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Chapter 3

Methanol Dehydrogenase, a PQQ-Containing Quinoprotein Dehydrogenase

Christopher Anthony

1. INTRODUCTION

Methanol dehydrogenase (MDH; EC 1.1.99.8) catalyses the oxidation of methanol to formaldehyde in the periplasm of methylotrophic bacteria during growth on methanol or methane. It was first described in *Methylobacterium extorquens* (Anthony and Zatman, 1964a,b) and has subsequently been shown to be the one feature that is common to almost all methylotrophs in which it often constitutes up to 15% of their soluble protein (see Anthony, 1986 for a review of the basic enzymology of a wide range of MDHs).

MDH is a soluble quinoprotein which has pyrroloquinoline quinone (PQQ) as its prosthetic group and it uses a specific cytochrome, cytochrome *c_L* as electron acceptor. It is usually assayed in a dye-linked system at high pH when ammonia is required as activator. It has an $\alpha_2\beta_2$ structure; each α

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subunit has one Ca^{2+} ion coordinated to the PQO. This is not covalently attached and so differs from the prosthetic groups of other quinoproteins which are derived from amino acids in the protein backbone of the enzyme and are shown in Figure 1 for comparison with PQO: Tryptophan tryptophylquinone (TTQ) is derived from two tryptophan residues and occurs in bacterial amine dehydrogenases (McIntire *et al.*, 1991; Davidson, 1993; Davidson, this book); Topa-quinone (TPQ) is a modified tyrosine residue and is the prosthetic group of the copper-containing amine oxidases found in bacteria, yeasts, plants and animals (Janes *et al.*, 1990; Klimman, 1995); and lysyl oxidase is a special type of copper-containing amine oxidase

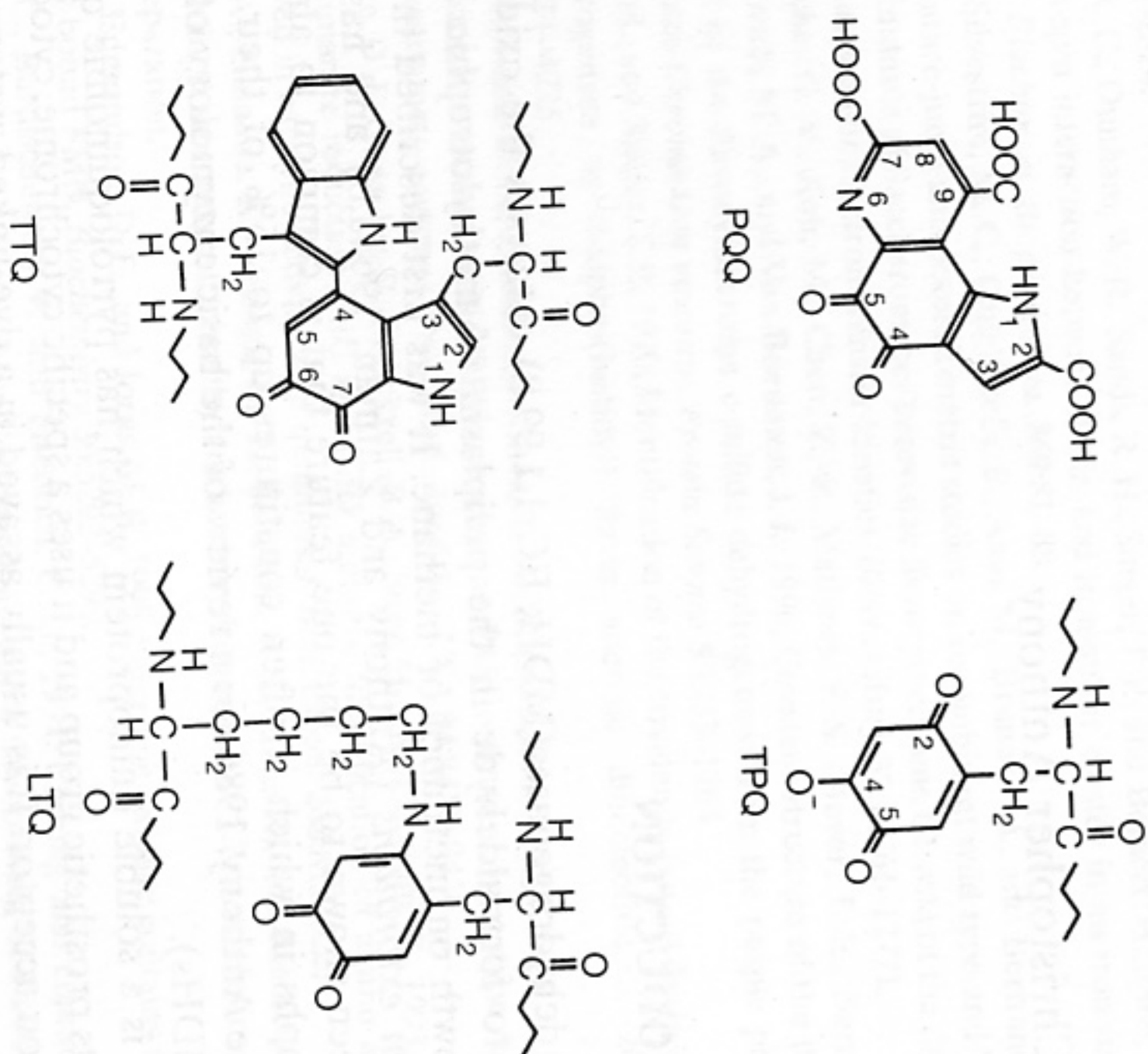


FIGURE 1. The prosthetic groups of quinoproteins. PQO (pyrrolo-quinoline quinone) is the prosthetic group of dehydrogenases for methanol, higher alcohols, aldose sugars, aldehydes and polyvinyl alcohol and for hydroxylation of lupanine. TTQ (tryptophan tryptophylquinone) is the prosthetic group of amine dehydrogenases. TPQ (6-hydroxyphenylalanine or topa quinone) is the prosthetic group of the copper-containing amine oxidases in bacteria, plants and animals. LTQ (lysine tyrosylquinone) is the prosthetic group of lysyl oxidase, a specific copper-containing amine oxidase occurring in animals.

whose prosthetic group is Lysyl tyrosylquinone (LTQ) (Wang *et al.*, 1996). The structures and mechanisms of all these enzymes have been reviewed by Anthony (1996, 1998), and the biochemistry, physiology and genetics of PQO and PQO-containing enzymes reviewed by Goodwin and Anthony (1998).

This chapter will review the general features of the enzymology of methanol dehydrogenase, its structure as determined by X-ray crystallography, the properties of the prosthetic group (PQO), the mechanism of its reduction by substrate, its oxidation by its specific cytochrome electron acceptor, and processes involved in its synthesis.

2. GENERAL ENZYMOLOGY

2.1. The Determination of MDH Activity

Although cytochrome c_1 is the physiological electron acceptor, it is most convenient to assay MDH in a dye-linked assay system with phenazine methosulphate or ethosulphate, linked to oxygen in an oxygen electrode, or linked to reduction of 2,6-dichlorophenol indophenol and measured spectrophotometrically (for convenient assay systems see Day and Anthony, 1990; Frank and Duine, 1990). The pH optimum in these assays is usually at least pH 9; in such conditions phenazine derivatives form free radicals and these are possibly the true electron acceptor (Duine *et al.*, 1978). This suggestion is supported by the demonstration that a better alternative to phenazine ethosulphate is Wurster's Blue which is the perchlorate salt of the cationic free radical of $\text{NNN}'\text{N}'$ -tetramethyl-*p*-phenylene diamine (Duine *et al.*, 1978; Frank and Duine, 1990). Use of Wurster's Blue avoids the problem of inactivation of MDH by phenazine dyes in the absence of protecting substrate; its only disadvantage is that it is not commercially available and must be synthesised from a commercially available precursor (Frank *et al.*, 1988; Harris and Davidson, 1994; Davidson *et al.*, 1992).

It should be noted that the dye-linked assay system is artificial in every way; the electron acceptor may also be an inhibitor; it has a high pH optimum (about pH 9); it requires ammonia as activator, but this may also inhibit; cyanide is a competitive inhibitor and may be used as a protective agent; in the absence of added substrate a high rate of dye reduction occurs which may or may not be taken into account when calculating rates of reaction. This complexity and confusion is mentioned here as an explanation for the length and complexity of some of the following discussion.

2.2. The Substrate Specificity of MDH

MDH is able to oxidise a wide range of primary alcohols as well as methanol (the best substrate), including, for example, crotyl alcohol, bromethanol, phenylethanol and cinnamyl alcohol. Their steric configuration is more important in determining whether or not they are oxidised than is the presence or absence of atoms or groups producing electron-displacement effects (Anthony and Zatman, 1965; Anthony, 1986). The general rule is that a second substituent on the C—2 atom appears to prevent binding, the general formula for an oxidizable substrate being $R\cdot CH_2OH$ where R may be H, OH (hydrated aldehydes), $R'\cdot CH_2-$ or $R'\cdot R''C\equiv CH-$ (Anthony and Zatman, 1965). The affinity for methanol and most other substrates is high (for methanol, $K_m = 3-20 \mu M$), the affinity tending to decrease with size of the alcohol, although maximum rates are usually similar to that for methanol (Duine and Frank, 1980b; Anthony, 1986). Analysis of the reaction with ^{13}C deuterated benzyl alcohols has demonstrated that the enzyme specifically removes the *pro-S* hydrogen at the C—1 carbon atom, the *pro-R* hydrogen being retained in the aldehyde product (Houck and Unkefer, cited in Anthony, 1993). Recent results using chemical models of the active site PQQ bound to Ca^{2+} in organic solvents confirm that the substrate specificity of this enzyme is not due to specific aspects of the catalytic process but is dictated by the dimensions of the active site (Itoh *et al.*, 1998). When alcohols are not substrates they are unable to act as a competitive inhibitors, suggesting that if the alcohol is able to bind then it is suitable as a substrate.

MDH is able to oxidise formaldehyde (probably in the gem-diol hydrated form), although this is very unlikely to be important physiologically, as a high proportion of the formaldehyde product is assimilated into cell material. Inappropriate formaldehyde oxidation is prevented by a periplasmic modifier protein (M-protein) which decreases the affinity of MDH for formaldehyde (Page and Anthony, 1986; Long and Anthony, 1990a, 1991). M-protein has a second effect on the activity of MDH; it increases its affinity for some substrates that, in its absence, have very low affinities (e.g. 1,2-propanediol and 4-hydroxybutyrate). This protein was first observed as a result of studies of the growth of *M. extorquens* on 1,2-propanediol which ceases to be a substrate in mutants lacking the dehydrogenase, and which is oxidised only in crude extracts when M-protein is also present (Bolbot and Anthony, 1980). That the role of this protein in growth on 1,2-propanediol and 4-hydroxybutyrate is fortuitous and secondary to its main role in regulation of formaldehyde oxidation is supported by the observation that M-protein occurs in a range of methylotrophs including obligate

methylotrophs which are unable to grow on multicarbon compounds such as 1,2-propanediol.

3. THE KINETICS OF METHANOL DEHYDROGENASE

3.1. The Reaction Cycle of MDH

It might be expected that the steady state kinetics of a dehydrogenase would be straightforward and informative with respect to the mechanism but this is not the case with MDH. When it is incubated at high pH with electron acceptor and activator (ammonia) in the absence of substrate, an unexpectedly large amount of electron acceptor becomes reduced by an unidentified "endogenous substrate" (Anthony and Zatman, 1964b; Duine *et al.*, 1978, 1980b) which is presumed to be very low concentrations of contaminating alcohols and aldehydes in buffers and reagents.

An extensive steady state kinetic analysis of MDH using phenazine ethosulphate by Duine and Frank (1980b) established many of the main features of the kinetics of MDH and solved the problem of isolating an oxidised form of the enzyme by including the competitive inhibitor cyanide in the reaction mixtures (also included at constant concentration during kinetic measurements). This study was then extended (with Wurster's Blue) using stopped flow kinetics with oxidised and reduced enzyme (Frank *et al.*, 1988). The reaction cycle proposed from this work (Figure 2) continues to be a generally valid description although modifications have been proposed by Harris and Davidson (1993a, 1994) who showed that ammonia is also an inhibitor at high concentrations (see Section 3.2). In their model, MDH binds substrate but can only form product when first activated by cyanide binding at a second (non-substrate) binding site. However, this can only be defined as an activator in the special context of demonstrating "methanol-dependent activity" which is not always seen because of the endogenous dye reduction. Cyanide, a competitive inhibitor, prevents oxidation of the very low concentration of endogenous substrate; when methanol is added then, of course, the activity resumes. In this context it should be noted that, as the endogenous substrate is oxidised by the same mechanism as added substrate which is oxidised in preference to the endogenous substrate, it is usually inappropriate to subtract the endogenous value in order to obtain a valid measure of enzyme activity (Beardmore-Gray *et al.*, 1983; Anthony, 1986).

Steady state, and stopped flow, kinetics using cytochrome c_1 as electron acceptor subsequently confirmed that the reaction cycle is essentially the same as with a dye electron acceptor (Figure 2) (Dijkstra *et al.*, 1989) and

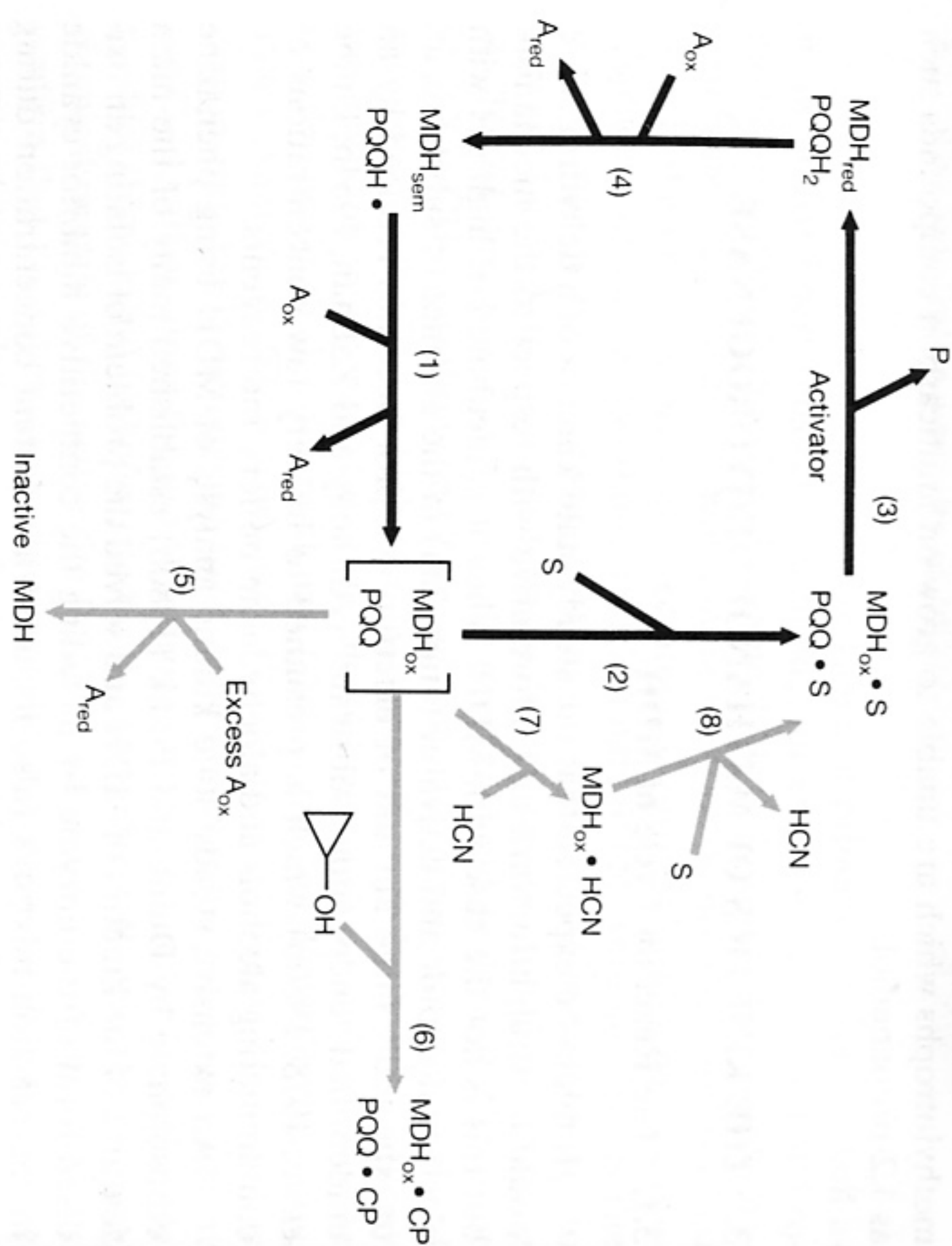


FIGURE 2. The reaction cycle of methanol dehydrogenase. This reaction cycle is based on the work of Frank and colleagues (Frank *et al.*, 1988; Dijkstra *et al.*, 1989). The electron acceptor (A) is either a dye such as phenazine ethosulphate or the natural electron acceptor cytochrome c_1 . The normal cycle is outlined using dark arrows. The starting point is the semi-quinone form of the enzyme as it is usually isolated MDH_{sem} . Reactions 1, 2 and 4 are reversible; reaction 3 is not. It should be emphasised that the enzyme does not require ammonia, or cyanide, or a high pH for full activity in the physiological reaction. Ammonia (and perhaps cyanide) may bind to MDH in a manner which induces conformational changes or modifies amino acid residues in such a way as to mimic the *in vivo* form of the enzyme when it is in contact with the modifier protein, cytochrome c_1 , or some other component of the cell.

further studies using this system have provided information for analysis by Marcus theory relating to reorganizational energy, electronic coupling and theoretical distances between redox centres (Harris and Davidson, 1993b; Harris *et al.*, 1994).

3.2. The Activation of MDH by Ammonia and Amines

As usually prepared, MDH has an absolute requirement for ammonium or methylammonium salts in the dye-linked assay system. The relatively higher concentrations required at lower pH values suggest that the

free base is the active species (Anthony and Zatman, 1964b). This can be replaced by esters of glycine or β -alanine but not by lysine esters nor by aliphatic amines or amino acids; the affinity for the alternative activators is usually higher than for ammonium salts. The enzyme does not always need ammonia when cytochrome c_1 is used as electron acceptor and sometimes anaerobically-prepared enzyme has no ammonia requirement (for references to the original literature see Anthony, 1986).

Kinetic analysis of the activation by ammonia of MDH is usually difficult because of its very high affinity for methanol and the endogenous dye reduction (see above) (Frank *et al.*, 1988; Harris and Davidson, 1993a). This problem has been overcome in a modified form of enzyme in which the Ca^{2+} ion in the active site has been replaced by Ba^{2+} , which decreases markedly the affinity for alcohol substrate and so has eradicated the "endogenous reductant" problem. These studies confirmed the previous conclusion (Frank *et al.*, 1988) that the main effect of the activator is to increase the V_{max} and that the apparent affinity for methanol decreased with increasing concentrations of activator (Goodwin and Anthony, 1996). The steady state kinetic data summarised in Figure 3 show that, although methanol and ammonia are essential for activity, each binds more strongly to the enzyme in the absence of the other. This suggests that the binding site for ammonia is likely to be independent of, but very close to, that for methanol; both activator and methanol must both be bound for formation of product. These studies showed that ammonia does not activate by

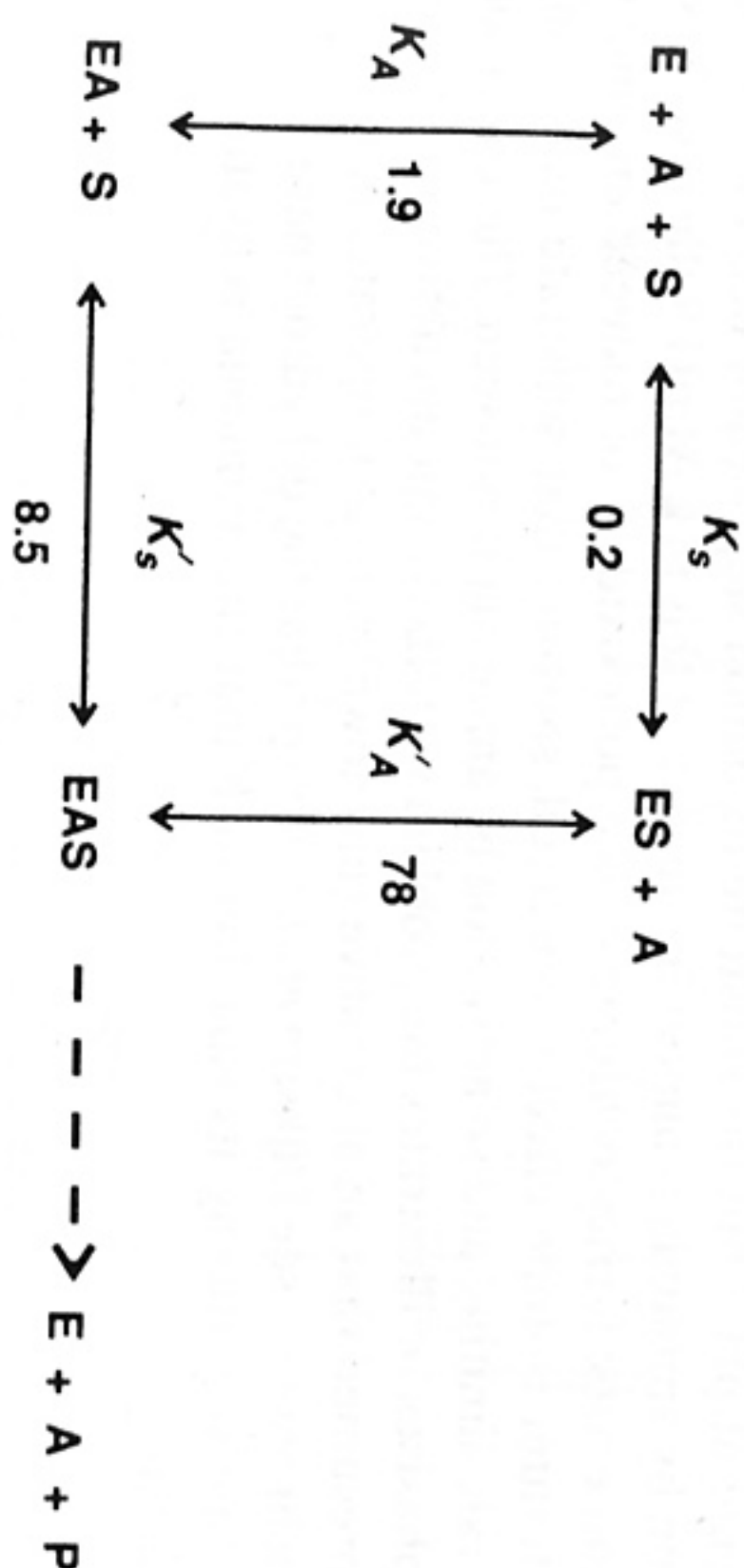


FIGURE 3. Kinetic scheme for the activation of Ba-MDH by ammonia. The combination of enzyme (E) with substrate (S) is not independent of its combination with activator (a), so E and EA have different affinities for the substrate. The equation for the reaction is as follows: $v = V_{\text{max}} / (1 + K'_S/S) [1 + K_A/a (1 + S/K_S) (1 + S/K'_S)]$. The values given are for the dissociation constants for each complex (mM).

encouraging the initial binding of substrate, or by affecting the formation of any initial PQO-methanol adduct, or by stimulating the re-oxidation of reduced PQO. It confirmed the previous suggestion (Frank *et al.*, 1988) that ammonia activates the reduction of PQO by methanol, the rate limiting step in this process being the transfer of the methyl hydrogen to PQO. The high deuterium isotope effect associated with this hydrogen transfer step is affected by ammonia; increasing ammonia concentration (30-fold) decreased the measured isotope effect from 6.7 to 1.4. Using the Ba-MDH this was subsequently shown to be because the K_A for deuterated methanol is 3.5 times higher than for normal substrate (Goodwin and Anthony, 1996). Kinetic analysis has also shown that there is a second (inhibitory) binding site for ammonia with K_i of 50–100 mM; spectroscopic evidence suggests that this may be due to formation of an imino adduct with the PQO (Harris and Davidson, 1993; Goodwin and Anthony, 1996).

3.3. The Low *in vitro* Rate of Cytochrome c_1 Reduction by MDH

Although cytochrome c_1 is the physiological electron acceptor for the dehydrogenase, the rate of its reduction *in vitro* is extremely slow compared with the rate measured in the artificial system with phenazine ethosulphate. This has been explained by a thorough study of the interaction of the enzyme with cytochrome c_1 using stopped flow kinetics, and studies of isotope effects. It appears that the differences in rates between electron acceptors are due to different rate-limiting steps in the reaction cycles (Dijkstra *et al.*, 1989). Ferricytochrome c_1 is an excellent oxidant of reduced enzyme at pH 7 but the substrate oxidation step is slow because the activation by ammonia is almost ineffective at this pH. At pH 9 the reverse situation exists: ferricytochrome c_1 is a poor oxidant of reduced enzyme; no deuterium isotope effect is observed, showing that substrate oxidation is not rate-limiting and so activation by ammonia is not seen. This conclusion emphasises or illustrates the problem with the ammonia activation. It must be presumed that an alternative (unknown) activator operates *in vivo* with cytochrome c_1 (see Dijkstra *et al.*, 1988) or that the dehydrogenase is altered in some way during its isolation such that the ammonia activator is now required.

4. THE ABSORPTION SPECTRA OF METHANOL DEHYDROGENASE

MDH is usually coloured olive-green, the spectrum of the isolated enzyme having a characteristic absorption due to the prosthetic group at

345 nm (Anthony and Zatman, 1967); the PQO in the isolated enzyme is usually in the semiquinone or reduced form (de Beer *et al.*, 1983; Frank *et al.*, 1988; Dijkstra *et al.*, 1989) (Figure 4). This tends to cause problems in all kinetic work and spectroscopy. For example, the effect of reduction by added substrate cannot be determined without first producing the oxidised form of the enzyme. This can only be produced by addition of small amounts of PES or Wurster's Blue in the presence of ammonia. However, the ubiquitous "endogenous substrate" rapidly re-reduces the enzyme. Incubation with an excess of PES or Wurster's Blue inactivates the enzyme unless a competitive inhibitor such as cyanide is included in the reaction mixture; as a result most spectra of the oxidised form are likely to be spectra of cyanide or ammonia adducts of the PQO (Duine and Frank, 1980b; Frank *et al.*, 1988). Addition of ammonia at the concentration needed for activation leads to no spectral perturbation, but higher inhibitory concentrations lead to changes in the spectrum (about 400 nm) suggesting that, at these concentrations of ammonia, an imine adduct of PQO may be formed (Harris and Davidson, 1993a).

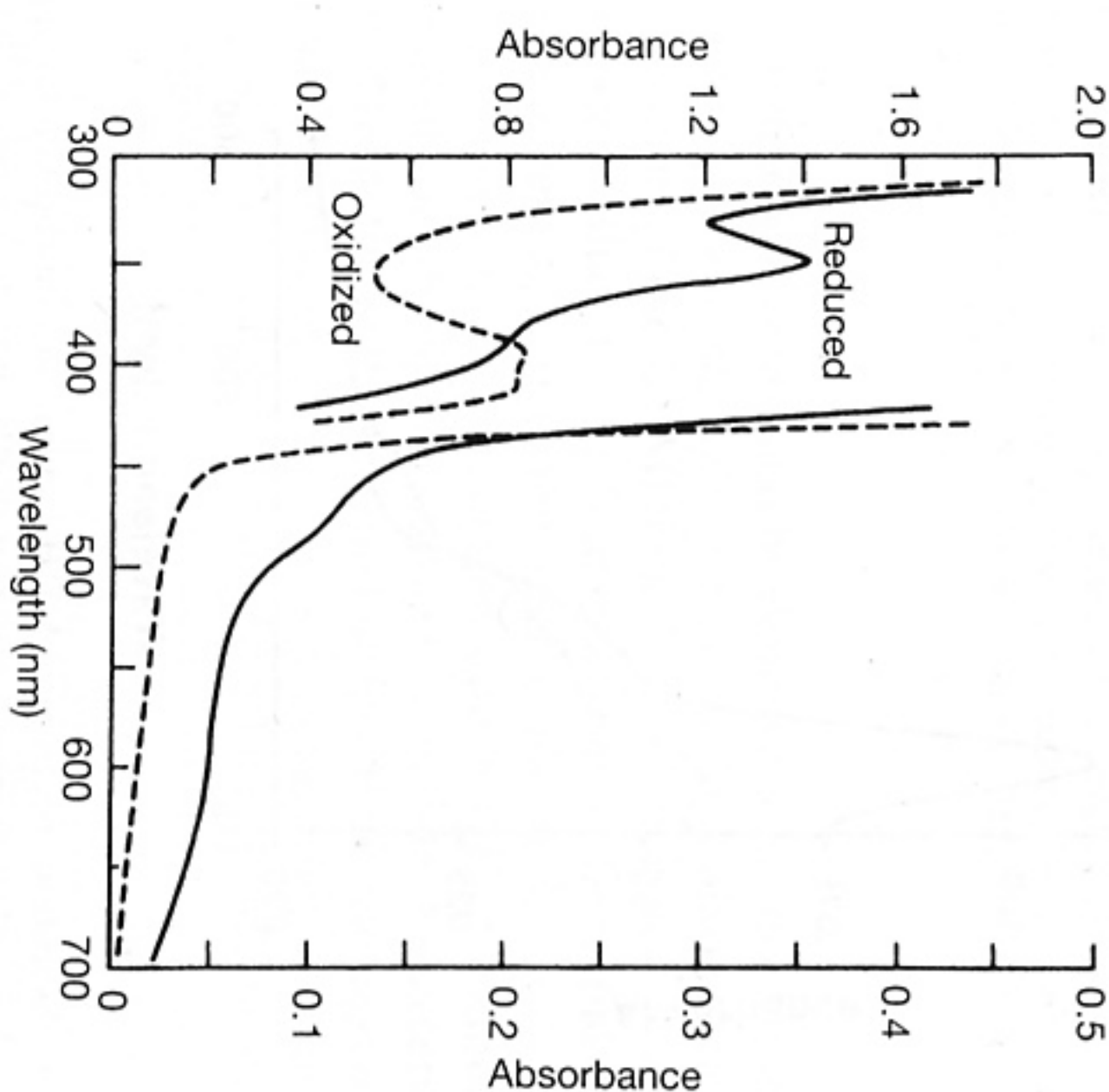


FIGURE 4. The absorption spectra of methanol dehydrogenase. The spectrum of the oxidised form is very difficult to determine and is often likely to be that of the cyanide or ammonia adducts. MDH is usually isolated in the reduced or half-reduced form (semiquinone).

The presence of the Ca^{2+} ion in the active site has a major effect on the spectrum as shown by using a mutant (*mxmA*) of *Methylobacterium extorquens* that produces both subunits of MDH and also the PQO, but is unable to process these in the periplasm to give the complete active enzyme. The result is an inactive enzyme containing PQO but no calcium and having a markedly different spectrum; incubation at pH 10 with Ca^{2+} led to incorporation of Ca^{2+} , and production of a normal spectrum together with a normally active enzyme (Figure 5) (Goodwin *et al.*, 1996). Before incorporation of Ca^{2+} the PQO was in the oxidised form but became immediately reduced by endogenous substrate as soon as Ca^{2+} was incorporated. When Ca^{2+} was replaced with Ba^{2+} the enzyme was more active but had a hugely decreased affinity for substrate (including endogenous substrate); consequently the oxidised enzyme could easily be produced and its subsequent reduction observed (Goodwin and Anthony, 1996). This provided probably the first example of a spectrum of the oxidised MDH without adducts (Figure 6).

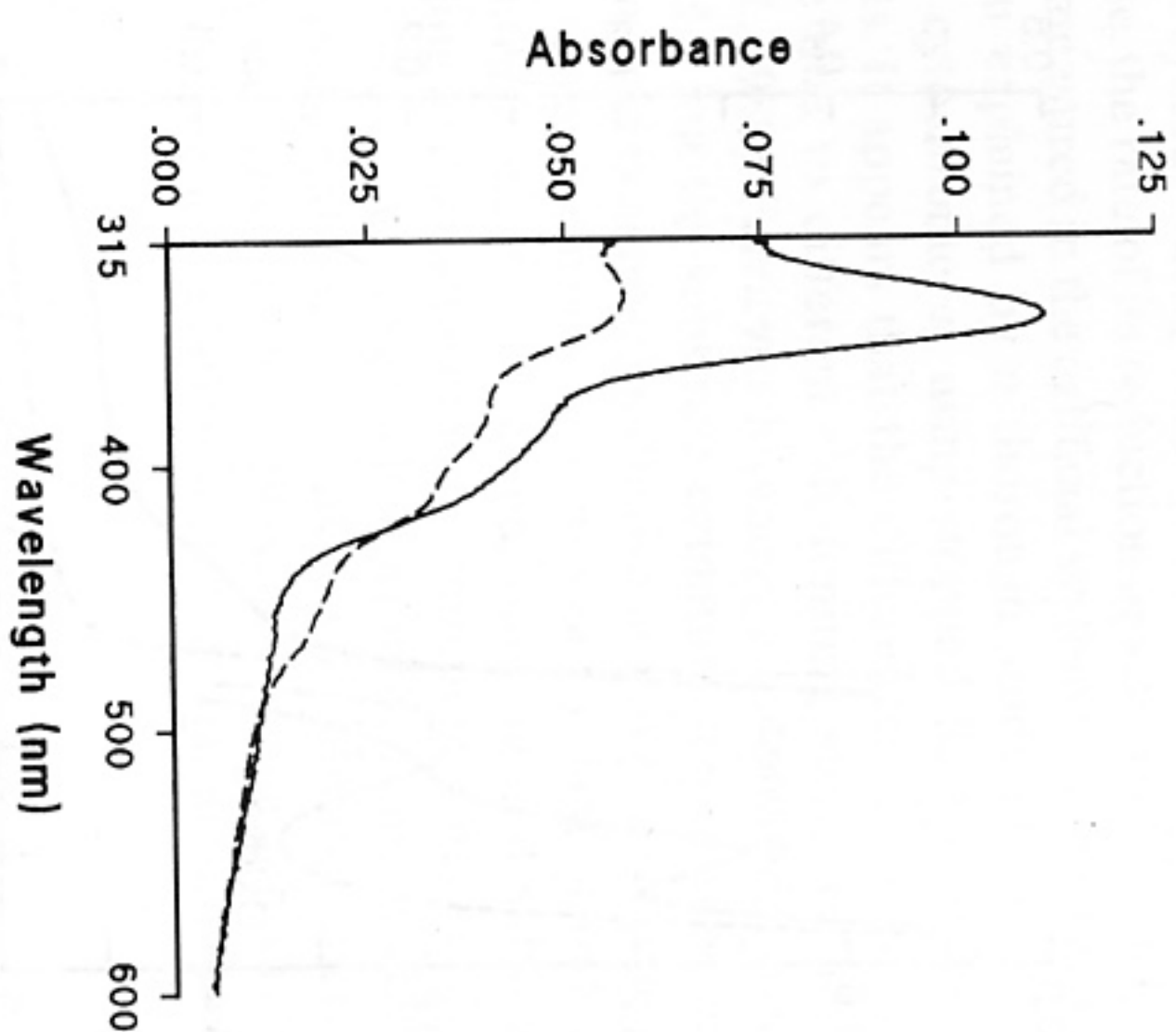


FIGURE 5. The absorption spectrum of MDH lacking calcium (broken line), isolated from *mxmA* mutant, and the effect of incorporation of calcium into the enzyme (solid line). Reproduced with permission from Goodwin *et al.* (1996), (*Biochemical Journal*, **319**, 839–842). © the Biochemical Society.

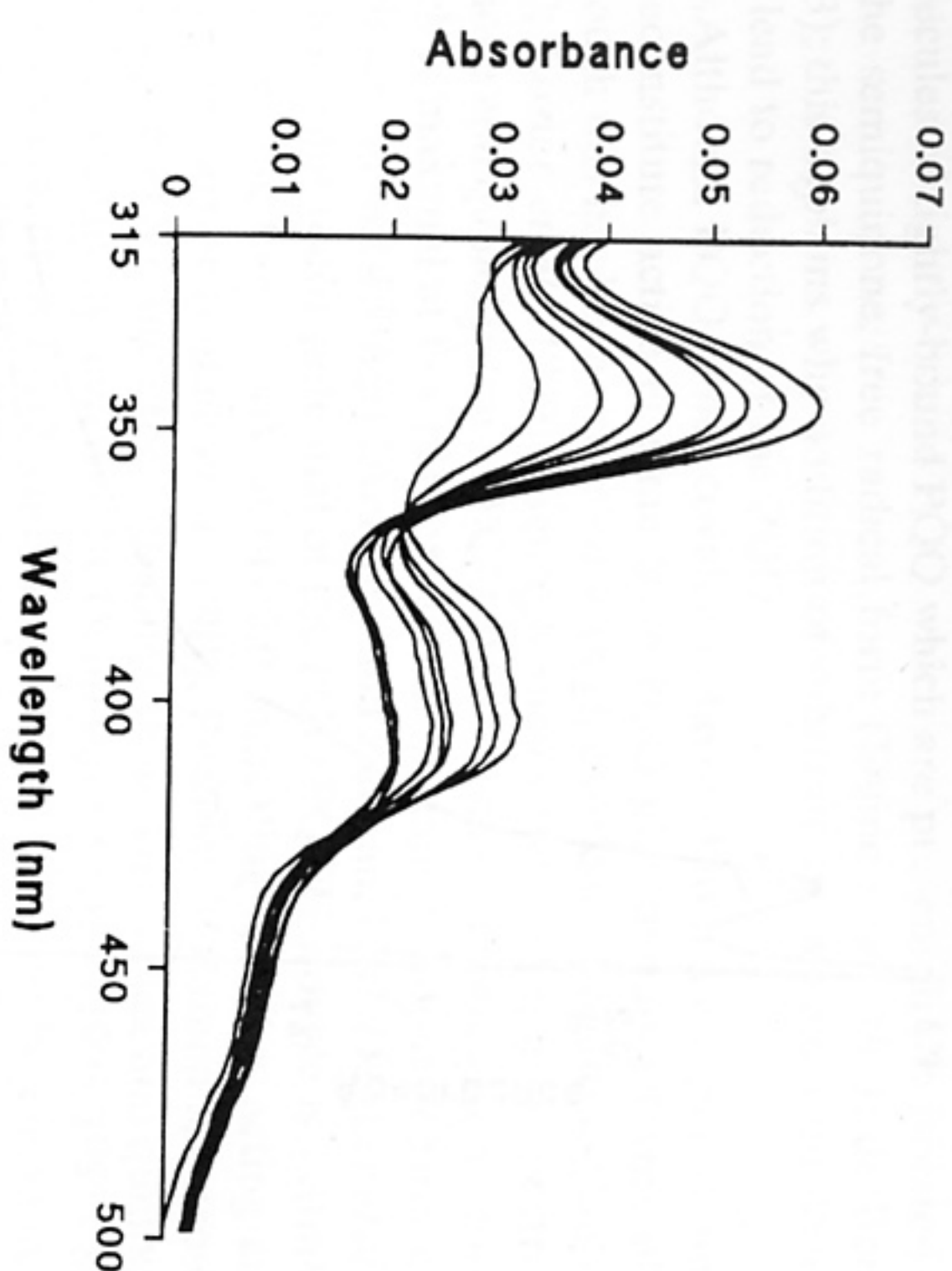


FIGURE 6. The reduction of oxidised Ba-MDH by endogenous substrate. MDH lacking any metal ion in its active site was produced from the *mxmA* mutant; it was incubated with Ba^{2+} to produce Ba-MDH which was then oxidised with a small excess of Wurster's Blue which was then removed by rapid gel filtration and spectra recorded. Reproduced with permission from Goodwin and Anthony (1996), (*Biochemical Journal*, **318**, 673–679). © the Biochemical Society.

The oxidation state was confirmed by demonstrating that its isolated prosthetic group was predominantly in the oxidised (quinone form). Figure 6 shows the spectrum of the Ba-MDH after oxidation at 4°C with a 1.5-fold excess of Wurster's Blue at pH 9. The spectrum of the initial oxidised form of the enzyme is very similar to that seen in Figure 4.

After Ca^{2+} has been incorporated into MDH it cannot usually be removed without completely denaturing the enzyme. An exceptional MDH is that isolated from the marine methylotroph *Methylobacterium marina* which requires at least 50 mM NaCl for stability. Sometimes, during storage, the enzyme lost Ca^{2+} , and became inactive and coloured red (due to absorbance at 520 nm) (Figure 7), the spectrum being similar to that in Ca^{2+} -deficient MDH (see above); in this case the normal spectrum and activity was regained by incubation with Ca^{2+} (Chan and Anthony, 1992b). It appears that the MDH of *M. marina* has a relatively low affinity for Ca^{2+} which is probably correlated with its normal seawater environment containing about 10 mM Ca^{2+} .

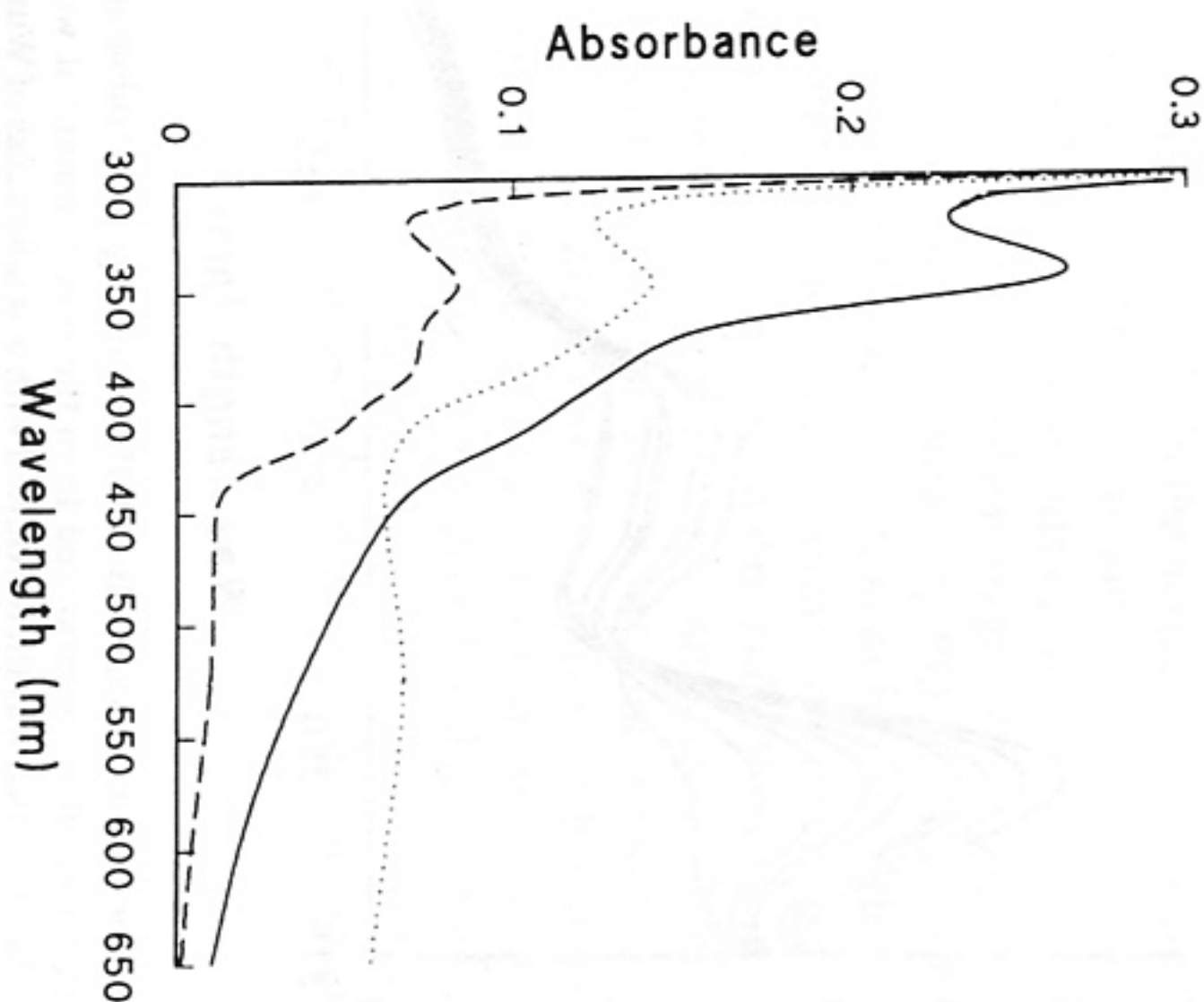


FIGURE 7. The spectra of the MDH from *Methylophaga marina*. The dotted line is the spectrum of the red form of the enzyme; the solid line is the active green form produced by incubation with Ca^{2+} , and the dashed line is the spectrum of an inactive green form produced by incubation at low pH in the absence of calcium. Reprinted from *FEMS Microbiology Letters*, 97, Chan and Anthony, Characterisation of a red form of methanol dehydrogenase from the marine methylotroph *Methylophaga marina*, 293–298, Copyright 1992, with permission from Elsevier Science.

5. PYRROLO-QUINOLINE QUINONE (PQQ): THE PROSTHETIC GROUP OF METHANOL DEHYDROGENASE

The novel prosthetic group from MDH was first isolated, purified and characterised by Anthony and Zatman (1967); it was readily removed from the enzyme by boiling or by treatment with acid or alkali, and shown to be reddish-brown in colour, highly polar and with a green fluorescence. The X-ray structure of an acetone adduct was eventually determined by Kennard and her colleagues (Salisbury *et al.*, 1979) and an extensive chemical characterisation was achieved by Frank and Duine and their co-workers (Duine and Frank, 1980a,b; Duine *et al.*, 1980, 1981; Dekker *et al.*, 1982; Duine, 1991). The structure of PQQ (full name: 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione is given in Figure 1. Each tetramer of MDH has two

molecules of tightly-bound PQQ which are present in the isolated enzyme in the semiquinone, free radical form (Duine *et al.*, 1981; de Beer *et al.*, 1983); this explains why addition of substrate (a two electron donor) does not lead to reduction of the PQQ.

Although PQQ is not covalently-bonded to the enzyme it is impossible to reconstitute active enzyme from PQQ plus the apoenzyme of MDH, although this has been achieved with glucose dehydrogenase (Duine *et al.*, 1980; Cozier *et al.*, 1999). Figure 8 shows the absorption spectra of the quinone and quinol forms of PQQ. It has a characteristic green fluorescence which is maximal at low pH (excitation maximum, about 365 nm; emission maximum, about 460 nm) (Anthony and Zatman, 1967; Dekker *et al.*, 1982). The midpoint redox potential of the PQQ/PQQH₂ couple is +90 mV at pH 7.0, increasing to +419 mV at pH 2.0, indicating that it is acting at a $2e^-/2H^+$ redox carrier (Duine *et al.*, 1981). Resonance Raman spectroscopy of the isolated PQQ, and other quinones, their derivatives, and quinoproteins has been extensively reviewed by Dooley and Brown (1993, 1995). Methods for measurement of PQQ, and the problems likely to be encountered are given in the review by Klinman and Mu (1994).

The crystal structures of the sodium and potassium salts of PQQ have shown that the tricyclic ring is planar and that the three carboxylate groups can be either coplanar with the ring or twisted out of the plane (van Koningsveld *et al.*, 1989). However it cannot be clear whether the observed

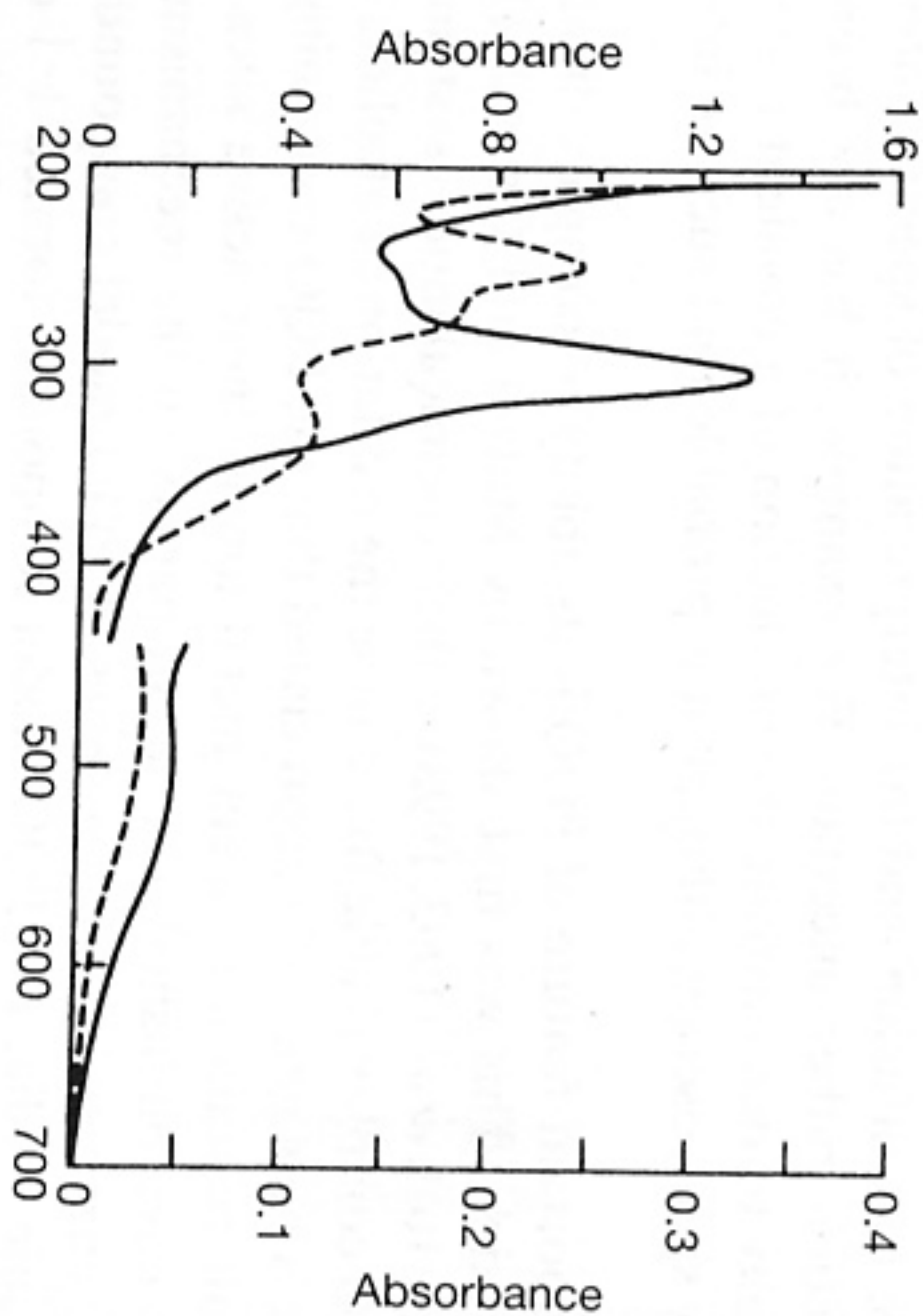


FIGURE 8. The absorption spectra of PQQ measured at pH 7.0. The solid line is the reduced, quinol form; the broken line is the oxidised quinone. The spectra were provided by J.A. Duine and J. Frank who have published the spectra of PQQ and those of many of its adducts (Figure 9) in Dekker *et al.* (1982).

planarity of the ring is an inherent property of PQO itself or a manifestation of crystal packing forces. The conformation of PQO and the role of Ca^{2+} were examined using a quantum mechanical method (Zheng and Bruice, 1997) which indicated that, in the presence or absence of Ca^{2+} , the tricyclic ring system will be essentially planar irrespective of its oxidation state, although the C—9 carboxyl group can be coplanar or twisted out of the plane of the ring, as seen in some X-ray structures of the enzyme (Ghosh *et al.*, 1995; White *et al.*, 1996).

Extensive studies of the chemical reactivity of the isolated PQO demonstrated the formation, by nucleophilic attack at the C—5 position, of a wide variety of adducts, many of which are potentially relevant to the assay and mechanism of the enzyme, including alcohols, aldehydes, cyanide, ammonia and amines (Dekker *et al.*, 1982; Ohshiro and Itoh, 1993) (Figure 9). Molecular orbital calculations (Zheng and Bruice, 1997) indicated that the two quinone carbonyl groups are not well coordinated with either the pyrrole or the pyridine ring while the adjacency of the pyrrole ring decreases the electron density of the quinone. As a result these two carbonyls are very reactive, the calculations supporting the experimental observations that the C5 carbonyl is expected to be more reactive than the C4 carbonyl. The same type of calculation indicated that the neutral and ionic forms of the free radical semiquinone are both planar, a result that is not consistent with the observed structures in the enzyme active site (Section 7.4.2). It is the relative ease of formation of such adducts that can make analysis and identification of PQO in proteins and biological tissues rather open to confusion, and the interpretation of spectral intermediates in the reaction rather uncertain. For example, it has not been possible unequivocally to demonstrate the production of a covalent reaction intermediate by spectroscopy, although it is probable that such an intermediate occurs.

An important feature of PQO is its ability to complex divalent metal ions in solution. This was first shown by Mutzel and Gorisch (1991) and exploited by Itoh *et al.* (1993, 1998) in their chemical model systems in which a Ca^{2+} -PQO complex is able to catalyse the oxidation of alcohols in organic solvents. It is now generally appreciated that the PQO-containing enzymes probably all contain a divalent metal ion in their active sites. Itoh and colleagues have published a series of papers on the mechanism of PQO-containing enzymes by using a series of PQO model compounds in anhydrous organic media. The PQO model compounds formed 1:1 complexes with a series of alkaline earth metal ions, the site of coordination with Ca^{2+} , Sr^{2+} and Ba^{2+} being the same as shown for Ca^{2+} in the known enzyme structures (see Section 7.4.2). Ca^{2+} ions bound much more tightly than the other ions, the affinity decreasing with size of ion ($\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+}$). Mg^{2+} binding

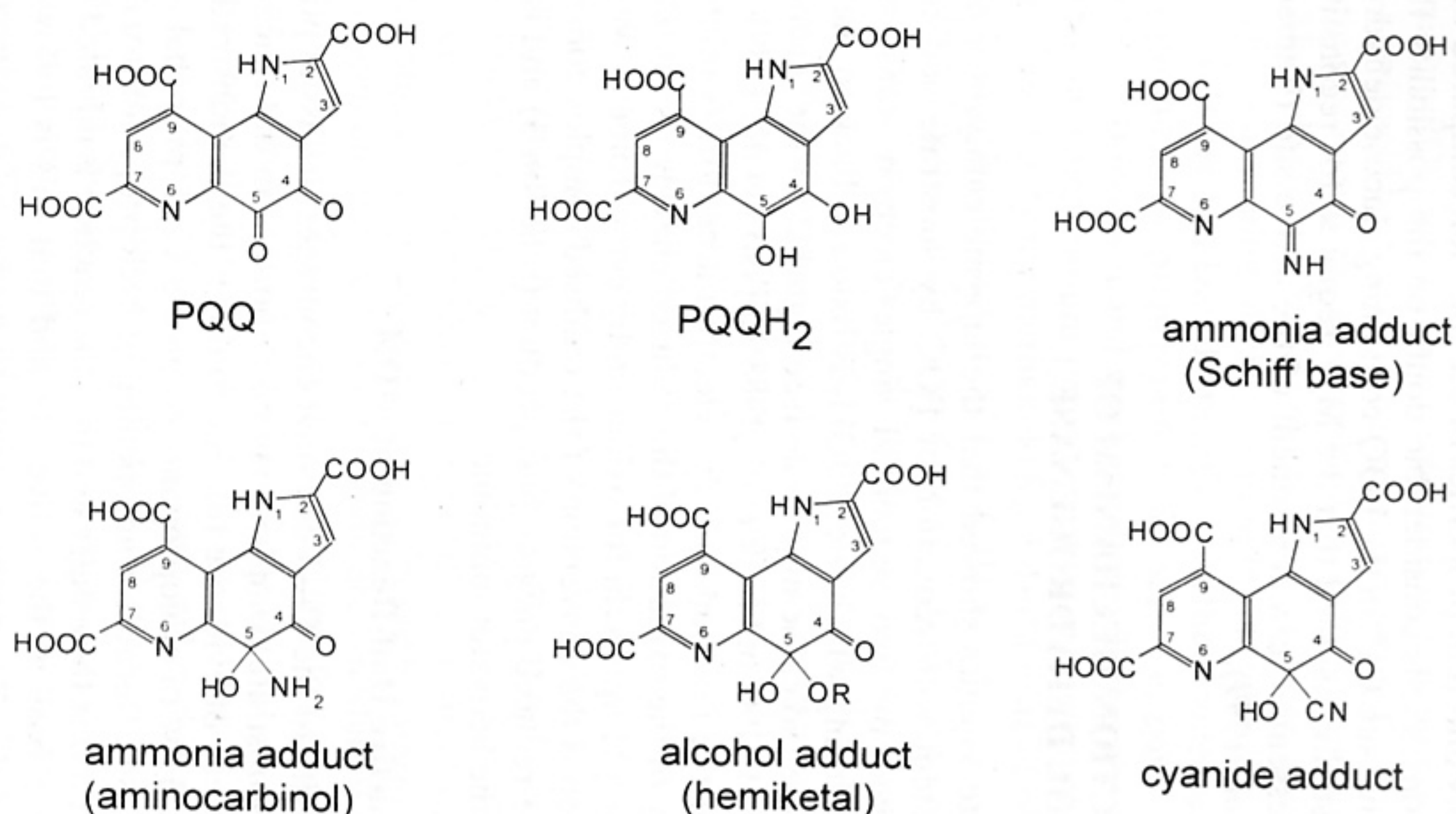


FIGURE 9. Adducts of PQO. The formation and properties of these adducts are discussed extensively in Dekker *et al.* (1982).

was extremely weak, and resulted in hydration of the *o*-quinone moiety, thus deactivating the PQO for redox reactions. Molecular orbital calculations suggested that the binding of Mg^{2+} is completely different from the other alkaline earths, bonding to both the O4 and O5 atoms. This, together with the generally different properties of the Mg^{2+} ion compared with other alkaline earth ions, sheds considerable doubt on the possibility that Mg^{2+} replaces the active site Ca^{2+} in the PQO-containing glucose dehydrogenase, although it is usually assumed that the Mg^{2+} required for reconstitution of this enzyme is essential as a constituent of the active site (Yamada *et al.*, 1998; Cozier *et al.*, 1999).

6. THE REACTION MECHANISM OF METHANOL DEHYDROGENASE

Steady state kinetics showed that the enzyme catalyses a ping-pong reaction, consistent with reduction of PQO by substrate and release of product, followed by two sequential single electron transfers to the cytochrome c_L , during which the PQOH₂ is oxidised back to the quinone by way of the free radical semiquinone (see Figure 2 for the reaction cycle) (Duine and Frank, 1980b; Frank *et al.*, 1988; Dijkstra *et al.*, 1989). The high pH optimum of the reaction with dye electron acceptors is related to the pH dependency of the oxidation of the reduced enzyme (reactions 1 and 4 in Figure 2); the pH optimum for oxidation by cytochrome c_L was 7.0. The rate-limiting step is the conversion of the oxidised complex containing the substrate to the reduced enzyme plus product (reaction 3) and is the only step requiring the activator ammonia.

6.1. The Reductive Half-Reaction of MDH

The reactivity of the C—5 carbonyl encourages the assumption that a covalent PQO-substrate complex may be important in the reaction mechanism. A key piece of evidence relating to this is the demonstration of the reaction of MDH with cyclopropanol to give a C—5 propanal adduct by Frank *et al.* (1989). Because ring opening of cyclopropanol usually occurs in alkaline conditions, they suggested that the reaction is initiated by proton abstraction by a base at the active site and that this is followed by rearrangement of the cyclopropoxy anion to a ring-opened carbanion, and attack of this on the electrophilic C—5 of PQO. It was concluded that during oxidation of methanol a similar proton abstraction from the alcohol by an active site base must occur.

The position of Asp-303 near PQO in the active site (see Section 7.4.2) suggests that this provides the catalytic base proposed above. Figures 10 and 11 illustrate two possible mechanisms, in which this aspartate (Asp-303) initiates the reaction by abstraction of a proton from the alcohol substrate. In these mechanisms the Ca^{2+} is given a role in addition to a structural role in maintaining PQO in an active configuration; it is proposed that the Ca^{2+} acts as a Lewis acid by way of its coordination to the C—5 carbonyl oxygen of PQO thus providing the electrophilic C—5 for attack by an oxyanion or hydride. The role of Ca^{2+} in the mechanism has been given support by a study of a Sr^{2+} -containing MDH produced by bacteria in a high concentration of Sr^{2+} (Harris and Davidson, 1994). Further evidence to support this has been provided by preparation of an active enzyme containing Ba^{2+} instead of Ca^{2+} (Goodwin and Anthony, 1996). This is the first example of any enzyme in which barium plays an active catalytic role; the Ba-MDH has a very low affinity for methanol (K_m , 3.4 mM instead of 10 μ M), but its activation energy is half that of the Ca-MDH and its V_{max} is doubled. It has been suggested that a further role of the Ca^{2+} might be to contribute to the mechanism by coordinating to the hydroxyl oxygen of the substrate, thus decreasing its pK and facilitating the initial proton abstraction step (Zheng and Bruice, 1997; Itoh *et al.*, 1998). However, this appears unlikely as the Ca^{2+} has now been shown to be too far away from the hydroxyl oxygen in the structure (Xia *et al.*, 1999).

Two general mechanisms for methanol oxidation have been proposed after the initial proton abstraction (Anthony, 1996, 1998). One is direct hydride transfer from the methanol α -methyl group to the PQO; in the more likely mechanism, however, the oxyanion produced by the initial proton abstraction attacks the electrophilic C—5, leading to formation of a covalent hemiketal intermediate (Figure 10). The direct evidence on this point is the slight change in spectrum of a possible intermediate (half-life 2 minutes) seen during reaction with deuterated methanol in the presence of excess dye electron acceptor (Frank *et al.*, 1988; Dijkstra *et al.*, 1989). The subsequent reduction of the PQO with release of product aldehyde is likely to require prior ionization of the C—4 carbonyl, a process facilitated by the pyrrole N atom. The involvement of this carbonyl in abstracting the methyl proton is consistent with the theoretical calculations and measurements with model PQO systems published by Itoh *et al.* (1998). In these systems the rates of hemiketal formation from alcohols plus metal ions showed that the hemiketal stabilisation was about 6-fold greater in the presence of Ca^{2+} . In the Ca^{2+} complex of the C5 hemiketal the Ca^{2+} is coordinated by both oxygen atoms of the hemiketal (OH and OMe) and this coordination forces the methyl group of the added methanol to flip towards the C4 carbonyl

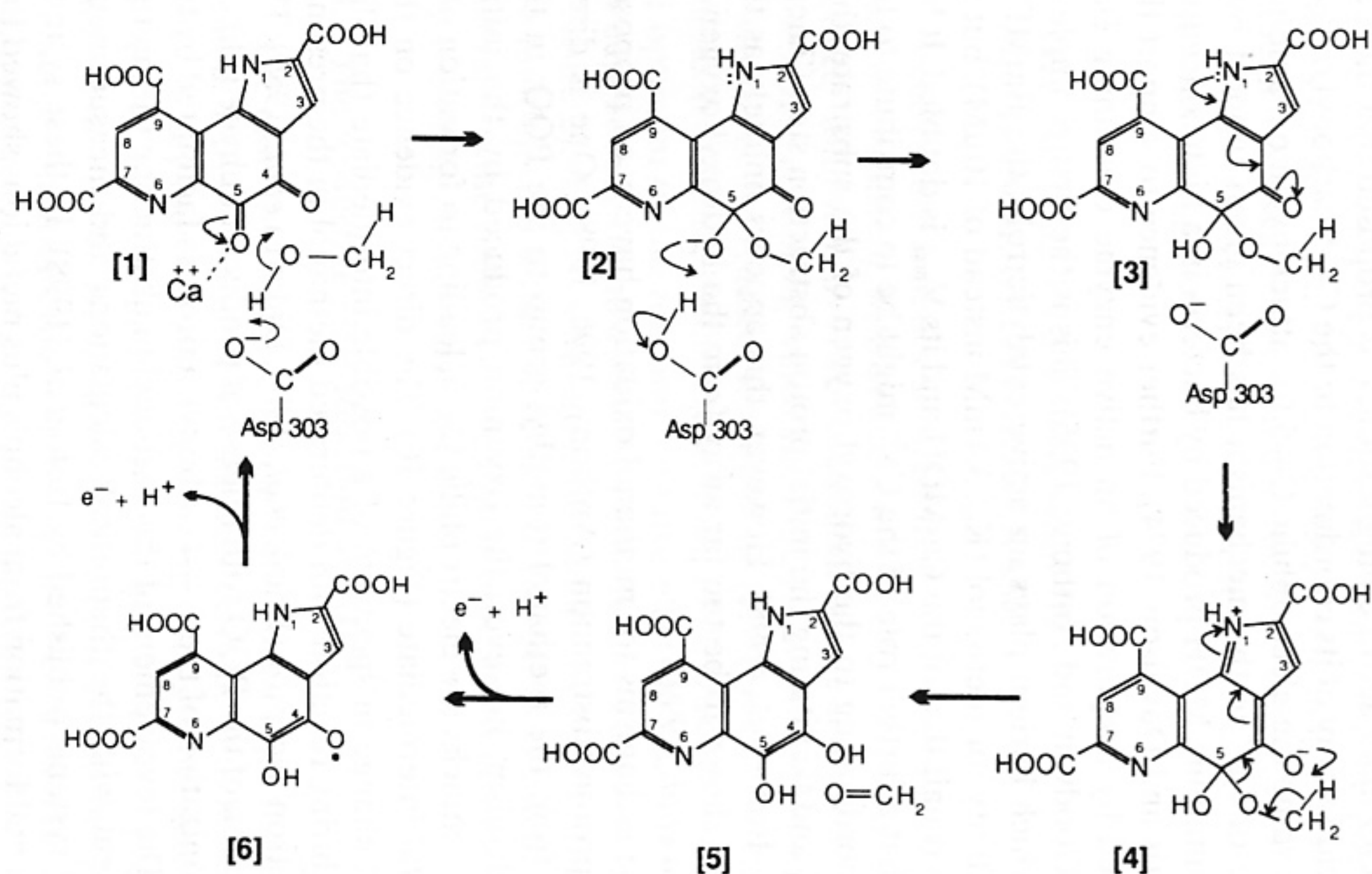


FIGURE 10. Proposed mechanism of MDH involving formation of hemiketal intermediate. It is suggested that Ca^{2+} plays a catalytic role by acting as a Lewis acid, facilitating attack on the electrophilic C5 of PQQ. Proton abstraction by the base (Asp303) leads to an oxyanion form of the substrate which attacks C5, giving the hemiketal intermediate [3] from which the methyl proton is abstracted by the C5 carbonyl oxygen whose ionisation is facilitated by the pyrrole nitrogen atom. The oxidative phase of the reaction cycle involves electron transfer to either PES or cytochrome c_L and involves the PQQ semiquinone free radical as an intermediate.

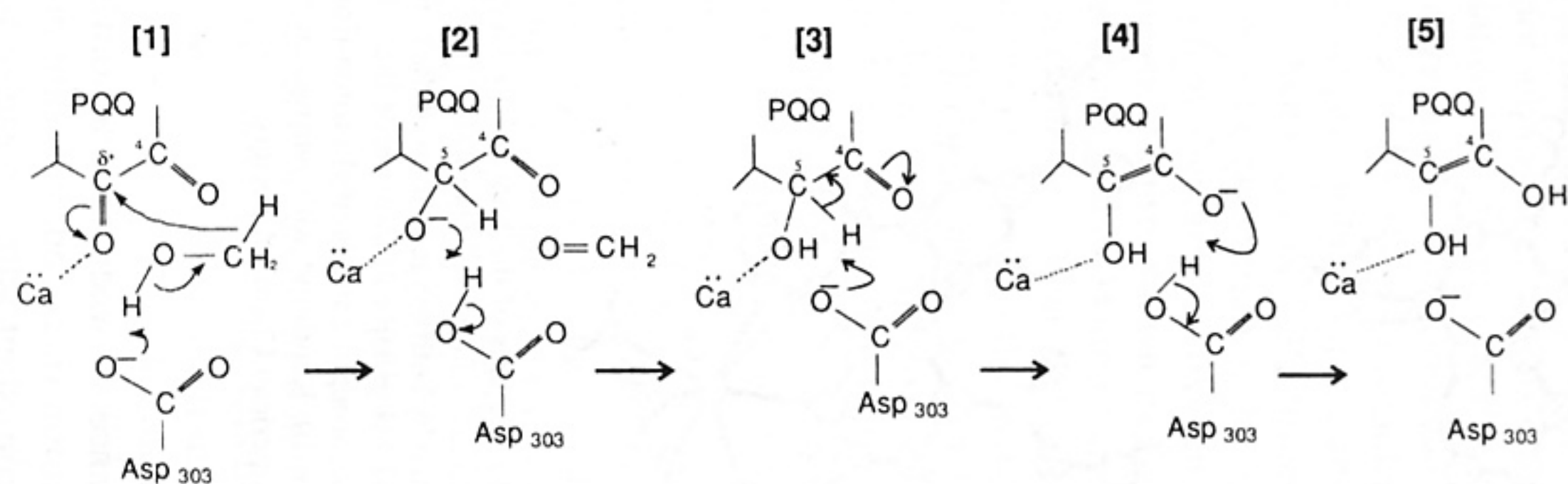


FIGURE 11. Alternative mechanism for MDH involving hydride transfer. In this case the initial proton abstraction is the same as in Figure 10, but the electrophilic C5 is involved directly in removal of the methyl hydrogen as a hydride. This mechanism is modified from those previously proposed (Anthony *et al.*, 1994) to emphasise the possible double involvement of the active site base (Asp303).

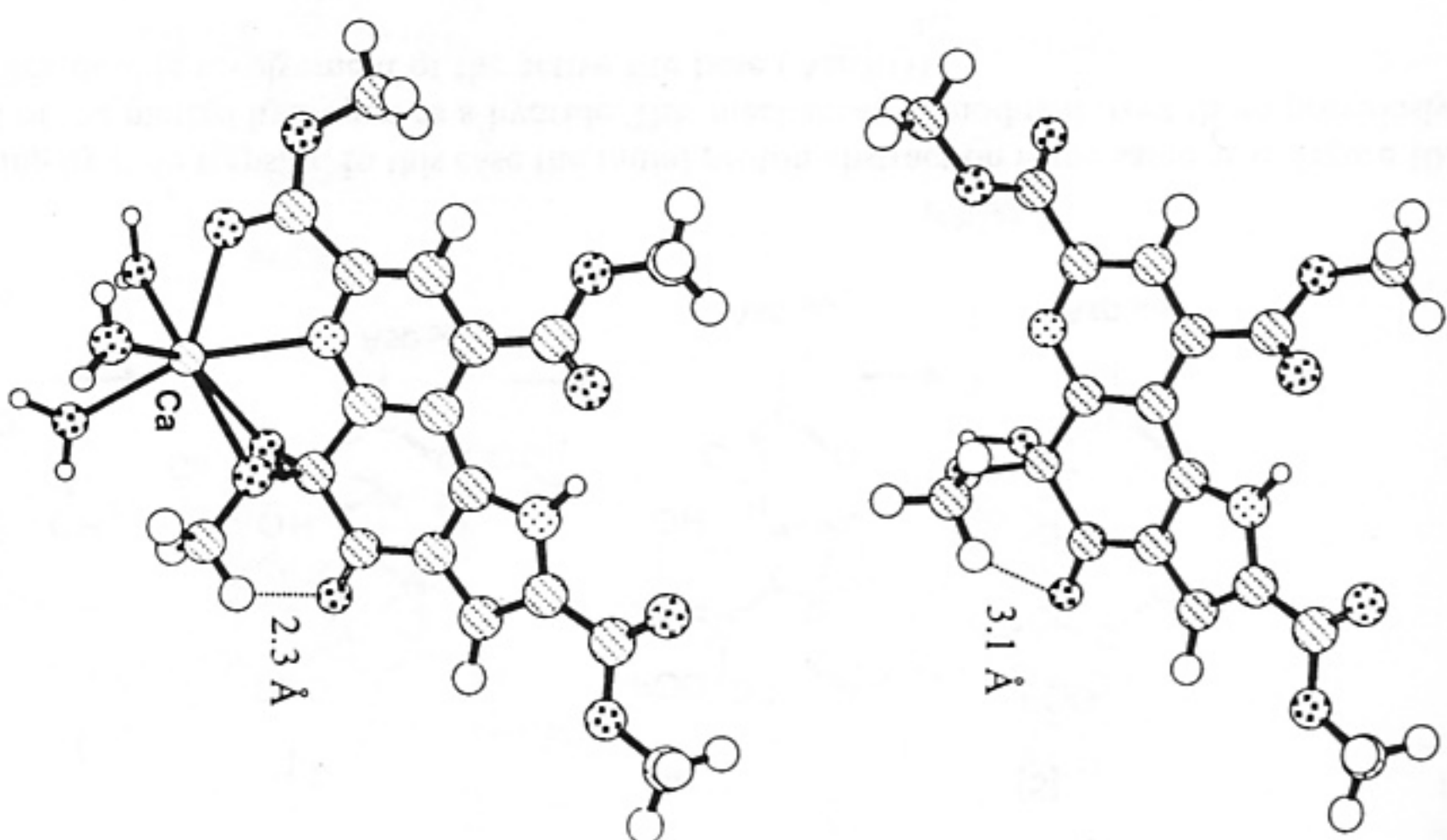


FIGURE 12. Computer-generated structures of the hemiketal adduct of the trimethyl ester of PQO and its Ca^{2+} complex. In the case of the Ca^{2+} complex of the C5 hemiketal (the lower structure) the metal ion is coordinated by both oxygen atoms of the hemiketal (OH and Ome), and this coordination forces the methyl group to flip towards the C4 carbonyl oxygen, suggesting that this might act as a base, accepting the methyl proton from the substrate as suggested in the mechanism in Figure 10. Reprinted with permission from Itoh *et al.* (1998), (*Biochemistry*, 37, 6562–6571). © American Chemical Society.

(Figure 12). Thus, the distance between the carbonyl oxygen at the 4-position and the methyl proton of added substrate in the Ca^{2+} complex decreases by about 0.8 Å, consistent with the suggestion that the neighbouring carbonyl oxygen at the 4-position could act as a general base to accept the α -proton of the substrate. In their studies of model systems, Itoh *et al.* (1998) showed that addition of DBU (a strong base, diazabicyclo-undecene) to an acetonitrile solution containing the metal

complexes of the PQO model compounds led to reduction of the PQO with production of the corresponding aldehyde. A large kinetic deuterium isotope effect was similar to that in the enzymic dehydrogenase reaction (about 6), clearly indicating that the base-catalyzed α -proton abstraction from the substrate is rate determining in the alcohol oxidation reaction. Divalent ions, but not monovalent cations or Mg^{2+} , accelerated the oxidative process, the relative effectiveness ($\text{Ba}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+}$) being the same as observed in the enzyme reaction (Goodwin and Anthony, 1996). It was suggested that this is consistent with intramolecular general base catalysis by the C4 carbonyl oxygen atom proposed above; namely, the binding of the larger Ba^{2+} to the hemiketal of PQO forces the added alcohol moiety closer to the C4 carbonyl oxygen than in the case of Ca^{2+} , thus facilitating the proton abstraction by the carbonyl oxygen.

A variant of the mechanism shown in Figure 10 is one in which the active site base (instead of the O4 atom) abstracts a methyl proton from the hemiketal adduct (Itoh *et al.*, 1998) but this possibility is not consistent with the position of the methanol in the active site as seen in the X-ray structure (Section 7.4.3). This orientation of methanol lends further support to the hemiketal mechanism. The hydroxyl oxygen is close to C5 (3.2 Å) while the methyl group is considerably further away (3.9 Å). This would favour covalent bond formation between C5 and the nearby hydroxyl over hydride ion transfer from the more distant methyl group of the substrate (Xia *et al.*, 1999).

In the alternative mechanism (Figure 11), a simple acid/base-catalysed hydride transfer occurs, and no covalent bond is formed between PQO and substrate; Asp-303 again provides the base and Ca^{2+} acts again as a Lewis acid. The large deuterium isotope effect (about 6) observed during the reductive phase of the reaction is consistent with either mechanism; in both cases the step affected will be the breaking of the C—H bond, and it is this step that is affected by the activator ammonia (Frank *et al.*, 1988; Goodwin and Anthony, 1996). Although the evidence on this matter is scarce, it slightly favours the mechanism involving a hemiketal intermediate (Figure 10). Itoh *et al.* (1998) point out that their kinetics, using the model system, might be interpreted in terms of a hydride transfer mechanism in which the C5 hemiacetal intermediate is a “bystander” in a side equilibrium; however, the orders of reactivity of their various model compounds and of alkaline earth metal ions ($\text{Ba}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+}$) can only be explained by taking into account the intramolecular base catalysis by the C4 carbonyl oxygen in the hemiketal intermediate, strongly supporting the validity of the mechanism involving the covalent addition of substrate to PQO.

6.2. The Role of Ammonia in the Reductive Half-Reaction Mechanism of Methanol Dehydrogenase

As mentioned above, activation of ammonia is confined to the substrate oxidation step but its mechanism of action is not known (Frank *et al.*, 1988; Dijkstra *et al.*, 1989; Goodwin and Anthony, 1996). The active form is the free base and ammonia can be replaced by methylamine or some glycine esters (Anthony, 1993a). An obvious suggestion for the involvement of ammonia would involve formation of a covalent iminoquinone adduct (Figure 9), and one of the first mechanisms proposed for this enzyme suggested that methanol binds as a methoxy group to the same carbon atom (C—4) as the ammonia and is then released as the aldehyde during reduction of the PQQ (Forrest *et al.*, 1980; Duine, 1991). However, although addition of ammonia to the C—5 position of isolated PQQ is known to occur (Dekker *et al.*, 1982; Ohshiro and Itoh, 1993), there is no convincing evidence that this occurs during the enzyme reaction. No nitrogen-containing adduct of PQQ has been isolated from the enzyme; added ammonia is easily removed by gel filtration to give inactive enzyme which must then be re-activated; in the reaction with cytochrome this activator is not always required; and some dehydrogenases for alcohol which are likely to have essentially similar mechanisms do not require ammonia as activator.

6.3. The Oxidative Half-Reaction of Methanol Dehydrogenase

6.3.1. The "Methanol Oxidase" Electron Transport Chain

In the electron transport chain for methanol oxidation, electrons are passed from MDH to cytochrome c_1 thence to cytochrome c_H which is then oxidised by cytochrome aa_3 or cytochrome co (Anthony, 1988, 1992a). This small electron transport chain is more like that involved in oxidation of inorganic substrates in not involving ubiquinone and the cytochrome b_{c_1} complex. It establishes a protonmotive force which drives ATP synthesis, yielding less than 1 ATP per mole of methanol oxidised. Except for the oxidase all the components of this system are in the periplasm. The concentrations of MDH and cytochrome c_1 in the periplasm are about 0.5 mM (Alefounder and Ferguson, 1981; Beardmore-Gray *et al.*, 1983). As these proteins are more or less globular in shape, they probably form a monolayer one molecule deep in the periplasm. Indeed if the distance between the periplasmic membrane and the outer membrane is about 70 Å then there is hardly space for the large dehydrogenase to move in more than two dimensions.

In terms of primary sequence and large size cytochromes of the cytochrome c_1 type constitute a novel class of acidic c -type cytochromes (Nunn and Anthony, 1988; Anthony, 1992a). The crystallisation of a ternary complex involving this cytochrome together with methylamine dehydrogenase and amicyanin from *Paracoccus denitrificans* has provided information on its tertiary structure (in this organism the cytochrome c_1 is usually referred to as cytochrome c_{511}) (Chen *et al.*, 1994). It contains five α -helices, the central three of which envelope the haem group and correspond to analogous helices in most other c -type cytochromes which share the "cytochrome c fold".

Cytochrome c_H is similar in most respects to other typical bacterial cytochromes c and c_2 . However its X-ray structure (Read *et al.*, 1999) shows a number of unusual features; it bears a closer gross resemblance to mitochondrial cytochrome c than to the bacterial cytochrome c_2 and the left hand side of the haem cleft is unique. In particular it is highly hydrophobic, the usual water is absent, and the "conserved" Tyr67 is replaced by tryptophan. A number of features of the structure demonstrate that the usual hydrogen bonding network involving water in the haem channel is not essential, and that other mechanisms for modulation of redox potentials may exist in this cytochrome. It should be emphasised that the unique character of this cytochrome does not appear to be related in any way to its special involvement in oxidising cytochrome c_1 in the methylotroph electron transport chain.

6.3.2. The Interaction of MDH with Cytochrome c_1

There is considerable evidence that the interaction between the dehydrogenase and cytochrome c_1 is electrostatic in nature, including the observation that respiration in whole bacteria is inhibited by high salt concentrations (50% inhibition by 200 mM-NaCl or about 25 mM-sodium phosphate) (Anthony, 1992a; Dijkstra *et al.*, 1989). Electron transfer activity between the MDH and cytochrome c_1 was inhibited by high ionic strength; there was an inverse linear relationship between reaction rate and the square root of the ionic strength, consistent with electrostatic interactions being involved (Chan and Anthony, 1991; Cox *et al.*, 1992).

The nature of the groups involved in the MDH / cytochrome c_1 interaction has been investigated by chemical modification using the proteins from a number of different classes of methylotroph including those that grow at low pH and those that grow in high salt concentrations. These studies indicated that the interaction is by way of a small number of lysyl residues on the α subunit of MDH and carboxylates on the cytochrome; modification of these residues prevents electron transfer between the

proteins, without however affecting activity in the PES-linked system, showing that the active site for reaction with substrate had not been altered (Chan and Anthony, 1991; Cox *et al.*, 1992; Anthony, 1992). Reagents which change the charge on the lysines led to inactive MDH, whereas those that modified MDH with retention of charge had relatively little effect. A range of experiments with various cross-linking reagents showed that cytochrome c_L dissociates from MDH before interaction with cytochrome c_H ; that is, the same site on cytochrome c_L interacts with the electron donor and the electron acceptor, and a three component electron transfer complex is not formed. Remarkably, similar results were obtained in chemical modification studies using MDH from the marine methylotroph *Methylophaga marina*; although 1 M NaCl had no effect on activity, chemical modification of only 4 lysine residues on the MDH led to complete loss of activity.

It has been suggested that hydrophobic interactions are also important between MDH and cytochrome c_L (Harris and Davidson, 1993b). PQO is buried within an internal chamber which communicates with the exterior of the protein by way of a hydrophobic funnel in the surface; the shortest distance for electron flow between PQO and the outside of the MDH is likely to be by way of this funnel, and so hydrophobic interactions may also be essential to hold cytochrome c_L in position for electron transfer. In order to distinguish between factors affecting electron transfer and those affecting the initial binding or docking process, a fluorescence method was developed (Dales and Anthony, 1995). This confirmed the role of electrostatic interactions in the initial binding but showed a surprising result with respect to the mode of action of the inhibitor EDTA. This has no effect on the dye-linked MDH activity and is not dependent on its chelating characteristics. Studies using a fluorescent analogue of EDTA (Indo-1) showed that it acts by binding tightly to MDH (I_{50} , about 3 μ M) but not to cytochrome c_L ; it was concluded that it inhibits methanol oxidation by binding to lysyl residues on MDH thus preventing docking with cytochrome c_L (Chan and Anthony, 1992). However, the fluorescence assay for measuring the interaction of MDH and cytochrome c_L showed, surprisingly, that the "docking" is not inhibited by 50 μ M-EDTA which completely inhibits the overall electron transfer process. It was therefore suggested that EDTA acts by binding to nearby lysyl residues, thus preventing movement of the "docked" cytochrome to its optimal position for electron transfer, which probably involves interaction with the hydrophobic funnel in the surface of the dehydrogenase (Dales and Anthony, 1995). A further extensive kinetic investigation of the interaction of the two proteins has led to Davidson and his colleagues to a similar conclusion; that is, after a non-optimal collision there is a rearrangement of the proteins to produce the most efficient orientation for electron transfer (Harris *et al.*, 1994). The structure of the cytochrome

c_L from *P. denitrificans* shows that most of the charged residues are located on the side of the molecule away from the hydrophobic edge of the haem cleft (Chen *et al.*, 1994). This provides a challenge to the model in which these residues are involved in the docking process. The MDH / cytochrome c_L complex has not been crystallized in order to determine its X-ray structure, and until this is achieved the route by which electrons pass from reduced PQO to the haem in cytochrome c_L must remain a matter for speculation.

6.3.3. The Oxidation of Reduced PQO in MDH

Very little is known about the process of electron transfer from the quinol form of PQO to the cytochrome electron acceptor. It is reasonable to assume that this occurs in two single electron transfer steps—the semiquinone form of PQO being produced after the first of these transfers (Figure 10). The protons are released from the reduced PQO into the periplasmic space thus contributing to the protonmotive force (Anthony, 1988, 1993b). It was once thought that an intermediary in this process was the novel disulphide bridge between adjacent cysteines in the active site; this novel structure was very readily reduced with dithiothreitol, yielding enzyme that was inactive with cytochrome but active with the phenazine ethosulphate (PES) (Blake *et al.*, 1994). However, it has now been shown that the activity with PES is because the dye very rapidly re-oxidises the two thiols back to the original disulphide. No free thiols were ever detected during the reaction cycle and the enzyme was active with cytochrome c_L even after the cysteines of the disulphide bridge had been carboxymethylated by reaction with iodoacetate, and so no longer able to undergo oxidation/reduction reactions (Avezoux *et al.*, 1995).

7. THE STRUCTURE OF METHANOL DEHYDROGENASE

MDH is the only PQO-containing enzyme for which a structure is available (Anthony and Ghosh, 1998); the structures have been determined for the MDH from the facultative methylotroph *M. extorquens* (1.94 Å) (Blake *et al.*, 1994; Ghosh *et al.*, 1995) and from the obligate methylotrophs *Methylophilus methylotrophus* (2.6 Å) (Xia *et al.*, 1992), and *Methylophilus* W3A1 (1.9 Å) (White *et al.*, 1993; Xia *et al.*, 1996, 1999). As expected from the similarities in protein sequence (Figure 13), all the structures are similar; the numbering system for MDH from *M. extorquens* will be used throughout this review. MDH has an $\alpha_2\beta_2$ tetrameric structure (Figure 14); the α -subunit containing the PQO is 66 kDa, and the β -subunit is very small

The α subunit of methanol dehydrogenase

AM1 1- NDKLVELSKSDDNWVMPGKNYDSNNFSDLKQINKGNVKQLRPAWTFSTGL
W3A1 1- DADLDKQVNTAGAWPIATGGYYSQHNSPLAQINKSNVKNVKAAWSFSTGV

AM1 51- LNGHEGAPLVVDGKMYIHTSFPNNTFALGLDDPGTILWQDKPKQNPAAARA
W3A1 51- LNGHEGAPLVIGDMMYVHSAFPNNTYALNLNDPGKIVWQHHPKQDASTKA

AM1 101- VACCDLVNRGLAYWPGDGKTPALILKTQLDGNVAALNAETGETVWKVENS
W3A1 101- VMCCDVVDRGLAYGAGQ-----IVKKQANGELLALDAKTGKINWEVEVC

AM1 151- DIKVGSTLTIAPYVVKDKVIIIGSSGAELGVRGYLTAYDVKTGEQVWRAYA
W3A1 145- DPKVGSTLTQAPFVAKDTVLMGCSGAELGVRGAVNAFDLKTGELKWRAFA

AM1 201- TGPDKDLLLASDFNIKNPHYGQKGLGTGTWEGDAWKIGGGTNWGWYAYDP
W3A1 195- TGSDDSVRLAKDFNSANPHYGQFGLGKTWEGDAWKIGGGTNWGWYAYDP

AM1 251- GTNLIYFGTGNPAPWNETMRPGDNKWTMTIFGRDADTGEAKFGYQKTPHD
W3A1 245- KLNLFYYGSGNPAPWNETMRPGDNKWTMTIWGRDLDTGMAKWGYQKTPHD

AM1 301- EWDYAGVNVMLLSEQKDKDGKARKLLTHPDRNGIVYTLDRTDGALVSANK
W3A1 295- EWDFAGVNQMVLTDQ-PVNGKMTPLLSHRDRNGILYTLNRENGNLIVA EK

AM1 351- LDDTVNVFKSVDLKTGQPVRDPEYGTRMDHLAKDICPSAMGYHNQGHDSY
W3A1 344- VDPVAVNVFKKVDLKTGTPVRDPEFATRMDHKGNTNICPSAMGFNHQGVDSY

AM1 401- DPKRELFFMGINHICMDWEPFMLPYRAGQFFVGATLNMYPGPKGDRQNYE
W3A1 394- DPESRTLYAGLNHICMDWEPFMLPYRAGQFFVGATLAMYPGPNGPTKK--

AM1 451- GLGQIKAYNAITGDYKWEKMERFAVWGGTMATAGDLVIFYGTLDGYLKARD
W3A1 442- EMGQIRAFDLTTGKAKWTKWEKFAAWGGTLYTKGGLVWYATLDGYLKALD

AM1 501- SDTGDLLWKFKIPSGAIGYPMTYTHKGTQYVAIYYGVGGWPGVGLVFDLA
W3A1 492- NKDGKELWNFKMPSSGIGSPMTYSFKGKQYIGSMYGVGGWPGVGLVFDLT

AM1 551- DPTAGLGAVGAFKKLANYTQMGGGVVVFSLDGKGPYDDPNVGEWKSAAK
W3A1 542- DPSAGLGAVGAFRELQNHTQMGGGLMVFSL

The β subunit of methanol dehydrogenase

AM1 1- YDGTKCKAAGNCWEPKPGFPKKIAGSKYDPKHDPKELNKQADSIKQME E
W3A1 1- YDGQNCKEPQNCWENKPQYPEKIAQSKYDPKHDPVELNKQEE SIKAMDA

AM1 51- RNKKRVENFKKTGKFEYDVAKISAN
W3A1 51- RNAKRIANAKSSGNTVFDVK

FIGURE 13. Amino acid sequence of the two subunits of methanol dehydrogenase. AM1 indicates the sequence for MDH from *Methylobacterium extorquens* AM1 (Anderson *et al.*, 1990; Nunn *et al.*, 1989; Anthony, 1992b); W3A1 indicates the sequence for *Methylophilus* W3A1 (Xia *et al.*, 1996).



FIGURE 14. The $\alpha_2\beta_2$ tetrameric structure of MDH. The small β subunit is the darker part of the structure. This Figure is based on the structure in Ghosh *et al.* (1995).

(8.5kDa). The subunits cannot be reversibly dissociated and no function has been ascribed to the small subunit. In the tetramer the two $\alpha\beta$ subunits are arranged with their pseudo 8-fold axes approximately perpendicular to each other, and the PQQ prosthetic groups are separated by about 45 Å.

7.1. The Structure of the α -Subunit

The large α subunit is a superbarrel made up of eight topologically-identical four-stranded twisted antiparallel β -sheets (W-shaped), stacked radially around a pseudo eight-fold symmetry axis running through the centre of the subunit. This structure has been referred to as a propeller fold, each W motif representing a propeller blade. The 32 β -strands that make up the superbarrel structure in MDH are shown in Figure 15, the sequence of the strands being the same as in the amino acid sequence as is also seen in other superbarrel structures. The normal twist of the β -sheets enables space to be efficiently packed in the subunit and allows the large polypeptide chain to be folded in a very compact form without any other typical structural domains. There is no 'hole' along the pseudosymmetry axis, which is filled with amino acid side chains from the eight A-strands of the β -sheets; where these come closest together, at the point of contact five of the eight residues are glycine, thus facilitating close-packing.

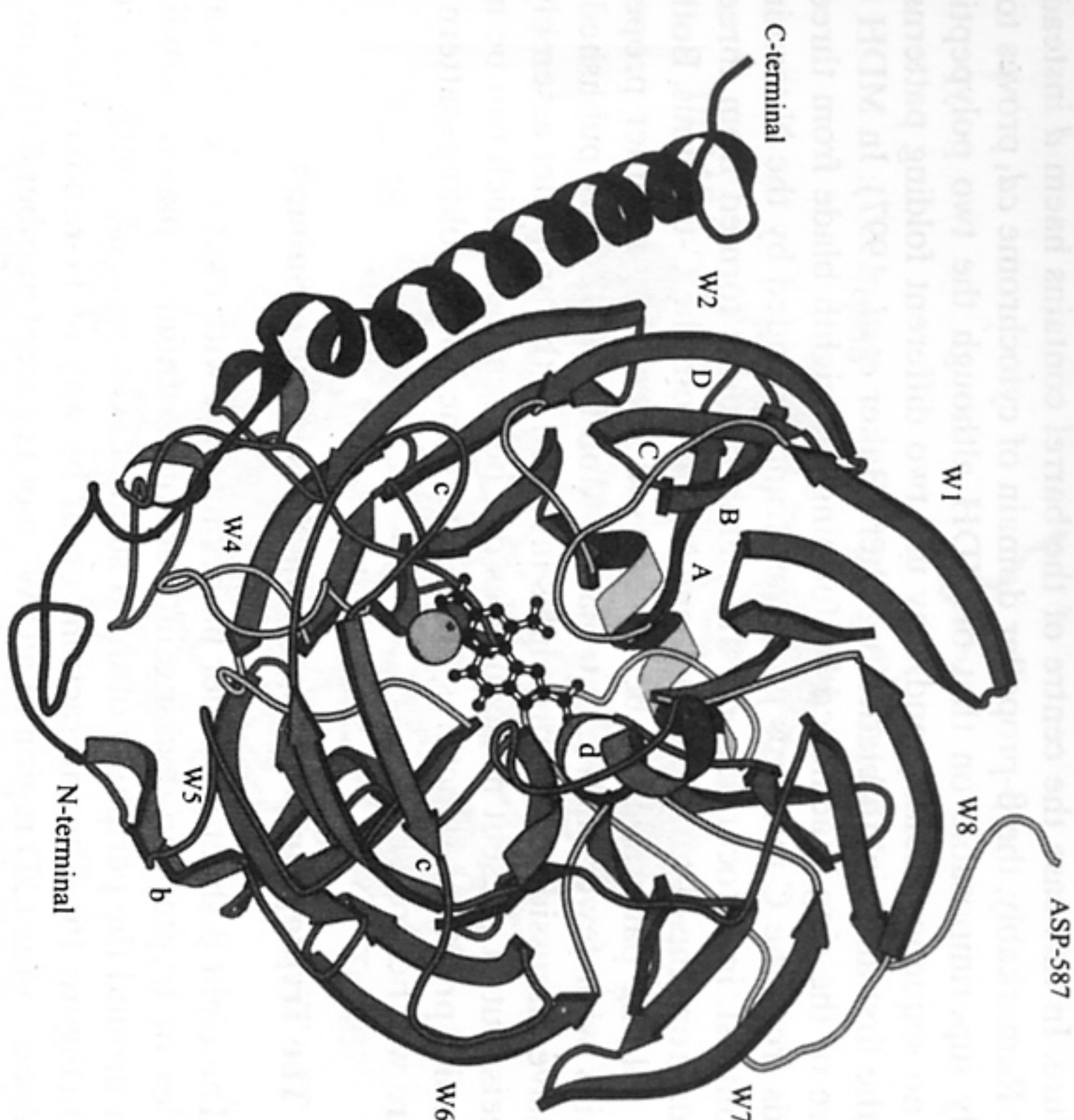


FIGURE 15. A drawing of an $\alpha\beta$ unit of MDH looking down the pseudo 8-fold axis, simplified to show only the β -strands of the "W" motifs of the α -chain, and the long α -helix of the β -chain, but excluding other limited β -structures and short α -helices. The PQQ prosthetic group is in skeletal form and the calcium ion is shown as a small sphere. The outer strand of each "W" motif is the D strand, the inner strand being the A strand. The "W" motifs are arranged in this view in an anti-clockwise manner. The exceptional motif W8 is made up of strands A—C near the C-terminus plus its D strand from near the N-terminus. This Figure is based on the structure in Ghosh *et al.* (1995).

The α subunits in methanol dehydrogenase contact one another in the region containing the D-strands of the seventh and eighth W motifs, associated over a large planar interface containing many hydrophobic and hydrophilic side chain interactions. These are augmented by the ten C-terminal residues of the α -chains which form extensions which associate with the symmetry-related subunit, again through hydrophobic and hydrophilic interactions.

Cytochrome *cd*₁ (nitrite reductase) is the only other protein, whose structure has been determined, that has an eight bladed β -propeller

structure. In this case the centre of the barrel contains haem *d* instead of PQQ. Remarkably, the β -propeller domain of cytochrome *cd*, proves to be closely superimposable on that of MDH although the two polypeptides have no sequence identity and they use two different folding patterns to bind the first and eighth blades together (Baker *et al.*, 1997). In MDH this closure of the ring is achieved by forming the eighth blade from three β -strands from the C-terminus plus one strand provided by the N terminus. By contrast, in cytochrome *cd*, the eighth blade is formed from three β -strands from the N-terminus plus one strand from the C-terminus. Both of these folding patterns have been observed previously in other propeller proteins with fewer than eight blades. Murzin (1992) has published an extensive discussion of the principles involved in the propeller assembly of β sheets, but at present no sequences can be identified which can be used to predict propeller structures or to determine which folding pattern for closure will be present.

7.2. The Tryptophan-Docking Interactions in the α -Subunit

The eight β sheet propeller blades interact with each other by way of a series of tryptophan-docking interactions forming a planar, stabilising girdle around the periphery of the α subunit (Ghosh *et al.*, 1995; Xia *et al.*, 1996) (Figure 16). The interactions occur by way of 11-residue consensus sequences in the C/D region of all "W" motifs except number 8 (Figure 17). The relevant characteristics of the tryptophan residues are their planar conjugated rings, and their ability to act as a hydrogen bond donor through the indole ring NH group (Figure 18). The tryptophan at position 11 is stacked between the alanine at position 1 of the same motif (W_n) and the peptide bond between residue 6 and the invariant glycine at position 7 of the next motif (W_{n+1}). The same tryptophan is also H-bonded between its indole NH and the main chain carbonyl of residue 4 in the next motif (W_{n+1}). The third type of interaction involving the conserved tryptophans is a β sheet hydrogen bond between its carbonyl oxygen and the backbone nitrogen of position 1 (usually alanine) of the same motif.

There are some exceptions to these interactions; for example, the tryptophan is replaced by Ser347 in W5 which makes a hydrogen bond with the peptide carbonyl of residue 4 in motif W6. In the other exception, Phe292 replaces tryptophan in W4 and makes only stacking interactions, with the usual glycine peptide bond of W5 on one side, and a second glycine peptide bond in position 1 of W4. Motif W8 is exceptional in having no consensus sequence (except for Trp44) and so there is no glycine to interact with the tryptophan in the previous motif (Trp508); in its place this tryptophan forms

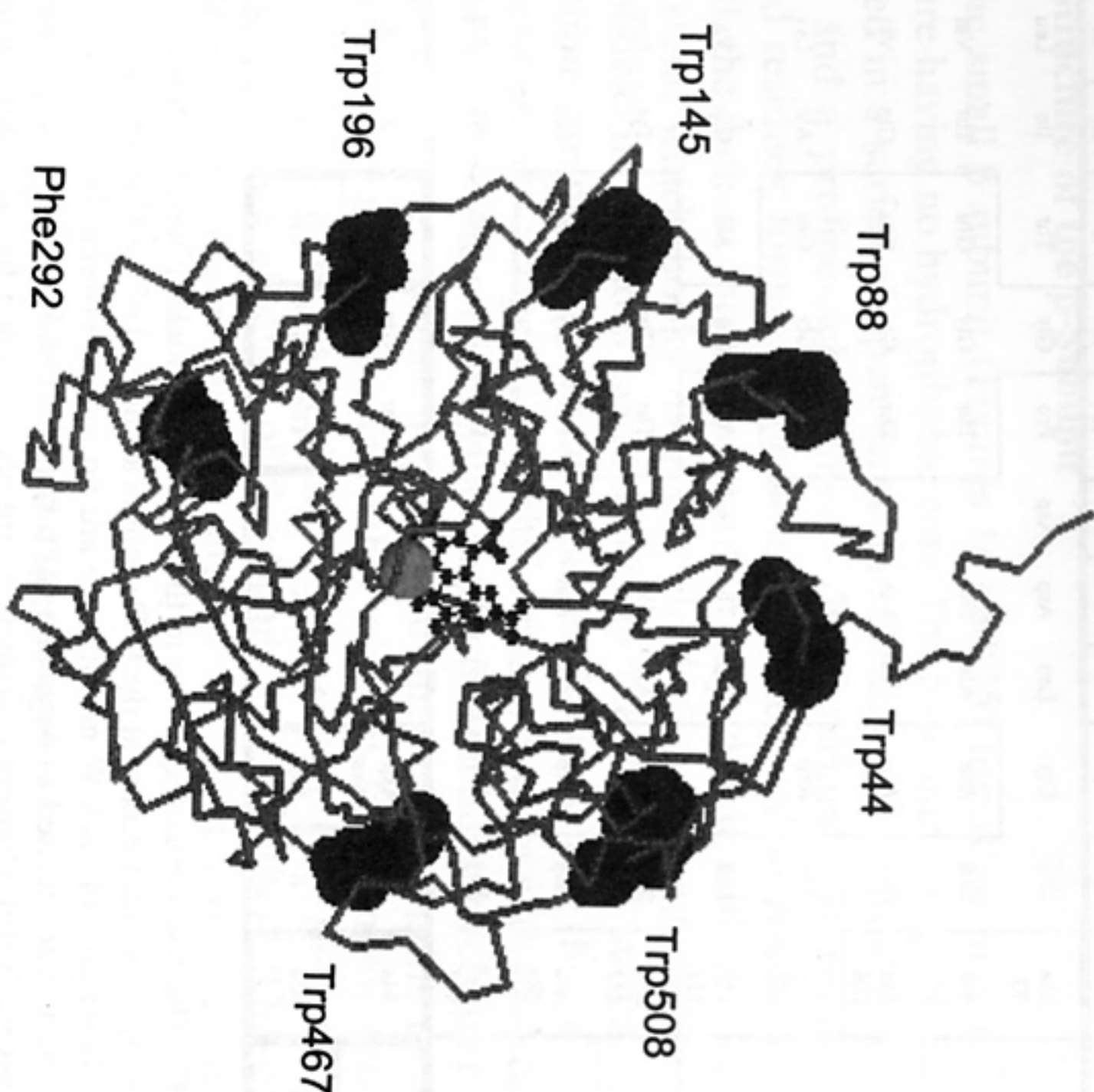


FIGURE 16. The girdle of tryptophan residues involved in docking the β -sheets together in the α subunit. The tryptophan residues involved in docking are shown in spacefill mode and the rest of the chain as backbone. The PQQ prosthetic group is in skeletal form and the calcium ion is shown as a small sphere. This Figure is based on the structure in Ghosh *et al.* (1995).

a hydrophobic interaction with the side chain of Leu40 and in the previous motif. The carbonyl of this leucine (position 7) replaces the usual carbonyl at position 4 in forming a hydrogen bond with the tryptophan indole NH. The 11-residue motif is extended in many cases by 2 further residues on the C and D strands which are joined by main chain hydrogen bonds (Xia *et al.*, 1996). The PQQ-containing dehydrogenases for glucose (Cozier and Anthony, 1995) and alcohols (Cozier *et al.*, 1995; Jongejan *et al.*, 1998) show the greatest similarity to the MDH in the sequences which form the W motifs which has facilitated modelling of the structure of these proteins.

No similar tryptophan docking motifs are present in the 7-bladed superbarrel proteins galactose oxidase and methylamine dehydrogenase, or in the 8-bladed nitrite reductase (cytochrome *cd*) (Fulop *et al.*, 1995; Baker *et al.*, 1997).

The small β subunit (Figures 14 and 15) forms an unusual extended structure having no hydrophobic core. The N-terminal region (30 residues) is folded in a series of open turns and includes one intrachain disulphide bridge and a proline-rich segment. The C-terminal 54 residues, rich in charged residues, form a single straight α -helix of 30 residues in 7 turns. Overall, the β -chain forms a planar "J" shaped unit, with the long α -helix as its stem, which hooks over the globular α -subunit. Although some hydrophobic interactions occur between the α and β subunits, ion-pair interactions are predominant, as expected from the fact that 40% of the β chain residues are charged. The β chain makes contact with the edges of the chain residues are charged. The β chain makes contact with the edges of the W1-W4 motifs of the α chain with ion pair interactions involving glutamate, arginine and lysine residues. It makes no direct contribution to the active site and, in the absence of any other obvious function for this unusual subunit, it has been suggested that it acts to stabilise the folded form of the large α -chain. The absence of β -subunits in the other PQO-containing quinoproteins, however, perhaps indicates that it has a more specific (unknown) function.

The active sites within each α subunit occur on the pseudo 8-fold symmetry axis, at the end of the superbarrel structure containing the loops which fold over the end of the superbarrel to enclose the active site chamber (Figure 15). Access is by way of a shallow funnel made up of hydrophobic surface residues (Anthony *et al.*, 1994) leading to a narrow entrance to the chamber containing the PQO coordinated to a Ca^{2+} ion, the novel disulphide ring structure and a potential active site base (Asp303). There is no obvious interaction between the two active sites in the two α subunits.

There is a remarkable novel structure within the α subunit produced by formation of a disulphide bridge between adjacent cysteine residues (Cys103-Cys104), the result being a strained 8-membered ring (Figure 19). Although such a disulphide bridge has been proposed to be present in the active site of the acetylcholine receptor (Kao and Karlin, 1986) and has been seen in the structure of an inactive, oxidised, form of mercuric ion reductase (Schiering *et al.*, 1991), this is the first time that this structure has been seen in an active enzyme. It had been predicted that if such a

Position		1	2	3	4	5	-	6	7	8	9	10	11
Motif		C	C	C				D	D	D	D	D	D
W1	Ala 77	Leu	Gly	Leu	Asp	Asp		Pro	Gly	Thr	Ile	Leu	Trp 88
W2	Ala 135	Leu	Asn	Ala	Glu	-		Thr	Gly	Glu	Thr	Val	Trp 145
W3	Ala 186	Tyr	Asp	Val	Lys	-		Thr	Gly	Glu	Gln	Val	Trp 196
W4	Gly 282	Arg	Asp	Ala	Asp	-		Thr	Gly	Glu	Ala	Lys	Phe 292
W5	Thr 337	Leu	Asp	Arg	Thr	-		Asp	Gly	Ala	Glu	Val	Ser 347
W6	Ala 457	Tyr	Asn	Ala	Ile	-		Thr	Gly	Asp	Tyr	Lys	Trp 467
W7	Ala 489	Arg	Asp	Ser	Asp	-		Thr	Gly	Asp	Leu	Leu	Trp 508
W8	Val 577	Phe	Ser	Leu	Asp	-		Gln 39	Leu	Arg	Pro	Ala	Trp 44
MIDH Consensus	Ala	X	Asp/Asn	X	X	-		Thr	Gly	Asp/Glu	X	X	Trp
GDIH/ADH Consensus	Ala	X	Asp/Asn	X	X	-		Thr	Gly	Lys	X	X	Trp

FIGURE 17. The consensus sequences in the tryptophan docking motif (Ghosh *et al.*, 1995; Xia *et al.*, 1996). This motif occurs at the C/D corners at the end of the C strands and the beginning of the D strands of each W motif; there are no loops between these strands. The C/D corners are best characterised as 4-residue (β) turns or 5-residue turns (comprising residues 3-6 or 3-7 respectively). Consensus sequences are also included for the quinoxaemoprotein alcohol dehydrogenase (ADH) and glucose dehydrogenase, which is a membrane quinoprotein (GDH).

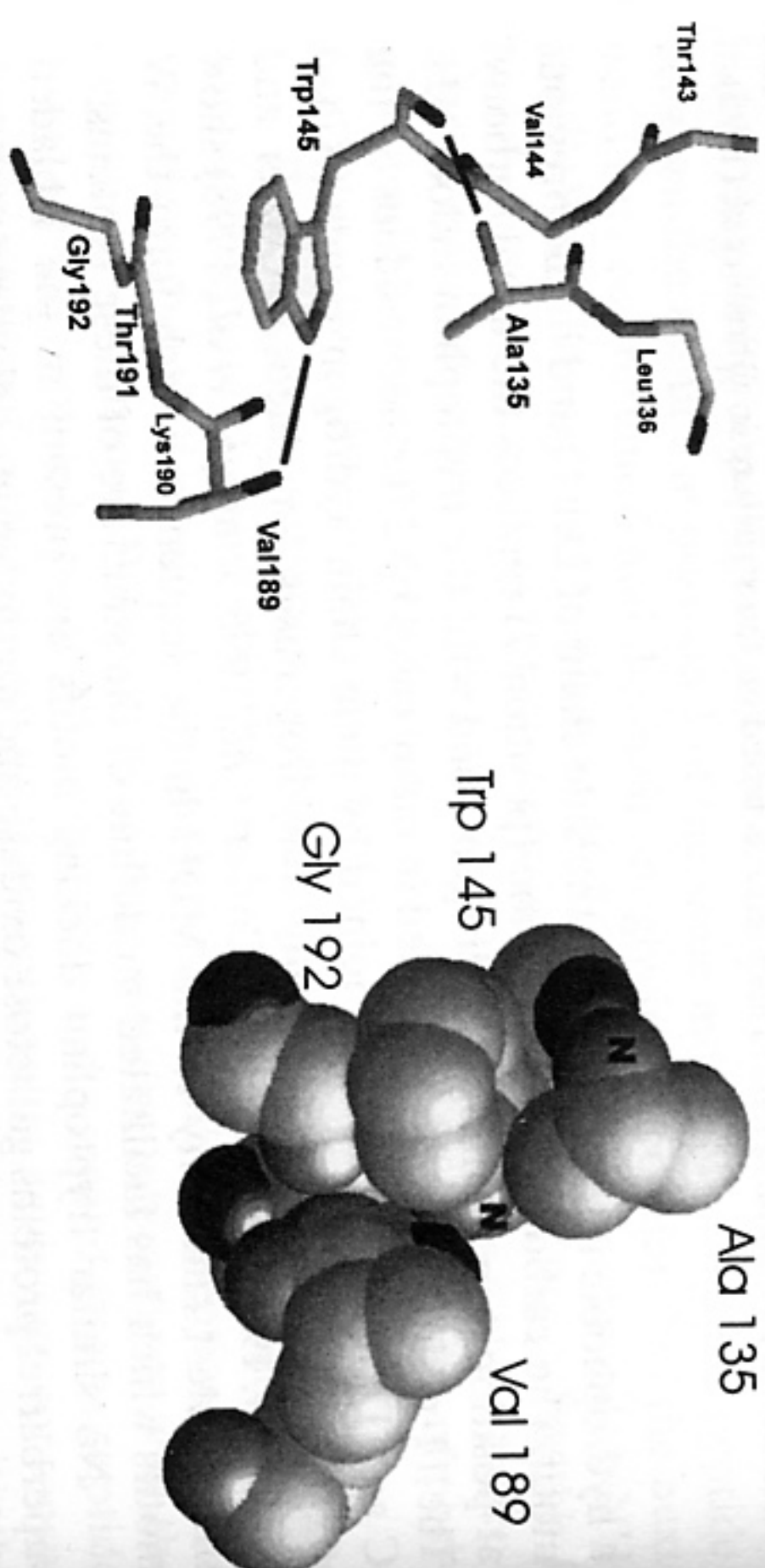


FIGURE 18. Part of a typical tryptophan-docking motif in stick format and spacefill format. This shows the interactions of Trp145 (in W2) with Ala135 (also in W2), and the interaction with the plane of the peptide bond between Thr191 and Gly192 (in W3). For clarity the remaining residues of the two motifs are omitted, and the side chains are omitted from all residues except for Trp145 and Ala135.

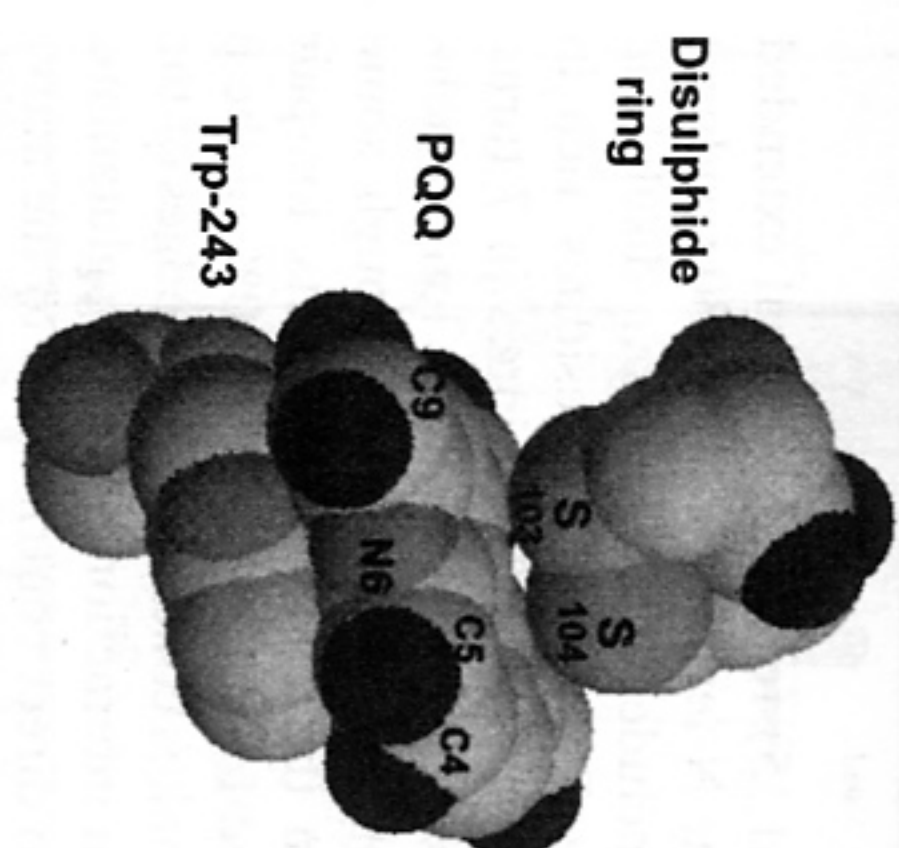


FIGURE 19. The novel disulphide ring in the active site of methanol dehydrogenase (Ghosh *et al.*, 1995; Xia *et al.*, 1996). The ring is formed by disulphide bond formation between adjacent cysteine residues. The PQQ is "sandwiched" between this ring and the tryptophan that forms the floor of the active site chamber. The calcium ion is coordinated between the C—9 carboxylate, the N—6 of the PQQ ring and the carbonyl oxygen at C—5. The oxygen of the C—4 carbonyl appears to be out of the plane of the ring.

structure should unexpectedly exist in a protein then a *cis* peptide bond would be present, forming part of a strained ring structure (Ramachandran and Sasisekharan, 1968; Chandrasekaran and Balasubramanian, 1969). In our 1.94 Å structure of the *M. extorquens* MDH it is clearly seen that the peptide bond is, as predicted, non-planar, but it is in the *trans* configuration (Ghosh *et al.*, 1995). The ω angle is 145° , giving a distortion from planarity of 35° . All of the other bond lengths and bond angles in the ring are standard values, including the distance between the sulphur atoms (2.06 Å). This structure is seen in all MDHs studied (Xia *et al.*, 1996).

The rarity of this type of ring structure would suggest some special biological function. Reduction of the disulphide bond leads to loss of activity but oxidation in air or carboxymethylation of the free thiols leads to return of activity. As the carboxymethylated thiols can no longer be involved in redox activity a possible role in electron transfer appears to be ruled out (see Section 6.3.3). The disulphide ring is not present in the quinoprotein glucose dehydrogenase in which electrons are transferred to membrane ubiquinone from the quinol PQQH₂, and in which the semiquinone free radical is unlikely to be involved as a stable intermediate. It is possible, therefore, that this novel structure might function in the stabilization of the free radical PQQ semiquinone or its protection from solvent at the entrance to the active site in MDH (Avezoux *et al.*, 1995). Free PQQ has been reported to catalyse disulphide bond formation both in model compounds and in proteins with adjacent thiols (Park and Churchich, 1992) and it is possible that, if the two cysteines are in the reduced form during processing of MDH, then PQQ might bind at the active site and catalyse disulphide bond formation, the disulphide ring then holding the PQQ in place (White

et al., 1993). The disulphide ring is not essential for incorporation of Ca^{2+} into the MDH purified from the processing mutants that require added Ca^{2+} for production of active enzyme (Avezoux *et al.*, 1995).

7.4.2. The Bonding of PQQ in the Active Site

The PQQ is sandwiched between the indole ring of Trp243 and the disulphide ring structure (Figure 19). The indole ring is within 15° of coplanarity with the PQQ ring and, on the opposite side, the two sulphur atoms of the disulphide bridge are within $3.75 \text{ \AA}$ of the plane of PQQ. In addition to these axial interactions, many amino acid residues are involved in equatorial interactions with the substituent groups of the PQQ ring system. These are exclusively hydrogen-bond and ion-pair interactions involving residues mostly on the A strands of the "W" motifs (Figure 20). Although the number of polar groups involved might indicate at first sight that the environment of the PQQ is polar, this is not the case. An oxygen of the 9-carboxyl forms a salt bridge with Arg109 and both groups are shielded from bulk solvent by the disulphide. The carboxyl group of Glu155 and a 2-carboxyl oxygen of PQQ are also shielded from solvent and it is probable

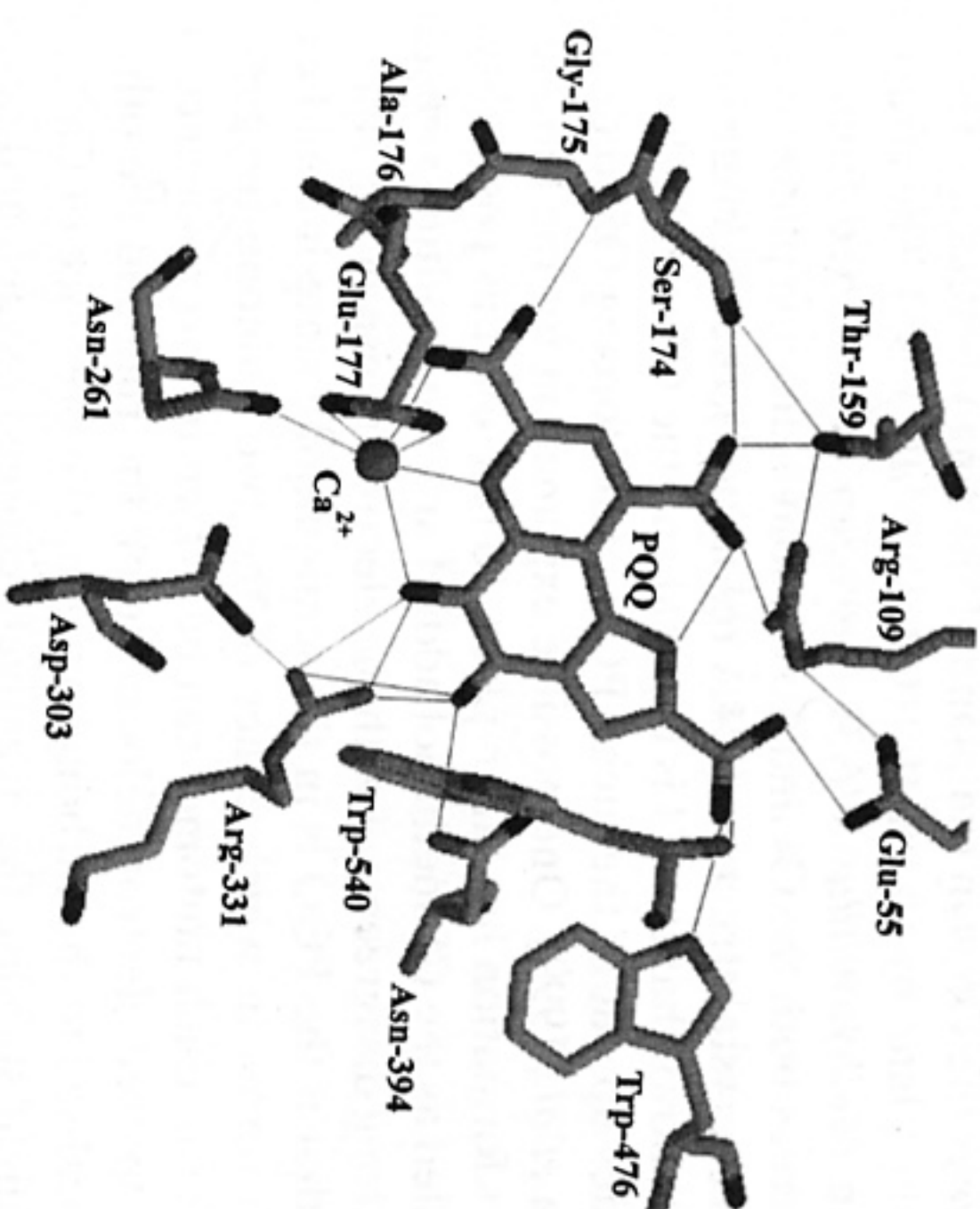


FIGURE 20. The equatorial interactions of PQQ and the coordination of Ca^{2+} in the active site of methanol dehydrogenase (Ghosh *et al.*, 1995). This figure also shows Asp303, which is likely to act as a base, and Arg331 which may also be involved in the mechanism.

that at least one is protonated, their interaction thus being stabilised through hydrogen bond formation.

The C4 and C5 oxygens are hydrogen bonded by the NH₁ and NH₂ atoms respectively of Arg331, and in addition the C4 oxygen makes a longer hydrogen bond interaction with the amide NH₂ of Asn394 whose amide CO is hydrogen bonded to its own main chain NH group. It is not known if the bonding of the C4 and C5 oxygen atoms is maintained in the fully oxidised quinone and fully reduced quinol forms of the prosthetic group, in which the C4 and C5 oxygen atoms are likely to be in the plane of the ring. As well as its hydrogen bonding to the PQQ, Arg331 also makes hydrogen bonds between its NH₂ and the carboxylate and main-chain carbonyl of Asp303. The two side chains lie side by side, permitting free access to the carboxyl group of Asp303, which is the most likely candidate for the base required by the catalytic mechanisms previously proposed for MDH (Section 6.1).

In the fully oxidised or reduced forms of PQQ the C4 and C5 carbonyl oxygen atoms would be expected to be in the plane of the ring (Itoh *et al.*, 1993, 1998; Zheng and Bruice, 1997). In the MDH as it is usually isolated the PQQ is in the semiquinone form and it is not so obvious what structure might be expected, especially in the environment of an enzyme active site, although Zheng and Bruice (1997) calculated that the semiquinone free radical coordinated to Ca²⁺ would be essentially planar. In the MDH from *M. extorquens* the O5 is in the plane of the ring whereas the O4 appears to be below the plane by about 40° (Ghosh *et al.*, 1995). The structure of the MDH from *Methylophilus* W3A1 has been determined from two crystal forms. Whereas both the C4 and C5 carbonyls are in a planar configuration in the form A crystal structure (2.4 Å resolution), the structure in the B crystals (1.9 Å) shows that the C4 is trigonal and the C5 tetrahedral. As a result C5 protrudes up out of the mean PQQ plane forcing O5 to deviate downwards (Xia *et al.*, 1999). One possible explanation for the tetrahedral C5 is that adduct formation has taken place. To rule out this possibility the PQQ was modelled as the C5-methanol adduct and the resulting structure shown to give a strong disagreement with the electron density map. Xia *et al.* (1999) point out that if the PQQ is in the semiquinone state it could exist in two tautomeric forms in which either of the two quinone oxygens is in the hydroxyl form. Each tautomer can be drawn in two resonance forms, one with the unpaired electron delocalised on the ring and the other with the electron localised to the carbonyl oxygen. The presence of Ca²⁺, 2.6 Å away from O5 might stabilise the negatively charged oxygen and permit distortion of C5 to a tetrahedral geometry.

The presence of the Ca²⁺ ion coordinated to the PQQ in the active site is consistent with predictions that it is likely to be intimately related to the

prosthetic group (Richardson and Anthony, 1992). Its co-ordination sphere contains PQQ and protein atoms (Figure 20), including both oxygens of the carboxylate of Glu177 and the amide oxygen of Asn261. The PQQ atoms include the C5 quinone oxygen, one oxygen of the C7 carboxylate and, surprisingly, the N6 ring atom which is only 2.45 Å from the metal ion. The five oxygen ligands have distances from the Ca²⁺ of 2.4–2.8 Å.

7.4.3. The Location of Substrate in the Active Site

In the first descriptions of the active site, the position of the substrate in the active site could not be unequivocally determined; the one or two water molecules which might occupy the same space as the substrate alcohol group are in slightly different locations in the two available structures (Ghosh *et al.*, 1995; Xia *et al.*, 1996, 1999). In the structure of A-form crystals of MDH from *Methylophilus* W3A1 a water molecule makes a hydrogen bond to both O5 of PQQ and to the side chain of Asp303. In the B-form crystal structure, the elongated electron density is consistent with the binding of methanol at this site instead of water. The substrate does not react with the MDH in this form because the PQQ is in the semiquinone (not oxidised) state. The hydroxyl of the methanol appears to be hydrogen bonded to the side chain of Asp303 (at 3.1 Å), but not to the O5 of PQQ (at 3.4 Å) (Figure 21). The methyl group is in a hydrophobic cavity bounded by side chains of Trp265, Trp540 and Leu556 as well as the disulphide ring. This raises the problem that this enzyme has a broad substrate specificity, and can oxidise primary alcohols including relatively large substrates such as pentanol and cinnamyl alcohol; it is not immediately obvious how these substrates could readily gain access to the active site and

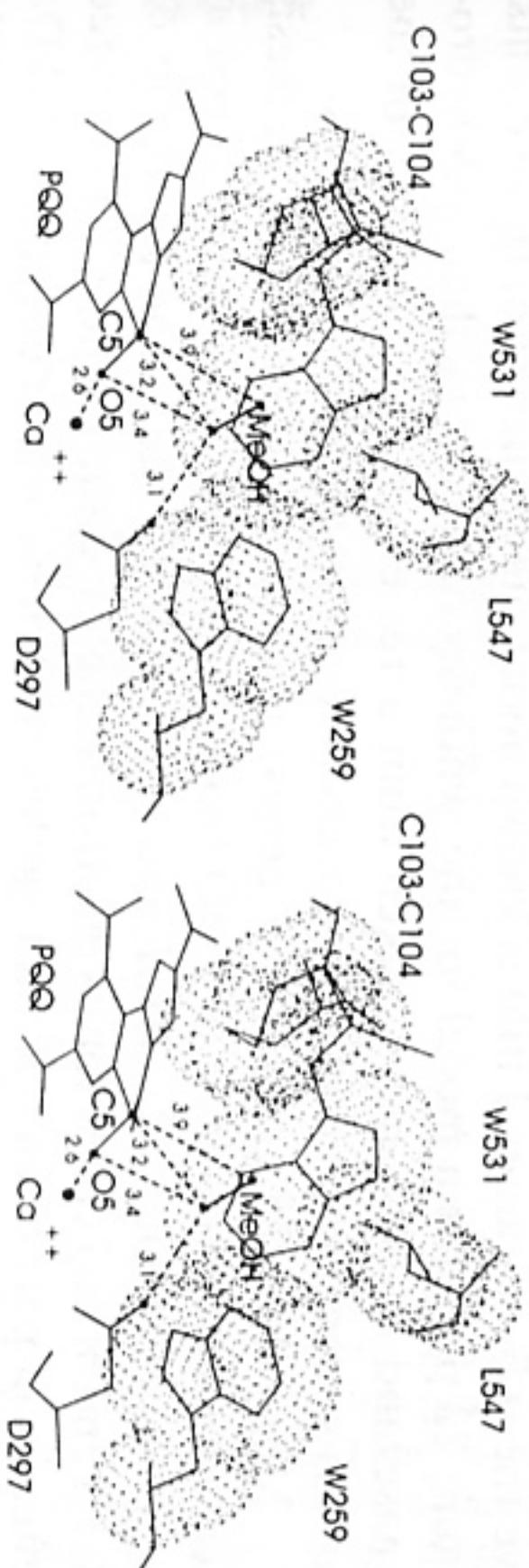


FIGURE 21. Stereo representation of the protein environment of the substrate methanol in the form B crystals of MDH from *Methylophilus* W3A1. The methyl group of methanol is above the hydroxyl group which is hydrogen bonded to Asp292 (Asp 303 in MDH from *M. extorquens*). The coordination bond between O5 and Ca²⁺ is also shown. Reprinted with permission from Xia *et al.* (1999), (*Biochemistry*, 38, 1214–1220). © American Chemical Society.

understanding of this awaits solution of a structure containing one of these larger substrates.

In the active site, Asp303 is well situated to serve as the catalytic base in the first step of abstracting a proton from the hydroxyl group of methanol, since one of its carboxyl oxygen atoms is 3.1 Å from the hydroxyl oxygen. This orientation of methanol in the active site lends further support to the hemiketal mechanism. The hydroxyl oxygen is close to C5 (3.2 Å) while the methyl group is considerably further away (3.9 Å). This would favour covalent bond formation between C5 and the nearby hydroxyl instead of hydride ion transfer from the more distant methyl group to the C5 atom of PQQ (see Section 6.1).

The possibility of an interaction between the methanol hydroxyl group and the Ca^{2+} ion has been discussed in an extensive study of the structure of PQQ and its possible mechanism by Zheng and Bruice (1997) using *ab initio* and molecular orbital calculations. However, the hydroxyl group of methanol appears to be too distant from the Ca^{2+} for coordination of the Ca^{2+} ion to substrate to have any importance in the mechanism.

8. PROCESSING AND ASSEMBLY OF METHANOL DEHYDROGENASE

Production of active periplasmic methanol dehydrogenase involves the following: synthesis and transport of PQQ; synthesis, transport and processing of pre-peptides for the α and β subunits; folding of these proteins in the periplasm; isomerisation of prolines; insertion of disulphide bridges (including the special one at the active site); insertion of calcium and PQQ; wrapping β chains around the α subunits; and association of the $\alpha\beta$ units to give the $\alpha_2\beta_2$ tetramer. Little is known about the sequence of these events. Figure 22 presents a model for the synthesis of active methanol dehydrogenase and cytochrome c_L taken from a review of the functions of the various genes involved in this process (Goodwin and Anthony, 1998).

More than 25 genes (*mox* genes) are required for MDH synthesis including seven involved in PQQ biosynthesis, and these are arranged in five clusters (Figure 22). *MxaF* and *mxaI* code for the α and β subunits of MDH and *mxaG* codes for its electron acceptor, cytochrome c_L . These three genes form part of an operon together with *mxaJ* which encodes a 30 kDa periplasmic protein which may play a role in stabilising or processing MDH (Amaratunga *et al.*, 1997ab). The sequence of genes from *mxaF* to *mxaD* (Amaratunga *et al.*, 1997ab). The sequence of genes from *mxaF* to *mxaD* form two well-defined groups. The intergenic regions in the first half of the cluster (*mxaF*—*mxaR*) are relatively large (105–261 bp), whereas those in the downstream half (between the putative *mxaS* and *mxaD*) are very small (0–8 bp). This draws attention to the possibility that this whole cluster might

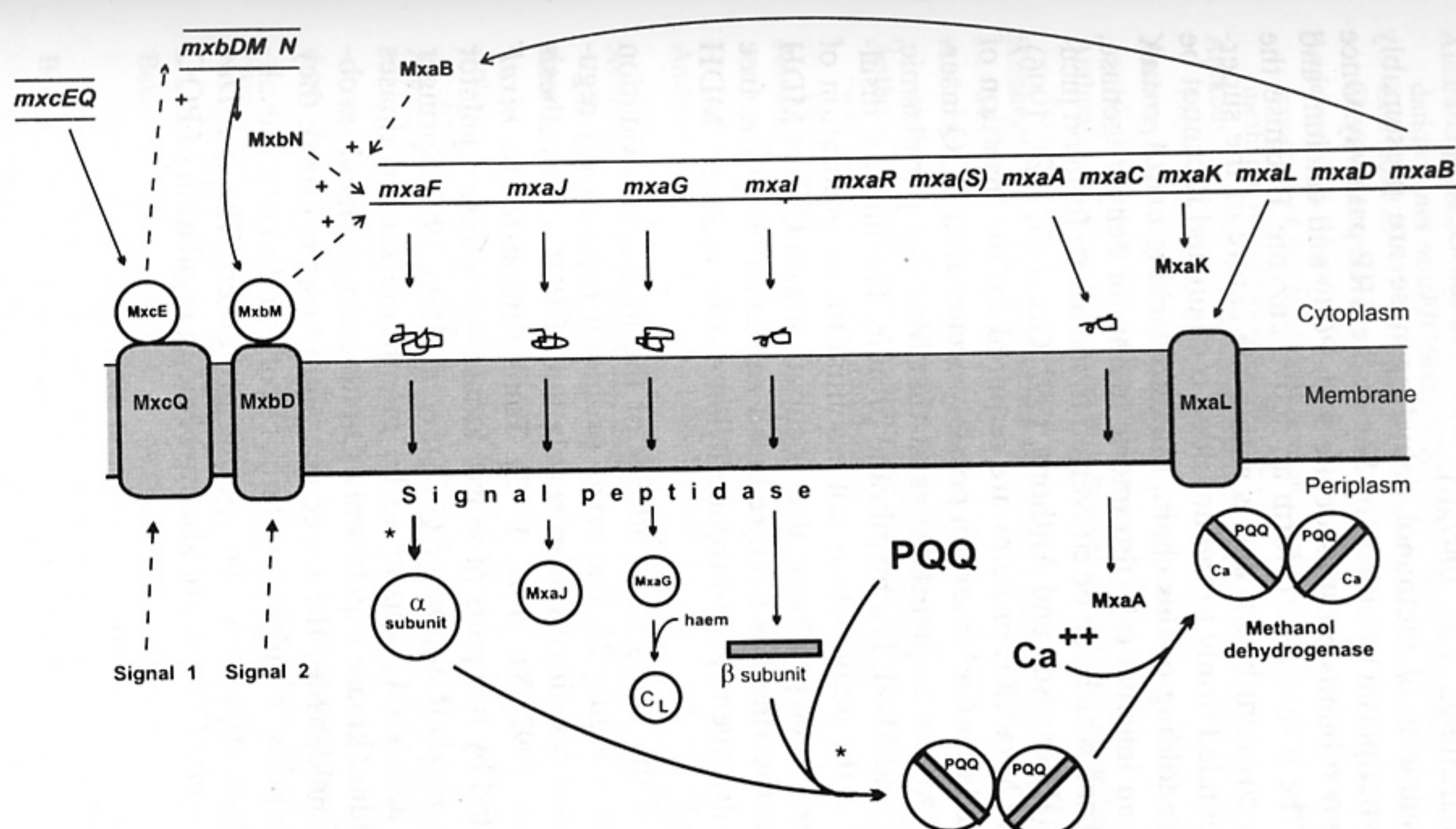


FIGURE 22. A model for the expression of the *mxaFJGIR(S)BACKLD* genes in *Methylobacterium extorquens*; it is essentially similar in *Paracoccus denitrificans* and related bacteria. A signal is transmitted by way of MxaX and MxaY, leading to activation of the *mxaF* promoter; 6 other genes are also involved in activating this promoter. One of the signals leading to increased expression of the *mox* genes is likely to be formaldehyde. The resulting preproteins, PQQ and haem are transported into the periplasm where they are assembled into the proteins that are specifically involved in methanol oxidation—methanol dehydrogenase and cytochrome c_L . The * indicates two possible steps at which PQQ may be inserted. At least three proteins (MxaA, MxaK and MxaL) are involved in incorporation of Ca^{2+} into the protein and there is some evidence that MxaC and MxaD are also involved in this. Reprinted from Goodwin and Anthony (1998), (*Advances in Microbial Physiology*, 40, 1–40) with permission from Academic Press.

be organised into two operons. However there is no obvious promoter between *mxaR* and *mxaA* and further work is required to ascertain whether or not the *mxaFGIR(S)ACKLD* cluster constitutes more than one transcriptional unit (Amarutunga *et al.*, 1997ab).

The α and β subunits of methanol dehydrogenase are presumably transported into the periplasm by either the Sec or the SRP pathway. Once in the periplasm the α subunits must associate with PQO and calcium and then assemble with the β subunits to form an active enzyme. Because the PQO is held by non-covalent bonds and is within the centre of the super-barrel it cannot be bonded firmly to the unfolded α chain and it cannot be added after complete folding of this chain. Mutants lacking *mxaA*, *mxaK* or *mxaL* synthesise an inactive $\alpha_2\beta_2$ tetrameric methanol dehydrogenase, which contains PQO and which can be converted to the active form on incubation with calcium (Richardson and Anthony, 1992; Goodwin *et al.*, 1996). This suggests that the MxaAKL proteins are required for the insertion of calcium into MDH and that Ca^{2+} insertion possibly occurs after PQO insertion; their predicted protein sequences suggest that MxaA is periplasmic, MxaK is cytoplasmic and MxaL is a membrane protein. The unusual disulphide ring structure in the active site is not essential for incorporation of Ca^{2+} , as active enzyme can be produced after incubation with Ca^{2+} of MDH from MxaA in which the ring has been reduced and even when the free thiols have been subsequently carboxymethylated the reduced MDH (Goodwin *et al.*, 1996).

The regulation of the genes for synthesis of the methanol oxidation system is summarised in Figure 22. The *mxaF* promoter is positively regulated by MxaB which is also involved in regulation of PQO biosynthesis (Morris and Lidstrom, 1992; Xu *et al.*, 1993). Transcription of the *mxaF* operon is also regulated by two pairs of sensor kinase—response regulator proteins, encoded by *mxdM* and *mxeQ* (Xu *et al.*, 1993, 1995; Springer *et al.*, 1997). MxdB and MxeQ appear to be membrane sensor kinases belonging to the histidine kinase superfamily. On detecting a signal (probably methanol or formaldehyde) these become autophosphorylated; they then react with the response regulator proteins (MxaE and MxaM) resulting in activation of transcription at the specific promoters. The *mxdM* genes are regulated by *mxeQ* and are also involved in regulation of PQO synthesis.

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