

Chapter 10

The PQQ-Containing Quinoprotein Dehydrogenases

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Summary

The quinoprotein dehydrogenases, which only occur in bacteria, have either modified tryptophan side chains forming the prosthetic groups (TTQ or CTQ), or non-covalently-bound pyrroloquinoline quinone (PQQ). The TTQ and CTQ dehydrogenases oxidize amines and the PQQ dehydrogenases oxidize alcohols and aldoses. The reactions are catalysed in the periplasm, either by membrane-bound enzymes, passing electrons to ubiquinone, or by soluble dehydrogenases, reacting with *c*-type cytochromes or blue copper proteins.

This review concentrates on PQQ-containing dehydrogenases. There are three classes of alcohol dehydrogenases. Type I are soluble and contain a single PQQ prosthetic group. Type II are soluble quinohemoproteins, having a C-terminal extension containing heme *c*. Type III are similar but have two additional subunits (including a multiheme cytochrome *c*), bound to the periplasmic membrane. There are two types of glucose dehydrogenase; an atypical soluble quinoprotein whose function is not understood a more widely-distributed glucose dehydrogenase, bound to the membrane by transmembrane helices at the N-terminus.

The X-ray structures show that they all have a propeller fold with 'propeller blades' made up of [*beta*]-sheets; soluble glucose dehydrogenase (sGDH) has six blades and all the others have eight. The structures provide considerable support to the suggestion that they all function by a general base-catalysis, hydride transfer

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mechanism, facilitated by bonding of a Ca^{2+} ion and an arginine or lysine to a carbonyl O atom of the PQQ. Of particular interest is the problem of electron flow from the prosthetic groups in the dehydrogenases to their electron acceptors. The quinohemoprotein alcohol dehydrogenases offer an excellent opportunity to investigate the problem of intra-protein electron flow, indicating that the novel disulphide ring structure seen in the active sites of the alcohol dehydrogenases plays an essential role in the flow of electrons from the reduced PQQ and heme *c*. The development of site-directed mutagenesis of the quinoproteins offers the challenge of further integrating the structure and function of this important group of enzymes.

I. Introduction

The term quinoprotein was coined in about 1980 to include a number of bacterial dehydrogenases which contain pyrroloquinoline quinone (PQQ) as their prosthetic group (Duine et al. 1979, 1980) (Fig. 1). The name is now used more widely to include all those enzymes whose catalytic mechanisms involve quinone-containing prosthetic groups in their active sites (Anthony, 1996). They have been recognized as the third most widespread class of redox enzymes after the pyridine nucleotide and flavin-dependent dehydrogenases and, like these enzymes, they are usually assayed with artificial electron acceptors such as phenazine ethosulphate or, in the case of quinohemoproteins which also have a heme prosthetic group, ferricyanide. The dehydrogenases for alcohols and aldoses contain PQQ, whereas the amine dehydrogenases contain prosthetic groups derived from modified tryptophan residues: TTQ (tryptophan tryptophylquinone) (Davidson, 1993a, 2000) or CTQ (cysteine tryptophylquinone) (Satoh et al., 2002) (Fig. 1). By contrast with TTQ and CTQ, which are derived from the amino acid chain of the proteins, PQQ is synthesized separately and is bound non-covalently into the active site of the PQQ-containing quinoproteins.

The main subjects of this review are the PQQ-containing dehydrogenases (Table 1). The history of these quinoproteins began in the 1960s with the characterization of the novel prosthetic groups of glucose dehydrogenase (GDH) (Hauge, 1964) and methanol dehydrogenase (MDH) (Anthony and Zatman, 1967). Duine, Frank and co-workers subsequently demonstrated that MDH contains a quinone structure with 2 nitrogen atoms (Duine et

al., 1979; Duine and Frank, 1980), and Kennard's group showed this to be pyrroloquinoline 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).

Table 1 lists the quinoprotein dehydrogenases. Among the most comprehensively studied of these enzymes are the three classes of PQQ-containing quinoprotein alcohol dehydrogenases; Type I are soluble, periplasmic enzymes containing a single PQQ prosthetic group; this group includes the methanol dehydrogenase of methylotrophs. Type II dehydrogenases are soluble, periplasmic, quinohemoproteins, having a C-terminal extension containing heme C. Type III dehydrogenases have similar quinohemoprotein subunits but have two additional subunits (one of which is a multiheme cytochrome *c*), bound in an unusual way to the periplasmic membrane. There are two types of glucose dehydrogenase; an atypical soluble quinoprotein which has little sequence similarity to the other dehydrogenases and whose function, if any, in energy transduction is not understood. The more widely-distributed glucose dehydrogenases are integral membrane proteins, bound to the membrane by transmembrane helices at the N-terminus.

The PQQ-dependent dehydrogenases are all involved in the oxidation of alcohols and aldoses in the periplasm of Gram-negative bacteria. This contrasts with oxidation systems involving the membrane flavoproteins which catalyze reactions on the inner face of the cytoplasmic membrane. The reason for the periplasmic location of the quinoprotein dehydrogenases is unclear, but it presumably relates to their nature and/or to their function. It might be that active enzymes must be assembled outside the cell because of some aspect of PQQ incorporation, or because proper folding of the protein will not

Abbreviations: ADH – alcohol dehydrogenase; CTQ – cysteine tryptophylquinone; EDH – ethanol dehydrogenase; MDH – methanol dehydrogenase; mGDH – membrane glucose dehydrogenase; PQQ – pyrroloquinoline quinone; QH-ADH – quinohemoprotein alcohol dehydrogenase; sGDH – soluble GDH (in *Acinetobacter*); TTQ – tryptophan tryptophylquinone

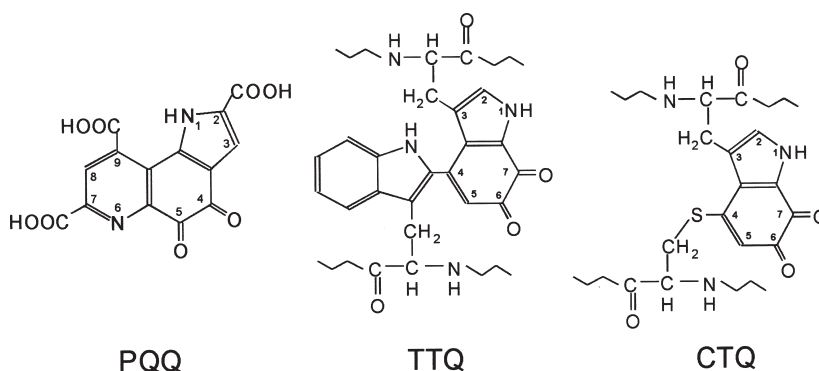


Fig. 1. The prosthetic groups of quinoprotein dehydrogenases. PQQ (pyrroloquinoline quinone) is the prosthetic group of dehydrogenases for alcohols and aldoses. TTQ (tryptophan tryptophylquinone) and CTQ (cysteine tryptophylquinone) are the prosthetic groups of amine dehydrogenases. See Table 1 for a list of these dehydrogenases. Not shown here are TPQ and LTQ which are the prosthetic groups of copper-containing amine oxidases (Anthony, 1996).

occur in a reducing environment. It is probable that all of the quinoproteins contain Ca^{2+} or Mg^{2+} , and perhaps their periplasmic location avoids the problem of transporting these ions into cells or of having high concentrations of them within the cells.

This chapter starts with a brief overview of the electron transport chains involving all the quinoprotein dehydrogenases, followed by an account of the structures and mechanisms of the better known examples of those having a PQQ prosthetic group. The respiratory chains of the acetic acid bacteria involve many soluble and membrane-bound quinoproteins and these are discussed in a separate Chapter of this book (Chapter 4, Matsushita Vol. 2) and useful surveys of biotechnological applications of the quinoproteins have been published (Davidson, 1993a). Reviews of the structure and mechanism of the TTQ-containing amine dehydrogenases have been published elsewhere (Davidson, 1993b, 2000; Anthony, 1996) and the structure of the CTQ-containing quinoxinoprotein amine dehydrogenase has been published by Satoh et al. (2002).

II. The Physiological Roles of the Quinoprotein Dehydrogenases and Their Electron Transport Chains

During growth on alcohols and amines as the sole source of carbon and energy the relevant periplasmic dehydrogenases usually play a key role in energy production, catalyzing oxidation of the substrate to the corresponding aldehyde, which is then either further oxidized to carbon dioxide or assimilated

into cell material. In the case of the acetic acid bacteria, however, the PQQ-dependent alcohol and aldose dehydrogenases catalyze the first step in the incomplete oxidation of the energy source and most of the products of these oxidations are released into the growth medium (Goodwin and Anthony, 1998; see also Chapter 4, Matsushita, Vol.2). The membrane glucose dehydrogenases sometimes play a role in energy production and (in enteric bacteria) they may also function in the rapid removal of potentially toxic oxygen during changes to nitrogen-fixing growth or to fermentative growth during which oxygen-labile enzymes are being synthesized in the presence of low concentrations of residual oxygen (Goodwin and Anthony, 1998).

Whatever the physiological role of a particular quinoprotein dehydrogenase, it must be coupled to an electron transport system which must be arranged so as to produce a protonmotive force across the inner cytoplasmic membrane to drive ATP synthesis by the membrane ATP synthase (Goodwin and Anthony, 1998). Electron transport chains involving the quinoproteins which are responsible for oxidation of alcohols, aldoses and amines are summarized in Fig. 2. The electron acceptor for the membrane-bound dehydrogenases is ubiquinone which is then oxidized by a quinol oxidase. By contrast, during the first step in the oxidation of alcohols and amines by the soluble dehydrogenases, electrons from the dehydrogenases are passed to the electron transport chain, while protons are liberated into the periplasm, thus contributing to the protonmotive force. The oxidase consumes protons on the inside face of the membrane and it may also act as a direct proton

Table 1. Summary of quinoprotein and quinohemoprotein dehydrogenases

Enzyme	Location	Prosthetic group	Electron acceptor	Organism ^(ref)
Type I Alcohol dehydrogenases				
Methanol dehydrogenase	Periplasm	PQQ	Cytochrome <i>c</i>	Methylophils ¹
Ethanol dehydrogenase	Periplasm	PQQ	Cytochrome <i>c</i>	<i>Pseudomonas sp.</i> ²
Type II alcohol dehydrogenases	Periplasm	PQQ heme C	Azurin	<i>Comamonas testosteroni</i> ³ <i>Pseudomonas putida</i> ⁴
Type III Alcohol dehydrogenases	Membrane	PQQ 4 hemes C	UQ	Acetic acid bacteria ⁵
Sorbitol dehydrogenase	Membrane	PQQ 4 hemes C	UQ?	Acetic acid bacteria ⁶
Membrane Glucose dehydrogenase (m-GDH)	Membrane	PQQ	UQ	Enteric bacteria ⁷ Acetic acid bacteria ⁸ <i>Acinetobacter calcoaceticus</i> ⁹
Soluble Glucose dehydrogenase (s-GDH)	Periplasm	PQQ	?	<i>Acinetobacter calcoaceticus</i> ¹⁰
Glycerol dehydrogenase*	Membrane	PQQ	UQ	Acetic acid bacteria ⁵
D-Arabitol dehydrogenase	Membrane	PQQ	UQ	Acetic acid bacteria ¹¹
D- Sorbitol dehydrogenase	Membrane	PQQ	UQ	Acetic acid bacteria ¹²
Lupanine hydroxylase	Periplasm	PQQ heme C	Cytochrome <i>c</i>	<i>Pseudomonas sp.</i> ¹³
Sorbose / sorbosone dehydrogenase	Periplasm	PQQ	Cytochrome <i>c</i>	Acetic acid bacteria ¹⁴
Methylamine dehydrogenase	Periplasm	TTQ	Amicyanin	Methylophils ¹⁵
Aromatic amine dehydrogenase	Periplasm	TTQ	Azurin	<i>Alcaligenes</i> ¹⁶
Amine dehydrogenase	Periplasm	CTQ 2 hemesC	Azurin	<i>Pseudomonas putida</i> ¹⁷ <i>Paracoccus denitrificans</i> ¹⁸

*Glycerol dehydrogenase in the membrane of acetic acid bacteria is able to oxidise a number of sugar alcohols including D-sorbitol and D-arabitol; this may be the same enzyme as the separate sorbitol and arabitol dehydrogenases described here (Matsushita et al., 2002, 2003). For general reviews covering most of these enzymes see: Davidson, 1993a; Anthony, 1996; Goodwin and Anthony, 1998; Matsushita et al., 2002, 2003. For references to preliminary descriptions of quinoprotein dehydrogenases for polyvinyl alcohol, polyethylene glycol, tetrahydrofurfuryl alcohol and quinate see review by Matsushita et al. (2002). For representative recent publications see: Davidson, 1993a¹⁵, 2000¹⁵; Choi et al., 1995⁶; Hyun and Davidson, 1995¹⁶; Anthony, 1996¹⁵, 2000¹; Adachi et al., 1998¹⁷; Hopper and Rogozinski, 1998¹³; Asakura and Hoshino, 1999¹⁴; Cozier et al., 1999⁷; Oubrie et al., 1999a¹⁰; Takagi et al., 1999¹⁸; Yoshida et al., 1999^{7,9}; Afolabi et al., 2001¹; Elias et al., 2000⁷, 2001⁷; Keitel et al., 2000²; Adachi et al., 2001¹¹; Datta et al., 2001¹⁸; Sugisawa and Hoshino, 2001¹²; Chen et al., 2002⁴; Matsushita et al., 2002⁵ 2003^{5,8}; Miyazaki et al., 2002¹²; Oubrie et al., 2002³; Satoh et al., 2002¹⁷; K. Matsushita, H. Toyama and O. Adachi, unpublished⁵.

Fig. 2. Bacterial electron transport chains involving quinoprotein dehydrogenases. References: a, Anthony (1992), Read et al. (1999); b, Schobert and Gorisch (1999); c, Matsushita et al. (1999); d, Matsushita et al. (1994, 2003); e, Matsushita et al. (1982b), Elias et al. (2001); f, Matsushita et al. (2002) and K. Matsushita, H. Toyama and O. Adachi (unpublished); g, Beardmore-Gray and Anthony (1986); h, Matsushita et al., (1989c); i, Anthony (1988, 1993b), Chen et al. (1994), Davidson (2000); j, Adachi et al. (1998); k, Takagi et al. (2001).

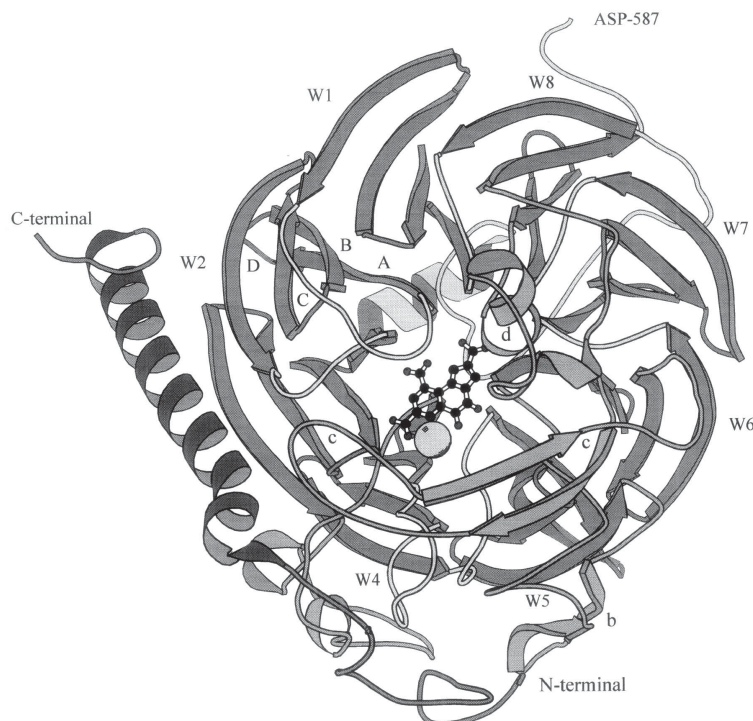


Fig. 3. The α/β unit of MDH looking down the pseudo 8-fold axis, simplified to show only the β -strands of the 'W' motifs of the α -chain, and the long α -helix of the β -chain, but excluding other limited β -structures and short α -helices (Ghosh et al., 1995). The PQQ prosthetic group is in skeletal 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. Figs. 7, 9 and 11 show the related structures of THE Type II quinohemoprotein AH-ADH, mGDH and the sGDH respectively.

pump. A critical point with respect to energy transduction is that all these periplasmic electron transport chains are similar to those operating in the oxidation of inorganic substrates in by-passing the low potential ubiquinone/cytochrome *b* parts of the chain and it likely that, during the first oxidation step, only about 1 mole of ATP is produced for every mole of substrate oxidized.

III. The Structures of the PQQ-Containing Quinoprotein Dehydrogenases

The primary sequences of mGDH, MDH and the other alcohol dehydrogenases show sufficient similarity to indicate some common structure and this has been confirmed by their X-ray structures. They all have a core structure similar to that first described for the α -subunit of MDH which contains the PQQ in the active site (Ghosh et al., 1995; Xia et al., 1996; Anthony and Ghosh, 1998). The quinoproteins have an additional C-terminal domain

containing heme C and the mGDH has an additional membrane-anchoring N-terminal sequence. The α -subunit of MDH is a superbarrel made up of eight topologically-identical four-stranded twisted anti-parallel β -sheets (W-shaped), stacked radially around a pseudo eight-fold symmetry axis running through the center of the subunit. This structure has been referred to as a propeller fold, each W motif representing a propeller blade (Fig. 3). The normal twist of the β -sheet enables space to be efficiently packed, this architecture allowing 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. A series of tryptophan-docking interactions (so far described only in these proteins) between the β sheet propeller blades stabilizes the structure (Xia et al., 1996; Anthony and Ghosh, 1998). The sequences of quinoproteins show the greatest similarity in the 11-residue motifs involved in the tryptophan docking

motifs and this has facilitated modeling of the structure of the quinoproteins whose structure has not been determined (e.g. mGDH and the Type III ADH (Cozier and Anthony, 1995; Cozier et al., 1995)).

The PQQ is within the center of the superbarrel, held by ionic interactions with the highly conserved amino acid side chains. In all cases where the X-ray structure has been determined there is a Ca^{2+} ion coordinated to three atoms of PQQ and to amino acid side chains. The Ca^{2+} is involved in the mechanism which is probably essentially the same in all quinoprotein dehydrogenases having a PQQ prosthetic group.

The amino acid sequence of the soluble GDH of *Acinetobacter calcoaceticus* shows very little similarity to that of the other dehydrogenases and its structure is, as expected, different; it will therefore be discussed separately from the others. Generalizations made about the quinoprotein structures do not necessarily apply to this protein, although the mechanism of all the dehydrogenases discussed here are likely to be similar in principle.

IV. The Dehydrogenases

This section describes the individual PQQ-containing dehydrogenases. It starts with a full account of methanol dehydrogenase which is the most extensively studied example, and much of the description of the other enzymes will be referred back to this.

A. Methanol Dehydrogenase: The Type I Alcohol Dehydrogenase of Methylobacteria

The MDH of methylotrophic bacteria oxidizes methanol to formaldehyde during growth of bacteria on methane or methanol (Anthony, 1982), during which its electron acceptor is a novel acidic cytochrome *c* (cytochrome c_L) (Anthony, 1992). MDH is also responsible for oxidation of ethanol to acetaldehyde during growth on ethanol. This enzyme has been reviewed extensively and these reviews should be used for full references to all aspects of this enzyme (Anthony, 1986, 1996, 2000; Anthony and Ghosh, 1998; Goodwin and Anthony, 1998). Using phenazine ethosulphate in a dye-linked assay system the pH optimum is about 9 and ammonia or methylamine is required as activator. MDH oxidizes a wide range of primary alcohols (very rarely

secondary alcohols), having a high affinity for these substrates; for example, the K_m for methanol is 5–20 μM . The pH optimum for the reaction with cytochrome c_L is 7.0, and ammonia is not usually required as activator.

1. The X-Ray Structure of Methanol Dehydrogenase

The X-ray structure has been determined for the MDH from *Methylobacterium extorquens* (Blake et al., 1994; Ghosh and Anthony, 1995; Afolabi et al., 2001), and from *Methylophilus* sp. (Xia et al., 1992, 1996, 1999; White et al., 1993; Zheng et al., 2001). MDH has an $\alpha_2\beta_2$ tetrameric structure; each α subunit (66 kDa) contains 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 (Fig. 3) (see Section III). The structure has several important novel features, including novel the ‘tryptophan-docking motifs’ that link together the eight beta sheets, and the presence in the active site of an unusual eight-membered disulphide ring structure formed from adjacent cysteine residues, joined by an atypical non-planar peptide bond. The PQQ is sandwiched between the indole ring of Trp243 and the disulphide ring structure (Fig. 4). The indole ring is within 15° of co-planarity with the PQQ ring and, on the opposite side, the two sulfur 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. Reduction of the disulphide bond leads to loss of activity but oxidation in air or carboxymethylation of the free thiols leads to return of activity. The activity of the carboxymethylated derivative rules out reduction to the thiols during the catalytic cycle. The disulphide ring is not present in the quinoprotein glucose dehydrogenase in which electrons are transferred to membrane ubiquinone from the quinol PQQH₂, and in which the semiquinone free radical is unlikely to be involved as a stable intermediate. It is possible, therefore, that this novel structure might function in the stabilization of the free radical PQQ semiquinone or its protection from solvent at the entrance to the active site in MDH (Blake et al., 1994; Avezoux et al., 1995). Recent work with the quinoxinoprotein (Type II) alcohol dehydrogenase suggests, however, that, although it

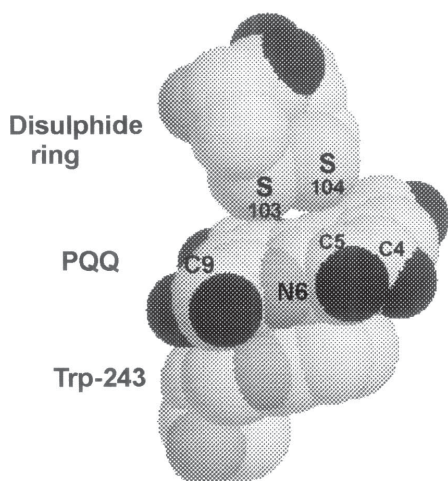


Fig. 4. The novel disulphide ring in the active site of methanol dehydrogenase (Ghosh et al. 1995). The ring is formed by disulphide bond formation between adjacent cysteine residues. The PQQ is 'sandwiched' between this ring and the tryptophan that forms the floor of the active site chamber. The calcium ion is coordinated between the C-9 carboxylate, the N-6 of the PQQ ring and the carbonyl oxygen at C-5. This structure is seen in all the alcohol dehydrogenases but not in aldose dehydrogenases.

does not become completely reduced, the disulphide ring is essential for intra-protein electron transfer in all the alcohol dehydrogenases (Oubrie et al., 2002).

In addition to the axial interactions, many amino acid residues are involved in equatorial interactions with the substituent groups of the PQQ ring system (Fig. 5). These are exclusively hydrogen-bond and ion-pair interactions. Although the number of polar groups involved might indicate at first sight that the environment of the PQQ is polar, this is not the case. An oxygen of the 9-carboxyl forms a salt bridge with Arg109 and both groups are shielded from bulk solvent by the disulphide. The carboxyl group of Glu155 and a 2-carboxyl oxygen of PQQ are also shielded from solvent and it is probable that at least one is protonated, their interaction thus being stabilized through hydrogen bond formation. The active site contains a single Ca^{++} ion whose co-ordination sphere contains PQQ and protein atoms, 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 (Fig. 5).

The C4 and C5 oxygen atoms, which become reduced during the catalytic cycle, are hydrogen bonded to Arg331, which 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.

2. The Mechanism of MDH

MDH 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 oxidized back to the quinone by way of the free radical semiquinone (Fig. 6). When isolated, the PQQ in the enzyme is in this semiquinone form. The rate-limiting step is the breaking of the methyl C-H bond, and it is this step that is affected by the unexplained activation by ammonia, and which leads to a large deuterium isotope effect (Frank et al., 1988; Goodwin and Anthony, 1996; Afolabi et al., 2001).

The first step in the reaction mechanism is likely to be abstraction of the proton from the alcohol group by an active site base, proposed to be Asp303 (Figs. 5 and 6). This proposal is supported by the properties of a modified MDH in which this aspartate was replaced by glutamate by site-directed mutagenesis (Afolabi et al., 2001).

The C-5 carbonyl of isolated PQQ is very reactive towards nucleophilic reagents, such as methanol, leading to the conclusion that a covalent PQQ-substrate complex (a hemiketal) may be important in the reaction mechanism (Frank et al., 1989). In the mechanism shown in Fig. 6a 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. Until recently, the balance of opinion tended to be in favor of this addition/elimination mechanism (Fig. 6a). The alternative proposal involves the same initial proton abstraction, but this is followed by a direct hydride transfer from the methyl group of methanol to the C-5 of PQQ (Anthony, 1993a, 1996, 2000; Anthony et al., 1994) (Fig. 6b). The evidence is strongly in favor of this hydride transfer mechanism in the case of the soluble glucose dehydrogenase (Oubrie et al., 1999a,b,c; Dewanti and Duine, 2000) and in the more closely related Type II alcohol dehydrogenase (Oubrie et al., 2002; Chen et al., 2002), and it has been argued that it is very likely that all these

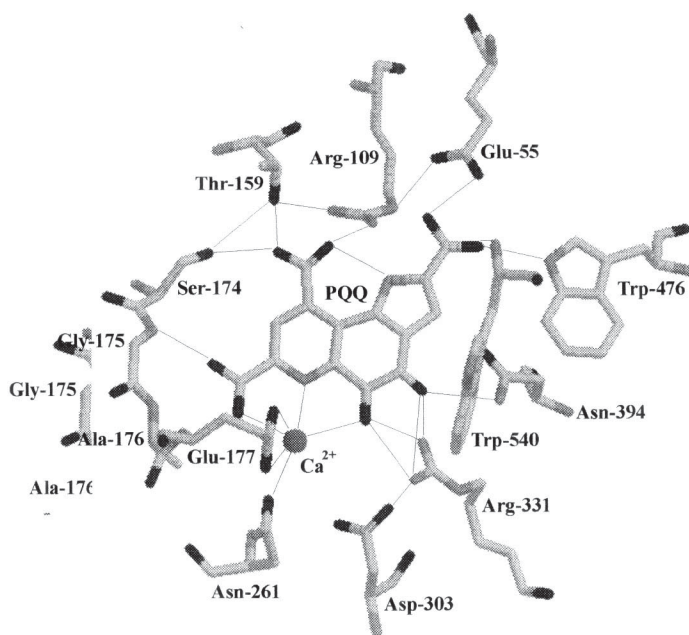


Fig. 5. The equatorial interactions of PQQ and the coordination of Ca^{2+} in the active site of methanol dehydrogenase (Ghosh et al., 1995). This Figure also shows Asp303, which is likely to act as the catalytic base, and Arg331 which may also be involved in the mechanism. The equatorial interactions of the quinohemoprotein alcohol dehydrogenase (QH-ADH) are almost identical to these, an important exception being that Arg331 is replaced by a lysine (Chen et al., 2002; Oubrie et al., 2002), as is also the case in mGDH (Fig. 10).

quinoprotein dehydrogenases have the same mechanism (Oubrie and Dijkstra, 2000). Recent structural, and quantum mechanical studies of MDH now also support the conclusion that the mechanism of MDH is our hydride transfer mechanism (Afolabi et al., 2001; Zheng et al., 2001).

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 stabilizing the electrophilic C-5 for attack by the hydride. In addition to the Ca^{2+} ion, the conserved arginine (Arg331) is also likely to be important in increasing the nucleophilicity of the C5 atom of PQQ. Ca^{2+} ions cannot usually be removed from MDH without its denaturation, but its role has been studied using mutants unable to incorporate Ca^{2+} into the enzyme. The assembly process occurs in the periplasm and requires specific processing proteins. In mutants lacking these proteins MDH is produced that contains PQQ but not Ca^{2+} , and incubation in high concentrations of Ca^{2+} are required to produce active enzyme (Goodwin et al., 1996). When Ba^{2+} ions are used, these are incorporated to produce

enzyme which has twice the maximum activity of the Ca^{2+} -MDH but with a much lower affinity for its substrates (Goodwin and Anthony, 1996).

The position of the substrate in the active site has not been determined. The hydroxyl of the methanol must necessarily be near enough to be hydrogen bonded to the side chain of Asp303 (the proposed active site base), and the methyl group must be near to the C5 of PQQ and within the hydrophobic cavity bounded by side chains of Trp265, Trp540 and Leu556 as well as the disulphide ring. This raises the problem that this enzyme has a broad substrate specificity, and can oxidize 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. The oxidation route for the reduced quinol form of PQQ is shown in Fig. 6a. The electrons are passed one at a time to the cytochrome c_L which is bound by carboxylate residues to lysyl residues on MDH (Anthony, 1992). The initial 'docking' of the two proteins is probably followed by movement of the cytochrome to its optimal position for electron transfer

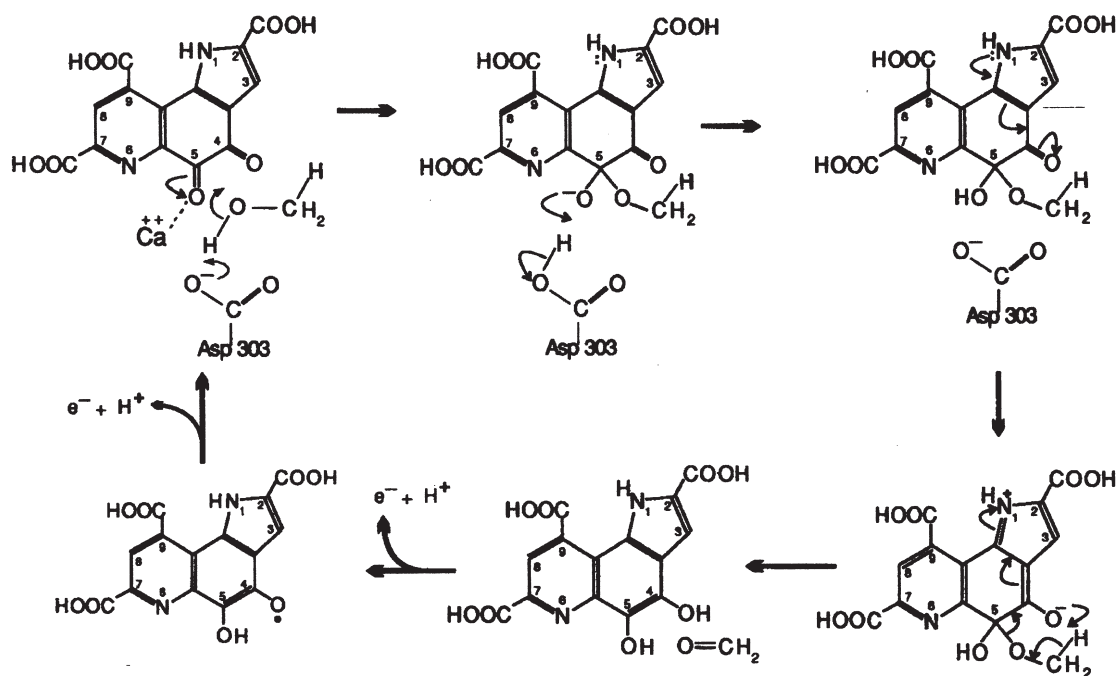
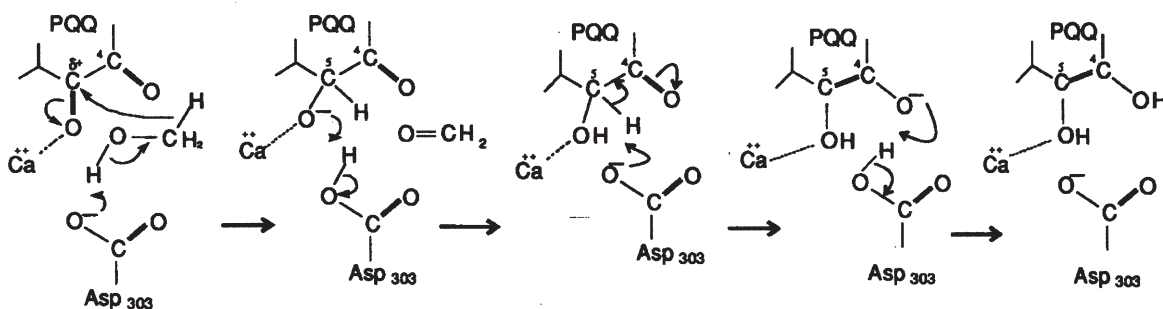
**Scheme a****Scheme b**

Fig. 6. The mechanisms proposed for methanol dehydrogenase. In both schemes the base (Asp303) abstracts a proton from methanol. The bonding of the Ca²⁺ ion and Arg331 to the O-5 of PQQ (Fig. 5) facilitates production of the electrophilic C5. The oxidative part of the cycle involves electron transfer to cytochrome *c*_L or an artificial dye electron acceptor. Scheme a involves formation of a hemiketal intermediate from which the methyl proton is abstracted; this is facilitated by ionization of the C-4 carbonyl oxygen which is made possible by the pyrrole nitrogen atom. Scheme b shows the alternative hydride transfer mechanism for which there is now more evidence, in which the electrophilic C-5 is involved directly in removal of the methyl hydrogen as hydride. This mechanism was adapted from that previously published (Anthony et al., 1994) in order to emphasize the possible double involvement of the active site base (Asp303). The same mechanism has now been shown in the Type II quinoxemoprotein and the sGDH.

(Dales and Anthony, 1995). 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 heme of cytochrome *c*_L. This route is likely to be similar to that calculated for

electrons in the Type II quinoxemoprotein alcohol dehydrogenase (QH-ADH) (Section C), involving water molecules, the conserved disulphide ring and Asp105 (Fig. 5). During the re-oxidation of reduced PQQ the protons from the quinol 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 QH-ADH (see Section C), involving Asp303, Glu177 and Arg331 (Fig. 5).

B. The Type I Ethanol Dehydrogenase of *Pseudomonas* species (QEDH)

This ethanol dehydrogenase (QEDH), induced during growth on ethanol of *Pseudomonas* or *Rhodopseudomonas*, is similar to MDH (Mutzel and Gorisch, 1991; Toyama et al., 1995; Keitel et al., 2000). It uses a specific cytochrome c_{550} as electron acceptor (Schobert and Gorisch, 1999), although this shows no sequence identity to cytochrome c_L , the electron acceptor for MDH. Like MDH, QEDH has a high pH optimum, requires ammonia or alkylamines as activator in the dye-linked assay system (ferricyanide is not used as electron acceptor), and is able to oxidize a wide range of alcohol substrates including secondary alcohols, but it differs in its very low affinity for methanol; the K_m for ethanol is about 15 μ M and that for methanol is about 1000 times higher. QEDH is homodimeric, the subunits being 65 kDa; it thus differs from MDH in lacking a small subunit. Unlike MDH, PQQ dissociates from QEDH after removal of Ca^{2+} with EDTA, this process being reversible after reconstitution in the presence of Ca^{2+} and PQQ (Mutzel and Goerisch, 1991). It is possible that the additional disulphide bridge in the α subunit of MDH and the complex with the small subunit may lead to a stronger stabilization of the native conformation of the enzyme.

The X-ray structure of the enzyme from *Pseudomonas aeruginosa* shows that, apart from differences in some loops, the folding pattern is very similar to the large (α) subunit of MDH (Keitel et al., 2000). There are different loops in the vicinity of the active site and several rather flexible loops protrude from the molecule surface and partly occupy the space filled by the small subunit of MDH. The PQQ is located in the center of the superbarrel, coordinated to a calcium ion. Most amino acid residues that make contact with the PQQ and the Ca^{2+} are similar to those in MDH. The main differences in the active-site region are a bulky tryptophan residue in the active-site cavity of MDH, which is replaced by a phenylalanine and a leucine side-chain in the QEDH, and a leucine residue right above the PQQ in MDH which is replaced by a tryptophan side-chain in

QEDH. Both amino acid exchanges appear to have an important influence, causing the different substrate specificities of these otherwise very similar enzymes. Docking calculations suggest that one of the tryptophans must be able to change its orientation in order to accommodate the higher primary alcohols in the active site (Keitel et al., 2000). In addition to the Ca^{2+} ion in the active-site cavity, QEDH contains a second Ca^{2+} -binding site at the N terminus, which contributes to its stability. Although the localization of the interaction surfaces between the subunits is identical in QEDH and MDH, the residues and the interactions involved are not conserved.

C. The Type II Soluble Quinohemoprotein Alcohol Dehydrogenase (QH-ADH)

The best-known quinohemoprotein ADH is that isolated from *Comamonas testosteroni* (Groen et al., 1986; Jongejan et al., 1998; Oubrie et al., 2002). It has also been described in *Pseudomonas putida* (Toyama et al., 1995; Chen et al., 2002) which produces two distinct forms, having different substrate specificities; ADH-IIB is induced during growth on butanol and ADH-IIG induced on glycerol. This same organism also produces a Type I alcohol dehydrogenase, induced during growth on ethanol. The electron acceptor for QH-ADH is a specific blue copper protein, azurin (Matsushita et al., 1999) which is probably oxidized directly by the membrane oxidase..

This periplasmic enzyme is a monomer (71 kDa) containing one molecule of PQQ and a single heme C. In the dye-linked assay system the pH optimum is 7.7 and there is no requirement for an amine activator. Because electron transfer from PQQ is by way of heme C this enzyme can also be assayed using ferricyanide. It has a wide specificity for primary and secondary alcohols, although it is unable to oxidize methanol; it also oxidizes aldehydes and can accept large molecules such as steroids as substrates. This has been exploited for enantiospecific oxidation of industrially important precursor molecules (synthons) (Geerlof et al., 1994). It may be isolated as the apoenzyme, containing the heme but lacking PQQ, reconstitution to the active holoenzyme requiring PQQ and Ca^{2+} ions. The enzyme has been extensively characterized by EPR, NMR and Raman resonance spectroscopy with respect to the nature of the heme and its relationship to PQQ (de Jong et al., 1995a,b), the conclusions being supported

by the X-ray structures of the enzymes from *Comamonas* (Oubrie et al., 2002) and from *Pseudomonas* (Chen et al., 2002).

QH-ADH comprises two domains connected by a long linker (23 amino acids) which spans the whole length of the enzyme (Fig. 7). The N-terminal dehydrogenase domain has the typical β barrel with its propeller fold, having the active site containing PQQ and a Ca^{2+} ion. The C-terminal domain, located on top of the dehydrogenase, is a type I cytochrome *c*, with 5 α helical segments, which enclose the C-type heme which is covalently bonded to cysteine residues and which has typical histidine and methionine heme-iron ligands. A channel leads from the periplasm to the region of the PQQ and a second channel contains a chain of hydrogen-bonded water molecules between the periplasm and the cavity between the two domains.

The N-terminal dehydrogenase domain is very similar to the α subunit of MDH, the PQQ being located at the top of the superbarrel in a hydrophobic

cavity that is accessible through a deep and narrow channel. It is 'sandwiched' between a co-planar tryptophan and the disulphide ring as in MDH (Fig. 4) and it has in-plane bonding interactions with almost exactly the same side chains as in MDH, the only significant difference is that Arg331 which is bonded to the O5 of PQQ in MDH (Fig. 5) is replaced by a lysine side chain in QH-ADH as it is in mGDH. The ligation of the Ca^{2+} ion with PQQ and with amino acid side chains is also exactly the same as in MDH (Fig. 5).

QH-ADH is the only alcohol dehydrogenase whose X-ray structure includes the substrate, or rather a product of substrate oxidation. In the case of the *Comamonas* enzyme this is tetrahydrofuran-2-carboxylic acid, presumably produced from the two step oxidation of tetrahydrofurfuryl alcohol (Oubrie et al., 2002). The tetrahydrofuran ring makes van der Waal's contacts with the hydrophobic walls of the substrate cavity. An oxygen atom of the substrate

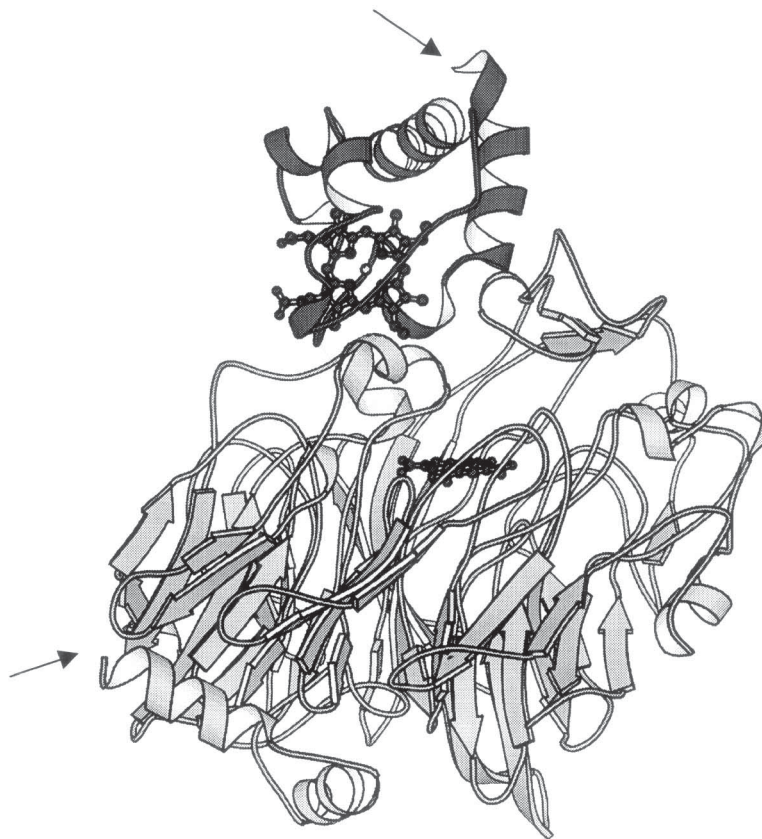


Fig. 7. The Type II soluble quinoxinohemoprotein alcohol dehydrogenase (QH-ADH. The structure is based on the coordinates of the *Comamonas testosteroni* enzyme (Oubrie et al., 2002). The PQQ-binding domain and the cytochrome *c* domain are joined by a flexible connecting linker of 23 amino acids (linking at the residues indicated by arrows). PQQ and heme *c* are shown as ball and stick models.

carboxylate is hydrogen bonded to the active site aspartate (Asp303 in MDH), and the glutamate carboxylate that coordinates to the Ca^{2+} (Glu177 in MDH), and to the two sulfur atoms of the disulphide ring. The enzyme from *Pseudomonas putida* was crystallized in the presence of isopropanol, and acetone, its oxidation product, was shown to be present in the active site, close to the O-4 and O-5 of PQQ and close to a carboxylate oxygen of the proposed active site aspartate (Asp303 in MDH; Fig. 5) (Chen et al., 2002). The side chains of the products lie in a cavity lined with mainly hydrophobic side chains—cysteines, phenylalanines, tyrosine and proline. The volume of this substrate cavity is about $120 \text{ \AA}^3$ which is about twice that of the Type I ethanol dehydrogenase and much larger than seen in the X-ray structure of MDH ($\sim 18 \text{ \AA}^3$) (Chen et al., 2002). MDH is unable to oxidize secondary alcohols or primary alcohols with substituents on the C2 atom but it is still able to oxidized a wide range of large alcohols (Anthony, 1986), and it is possible that the entrance to the active site might be flexible in order to accommodate these substrates.

Because the catalytic machinery of MDH is strictly conserved in QH-ADH, the mechanism of alcohol oxidation is most likely identical for the two enzymes (Oubrie et al., 2002). The positions of the substrate products together with the results obtained by sited directed mutagenesis of Asp303 in MDH (Afolabi et al., 2001), all suggest that Asp308 is the catalytic base.

The mechanism for aldehyde oxidation is presumably essentially similar to that for alcohol oxidation: it is proposed that Asp308 abstracts a proton from a hydrogen-bonded water and the resulting hydroxyl ion performs a nucleophilic attack on the aldehyde C1 atom in concert with hydride transfer from this atom to the C5 of PQQ, to give the carboxylic acid product (Oubrie et al., 2002).

The shortest distance between PQQ and the heme is $13\text{--}15 \text{ \AA}$ which is close to the maximum travel distance for electrons but the predicted rate of transfer through the protein is much higher than the measured rate of substrate oxidation. A number of paths are possible for the electron flow but they all involve the disulphide bridge and probably at least one water molecule (for example, see Fig. 8) (Chen et al., 2002; Oubrie et al., 2002). During oxidation of the reduced PQQ, protons are released into the periplasm. This is likely to be by way of a hydrogen bonded network involving a water filled chamber between the two

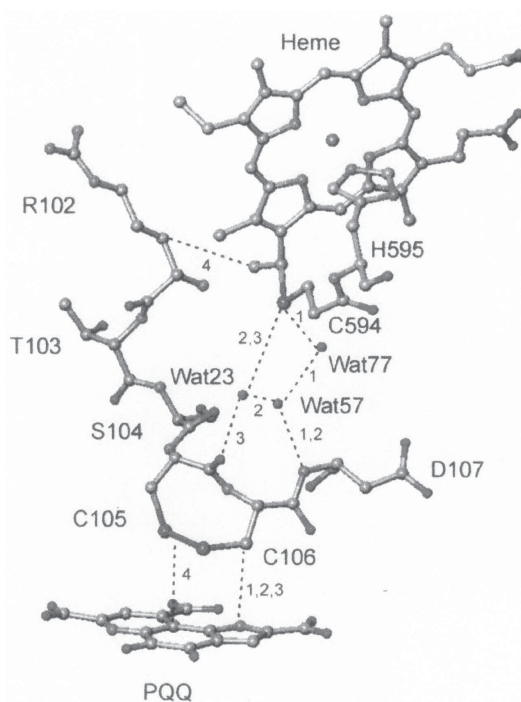


Fig. 8. The likely pathways for electron flow from the C5 of PQQ to the haem iron in the QH-ADH of *Pseudomonas putida* (Chen et al., 2002); similar routes and some alternative routes have been proposed by Oubrie et al. (2002).

domains, Lys335, Asp308 and Glu185 (Oubrie et al., 2002); these are equivalent to the MDH residues Arg331, Asp303 and Glu177 (Fig. 5).

Azurin isolated from *P. putida* is a good electron acceptor for the QH-ADH, the interaction being mediated by hydrophobic forces (Matsushita et al., 1999). The heme is buried within the cytochrome domain (Fig. 7) except for one edge which is surrounded by a charge-neutral surface area which may form a binding site for azurin, in which one of the histidine ligands to the buried copper is exposed to the surface and is surrounded by a surface patch of hydrophobic residues (Chen et al., 2002).

D. Membrane Associated Quinohemoprotein Alcohol Dehydrogenase (Type III Alcohol Dehydrogenase)

This enzyme is a quinohemoprotein-cytochrome *c* complex and has only been described in the acetic acid bacteria *Acetobacter* and *Gluconobacter* (Matsushita and Adachi, 1993; Matsushita et al., 1994, 2003; Goodwin and Anthony, 1998). Together with the membrane-bound aldehyde dehydrogenase,

it is responsible for the oxidation of alcohol to acetic acid in vinegar production. It does not require ammonia as activator and has a pH optimum of 4-6. Its substrate specificity is relatively restricted, oxidizing only a few primary alcohols (chain length, C2-C6) (but not methanol), or secondary alcohols and has some activity with formaldehyde and acetaldehyde.

The Type III ADH has 3 subunits and is tightly bound to the periplasmic membrane, requiring detergent for its isolation. Translation of the gene sequences shows that all the subunits have N-terminal signal peptides typical of periplasmic proteins. Its natural electron acceptor is ubiquinone in the membrane. Subunit I (72-80 kDa) is a quinohemoprotein similar to the soluble (Type II) QH-ADH, with a single molecule of PQQ and a single heme C. Its N-terminal region has sequence similarity to the soluble methanol dehydrogenase but with a C-terminal extension having a single heme binding site. Subunit II (48k-53 kDa) has 3 hemes that can be distinguished by biochemical techniques in the pure protein (Matsushita et al., 1996). Subunits I + II therefore have a total of 4 hemes. Most Type III ADHs have a third subunit (subunit III, 14-17 kDa) whose gene is not linked to the genes encoding the other two subunits, and whose predicted amino acid sequence indicates that its processed size is greater (about 20 kDa) than that obtained by SDS -PAGE (14 kDa). The Type III ADH may be assayed with phenazine methosulfate, or with ferricyanide which reacts at the level of one or more of the heme C prosthetic groups on subunits I and II. It differs from all other ADHs in using short-chain ubiquinone homologues (Q_1 and Q_2) as electron acceptors and native ubiquinone (Q_9 and Q_{10}) when reconstituted in membrane vesicles (Matsushita et al., 1992). There is good evidence that electron transfer from reduced PQQ to the membrane ubiquinone takes place by way of the hemes on the cytochrome subunit II but that only two of them may be involved in this electron transfer process (Matsushita et al., 1996; Frebortova et al., 1998). It has been suggested that the cytochrome subunit II is firmly embedded in the membrane, that subunits I and III are firmly attached to each other and that this attachment helps the dehydrogenase subunit I couple with the cytochrome *c* (subunit II). This raises the question of how the ubiquinone in the membrane reacts with subunit II to accept electrons from its heme. Clearly part of the protein must be embedded in the membrane for this to occur but

subunit II does not appear to have typical hydrophobic transmembrane helices (Kondo and Horinouchi, 1997)

The Type III ADH thus appears to be unique in a number of ways; it requires detergent for its isolation from membranes and so seems to be a typical integral membrane protein, although none of the subunits appears (from their gene sequences) to have characteristic membrane protein structural domains. Furthermore, the electron acceptor for the quinohemoprotein/cytochrome *c* complex is membrane ubiquinone, so we have the unusual situation where a *c*-type cytochrome precedes ubiquinone in the electron transport chain (K. Matsushita, H. Toyama and O. Adachi, unpublished).

E. The Membrane-bound Glucose Dehydrogenase (mGDH)

The first description of glucose dehydrogenase and its prosthetic group was by Hauge (1964), PQQ being subsequently identified as its prosthetic group by Duine and colleagues in a paper in which they first introduced the term quinoproteins (Duine et al., 1979). The organism used for this work was *Acinetobacter calcoaceticus* which was later shown to be unique in having two different quinoprotein glucose dehydrogenases, one periplasmic (sGDH) and the other an integral membrane protein (mGDH).

This mGDH has been described in a wide range of bacteria including *Acinetobacter calcoaceticus*, enteric bacteria, pseudomonads and acetic acid bacteria (Table 1). Although the enzymes differ slightly in some properties such as substrate specificity and stability, they are similar in most essential respects (Matsushita and Adachi, 1993; Matsushita et al., 1994; Anthony, 1996; Goodwin and Anthony, 1998). This enzyme catalyses the oxidation, to the lactone, of the pyranose form of D-glucose and other monosaccharides, usually including mannose, galactose, rhamnose and xylose. The substrate specificity suggests that the pentose sugars that are oxidized are also in the pyranose form (Cozier and Anthony, 1995; Cozier et al., 1999). It differs from sGDH in that it is unable to oxidize disaccharides, but mutation of a single histidine to asparagine was sufficient to confer on the enzyme from *Gluconobacter suboxydans* the ability to oxidize maltose (Cleton-Jansen et al., 1991); modeling studies indicate that this change increases the width of the entrance to the active site region of the enzyme

(Cozier and Anthony, 1995). The oxidation of glucose to the lactone occurs in the periplasm and the electron acceptor is ubiquinone in the membrane (Matsushita et al., 1982a,b, 1989a,b,c; Beardmore-Gray and Anthony, 1986).

After solubilization from the membrane, mGDH is isolated as a monomer of about 87 kDa, containing one PQQ molecule. Topological and sequence analysis of the protein reveals that the N-terminal region (154 amino acids) is likely to have five membrane-spanning regions which was thought to be the most likely region for the ubiquinone binding site, but this appears not to be the case (Yamada et al., 1993; Elias et al., 2001). Expression of the C-terminal periplasmic part of mGDH fused to the signal sequence of beta-lactamase showed that the periplasmic domain was transported to the periplasm but remained bound as a peripheral membrane protein. The periplasmic domain had ubiquinone reductase activity nearly equivalent to that of complete mGDH, suggesting that the periplasmic domain possesses the ubiquinone binding site. In the model of this domain there is a sequence of about 80 amino acids that is absent from alcohol dehydrogenases

which is possibly involved in electron transfer from the PQQ quinol to ubiquinone (Cozier and Anthony, 1995). This suggestion is supported by the discovery of a similar sequence within the equivalent catalytic domain of the membrane sorbitol dehydrogenase, a quinoprotein that also donates electrons to a membrane quinone rather than to a cytochrome *c* (Sugisawa and Hoshino, 2001). It is hoped that the production of the separate C-terminal domain of mGDH will facilitate its crystallization and production of an X-ray structure.

The periplasmic domain (residues 155-796) shows 26% identity of sequence to that of the α -subunit of MDH the coordinates of which have been used to model the mGDH structure (Cozier and Anthony, 1995; Cozier et al., 1999). In the model structure, the novel disulphide ring (Fig. 4) is replaced by a histidine residue which maintains the position of PQQ in the active site). There are fewer equatorial interactions between the protein and PQQ (Fig. 9), perhaps explaining why it is possible to effect the reversible dissociation of PQQ from mGDH but not from MDH. One clear difference is that there is more 'space' in the glucose dehydrogenase active site,

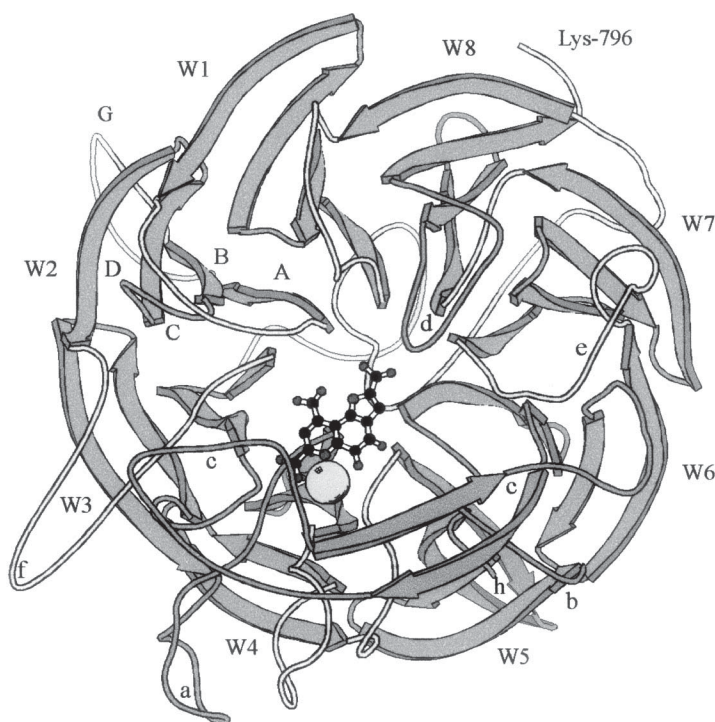


Fig. 9. The superbarrel structure of the C-terminal domain of membrane glucose dehydrogenase (mGDH). This is a model based on the MDH coordinates (Cozier and Anthony, 1995). The position marked h is where a sequence of 83 residues (497-579), absent from MDH, would join the superbarrel structure; it is in this sequence that there is a possible PQQ binding site (Elias et al., 2001).

perhaps to accommodate the larger substrate, and Arg331 in MDH which may play a role in catalysis is replaced by Lys493 in mGDH. The ligation of the divalent metal ion (Ca^{2+} or Mg^{2+}) 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 stabilizing the electrophilic C-5 of PQQ. By analogy with the MDH structure, Asp466 is likely to be involved in abstraction of a proton from the anomeric hydroxyl of the pyranose ring. This would be followed by attack by a hydride from the glucose oxyanion, leading directly to formation of the lactone and the quinol form of PQQ. This would be similar to the mechanism of sGDH in which the initial proton abstraction is however catalysed by a histidine residue (see Section IVF). Characterization of mGDHs produced by site-directed mutagenesis have supported the conclusion that the model mGDH is appropriate for understanding its mechanism (Sode et al., 1995a,b; Cozier et al., 1999; Yoshida et al., 1999; Elias et al., 2000).

In all the PQQ-containing quinoproteins for which X-ray structures are available, including sGDH, there is a Ca^{2+} ion in the active site, suggesting that this will be a feature of all such dehydrogenases, but mGDH appears to be an exception. It is usually isolated as the apo-enzyme and requires PQQ plus a divalent metal ion for reconstitution. For this process Mg^{2+} or other divalent metal ions can be used instead of Ca^{2+} (Matsushita et al., 1995). The metal content of the reconstituted holoenzyme cannot be determined because removal of the excess Mg^{2+} required for reconstitution also removes PQQ and metal ions from the active site. If the function of the Mg^{2+} during reconstitution is to provide a metal ion at the active site, then this will have implications for our understanding of the mechanism of mGDH, as it is unlikely that Mg^{2+} could replace Ca^{2+} for some functions. Modeling studies, based on the coordinates of MDH, have shown that some of the residues important in coordination with the Ca^{2+} are different in the glucose dehydrogenase (Cozier and Anthony, 1995) (Fig. 10). Remarkably, site directed mutagenesis of the residues predicted to be involved in binding the ion in the active site led to GDH with the same activity as the WT-GDH but where the preferred ion for reconstitution had changed from Mg^{2+} to Ca^{2+} or Ba^{2+} , reconstitution with Ca^{2+} being competitively inhibited by Mg^{2+} (James and Anthony, 2002). The simplest conclusion of this study is that mGDH really is exceptional in having Mg^{2+} at the active site

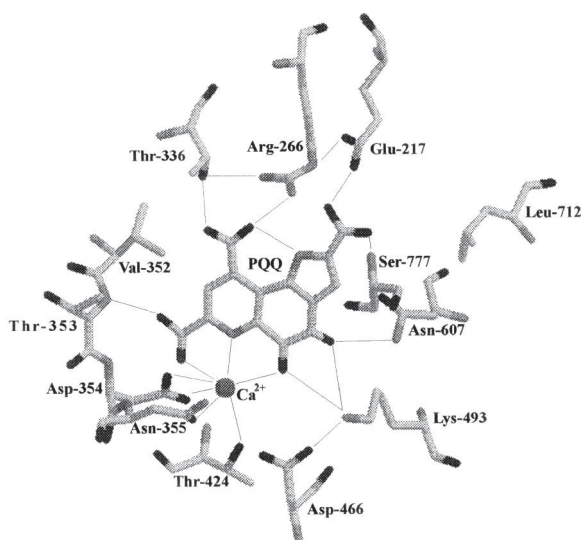


Fig. 10. The equatorial interactions of PQQ and the coordination of Ca^{2+} in the active site of mGDH (a model based on the MDH coordinates; Cozier and Anthony, 1995). This Figure also shows Asp466, which is likely to act as the catalytic base, and Lys493 which may also be involved in the mechanism.

ion, but confirmation and understanding of this awaits an X-ray structure.

The mGDHs from different bacteria differ with respect to their stability and the ease with which PQQ may be dissociated from them and it has been suggested that they can be considered to be in two classes, depending on their stability with respect to EDTA (Dokter et al., 1986; Sode et al., 1995a,b). The first type is easily denatured and occurs in *Escherichia coli* and *Pseudomonas* sp. whereas the more stable second type occurs in *Acinetobacter* and *Gluconobacter*. The stability of the *Escherichia coli* enzyme has been modified by a single amino acid substitution and by formation of a chimeric enzyme using the glucose dehydrogenase structural genes from *Escherichia coli* and *Acinetobacter calcoaceticus* (Sode et al., 1995a,b; Yoshida et al., 1999).

F. The Soluble Glucose Dehydrogenase of *Acinetobacter calcoaceticus* (sGDH)

This soluble, periplasmic glucose dehydrogenase has only been described in *Acinetobacter calcoaceticus* (Goodwin and Anthony, 1998; Oubrie et al., 1999b). Its primary sequence is completely different from all other PQQ-containing dehydrogenases, although recent database searches have suggested

that similar proteins may exist in other bacteria (Oubrie et al., 1999b). It is a dimer of identical subunits of about 50 kDa, each having an active site containing PQQ and a Ca^{2+} ion. It catalyses an exceptionally high rate of oxidation of a wide range of aldose sugars including D-glucose, arabinose, galactose, xylose and also the disaccharides lactose, cellobiose and maltose. It is active with phenazine ethosulphate, Wurster's blue (pH optimum 9.0) and 2,6-dichlorophenolindophenol (optimum pH 6.0), but not with ferricyanide. The soluble enzyme does not react with ubiquinone (Matsushita et al., 1993), and although it slowly reduces a soluble cytochrome *b* there is no evidence that this cytochrome interacts with the electron transport chain (Dokter et al., 1998). It is not obvious why this very active enzyme is present in *Acinetobacter calcoaceticus* as this organism is also able produce mGDH which is able to couple glucose oxidation to energy production, although this does not support growth on glucose.

The Ca^{2+} can be removed from the holoenzyme, or the apoenzyme can be produced by bacteria unable to synthesize PQQ; subsequent reconstitution with PQQ requires Ca^{2+} , Mn^{2+} or Cd^{2+} , but Mg^{2+} is ineffective (Gorisch et al., 1989; Matsushita et al., 1995; Olsthoorn et al., 1997). From a study of reconstitution using the monomer and dimer, it appears that Ca^{2+} plays a dual role, as it is required

for dimerization as well as for incorporation of PQQ in a functional form. After reconstitution, the holoenzyme resembles reconstituted MDH in that Ca^{2+} can no longer be removed by chelating agents (Olsthoorn and Duine, 1996).

Remarkably, the crystal structure of sGDH (Oubrie et al., 1999a,b,c) shows that it is similar to the other PQQ-containing dehydrogenases in having a β -propeller superbarrel structure (Fig. 11). However, it has six (not eight) four-stranded antiparallel β -sheets linked together and it is stabilized, not by tryptophan docking motifs as in MDH but by two novel types of interaction. Each monomer has three Ca^{2+} ions, two of which are located in the dimer interface, the third being located in the active site. The PQQ resides in a deep, broad, positively-charged cleft in the center of each monomer, near the top of the β -propeller structure. The overall binding of PQQ (Fig. 12) is similar to that in the active site of MDH (Fig. 5). In both proteins equatorial reactions are polar whereas hydrophobic stacking interactions are formed on both sides of the PQQ. The active site Ca^{2+} ion and one arginine side chain are ligated to PQQ in almost identical fashion, both of these ligations being important in facilitating attack by a hydride on the electropositive C5 of PQQ (Figs. 12 and 13). The structure containing glucose and reduced PQQ (Oubrie et al., 1999a) shows that the glucose binding

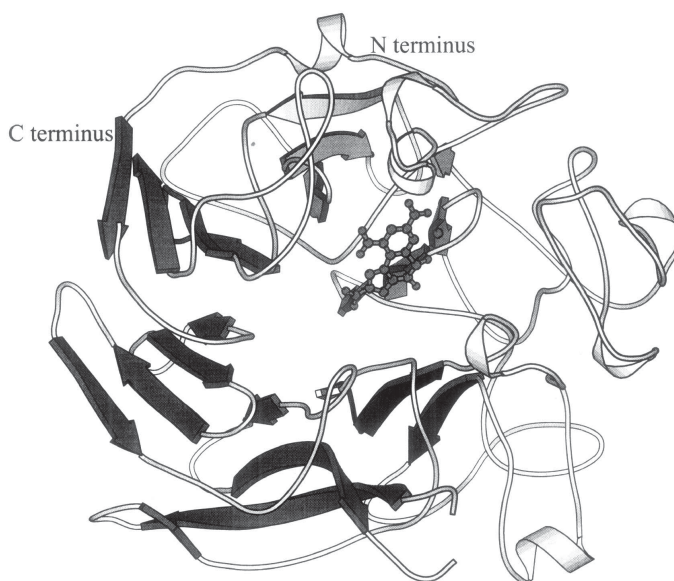


Fig. 11. The overall structure of a single monomer of soluble GDH. sGDH has a 6-bladed propeller structure, contrasting with all other PQQ-containing dehydrogenases which have eight blades. This structure is taken from the coordinates published by Oubrie et al. (1999a).

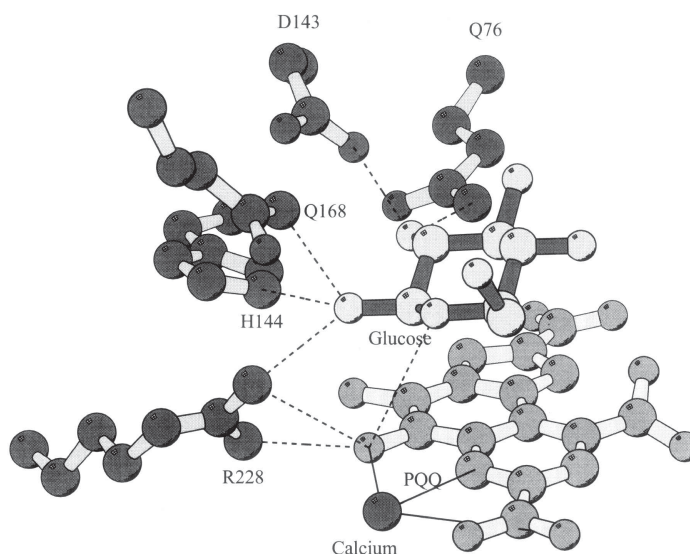


Fig. 12. Substrate binding in the active site of soluble glucose dehydrogenase. This structure is taken from the coordinates published by Oubrie et al. (1999a). His144 is the active site base. The Ca^{2+} ion and Arg228 coordinate to the O5 of PQQ as in MDH (see Fig. 5).

site is a wide and solvent accessible crevice which is located directly above PQQ. Glucose docks onto the PQQ surface making extensive hydrophobic interactions (Fig. 12). The interactions of the protein with the glucose O1 hydroxyl group are only possible if it is in an equatorial position and this explains the absolute β -anomer preference of the enzyme. Three of the hydroxyl groups make no hydrogen bonding interactions with the protein which explains its lack of specificity. The C1 atom is positioned directly above the PQQ C5 atom and close to the side chain of His144 which can therefore act as the general base that abstracts a proton from the glucose O1 atom. The active site calcium is appropriately located for it to contribute to polarization of the C5 carbonyl bond thus improving its reactivity with nucleophiles.

It was shown that for an addition/elimination reaction to occur (which involves covalent attachment of the glucose to PQQ—analogue to the MDH mechanism in Fig. 6a), three favorable interactions of the negatively charged O1 atom in the oxyanion would have to take place. This is energetically expensive and suggests that this mechanism is unlikely (Oubrie et al., 1999a). By contrast, the orientation of glucose is ideal for direct hydride transfer from the glucose C1 atom to the PQQ C5 atom, the distance between the atoms being only 3.2 Å; a hydride has to travel only 1.2 Å for covalent addition to the C5 atom (Fig. 13). It was therefore concluded that the catalytic mechanism proceeds

through general base-catalysed proton abstraction in concert with direct hydride transfer from substrate to PQQ (Fig. 13). This conclusion has been supported by extensive spectral and kinetic studies on s-GDH in which the PQQ-activating Ca^{2+} was absent or was replaced with Ba^{2+} or in which PQQ was replaced with an analogue or derivatives (Dewanti and Duine, 2000). The mechanism for this enzyme, and very likely all PQQ-containing dehydrogenases is thus a general base-catalysed hydride transfer with subsequent tautomerization of the reduced PQQ to the quinol form (Oubrie and Dijkstra, 2000), as first proposed for MDH (Anthony, 1993a, 1996, 2000), and similar to that of nicotinamide and flavin-dependent oxidoreductases.

Acknowledgments

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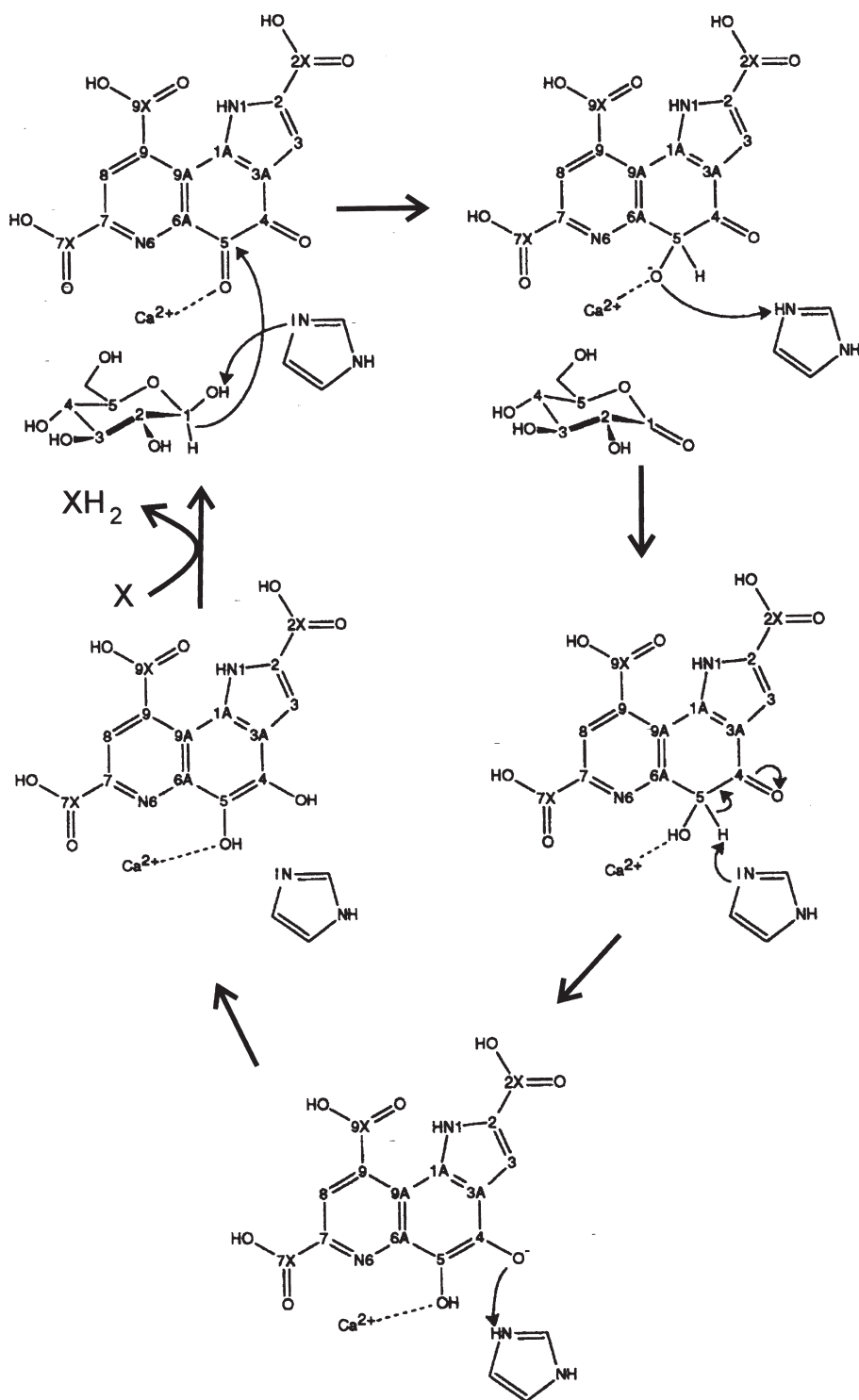


Fig. 13. The mechanism of soluble glucose dehydrogenase (sGDH). The mechanism is essentially similar to that proposed for MDH (Fig. 6b). It comprises a general base-catalysed proton abstraction in concert with direct hydride transfer for glucose to PQQ, followed by tautomerization to PQH₂ (Oubrie et al., 1999a). The re-oxidation of the quinol back to PQQ involves an electron acceptor which may be a hydrogen carrier (indicated here as 'X') or it might involve two sequential electron transfer reactions to a cytochrome as occurs with MDH.

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The above reference was removed as the book is not in press yet and will not be until 2004 or 2005. The in-text references have been changed to: (K. Matsushita, H. Toyama and O. Adachi, unpublished). In other places, the in-text reference is Chapter 4, Vol. 2, Matsushita et al. Perhaps the unpublished refs should follow that example. Please advise.