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The structure and function of the PQQ-containing quinoprotein dehydrogenases

Christopher Anthony[a,*], Minakshi Ghosh[b]

[a]*Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton SO16 7PX, U.K.*

[b]*Laboratory of Molecular Biophysics, University of Oxford, U.K.*

Abstract

Bacterial methanol and glucose dehydrogenases containing a novel type of prosthetic group, subsequently identified as pyrrolo-quinoline quinone (PQQ), were first described about 30 years ago. Quinoproteins were originally defined as proteins containing PQQ but this definition has since been broadened to include those proteins containing other types of quinone-containing prosthetic groups, and the X-ray structures of representatives of each type of quinoprotein have recently been published. This review is mainly concerned with the structure and function of the PQQ-containing methanol dehydrogenase, whose structure has been determined at high resolution, and related proteins. Their basic structure consists of a 'propeller' fold superbarrel made up of 8-sheet 'propeller blades' which are held together by novel tryptophan-docking motifs. In methanol dehyhdrogenase the PQQ in the active site is coordinated to a Ca^{2+} ion and is maintained in position by a stacked tryptophan and a novel 8-membered ring structure made up of a disulphide bridge between adjacent cysteine residues. This review describes these features and discusses them in relation to previously proposed mechanisms for this enzyme. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Pyrrolo-quinoline quinone; Methanol dehydrogenase; Glucose dehydrogenase; Propeller fold

1. Introduction

In about 1980 the term quinoprotein was coined to include a number of bacterial dehydrogenases which contain pyrrolo-quinoline quinone (PQQ) as their prosthetic group (Duine et al., 1979, 1980) (Fig. 1). The name is now used more widely to include all those

* Corresponding author. Tel.: 01703 594280; fax: 01703 594459; e-mail: c.anthony@soton.ac.uk.

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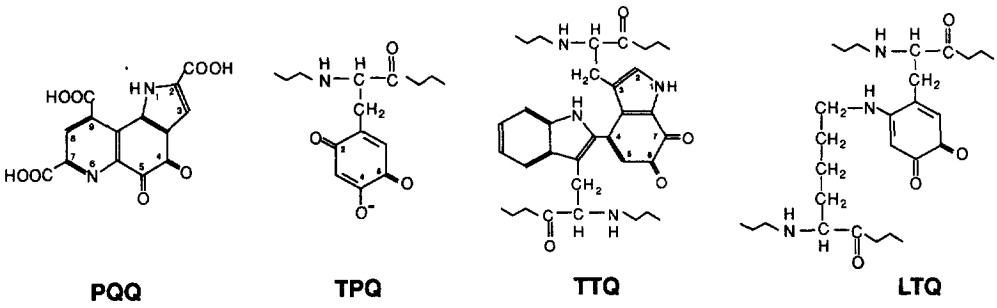


Fig. 1. The prosthetic groups of quinoproteins. PQQ (pyrrolo-quinoline quinone) occurs in the dehydrogenases for methanol, higher alcohols, aldose sugars and polyvinyl alcohol, and for hydroxylation of lupanine. TTQ (tryptophan tryptophylquinone) is in bacterial amine dehydrogenases. TPQ (6-hydroxyphenylalanine quinone or topa quinone) is in 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.

enzymes whose catalytic mechanisms involve quinone-containing prosthetic groups in their active sites. Except for PQQ, these are derived from amino acids in the protein backbone of the enzyme (Fig. 1): Tryptophan tryptophylquinone (TTQ) is derived from two tryptophan residues and occurs in bacterial amine dehydrogenases (McIntire et al., 1991; Davidson, 1993); 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; Klinman, 1995); and lysyl oxidase is a special type of copper-containing amine oxidase whose prosthetic group is Lysyl tyrosylquinone (LTQ) (Wang et al., 1996).

The main subjects of this review are the PQQ-containing dehydrogenases of which the methanol dehydrogenase of methylotrophic bacteria is the best-characterized example (Anthony, 1993a; Anthony, 1996). The history of the quinoproteins began in the 1960s with the characterisation of the novel prosthetic group of this enzyme (Anthony and Zatman, 1967), and of glucose dehydrogenase (Hauge, 1964). More than 10 years later Duine, Frank and co-workers demonstrated that it contains a quinone structure with 2 nitrogen atoms (Duine et al., 1978; Duine and Frank, 1980), and Kennard's group showed this to be pyrrolo-quinoline quinone (PQQ), by X-ray diffraction analysis (Salisbury et al., 1979). A number of other bacterial dehydrogenases were subsequently shown to contain PQQ by the groups of Duine and Frank in Delft (Duine, 1991), and Ameyama and Adachi in Yamaguchi (Matsushita and Adachi, 1993; Matsushita et al., 1994). These quinoproteins usually catalyse the first step in the oxidation of alcohols and sugars in the periplasm of bacteria, thus contributing to the formation of a protonmotive force and hence the formation of ATP (Anthony, 1993b). They are usually assayed with artificial electron acceptors such as phenazine ethosulphate (PES). The physiological electron acceptor is a soluble cytochrome *c* in the case of methanol dehydrogenase and some ethanol dehydrogenases; it is protein-bound haem C in the quinohaemoprotein alcohol dehydrogenases, and ubiquinone in the membrane-bound glucose dehydrogenase. For many years it was known that a divalent cation may be involved in the structure or activity of some alcohol dehydrogenases because Ca^{2+} or Mg^{2+} are required for reconstitution of active enzyme from apoenzyme plus PQQ. By contrast, it is not possible to

dissociate PQQ from methanol dehydrogenase without irreversible denaturation and it is only relatively recently that calcium has been shown to be present and implicated in the activity of this enzyme (Adachi et al., 1990; Richardson and Anthony, 1992).

2. Methanol dehydrogenase

The oxidation of methanol to formaldehyde is catalysed by this enzyme in the periplasm of methylotrophic bacteria and is the only PQQ-containing enzyme for which a structure is available (Xia et al., 1992; White et al., 1993; Anthony et al., 1994; Ghosh et al., 1995; Xia et al., 1996), the highest resolution structure (1.94Å) being that of the enzyme from *Methylobacterium extorquens* (Ghosh et al., 1995), which is the one referred to throughout this review. It has an $\alpha_2\beta_2$ tetrameric structure; the α-subunit containing the PQQ is 66 kDa, and the β-subunit is very small (8.5 kDa). The subunits cannot be reversibly dissociated and no function has been ascribed to the small subunit. In the tetramer the two subunits $\alpha\beta$ are arranged with their pseudo 8-fold axes approximately perpendicular to each other, and the PQQ prosthetic groups are separated by about 45Å.

2.1. The propeller fold superbarrel structure of the α subunit of methanol dehydrogenase

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 and examples with four, six, seven or eight propeller blades have been described. Haemopexin (Faber et al., 1995) and sinovial collagenase (Li et al., 1995) have four blades; viral neuraminidase (Varghese et al., 1983) and a related bacterial sialidase (Crennell et al., 1993) have six blades; galactose oxidase (Ito et al., 1994), methylamine dehydrogenase (Vellieux et al., 1989) and the β subunit of the G protein transducin (Sondek et al., 1996; Lambright et al., 1996) have seven blades, while methanol dehydrogenase and nitrite reductase (Fulop et al., 1995; Baker et al., 1997) both have eight blades. It is intriguing that two of the proteins having the propeller fold structure are quinoproteins, although the related structures have no related catalytic function. Thus, in methylamine dehydrogenase (also from methylotrophic bacteria) the TTQ prosthetic group is in the smaller subunit, the centre of the larger superbarrel being filled with side chains (Vellieux et al., 1989); but, by contrast, in methanol dehydrogenase the active site containing PQQ lies within the large superbarrel structure.

The 32 β-strands that make up the superbarrel structure in MDH are shown schematically in Fig. 2, labelled according to the 'W' motif in which they occur (1–8) and the position they take within the motif (A–D). The sequence of the strands in the structure is the same as in the amino acid sequence (this is also seen in other superbarrel structures), with the sole exception that the final strand of the eighth motif (D8) is derived from the N-terminus, not the C-terminus. The short A strands are closest to the pseudo 8-fold axis, and the D strands are on the surface of the subunit. Fig. 2 shows 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

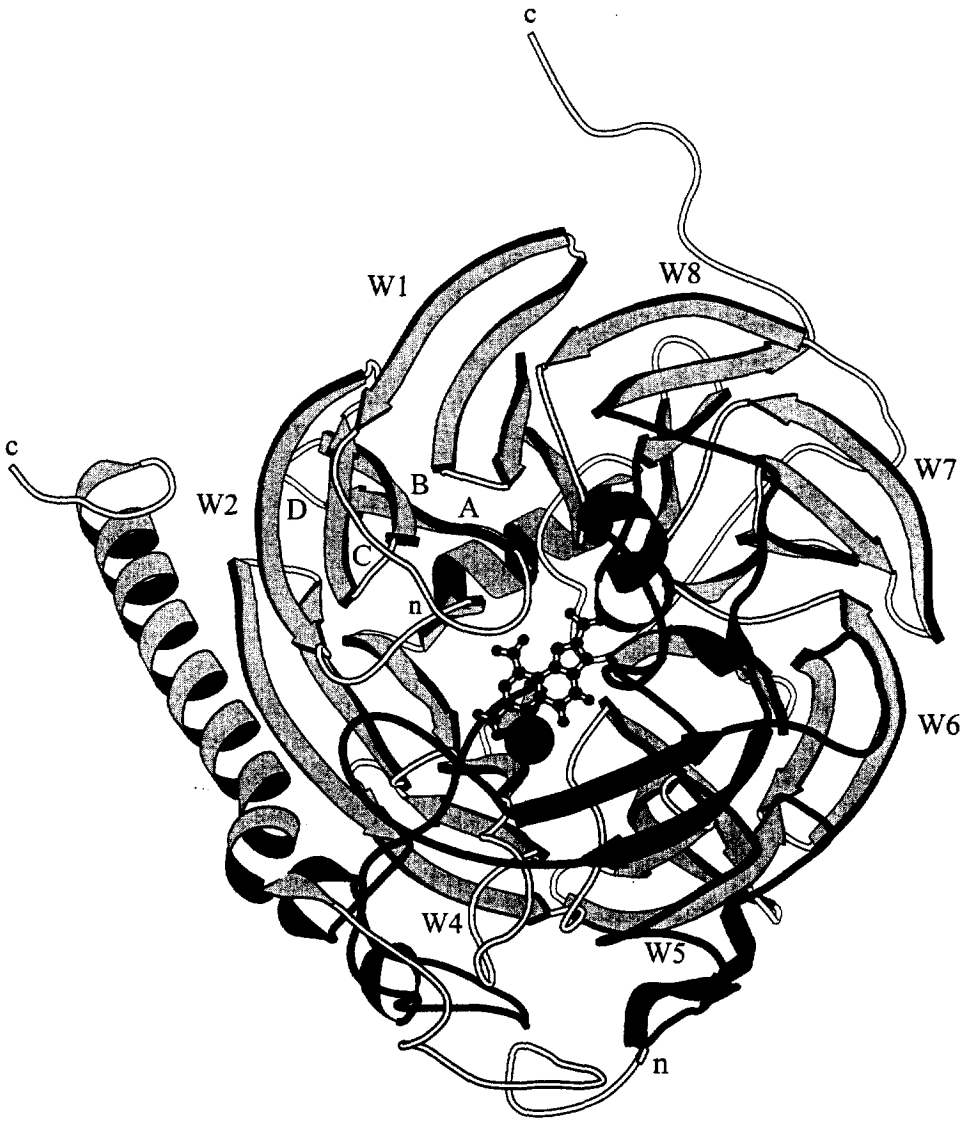


Fig. 2. A drawing of an $\alpha\beta$ unit of MDH looking down the pseudo 8-fold axis, simplified to show only the β-strands of the 'W' motifs of the α-chain, and the long α-helix of the β-chain, but excluding other limited β-structures and short α-helices (Ghosh et al., 1995). The PQQ prosthetic group 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.

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 the B- and C-strands. Strand A runs more or less antiparallel to strand B, but makes only three β-type

hydrogen bond interactions with it, all of which are close to the A/B turn. It is likely that the conformations of the A-strands are controlled by the close packing of side-chains in the very centre of the molecule, where all eight A-strands come close together; at the point of contact five of the eight residues are glycine, thus facilitating close-packing. The remaining B, C and D strands in each motif form regular twisted β-sheets of sequences of eight to nine residues. In all motifs the A–B and C–D corners are short, but the B–C corners are more variable and in three cases (motifs 4, 6 and 8) they form extensions containing 24–30 residues.

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_1 proves to be closely superimposable on that of methanol dehydrogenase 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 methanol dehydrogenase 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_1 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. 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. Murzin (1992) has published a valuable discussion of the principles involved in propeller assembly of β-sheets.

2.2. The tryptophan-docking interactions in the α-subunit of methanol dehydrogenase

A series of tryptophan-docking interactions between the β sheet propeller blades make planar, stabilising girdles around the periphery of the subunits (Ghosh et al., 1995; Xia et al., 1996) (Fig. 3). This method of stabilising protein structures has not been described previously and is therefore described here in some detail. The interactions occur by way of 11-residue consensus sequences in the C/D region of all 'W' motifs except number 8 (Fig. 4). 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 (Fig. 5). 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.

Exceptions to these interactions with tryptophan occur in W1 and W7. In W1 the glycine at position 7 is replaced in the stacking interaction by proline at position 6; and in W7 the alanine at position 1 (Ala498) does not form the expected interaction with Trp508. There are two exceptions to tryptophan in position 11. 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

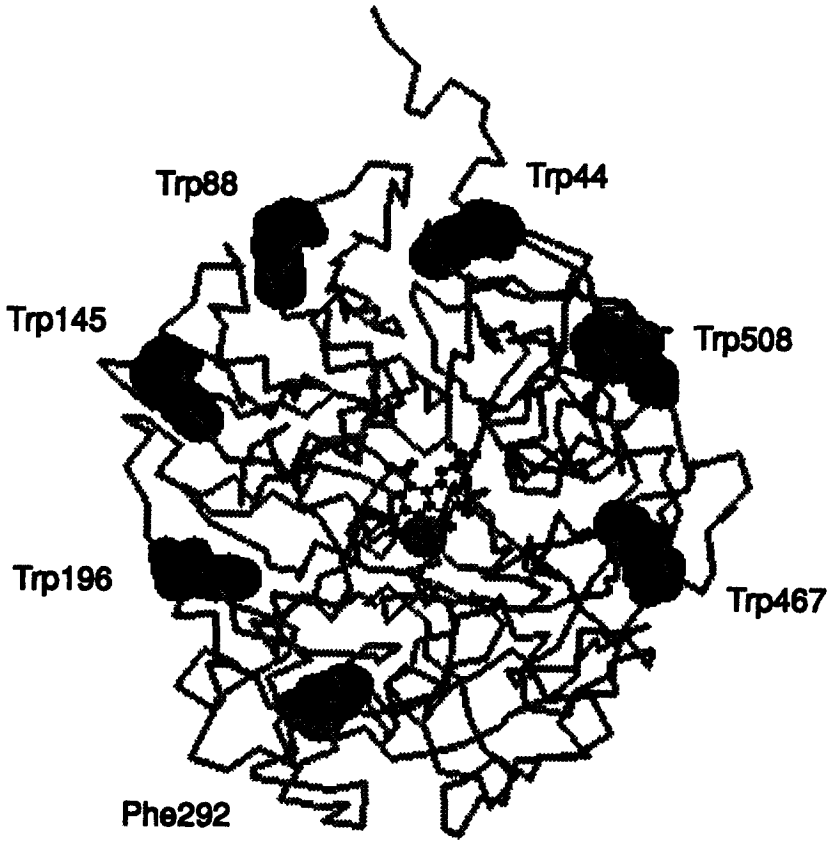


Fig. 3. The girdle of tryptophan residues involved in docking the β-sheets together (Ghosh et al., 1995). The tryptophan residues involved in docking are shown in spacefill mode and the rest of the chain as backbone.The PQQ prosthetic group is in skeletal form and the calcium ion is shown as a small sphere.

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 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 two 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 (GDH) and alcohol (ADH) show the greatest similarity to the MDH in the sequences which form the W motifs. This has facilitated modelling of the structure of these proteins which has suggested that they have an almost identical tryptophan docking motif at the C/D corners as indicated for MDH (Fig. 4).

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_1) (Fulop et al., 1995; Baker et al., 1997) in which the centre of the superbarrel provides a pocket for the d_1 haem. This is particularly remarkable for the nitrite reductase

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 581	-	Gln 39	Leu	Arg	Pro	Ala	Trp 44
MDH Consensus	Ala	X	Asp/ Asn	X	X	-	Thr	Gly	Asp/ Glu	X	X	Trp
GDH/ADH Consensus	Ala	X	Asp/ Asn	X	X	-	Thr	Gly	Lys	X	X	Trp

Fig. 4. The consensus sequences in the tryptophan docking motif (Ghosh et al., 1995). This 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 quinohaemoprotein alcohol dehydrogenase (ADH) and glucose dehydrogenase, which is a membrane quinoprotein (GDH).

because the structure of the superbarrel domain of this enzyme can be superimposed on that of methanol dehydrogenase.

The 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.

2.3. The structure of the β-subunit of methanol dehydrogenase

The β subunit (Fig. 2) is most unusual as it forms a very extended structure having no hydrophobic core. The N-terminal 30 residues, which include one intrachain disulphide bridge (Cys6–Cys12) and a proline-rich segment (residues 14–20), is folded in a series of open turns. The C-terminal 54 residues, rich in charged residues, form a single straight α-helix of 30 residues in seven turns followed by a C-terminal segment folded back on the helix. 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,

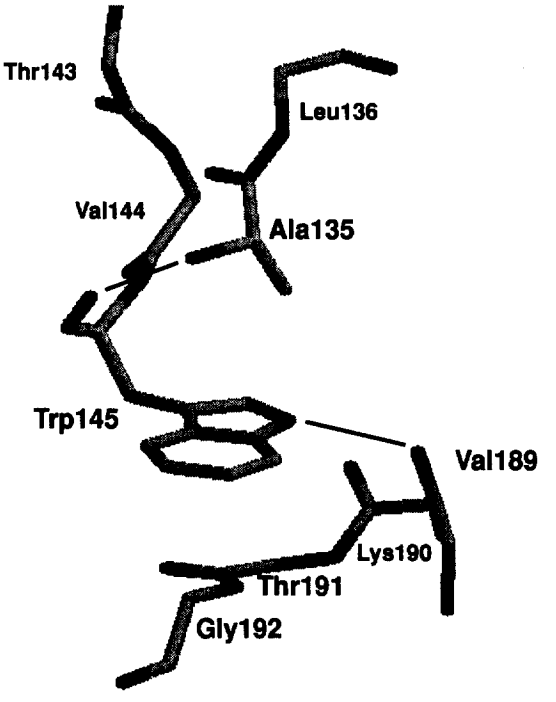


Fig. 5. Part of a typical tryptophan-docking motif. This shows the interactions of Trp 145 (in W2) with Ala135 (also in W2), and the interaction with the plane of the peptide bond between Thr191 and Gly192 (in W3). For clarity the remaining residues of the two motifs are omitted, and the side chains are omitted from all residues except for Trp145 and Ala135.

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 W1–W4 motifs of the α-chain with ion pair interactions involving Glu148, Glu193, Arg197, Lys236, Glu267 and Glu301 on the α-chain, with Lys16, Glu48, Arg50, Arg54, and Lys59 of the β-chain. 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 PQQ-containing quinoproteins, however, perhaps indicates that it has a more specific (unknown) function.

2.4. The novel disulphide ring structure in the active site of methanol dehydrogenase

Within the α subunit there is a remarkable novel structure derived by formation of a disulphide bridge between adjacent cysteine residues (Cys103–Cys104); the result is a strained 8-membered ring (Fig. 6). 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 has been predicted that if such a structure should unexpectedly exist in a protein then a strained ring would be produced in which a normal planar *trans* peptide bond would not be possible, and that a *cis* peptide

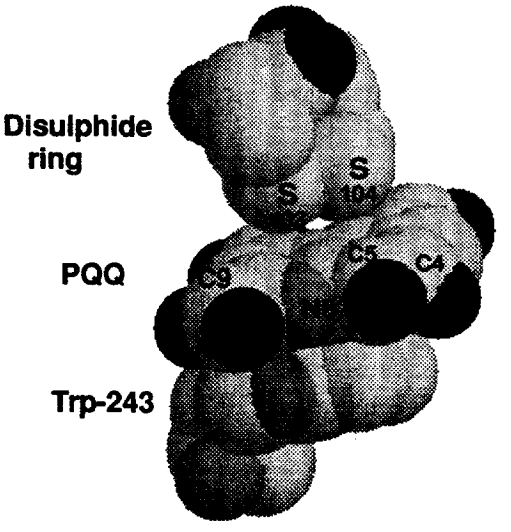


Fig. 6. The novel disulphide ring in the active site of methanol dehydrogenase (Ghosh et al., 1995). The ring is formed by disulphide bond formation between adjacent cysteine residues. The PQQ is 'sandwiched' between this ring and the tryptophan that forms the floor of the active site chamber. The calcium 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.

bond would be more likely (Ramachandran and Sasisekharan, 1968; Chandrasekaran and Balasubramanian, 1969). In our 1.94Å structure of the enzyme it is clearly seen that the peptide bond is, as predicted, non-planar, but it is in the *trans* configuration. 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); the distortion in the polypeptide chain within the disulphide ring structure is thus different from that observed in the isolated Cys–Cys dipeptide model compounds (Capasso et al., 1977; Mez, 1974; Sukumaran et al., 1991).

2.5. The active site of methanol dehydrogenase

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 between the B and C strands of motifs W6 and W8 which fold over the end of the superbarrel to enclose the active site chamber. 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 non-covalently bound PQQ, a bound Ca^{2+} ion, the novel disulphide ring structure and a potential active site base (Asp-303). There is no obvious interaction between the two active sites in the two α subunits, and the β subunits make no direct contribution to the structure of the active sites.

The PQQ is held in place by way of two non-polar axial interactions, the PQQ ring being sandwiched between the indole ring of Trp243 and the disulphide ring structure (Fig. 6). The indole ring is within 15° of coplanarity with the PQQ ring and in contact with it and, on

the opposite side the two sulphur atoms of the disulphide bridge, are within 3.75Å 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 (Fig. 7). Although the number of polar groups involved might indicate at first sight that the environment of the PQQ is polar, earlier ENDOR experiments had previously indicated a relatively hydrophobic environment for PQQ and a closer look at the interactions confirms this. 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 that at least one is protonated, their interaction thus being stabilised through hydrogen bond formation.

Of particular interest are the interactions of the C4 and C5 oxygen atoms; whereas the C5 oxygen is in the plane of the PQQ ring system, the C4 oxygen appears to be out of the plane by about 40° (Ghosh et al., 1995). This is inconsistent with PQQ being in either the oxidised quinone form or the reduced quinol form; this is consistent with the observation that, when isolated, the PQQ is always in the semiquinone state (Duine and Frank, 1980; Frank et al.,

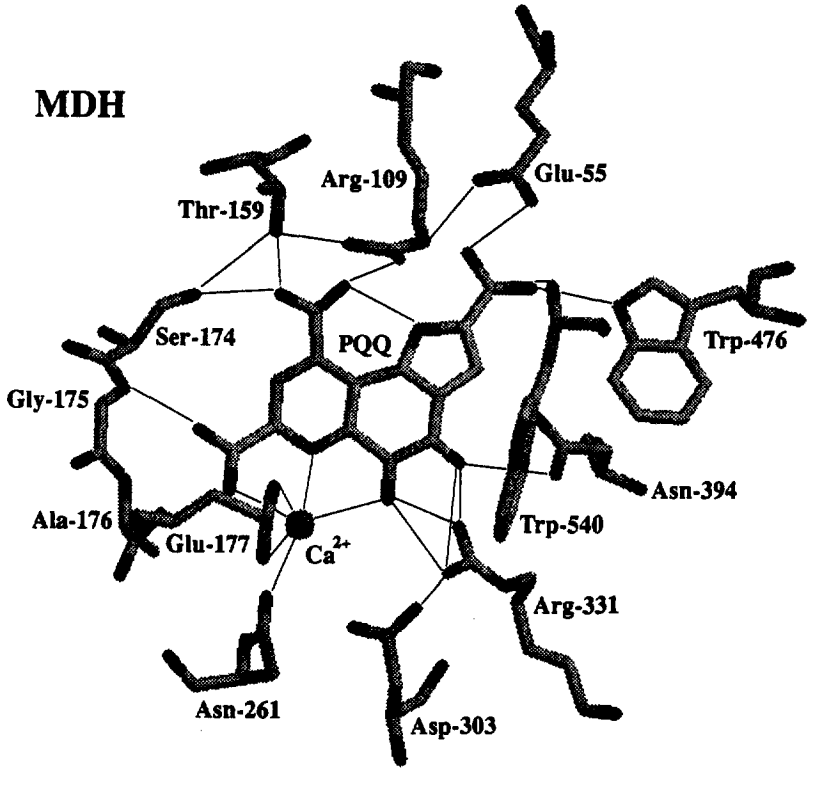


Fig. 7. The equatorial interactions of PQQ and the coordination of Ca^{2+} in the active site of methanol dehydrogenae (Ghosh et al., 1995). This figure also shows Asp303, which is likely to act as a base, and Arg331 which may also be involved in the mechanism. Figure 6 shows the axial interactions that are also involved in holding PQQ in place in the active site.

1988; Dijkstra et al., 1989; Richardson and Anthony, 1992; Avezoux et al., 1995). The C4 and C5 oxygens are hydrogen bonded by the NH_1 and NH_2 atoms respectively of Arg331, and in addition the C4 oxygen makes a longer hydrogen bond interaction with the amide NH_2 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 NH_2 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 some catalytic mechanisms previously proposed for MDH (Anthony, 1996, 1997).

A Ca^{2+} ion is clearly seen in the active site, confirming predictions that it is likely to be intimately related to the prosthetic group (Richardson and Anthony, 1992). The co-ordination sphere of the calcium ion in the active site contains PQQ and protein atoms (Fig. 7), 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 2.45Å from the metal ion. The five oxygen ligands have distances from the Ca^{2+} of 2.4–2.8Å.

The position of the substrate in the active site has not been unequivocally determined; the one or two solvent 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). Immediately adjacent to the more polar region containing the active site base there is a hydrophobic cavity, which could accommodate a small alkyl group, bounded by two tryptophans, a leucine and 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 understanding of this awaits solution of a structure containing one of these larger substrates.

2.6. The mechanism of methanol dehydrogenase

The enzyme catalyses a Ping–Pong reaction, consistent with reduction of PQQ by substrate and release of product, 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 free radical semiquinone (Duine and Frank, 1980; Frank et al., 1988; Dijkstra et al., 1989). The rate-limiting step is the conversion of the oxidised complex, containing the substrate, into the reduced enzyme plus product, and is the only step requiring the activator ammonia.

The C-5 carbonyl of isolated PQQ is very reactive towards nucleophilic reagents, such as methanol, leading to the obvious conclusion that a covalent PQQ-substrate complex may be important in the reaction mechanism. Support for this has come from the reaction of MDH with cyclopropanol which gives a C-5 propanal adduct, indicating that the mechanism consists of proton abstraction by a base, giving a ring-opened carbanion, which then attacks the electrophilic C-5 of PQQ (Frank et al., 1989a,b). It was suggested that during oxidation of methanol a similar proton abstraction must occur, followed by formation of a carbon/oxygen

bond to give a hemiketal intermediate. It is probable that Asp-303 (Fig. 7) provides the catalytic base which initiates the reaction by abstraction of a proton from the alcohol substrate (Fig. 8). In the mechanism shown in Fig. 7 the oxyanion produced by proton abstraction attacks the electrophilic C-5, leading to formation of the proposed hemiketal intermediate, the subsequent reduction of the PQQ with release of product aldehyde being facilitated by prior ionization of the hemiketal complex which might involve the pyrrole N atom. An alternative mechanism, 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 (Fig. 9). 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, although the mechanism of this activation is not understood (Frank et al., 1988; Goodwin and Anthony, 1996). Recent studies using PQQ analogues bonded to Ca^{2+} in organic solvents have provided supporting evidence for mechanisms involving hemiketal formation (Fig. 8) (Itoh et al., 1997).

In the mechanisms described here the Ca^{2+} ion is given a role in addition to a 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 coordination to the C-5 carbonyl oxygen of PQQ thus stabilising the electrophilic C-5 for attack by an oxyanion or hydride (Anthony et al., 1994; Anthony,

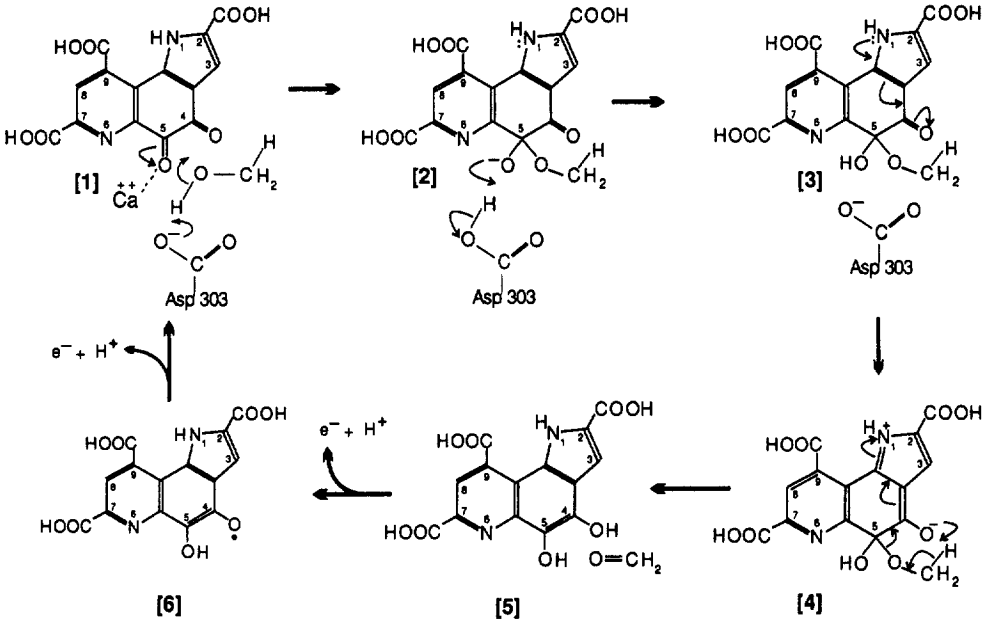


Fig. 8. Mechanism for methanol dehydrogenase involving formation of a hemiketal intermediate (Anthony, 1996, 1997). It is suggested that the base (Asp303) abstracts a proton from methanol and that the Ca^{2+} ion facilitates attack by the resulting oxyanion on the electrophilic C-5, to give the hemiketal from which the methyl proton is abstracted; this is facilitated by ionizationof the C-4 carbonyl oxygen which is made possible by the pyrrole nitrogen atom. The oxidative part of the cycle involves electron transfer to cytochrome c_L or an artifical dye electron acceptor.

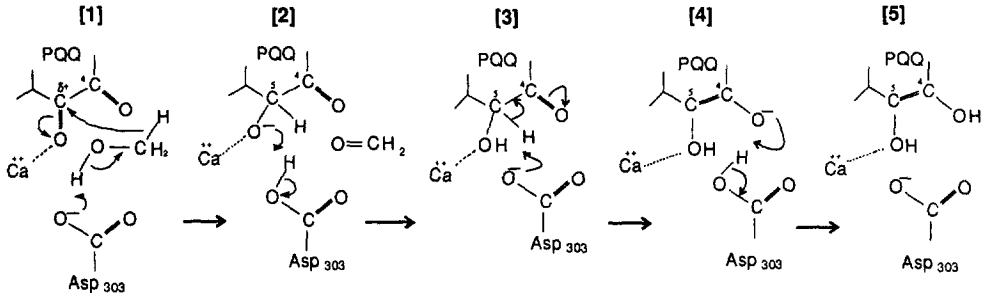


Fig. 9. An alternative hydride transfer mechanism. The initial proton abstraction is the same but the electrophilic C-5 is involved directly in removal of the methyl hydrogen as hydride. This mechanism was adapted from those previously published (Anthony et al., 1994a) in order to emphase the probable double involvement of the active site base (Asp303). The oxidative phase is the same as in Figure 8.

1996, 1997). It is also possible that the Ca^{2+} ion coordinates to the substrate oxygen atom. The role of Ca^{2+} in the mechanism has been given support by a study of a Sr^{2+}-containing methanol dehydrogenase produced by growing bacteria in a high concentration of Sr^{2+} (Harris and Davidson, 1994), and by investigations using an active enzyme containing Ba^{2+} instead of Ca^{2+} (Goodwin and Anthony, 1996). This is the first example of an enzyme in which barium plays an active catalytic role; the modified enzyme has a relatively low affinity for methanol (K_m, 3.4 mM instead of 10 μM), and for its activator ammonia, but its activation energy is half (and its V_{max} twice) that of the normal Ca^{2+} enzyme. We have suggested that this may be due to a change in conformation at the active site, leading to a decrease in free energy of binding and hence to a decrease in activation energy (Goodwin and Anthony, 1996). It should be noted that, as an alternative to Ca^{2+} acting as activator of the C5 atom, it has been suggested that Arg-331, which hydrogen bonds to the C5 oxygen, might be involved as an electrophile leading to build up of positive charge density on this oxygen (Xia et al., 1996).

2.7. The reaction of methanol dehydrogenase with its electron acceptor, cytochrome c_L

Cytochrome c_L is a specific large acidic *c*-type cytochromes (Nunn and Anthony, 1988; Anthony, 1992; Chen et al., 1994). There is considerable evidence that the dehydrogenase and cytochrome c_L 'dock' together, initially by electrostatic interactions between a small number of lysyl residues on the dehydrogenase and carboxylates on the cytochrome (Dijkstra et al., 1989; Anthony, 1992; Chan and Anthony, 1991; Cox et al., 1992). A study of this initial interaction confirmed the role of electrostatic inter actions but surprisingly showed that it is not inhibited by 50 μM-EDTA which is sufficient to inhibit the overall electron transfer process between the proteins (Dales and Anthony, 1995). It is possible, therefore, that EDTA inhibits 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 (Harris and Davidson, 1993; Anthony et al., 1994; Ghosh et al., 1995; Dales and Anthony, 1995; Harris et al., 1994).

Electron transfer from the quinol form of PQQ to the cytochrome electron acceptor occurs in two single electron transfer steps—the semiquinone form of PQQ being produced after the first of these transfers (Fig. 8). The protons are released from the reduced PQQ into the periplasmic space thus contributing to the protonmotive force (Anthony, 1988, 1993b). An obvious candidate as an intermediary in this process is the novel disulphide bridge between adjacent cysteines in the active site. This possibility appeared to be supported by the demonstration that this novel structure is very readily reduced with dithiothreitol, yielding enzyme that is inactive with cytochrome. However, reaction of the inactive reduced enzyme with iodoacetate produces carboxymethylated cysteine residues that would not be available to take part in oxidation/reduction reactions, and yet this carboxymethylation leads to re-formation of active enzyme (Avezoux et al., 1995). This type of disulphide ring structure has not been observed previously in an active enzyme and its rarity would suggest some special biological function. It is not present in the quinoprotein glucose dehydrogenase in which electrons are transferred to membrane ubiquinone from the quinol $PQQH_2$, and in which the semiquinone free radical is unlikely to be involved as a stable intermediate. It has been suggested, therefore, that this novel structure might function in the stabilization or protection from solvent at the entrance to the active site of the free radical PQQ semiquinone in methanol dehydrogenase (Avezoux et al., 1995).

3. The PQQ-containing dehydrogenase for alcohols

There are three types of PQQ-containing alcohol dehydrogenase that are distinct from methanol dehydrogenase (Matsushita and Adachi, 1993) but they all contain a calcium ion, and the reductive parts of their mechanisms are likely to be similar. The first type, such as that in *Pseudomonas aeruginosa* is almost identical except for its substrate specificity (Schrover et al., 1993). The other two types are quinohaemoproteins, having an in-built electron acceptor in the form of haem C which occurs on a C-terminal extension of the primary sequence. This is illustrated in Fig. 10, which also shows the membrane glucose dehydrogenase with its N-terminal additional sequence likely to be involved in binding the enzyme in the membrane.

The quinohaemoprotein alcohol dehydrogenase from acetic acid bacteria is membrane-bound and contains three types of subunit but no subunit equivalent to the small *v* subunit of methanol dehydrogenase (Matsushita and Adachi, 1993; Matsushita et al., 1994). The primary sequence of the catalytic subunit shows an *N*-terminal region (600 residues) with an additional *C*-terminal extension containing a haem-binding site (Inoue et al., 1989, 1990). In the *N*-terminal region there was 31% identity to the sequence of methanol dehydrogenase and it was possible to model its structure using the coordinates of methanol dehydrogenase (Fig. 11) (Cozier et al., 1995). Although there are considerable differences in the external loops, particularly those involved in the formation of the shallow funnel leading to the active site, the active site region was highly conserved, including the tryptophan and the disulphide ring on opposite sides of the plane of the PQQ, and also most of equatorial coordinations to the PQQ (Fig. 7). Especially important with respect to the mechanism was conservation of the active site base (Asp-303 in methanol dehydrogenase) and all the coordinations to the calcium

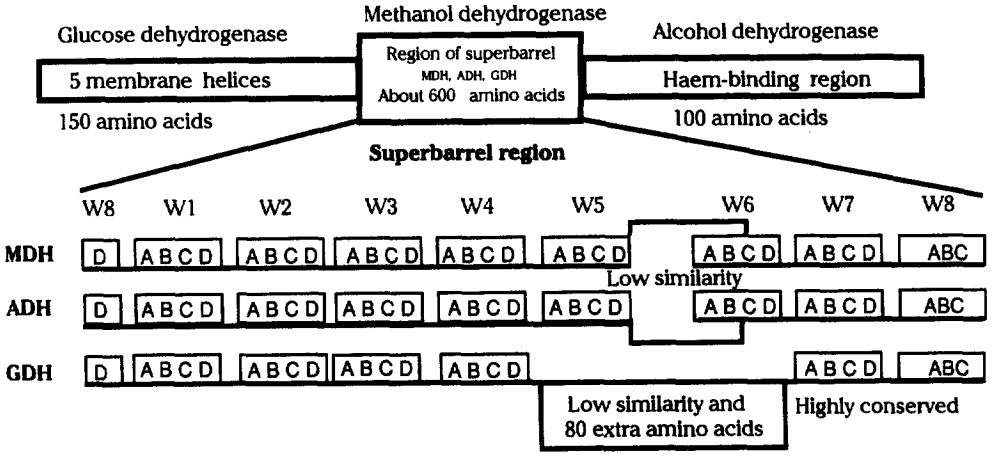


Fig. 10. Sequence alignment of quinoprotein dehydrogenases. Each 'W' is a 4-stranded β-sheet (or propeller blade). These are the regions showing greatest similarity of sequence between the quinoproteins. There are many loops between, and within, the β-sheets which show least similarity. for example, 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 end of the D-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 and the reason for the high level of conservation is not understood.

ion. This suggests that the mechanism of this alcohol dehydrogenase is essentially similar to that of the methanol dehydrogenase. Comparison of the protein sequence of the soluble quinohaemoprotein ethanol dehydrogenase from *Comomonas testosteroni* leads to a similar conclusion for that enzyme (Stoorvogel et al., 1996).

4. The PQQ-containing membrane-bound glucose dehydrogenase

The membrane-bound glucose dehydrogenase catalyses the oxidation of the pyranose form of D-glucose (at the C1 position) and other monosaccharides to the lactone. The reaction occurs in the periplasm and the electron acceptor is ubiquinone in the membrane (Matsushita et al., 1986, 1989). It is an intrinsic monomeric membrane protein (about 87 kDa) in which a divalent cation is necessary for reconstitution of active enzyme from apoenzyme plus PQQ. The *N*-terminal region (residues 1–154) forms a membrane anchor with 5 trans-membrane segments, and this region is likely to contain the ubiquinone-binding site. The remaining periplasmic region (residues 155–796) shows 26% identity of sequence to that of the α-subunit of methanol dehydrogenase and it has been possible to model its structure using the coordinates of methanol dehydrogenase (Fig. 11) (Cozier and Anthony, 1995). In the model structure, the novel disulphide ring is replaced by a histidine residue which maintains the position of PQQ in the active site, consistent with the previous demonstration that a histidine residue is essential for binding PQQ (Imanaga, 1989) (Fig. 12). There are fewer equatorial interactions between the protein and PQQ (Fig. 13), perhaps explaining why it is possible to effect the reversible dissociation of PQQ from the glucose dehydrogenase but not from

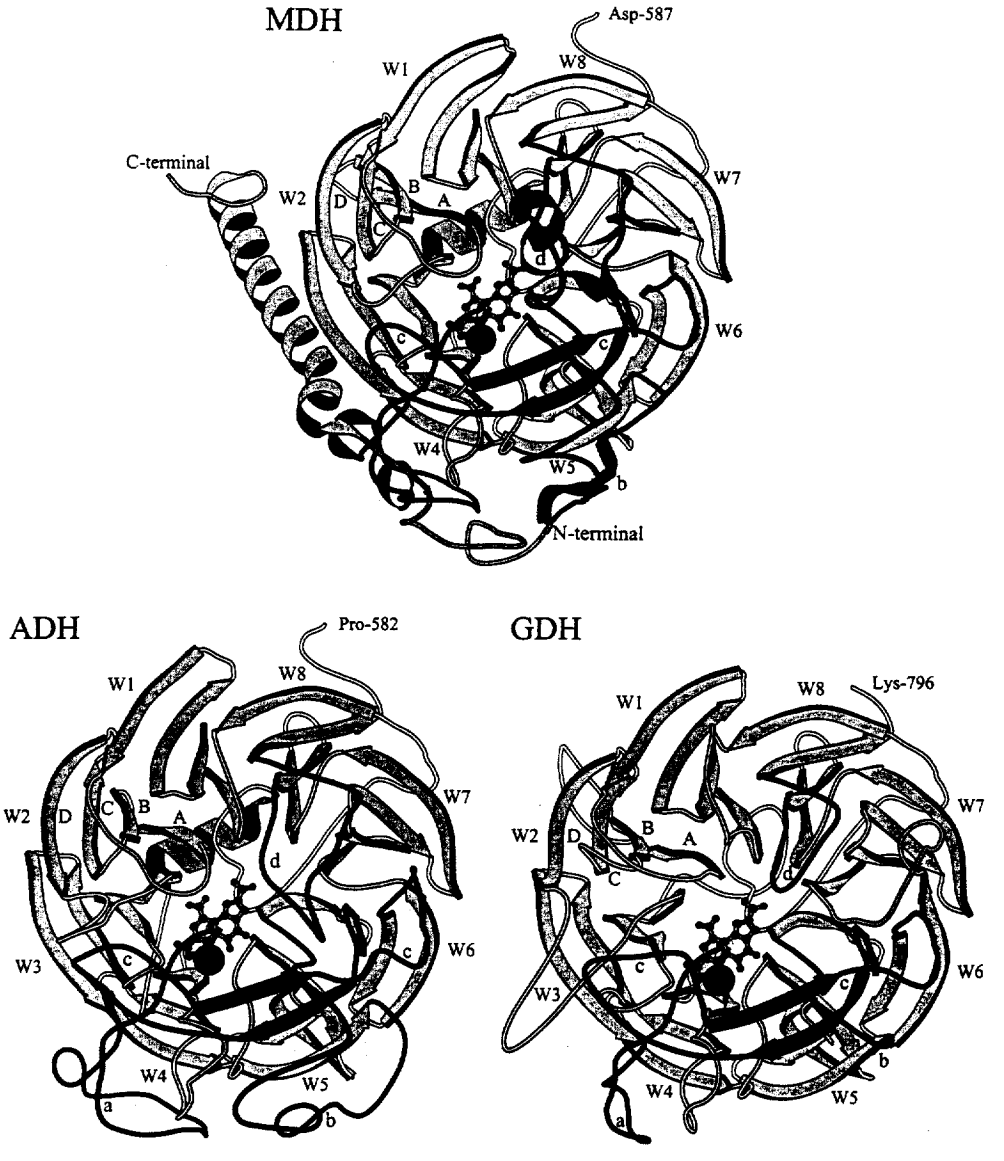


Fig. 11. Schematic representation of the backbone of glucose dehydrogenase (GDH), methanol dehydrogenase (MDH) and alcohol dehydrogenase (ADH) showing their major secondary structure. The MDH was determined by X-ray diffraction (Ghosh et al., 1995).The model GDH structure is of the C-terminal section of the membrane-bound GDH (residues 155–796) (Cozier and Anthony, 1995). The model ADH structure is of the N-terminal region of the quinohaemoprotein subunit I of the membrane complex isolated from acetic acid bacteria (residues 1–590) (Cozier et al., 1995). The prosthetic group is shown as a ball and stick structure, and the Ca^{2+} as a van der Waal's sphere. The major loops are in black. The position marked (h) shows the position where residues 497–579 (GDH) would join the main superbarrel structure. These residues are not present in MDH or ADH and the sequence on GDH is too long to model.

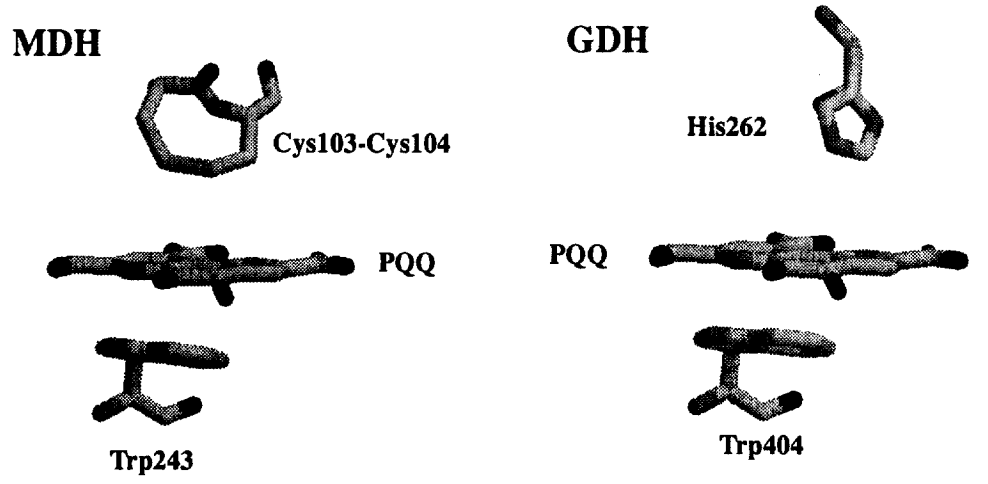


Fig. 12. Comparison of the stacking interactions of the PQQ in MDH and the model GDH. In MDH the PQQ is stacked between the coplanar Trp-243 and the disulphide ring system of Cys103 and Cys104 (Ghosh et al., 1995). In GDH the coplanar tryptophan is retained (Trp-404) but the disulphide is not conserved. Instead, His-262 may perform a similar role in helping to bind the PQQ into the active site region.

methanol dehydrogenase (Ameyama et al., 1985; Matsushita and Adachi, 1993). By analogy with the methanol dehydrogenase structure Asp466 is likely to be involved in base catalysis. One clear difference is that there is more 'space' in the glucose dehydrogenase active site, perhaps to accommodate the larger substrate, and the Arg331 in MDH which may play a role in catalysis is replaced by Lys493 in glucose dehydrogenase (Cozier and Anthony, 1995). The ligation of the (presumed) calcium is similar, suggesting that it plays a similar role in the two enzymes—that of a Lewis acid through coordination to the C-5 carbonyl oxygen, thus stabilising the electrophilic C-5 of PQQ. The proposed active site base is conserved, suggesting that the reaction is initiated by abstraction of a proton from the anomeric hydroxyl of the pyranose ring. This would be followed by attack by the resulting oxyanion to form a hemiketal intermediate; or attack by a hydride from the glucose oxyanion, leading directly to formation of the lactone and the quinol form of PQQ. When active holoenzyme is reconstituted from inactive apoenzyme plus PQQ a divalent metal is required; for this purpose Mg^{2+} is usually better than Ca^{2+} (Ameyama et al., 1985; Buurman et al., 1994). If this reconstitution leads to incorporation of Mg^{2+} instead of Ca^{2+} at the active site then this might imply some difference in mechanism between the glucose dehydrogenase and methanol dehydrogenase.

The oxidative half reaction of glucose dehydrogenase is completely different from that in methanol dehydrogenase in which there must be two single electron transfers to two separate cytochrome molecules. By contrast, in glucose dehydrogenase two hydrogen atoms must be transferred to the acceptor ubiquinone. Although this may also involve transfer of the electrons one at a time it is not necessary for a stable semiquinone to be formed, and indeed no semiquinone has ever been observed in glucose dehydrogenase. The active site funnel is not hydrophobic and there is no suggestion from the model structure or from the primary sequence that there is a hydrophobic region of the protein that could interact with the membrane except the N-terminal transmembrane segments.

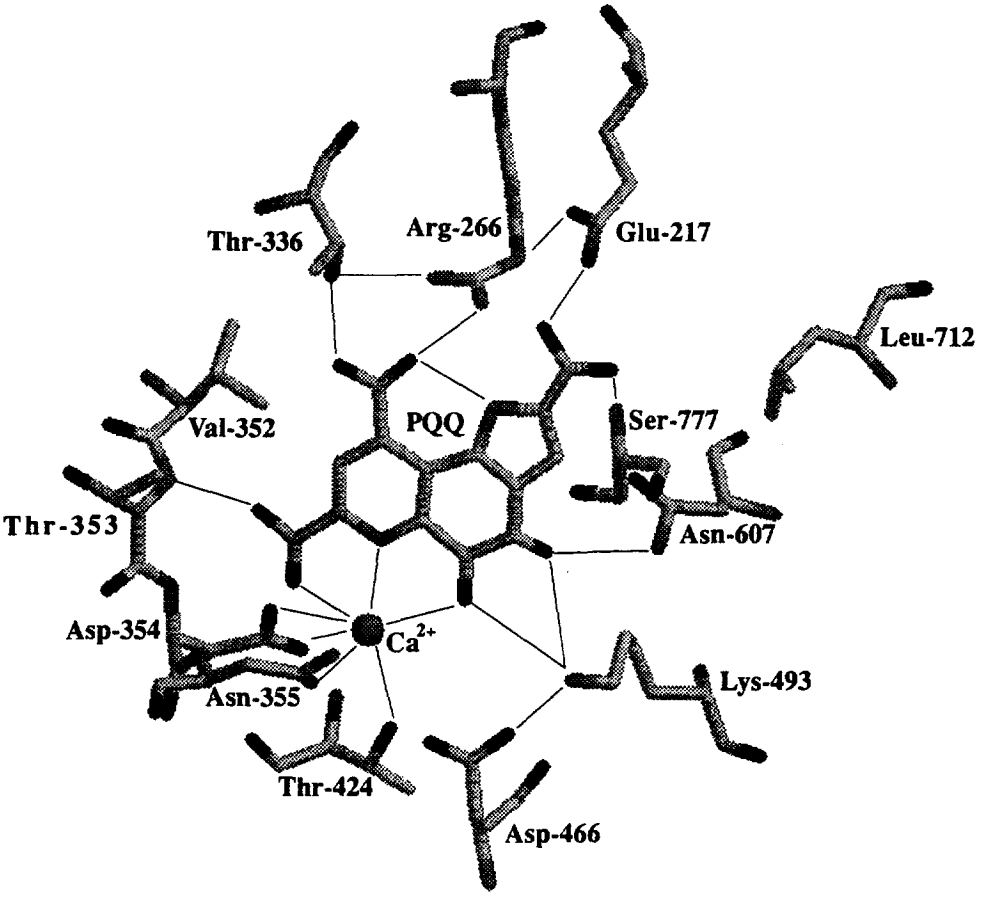


Fig. 13. The coordination of Ca^{2+} and the bonding of PQQ in the active site of the model glucose dehydrogenase. Of the equatorial interactions with PQQ the significant differences between the two enzymes are that residues Ser-174, Arg-331 and Trp-476 of MDH are replaced by Val-352, Lys-493 and Leu-712 in GDH; this results in fewer H-bonds to the PQQ in GDH. Ca^{2+} is included in the model GDH, although this may be replaced by Mg^{2+} in the GDH from some bacteria. By analogy with the mechanism proposed for MDH (Anthony, 1993a, 1996), Asp-466 may act as a base, initiating the reaction by abstraction of a proton from glucose; in this mechanism the Ca^{2+} acts as a Lewis acid, co-ordinating with the C-5 carbonyl oxygen, stabilising the electrophilic C-5 carbon of PQQ.

5. Future prospects

Now that the structure of the PQQ-containing quinoprotein methanol dehydrogenase has been determined, the most important remaining questions concern details of the mechanism of its action, including the role of the activator ammonia, the 'docking' system for interaction with its specific cytochrome, and determination of the route for electron transfer within the enzyme and the transfer of electrons to the cytochrome. Similar studies will be extended to the related alcohol and glucose dehydrogenases. The approaches to address these questions involve analysis of structures of multiprotein complexes, and of proteins containing activator and substrate, and the use of site directed mutagenesis together with kinetic and structural studies of the mutant proteins.

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