprotein. This would be consistent with the demonstration that PQQ is readily released from glucose and alcohol dehydrogenases but not from methanol dehydrogenase. It should also be noted that metal ions can be removed from other quinoproteins by chelating agents and this leads to loss of PQQ. By contrast, treatment of MDH with EDTA did not remove calcium and did not lead to loss of PQQ or loss of activity.

The characterization of MDH isolated from MoxA, K and L mutants sheds some light on the role of calcium. In these mutants the structural genes *moxF* and *moxI* code for the normal α and β subunits, which were present in the normal $\alpha_2\beta_2$ tetrameric configuration. Beside the absence of calcium, the only observable difference in the MDH from the mutants was in their absorption, CD and esr spectra, and their reactivity with cyclopropanol. This is consistent with the proposal that the calcium in normal MDH is responsible either for binding PQQ directly in the active site or for maintaining the correct conformation of MDH responsible for binding PQQ. The experiments on the denaturation of the mutant MDHs under a variety of conditions showed that the PQQ in these enzymes is still tightly bound (Chapter 4). Therefore the calcium in MDH forms a bond with PQQ that is absolutely essential for the formation of active enzyme, although the PQQ makes additional bonds in the mutant MDHs that hold it in almost the correct configuration. The earlier observation that borate can

affect the absorption spectra of the mutant MDHs, but not that of wild-type MDH (Section 4.9.a), may provide some information about the nature of the binding between calcium and PQQ in MDH. Borate is known to form a spectrally unique complex with the *cis*-hydroxyls of hydrated PQQ in solution (Dekker *et al.*, 1982). The fact that the spectra of the mutant MDHs are also affected by borate suggests that a similar complex may be formed in these enzymes. This suggests that the PQQ in the mutant MDHs is hydrated whereas that in the wild-type enzyme is not. It is possible, therefore, that the calcium in MDH binds PQQ in such a way as to prevent hydration of the C-4 and C-5 carbonyl groups, and that this may occur by the formation of a bond between calcium and the oxygen atom of one or other of the carbonyl groups.

CHAPTER 7

General Summary and Discussion

The results presented in this thesis have been discussed in detail at the end of each chapter. The aim of this final chapter is to bring together and summarise these discussions, and to provide a model for the function of the *moxA*, *K* and *L* genes. A section relating to future work is also included.

7.1. The MoxC and MoxH mutants - Chapter 3

(a) Both of these mutants were shown to synthesise a protein cross-reactive to anti-MDH antibodies. This MDH was not in the typical $\alpha_2\beta_2$ tetrameric configuration, although it did contain at least the α-subunit.

(b) Supplementation of MoxC and MoxH with PQQ restored the ability to grow on methanol. This suggested that these mutants were impaired in either PQQ biosynthesis or PQQ transport.

(c) The crude extracts of MoxC and MoxH did not contain any material able to reconstitute activity in apo-alcohol dehydrogenase from *Pseudomonas testosteroni*. Therefore these mutants are unable to synthesise PQQ and, hence, the *moxC* and *moxH* genes are both involved in PQQ biosynthesis.

7.2. MDH from the MoxA, K and L mutants - Chapter 4

(a) These mutants were seen to produce MDH to similar levels as that produced by wild-type bacteria. These MDHs were in the typical $\alpha_2\beta_2$ tetrameric configuration, and both subunits had the same *N*-terminal sequence and amino acid composition as that for wild-type MDH. This confirmed that the *moxA*, *K* and *L* genes do not affect the primary sequence of MDH.

(b) All of the cystyl residues in MDH are involved in disulphide bridges. These bridges are intramolecular, ie they are formed within and not between the subunits.

(c) The mutant MDHs had very low activties (< 5% of that for wild-type MDH) in both dye-linked and cytochrome *c*-linked assay systems. Although the activity was low, it was still ammonia- and methanol-dependent. This lack of activity

was not due to the absence of a prosthetic group in the mutant MDHs.

(d) The mutant MDHs were able to cross-link with cytochrome c_L forming the same α-subunit/cytochrome complex as seen with wild-type MDH. This suggested that there was no conformational difference between the mutant and wild-type enzymes in the region of cytochrome c_L interaction.

(e) Denaturation of the wild-type and mutant MDHs under a variety of conditions showed that the proteins unfolded at a rate similar to that for release of prosthetic group. This suggested that there was no gross conformational differences between the MDHs.

(f) It was not possible to reconstitute active MDH by incubation of unfolded (by guanidinium chloride) MDH with an excess of PQQ. This suggested that MDH cannot self-assemble.

(g) Absorption spectra of the mutant MDHs did not conform to any of those for the known redox forms of the wild-type enzyme. The spectra were altered in the region due to the prosthetic group and varied from batch to batch. This suggested that the prosthetic group in the mutant MDHs might be different from that in the wild-type enzyme. This suggestion was supported by the observation that the mutant MDHs could not react with cyclopropanol under oxidising conditions.

(h) The absorption spectra varied with the nature of the buffer whereas the wild-type enzyme was unaffected. Circular dichroism of the mutant MDHs revealed a difference in the region of the spectrum due to aromatic amino acid residues. These results suggested that the the prosthetic group in the mutant MDHs was bound differently from that in the wild-type enzyme.

7.3. <u>The prosthetic group in the MoxA, K and L mutant MDHs - Chapter 5</u>

(a) The prosthetic group was shown to be normal PQQ, in terms of spectral properties and reconstitution of apo-quinoproteins, and was present in the usual stoichiometry of 2 moles per $\alpha_2\beta_2$ tetramer.

(b) Electron spin resonance (ESR) spectroscopy showed that, in wild-type MDH, some of the PQQ (about 28%) was in the semiquinone form. In the mutant MDHs, however, there was no detectable ESR signal. This suggested that the PQQ in these enzymes was either in the fully oxidised or fully reduced states.

(c) HPLC analysis of the extracted prosthetic group from wild-type MDH showed

the presence of roughly equimolar amounts of the quinone and quinol forms of PQQ. By contrast, the extracted prosthetic group from the mutant MDHs was predominantly (> 90%) in the oxidised state. Although the PQQ in the mutant MDHs was oxidised, the enzymes could not react with substrate. This observation supported the earlier suggestion that the PQQ in the mutant MDHs was bound differently from that in the wild-type enzyme.

7.4. Calcium in MDH - Chapter 6

(a) MDHs purified from wild-type *Methylobacterium extorquens*, *Hyphomicrobium* X, *Methylophilus methylotrophus* and *Paracoccus denitrificans* were all shown to contain 1 atom of calcium per $\alpha_2\beta_2$ tetramer.

(b) The MDHs from the MoxA, K and L mutants of *Mb. extorquens* were shown to contain virtually no calcium. Active enzyme could be reconstituted from the mutant MDHs by incubation with calcium ions at pH 9.0. Neither ammonia nor methanol were necessary for reconstitution.

(c) The reconstituted MDHs had full activity (as compared to the wild-type enzyme) in both dye-linked and cytochrome *c*-linked assay systems, and were able to react with cyclopropanol under oxidising conditons.

(d) The PQQ extracted from the reconstituted MDHs was shown to be almost exclusively in the reduced state.

(e) The calcium could not be removed from the wild-type or reconstituted mutant MDHs by gel filtration or by treatment with chelating agents.

(f) It was concluded that the single calcium atom in MDH is probably located at the interface between the two $\alpha\beta$ dimers, and that it serves to bind PQQ in the correct configuration for reaction with substrate in the active site.

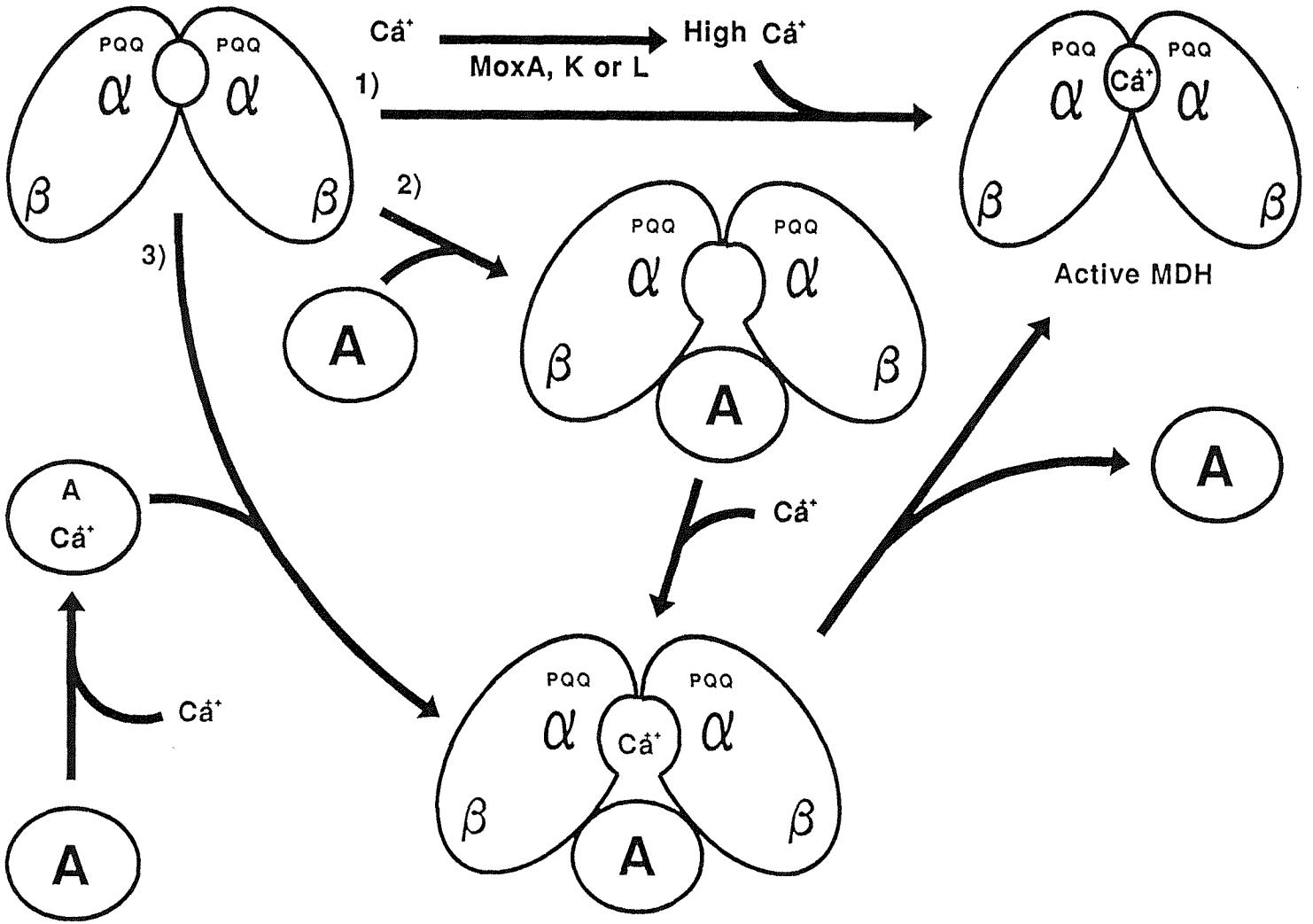
7.5. The role of the *moxA*, *K* and *L* genes in MDH biosynthesis

The work decribed in this thesis has investigated the nature of the MDHs from the MoxA, K and L mutants of *Mb. extorquens*. These inactive MDHs were shown to be lacking a calcium atom that is normally present in the wild-type enzyme. Incubation of the mutant MDHs with calcium restored activity. Therefore, the *moxA*, *K* and *L* genes are clearly implicated in the insertion of calcium into MDH. Fig. 7.1 summarises possible roles for the proteins coded by these genes. A key feature of the 3 models included in Fig. 7.1 is that they are based on the observation that PQQ is tightly bound

Fig. 7.1. The role of the *moxA, K* and *L* gene products in MDH assembly

Fig. 7.1. The role of the *moxA*, *K* and *L* gene products in MDH assembly

The three possible roles summarised here are for the MoxA protein; an identical role could be described for MoxK and/or MoxL proteins. Nothing in the present work has distinguished the phenotype of the three mutants. 1) the three genes are involved merely in maintaining a sufficiently high local concentration of calcium in the periplasm. 2) the MoxA protein stabilises the $\alpha_2\beta_2$ tetramer together with the 2 molecules of PQQ in a conformation able to bind low concentrations of calcium, after which the MoxA protein dissociates. 3) This proposal is the same as the second, except that the MoxA protein carries the calcium ion to its location in the MDH.


PQQ
α
α
PQQ
β
β
Ca2+
MoxA, K or L
High Ca2+
1)
2)
3)
A
Ca2+
PQQ
α
Ca2+
α
PQQ
β
β
A
Active MDH

to the $\alpha_2\beta_2$ tetramer before insertion of Ca^{2+}; and that the conformation in the absence of calcium is sufficiently similar to that of the holoenzyme for this to be formed when the concentration of calcium in the medium is artificially high. The concentration of free calcium in the periplasm where the MDH is assembled is presumably the same as that in the surrounding growth medium. The concentration in normal growth media is at least 20 μM. Incubation of pure MDH from the MoxA mutant in this concentration of calcium led to reconstitution of active MDH but only 25% of maximum activity was achieved and the rate was extremely slow (10% reconstitution after 2 days). The rate with higher concentrations was very much greater and full activity could be achieved (100% activity in 1 h in 10 mM calcium). The simplest description of the role of the MoxA, K and L proteins might therefore be that they provide a high local concentration of calcium in the periplasm (model 1). However, as it is probable that calcium flux in and out of the periplasm by way of porins in the outer membrane would be rapid it is difficult to conceive of such a mechanism. Alternative functions of the proteins are suggested in Fig. 7.1. These include a calcium-binding role in which the MoxA protein carries calcium to the active site and is then released (model 3). Alternatively, the MoxA protein might be involved in stabilising a conformation of MDH that is then able to bind calcium, after which the MoxA protein is released and does not form part of the final structure (model 2). In this respect these proteins would be fulfilling a molecular chaperone function (see Section 4.8.a). The use of the MoxA protein in these models is for example only; identical functions could be described for the MoxK and MoxL proteins. In view of the phenotypic similarity of these mutants, some important questions are raised: why have three genes involved in the same, apparently simple function; do the gene products form a complex in the periplasm; or do the gene products act in a 'linear' sequence in processing MDH.

7.6. Future work

(a) The MoxC and MoxH mutants. Perhaps the obvious first step in identifying the precise function(s) of the *moxC* and *moxH* genes in PQQ biosynthesis would be to sequence these genes. The deduced amino acid sequences could then be compared with those for the PQQ genes identified from other organisms. It would be interesting to determine the nature of the MDH produced by these mutants, and whether it contained the β-subunit. Clearly the established method for the purification of MDH was not successful, and so a new protocol would have to be developed. If this MDH does

contain both α- and β-subunits, the reconstitution of active enzyme could be studied by incubation of the isolated protein with PQQ and calcium. This would show whether PQQ could be incorporated spontaneously into MDH.

(b) The MoxA, K and L mutants. Presently there is little genetic information on the *moxA*, *K* and *L* genes, although it has been suggested that *moxL* encodes a protein of 19 kDa (Lidstrom and Tsygankov, 1991). The three genes are clustered, lying in a 4.55 kbp fragment, but are not co-transcribed (Nunn and Lidstrom, 1986b). Therefore, the sequencing of these genes should be relatively straightforward and will allow amino acid sequence analysis to be performed. In view of their probable role in the insertion of calcium into MDH, the three gene products should be located in the periplasm and hence the genes will have regions encoding a signal peptide. The sequencing of these genes is currently in progress in our laboratory.

Recent work by Adachi *et al.* (1990a) showed that the calcium in MDH from *Methylobacillus glycogenes* could be replaced by other divalent cations (such as strontium or barium), by substituting the alternative ion for calcium in the growth medium. Similar studies could be performed with *Mb. extorquens*, although the metal ion specificity of MDH could be more readily tested by incubation of the metal-free MDHs from the MoxA, K and L mutants with a wide variety of alternative metal ions. The incorporation of a relatively heavy divalent metal ion, for example barium, into MDH would result in a 'natural' heavy metal derivative that could be used for the determination of the 3-dimensional structure of MDH, and that could provide a simple direct demonstration of the location of the metal ion (see Appendix).

Further work on the reconstitution of the mutant MDHs should include studying the effect of pH. The reconstitution studies described in Chapter 6 were performed at pH 9.0, since this is the optimum pH for the dye-linked assay system and the reconstitution mixtures were used directly in the assays. *Mb. extorquens* does not grow at pH 9.0 (its optimum is pH 7.0), and so calcium would have to be inserted into MDH at pH 7.0 in the periplasm. The pI of MDH is 8.8 and so the change in pH will probably have an effect on the charges of some or all of the side-groups involved in the interaction between MDH, PQQ and calcium. It is possible that this charge is important in the insertion of calcium, and the *moxA*, *K* and *L* gene products may function by masking unfavourably charged residues.

Other possible avenues for further study include:

(a) The effect of temperature on the reconstitution of the mutant MDHs.

(b) Do the reconstituted MDHs have the same CD spectra as that for the wild-type enzyme?

(c) Do the reconstituted MDHs have an ESR signal, and is it the same as that seen for the wild-type enzyme?

(d) Can the normal level of calcium in MDH be achieved simply by increasing the concentration of calcium in the growth medium?

(e) To test for a calcium-binding protein in wild-type *Mb. extorquens* that may not be present in the MoxA, K and L mutants, crude extracts of the various strains could be incubated with ^{45}Ca, and then run on non-denaturing PAGE to maintain the native conformation of the proteins. Obviously a control using a known calcium-binding protein, such as calmodulin, would have to be included.

(f) A molecular chaperone function for the MoxA, K and L proteins could be tested for by incubating wild-type and mutant strains, grown under conditions that induces the methanol oxidising system, with ^{35}S-methionine. The presence of any proteins that bind to MDH during the synthesis of this enzyme in wild-type bacteria could then be detected by non-denaturing PAGE and autoradiography. A similar approach was used for the identification of the involvement of GroEL in the assembly of Rubisco (Ellis, 1990).

References

Adachi, O., Matsushita, K., Shinagawa, E.and Ameyama, M. (1988) *Agric. Biol. Chem.* **52**, 2081-2082

Adachi, O., Matsushita, K., Shinagawa, E.and Ameyama, M. (1990a) *Agric. Biol. Chem.* **54**, 2833-2837

Adachi, O., Matsushita, K., Shinagawa, E. and Ameyama, M. (1990b) *Agric. Biol. Chem.* **54**, 3123-3129

Akhtar, M. and Wilton, D.C. (1973) *Ann. Rep. Chem. Soc.* **70**, 98-118

Alefounder, P.R. and Ferguson, S.J. (1981) *Biochem. Biophys. Res. Comm.* **98**, 778-784
Biol. Chem. **48**, 561-565

Ameyama, M., Matsushita, K., Shinagawa, E., Hayashi, M. and Adachi, O. (1988) *Biofactors* **1**, 51-53

Ameyama, M., Nonobe, M., Shinagawa, E., Matsushita, K. and Adachi, O. (1985a) *Anal. Biochem.* **151**, 263-267

Ameyama, M., Nonobe, M., Hayashi, M., Shinagawa, E., Matsushita, K. and Adachi, O. (1985b) *Agric. Biol. Chem.* **49**, 1227-1231

Ameyama, M., Nonobe, M., Shinagawa, E., Matsushita, K., Takimoto, K. and Adachi, O. (1986) *Agric. Biol. Chem.* **50**, 49-57

Ameyama, M., Shinagawa, E., Matsushita, K. and Adachi, O. (1984) *Agric. Biol. Chem.* **48**, 2909-2911

Anderson, D.J. and Lidstrom, M.E. (1988) *J. Bact.* **170**, 2254-2262

Anderson, D.J., Morris, C.J., Nunn, D.N., Anthony, C. and Lidstrom, M.E. (1990) *Gene.* **90**, 173-176

Anthony, C. (1975) *Biochem. J.* **146**, 289-298

Anthony, C. (1982) The Biochemistry of Methylotrophs, Academic Press, London,

Anthony, C. (1986) *Adv. Microbial. Physiol.* **27**, 113-210

Anthony, C. (1988) in Bacterial Energy Transduction (Anthony, C. ed.), pp. 293-316, Academic Press , London

Anthony, C. (1992a) *Int. J. Biochem.* **24**, 29-39

Anthony, C. (1992b) *Biochim. Biophys. Acta* **1099**, 1-15

Anthony, C. (1992c) in Principles and applications of quinoproteins (Davidson, V.L. ed.), Marcel Dekker, New York

Anthony, C. and Zatman, L.J. (1964a) *Biochem. J.* **92**, 609-614

Anthony, C. and Zatman, L.J. (1964b) *Biochem. J.* **92**, 614-621

Anthony, C. and Zatman, L.J. (1965) *Biochem. J.* **96**, 808-812

Anthony, C. and Zatman, L.J. (1967a) *Biochem. J.* **104**, 953-959

Anthony, C. and Zatman, L.J. (1967b) *Biochem. J.* **104**, 960-969

Bamforth, C.W. and Quayle, J.R. (1978a) *Arch. Microbiol.* **119**, 91-97

Bamforth, C.W. and Quayle, J.R. (1978b) *Biochem. J.* **169**, 677-686

Bamforth, C.W. and Quayle, J.R. (1979) *Biochem. J.* **181**, 517-524

Bastien, C.A., Machlin, S.M., Zhang, Y., Donaldson, K. and Hanson, R.S. (1989) *Appl. Environ. Microbiol.* **55**, 3124-3130

Beardmore-Gray, M. (1982) Ph.D. Thesis, University of Southampton

Beardmore-Gray, M. and Anthony, C. (1984) in Microbial Growth on C-1 Compounds (Crawford, R.L. and Hanson, R.S. eds.), pp. 97-105, American Society of Microbiology, Washington, D.C.

Beardmore-Gray, M., O'Keeffe, D.T. and Anthony, C. (1983) *J. Gen. Microbiol.* **129**, 923-933

Biville, F., Mazodier, P., Gasser, F., van Kleef, M.A.G. and Duine, J.A. (1988) *FEMS Microbiol. Lett.* **52**, 53-58

Biville, F., Turlin, E. and Gasser, F. (1989) *J. Gen. Microbiol.* **135**, 2917-2929

Biville, F., Turlin, E. and Gasser, F. (1991) *J. Gen. Microbiol.* **137**, 1775-1782

Bolbot, J.A. and Anthony, C. (1980) *J. Gen. Microbiol.* **120**, 245-254

Brahms, S. and Brahms, J. (1980) *J. Mol. Biol.* **138**, 149-178

Branden, C. and Tooze, J. (1991) Introduction to Protein Structure, Garland Publishing, New York

Breslow, R. and Guo, T. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 167-169

Buechi, G., Botkin, J.H., Lee, G.C.M. and Yakushijin, K. (1985) *J. Am. Chem. Soc.* **107**, 5555-5557

Burton, S.M., Byrom, D., Carver, M.A., Jones, G.D.D. and Jones, C.W. (1983) *FEMS Microbiol. Lett.* **17**, 185-190

Carver, M.A., Humphrey, K.M., Patchett, R.A. and Jones, C.W. (1984) *Eur. J. Biochem.* **138**, 611-615

Carver, M.A. and Jones, C.W. (1984) *Arch. Microbiol.* **139**, 76-82

Chan, H.T.C. and Anthony, C. (1991) *Biochem. J.* **280**, 139-146

Chistoserdov, A.Y., Tsygankov, Y.D. and Lidstrom, M.E. (1990) *Biochem. Biophys. Res. Comm.* **172**, 211-216

Cleton-Jansen, A-M., Goosen, N., Fayet, O. and van de Putte, P. (1990) *J. Bacteriol.* **172**, 6308-6315

Cleton-Jansen, A-M., Goosen, N., Odle, G. and van de Putte, P. (1988) *Nucleic. Acids. Res.* **16**, 6228

Cleton-Jansen, A-M., Goosen, N., Vink, K. and van de Putte, P. (1989) *Mol. Gen. Genet.* **217**, 430-436

Colby, J. and Zatman, L.J. (1972) *Biochem. J.* **128**, 1373-1376

Compton, L.A. and Johnson, W.C. (1986) *Anal. Biochem.* **155**, 155-167

Corey, E.J. and Tramontano, A. (1981) *J. Am. Chem. Soc.* **103**, 5599

Cox, J.M. (1992) Ph.D. Thesis, University of Southampton

Cox, J.M., Day, D.J. and Anthony, C. (1992) *Biochim. Biophys. Acta* **1119**, 97-106

Creighton, T.E. (1984) The Proteins, Freeman, New York,

Davidson, V.L., Neher, J.W. and Cecchini, G. (1985) *J. Biol. Chem.* **260**, 9642-9647

Dawson, A., Southgate, G. and Goodwin, P.M. (1990) *FEMS Microbiol. Lett.* **68**, 93-96

Day, D.J. (1990) Ph.D. Thesis, University of Southampton

Day, D.J. and Anthony, C. (1990a) *Methods in Enzymology* **188**, 210-216

Day, D.J. and Anthony, C. (1990b) *Methods in Enzymology* **188**, 298-303

Day, D.J., Nunn, D.N. and Anthony, C. (1990) *J. Gen. Microbiol.* **136**, 181-188

de Beer, R., Duine, J.A. and Frank, J. (1983) *Eur. J. Biochem.* **130**, 105-109

de Beer, R., Duine, J.A., Frank, J. and Large, P.J. (1980) *Biochem. Biophys. Acta* **622**, 370-374

de Beer, R., van Ormmondt, D., van Ast, M.A., Banen, R., Frank, J. and Duine, J.A. (1979) *J. Chem. Phys.* **70**, 4491-4495

De Biase, D., Maras, B. and John, R.A. (1991) *FEBS Lett.* **278**, 120-122

de Vries, G.E. (1986) *FEMS. Microbiol. Revs.* **39**, 235-258

de Vries, G.E., Harms, N., Maurer, K., Papendrecht, A. and Stouthamer, A.H. (1988) *J. Bact.* **170**, 3731-3737

de Vries, G.E., Kues, U. and Stahl, U. (1990) *FEMS. Microbiol. Letts.* **75**, 57-102

Dekker, R.H., Duine, J.A., Frank, J., Verwiel, P.E.J. and Westerling, J. (1982) *Eur. J. Biochem.* **125**, 69-73

Dijkstra, B.W., Kalk, K.H., Hol, W.G.J. and Drenth, J. (1981) *J. Mol. Biol.* **147**, 97-123

Dijkstra, B.W., Renetseder, R., Kalk, K.H., Hol, W.G.J. and Drenth, J. (1983) *J. Mol. Biol.* **168**, 163-179

Dijkstra, M., Frank, J. and Duine, J.A. (1988) *FEBS Lett.* **227**, 198-202

Dijkstra, M., Frank, J., Jongejan, J.A. and Duine, J.A. (1984) *Eur. J. Biochem.* **140**, 369-373

Duine, J.A. (1990) *TIBS* **15**, 96-96

Duine, J.A. (1991) *Eur. J. Biochem.* **200**, 271-284

Duine, J.A. and Frank, J. (1980a) *Biochem. J.* **187**, 213-219

Duine, J.A. and Frank, J. (1980b) *Biochem. J.* **187**, 221-226

Duine, J.A., Frank, J. and de Beer, R. (1984) *Arch. Biochem. Biophys.* **233**, 708-711

Duine, J.A., Frank, J. and Jongejan, J.A. (1983) *Anal. Biochem.* **133**, 239-243

Duine, J.A., Frank, J. and Jongejan, J.A. (1987) in Advances in Enzymology (pp. 169-212, John Wiley & Sons, New York

Duine, J.A., Frank, J. and Ruiter, L.G. (1979) *J. Gen. Microb.* **115**, 523-526

Duine, J.A., Frank, J. and Verwiel, P.E.J. (1980) *Eur. J. Biochem.* **108**, 187-192

Duine, J.A., Frank, J. and Verwiel, P.E.J. (1981) *Eur. J. Biochem.* **118**, 395-399

Duine, J.A., Frank, J. and Westerling, J. (1978) *Biochim. Biophys. Acta* **524**, 277-287

Duine, J.A. and Jongejan, J.A. (1989) *Annu. Rev. Biochem.* **58**, 403-426

Duine, J.A., van der Meer, R.A. and Groen, B.W. (1990) *Annu. Rev. Nutr.* **10**, 297-318

Dunstan, P.M. and Anthony, C. (1973) *Biochem. J.* **132**, 797-801

Dunstan, P.M., Anthony, C. and Drabble, W.T. (1972a) *Biochem. J.* **128**, 99-106

Dunstan, P.M., Anthony, C. and Drabble, W.T. (1972b) *J. Gen. Microbiol.* **128**, 107-115

Eady, R.R. and Large, P.J. (1968) *Biochem. J.* **106**, 245-255

Eady, R.R. and Large, P.J. (1971) *Biochem. J.* **123**, 757-771

Elliott, E.J. and Anthony, C. (1988) *J. Gen. Microbiol.* **134**, 369-377

Ellis, R.J. (1990) *Science* **250**, 954-959

Ellis, R.J. (1991) *Plant J.* **1**, 9-13

Ellis, R.J. and Hemmingsen, S.M. (1989) *TIBS* **14**, 339-342

Emmons, W.D. and Lucas, G.B. (1955) *J. Am. Chem. Soc.* **77**, 2287-2288

Flynn, G.C., Pohl, J., Flocco, M.T. and Rothman, J.E. (1991) *Nature* **353**, 726-730

Ford, S., Page, M.D. and Anthony, C. (1985) *J. Gen. Microbiol.* **131**, 2173-2182

Forrest, H.S., Salisbury, S.A. and Kilty, C.G. (1980) *Biochem. Biophys. Res. Comm.* **97**, 248-251

Frank, J., Dijkstra, M., Duine, A.J. and Balny, C. (1988) *Eur. J. Biochem.* **174**, 331-338

Frank, J., van Krimpen, S.H., Verwiel, P.E.J., Jongejan, J.A., Mulder, A.C. and Duine, J.A. (1989a) *Eur. J. Biochem.* **184**, 187-195

Frank, J., Dijkstra, M., Balny, C., Verwiel, P.E.J. and Duine, J.A. (1989b) in PQQ and Quinoproteins (Jongejan, J.A. and Duine, J.A. eds.), pp. 13-22, Kluwer Academic Publishers, Dordrecht

Fukumori, Y., Nakayama, K. and Yamanaka, T. (1985) *J. Biochem.* **98**, 493-499

Fukumori, Y., Nakayama, K. and Yamanaka, T. (1985) *J. Biochem.* **98**, 1719-1722

Gainor, J.A. and Weinreb, S.M. (1981) *J. Org. Chem.* **46**, 4317

Gainor, J.A. and Weinreb, S.M. (1982) *J. Org. Chem.* **47**, 2833

Gallop, P.M. (1990) *TIBS* **15**, 96-97

Gallop, P.M., Paz, M.A., Fluckiger, R. and Kagan, H.M. (1989) *TIBS* **14**, 343-346

Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) *J. Mol. Biol.* **120**, 97-120

Gatenby, A.A. and Ellis, R.J. (1990) *Annu. Rev. Cell Biol.* **6**, 125-149

Geiger, O. and Görisch, H. (1987) *Anal. Biochem.* **164**, 418-423

Geiger, O. and Görisch, H. (1989) *Biochem. J.* **261**, 415-421

Ghosh, R. and Quayle, J.R. (1981) *Biochem. J.* **199**, 245-250

Goldberg, I. (1976) *Eur. J. Biochem.* **63**, 233-240

Goldenberg, D.P. (1989) in Protein structure: a functional approach (Creighton, T.E. ed.), pp. 225-250, IRL Press, Oxford

Goodwin, P.M. (1990) in Autotrophic Micribiology and One-Carbon Metabolism (Codd, G.A., Dijkhuizen, L. and Tabita, F.R. eds.), pp. 143-162, Kluwer academic publishers, Dordrecht

Goosen, G., Horsman, H.P.A., Huinen, R.G.M. and van der Putte, P. (1989) *J. Bacteriol.* **171**, 447-445

Goosen, N., Vermaas, D. and van de Putte, P. (1987) *J. Bact.* **169**, 303-307

Görisch, H., Geiger, O. and Adler, M. (1989) in PQQ and Quinoproteins (Jongejan, J.A. and Duine, J.A. eds.), pp. 100-102, Kluwer Academic Publishers, Dordrecht

Grabarek, Z. and Gergely, J. (1990) *Anal. Biochem.* **185**, 131-135

Green, P.N. and Bousfield, I.J. (1982) *J. Gen. Microbiol.* **128**, 623-638

Greenwood, J.A. and Jones, C.W. (1986) *J. Gen. Microbiol.* **132**, 1247-1256

Groen, B.W. and Duine, J.A. (1990) *Methods in Enzymology* **188**, 33-39

Groen, B.W., van Kleef, M.A.G. and Duine, J.A. (1986) *Biochem. J.* **234**, 611-615

Harms, N., de Vries, G.E., Maurer, K., Hoogendijk, J. and Stouthamer, A.H. (1987) *J. Bact.* **169**, 3969-3975

✳ Hartmann, C. and Klinmann, J.P. (1988) *Biofactors* **1**, 41-49

Hayashi, S.I. and Lin, E.C.C. (1965) *J. Mol. Biol.* **14**, 515-521

Hendricksen, J.B. and de Vries, J.G. (1982) *J. Org. Chem.* **47**, 1148

Hendricksen, J.B. and de Vries, J.G. (1985) *J. Org. Chem.* **50**, 1688

Heptinstall, J. and Quayle, J.R. (1969) *J. Gen. Microbiol.* **55**, xvi-xvii

Holmgren, A. and Branden, C-I. (1989) *Nature* **342**, 248-251

Hommel, R. and Kleber, H.P. (1990) *J. Gen. Microbiol.* **136**, 1705-1711

Houck, D.R., Hanners, J.L. and Unkefer, C.J. (1988) *J. Am. Chem. Soc.* **110**, 6920-6921

Houck, D.R., Hanners, J.L. and Unkefer, C.J. (1991) *J. Am. Chem. Soc.* **113**, 3162-3166

✳ Harms, N. and van Spanning, R.J.M. (1991) *J. Bioenerg. Biomemb.* **23,** 187-210

Houck, D.R., Hanners, J.L., Unkefer, C.J., van Kleef, M.A.G. and Duine, J.A. (1989) *Ant. van. Leeuw.* **56**, 93-101

Hultgren, S.J., Normark, S. and Abraham, S.N. (1991) *Ann. Rev. Microbiol.* **45**, 383-415

Inoue, T., Sunagawa, M., Mori, A., Imai, C., Fukuda, M., Takagi, M. and Yano, K. (1989) *J. Bact.* **171**, 3115-3122

Inoue, T., Sunagawa, M., Mori, A., Imai, C., Fukuda, M., Takagi, M. and Yano, K. (1990) *J. Ferment. Bioeng.* **70**, 58-60

Ishii, I., Hase, T., Fukumori, Y., Matsubara, H. and Tobari, J. (1983) *J. Biochem.* **93**, 107-119

Ito, N., Phillips, S.E.V., Stevens, C., Ogel, Z.B., Mcpherson, M.J., Keen, J.N., Yadav, K.D.S. and Knowles, P.F. (1991) *Nature* **350**, 87-90

Jaenicke, R. and Rudolph, R. (1989) in Protein structure: a functional approach (Creighton, T.E. ed.), pp. 191-223, IRL Press, Oxford

Janes, S.M., Mu, D., Wemmer, D., Smith, A.J., Kaur, S., Maltby, D., Buringame, A.L. and Klinman, J.P. (1990) *Science* **248**, 981-987

Janvier, M. and Gasser, F. (1987) *Biochimie* **69**, 1169-1174

Jensen, K., Hertz, J.B., Samra, J., Magiar, C., Larsen, F.S., Selmer, J. and Sompolinsky, D. (1985) *FEMS Microbiol. Lett.* **29**, 129-133

Jones, C.W., Greenwood, J.A., Burton, S.M., Santos, H. and Turner, D.L. (1987) *J. Gen. Microbiol.* **133**, 1511-1519

Jones, C.W., Kingsbury, S.A. and Dawson, M.J. (1982) *FEMS Microbiol. Lett.* **13**, 195-200

Jongejan, J.A. and Duine, J.A. (1987) *Tetrahedron Lett.* **28**, 2767-2768

Jongejan, J.A., Groen, B.W. and Duine, J.A. (1989) in PQQ and quinoproteins (Jongejan, J.A. and Duine, J.A. eds.), pp. 205-216, Kluwer Academic Publishers, Dordrecht

Kasprzak, A.A. and Steenkamp, D.J. (1983) *J. Bact.* **156**, 348-353

Kenney, W.C. and Mcintire, W.S. (1983) *Biochemistry* **22**, 3858-3868

Killgore, J., Smidt, C., Duich, L., Romero-Chapman, N., Tinker, D., Reiser, K., Melko, M., Hyde, D. and Rucker, R.B. (1989) *Science* **245**, 850-852

King, J., Griffin-Shea, R. and Fuller, M.T. (1980) *Quart. Rev. Biol.* **55**, 369-393

Kohler, J. and Scwartz, A.C. (1981) *Can. J. Microbiol.* **28**, 65-72

Kornberg, H.L. and Morris, J.G. (1965) *Biochem. J.* **95**, 557-586

Kretsinger, R.H. and Nockolds, E.E. (1973) *J. Biol. Chem.* **248**, 3313-3326

Ladner, A. and Zatman, L.J. (1969) *J. Gen. Microbiol.* **55**, xvi-xvi

Laemmli, U.K. (1970) *Nature* **227**, 680-685

Landry, S.J. and Gierasch, L.M. (1991) *Biochemistry* **30**, 7359-7362

Lawton, S.A. and Anthony, C. (1985) *J. Gen. Microbiol.* **131**, 2165-2171

Lee, K.E., Stone, S., Goodwin, P.M. and Holloway, B.W. (1991) *J. Gen. Microbiol.* **137**, 895-904

Lidstrom, M.E. (1990) *FEMS Microbiol. Rev.* **87**, 431-436

Lidstrom, M.E. and Stirling, D.I. (1990) *Ann. Rev. Microbiol.* **44**, 27-58

Lidstrom, M.E. and Tsygankov, Y.D. (1991) in Biology of Methylotrophs (Goldberg, I. and Rokem, J.S. eds.), pp. 273-304, Butterworth-Heinemann, Boston

Lobenstein-Verbeek, C.L., Jongejan, J.A., Frank, J. and Duine, J.A. (1984) *FEBS Lett.* **170**, 305-309

Long, A.R. and Anthony, C. (1991) *J. Gen. Microbiol.* **137**, 2353-2360

Machlin, S.M. and Hanson, R.S. (1988) *J. Bact.* **170**, 4739-4747

Machlin, S.M., Tam, P.E., Bastien, C.A. and Hanson, R.S. (1988) *J. Bact.* **170**, 141-148

MacKenzie, A.R., Moody, C.J. and Rees, C.W. (1986) *Tetrahedron* **42**, 3259

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory, New York,

Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A.L. and Hartl, F.U. (1991) *Nature* **352**, 36-42

Mazodier, P., Biville, F., Turlin, E. and Gasser, F. (1988) *J. Gen. Microbiol.* **134**, 2513-2524

Mcintire, W.S. and Stults, J.T. (1986) *Biochem. Biophys. Res. Comm.* **141**, 562-568

Mcintire, W.S., Wemmer, D.E., Chistoserdov, A. and Lidstrom, M.E. (1991) *Science* **252**, 817-824

McNerney, T. and O'Connor, M.L. (1980) *Appl. Environ. Microbiol.* **40**, 370-375

Meulenberg, J.J.M., Sellink, E., Riegman, N.H. and Postma, P.W. (1992) *Mol. Gen. Genet.* **232**, 284-294

Meulenberg, J.J.M., Sellink, E., Loenen, W.A.M., Riegman, N.H., van Kleef, M.A.G. and Postma, P.W. (1990) *FEMS Microbiol. Lett.* **71**, 337-344

Michaelis, L. and Granick, S. (1944) *J. Am. Chem. Soc.* **65**, 1747-1755

Mincey, T., Bell, J.A., Mildran, A.S. and Abeles, R.H. (1981) *Biochem.* **20**, 7502-7509

Mutzel, A. and Görisch, H. (1991) *Agric. Biol. Chem.* **55**, 1721-1726

Nunn, D.N. and Anthony, C. (1988a) *Biochem. J.* **256**, 673-676

Nunn, D.N. and Anthony, C. (1988b) *Nucl. Acids. Res.* **16**, 7722-7722

Nunn, D.N., Day, D.J. and Anthony, C. (1989) *Biochem. J.* **260**, 857-862

Nunn, D.N. and Lidstrom, M.E. (1986a) *J. Bact.* **166**, 581-590

Nunn, D.N. and Lidstrom, M.E. (1986b) *J. Bact.* **166**, 591-597

O'Connor, M.L. (1981) in Microbial Growth on C1 Compounds (Dalton, H. ed.), pp. 294-300, Heyden, London

O'Connor, M.L. and Hanson, R.S. (1977) *J. Gen. Microbiol.* **101**, 327-332

O'Keeffe, D.T. (1980) Ph.D. Thesis, University of Southampton

O'Keeffe, E.T., Hill, R.L. and Bell, J.E. (1980) *Biochemistry* **19**, 4954-4962

Oefner, C. and Suck, D. (1986) *J. Mol. Biol.* **192**, 605-632

Page, M.D. and Anthony, C. (1986) *J. Gen. Microbiol.* **132**, 1553-1563

Page, M.D. and Ferguson, S.J. (1989) *Mol. Microbiol.* **3**, 653-631

Page, M.D. and Ferguson, S.J. (1990) *Mol. Microbiol.* **4**, 1181-1192

Parkes, C. and Abeles, R.H. (1984) *Biochemistry* **23**, 6355-6363

Patel, R.N., Hou, C.T. and Felix, A. (1978) *J. Bact.* **133**, 641-649

Paz, M.A., Flückiger, R., Boak, A., Kagan, H.M. and Gallop, P.M. (1991) *J. Biol. Chem.* **266**, 689-692

Paz, M.A., Flückiger, R. and Gallop, P.M. (1990) *FEBS Lett.* **264**, 283-284

Paz, M.A., Flückiger, R., Henson, E. and Gallop, P.M. (1989) in PQQ and Quinoproteins (Jongejan, J.A. and Duine, J.A. eds.), pp. 131-144, Kluwer Academic Publishers, Dordrecht

Peel, D. and Quayle, J.R. (1961) *Biochem. J.* **81**, 465-469

Pelham, H.R.B. (1989) *EMBO J.* **8**, 3171-3176

Provencher, S.W. and Glöckner, J. (1981) *Biochemistry* **20**, 33-37

Quilter, J.A. and Jones, C.W. (1984) *FEBS Lett.* **174**, 167-172

Redinbaugh, M.G. and Turley, R.B. (1986) *Anal. Biochem.* **153**, 267-271

Riddles, P.W., Blakeley, R.L. and Zerner, B. (1983) *Methods in Enzymology* **91**, 49-60

Robertson, J.G., Kumar, A., Mancewicz, J.A. and Villafranca, J.J. (1989) *J. Biol. Chem.* **264**, 19916-19921

Roitsch, T. and Stolp, H. (1986) *Arch. Microbiol.* **144**, 245-247

Sahm, H., Cox, R.B. and Quayle, J.R. (1976) *J. Gen. Microbiol.* **94**, 313-322

Salisbury, S.A., Forrest, H.S., Cruse, W.B.T. and Kennard, O. (1979) *Nature* **280**, 843-844

Schmid, F.X. (1989) in Protein structure: a functional approach (Creighton, T.E. ed.), pp. 251-285, IRL Press, Oxford

Sears, D.W. and Beychok, S. (1973) in Physical Principles and Techniques of Protein Chemistry (Leach, S.J. ed.), pp. 445-593, Academic Press, New York

Shinagawa, E., Matsushita, K., Adachi, O. and Ameyama, M. (1989) *Agric. Biol. Chem.* **53**, 1823-1828

Shinagawa, E., Matsushita, K., Nonobe, M., Adachi, O., Ameyama, M., Ohshiro, Y., Itoh, S. and Kitamura, Y. (1986) *Biochem. Biophys. Res. Comm.* **139**, 1279-1284

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Garther, F.H., Provenzano, M.D., Fujimoto, D.C., Goeke, N.M., Olsen, B.J. and Klesk, D.C. (1985) *Anal. Biochem.* **150**, 76-85

Sohngen, N.L. (1906) *Zenttralblat. fur. Bakteriologie. ,Parasitenkunde. ,Infektions. krankheiten. und. Hygeine. ,Abteilung. II.* 513-513

Sperl, G.T., Forrest, H.S. and Gibson, D.T. (1974) *J. Bact.* **118**, 541-550

Stanier, R.Y. and Ornston, L.N. (1973) *Adv. Microbial. Physiol.* **9**, 89-151

Strynadka, N.C.J. and James, M.N.G. (1989) *Annu. Rev. Biochem.* **58**, 951-998

Tatra, P.K. and Goodwin, P.M. (1983) *J. Gen. Microbiol.* **129**, 2629-2634

Thomas, P.E., Ryan, D. and Levin, W. (1976) *Anal. Biochem.* **75**, 168-176

Thomson, A.J. (1991) *Nature* **350**, 22-23

Turlin, E., Biville, F. and Gasser, F. (1991) *FEMS Microbiol. Lett.* **83**, 59-64

van der Meer, R.A., Groen, B.W., Jongejan, J.A. and Duine, J.A. (1990a) *FEBS Lett.* **264**, 284-284

van der Meer, R.A., Groen, B.W., van Kleef, M.A.G., Frank, J., Jongejan, J.A. and Duine, J.A. (1990b) *Methods in Enzymology* **188**, 260-283

van der Meer, R.A., Groen, B.W., Jongejan, J.A. and Duine, J.A. (1990c) *FEBS Lett.* **261**, 131-134

van Kleef, M.A.G., Dokter, P., Mulder, A.C. and Duine, J.A. (1987) *Anal. Biochem.* **162**, 143-149

van Kleef, M.A.G. and Duine, J.A. (1988a) *FEBS Lett.* **237**, 91-97

van Kleef, M.A.G. and Duine, J.A. (1988b) *Arch. Microbiol.* **150**, 32-36

van Kleef, M.A.G. and Duine, J.A. (1989) *Biofactors* **1**, 297-302

van Schie, B.J., van Dijken, J.P. and Kuenen, J.G. (1984) *FEMS. Microbiol. Letts.* **24**, 133-138

van Spanning, R.J.M., Wansell, C.W., de Boer, T., Hazelaar, M.J., Hideharu, A., Harms, N., Oltmann, L.F. and Stouthamer, A.H. (1991) *J. Bact.* **173**, 6948-6961

Veldink, G.A., Boelens, H., Maccaroni, M., van der Lecq, F., Vliegenthart, J.F.G., Paz, M.A., Flückiger, R. and Gallop, P.M. (1990) *FEBS Lett.* **270**, 135-138

Vellieux, F.M.D. and Hol, W.G.J. (1989) *FEBS Lett.* **255**, 460-464

Vellieux, F.M.D., Huitema, F., Groendijk, H., Kalk, K.H., Frank, J., Jongejan, J.A., Duine, J.A., Petratos, K., Drenth, J. and Hol, W.G.J. (1989) *EMBO. J.* **8**, 2171-2178

Weaver, C.A. and Lidstrom, M.E. (1985) *J. Gen. Microbiol.* **131**, 2183-2197

Weber, K. and Osborn, M. (1975) *The. Proteins. 3rd. edn.* **1**, 179-223

Westerling, J., Frank, J. and Duine, J.A. (1979) *Biochem. Biophys. Res. Comm.* **87**, 719-724

Zatman, L.J. (1981) in Microbial growth on C1 compounds (Dalton, H. ed.), pp. 42-54, Heyden, London

Zeilstra-Ryalls, J., Fayet, O. and Georgopoulos, C. (1991) *Ann. Rev. Microbiol.* **45**, 301-325

APPENDIX

Crystallization and Preliminary Crystallographic Investigation of Methanol Dehydrogenase from *Methylobacterium extorquens* AM1

M. Ghosh[1], K. Harlos[1], C.C.F. Blake[1], I. Richardson[2] and C. Anthony[2]

[1] *Laboratory of Molecular Biophysics*
University of Oxford
South Parks Road, Oxford OX1 3QU, U.K.

[2] *SERC Centre for Molecular Recognition*
Biochemistry Department, School of Biological Sciences
University of Southampton
Southampton SO9 3TU, U.K.

I have been involved in the provision of a consistently high standard of MDH from Methylobacterium extorquens that is suitable for the reproducible preparation of crystals used in the X-ray crystallography. This, and the following pages are a copy of a manuscript submitted to The Journal of Molecular Biology that reports progress in the elucidation of the 3-dimensional structure of MDH. These pages are included as an appendix because the work is not included in the original aims of my project, and I have not been involved in the preparation of native and heavy metal-derivitive crystals, data collection and data processing.

Abstract:

Single crystals of methanol dehydrogenase from *Methylobacterium extorquens* AM1 have been grown by the vapour diffusion method. These crystals diffract to beyond 2 Å resolution and are suitable for X-ray crystallography. They belong to the orthorhombic space group $P2_12_12_1$ and have the following unit cell parameters: a = 66.79 Å, b = 108.9 Å, c = 188.9 Å. One asymmetric unit contains a $\alpha_2\beta_2$ tetramer of MDH and the location of the non-crystallographic two-fold symmetry axis of this tetramer is defined by the paired positions of the binding sites of heavy atoms in four MDH-derivatives.

Keywords : crystallization, methanol dehydrogenase, quinoprotein, PQQ, oxido-reductase.

Introduction:

Methylobacterium extorquens AM1 is a facultative methylotroph in which methanol is oxidised to formaldehyde by a soluble, periplasmic methanol dehydrogenase (MDH, EC 1.1.99.8); this is a quinoprotein, having pyrrolo-quinoline quinone (PQQ) as its prosthetic group (Anthony, 1986,1992a). MDH has an $\alpha_2\beta_2$ tetrameric structure (Nunn *et al.*, 1989), there being a single calcium ion bound per tetramer (Richardson & Anthony, unpublished data). The protein sequences of the two subunits have been determined from the gene sequences; the large α subunit is about 66 kDa (Anderson *et al.*, 1990; Anthony, 1992a) and the very small β subunit is about 8.5 kDa (Nunn *et al.*, 1989; Anthony, 1992a). The electron acceptor for this enzyme is a novel specific cytochrome c_L, whose sequence is known (Anthony, 1986; Nunn & Anthony, 1988). These proteins interact by way of carboxylate residues on the cytochrome c_L and lysyl residues on the α subunit of MDH (Cox *et al.*, 1992). The wealth of knowledge available for MDH makes it ideally suited for studies of the relationship between structure and function of quinoproteins. Although the structure of PQQ was originally determined by X-ray crystallography some years ago (Salisbury *et al.*, 1979), the only quinoprotein whose high resolution X-ray structure has been determined is methylamine dehydrogenase, whose prosthetic group is not PQQ but tryptophan tryptophylquinone (TQQ) (Vellieux *et al.*, 1989; McIntire *et al.*, 1991; Chen *et al.*, 1991).

Reports of crystallization of MDH, together with characterization of the unit cells, have been published previously for the enzymes from *Methylophilus methylotrophus* (Lim *et al.*, 1986), *Methylosinus trichosporium* OB3b (Parker *et al.*, 1987), *bacterium* W3A1 (Xia *et al.*, 1989). A short abstract of the structure of MDH from *Methylophilus methylotrophus* (Xia *et al.*, 1991) has been reported recently. The present paper describes the crystallization of MDH from *Methylobacterium extorquens* AM1 and the preparation of four isomorphous heavy metal derivatives. The primary amino acid sequences of both subunits of this enzyme are known and a genetic system is available, facilitating further investigation, by site-directed mutagenesis, of the relationship between structure and function of this important quinoprotein.

Results:

Methylobacterium extorquens AM1 (NCIMB 9133) was grown, harvested and disrupted, and methanol dehydrogenase purified as described by Day and Anthony (1990). Initial screening of the crystallization conditions for MDH from *M. Extorquens* was carried out using the hanging drop vapour diffusion technique at three different temperatures (22°C, 16°C and 4°C). The protein solution contained 16–22 mg/ml of MDH in 5 mM phosphate buffer (pH=6.8). Small crystals (0.3 mm in maximum dimension) of two different forms appeared within a week. Crystals of form I were irregular in shape and were grown from 16% (w/v) polyethelene glycol (PEG) 8000, pH 8.0; form II crystals were elongated prisms and were grown from 14.4% PEG 8000, pH 7.0. Larger crystals of form II, typically 2 mm in length could be grown in 10 μl hanging drops, containing protein and reservoir solution (1:1 ratio) and equilibrating against a reservoir of 14.2–14.5% PEG 6000 in 100 mM Tris-HCl buffer, pH 9.0 at 16°C. Form II crystals grew in an interesting shape: they were solid rectangular prism at the base and open tube of very thin wall, frequently of a triangular cross section, at the top (Fig. 1). Even in the optimum condition most crystals were twinned and were not stable in the drop for more than a week. They tended to disintegrate, dissolve and reappear as form II or as small rectangular plates (form III).

All crystal forms were examined using a Siemens/Xentronics area-detector attached to a Rigaku rotating anode X-ray generator. The form I crystals diffracted to about 3.5 Å in this system. Unit cell parameters were determined as a = 61.5 Å, b = 72.7 Å, c = 86.8 Å, α = 85.9°, β = 103.5°, γ = 108.4° and the space group as triclinic P1. This cell is similar to that recorded in a preliminary report of MDH from *Pseudomonas maltovidia* (Vellieux, 1990). Assuming one $\alpha_2\beta_2$ tetramer of 149,000 Da in the unit cell, the volume per unit protein molecular mass is 2.4 Å^3/Da (Matthews, 1968). The larger crystals of form II diffracted to 2.5 Å using the rotating anode generator and to better then 2 Å on the Daresbury Synchrotron Radiation Source. The unit cell is orthogonal, with a = 66.8 Å, b = 108.9 Å, c = 188.9 Å. Systematic absences were observed for odd reflections along each of the h00, 0k0, 00l axis, defining the space group as $P2_12_12_1$. One $\alpha_2\beta_2$ tetramer in the asymmetric unit would give a V_m=2.2 Å^3/Da, which is within the normal range for proteins (Matthews, 1968). Form III crystals belong to the monoclinic system with unit cell dimensions a = 61.3 Å, b = 130.4 Å, c = 177.7 Å, β = 96.3°. Both form I and form

III crystals could not be grown reproducibly.

Form II crystals were used for native and derivative data collection with the Xentronics area-detector. Indexing, integration and scaling of the intensity data were carried out using the XENGEN (Howard, 1987) processing program. Isomorphous derivatives were prepared by soaking crystals in heavy metal solutions, four of which gave interpretable difference Patterson maps and were suitable for phasing. Table 1 summarizes the derivative soaking conditions and the data collection and refinement statistics.

All the major heavy atom binding sites of the four derivatives occur in pairs with one exception in the $PtCl_4$ derivative where we could identify two pairs and a single unpaired site, five sites in total. As shown in Fig. 2 all paired sites are related by the same non-crystallographic two-fold symmetry axis. This symmetry axis is inclined to all three cell edges and we assume it to mark the molecular symmetry axis of the $\alpha_2\beta_2$ tetrameric MDH molecule. The position of the non-crystallographic two-fold symmetry axis has been confirmed by the low resolution electron density map of the protein, calculated with the isomorphous derivative phases and after application of solvent flattening (Wang, 1985).This electron density map clearly reveals the boundaries of a molecule composed of two compact units related by a two-fold axis of symmetry, which coincides with the axis relating the heavy atom binding sites. We are currently applying symmetry averaging and extending the resolution of this electron density map to 3.0 Å.

Acknowledgements:

We thank The Science and Engineering Research Council, UK., The Wellcome Trust (M.G.) and Oxford Centre for Molecular Sciences (K.H.) for financial support. We also thank S. Gover, E. Y. Jones and D. Stuart for the use of their programs, A. Basak, A. Cleasby and P. Nordlund for discussions, E. Garman, R. Bryan and S. Lee for their assistance.

References:

Anderson, D.J., Morris, C.J., Nunn, D.N., Anthony, C. & Lidstrom, M.E. (1990) Nucleotide sequence of the *Methylobacterium extorquens* AM1 moxF and moxJ genes involved in methanol oxidation. *Gene.* **90**, 173-176.

Anthony, C. (1986). The bacterial oxidation of methane and methanol. *Adv. Microbial. Physiol.* **27**, 113-210.

Anthony, C. (1992). The structure of bacterial quinoprotein dehydrogenases. *Int. J. Biochem.* **24**, 29-39.

Anthony, C. (1992). The c-type cytochromes of methylotrophic bacteria. *Biochim. Biophys. Acta* **1099**, 1-15.

Chen, L.Y., Mathews, F.S., Davidson, V.L., Huizinga, E.G., Vellieux, F.M.D., Duine, J.A. & Hol, W.G.J. (1991). Crystallographic investigations of the Tryptophan-derived cofactor in the quinoprotein methylamine dehydrogenase. *FEBS Lett.* **287**, 163-166.

Cox, J.M., Day, D.J. & Anthony, C. (1992). The interaction of methanol dehydrogenase and its electron acceptor, cytochrome c_L, in the facultative methylotroph *Methylobacterium extorquens* AM1 and in the obligate methylotroph *Methylophilus methylotrophus. Biochim. Biophys. Acta* **1119**, 97-106.

Day, D.J. & Anthony, C. (1990). Methanol dehydrogenase from *Methylobacterium extorquens* AM1. *Methods. in Enzymology.* **188**, 210-216.

Howard, A.J., Gilliland, G.L., Finzel, B.C., Poulos, T.L., Ohlendorf, D.H., & Salemme, F.R. (1987). The use of an imaging proportional counter in macromolecular crystallography. *J. Appl. Cryst.* **20**, 383-387.

Mcintire, W.S., Wemmer, D.E., Chistoserdov, A. & Lidstrom, M.E. (1991). A new cofactor in a prokaryotic enzyme – tryptophan tryptophylquinone as the redox prosthetic group in methylamine dehydrogenase. *Science* **252**, 817-824.

Nunn, D.N. & Anthony, C. (1988). The nucleotide sequence and deduced amino acid sequence of the cytochrome c_L gene of *Methylobacterium extorquens* AM1: a novel class of c-type cytochromes. *Biochem. J.* **256**, 673-676.

Nunn, D.N., Day, D.J. & Anthony, C. (1989). The second subunit of methanol dehydrogenase of *Methylobacterium extorquens* AM1. *Biochem. J.* **260**, 857-862.

Parker, M.W., Cornish, A., Gossain, V. and Best, D.J. (1987). Purification,

crystallisation and preliminary X-ray diffraction charactisation of methanol dehydrogenase from *Methylosinus trichosporium* OB3b. *Eur. J. Biochem.* **164**, 223-227.

Salisbury, S.A. Forrest, H.S., Cruse, W.B.T. & Kennard, O. (1979). A novel coenzyme from bacterial primary alcohol dehydrogenase. *Nature* **280**, 843-844.

Vellieux, F.M.D. (1990). Three-dimensional structure of quinoprotein methylamine dehydrogenase. *Ph. D. Thesis, Groningen University.*

Vellieux, F.M.D., Huitema, F., Groendijk, H., Kalk, K.H., Frank, J., Jongejan, J.A., Duine, J.A., Petratos, K., Drenth, J. & Hol, W.G.J. (1989). Structure of quinoprotein methylamine dehydrogenase at 2.25 Å resolution. *EMBO. J.* **8**, 2171-2178.

Wang, B. C. (1985). Resolution of Phase Ambiguity in Macromolecular Crystallography. *Methods. in Enzymology.* **115**, 90-112.

Xia, Z. X., Hao, Z. P., Mathews, F. S. and Davidson, V. L. (1989). Crystallization and preliminary X-ray crystallographic study of the quinoprotein methanol dehydrogenase from *bacterium* W3A1. *FEBS Lett.* **258**,175-176.

Xia, Z., Xiong, J., Dai, W., Davidson, V. L., White, S. and Mathews, F. S., (1991)*Abstract I-5, 2nd International Symposium on PQQ and Quinoproteins.*

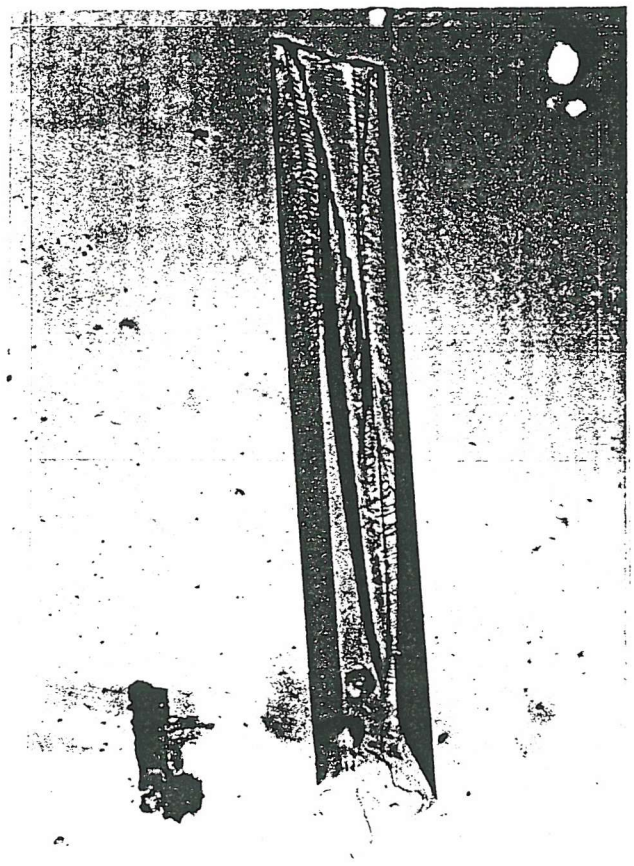


Fig. 1. Crystals of MDH from *Methylobacterium extorquens* AM1, grew to an interesting shape: one shown above is typical. One end is a solid rectangular prism with base dimension up to 0.3 x 0.3 mm, the other end is a thin walled tube often of triangular section and there is a gradual transition in cross-section between the two ends. The length of the crystal is 2.3 mm.

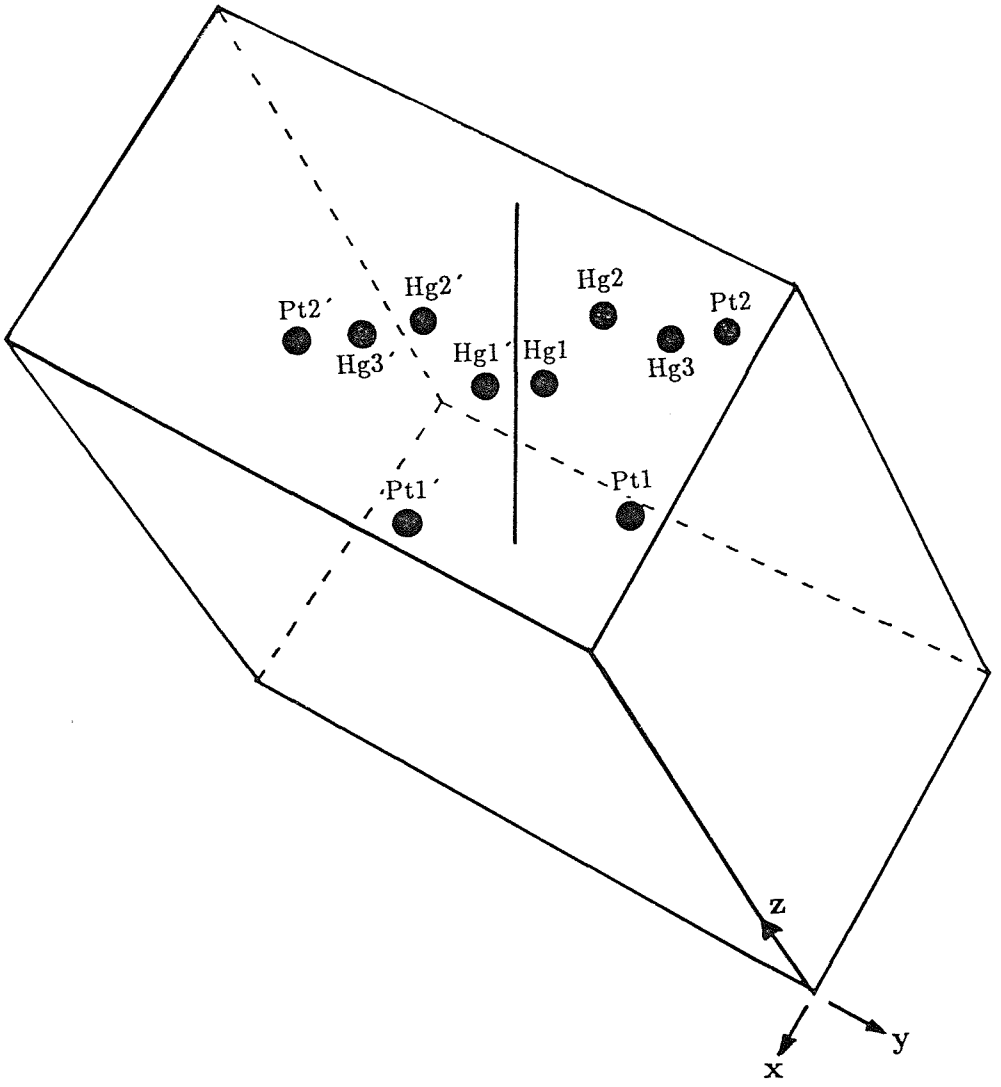


Fig. 2. A drawing showing the positions of the heavy atom binding sites of the four derivatives in the table I. A total of 10 sites occur in two sets (indicated by the primed and unprimed labels) related by a non-crystallographic two-fold symmetry axis shown by the short solid line. Sites Hg1, Hg2 and their symmetry equivalents are occupied by EMP, site Hg3 and its symmetry equivalent is occupied by PCMBS. Sites Pt1, Pt2 and their symmetry equivalents are occupied by both K_2PtCl_4 and K_2PtCl_6, but with different relative occupancies. The unpaired site in K_2PtCl_4 has not been shown above.

Table I. Data collection and refinement statistics

Dataset	Soaking time (days)	Concentration	max. Resolution (Å)	Unique Reflections (% complete)	R_{merge} (%)	MFID (%)	Number of sites	Phasing $\|F_H\|/E$ (all data)	Statistics* R_C(%)
Native			2.75	31542 (87)	7.9				
EMP	6	23 mM	5.0	5131 (82)	5.4	12.1	4	1.57	43
$PtCl_6$	6	24 mM	5.0	3820 (61)	6.3	17.5	4	1.51	50
$PtCl_4$	11	20 mM	5.4	3901 (79)	6.3	10.6	5	1.54	45
PCMBS	12	saturated	5.4	3118 (65)	6.6	9.4	4	1.46	58

$$R_{merge} = \frac{\sum_h \sum_i |I_{i,h} - < I_h >|}{\sum_h \sum_i < I_h >}$$, where $I_{i,h}$ is the individual intensity measurement for each reflection h and $<I_h>$ is the mean intensity for this reflection

MFID = mean fractional isomorphous difference $\sum ||F_{PH}| - |F_P|| / \sum |F_P|$,

F_P, F_{PH} are the structure factors of native and derivative respectively.

R_C = Cullis R-factor for centric reflections $\sum |F_{H(o)}| - \sum |F_H| / \sum |F_{H(o)}|$,

where $|F_{H(o)}|$ and $|F_H|$ are observed and calculated heavy atom structure factor amplitudes respectively.

E = residual lack of closure error.

*Heavy atom phasing and refinement were carried out using the programs PHASE and REFINE coded by Dr. P. Shaw and modified by Dr. D. Stuart (unpublished).