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Forum Review

Pyrroloquinoline Quinone (PQQ) and Quinoprotein Enzymes

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ABSTRACT

This review summarises the characteristics, identification, and measurement of pyrroloquinoline quinone, the prosthetic group of bacterial quinoprotein dehydrogenases whose structures, mechanisms, and electron transport functions are described in detail. Type I alcohol dehydrogenase includes the "classic" methanol dehydrogenase; its x-ray structure and mechanism are discussed in detail. It is likely that its mechanism involves a direct hydride transfer rather than a mechanism involving a covalent adduct. The x-ray structure of a closely related ethanol dehydrogenase is also described. The type II alcohol dehydrogenase is a soluble quinohaemoprotein, having a C-terminal extension containing haem C, which provides an excellent opportunity for the study of intraprotein electron transfer processes. The type III alcohol dehydrogenase is similar but it has two additional subunits (one of which is a multihaem cytochrome c) bound in an unusual way to the periplasmic membrane. One type of glucose dehydrogenase is a soluble quinoprotein whose role in energy transduction is uncertain. Its x-ray structure (in the presence and absence of substrate) is described together with the detailed mechanism, which also involves a direct hydride transfer. The more widely distributed glucose dehydrogenases are integral membrane proteins, bound to the membrane by transmembrane helices at the N-terminus. Antioxid. Redox Signal. 3, 757–774.

INTRODUCTION

PYRROLOQUINOLINE QUINONE (PQQ) is the prosthetic group of quinoprotein dehydrogenases that catalyse the oxidation of alcohols and aldose sugars in the periplasm of gram-negative bacteria. Although PQQ is never covalently bound to the dehydrogenases and can sometimes be reversibly dissociated from them, this dissociation is never important for their metabolic function. They may contain only PQQ (the quinoproteins), or they may also contain haem as a second prosthetic group (the quinohaemoproteins); they may be monomeric or multimeric; they may be freely soluble in the periplasm or bound to membranes; some are firmly associated with other redox components, whereas others are not; their electron acceptors may be cytochromes, blue copper proteins, or membrane ubiquinone; the PQQ may be tightly bound or easily dissociated. They may be assayed using their physiological electron acceptors but, as with flavoprotein dehydrogenases, it is often more convenient to assay them by using artificial dye electron acceptor systems such as phenazine methosulphate, phenazine ethosulphate or Wurster's blue. The quinohaemoproteins may also be assayed with ferricyanide, which accepts electrons from the higher potential haem groups.

This review will cover the main types of quinoprotein dehydrogenase. There are three

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classes of alcohol dehydrogenases: type I includes the "classic" methanol dehydrogenase (MDH); type II is a soluble quinohaemoprotein, having a C-terminal extension containing haem C; type III is similar but is membrane-bound, and it has two additional subunits (one of which is a multihaem cytochrome *c*), bound in an unusual way to the periplasmic membrane. There are two types of glucose dehydrogenase (GDH); one is an atypical soluble quinoprotein whose role in energy transduction is uncertain. The more widely distributed GDHs are integral membrane proteins, bound to the membrane by transmembrane helices at the N-terminus. See the following extensive reviews for comprehensive bibliographies (3–6, 24, 25, 31, 45).

PQQ

PQQ was first isolated from GDH and MDH (7,36). It was readily released by denaturation of the enzymes, purified, and shown to be a red, highly polar, acidic compound with a very characteristic green fluorescence (7). Its structure was subsequently elucidated by x-ray crystallography, and it was originally named methoxatin (58). Its full name is 2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione, hence its more convenient short name of pyrroloquinoline quinone (PQQ). Its chemistry and function in dehydrogenases was described in detail by Frank and Duine and their coworkers (24, 25) who showed that a key feature of its structure is the *ortho*-quinone at the C4 and C5 positions of the quinoline ring, which becomes reduced to the quinol during catalysis (Fig. 1). PQQ very readily forms adducts at the C5 position, which may be important in mechanisms and in measurement and identification. The midpoint redox potential at pH 7 of the isolated PQQ is about +90 mV, but this is likely to be influenced by its environment in the dehydrogenases. An important feature of PQQ is its ability to complex divalent cations in solution (38, 52). Model compounds containing these complexes can catalyse oxidation reactions (38, 39), and it is now clear that all the PQQ-containing enzymes are likely to have PQQ complexed with a divalent metal ion in their active sites.

The biosynthesis of PQQ has been shown by Unkefer and colleagues to involve the condensation of one molecule of glutamate with one of tyrosine; however, although the relevant biosynthetic genes have been identified, the enzymes encoded by them have not been described (for review and bibliography, see 31).

IDENTIFICATION AND MEASUREMENT OF PQQ

Identification of PQQ is not straightforward, and before some of the pitfalls were appreciated, a number of enzymes were identified as containing covalently bound PQQ. These have subsequently been found to be quinoproteins whose quinone prosthetic groups are derived from modification of main chain amino acids: tryptophan tryptophylquinone, 2,4,5-trihydroxyphenylalanine quinone, or lysine tyrosylquinone (for reviews, see 3, 4, 17, 24, 40).

When the primary sequences of PQQ-containing dehydrogenases were first compared, it was seen that there was one region of greater identity than any other, and it was reasonably concluded that this might represent the one feature known to be common to all PQQ-containing enzymes—their ability to bind PQQ; this region was therefore designated a putative PQQ-binding domain and has subsequently been used as evidence that a protein is able to bind PQQ. However, remarkably, this sequence constitutes part of the main superbarrel structure and is not in any way directly involved in PQQ binding. Why this region has such a relatively high level of identity is not known, but it provides the most obvious region for designing DNA probes for use in the identification of the genes for PQQ-dependent quinoproteins.

The spectra of PQQ-containing quinoproteins have a characteristic absorption band between 300 and 420 nm, due to the bound PQQ, but this is markedly affected by the environment and cannot be relied upon for definitive identification. The fluorescence spectrum of the isolated prosthetic group provides a further indication of its identity as PQQ (21), but this should be confirmed by HPLC with absorbance or fluorescence detection of a known adduct

(24). A second valuable method for measurement of PQQ is to use its ability to reconstitute active holoenzyme (alcohol or glucose dehydrogenases) from apoenzyme (for references, see 31).

A controversial method of measuring PQQ depends on its redox cycling properties, which provide a method that may be useful to indicate the presence of PQQ (27); this is inappropriate as the only way of demonstrating that PQQ is the prosthetic group of a dehydrogenase because the property of redox recycling is not specific for PQQ. A modified form of the assay is useful as a "stain" for quinoproteins after polyacrylamide gel electrophoresis. This nonspecific method has been used in attempts to demonstrate the presence of low concentrations of PQQ in animal tissues and fluids, and this issue remains a matter of debate. Some experiments on the effects of PQQ as a dietary supplement, and experiments with many different types of tissue and cell preparation in which PQQ is used as reagent, have led to suggestions that PQQ has many functions in mammalian systems and could be considered to be a vitamin. This work has been summarised (11, 63), but it should be emphasised that many of the conclusions have not yet achieved general acceptance.

SOLUBLE (TYPE I) QUINOPROTEIN ALCOHOL DEHYDROGENASES

MDH

The most fully-described alcohol dehydrogenase is the MDH of methylotrophic bacteria, which oxidises methanol to formaldehyde during growth of bacteria on methane or methanol (1, 3–5). MDH is a soluble periplasmic enzyme that uses, as physiological electron acceptor, a novel acidic cytochrome *c* (cytochrome c_L) (2). It can be assayed with phenazine ethosulphate or Wurster's blue, but not with ferricyanide. Using phenazine ethosulphate in this dye-linked assay system, the pH optimum is ~9, and ammonia or methylamine is required as activator. It oxidises 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. 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 oxidised back to the quinone by way of the free radical semiquinone (Fig. 1). When isolated, the PQQ in the enzyme is in this semiquinone form.

The C5 carbonyl group of isolated PQQ is very reactive towards nucleophilic reagents, such as methanol, leading to the initial conclusion that a covalent PQQ–substrate complex (a hemiketal) may be important in the reaction mechanism. It is probable that an aspartate ion (Asp^{303}) provides a catalytic base that initiates the reaction by abstraction of a proton from the methanol; this proposal is supported by the kinetics and x-ray structure of MDH in a site-directed mutant in which Asp^{303} is replaced by glutamate (author, unpublished observations). In the mechanism shown in Fig. 1, the oxyanion produced by proton abstraction attacks the electrophilic C5, 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 to this addition/elimination mechanism is a simple acid/base-catalysed hydride transfer (Fig. 2). The large deuterium isotope effect (~6) observed during the reductive phase of the reaction is consistent with either mechanism for MDH; in both cases, the step affected will be the breaking of the C-H bond, and it is this rate-limiting step that is activated by ammonia, although the mechanism of this activation is not understood (5, 28, 30). Until recently, the small amount of evidence available tended to support the addition/elimination mechanism (Fig. 1), but more recent evidence favours our hydride transfer mechanism (Fig. 2). A revised analysis of the active-site region by x-ray crystallography together with a computerised quantum mechanical approach (72) has concluded that the distorted PQQ (with the C5 carbon atom in the tetrahedral configuration) is actually the C5-reduced intermediate, implying that MDH follows a hydride transfer mechanism. This conclusion is also supported by our

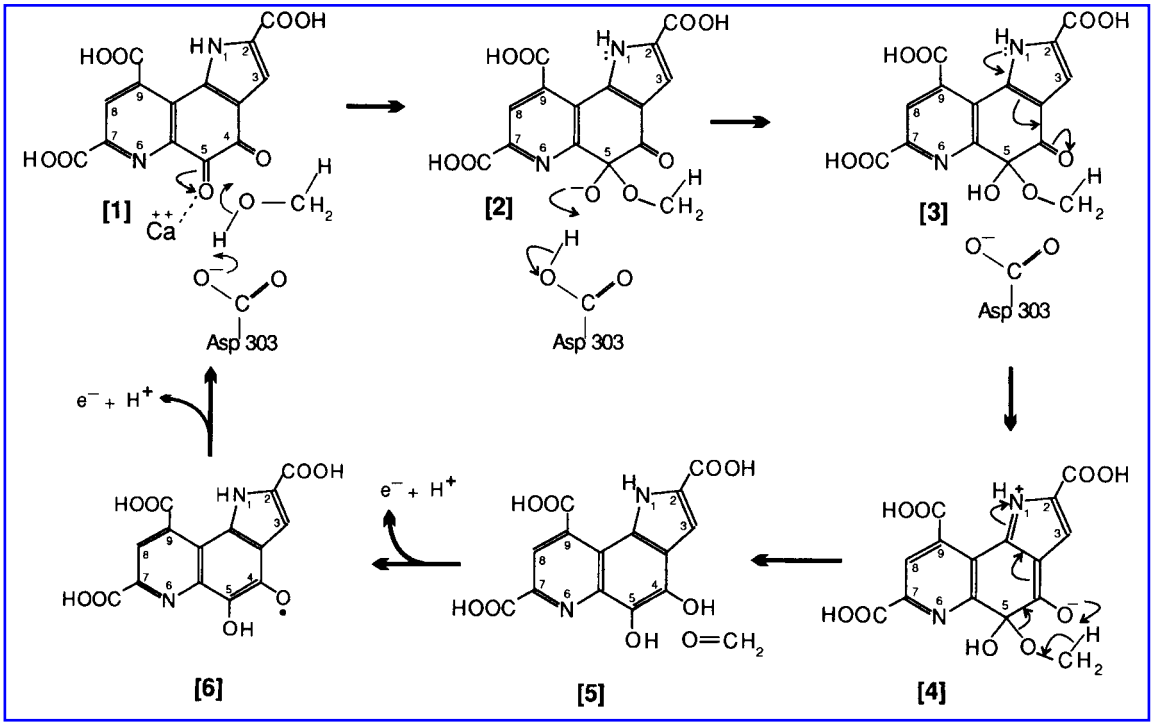

FIG. 1. A proposed reaction mechanism for MDH. In this addition/elimination reaction, a covalent hemiketal adduct **[3]** is formed between the substrate and the C5 of the PQQ. Intermediate **[5]** is the reduced quinol, and **[6]** is the semiquinone free radical form, produced after a single electron has passed to the electron acceptor.

results (unpublished data) on the crystal structure of a D303E-MDH produced by site-directed mutagenesis. Both the mechanisms have also been considered for the soluble GDH (s-GDH) and in this case the evidence is strongly in favour of the hydride transfer mechanism (see below) (22, 57).

In both mechanisms for MDH, 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 C5 carbonyl oxygen of PQQ, thus stabilising the electrophilic C5 for attack by an oxyanion or hydride (3, 5, 8). Recent studies using PQQ analogues bonded to Ca^{2+} in organic solvents have provided supporting evidence for the role of Ca^{2+}, and mechanisms involving hemiketal formation (38, 39). That Ca^{2+} might play some catalytic role in MDH was indicated by work using some unusual mutants (including *mxaA*) of *Methylobacterium extorquens*. These are defective in calcium insertion, and inactive enzyme is produced containing PQQ, but no metal ion. Reconstitution using Ca^{2+} or Ba^{2+} involved a large conformational change, re-

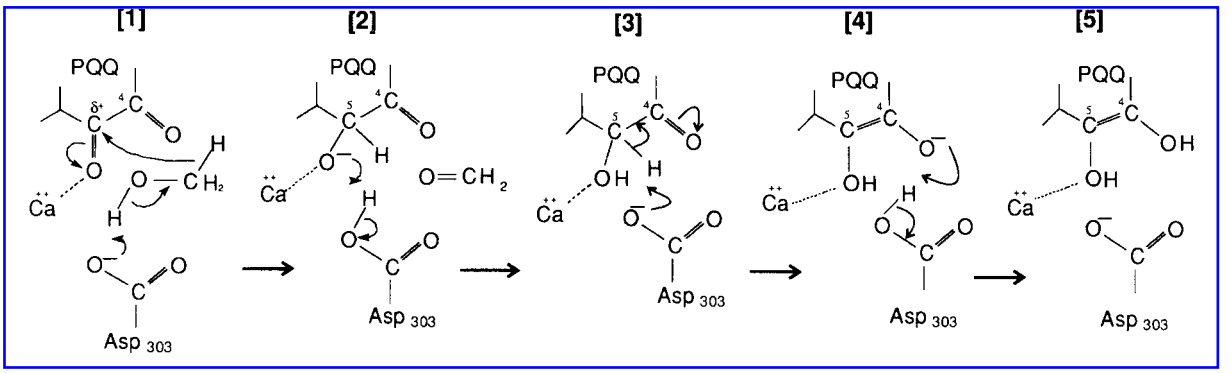

FIG. 2. A proposed hydride-transfer mechanism for MDH. The subsequent oxidation of the quinol form of PQQ is the same as that described in Fig. 1. This mechanism is similar to that demonstrated for s-GDH (Fig. 8).

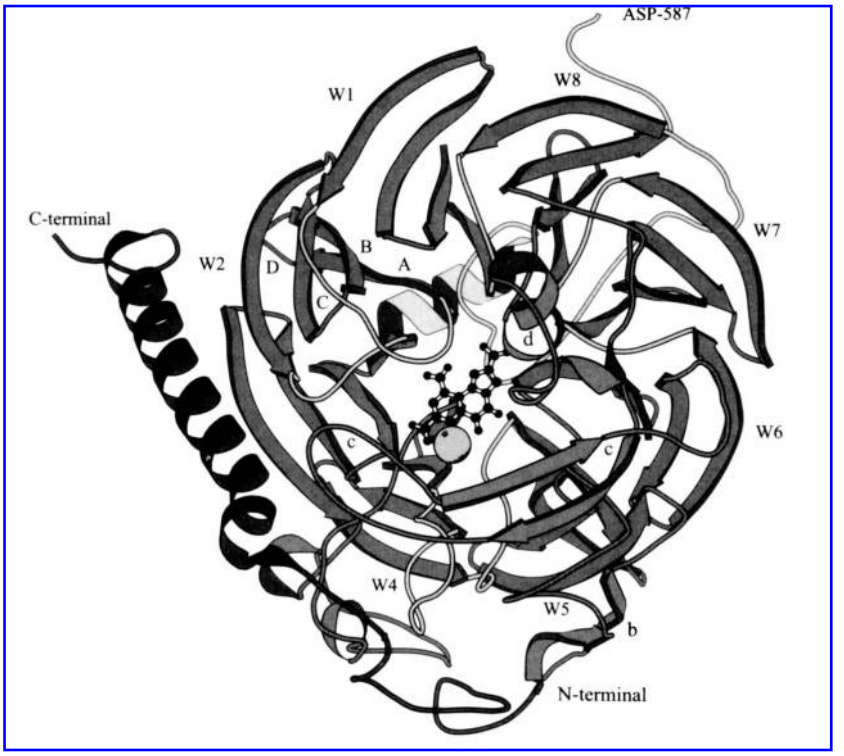

FIG. 3. The $\alpha\beta$ unit of MDH. This is simplified to show "propeller-fold" structure of the superbarrel and the long α-helix of the β-subunit.

sulting in active holoenzyme from which Ca^{2+} could not subsequently be removed, and was optimal at high pH and high Ca^{2+} concentrations (30). This contrasts with the *in vivo* situation where the pH in the periplasm is neutral and the concentrations of Ca^{2+} are likely to be relatively low. Presumably assembly of the holoenzyme in the periplasm is facilitated by proteins including MxaA (31). The MDH reconstituted with Ba^{2+} (Ba^{2+}-MDH) provides 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 m*M* instead of 10 μM), and for its activator ammonia, but its activation energy is less than 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 (30). The Ba^{2+}-MDH was subsequently used to prepare fully oxidised MDH for spectroscopy, but these studies failed to provide any evidence for production of a spectroscopically identifiable covalent adduct intermediate in the reaction mechanism (note that a Ba^{2+}-enzyme has been used for similar studies with the s-GDH see below). Similar experiments with a D303E site-directed mutant MDH have also failed to provide evidence for a covalent adduct.

The x-ray structure of MDH has been determined for the MDH from *Methylobacterium extorquens* (12, 29), from *Methylophilus methylotrophus* (68), and from *Methylophilus* W3A1 (66, 67, 69, 72). As expected from the similarities in protein sequence, all the structures are similar; unless otherwise stated, in this review the discussion and the numbering system will be for the MDH from *M. extorquens*. 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 has no known function and, like PQQ, it cannot be reversibly dissociated from the subunit. The large α subunit is a superbarrel made up of eight four-stranded antiparallel twisted β-sheets (W-shaped), stacked radially around a pseudo eightfold symmetry axis running through the centre of the subunit (Fig. 3). This structure has been referred to as a propeller fold, each twisted W motif representing a pro-

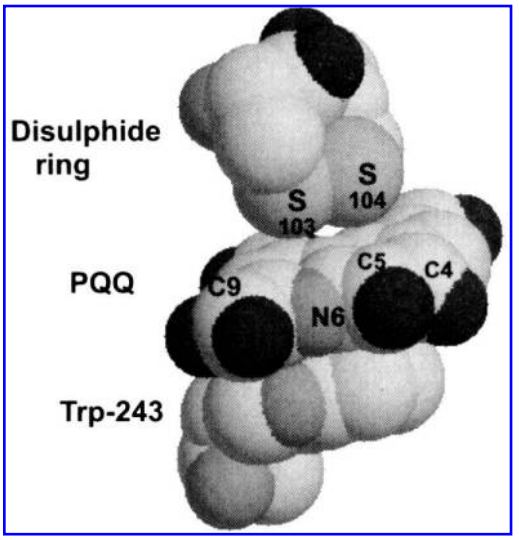

FIG. 4. The novel disulphide ring in the active site of MDH. The Ca^{2+} ion (not shown) is packed between the oxygen atoms attached to C5 and C9 and to the N6 nitrogen atom of PQQ.

peller blade. The structure has several important novel features, including novel "tryptophan-docking motifs" that link together the eight β-sheets, the presence in the active site of an unusual disulphide ring structure formed from adjacent cysteine residues, and a Ca^{2+} ion coordinated to PQQ. The PQQ is sandwiched between the indole ring of Trp^{243} and the disulphide ring structure. The indole ring is within 15% of coplanarity with the PQQ ring and, on the opposite side, the two sulphur atoms of the disulphide bridge are within 3.75 Å of the plane of PQQ (Fig. 4). The rarity of this 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. As the carboxymethylated thiols can no longer be involved in redox activity, a possible role in electron transfer appears to be ruled out. The disulphide ring is not present in the membrane-associated quinoprotein GDH 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 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 (9).

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 Arg^{109}, and both groups are shielded from bulk solvent by the disulphide. The carboxyl group of Glu^{55} 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. The coordination sphere of the Ca^{2+} ion contains PQQ and protein atoms, including both oxygen atoms of the carboxylate of Glu^{177} and the amide oxygen of Asn^{261}. The PQQ atoms include the C5 quinone oxygen, one oxygen of the C7 carboxylate, and, surprisingly, the N6 ring atom, which is only 2.45 Å from the metal ion (Fig. 5). The C4 and C5 carbonyl oxygen atoms of PQQ are hydrogen-bonded to Arg^{331}; this also makes hydrogen bonds between its NH_2 and the carboxylate of Asp^{303}, which is well-positioned to act as the base required by the catalytic mechanism.

In the fully oxidised or reduced forms of PQQ, the C4 and C5 carbonyl oxygen atoms would be expected to be in the plane of the ring. In MDH as it is usually isolated, the PQQ is in the semiquinone form, and it is not so obvious what structure might be expected, especially in the environment of an enzyme active site, and the published structures all differ (29, 67, 69, 72). In one published structure of the MDH from *Methylophilus*, the C4 atom is trigonal and the C5 is tetrahedral (67, 69). It has now been concluded that this represents the C5-reduced intermediate, which is only compatible with the hydride-transfer mechanism (see above) (72).

The position of the substrate in the active site has not been determined. A theoretical analysis and refinement of the 1.9 Å resolution crystal structure of MDH (produced in the presence of methanol) (69) has subsequently been shown to have no methanol in the active site (72). Al-

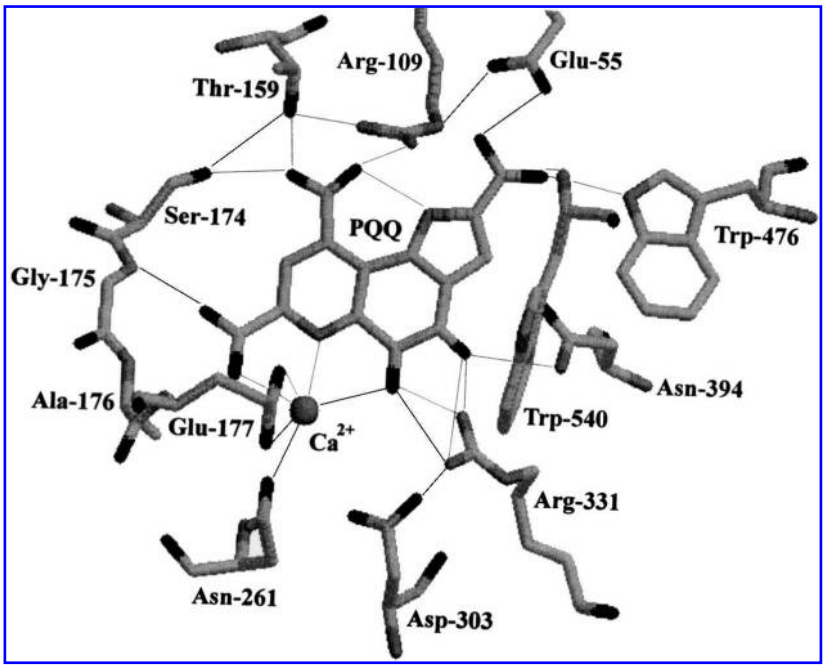

FIG. 5. The equatorial interactions and the coordination of Ca^{2+} in the active site of MDH of *Methylbacterium extorquens*.

though its exact location is unknown, the hydroxyl of methanol must necessarily be near Asp^{303} and the C5 carbonyl group of PQQ, and this would place the methyl group in a hydrophobic cavity bounded by side chains of Trp^{265}, Trp^{540}, and Leu^{556}, as well as the disulphide ring. This raises the problem that this enzyme has a broad substrate specificity, and can oxidise primary alcohols, including relatively large substrates such as pentanol and cinnamyl alcohol; it is not immediately obvious how these substrates could readily gain access to the active site, and understanding of this awaits solution of a structure containing one of these larger substrates.

Ethanol dehydrogenase (type I alcohol dehydrogenase or QEDH)

An ethanol dehydrogenase similar to MDH has been described in *Pseudomonas* strains (32, 34, 52, 65) and in *Rhodopseudomonas* (10); this enzyme is sometimes referred to as QEDH. Like MDH, it 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 oxidise a wide range of alcohol substrates, including secondary alcohols. Its absorption spectrum is very similar to that of MDH but it differs in its very low affinity for methanol; the K_m for ethanol is about 15 μM and that for methanol about ~1,000 times higher. This enzyme is unusual in being inhibited by EDTA (measured in the dye-linked assay), which leads to release of PQQ and formation of inactive monomers, as seen by gel filtration (65). The first demonstration of a metal ion in a quinoprotein alcohol dehydrogenase was in the ethanol dehydrogenase (type I) of *Pseudomonas aeruginosa* (52). This could be removed by treatment with a chelating agent and reconstitution achieved by incubation with PQQ and Ca^{2+} or Sr^{2+}, but not with Mg^{2+}, Mn^{2+}, or Cd^{2+}. It uses a specific *c*-type cytochrome (cytochrome c_{QEDH}) as electron acceptor (59,60). There has been some debate as to whether or not the enzyme has a small subunit as in MDH (see 31 for references to original work). The recent x-ray structure of the enzyme from *Pseudomonas aeruginosa*, however, confirms that this enzyme is homodimeric (42). Apart from differences in some loops, the folding pattern is very similar to the large α sub-

unit of MDH. PQQ is located in the centre of the superbarrel, coordinated to a Ca^{2+} 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 that a bulky tryptophan residue (Trp^{540}) in the active-site cavity of MDH is replaced by side chains of Phe^{408} and Leu^{409} in QEDH; and a leucine residue (Leu^{556}) immediately above the PQQ in MDH is replaced by a tryptophan side chain (Trp^{557}) in QEDH, forming a "lid" closing the active site cavity. These amino acid exchanges appear to have an important influence, causing the different substrate specificities of these otherwise very similar enzymes. In addition to the Ca^{2+} ion in the active-site cavity, found also in MDH, the QEDH contains a second Ca^{2+}-binding site at the N-terminus, which contributes to its stability.

SOLUBLE QUINOHAEMOPROTEIN ALCOHOL DEHYDROGENASES (TYPE II ALCOHOL DEHYDROGENASES OR QH-ADH)

This type of periplasmic alcohol dehydrogenase has been described in *Pseudomonas putida* (65) and in *Comamonas testosteroni* in which it has been shown to be a monomer (71 kDa) containing two prosthetic groups—one molecule of PQQ and a single haem C (19, 20, 35). In the assay system with phenazine ethosulphate, the pH optimum is 7.7 and there is no requirement for an amine activator. Because electron transfer from $PQQH_2$ is by way of haem C, this enzyme can also be assayed using ferricyanide. It has a wide specificity for primary and secondary alcohols, although it is unable to oxidise methanol; it also oxidises aldehydes and can accept large molecules such as sterols as substrates. It may be isolated as the apoenzyme, containing the haem, which is covalently bound, but lacking PQQ. Although calcium has not been measured in this enzyme, reconstitution to the active holoenzyme requires one molecule of PQQ and Ca^{2+} ions, indicating that its structure and function at the active site might be similar to MDH. Electron paramagnetic resonance (EPR) spectroscopy has been used to demonstrate the presence of the semiquinone form of PQQ in the active site; haem C has also been detected by EPR spectroscopy, and this, together with the absorption spectra, indicates that the iron is similar to that in other low-spin cytochromes *c* in being coordinated by histidine and methionine (19). Because this quinohaemoprotein enzyme is soluble and requires addition of PQQ for activity, it is likely to provide an excellent system for the study of intraprotein electron transport. Studies with nuclear magnetic resonance and Raman resonance spectroscopy have shown that binding of PQQ induces a conformational change in the protein, a reorientation of the methionine ligand of haem C, an increase of electron density on one of the pyrrole rings, and an increase in midpoint redox potential of the haem. Although this clearly indicates that the presence of PQQ in the enzyme affects the properties of the haem, it is unclear whether the interactions between the two cofactors are direct or indirect (20). All the available evidence is consistent with the obvious interpretation, that electrons pass from the reduced form of PQQ to the haem (midpoint redox potential, 140 mV) and thence to an external electron acceptor. The nature of the electron acceptor for this enzyme has not been reported but, because it is a periplasmic enzyme, this is likely to be a high-potential *c*-type cytochrome or a blue copper protein.

The deduced amino acid sequence of the N-terminal domain is similar to that of MDH and shows conservation of the tryptophan docking motifs, the adjacent cysteine residues involved in formation of the disulphide ring, and residues involved in binding PQQ, and coordination of the Ca^{2+} in the active site (64). The C-terminal domain includes a single haem C site. By using the deduced protein sequence, together with the x-ray coordinates of MDH and those of two *c*-type cytochromes, a convincing homology model has been published for the enzyme from *C. testosteroni* (41). The overall structure of the dehydrogenase domain was, as expected, similar to that of MDH, retaining the main structural features, including the mode of binding of PQQ and the Ca^{2+} ion, and the novel disulphide ring structure in the active site. Due to the absence of one loop (containing Trp^{477} in MDH), the active-site entrance of QH-ADH is

widened into a cleft-like structure instead of the funnel-shaped entrance of MDH. This provides a suitable explanation for the observed difference in substrate specificity; for example, the conversion of bulky alcohols and even sterols by QH-ADH as opposed to the more restricted substrate specificity of MDH. Although the haem part of the model was more difficult to achieve because of the low sequence identity with any known cytochromes, the final model was consistent with the proposals for conformational changes due to PQQ binding (see above), and the necessity for appropriate rates of electron transfer between PQQ (reduced) and the haem prosthetic group.

MEMBRANE-ASSOCIATED QUINOHAEMOPROTEIN ALCOHOL DEHYDROGENASE (TYPE III ALCOHOL DEHYDROGENASE)

This enzyme is a quinohaemoprotein–cytochrome *c* complex and has only been described in the acetic acid bacteria *Acetobacter* and *Gluconobacter* where it is responsible for the characteristic oxidation of alcohol (45, 48). It does not require ammonia as activator and has a pH optimum of 4–6. Its substrate specificity is relatively restricted compared with that of other quinoprotein alcohol dehydrogenases; it oxidises primary alcohols (chain length, C2–C6), but does not oxidise methanol or secondary alcohols, and it has some activity with formaldehyde and acetaldehyde.

It has three subunits and is tightly bound to the periplasmic membrane, requiring detergent for its isolation. Its natural electron acceptor is ubiquinone in the membrane (Fig. 6). Subunit I (72–80 kDa) is a quinohaemoprotein similar to the soluble (type II) quinohaemoprotein alcohol dehydrogenase, in that it has a single molecule of PQQ and a single haem C. The predicted amino acid sequence of subunit I indicates that it is a typical soluble protein, the first part of which has sequence similarity to the soluble MDH, but with a C-terminal extension having a single haem binding site. The predicted amino acid sequence of subunit II (48–53 kDa) indicates that it has three haem C binding motifs corresponding to the three haems that can be distinguished by biochemical techniques in the pure protein (50). Subunits I and II therefore have a total of four haems. Most of these enzymes from acetic acid bacteria have a third subunit (subunit III, 14–17 kDa). This dehydrogenase can be assayed with ferricyanide (as well as phenzine ethosulphate), which reacts at the level of one or more of the haem C prosthetic groups on subunits I and II. The rate of reaction with ferricyanide is about ~10 times greater than observed with the soluble quinohaemoprotein, and it has therefore been suggested that the cytochrome subunit II is needed for this high rate of electron transport. The quinohaemoprotein of acetic acid bacteria differs from all other alcohol dehydrogenases 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 (47). It 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 to have characteristic membrane protein structural domains. Furthermore, the electron acceptor for the quinohaemoprotein/cytochrome *c* complex is membrane ubiquinone, so we have the unusual situation where a *c*-type cytochrome precedes ubiquinone in the electron transport chain (Fig. 6).

Reconstitution experiments using subunits isolated from different acetic acid bacteria have led to a tentative model for electron transport in this enzyme system (43, 44, 50). It is probable 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), thereby keeping the correct conformation for electron transport of the alcohol dehydrogenase complex on the periplasmic surface of the membrane. Internal electron transport is presumably from $PQQH_2$ to the haem in subunit I and thence by way of two or three of the haems in the cytochrome subunit II to the membrane ubiquinone (Fig. 6). This raises the question of how the ubiquinone in the membrane reacts with subunit II to accept electrons from its haem. Clearly, part of the protein must be

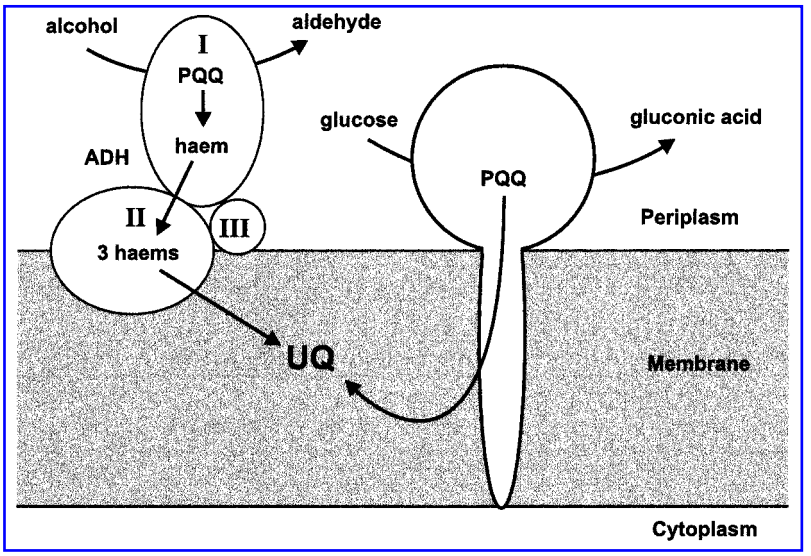

FIG. 6. The arrangement of the quinohaemoprotein type III alcohol dehydrogenase in acetic acid bacteria and the m-GDH. The ubiquinone electron acceptor is subsequently oxidised by a quinol oxidase (for example, cytochrome *bo*).

embedded in the membrane for this to occur, but subunit II does not appear to have typical hydrophobic transmembrane helices.

The deduced amino acid sequence of the N-terminal region of subunit I is sufficiently similar to that of the catalytic large α subunit of MDH that it could be used to build a model of its structure based on the MDH x-ray coordinates (15). In this structure, there are considerable differences in the external loops, particularly those involved in the formation of the shallow funnel leading to the active site in MDH. However, the active-site region is highly conserved, including the tryptophan and the disulphide ring on opposite sides of the plane of the PQQ, and most of the equatorial interactions with the PQQ. Especially important with respect to the mechanism is the conservation of the active-site base (Asp^{303} in MDH) and all the coordinations to the (assumed) Ca^{2+} ion. This suggests that the mechanism of this alcohol dehydrogenase is essentially similar to that of the MDH.

THE MEMBRANE-BOUND GDH (m-GDH)

This m-GDH has been described in a wide range of bacteria, including *Acinetobacter calcoaceticus*, enteric bacteria, pseudomonads, and acetic acid bacteria. Although the enzymes differ slightly in some properties, such as substrate specificity and stability, they are similar in most essential respects (for reviews, see 3, 25, 45, 48). GDH catalyses the oxidation of aldose sugars (mainly monosaccharides) in the periplasm, to the lactone, the electron acceptor being ubiquinone in the membrane. The substrate is the pyranose form of hexoses or pentoses. By using the enzyme from *E. coli*, it was shown that, remarkably, all pentoses tested are able to bind, acting as either substrates or competitive inhibitors; by contrast only D-hexoses were oxidised and substrates that were not substrates were not able to bind and act as competitive inhibitors (16). Good substrates include D-glucose, D-mannose, D-xylose and L-arabinose. After solubilization from the membrane, the enzyme is isolated as a monomer of ~87 kDa, containing one PQQ molecule. Topological and sequence analysis of the protein reveals that it is likely to have five membrane-spanning regions in the N-terminal region (residues 1–154), and this region is likely to contain the ubiquinone binding site (Fig. 6) (51, 70). The remaining C-terminal domain (assumed to be located in the periplasm) has considerable identity to the MDH sequence.

The enzymes from different bacteria vary with respect to their stability and the ease with which PQQ may be dissociated from them; it has been suggested that they can be considered

to be in two classes, depending on their stability in the presence of EDTA (61, 62, 71). The type I enzyme is easily denatured and occurs in *Escherichia coli* and *Pseudomonas* sp., whereas the more stable type II enzyme occurs in *Acinetobacter* and *Gluconobacter*. The stability of the *Escherichia coli* enzyme has been modified by formation of a chimeric enzyme using the GDH structural genes from *Escherichia coli* and *Acinetobacter calcoaceticus* (61, 62, 71).

Based on the coordinates of the MDH structure, a model structure of the periplasmic portion of the m-GDH of *Escherichia coli* (14) has been produced. The novel disulphide ring is replaced by a histidine residue that maintains the position of PQQ in the active site, consistent with the previous demonstration that a histidine residue is essential for binding PQQ (37). There are fewer equatorial interactions between the protein and PQQ, perhaps explaining why it is possible to effect the reversible dissociation of PQQ from GDH, but not from MDH. One clear difference between these proteins is that there is more "space" in the GDH active site, perhaps to accommodate the larger substrate. By analogy with the MDH structure, Asp^{466} is likely to be involved in base catalysis, initiation of the reaction being by abstraction of a proton from the anomeric hydroxyl of the pyranose ring. The model structure for the m-GDH has been used to design and interpret many site-directed mutagenesis studies, which have largely confirmed ideas about the structure and mechanism of the quinoprotein dehydrogenases (*e.g.*, 16, 26, 71).

It is difficult to come to a firm conclusion about the role of the divalent metal ion in m-GDHs. The metal ion content cannot be readily determined because dialysis, which is needed prior to assay, removes the PQQ and any bound metal ion. The only evidence available is from reconstitution studies. For this process, Mg^{2+} is usually better than Ca^{2+}, and many other metals can be used instead of Ca^{2+} (49). If the function of the metal ion required for reconstitution is to provide a metal ion at the active site, then this will have implications for our understanding of the mechanism of GDH, as it is unlikely that Mg^{2+} could replace Ca^{2+} in some aspects of the mechanism. Modelling studies using the predicted amino acid sequence of GDH, with the coordinates of MDH, have shown that some of the residues important in coordination with the Ca^{2+} are different in the GDH (14). Contrary to the usual assumptions, it is possible that the active site does contain Ca^{2+} bound to PQQ as in other quinoproteins, and that the requirement for Mg^{2+} merely indicates that it is essential for some part of the reconstitution process that it is not bound in the active site.

Relatively little is known about the mechanism of the m-GDH, but some information is available from chemical modification studies (37), and the modelling studies have suggested that many features of its mechanism are likely to be similar to that of MDH (3, 4, 14). One key difference is that electron transfer from the reduced PQQ does not occur in two stages to cytochrome *c* as in MDH; instead two electrons must pass through the protein to the ubiquinone in the membrane. Although this must also involve transfer of the electrons one at a time, this can be by a rapid direct route between the two redox centres; it is not necessary for a stable semiquinone to be formed, and indeed no semiquinone has ever been observed in GDH. This is perhaps consisent with the absence of the novel disulphide ring structure that is always present in the alcohol dehydrogenases.

THE SOLUBLE GDH (s-GDH) OF *ACINETOBACTER CALCOACETICUS*

This s-GDH has only been described in *Acinetobacter calcoaceticus* (for reviews, see 31, 56), although recent database searches have suggested that similar proteins may exist in other bacteria (56). It is a dimer of identical subunits of ~50 kDa, each containing one molecule of PQQ, and the sequence of the structural gene indicates that it is a periplasmic protein (13). 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 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 (46), and although it slowly reduces a soluble cytochrome *b*, there is no evidence that this cytochrome interacts with the electron transport chain (23). The presence of a metal ion (Ca^{2+}) in a GDH was first demonstrated in this s-GDH from *Acinetobacter calcoaceticus* (33). The Ca^{2+} could be removed by treatment with high salt concentrations, low pH, or high temperature, subsequent reconstitution requiring PQQ plus Ca^{2+}, Mn^{2+}, or Cd^{2+} (Mg^{2+} was not effective, and Sr^{2+} was not tested). This study has been extended by using a mutant of *Acinetobacter calcoaceticus* that is unable to synthesize PQQ and so only produces the apoenzyme (49). Active enzyme could be formed by incubation with PQQ and a divalent cation, the most effective ions being Cd^{2+} and Ca^{2+}, followed by Sr^{2+} and Mn^{2+}; no reconstitution occurred with Mg^{2+}. The K_m value for PQQ was 1.3 n*M* (it is 50–200 times higher in m-GDH). A second approach to the study of the metal ion in the s-GDH involved its expression in *E. coli*, which does not produce PQQ (54). The soluble apoenzyme was isolated in the dimeric form, monomerisation occurring during gel filtration in the presence of a chelating agent. From a study of reconstitution using the monomer and dimer, it appears that Ca^{2+} plays a dual role in this enzyme as it is required for dimerization as well as for incorporation of PQQ in a functional form. After reconstitution, the holoenzyme resembled reconstituted MDH in that Ca^{2+} could no longer be removed by chelating agents (30, 53).

The predicted amino acid sequence of s-GDH is different from that of other PQQ-containing dehydrogenases and lacks all their characteristic features, including the conserved tryptophan docking motifs (2, 3). Its crystal structure has now been determined (55–57). Remarkably the s-GDH is similar to the other PQQ-containing dehydrogenases in having a β-propeller superbarrel structure. However, by contrast with the other enzymes, it has six (not eight) four-stranded antiparallel β-sheets. 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 centre of each monomer, near the top of the β-propeller structure. The overall binding of PQQ is

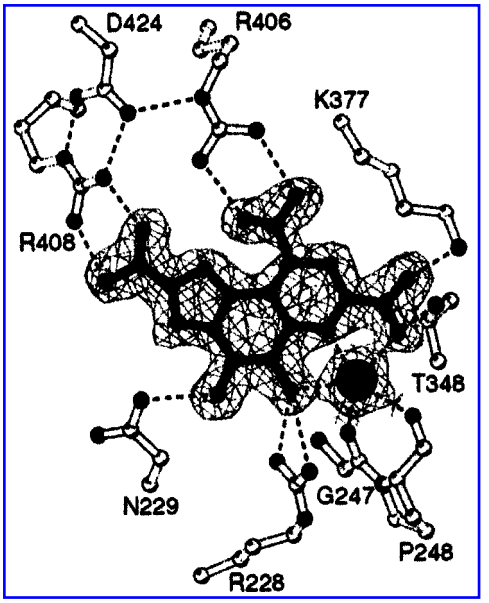

FIG. 7. The active site of the s-GDH of *Ac. calcoaceticus*. The dark sphere represents the Ca^{2+} ion.

similar to that in the active site of MDH (Figs. 5 and 7). In both proteins, equatorial interactions 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 (Fig. 7). When methylhydrazine (a competitive inhibitor) is present during crystallization, the condensation adduct is covalently attached to the C5 of the PQQ, thereby confirming that the C5 carbonyl group is the most reactive moiety of PQQ and that there is sufficient space in the structure of the active site for reaction at the C5 carbonyl to occur (55). It does not in itself provide evidence that the mechanism includes formation of a covalent adduct with PQQ. The structure (determined at 1.9 Å) containing glucose and reduced PQQ (57) shows that the glucose binding site is a wide and solvent accessible crevice that is located directly above PQQ. Glucose docks onto the PQQ surface making extensive hydrophobic interactions. 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. The O3, O4, and O6 hydroxyl groups make no hydrogen-bonding interactions with the protein, which explains its lack of specificity. The C1 atom is positioned di-

rectly above the PQQ C5 atom and close to the side chain of His^{144}. This 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 polarisation of the C5 carbonyl bond, thus improving its reactivity with nucleophiles.

It was shown that for an addition/elimination reaction mechanism, involving covalent attachment of the glucose to PQQ (analogous to the MDH mechanism in Fig. 1), three favourable interactions of the negatively charged O1 atom in the oxyanion would be required. This is energetically expensive and suggests that this mechanism is unlikely (57). 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. A similar proximity and geometry of substrate and prosthetic group have been observed in several nicotinamide and flavin-dependent oxidoreductases. 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. 8). 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 (22).

It should be noted that until recently there was insufficient evidence to come to any conclusions on whether or not a similar hydride mechanism operates in the membrane GDH and alcohol dehydrogenases (3–5, 22), but the balance of evidence is now also favouring a hydride-transfer mechanism for MDH (see above) (72).

THE ROLE OF QUINOPROTEINS IN ENERGY TRANSDUCTION

Whatever the specific role for each of the quinoprotein dehydrogenases, they must be coupled to an electron transport system, and for ATP production this must be arranged so as to produce a protonmotive force across the inner cytoplasmic membrane to drive ATP synthesis by the membrane ATP synthase. The type of electron transport systems for the quinoprotein dehydrogenases depends on the type of enzyme and on the type of terminal oxidase present (for complete review of this topic, see 31).

The alcohol dehydrogenases

The soluble periplasmic alcohol dehydrogenases (MDH, type I and type II alcohol dehydrogenases) are all involved in the first step in the complete oxidation to carbon dioxide of the alcohol growth substrates. In most cases, a specific soluble cytochrome *c* is the first electron acceptor, but this might be replaced in some systems with a small blue copper protein. Oxidation of the specific cytochrome *c* by the terminal oxidase is then mediated by a second (typical) cytochrome *c* or blue copper protein; for example, the electron transport chain for methanol oxidation is:

$$\text{Methanol} \rightarrow \text{MDH} \rightarrow \text{cytochrome } c_L \rightarrow \text{cytochrome } c_H \rightarrow \text{oxidase} \rightarrow O_2$$

These periplasmic electron transport chains differ from most other electron transport chains as they do not involve NADH dehydrogenase, ubiquinone, or cytochrome bc_1 complexes.

The acetic acid bacteria (*Acetobacter* and *Gluconobacter*) can obtain their energy from the incomplete oxidation of ethanol catalysed by a type III alcohol dehydrogenase (quinohaemoprotein), which oxidises ethanol to acetaldehyde. A membrane aldehyde dehydrogenase then oxidises the acetaldehyde to acetic acid, which is excreted into the growth medium. These bacteria have long been exploited because of such incomplete oxidation reactions, many of which are catalysed by quinoproteins. The membrane-bound alcohol dehydrogenase passes electrons to membrane ubiquinone which is oxidised by a quinol oxidase (*e.g.*, cytochrome *bo*) (Fig. 7) (48).

GDHs

There is no evidence that the s-GDH in *Acinetobacter calcoaceticus* is involved in electron

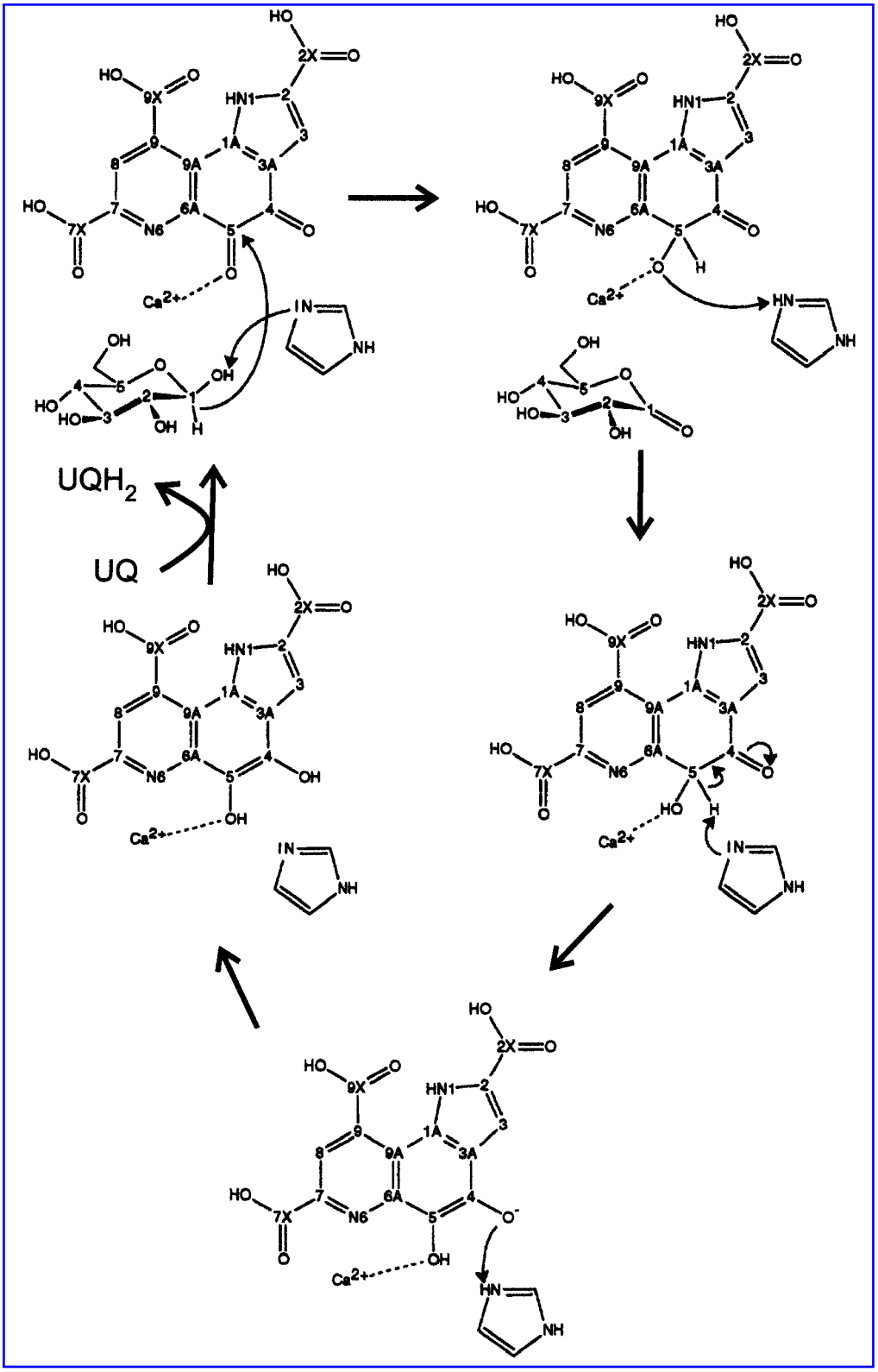

FIG. 8. The proposed hydride transfer mechanism of s-GDH. This mechanism is based on structural (57) and mechanistic (22) evidence. In essence, it is similar to that proposed for MDH (Fig. 2).

transport linked to energy transduction, but it does have a high affinity for PQQ and it has been suggested that this enzyme functions as a PQQ carrier, accumulating PQQ derived either from the external medium or endogenously, and then transferring it to the membrane-bound apoenzyme on the outer surface of the cytoplasmic membrane (49). If this is the case, then it is not obvious why the enzyme should be able to catalyse such a high rate of glucose oxidation.

Electron transport from the m-GDH, which interacts directly with membrane ubiquinone, varies from organism to organism, depending on the nature of the oxidase(s) produced. In *Pseudomonas aeruginosa*, the oxidation of ubiquinol is by way of the cytochrome bc_1 complex, which is subsequently oxidised by a typical periplasmic cytochrome *c*, the terminal oxidase being a cytochrome *co*. In other bacteria, the ubiquinol is oxidised directly by a ubiquinol oxidase. The function of the m-GDH is not always immediately obvious (for extensive discussion, see reference 31). It oxidises glucose in the periplasm to gluconate, which is usually further oxidised to ketogluconate, and these products are often excreted into the growth medium. This direct, nonphosphorylating, dissimilatory route is sometimes present as well as the apparently more efficient pathway in which glucose is taken up with concomitant phosphorylation by the phosphotransferase system. In acetic acid bacteria, the direct route involving m-GDH is the major route of energy production from glucose. In some pseudomonads, the gluconate and 2-ketogluconate may be subsequently taken up and metabolised by the Entner–Doudoroff pathway. The enzyme has a broad substrate specificity and may enable energy to be produced from the oxidation of sugars other than glucose, which cannot be used as carbon sources. In some bacteria, the acidic products gluconic acid and 2-ketogluconic acid are important in mineral phosphate solubilisation. In addition to these possible roles in energy metabolism in enteric bacteria, the respiratory chain involving the GDH and cytochrome *bd* might also play some role in respiratory protection during transfer from aerobic growth to anaerobic growth, when induction of oxygen-sensitive fermentative or nitrogen-fixing enzymes occurs.

Why have quinoprotein dehydrogenases?

In summary, the PQQ-dependent dehydrogenases all function in respiration and usually also in energy transduction, catalysing the oxidation of substrates in the periplasm of gram-negative bacteria. This contrasts with the membrane flavoproteins, which catalyse 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 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. In the case of some of the alcohol dehydrogenases, electrons are passed directly to high potential *c*-type cytochromes that are only found in the periplasm, and in these systems it is more appropriate to have the substrate oxidised in the periplasm of the bacteria.

ABBREVIATIONS

EPR, electron paramagnetic resonance; GDH, glucose dehydrogenase; MDH, methanol dehydrogenase; m-GDH, membrane-associated glucose dehydrogenase; PQQ, pyrroloquinoline quinone; QEDH, type I alcohol dehydrogenase; QH-ADH, quinohaemoprotein alcohol dehydrogenases; s-GDH, soluble glucose dehydrogenase.

Note added in proof: The following paper (referred to in the text as unpublished is now published): Afolabi PR, Mohammed F, Amaratunga K, Majekodunmi O, Dales SL, Gill R, Thompson D, Cooper JB, Wood SP, Goodwin PM, and Anthony C. Site-directed mutagenesis and X-ray crystallography of the PQQ-containing quinoprotein methanol dehydrogenase and

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