

Minireview

The quinoprotein dehydrogenases for methanol and glucose

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Abstract

This review summarises our current understanding of two of the main types of quinoprotein dehydrogenase in which pyrroloquinoline quinone (PQQ) is the only prosthetic group. These are the soluble methanol dehydrogenase and the membrane glucose dehydrogenase (mGDH). The membrane GDH has an additional N-terminal domain by which it is tightly anchored to the membrane, and a periplasmic domain whose structure has been modelled on the X-ray structure of the α -subunit of MDH which contains PQQ in the active site. This review discusses their structures and mechanisms, concentrating particularly on the pathways for electron transfer from the reduced PQQ, through the protein, to their electron acceptors. In MDH, this is the specific cytochrome c_L , the electron transfer pathway probably involving the unique disulphide ring in the active site. By contrast, mGDH contains a permanently bound ubiquinone, which acts as a single electron carrier, mediating electron transfer through the protein to the membrane ubiquinone.

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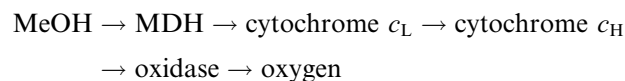
The dehydrogenases for methanol and glucose represent the two main types of quinoprotein in which pyrroloquinoline quinone (PQQ)¹ is the only prosthetic group (for extensive reviews of all the PQQ-dependent quinoproteins, see [1–4]). These enzymes catalyse their reactions in the periplasm of Gram-negative bacteria. This contrasts with oxidation systems involving membrane flavoproteins, which catalyse reactions on the inner face of the cytoplasmic membrane. Like these enzymes, the quinoproteins are assayed with artificial electron acceptors such as phenazine ethosulphate (PES). They are linked to energy-transducing electron transport chains by way of their physiological electron acceptors; for the soluble methanol dehydrogenase (MDH), this is a specific cytochrome c (cytochrome c_L), whereas the glucose dehydrogenase (mGDH), which is tightly anchored to the membrane, uses ubiquinone in the membrane.

The primary sequences of PQQ-containing dehydrogenases show sufficient similarity to indicate some common structure, and this has been confirmed for many of

the proteins by their X-ray structures. [An exception is the soluble glucose dehydrogenase of *Acinetobacter* [5] which will not be discussed further here.] They all have a core structure similar to that first described for the α -subunit of MDH which contains PQQ in the active site. The sequence of mGDH shows that it has a membrane-anchoring N-terminal sequence in addition to the periplasmic core structure, which in the absence of an X-ray structure has been successfully modelled on MDH. A very closely related group of dehydrogenases are the quinohaemoprotein alcohol dehydrogenases which have a C-terminal cytochrome domain covalently attached to the PQQ-containing superbarrel structure [6,7].

Methanol dehydrogenase

MDH catalyses the first reaction of an unusual periplasmic electron transport chain responsible for oxidation of methanol to formaldehyde in methylotrophic bacteria during growth on methane or methanol [8]:



Although its physiological electron acceptor is cytochrome c_L , with which it usually reacts at pH 7, it is

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¹ Abbreviations used: mGDH, membrane glucose dehydrogenase; PQQ, pyrroloquinoline quinone; PES, phenazine ethosulphate; MDH, methanol dehydrogenase; MD, molecular dynamics.

usually assayed at high pH (about pH 9) when ammonia (not ammonium) is required as activator [9]. It oxidises a wide range of primary alcohols, often having a very high affinity for substrate; e.g., for methanol the K_m value is about 20 μM [10]. MDH and its novel prosthetic group were first described 40 years ago [11,12] and is probably the most thoroughly studied of the quinoprotein dehydrogenases; it has been extensively reviewed and much of the following material is taken directly from these reviews [3,4,13].

The structure of methanol dehydrogenase

The X-ray structure has been determined for the MDH from *Methylobacterium extorquens* [14–16], *Methylophilus sp.* [17–21], and *Paracoccus denitrificans* [22]; the amino acid numbers in the text refer to those for the MDH of *M. extorquens*. MDH has an $\alpha_2\beta_2$ tetrameric structure, each α -subunit (66 kDa) containing one molecule of PQQ and one Ca^{2+} ion. The β -subunit is very small (8.5 kDa), it cannot be reversibly dissociated, its function is unknown, and it is not present in any other quinoproteins. The large α -subunit has a propeller fold making up a superbarrel of eight radially arranged β -sheets (the ‘propeller blades’) (Fig. 1). This structure has several important unusual features, including the novel ‘tryptophan-docking motifs’ that link together the eight β -sheets, and the presence in the active site of a unique eight-membered disulphide ring structure formed from adjacent cysteine residues (Cys 103–104), joined by an atypical non-planar peptide bond. The PQQ is sandwiched between the indole ring of Trp243 and the disulphide ring structure (shown later in Fig. 4). The indole ring is almost co-planar with the PQQ ring and, on the opposite side, the two sulphur atoms of the disulphide bridge are within 3.75 Å of the plane of PQQ. The rarity of the disulphide ring structure would suggest some special biological function, which is supported by the demonstration that inactive enzyme was produced when Cys103 or Cys104 was altered by site-directed mutagenesis [16]. Reduction of the disulphide bond leads to loss of activity but subsequent oxidation in air, or irreversible carboxymethylation of the free thiols, leads to return of activity [23], thus ruling out 2-electron reduction to the thiols as part of the catalytic cycle. Recent work with the quinohaemoprotein alcohol dehydrogenase [6,7] suggests, however, that the disulphide ring structure is involved in the single electron transfer between reduced PQQ and the haem of the electron acceptor in all the quinoprotein alcohol dehydrogenases [24] (see below).

In addition to the axial interactions, involving Trp243 and the disulphide ring, many amino acid residues are involved in equatorial interactions with the substituent groups of the PQQ ring system (Fig. 2). At first sight the

environment of the PQQ appears to be polar, but this is not the case; an oxygen of the 9-carboxyl group 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 at least one is probably protonated, thus stabilising interactions through hydrogen bond formation. The active site contains a single Ca^{2+} ion whose coordination sphere contains PQQ and protein atoms (Fig. 2). The C4 and C5 oxygen atoms, which become reduced during the catalytic cycle, are hydrogen bonded to Arg331; this residue also makes hydrogen bonds between its NH_2 and the carboxylate of Asp303 which is the most likely candidate for the base required by the catalytic mechanism. An alternative candidate for the catalytic base is Glu177 (see below) which is coordinated to the Ca^{2+} .

The mechanism of MDH

The MDH reaction mechanism involves reduction of PQQ by methanol, and release of product formaldehyde, followed by two sequential single-electron transfers to the cytochrome c_L , during which the PQQH_2 is oxidised back to the quinone by way of the semiquinone (Fig. 3). The rate-limiting step is the breaking of the methyl C–H bond, and it is this step that leads to a very large kinetic isotope effect (KIE), and which is affected by the unexplained activation by ammonia [11,16,25,26]. It should be emphasised that there are many difficulties in experimental design and interpretation when using spectroscopy and kinetics with MDH [3] and this is illustrated in a recent analysis of this enzyme [27]. It was shown that the high KIE effect observed in steady state studies is not seen when using stopped-flow kinetics. It was concluded that reduction of the oxidised enzyme generated in stopped-flow analyses is gated by conformational change or ligand exchange, which accounts for the slow reduction of PQQ after addition of methanol, and for the KIE of 1. This difference in KIEs emphasised the need for caution when using inflated KIEs, and their temperature dependence, as a probe for hydrogen tunneling [27].

The reaction starts by abstraction of the proton from the alcohol group by an active site base, proposed to be Asp303 (Figs. 2 and 3); this proposal is supported by the properties of a modified MDH in which this aspartate was replaced by glutamate by site-directed mutagenesis [16]. The proton abstraction is followed immediately by a direct hydride transfer from the methyl group of methanol to the C-5 of PQQ to give the C-5 reduced form, followed by tautomerism to the PQQH_2 ; these tautomerisation reactions may also involve the pyrrole ring of PQQ or Asp303 [3,28–30] (Fig. 3). A previously considered alternative mechanism, involving formation

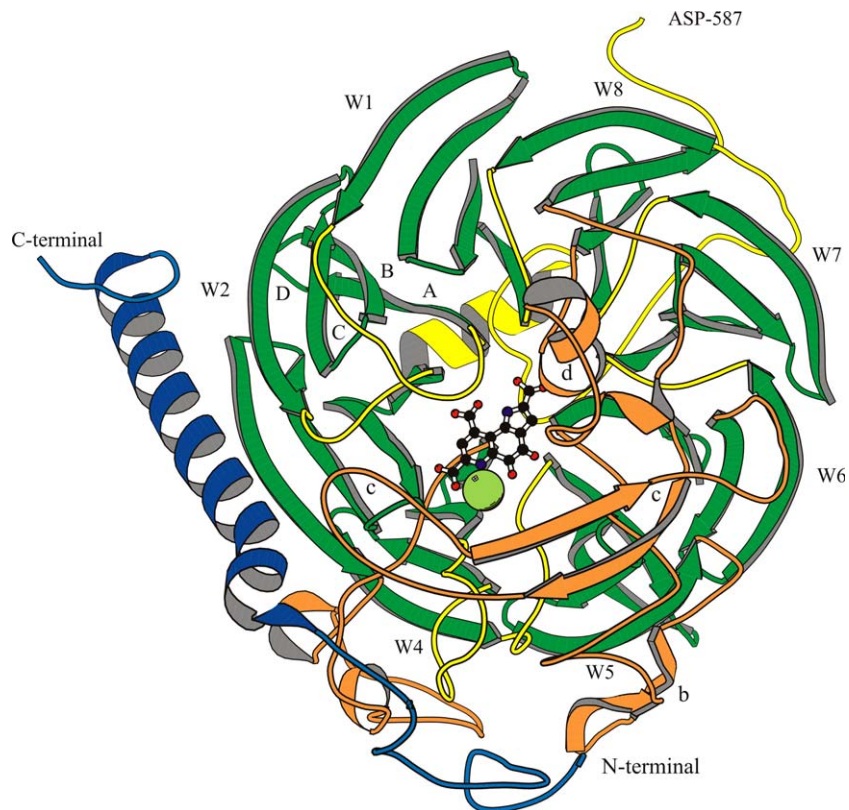


Fig. 1. The $\alpha\beta$ -heterodimer of MDH, simplified to show the eight β -strands in the large α -chain (the 'propeller blades') and the long α -helix in the small β -subunit. The PQQ is shown in skeletal form and the Ca^{2+} as a small sphere. This figure is based on the structure in [15].

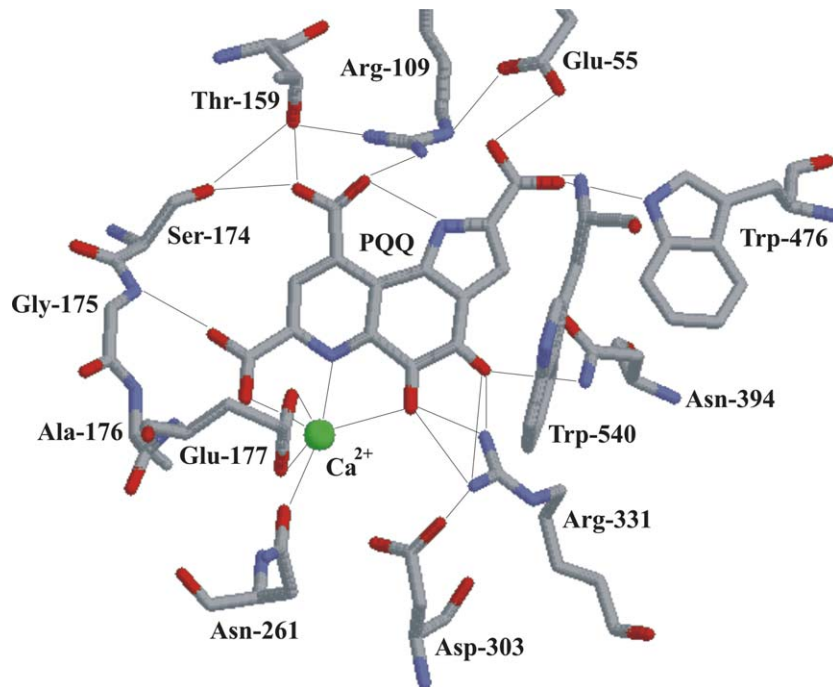


Fig. 2. The equatorial interactions of PQQ and the coordination of Ca^{2+} in the active site of MDH of *Methylobacterium extorquens* [15]. The figure also shows the proposed catalytic base (Asp303), and Arg331 which may also be involved in the mechanism. It has recently been suggested [32] that Glu177, rather than Asp303, may be the catalytic base. The side view, showing the disulphide ring structure in relation to PQQ, is shown in Fig. 4.

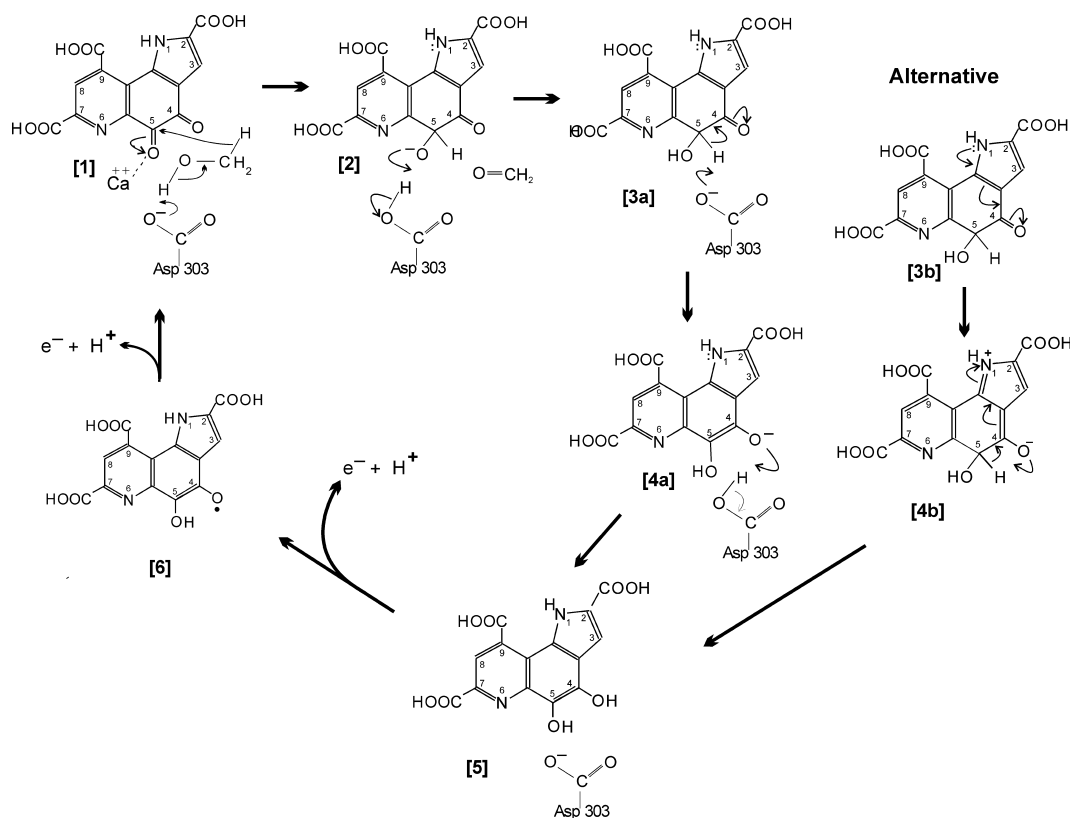


Fig. 3. The direct hydride transfer mechanism for MDH. In the alternative route involving intermediates [3b] and [4b], the pyrrole nitrogen contributes to the ionisation of the oxygen atom on C4, perhaps contributing to the tautomerism of intermediates [3–5].

of a covalent hemiketal intermediate [3,13], appears unlikely [24]. On balance, the evidence is now in favour of the hydride transfer mechanism in MDH and in the closely related quinohaemoprotein alcohol dehydrogenase [6,7], and it is very likely that all the quinoprotein dehydrogenases have the same mechanism [24], the evidence for which is most complete for the soluble glucose dehydrogenase of *Acinetobacter* [5]. Recent structural, and quantum mechanical, studies of MDH now also support the conclusion that the mechanism of MDH involves our hydride transfer mechanism [16,21].

It is proposed that the Ca²⁺ ion acts as a Lewis acid by way of its coordination to the C-5 carbonyl oxygen of PQQ, thus stabilising the electrophilic C-5 for attack by the hydride. The conserved arginine (Arg331) is also likely to be important in increasing the nucleophilicity of the C5 atom of PQQ (Figs. 2 and 3). The role of the Ca²⁺ ions has been studied using mutants unable to incorporate Ca²⁺ by the usual periplasmic assembly process, which requires specific processing proteins. In mutants lacking these, MDH is produced that contains PQQ but not Ca²⁺, and incubation with high concentrations of Ca²⁺ (at high pH) is required to produce active enzyme [31]. When Ba²⁺ ions are used, these are incorporated to produce Ba-MDH which has twice the maximum activity of the Ca-MDH but with a much lower affinity for its substrates [26].

A recent molecular dynamics (MD) study of reduction of PQQ by way of the hydride transfer mechanism [32] has led to the suggestion that Glu177 plays the role of general base catalyst rather than Asp303 (Figs. 2 and 3), and that the rearrangement of the C5-reduced intermediate (intermediate 3 in Fig. 3) to produce the hydroquinone PQQH₂ (intermediate 5) is also assisted by proton abstraction by Glu177 instead of by Asp303. The characteristics of the altered MDH produced by site-directed mutagenesis of Asp303 [16] to glutamate were consistent with the proposed role of Asp303 in the initial proton abstraction, and it will now clearly be of interest to characterise a protein in which Glu177 is changed by site-directed mutagenesis.

Results of recent kinetic studies have led to the suggestion that ammonium ions (added as activator) might form a salt bridge with the second carboxyl oxygen of Glu177 which coordinates the Ca²⁺ in the active site (Fig. 2), hence withdrawing negative charge from Glu177, resulting in Ca²⁺ becoming more positive, thus allowing it to function more effectively as a Lewis acid, thereby accelerating PQQ reduction by methanol [27]. However, it is difficult to reconcile this suggestion with the demonstration that ammonia (or methylammonia) is the activating species rather than ammonium (or methylammonium) ions [3,11].

The position of methanol in the active site has not been determined. Its hydroxyl group must be near to the side chain of Asp303 (or an alternative active site base) for hydrogen bonding to occur, and the methyl group must be near to the C5 of PQQ, and within the hydrophobic cavity bounded by side chains of the disulphide ring, Trp265, Trp540, and Leu556. This is well illustrated in the results of an MD analysis which indicated appropriate locations for methanol, and for the channel for substrate and water into the active site entrance [32]. The relatively narrow entrance to the active site 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 [10]; it is not immediately obvious how these substrates could readily gain access to the active site, and understanding of this awaits solution of a structure containing one of these larger substrates. Whatever the structure, it is unlikely to accommodate the suggestion [27] that the reason for the competitive kinetics, with respect to methanol, shown by the artificial electron acceptor PES is because it binds at the same place in the active site as methanol; there must be some other reason for this as PES is similar in size to PQQ itself.

The oxidation of the reduced quinol form of PQQ (PQQH₂) involves passing single electrons sequentially to two separate molecules of cytochrome *c*_L (Fig. 3). Chemical modification and cross-linking studies have shown that the initial ‘docking’ of the two proteins involves carboxylate residues on cytochrome *c*_L and lysyl (or arginyl) residues on MDH [8,33,34]. This is probably followed by movement of the cytochrome to its optimal position for electron transfer [35]. No structure of the MDH/cytochrome *c*_L complex is available but it is reasonable to conclude that electrons pass by the shortest route from the reduced PQQ to the haem of cytochrome *c*_L. This route is likely to be similar to that calculated for electrons in the quinohaemoprotein alcohol dehydrogenase [6,7], and likely to involve water molecules, the conserved disulphide ring and Asp105 (Fig. 4). During re-oxidation of the PQQH₂, protons must be released into the periplasm and this is likely to be by way of a similar hydrogen bonded network of proton donor and acceptor groups, as has been shown in quinohaemoprotein alcohol dehydrogenase [6,7], involving Asp303, Glu177, and Arg331 (Fig. 4). The interactions of the MDH from *Paracoccus denitrificans* and cytochrome

*c*_{551i} (the equivalent of cytochrome *c*_L in this organism) have recently been investigated by molecular modelling using the X-ray coordinates of the two proteins, previously determined separately [22]. The starting point for the model was the covalently attached cytochrome and PQQ domains of the quinohaemoprotein alcohol dehydrogenase [7]. The interprotein ionic interactions were shown to be consistent with previous proposals based on chemical modification and cross-linking studies [33,34] (see above). The best electron transfer route in the favoured model involves a through-space jump from PQQ to the disulphide bridge above the plane of the PQQ (as suggested in Fig. 4), passage through 13 covalent bonds, and a second interprotein space jump from the MDH to the haem edge. The 17 Å distance between the edges of PQQ and haem is almost beyond the usual limits considered for such electron transfer reactions, but it was suggested that it may be reasonable to treat the through-space jump from PQQ to the disulphide in MDH and alcohol dehydrogenases as being more highly coupled than a typical non-bonded jump, because structural analysis shows that their orbits are nearly in contact [22].

The membrane glucose dehydrogenase

Membrane glucose dehydrogenases (mGDH) have been described in a wide range of bacteria including *Acinetobacter*, enteric bacteria, pseudomonads and acetic acid bacteria, in which they are responsible for the direct oxidation of D-glucose to the lactone, and thence gluconic acid. In all cases the physiological electron acceptor is ubiquinone which is subsequently oxidised by a quinol oxidase (as in *E. coli*) or by way of a cytochrome *bc*₁ complex, cytochrome *c*, and a cytochrome oxidase (for reviews, see [1,2,4]). Although the mGDHs are similar in most respects, they differ in their stability and the ease with which PQQ may be dissociated from them. It has therefore been suggested that they can be placed in two classes, depending on their stability with respect to EDTA; type I is easily denatured and occurs in *E. coli* and *Pseudomonas* sp., whereas the more stable enzyme occurs in *Acinetobacter* and *Gluconobacter* (see [1]).

The mGDH catalyses the oxidation of a wide range of hexoses and pentoses, but rarely oxidises disaccha-

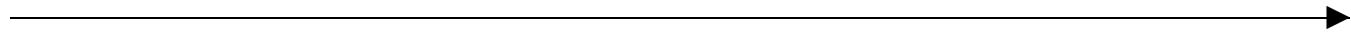
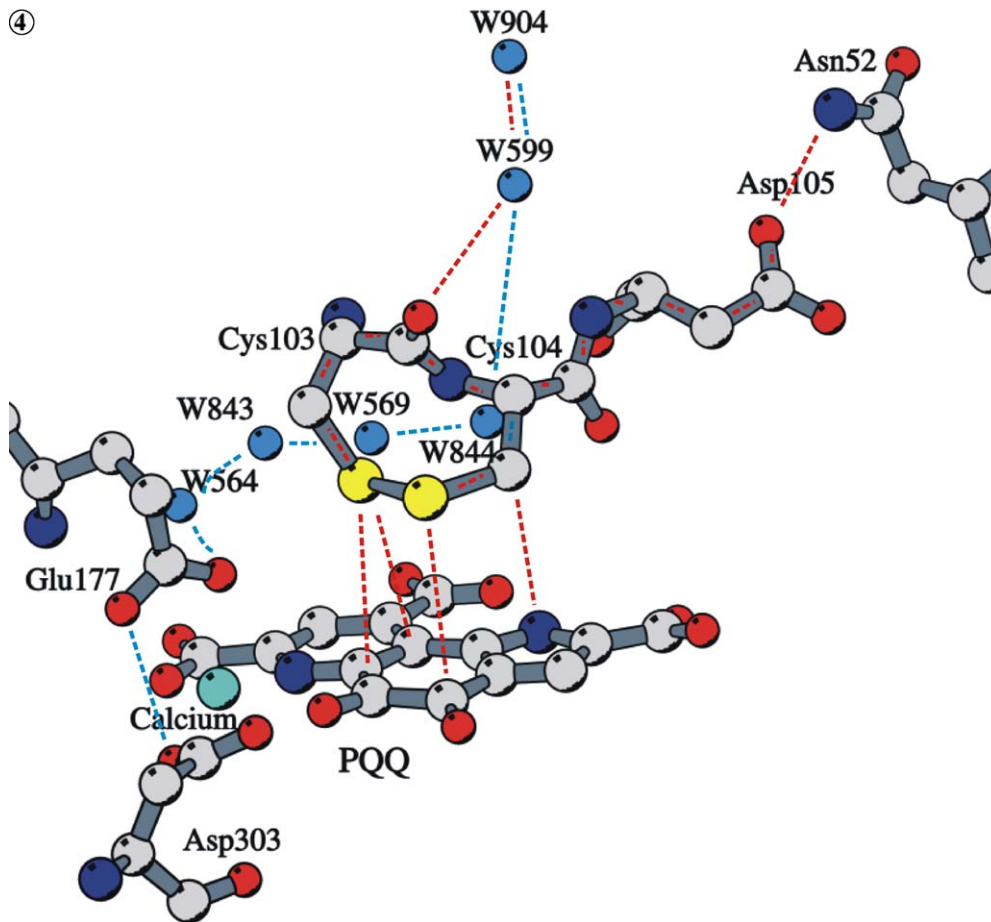


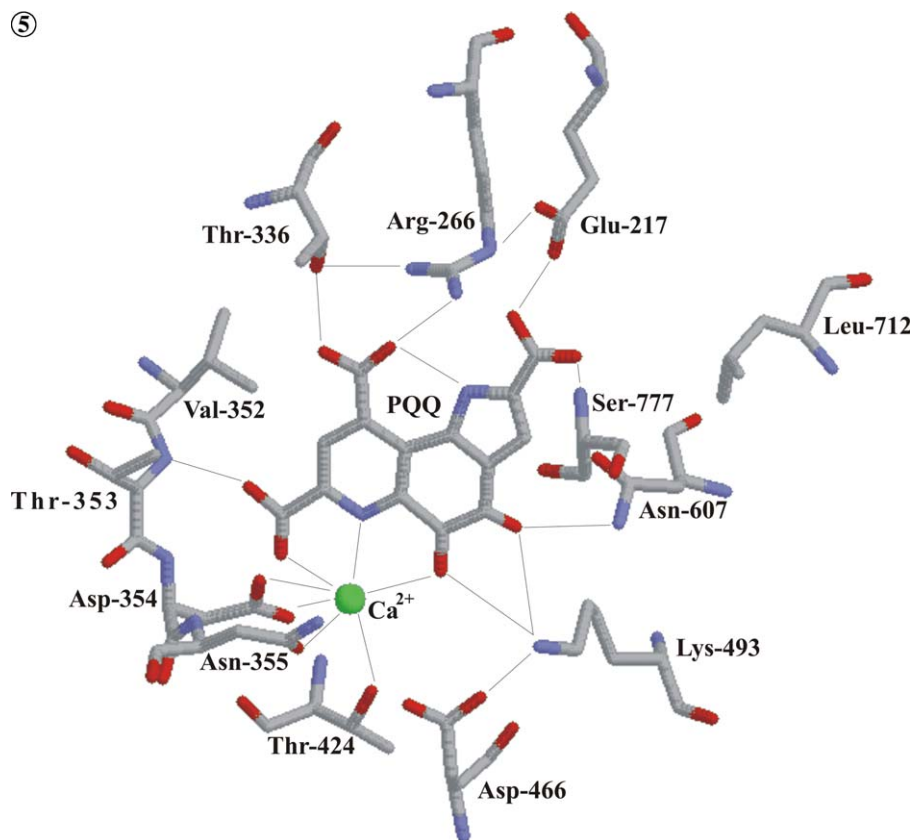
Fig. 4. The active site of MDH, indicating pathways from the reduced PQQ for electrons towards the electron acceptor, cytochrome *c*_L, and protons to the surrounding medium. This suggestion [13] is based on calculations for the quinohaemoprotein alcohol dehydrogenase [6,7]. Red dotted lines indicate probable electron transfer routes; blue dotted lines indicate the hydrogen-bonding network between the hydroxyls of PQQH₂ and the water (W904) that is accessible to the periplasmic solvent (the pathway is disrupted once by a 5 Å gap between W844 and W599). For pathways based on modelling the interaction of MDH with its cytochrome electron acceptor, see [22].

Fig. 5. The active site of mGDH of *E. coli*; this was modelled on the coordinates of MDH [39]. In MDH, the metal ion (M²⁺) is Ca²⁺; this is possibly replaced by Mg²⁺ in mGDH [43].

④



⑤



rides, as shown in an extensive survey of the substrate specificity of the *E. coli* enzyme [36]. The activation energies for hexoses and pentoses were the same and all results indicated that the mechanism for their oxidation is the same. L-hexoses were not oxidised and none was a competitive inhibitor, suggesting that these sugars do not bind at the active site; this is probably because of steric hindrance due to the C-6 hydroxymethyl (or methyl) group below the plane of the ring which is their only common feature. All of the D-hexoses are good substrates, the catalytic efficiency being determined mainly by their affinity for the enzyme. No particular hydroxy group is absolutely essential for binding; the hydroxy groups on C-2, C-3, and C-4 can be in either orientation, but any substituent on C-5 must be above the plane of the ring, the nature of the substituent (methyl, hydroxyl or a second saccharide) only affecting affinity and not the rate of reaction.

As with the hexoses, the pyranose forms of pentoses predominate in solution, but they lack a hydroxymethyl substituent at the C-5 position; as this group is not essential for binding hexoses, it is not surprising, therefore, that some pentoses are good substrates. Because the major distinction between the D- and L-isomers of the hexoses (that is, the orientation of the C-6 hydroxyl) does not apply in the pentoses, it might be expected that they would all be able to bind to the enzyme, and this appears to be the case; all pentoses are either substrates (e.g., D-xylose, D-ribose, and L-arabinose) or competitive inhibitors (e.g., L-xylose, L-ribose, and D-arabinose). Inexplicably, although all pentoses are able to bind to the enzyme, those hexoses that are not substrates are not competitive inhibitors and so presumably were unable to bind to the active site.

The structure of mGDH

The N-terminal domain (154 amino acids) anchors the monomeric enzyme by way of five transmembrane helices to the membrane [37,38], and the remaining amino acids (155–796) form a periplasmic superbarrel domain. This catalytic domain has been modelled [39] on the X-ray structure of the α -subunit of MDH, and has been used successfully in interpreting the characteristics of many mGDHs modified by site-directed mutagenesis [36–41]. There are two key structural features that are different in the mGDH and MDH structures, and both are likely to be related to the different electron acceptors used by these enzymes. The mGDH lacks the remarkable disulphide ring structure in the active site of MDH, which is probably involved in electron transfer from reduced PQQ to its cytochrome c_L electron acceptor (Fig. 4); this is not needed in mGDH or in the related membrane sorbitol dehydrogenase [38], in both of which the electron acceptor is

membrane ubiquinone (UQ₈). The second key difference is that there is a sequence of about 80 amino acids in mGDH and sorbitol dehydrogenase that is absent from MDH; this sequence includes an amphiphilic segment that possibly forms a domain close to the membrane which may contain the site for binding the membrane electron acceptor UQ₈ [38,42].

The mechanism of mGDH

The first part of the mechanism of mGDH is very likely to be the same as that for methanol oxidation by MDH (Fig. 3). The reaction is initiated by Asp466 (Fig. 5) which abstracts a proton from the anomeric (C-1) hydroxyl group of glucose, the subsequent hydride transfer from the C-1 of the glucose to the electrophilic C-5 carbon atom in PQQ leading to the production of gluconolactone and PQQH₂. Analysis of the kinetics of mGDHs produced by site-directed mutation of Asp466 and Lys493 is consistent with this proposed mechanism and indicates that Lys493 is involved in the subsequent oxidation of PQQH₂ [41].

It has recently been shown [42] that mGDH bears two ubiquinone-binding sites; one site (Q_I), close to the PQQ in the active site, retains a tightly bound ubiquinone (UQ₈) which acts as a single electron mediator, alternating between its oxidised and semiquinone forms. The second site (Q_{II}) probably occurs in the amphiphilic segment that is absent from MDH, and reversibly binds ubiquinone from the membrane pool. During the oxidative phase of the reaction mechanism, Lys493 probably passes electrons one at a time from PQQH₂ to the single electron mediator UQ₈ (at the Q_I site) and hence to a molecule of membrane UQ₈ bound loosely at the Q_{II} site.

The metal ion at the active site of mGDH

The mGDH in *E. coli* occurs as an apoenzyme because this organism is unable to synthesise PQQ; the active holoenzyme is readily reconstituted with PQQ and Mg²⁺ ions (but not Ca²⁺ ions). In MDH, the Ca²⁺ ion plays a key role in the enzyme mechanism (see above), and all the PQQ-containing dehydrogenases whose structures have been determined also contain a Ca²⁺ ion bonded to the PQQ at the active site, and are likely to have a similar mechanism of action [24]. There is no reason why the mGDH discussed here might be expected to be different. In the modelled mGDH, PQQ is ligated to the metal ion in the same manner as in MDH and three residues from mGDH form an additional four interactions with the metal ion; they are Asp354, Asn355, and Thr424 (Fig. 5). This raises the question of the role of the Mg²⁺ ions that are essential

for production of the active holo-mGDH from apoenzyme. Either the metal ion becomes incorporated into the active site of the holo-mGDH, or perhaps it is required for some other purpose. To address this question and to confirm the proposed ‘model’ active site with respect to metal ion binding, site-directed mutants were produced in which the amino acids proposed to be involved in binding the metal ions were modified [43]. Remarkably, the reconstitution of the mutant enzymes was not supported by Mg^{2+} ions as in the wild-type enzyme, but it could be supported by Ca^{2+} , Sr^{2+} or Ba^{2+} ions, this reconstitution being competitively inhibited by Mg^{2+} ions. This result, together with studies on the kinetics of the modified enzymes, has led to the conclusion that, although a Ca^{2+} ion is able to form part of the active site of the genetically modified mGDHs, as in all other PQQ-containing quinoproteins, a Mg^{2+} ion can surprisingly replace this in the active site of the wild-type enzyme.

It is expected that future studies will concentrate on the pathways within MDH and mGDH for electron transfer from PQQH₂ to their respective electron acceptors, the role of the metal ion in mGDH, and the determination of the X-ray structure of its periplasmic domain.

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