REVIEW ARTICLE

The structure and function of methanol dehydrogenase and related quinoproteins containing pyrrolo-quinoline quinone

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INTRODUCTION

By analogy with flavins and flavoproteins, the term quinoprotein was first coined about 1980 by Duine, Frank and co-workers [1,2] to include proteins containing pyrrolo-quinoline quinone (PQQ) as their prosthetic group (Figure 1). These enzymes are NAD(P)-independent and, for convenience, are usually assayed with the dye phenazine ethosulphate. The first quinoproteins to be described were glucose dehydrogenase (GDH) [3], and methanol dehydrogenase (MDH) [4], the prosthetic group of which was first isolated and characterized in 1967 [5]. We then concluded that it was either a previously undescribed type of pteridine or a completely novel type of prosthetic group. It was more than ten years later that this latter prediction was confirmed when it was demonstrated by X-ray crystallography [6] and chemical methods [2,7] to be the novel prosthetic group PQQ. Around this time (about 1980), a number of other bacterial dehydrogenases were also shown to contain PQQ by the groups of Duine and Frank in Delft, and Ameyama and Adachi in Yamaguchi (see [8–12] for extensive reviews).

During the next 10 years, premature unconfirmed 'identification' of the prosthetic groups of many other enzymes as PQQ led to considerable confusion [12]. Much of this has now been eradicated by full characterization of three novel prosthetic groups (Figure 1). Two of these are related to PQQ in having a quinone moiety as their essential feature, and so the enzymes containing them happily retain their designation as quinoproteins. So far, the only enzymes containing PQQ are bacterial enzymes in which the PQQ is tightly, but not covalently, bound (for reviews see [8,10–15]).

The other types of quinoprotein all have amino-acid-derived prosthetic groups which retain their covalent linkage to the enzyme. The first of this type has only been reported in bacterial dehydrogenases for methylamine [16,17] and for aromatic amines [18] which catalyse the oxygen-independent oxidation of primary amines to aldehydes plus ammonia. The prosthetic group, derived from two tryptophan residues, is tryptophan tryptophylquinone (TTQ) (Figure 1) [19–21]. The second type of quinoprotein whose prosthetic group is derived from amino acids is the copper-containing amine oxidase found in bacteria, yeasts, plants

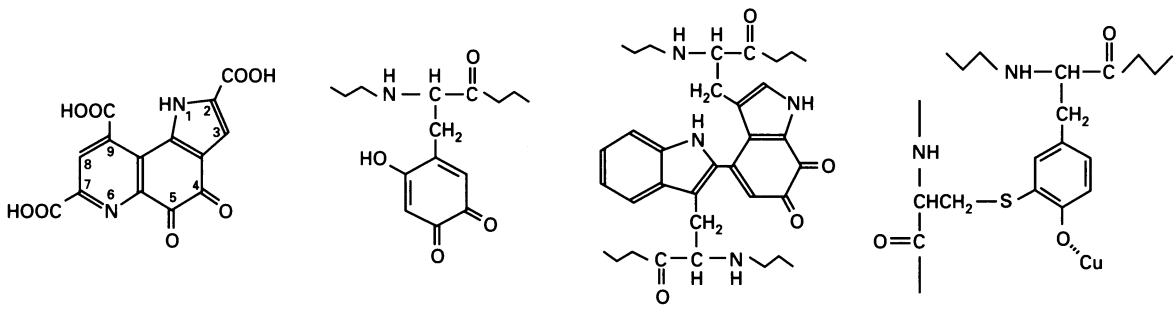

Figure 1 The prosthetic groups of quinoproteins

PQQ is pyrrolo-quinoline quinone or, more accurately, 2,7,9-tricarboxypyrrolo-quinoline quinone [2,5–7]. It is the prosthetic group of dehydrogenases for methanol, higher alcohols, aldose sugars, quinate, lupanine, aldehydes and polyvinyl alcohol (see [8–11] for extensive reviews). Some quinoproteins also have bound haem and so are designated quinohaemoproteins; these include some alcohol dehydrogenases [10,81] and lupanine hydroxylase [82]. TTQ is tryptophan tryptophylquinone and so far has only been described in bacterial dehydrogenases for methylamine [19–21] and aromatic amines [18]. TPQ is 6-hydroxydopa quinone; it may exist as the *para* or *ortho* form (as shown here). It is the prosthetic group of the universally distributed copper-containing amine oxidases [22–24]. Cys-Tyr-Cu is the prosthetic group of galactose oxidase [25,26]; this is included for reference although the enzyme is not a quinoprotein. (NB: We have used the expression prosthetic group throughout this review rather than cofactor or co-enzyme. The convention is that the various forms of prosthetic groups recycle while tightly bound to the same enzyme. Co-enzymes must leave the enzyme for part of their cycle. Cofactors are traditionally factors that need to be added to an enzyme; in quinoproteins it is very rare that the enzyme can be made active by addition of PQQ, TPQ or TTQ.)

Abbreviations used: ADH, alcohol dehydrogenase; GDH, glucose dehydrogenase; MDH, methanol dehydrogenase; PQQ, pyrrolo-quinoline quinone; TTQ, tryptophan tryptophylquinone.

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and animals. It catalyses the oxygen-dependent oxidation of amines to aldehydes plus hydrogen peroxide. Its prosthetic group, identified by Klinman and her colleagues [22–24], is derived from tyrosine; it is 6-hydroxydopaquinone, also known as TOPA quinone or TPQ (Figure 1).

Galactose oxidase, which catalyses the oxidation of a range of primary alcohols including the C-6 position of D-galactose, was previously thought to be a quinoprotein but has now lost this honourable status with the identification by Itoh, Knowles and their colleagues of its remarkable prosthetic group. This consists of a tyrosine, covalently linked to a cysteine, the linkage stabilizing a free radical on the tyrosine which is co-ordinated to the essential copper atom (Figure 1) [25,26].

THE QUINOPROTEINS TO BE CONSIDERED IN DETAIL IN THIS REVIEW

The main subjects of this review are the PQQ-containing quinoproteins of which MDH is the best-characterized example [8,11]. Its PQQ is non-covalently bound within the α-subunit and its electron acceptor is cytochrome c_L [27]. The first quinoprotein whose X-ray structure was determined, however, was methylamine dehydrogenase. Remarkably, this was first described by Eady and Large [16,17] in the same methylotrophic bacterium in which MDH was described (*Methylobacterium extorquens*). By contrast with MDH, the prosthetic group of methylamine dehydrogenase is TTQ (Figure 1), derived from two tryptophan residues in the light subunit; its electron acceptor is the blue copper protein amicyanin [28].

MDH [29–32] and methylamine dehydrogenase [33,34] are both $\alpha_2\beta_2$ tetramers in which the heavy α-subunit is a superbarrel made up of topologically identical four-stranded antiparallel β-sheets (W-shaped) arranged with radial symmetry like the petals of a flower or the blades of a propeller (Figure 2). However, this is the only structural similarity between these quinoproteins. This rare feature was first described in influenza virus neuraminidase [35] and subsequently in a related bacterial sialidase [36]; and, remarkably, it is also seen in galactose oxidase [25,26]. The number of propeller blades varies; there are six in neuraminidase, seven in galactose oxidase [25,26] and methylamine dehydrogenase [33,34], and eight in MDH [29,31,32] (Figure 2). Although this superbarrel or propeller structure is not directly related to the function of the proteins, it has been pointed out recently that "given its complex arrangement the possibility that all propeller folds share a remote ancestor cannot be excluded, even though their amino acid sequences have diverged to the point where their heritage is not readily apparent" [37]. In particular, the superbarrel or propeller structure is not obviously relevant to the fact that the three oxidizing enzymes have unusual prosthetic groups. There is no similarity in their amino acid sequences, and their structures differ in further important respects. The centre of the superbarrel contains the PQQ in MDH, whereas in galactose oxidase the centre is pierced by two β-strands of a separate domain to provide a ligand to the copper ion [25,26]. In methylamine dehydrogenase the centre is filled with side-chains, the prosthetic group (TTQ) being derived from the small β-subunit.

Although not discussed further in this review, the methylamine dehydrogenase remains relevant to the discussion of MDH because of the questions about inter-protein electron transfer that can be addressed in relation to the X-ray structure of the complex between the dehydrogenase and its electron acceptor, amicyanin [28,38].

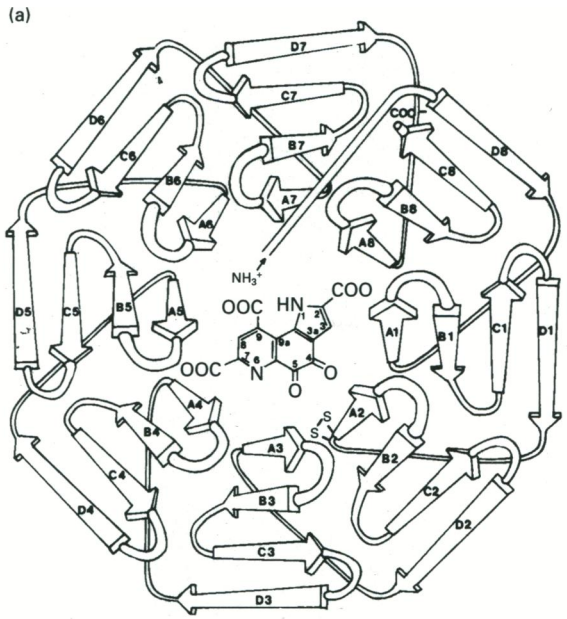
The remainder of this review will consider the structure and function of MDH, and then discuss this in relation to two other PQQ-containing quinoproteins, i.e. alcohol dehydrogenase and glucose dehydrogenase.

THE STRUCTURE OF MDH

Our description [31,32] of the structure of MDH from *Methylobacterium extorquens* which forms the basis of this review is very similar to that published for MDH from *Methylophilus methylotrophus* and *Methylophilus* W3A1 [29,30]. MDH has an $\alpha_2\beta_2$ tetrameric structure, each α-subunit (66 kDa) having a single molecule of PQQ and a Ca^{2+} ion (Figure 3). The genes for the subunits are together (but not adjacent) on a single operon, together with the gene for the specific electron acceptor, cytochrome c_L [39–41]. This operon has a fourth gene (*moxJ*) coding for a periplasmic 32 kDa protein [41]. This protein has been identified as a protein binding to the usual $\alpha_2\beta_2$ tetramer in the MDH from *Acetobacter methanolicus*; it does not, however, markedly alter its activity and it may function in the periplasmic assembly of MDH [42,43].

As seen in Figure 3, there is no interaction between the β-subunits (8.5 kDa) which are, as predicted from their primary structures [40], folded around the surface of the α-subunits. They are bonded to them by hydrogen bonds and by hydrophobic and ionic interactions, which also bind the $\alpha\beta$ subunits together. This is as expected from the observation that it is not possible to reversibly dissociate the α- and β-subunits, or the $\alpha\beta$-subunits from each other. The N-terminal half of the β-chain is irregular and includes one intrachain disulphide bridge, but the C-terminal half is largely in the form of a single long α-helix which lies on the surface of the α-chain. Because the β-subunit does not form a well-defined domain on MDH it might be assumed that the α- and β-subunits arise from a single polypeptide chain. This, however, is not the case; the genes for the two subunits are not adjacent on the operon and each codes for a separate signal peptide leading to separate transport into the periplasm prior to assembly of the whole protein. Although initially proposed to be involved in binding of cytochrome c_L (see below), there is some evidence against this and the function of the β-subunit remains obscure.

As mentioned above, the α-subunit is a superbarrel made up of eight topologically identical four-stranded antiparallel β-sheets (W-shaped), arranged with radial symmetry like the blades of a propeller. The structure is shown schematically in Figure 2, where it can be seen that the normal twist of the β-sheet enables space to be efficiently packed in the subunit. This architecture 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 which are, however, more hydrophilic than those on, particularly, the B- and C-strands of each of the β-sheet motifs. Prior to the description of the 8-fold propeller structure of MDH, Murzin proposed a model for multisheet packing in a β-propeller fold, stating that the 7-fold symmetry is preferable to a 6-fold or 8-fold symmetry; he concluded that "the β-propeller fold accommodates seven motifs and any additional β-structure would not be included in the propeller domain" [44]. The MDH structure has shown that this is not the case but the model does draw attention to the requirement for some special packing arrangement, the most obvious solution being some overlap between the outer (D) strands of adjacent β-sheet propeller blades. This is clearly seen in the MDH structure, in which glycine residues on each D-strand interact closely with tryptophan residues on the subsequent strand D (Figure 4). This was first reported by Scott Mathews at the 3rd Symposium on PQQ and Quinoproteins held in Capri in 1994.



(b)

Figure 2 The superbarrel or 'propeller' structure of the α-subunit of MDH

The view is of a single α-subunit showing that it is made up of topologically identical antiparallel β-sheets arranged with radial symmetry like the blades of a propeller. The PQQ in the diagrammatic representation is not to scale. The arrows represent β-strands, labelled ABCD, in each motif or propeller 'blade' which are numbered 1 to 8. Neither extensive loops between the β-sheets, nor the C-terminal or N-terminal sequences are indicated in the diagrammatic representation.

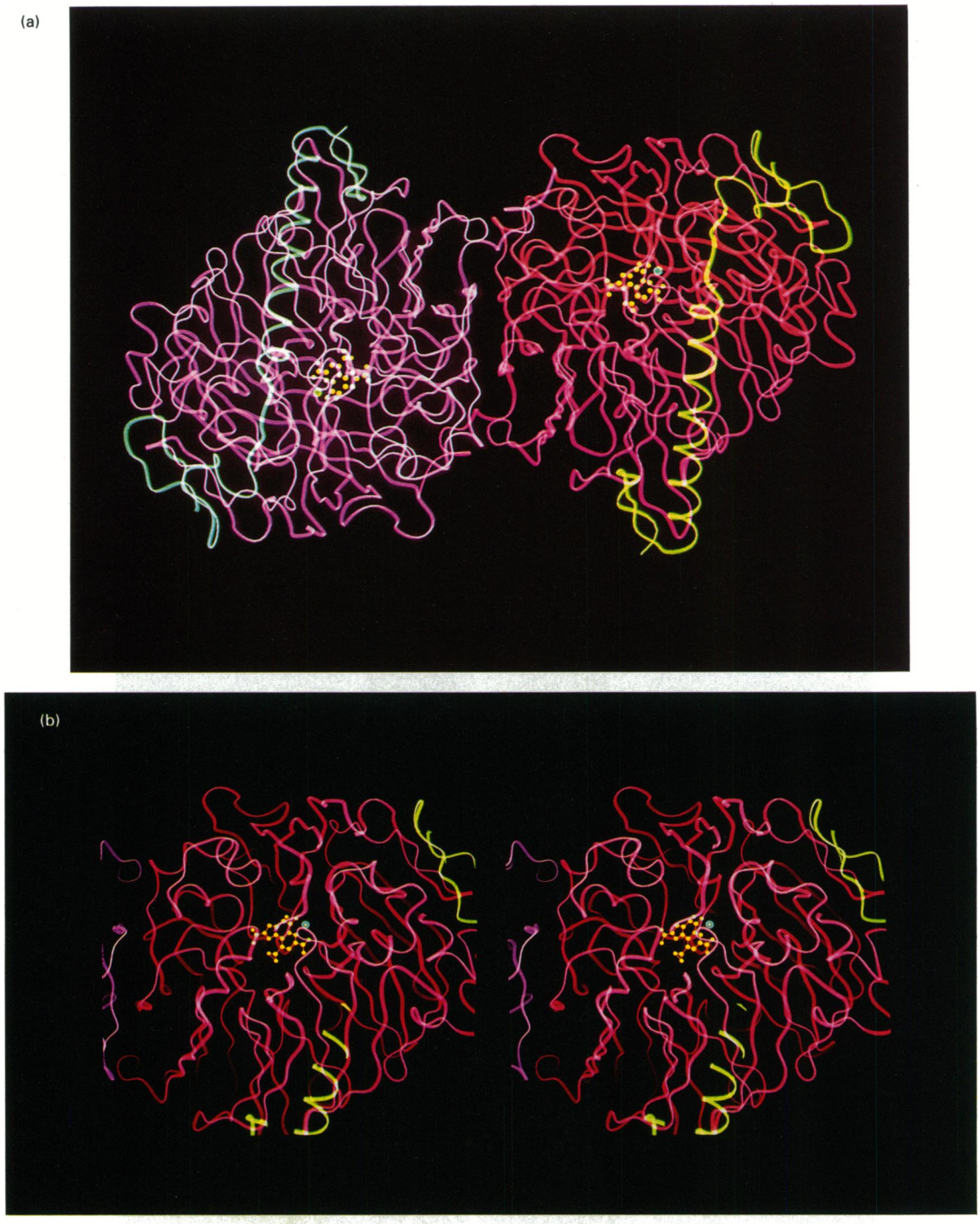


Figure 3 The three-dimensional structure of MDH

(**a**) The $\alpha_2\beta_2$ tetramer viewed down the 2-fold axis. The two α-chains are coloured pink and purple, while the two non-globular β-chains are both shown in green. PQQ is shown in orange, and the calcium ion as a blue sphere. (**b**) A close-up stereo view in the same orientation as (**a**).

THE STRUCTURE OF THE ACTIVE SITE OF MDH

The PQQ prosthetic group is buried quite deeply in the interior of the α-subunit and is located on the pseudo 8-fold molecular symmetry axis, in a non-covalent association with a number of side-chains mostly from the A-strands of the eight β-sheet motifs (Figures 3 and 5). In effect, the PQQ is buried within an internal chamber, communicating with the exterior through a funnel-shaped depression in the surface which is quite narrow where it meets the chamber. A remarkable feature of the 'funnel' (Figure 6) is that the surface-accessible residues are almost exclusively hydrophobic; the surface-accessible residues are indicated in **bold type** in the following sequences which are numbered for MDH from *M. extorquens*:

MDH: 100-**AVACCDL**; 420-**PFMLP**; 430-**FFV**; 540-**WPGVGLVFDLADPTAGL**

The PQQ lies within a hydrophobic chamber as previously predicted by Duine and colleagues [45]. The floor of this active-site chamber is formed by Trp-243, whose indole group is parallel to, and in contact with, the planar ring system of PQQ (Figure 5) [31,32]. The ceiling of the chamber is formed by a novel ring structure arising from a disulphide bridge between adjacent cysteine residues (Cys-103–Cys-104), both S atoms being within 4 Å of the plane of the PQQ (Figure 5). This is the first active enzyme shown to have such a ring structure. A disulphide bond does form between adjacent cysteine residues at the C-terminus of an inactive form of mercuric ion reductase but this is a preparation artefact and for activity a reduction step is first necessary to provide the two thiols which are essential for co-ordination of the mercuric ion [46]. A similar disulphide bridge has been proposed (but not seen in an X-ray structure) for the agonist-binding site of the acetylcholine receptor and, as in MDH, this disulphide bridge is very easily reduced [47]. Theoretical analysis, by Ramachandran and others [48,49], of the conformation of such a disulphide suggested that the adjacent cysteines would be joined by a non-planar *cis* peptide bond

	D8	D1		D2		D3		D4		D5		D6		D7		D8
	44	84	88	141	145	192	196	288	292	343	347	463	467	504	508	40
MDH:	W	G	W	G	W	G	W	G	F	G	S	G	W	G	W	L
ADH:	W	G	W	G	W	G	W	G	W	G	S	M	W	G	Y	L
GDH:	W	G	W	G	–	G	W	G	W	G	V	F	L	G	W	L

Figure 4 Bonding between tryptophans and glycines in consecutive D-strands of the α-chain of MDH and related quinoproteins

The layout of the strands is indicated in Figure 2. In some strands tryptophan is replaced with phenylalanine or tyrosine; in strand D8 leucine replaces glycine in all proteins. This sequence pattern is not seen in propeller structures with only six or seven blades where there is no need for overlap in the D-strands.

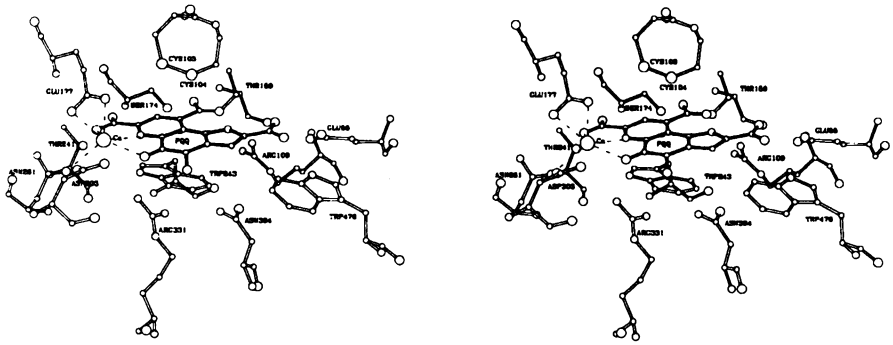


Figure 5 A stereo diagram of the active site of MDH of *Methylobacterium extorquens*

This shows the relative locations of the PQQ, the Cys-103–Cys-104 disulphide bridge and the calcium ion. Trp-243 can be seen forming the floor of the active-site chamber beneath the plane of the PQQ. The relative orientations of these key components are also seen in Figure 10, and the disulphide ring structure can be seen in surface view in Figure 6. The co-ordination to the Ca^{2+}, and interactions of the PQQ are similar to those seen in MDH from *Methylophilus methylotrophus* [30].

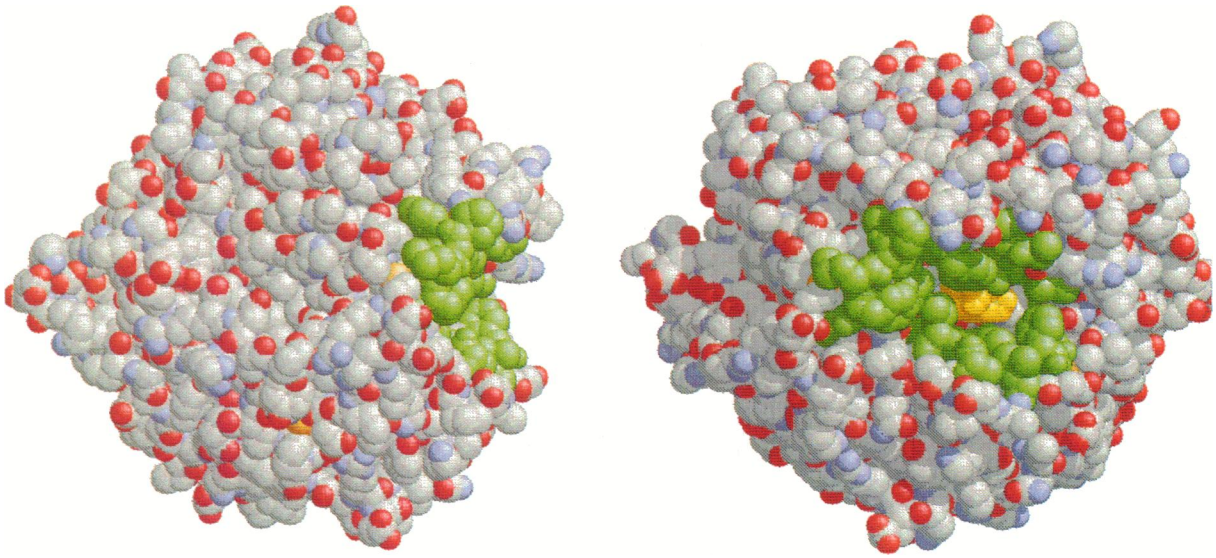


Figure 6 The hydrophobic funnel at the entrance to the active site in the α-subunit of MDH (side and front views)

The surface-accessible hydrophobic residues (listed in text) are coloured green and the disulphide bridge is yellow. For clarity a single α-subunit is shown; the active-site 'funnel' is neither near the β-subunit nor near the interface between the subunits.

within a strained eight-membered ring structure. This has since been demonstrated in a model L-Cys-L-Cys dipeptide [50], and modelling of its co-ordinates on to the electron density map of MDH initially suggested that a similar *cis* peptide bond occurs in this enzyme [31]. However, further refinement of our MDH structure has shown that the adjacent cysteines are bonded by an unusual non-planar *trans* peptide bond within a strained eight-membered ring in which the S–S distance is considerably less than in a typical disulphide bond.

After reduction of MDH with 5 mM dithiothreitol for 1 h all activity with cytochrome c_L is lost, whereas activity with phenazine ethosulphate is retained, indicating that this structure may have particular importance in electron transport from $PQQH_2$ to cytochrome c_L [31], although this may only be to maintain the PQQ in an appropriate configuration for electron transfer to occur. In addition to such an electron transport function the disulphide ring might also have a role in facilitating insertion of PQQ into the protein during assembly in the periplasm.

A second important feature seen in the active site is the Ca^{2+} ion, which clearly plays a role in maintaining PQQ in the correct configuration, as predicted from spectroscopic and reconstitution studies of a mutant form of MDH that lacks Ca^{2+} and is unable to oxidize substrates [51]. The Ca^{2+} ion is co-ordinated by Glu-177 (both carboxylate O atoms) and Asn-261, as well as the N-6, the C-5 quinone oxygen and the C-7 carboxyl group of PQQ which also interacts with Thr-241 (Figure 5). The other two carboxylates of PQQ are shielded from solvent and are bonded to Ser-174, Thr-159, Arg-109, Glu-55 and Trp-476. All these residues are conserved in the three MDH sequences so far published [14], and are also seen in the active site of the MDH of *Methylophilus methylotrophus* [30].

THE MECHANISM OF MDH

Mechanistic studies on MDH have been limited by the lack of intermediates in the reaction that can be readily detected spectroscopically (see [9,11] for reviews of the evidence relating to the mechanism of MDH). All the evidence is consistent with reduction by methanol of PQQ to the quinol ($PQQH_2$), followed by two sequential single-electron transfers to the cytochrome c_L; during this process the semiquinone is produced and it is this free radical form of the enzyme that is usually isolated [51–53].

Of particular relevance in proposing reaction mechanisms are the extensive studies with isolated PQQ, which have demonstrated that the C-5 carbonyl is very reactive towards nucleophilic reagents, adducts being formed with methanol, aldehydes, ketones, urea, cyanide, ammonia and amines [8,9,54,55]. It is this feature of the chemistry of PQQ that encourages the assumption that a covalent PQQ–substrate complex may be important in the reaction mechanism. The most important piece of evidence relating to this is the reaction of MDH with cyclopropanol to give a C-5 propanal adduct [56]. It has been suggested that the mechanism of reaction with cyclopropanol consists of proton abstraction by a base at the active site, followed by rearrangement of the cyclopropoxy anion to a ring-opened carbanion, and attack of this on the electrophilic C-5 of PQQ [56,57]. It was concluded that during oxidation of methanol a similar proton abstraction by a base must occur but that this is followed by formation of a carbon/oxygen bond to give a hemiketal intermediate. The direct evidence on this point is the slight change in spectrum of a possible intermediate (half-life 2 min) seen during reaction with deuterated methanol in the presence of excess dye electron acceptor [53]; and the large deuterium isotope effect during reduction of MDH by substrate is also consistent with a mechanism involving hemiketal formation.

Identification of the Asp-303 and Glu-177 in the active site [31] raises the possibility that one of these groups provides the catalytic base proposed above. Figures 7 and 8 illustrate two possible mechanisms, both involving Asp-303 acting as a base, initiating the reaction by abstraction of a proton from the alcohol substrate. In these mechanisms the Ca^{2+} is given a role in addition to its structural role in maintaining PQQ in an active configuration; it is proposed that the Ca^{2+} acts as a Lewis acid by way of its co-ordination to the C-5 carbonyl oxygen of PQQ, thus providing the electrophilic C-5 for attack by an oxyanion or hydride. It is also possible that the Ca^{2+} ion co-ordinates to the substrate oxygen atom. This dual structural and catalytic role is similar to that proposed for phospholipase A_2 [58], in which Ca^{2+} is essential, both for the binding of substrate and for catalysis; it co-ordinates to the carbonyl oxygen in an ester bond, facilitating nucleophilic attack by water.

In the first mechanism (Figure 7) the oxyanion produced by proton abstraction attacks the electrophilic C-5, leading to formation of the proposed hemiketal intermediate. The second mechanism (Figure 8), is a simple acid/base-catalysed hydride transfer in which Asp-303 again provides the base and Ca^{2+} acts again as a Lewis acid. Although the evidence on this matter is scarce, it slightly favours a mechanism involving a hemiketal intermediate but, as pointed out by Duine and his colleagues, the scarce evidence does not rule out more unusual mechanisms that

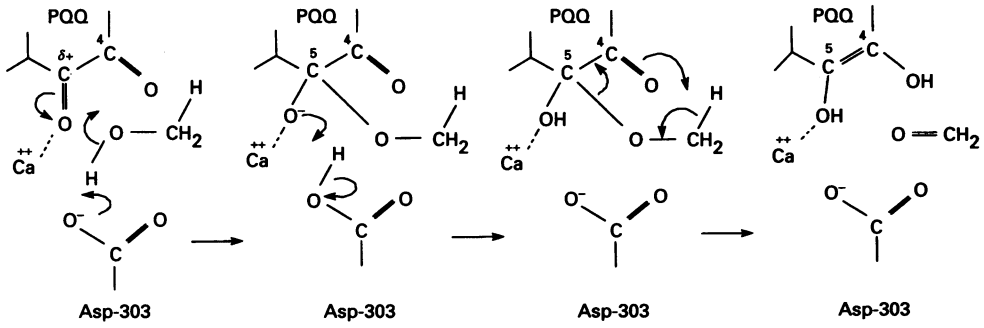


Figure 7 MDH mechanism involving a hemiketal intermediate

Proton abstraction by a base (possibly Asp-303) is followed by attack by the oxyanion on the electrophilic C-5, leading to the formation of a hemiketal intermediate. This mechanism was first proposed by Frank and Duine and their colleagues [56,57].

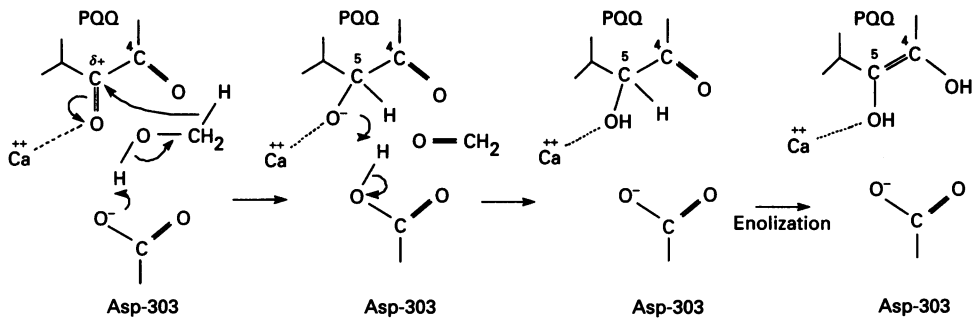


Figure 8 Acid/base-catalysed hydride transfer mechanism for MDH

As in Figure 7, Asp-303 provides the base for proton abstraction and Ca^{2+} acts as a Lewis acid, facilitating hydride transfer to the electrophilic C-5.

might involve, for example, the PQQ acting as a *p*-quinone [9]; this would be consistent with the demonstration of the importance of the pyrrole nitrogen for activity of reconstituted ethanol dehydrogenase [59]. Such a mechanism has been further elaborated as a result of an extensive model study of PQQ and its adducts by Itoh and his colleagues [55]. This mechanism involves formation of a C-4 adduct and a covalent ether linkage to serine or threonine; however, this model was partly based on the initial erroneous placing of the PQQ in the active site [29] and cannot be readily modified to conform with the correct active-site structure.

It should be noted that the proximity of Asp-303 and Arg-331 in the active site might diminish the possibility that Asp-303 acts as a catalytic base. However, there remains the possibility that in the Michaelis complex the arginine might be more separate, or involved in other interactions, thus permitting the pK of the aspartate to be sufficiently low for it to catalyse the initial proton abstraction.

INTERACTION OF MDH WITH CYTOCHROME c_L

Because 15 of the 74 amino acids of the β-subunit of MDH of *Methylobacterium extorquens* are lysines [40], it was suggested that this small subunit might play a role in interaction with the acidic electron acceptor, cytochrome c_L [11,60]; this now appears less likely because these lysines are not conserved in all MDHs. Furthermore, extensive chemical modification and cross-linking studies have suggested that interaction with cytochrome c_L is by way of lysyl residues on the α-subunit interacting with carboxylates on the cytochrome c_L [61,62]. That ionic interactions are important is confirmed by the sensitivity to ionic strength of the medium during electron transfer between MDH and cytochrome c_L; 10 mM salt is sufficient to cause 50% inhibition when measured with pure proteins, and 50 mM salt has a marked effect on respiration by whole bacteria. Measurements of electron transfer in low-salt concentrations have given K_m values of 1–7 μM cytochrome c_L [61,62].

This conclusion contrasts with that recently published by Davidson's group, who concluded that interaction is exclusively by way of hydrophobic residues [63]. Their conclusion was based on direct binding studies of the two proteins, a K_d value of about 375 μM being published. However, this sort of conclusion was inevitable because of the experimental design, all measurements being made in a medium of ionic strength 0.334 (equivalent to 334 mM NaCl). Although the two proteins will bind at this high ionic strength, our results indicate that the affinity will clearly be very much lower at high ionic strength [61,62]. For rapid electron transfer between $PQQH_2$ and the haem of cytochrome c_L, these prosthetic groups need to be as close as possible and the shortest path between the active site and the outside of the protein is probably by way of the hydrophobic funnel (Figure 6). There are no lysine residues in this immediate region and it is probable that alignment and initial 'docking' of the two proteins is by electrostatic interactions involving, as previously proposed, specific lysyl residues on MDH and carboxylates on cytochrome c_L; this initial alignment ('docking') is possibly then followed by close interaction by way of the hydrophobic residues in the active-site funnel.

COMPARISON OF THE STRUCTURE OF MDH WITH THAT OF THE QUINOHAEMOPROTEIN ALCOHOL DEHYDROGENASE (ADH) AND GLUCOSE DEHYDROGENASE (GDH)

Besides the MDHs [14,41,64,65], the PQQ-containing quinoproteins whose primary sequences are known, and which are discussed in detail here, are the quinohaemoprotein ADH isolated from membranes of acetic acid bacteria [14,66–68], and the membrane-bound GDH which occurs in many bacteria including *Acinetobacter calcoaceticus* [69], enteric bacteria [70], pseudomonads and acetic acid bacteria [71]. *A. calcoaceticus* also contains a second, soluble GDH which shows little sequence similarity to these other quinoproteins [72]. Although these dehydrogenases have PQQ as prosthetic group, they differ in their electron acceptors. MDH is a soluble enzyme and reacts with the specific periplasmic, cytochrome c_L [27]. ADH is a membrane complex, in which subunit I is similar to the α-subunit of MDH; electrons probably pass from $PQQH_2$ to ubiquinone by way of haem C on subunit I and a separate cytochrome *c* in the complex [10]. GDH is an integral membrane protein which passes electrons direct to ubiquinone [10,73].

There are sequences in subunit I of ADH (1–594) and in GDH (153–C-terminus) with sufficient similarity to the α-subunit of MDH (1–599) to suggest that they have essentially the same type of superbarrel or propeller structure (ADH being the more similar) [14] (Figure 9). This is supported by the observation that the pattern of Trp and Gly residues that are important in the interaction of the D-strands of adjacent propeller blades are highly conserved in all three proteins; the D-strands lacking the potential for these bands are in the regions with least overall sequence similarity (D5/D6 and D8 in ADH; D5–D7 in GDH).

In addition to the superbarrel region of about 600 amino acids, ADH has a C-terminal extension that includes the haem C site and which shows some similarity in sequence to cytochrome c_L [14,66,68]. The N-terminal sequence of GDH (1–152) provides

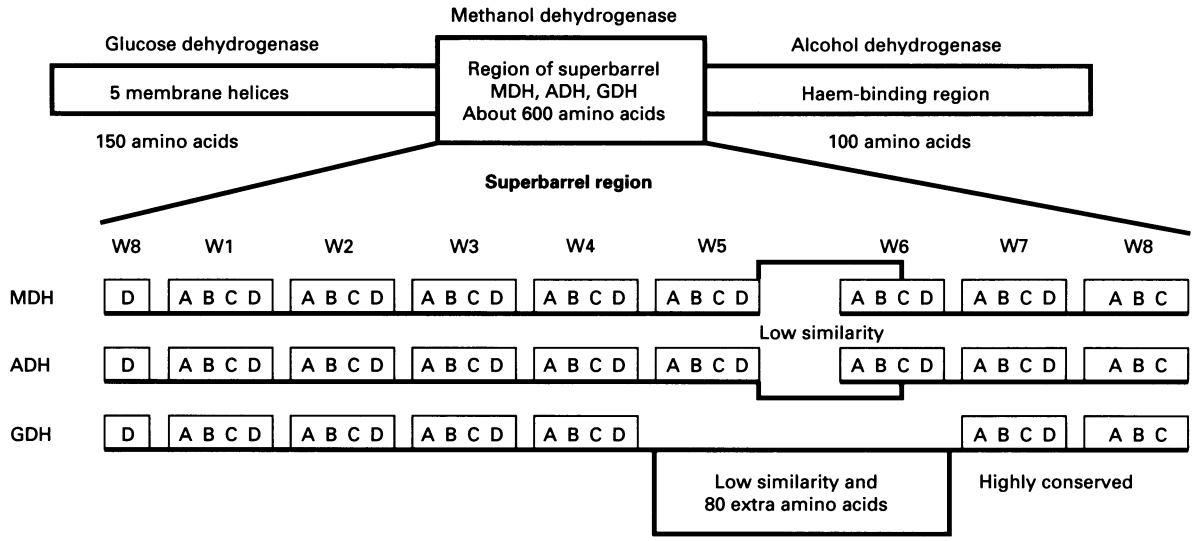


Figure 9 Sequence alignment of quinoprotein dehydrogenases

Each 'W' is a four-stranded β-sheet (or propeller blade). These are the regions showing greatest similarity of sequence between the quinoproteins. There are many loops between, and within, the β-sheets which show least similarity. For example, there is a long region with little conservation of sequence (including a large loop) between the end of the D-strand in W5 and the start of the C-strand of W6. The highly conserved region between strand A in W7 and the end of strand B in W8 was originally proposed to be a PQQ-binding domain; this is not the case, as can be seen in Figure 10.

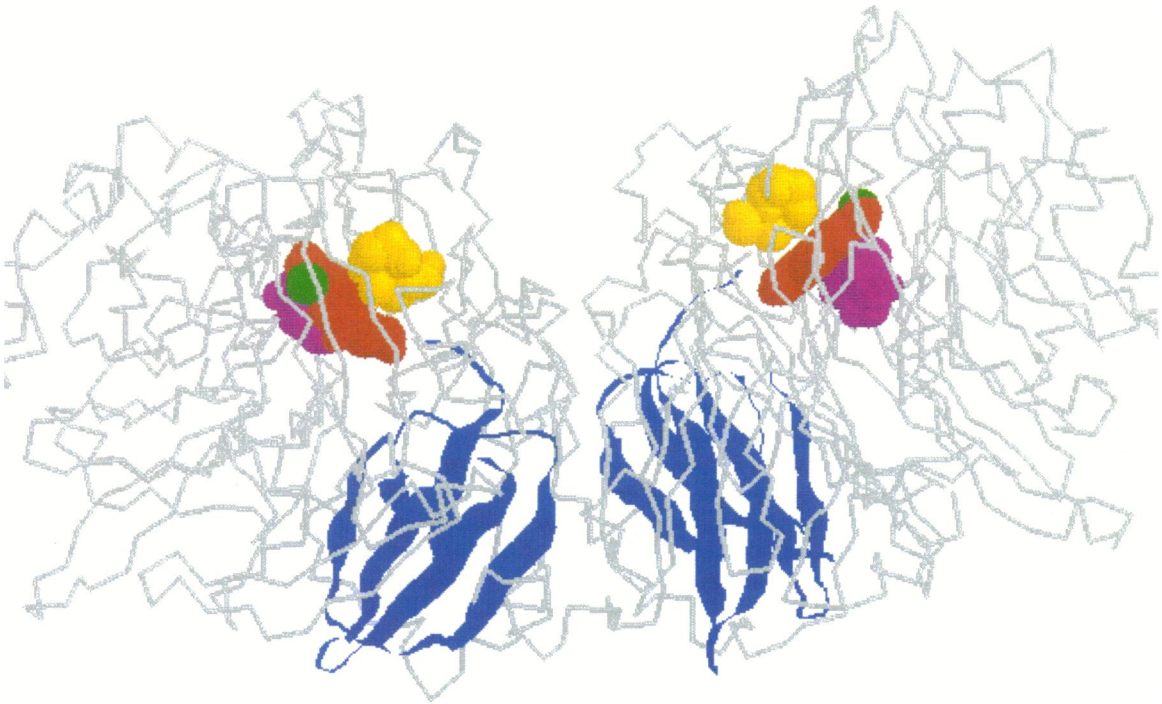


Figure 10 The α_2 dimer of MDH showing the highly conserved region

The β-subunits are omitted for clarity. The highly conserved region, between strand A in W7 and the end of strand B in W8, shown by the blue ribbon, was originally proposed to be a PQQ-binding domain; this is clearly not the case. The Ca^{2+} is shown as a green sphere. The PQQ prosthetic group (in orange) is shown in the active site between the hydrophobic Trp-243 (magenta) and the disulphide ring (in yellow).

four or five membrane-spanning helices, consistent with its membrane location [70], and this region has been discussed as a possible location of the ubiquinone-binding site in GDH [74–76].

Topological studies of the *Escherichia coli* enzyme have confirmed that the N-terminal region is on the cytoplasmic side of the bacterial membrane while the C-terminal superbarrel region

(containing the active site) is on the periplasmic side of the membrane [76]. This is consistent with the observation that all three quinoproteins discussed here catalyse their reactions in the periplasm [77].

Neither ADH nor GDH has any sequence corresponding to the small β-subunit of MDH, the function of which remains obscure. It cannot be prepared separately from the α-subunit of MDH and no mutants are at present available for this subunit. Because a key distinguishing feature of MDH compared with ADH and GDH is its reaction with cytochrome c_L there remains the possibility that it does play some role in stabilizing the complex between these proteins or, because of its very basic character, that it might bind the MDH loosely to the phospholipid membrane.

The suggestion that the novel disulphide bridge in MDH might be involved in electron transfer to cytochrome c_L is supported by the fact the adjacent cysteines are conserved in other MDHs and in ADHs, in which electron transfer is also probably to a haem C, whereas the disulphide bridge is not conserved in GDH, in which the immediate electron acceptor is ubiquinone, in the membrane (sequences from [14]): i.e.

MDH: AVA**CC**DLVNRGLAYWP

ADH: DKG**CC**DTVNRGAGYWN

GDH: HLT**C**-----RGVMYYD

A feature of all the PQQ-containing quinoproteins is that they have been shown to contain Ca^{2+}, or that they require a bivalent metal ion for reconstitution of apoenzyme with PQQ to form active holoenzyme (see [51] for complete references on the importance of Ca^{2+} in quinoproteins). This generalization includes the soluble ethanol dehydrogenase (sequence not available), which is very like MDH except for its substrate specificity, and the fact that an active enzyme can be produced by reconstitution with PQQ in the presence of Ca^{2+} after its dissociation by incubation in EDTA [78]; this dissociation is not possible with MDH [79]. That MDH, ADH and GDH have a similar structural requirement for Ca^{2+} at their active sites is implied by the observation that both of the residues involved in coordinating Ca^{2+} in MDH (Glu-177 and Asn-261) are conserved in ADH; and in GDH Glu-177 is replaced by aspartate (Asn-261 is absent). It is also possible that the mechanisms of these oxidation reactions are similar to that of MDH; this is especially so if a hydride transfer is involved because the reactions are all, in effect, alcohol oxidation reactions; the substrate for GDH is the C-1 anomeric hydroxyl group, giving the lactone and subsequently the gluconate end product.

In the absence of an X-ray structure the primary sequences of proteins provide material for speculation on their functional domains. In previous discussions of the sequences of these three proteins it has been suggested that the region with the highest degree of similarity (residues 477–539 in MDH) must constitute the PQQ domain [14,66,70]. This appears, however, not to be the case. This region, formed from the β-sheet motif W7 plus part of W8, has no direct relationship to binding PQQ or Ca^{2+}. One small part is important in the interface between the α-subunits of MDH (509–512) and this perhaps suggests that ADH and GDH might also have an α_2 structure. The last two amino acids in this long conserved sequence are the only ones that approach the PQQ in the active site (Figure 10). A single change of one of these leads to a change in substrate specificity in GDH; changing His-787 to Asn leads to GDH having the extra ability to oxidize xylose [71]. The 'identification' of the PQQ domain from sequence similarity studies has led to the conclusion that Lys-748 in GDH of *Acinetobacter calcoaceticus* might be responsible for the relatively tight binding of PQQ in this GDH; in *E. coli* the equivalent amino acid is Glu-742 and the PQQ is readily lost by treatment with EDTA. Site-directed mutagenesis of the *E. coli* enzyme appeared to confirm the proposal because the mutant enzyme E742K showed higher tolerance towards EDTA inactivation [80]. However, the reason for this change will need to be reconsidered because in this region of high similarity the equivalent of Glu-742 in GDH is Asp-505 in MDH and this residue is near the beginning of the D7 strand on the outer surface, far removed from the active site (Figure 10).

A remarkable feature of the outer face of MDH is the entrance to the funnel leading to the hydrophobic active-site 'chamber'; the funnel mouth is also almost exclusively derived from hydrophobic residues (Figure 6). The sequences comprising these are summarized here, with the **surface-accessible** residues of MDH in **bold type**; these are almost all hydrophobic residues and are identical in all MDHs:

```
MDH: 100-AVACCDL;  420-PFMLP;  430-FFV; 540-WPGVGLVFDLADPTAGL-
ADH:     DKGCCDT       NQVGG;       WNV;      I--missing--YPISM
GDH:     HLTC---;  no alignment                 no alignment
```

GDH is clearly completely different and, besides the adjacent cysteines, the only conserved region (i.e. similar 'visible' hydrophobic residues) in ADH occurs near Phe-430 (WNV in ADH). This lack of conservation between the three types of quinoprotein suggests that this region must have some special function in MDH. As it is sufficiently distant from the PQQ, it is unlikely to be especially important in determining substrate specificity and so the most likely interpretation of this rather obvious feature is that it is involved in the one 'special' feature of MDH, that is its reaction with cytochrome c_L. It is an obvious location for hydrophobic bonding of the cytochrome to the MDH after initial 'docking' by way of the electrostatic interactions previously shown to be essential (see above).

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