The Biochemistry, Physiology and Genetics of PQQ and PQQ-containing Enzymes

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ABSTRACT

Pyrrolo-quinoline quinone (PQQ) is the non-covalently bound prosthetic group of many quinoproteins catalysing reactions in the periplasm of Gram-negative bacteria. Most of these involve the oxidation of alcohols or aldose sugars. PQQ is formed by fusion of glutamate and tyrosine, but details of the biosynthetic pathway are not known; a polypeptide precursor in the cytoplasm is probably involved, the completed PQQ being transported into the periplasm. In addition to the soluble methanol dehydrogenase of methylotrophs, there are three classes of alcohol dehydrogenases; type I is similar to methanol dehydrogenase; type II is a soluble quinohaemoprotein, having a C-terminal extension containing haem C; type III 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. There are two types of glucose dehydrogenase; one is an atypical soluble quinoprotein which is probably not involved in energy transduction. The more widely distributed glucose dehydrogenases are integral membrane proteins, bound to the membrane by transmembrane helices at the N-terminus. The structures of the catalytic domains of type III alcohol dehydrogenase and membrane glucose dehydrogenase have been modelled successfully on the methanol dehydrogenase structure (determined by X-ray crystallography). Their mechanisms are likely to be similar in many ways and probably always involve a calcium ion (or other divalent cation) at the active site. The electron

ADVANCES IN MICROBIAL PHYSIOLOGY VOL 40
ISBN 0-12-027740-9

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transport chains involving the soluble alcohol dehydrogenases usually consist only of soluble *c*-type cytochromes and the appropriate terminal oxidases. The membrane-bound quinohaemoprotein alcohol dehydrogenases pass electrons to membrane ubiquinone which is then oxidized directly by ubiquinol oxidases. The electron acceptor for membrane glucose dehydrogenase is ubiquinone which is subsequently oxidized directly by ubiquinol oxidases or by electron transfer chains involving cytochrome bc_1, cytochrome *c* and cytochrome *c* oxidases. The function of most of these systems is to produce energy for growth on alcohol or aldose substrates, but there is some debate about the function of glucose dehydrogenases in those bacteria which contain one or more alternative pathways for glucose utilization. Synthesis of the quinoprotein respiratory systems requires production of PQQ, haem and the dehydrogenase subunits, transport of these into the periplasm, and incorporation together with divalent cations, into active quinoproteins and quinohaemoproteins. Six genes required for regulation of synthesis of methanol dehydrogenase have been identified in *Methylobacterium*, and there is evidence that two, two-component regulatory systems are involved.

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1. INTRODUCTION

Pyrrolo-quinoline quinone (PQQ) (Fig. 1) is the prosthetic group of most of the quinoprotein dehydrogenases that catalyse reactions in the periplasm of Gram-negative bacteria. It is synthesized independently of the apoenzyme dehydrogenase and transported to the periplasm where it is incorporated into the structure of the enzyme, forming active holoenzyme. It is always non-covalently bound to the enzyme. This contrasts with the prosthetic groups of other types of bacterial quinoprotein; the amine dehydrogenases which contain tryptophan–tryptophylquinone and the amine oxidases which contain topa quinone (Fig. 1). A fourth type of quinoprotein is the mammalian lysyl oxidase which contains lysine tyrosylquinone. In all quinoproteins except for those containing PQQ the prosthetic groups are covalently bonded to the enzyme, being derived by modification of tryptophan, tyrosine or lysine residues in the amino acid backbone of the protein (Anthony, 1996, 1998).

PQQ was first isolated from glucose and methanol dehydrogenases (Hauge, 1964; Anthony and Zatman, 1967). 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. At that time it was concluded that it might be a novel flavin or pteridine derivative. Its structure was subsequently elucidated by X-ray crystallography (Salisbury *et al.*, 1979) and its chemistry described in detail by Frank and Duine and their co-workers (Duine *et al.*, 1987; Duine, 1991). They showed that a key

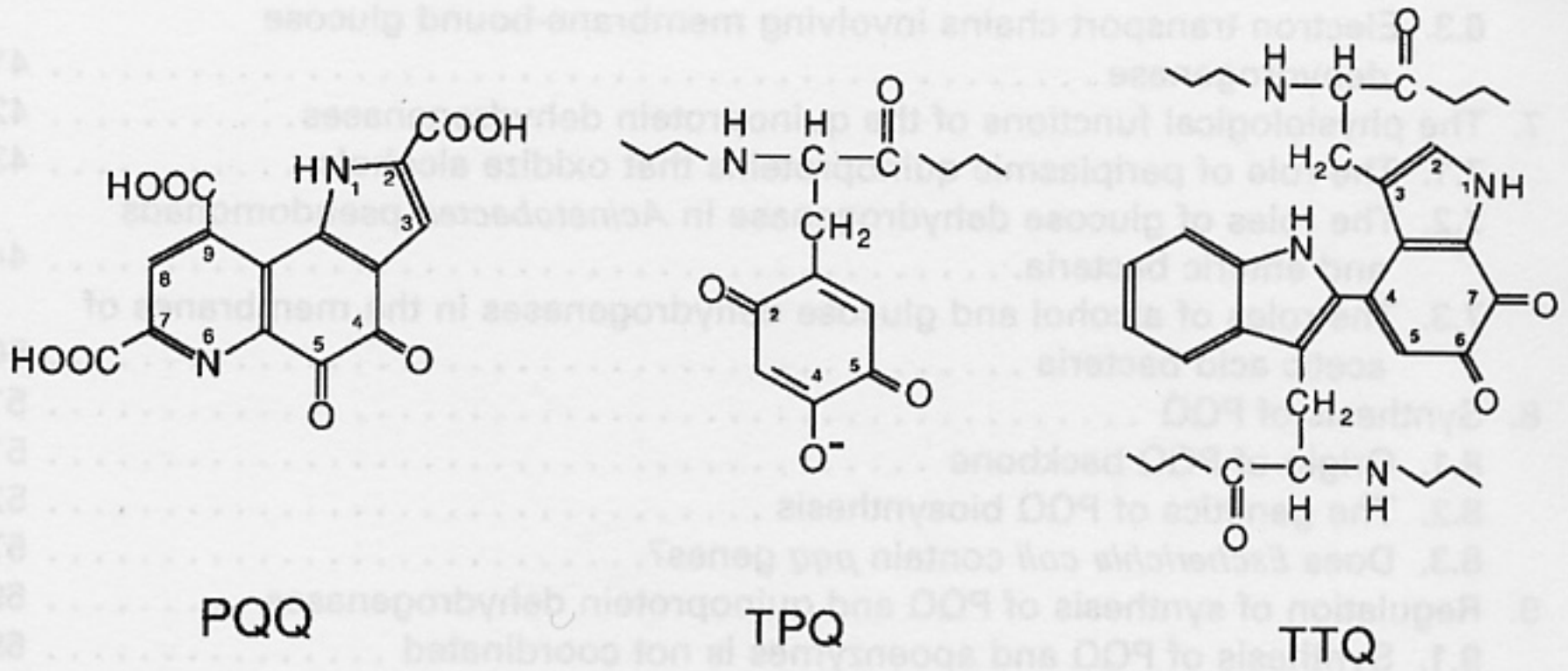
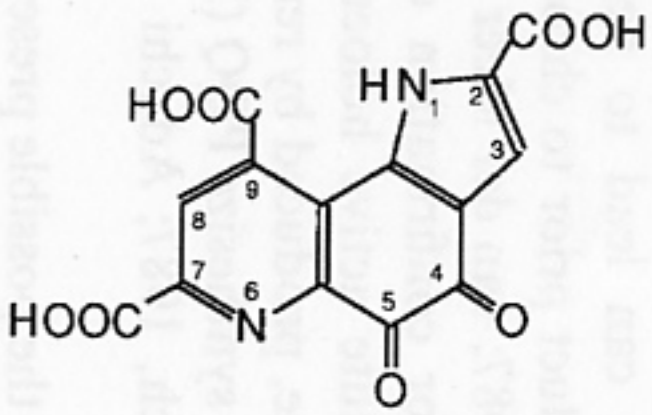


Figure 1 The prosthetic groups of quinoproteins. PQQ (pyrroloquinoline quinone) is the prosthetic group of the dehydrogenases discussed in this review. TTQ (tryptophan tryptophylquinone) is the prosthetic group of amine dehydrogenases. TPQ (6-hydroxyphenylalanine or topa quinone) is the prosthetic group of the copper-containing amine oxidases.

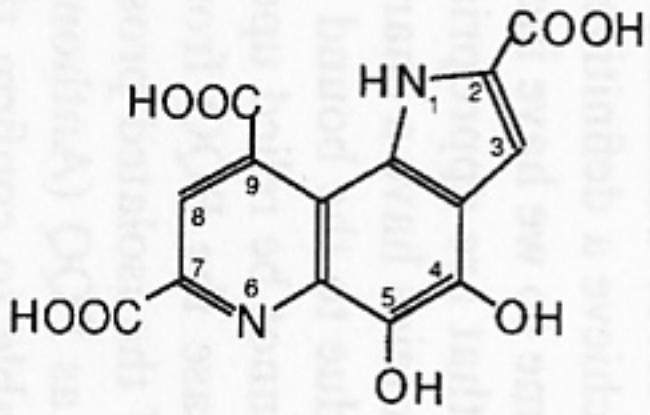
feature of the structure of PQQ is the *ortho* quinone at the C4 and C5 positions of the quinoline ring, which becomes reduced to the quinol during catalysis. The C5 carbonyl in the oxidized form is very reactive towards nucleophiles such as alcohols, ammonia, amines, cyanide and amino acids (Fig. 2) and this reactivity must be taken into account when interpreting mechanisms of enzyme action, when devising assay systems and when measuring PQQ in complex growth media. 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, only appreciated more recently, is its ability to complex Ca^{2+} or Mg^{2+} in solution. This was first shown by Mutzel and Gorisch (1991) and exploited by Itoh *et al.* (1997) in their chemical model systems in which a Ca^{2+}–PQQ complex is able to catalyse the oxidation of alcohols in organic solvents. It is now generally appreciated that the PQQ-containing enzymes probably all contain a divalent metal ion in their active sites (Section 4).

Although PQQ is not covalently bound to the apoenzyme dehydrogenases and can be readily dissociated from some holoenzymes, this dissociation is never important for its metabolic function. In this way PQQ is like FMN and FAD in flavoprotein dehydrogenases and, like them, quinoprotein dehydrogenases are most conveniently assayed by using artificial electron acceptors such as phenazine ethosulphate.

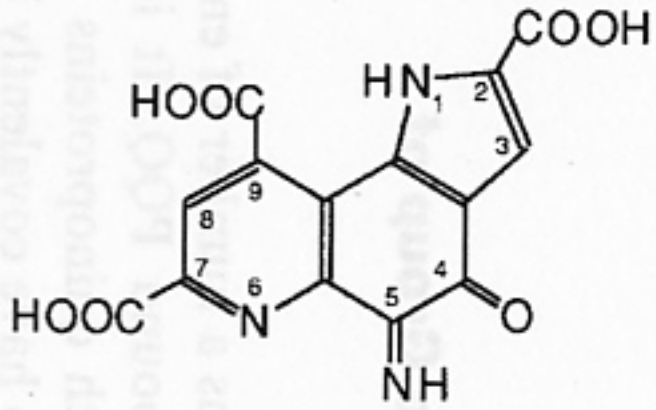
This review discusses the structure and function of the PQQ-containing dehydrogenases, the electron transport systems of which they form a part,



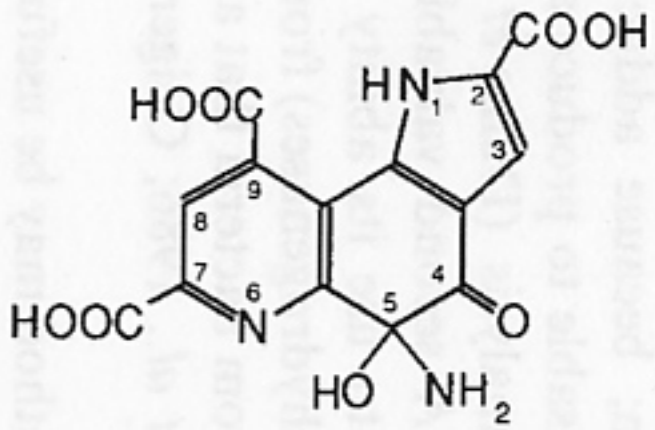
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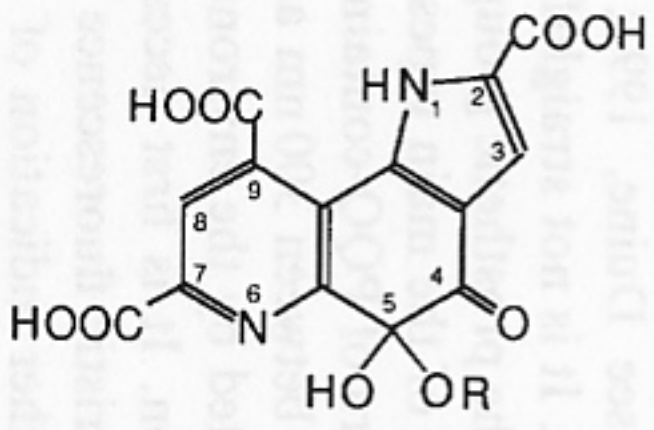
$PQQH_2$



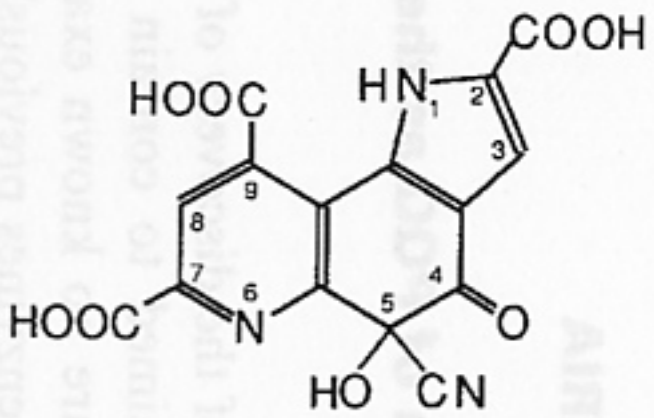
ammonia adduct
(Schiff base)



ammonia adduct
(aminocarbinol)



alcohol adduct
(hemiketal)



cyanide adduct

Figure 2 Adducts of PQQ. The ease of formation of these adducts has considerable importance in discussion of mechanisms, determination of the prosthetic group, and measurements of PQQ in biological samples.

their physiological functions and the genetics and regulation of synthesis of the dehydrogenases and of PQQ itself.

2. PQQ IN BACTERIA

2.1. Identification of PQQ as the Prosthetic Group of Quinoproteins

Early in the history of the discovery of quinoproteins a number of enzymes were erroneously claimed to contain covalently bound PQQ. It is now accepted that there are no known examples of such quinoproteins and it has been shown that enzymes previously thought to have covalently bound PQQ contain the other types of quinone prosthetic group, tryptophan tryptophylquinone, topa quinone or lysine tyrosylquinone (for reviews of these other quinoproteins see Duine, 1991; Davidson, 1993; Klinman, 1995; Anthony, 1996, 1998). It is not straightforward to achieve a definitive identification of PQQ as the prosthetic group of an enzyme so we have included here a brief summary of the main types of method that are appropriate.

Although the spectra of PQQ-containing quinoproteins have a characteristic absorption band between 300 nm and 420 nm, due to the bound PQQ, this is markedly affected by the environment and cannot be relied upon for definitive identification. It is first necessary to release the PQQ from the protein. The characteristic fluorescence spectrum of the isolated prosthetic group provides a further indication of the identity as PQQ (Anthony and Zatman, 1967; Dekker *et al.*, 1982) but it is advisable to confirm this by chromatographic methods, with appropriate standards. This is best done by HPLC using a reversed-phase column in combination with absorbance or fluorescence detection; because adduct formation can lead to negative results it may be advisable to produce a known adduct prior to chromatography or biological analysis (Duine *et al.*, 1983, 1987; van der Meer *et al.*, 1990; Duine, 1991). A second valuable method for confirmation of the presence of PQQ is to use its ability to reconstitute active holoenzyme (alcohol or glucose dehydrogenases) from apoenzyme, produced by removal of PQQ, or isolated from bacteria that are unable to synthesize PQQ (Duine *et al.*, 1983; Groen *et al.*, 1986; Geiger and Gorisch, 1987; Adachi *et al.*, 1988, 1990b).

A redox cycling method may be useful to indicate the possible presence of PQQ (Fluckiger *et al.*, 1995), but this is inappropriate as the only way of demonstrating that PQQ is the prosthetic group of a dehydrogenase. A modified form of the assay is useful as a 'stain' for quinoproteins after polyacrylamide gel electrophoresis. This non-specific method has been

used in attempts to demonstrate the presence of very low concentrations of PQQ in animal tissues and fluids, but although it appeared from such experiments that PQQ occurs in eukaryotic organisms, this conclusion has not yet achieved general acceptance (van der Meer *et al.*, 1990; Duine, 1991).

2.2. The Effect of Exogenous PQQ on Bacterial Growth

Many of the bacteria which can synthesize PQQ excrete it into the medium under certain growth conditions (Ameyama *et al.*, 1988; van Kleef and Duine, 1989). This may be important in microbial communities in natural environments because in some circumstances PQQ can stimulate bacterial growth. Some organisms which cannot synthesize PQQ make an apoenzyme dehydrogenase which can be converted to the active holoenzyme if PQQ is present in the growth medium (Groen *et al.*, 1986; van Schie *et al.*, 1987c). These bacteria may then be able to use additional substrates as energy sources, and possibly also as carbon sources. For example, a *Pseudomonas* sp. which makes the apoenzyme of polyvinyl alcohol dehydrogenase is only able to grow on polyvinyl alcohol when exogenous PQQ is supplied (Matsushita and Adachi, 1993b). The presence of PQQ can also enable more efficient use of a growth substrate, as in the case of *Comamonas testosteroni* which grows slowly on ethanol and some other alcohols, probably by using an NAD^+-dependent alcohol dehydrogenase. This organism also synthesizes an apo-alcohol dehydrogenase; addition of PQQ to the growth medium leads to formation of an active quinohaemoprotein and there is a concomitant increase in growth rate (Groen *et al.*, 1986). Synthesis of PQQ is 'expensive' in energy terms and organisms which can take advantage of exogenous PQQ to convert their apoenzymes into active enzymes may be at an advantage under natural conditions. In this context it is interesting to note that PQQ can act as a chemoattractant to *Escherichia coli* (de Jonge *et al.*, 1996), *Rhizobium meliloti* and *Bradyrhizobium japonicum* (Boiardi *et al.*, 1996).

PQQ can also stimulate growth of bacteria which synthesize active PQQ-dependent dehydrogenases. In this case, there is no effect on the growth rate or growth yield, but there is a decrease in the lag time on inoculation into fresh medium (Ameyama *et al.*, 1988).

3. THE QUINOPROTEIN DEHYDROGENASES CONTAINING PQQ

There is considerable diversity in the nature of the quinoprotein dehydrogenases containing PQQ as their prosthetic group. 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; freely soluble in the periplasm or bound to membranes; some are firmly associated with other redox components while others are not; their electron acceptors may be cytochromes, blue copper proteins or membrane ubiquinone; the PQQ may be tightly bound or easily dissociated; the enzyme may sometimes be produced in the apo form, requiring addition of PQQ, either during or after growth, for activity. The quinoproteins may be assayed using their physiological electron acceptors, for example, cytochromes, blue copper proteins or soluble ubiquinone analogues. It is more usual to assay them using artificial dye electron acceptor systems such as phenazine methosulphate, phenazine ethosulphate or Wurster's blue. It is assumed that these either interact directly with the PQQ or accept electrons from the surface of the enzyme near the PQQ. In addition to using these electron acceptors, the quinohaemoproteins may also be assayed with ferricyanide, which accepts electrons from the higher potential haem groups. For references to assay systems see reviews of Anthony (1986, 1996, 1998), Duine *et al.* (1987) and Duine (1991).

The best characterized enzymes are described below; key references are given in Table 1 and their structures, roles in electron transport and physiological functions discussed in Sections 5, 6 and 7 respectively. In addition to those listed in Table 1, the enzymes described below have also been identified as PQQ-containing quinoproteins.

A membrane-bound polyvinyl alcohol dehydrogenase (a type II alcohol dehydrogenase – a quinohaemoprotein) occurs in a *Pseudomonas* species during growth on polyvinyl alcohol; this organism cannot synthesize PQQ, which must be provided in the growth medium, or added to the apoenzyme after its isolation in order to obtain active holoenzyme (Shimao *et al.*, 1989, 1996).

A soluble alcohol dehydrogenase which is very similar to methanol dehydrogenase is produced by *Rhodopseudomonas acidophila* during growth on ethanol (Bamforth and Quayle, 1978). This organism also produces a soluble quinohaemoprotein aromatic alcohol dehydrogenase (Yamanaka, 1989); and a polyethylene glycol dehydrogenase which is likely to be a quinoprotein and is responsible for growth on this substrate (Kawai *et al.*, 1985; Yamanaka, 1991).

A number of other possible PQQ-containing dehydrogenases have been described in the membranes of acetic acid bacteria, in addition to the well-established alcohol and glucose dehydrogenases; these include dehydrogenases for aldehydes (see Section 4), glycerol and fructose (Matsushita *et al.*, 1994).

Table 1 PQQ-containing quinoproteins that oxidize alcohols.*

Enzyme	Prosthetic groups	Location	Organism	References
Methanol dehydrogenase; soluble quinoprotein	PQQ	Periplasm	Methylotrophs	Anthony and Zatman (1967); Anthony (1986, 1993b, 1996, 1997); Frank *et al.* (1989)
Alcohol dehydrogenase (type I); soluble quinoprotein	PQQ	Periplasm	*Pseudomonas aeruginosa*	Groen *et al.* (1984); Gorisch and Rupp (1989); Mutzel and Gorisch (1991); Schrover *et al.* (1993)
			Pseudomonas putida	Gorisch and Rupp (1989); Toyama *et al.* (1995)
Alcohol dehydrogenase (type II); soluble quinohaemoprotein	PQQ, haem C	Periplasm	*Comamonas testosteroni*	Groen *et al.* (1986); de Jong *et al.* (1995a,b); Geerlof *et al.* (1994); Stoorvogel *et al.* (1996)
			Pseudomonas putida (two types)	Toyama *et al.* (1995)
Alcohol dehydrogenase (type III); membrane quinohaemoprotein	PQQ, haem C	Membrane	*Gluconobacter*	Ameyama and Adachi (1982); Shinagawa *et al.* (1989); Kondo and Horinouchi (1997); Matsushita and Adachi (1993a); Matsushita *et al.* (1994, 1996)
			Acetobacter	Ameyama and Adachi (1982); Inoue *et al.* (1989, 1990, 1992); Kondo *et al.* (1995); Kondo and Horinouchi (1997); Matsushita and Adachi (1993a); Matsushita *et al.* (1994); Takemura *et al.* (1993); Tamaki *et al.* (1991); Tayama *et al.* (1989)

*Only those enzymes that have been studied extensively are included here; see text for mention of others.

Finally, a soluble haemoquinoprotein lupanine hydroxylase which is used during growth on alkaloids has been purified from a *Pseudomonas* species (Hopper *et al.*, 1991).

3.1. Soluble Quinoprotein Alcohol Dehydrogenases

3.1.1. Methanol Dehydrogenases

The most fully described alcohol dehydrogenases are the methanol dehydrogenases of methylotrophic bacteria which oxidize methanol to formaldehyde during growth of bacteria on methane or methanol (for reviews of the many examples of this enzyme see Anthony, 1986; for reviews of the work of the Delft group on mechanism see Duine *et al.*, 1987; Frank *et al.*, 1989; Duine, 1991; for reviews of structure and mechanism see Anthony, 1993b, 1996, 1998). Methanol dehydrogenase is a soluble periplasmic enzyme having an $\alpha_2\beta_2$ tetrameric structure; each α subunit (66 kDa in *Methylobacterium extorquens*) contains one molecule of PQQ and one Ca^{2+} ion. The β subunit is very small (8.5 kDa in *Methylobacterium extorquens*); it has no known function and, like PQQ, it cannot be reversibly dissociated from the α subunit. The structure of this enzyme has several important novel features, including eight 'tryptophan-docking motifs' that maintain the structure of the α subunit, 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 (see Sections 4 and 5 for further discussion of its structure and function). In *Methylobacterium extorquens* the genes encoding the α and β subunits (*mxaF* and *mxaI*) are located in a cluster of 12 methanol oxidation genes (*mxaFJGIR(S)ACKLDB)* (Nunn and Lidstrom, 1986b; Amaratunga *et al.*, 1997a,b). MxaG is cytochrome c_L, the electron acceptor for the dehydrogenase. Between *mxaF* and *mxaG* is *mxaJ* which codes for a periplasmic protein (30 kDa in *Methylobacterium extorquens*) of unknown function. Following studies with the enzyme from *Acetobacter methanolicus* it has been suggested that this might be a third enzyme subunit (Matsushita *et al.*, 1993), but this has not been confirmed, either for this enzyme or for other methanol dehydrogenases. The *mxaFJGI* genes are cotranscribed from a promoter upstream of *mxaF*. They have been sequenced and each of the predicted polypeptide products contains a typical signal sequence, which is cleaved on translocation into the periplasm (Nunn and Anthony, 1988; Nunn *et al.*, 1989; Anderson *et al.*, 1990).

Methanol dehydrogenase oxidizes a wide range of primary alcohols, having a high affinity for these substrates; for example, the K_m, for methanol is 5–20 μM. Secondary alcohols are rarely oxidized. The enzyme can be

assayed with phenazine ethosulphate or Wurster's blue, but not with ferricyanide. Using phenazine ethosulphate in the dye-linked assay system the pH optimum is about 9 and ammonia or methylamine is required as activator. Its physiological electron acceptor is a novel acidic cytochrome *c* (cytochrome c_L). Apart from its haem-binding site it has little homology with other cytochromes and in particular it lacks the conserved lysine residues which, in most *c*-type cytochromes, interact with cytochrome oxidase. This is not surprising in view of the fact that cytochrome c_L mediates electron transfer between methanol dehydrogenase and a typical Class I *c*-type cytochrome and it has been shown that the reaction of methanol dehydrogenase and cytochrome c_L is initially by way of electrostatic interactions between lysine residues on the basic dehydrogenase and carboxylate residues on the acidic cytochrome (Chan and Anthony, 1991b; Cox *et al.*, 1992); after initial 'docking' it is possible that an electron transfer complex is subsequently formed in which there is a hydrophobic component to the bonding (Harris *et al.*, 1994; Dales and Anthony, 1995).

3.1.2. *Ethanol Dehydrogenases (Type I Alcohol Dehydrogenases)*

An ethanol dehydrogenase similar to methanol dehydrogenase has been described in *Pseudomonas aeruginosa* and *Pseudomonas putida* (Groen *et al.*, 1984; Gorisch and Rupp, 1989; Mutzel and Gorisch, 1991; Toyama *et al.*, 1995). Like methanol dehydrogenase, 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 oxidize a wide range of alcohol substrates including secondary alcohols. Its absorption spectrum is very similar to that of methanol dehydrogenase but it differs in its very low affinity for methanol; the K_m for ethanol is about 15 μM and that for methanol about 1000 times higher. In the first description, the enzyme from *P. aeruginosa* was said to be monomeric with two molecules of PQQ per monomer (101 kDa) (Groen *et al.*, 1984). Subsequently it has been claimed to have the same $\alpha_2\beta_2$ tetrameric structure as in methanol dehydrogenase (Schrover *et al.*, 1993); no β subunit could be seen, however, in the pure enzyme from *P. putida* (Toyama *et al.*, 1995). 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 (Toyama *et al.*, 1995). This type I alcohol dehydrogenase uses a specific *c*-type cytochrome (cytochrome c_{QEDH}) as electron acceptor (Schrover *et al.*, 1993).

3.1.3. *Soluble Quinohaemoprotein Alcohol Dehydrogenases (Type II Alcohol Dehydrogenases)*

A periplasmic alcohol dehydrogenase has been described in *Comamonas testosteroni*. It is a monomer (71 kDa) containing two prosthetic groups – one molecule of PQQ and a single haem C (Groen *et al.*, 1986; de Jong *et al.*, 1995a,b). 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 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 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 was first isolated from *C. testosteroni* as the apoenzyme, containing the haem, which is covalently bound, but lacking PQQ. Reconstitution to the active holoenzyme requires one molecule of PQQ and calcium ions, indicating that its structure and function at the active site might be similar to that of methanol dehydrogenase. 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 (de Jong *et al.*, 1995a). 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 NMR 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 (de Jong *et al.*, 1995b). 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 gene encoding this dehydrogenase has been recently isolated, sequenced and expressed in *E. coli* to give the apoenzyme, lacking PQQ (Stoorvogel *et al.*, 1996); it was necessary to grow the host *E. coli* in a low concentration of oxygen to obtain synthesis of the haem required for the haem component of the quinohaemoprotein. The DNA sequence

indicates that the preprotein has a signal peptide typical of periplasmic enzymes. Part of the deduced amino acid sequence is similar to that of methanol dehydrogenase 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.

A type II alcohol dehydrogenase (soluble quinohaemoprotein) has also been described in *P. putida*, in which it is formed as the holoenzyme (Toyama *et al.*, 1995). Remarkably, this organism has two immunologically distinct forms of the enzyme; one is induced during growth on medium-chain length alcohols (e.g. butanol) and the other during growth only on 1,2-propanediol or glycerol. The same organism also has a type I alcohol dehydrogenase which is present during growth on all substrates but induced to the highest level during growth on ethanol.

3.2. Membrane-associated Quinohaemoprotein Alcohol Dehydrogenases (Type III Alcohol Dehydrogenases)

The type III alcohol dehydrogenase is a quinohaemoprotein–cytochrome *c* complex and has only been described in the acetic acid bacteria *Acetobacter* and *Gluconobacter* (for reviews see Beppu, 1993; Matsushita and Adachi, 1993a; Matsushita *et al.*, 1994). Together with the membrane-bound aldehyde dehydrogenase (see below), it is responsible for the characteristic 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 compared with other quinoprotein alcohol dehydrogenases; it oxidizes primary alcohols (chain length, C_2–C_6) but does not oxidize methanol or secondary alcohols and has some activity with formaldehyde and acetaldehyde.

It is distinguished from other alcohol dehydrogenases in usually having three subunits and in being tightly bound to the periplasmic membrane, requiring detergent for its isolation. Its natural electron acceptor is ubiquinone in the membrane. 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 structural genes of several acetic acid bacteria have been sequenced and the genes encoding subunits I and II are adjacent on the genome with the same polarity and in the same reading frame (Tamaki *et al.*, 1991; Kondo and Horinouchi, 1997). Translation of the gene sequences shows that all the subunits have N-terminal signal peptides typical of periplasmic proteins. 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

methanol dehydrogenase but with a C-terminal extension having a single haem binding site (Inoue *et al.*, 1989, 1990; Tamaki *et al.*, 1991). The predicted amino acid sequence of subunit II (48–53 kDa) (Tamaki *et al.*, 1991; Inoue *et al.*, 1992) 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 (Matsushita *et al.*, 1996). Subunits I + 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), although this is absent from *Acetobacter polyoxogenes* (Tayama *et al.*, 1989). The gene coding for this small subunit is not linked to the genes encoding the other two subunits (Takemura *et al.*, 1993; Kondo *et al.*, 1995; Kondo and Horinouchi, 1997). The predicted amino acid sequence for subunit III indicates that its processed size is greater (about 20 kDa) than that estimated by SDS–PAGE (14 kDa).

As with all other quinoprotein dehydrogenases, this enzyme may be assayed with phenazine methosulphate, which presumably reacts at the level of PQQ; in addition it can be assayed with ferricyanide 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 quniohaemoprotein 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 (Matsushita *et al.*, 1992b). 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.

It has recently proved possible to isolate subunit II (the tri-haem cytochrome *c*) and a separate complex containing subunits I and III from *Gluconobacter suboxydans* (Matsushita *et al.*, 1996). Subunits I + III had activity in the dye-linked assay, and some activity (only at low pH) with ferricyanide, presumably by way of the haem C of the quinohaemoprotein subunit I. There was no activity using coenzyme Q_1 but this activity was restored by addition of subunit II, leading to the conclusion that electron transfer to the quinone takes place by way of the haems on this cytochrome component. As expected, subunit II had no catalytic activity alone. Kinetic analysis of ferricyanide reduction at various pH values indicated that only two of the haems in subunit II may be involved in electron transfer from

reduced PQQ in subunit I to the ubiquinone, but further work on this complex system is needed to be certain about this.

The work described above has recently been complemented by a different experimental approach using the enzymes from *Gluconobacter suboxydans* and *Acetobacter pasteurianus* (Kondo *et al.*, 1995; Kondo and Horinouchi, 1997). Previous work with the enzyme from *Acetobacter polyoxogenes*, which has no small subunit III, had indicated that this subunit is not essential for activity (Tamaki *et al.*, 1991). Subsequently it was shown that spontaneous mutants of *A. pasteurianus*, lacking subunit III, contained inactive enzyme and could be complemented by the gene encoding subunit III. Thus, in this organism at least, subunit III is essential for activity (Kondo *et al.*, 1995). Analysis of the location of the subunits in *G. suboxydans* and *A. pasteurianus*, and in the mutants led to the suggestion 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 (Fig. 3) (Kondo

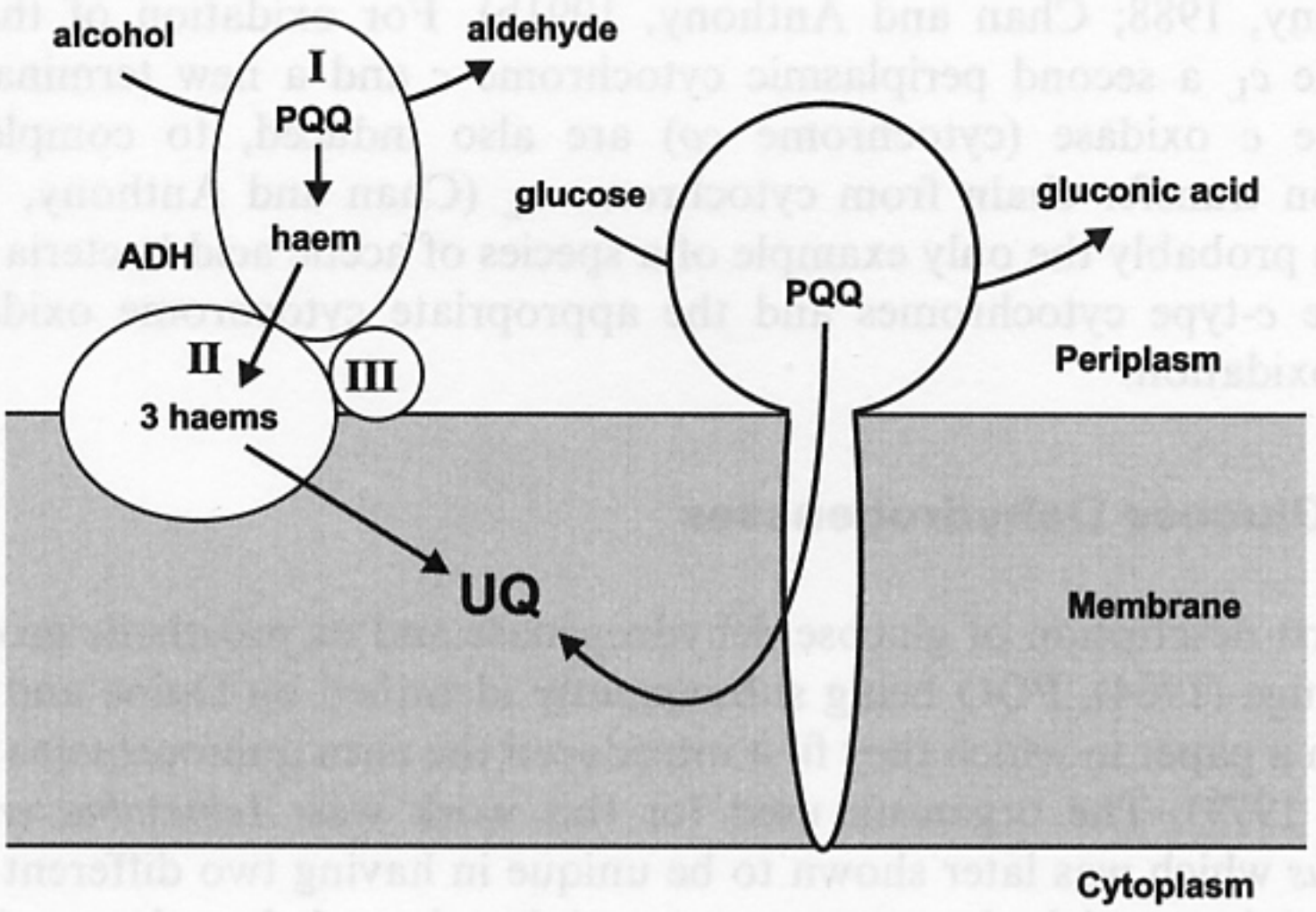


Figure 3 The arrangement of the quinohaemoprotein type III alcohol dehydrogenase (ADH), and glucose dehydrogenase (GDH) in the membrane of acetic acid bacteria. The interaction of subunit II of the alcohol dehydrogenase with the membrane is unusual; although firmly attached, it does not have typical transmembrane helices. The membrane glucose dehydrogenase is likely to be organized in the same way in all bacteria.

and Horinouchi, 1997). This is consistent with the conclusions of Matsushita *et al.* (1996). Internal electron transport is presumably from $PQQH_2$ to the haem in subunit I and hence by way of two or three of the haems in the cytochrome subunit II to the membrane ubiquinone. 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 embedded in the membrane for this to occur but subunit II does not appear to have typical hydrophobic transmembrane helices.

In some growth conditions (high aeration, low pH), the arrangement of the subunits appears to become altered so that an inactive form of the enzyme is produced; a change in growth conditions (low aeration, neutral pH) then leads to an alteration to the active form, a process that requires expenditure of energy (Matsushita *et al.*, 1995b).

Acetobacter methanolicus is an unusual type of acetic acid bacterium, being able to grow on methanol as well as on glycerol and sugars. During growth on glycerol a membrane quinohaemoprotein (type III alcohol dehydrogenase) is produced which is similar to that in other species of *Acetobacter*, together with a ubiquinol oxidase, cytochrome *bo* (Matsushita *et al.*, 1992a). During growth on methanol, by contrast, a typical methanol dehydrogenase and cytochrome c_L are induced, which are unusual only in the optimum pH (pH 4) at which they react (Elliott and Anthony, 1988; Chan and Anthony, 1991b). For oxidation of the cytochrome c_L a second periplasmic cytochrome *c* and a new terminal cytochrome *c* oxidase (cytochrome *co*) are also induced, to complete the electron transfer chain from cytochrome c_L (Chan and Anthony, 1991a). This is probably the only example of a species of acetic acid bacteria having soluble *c*-type cytochromes and the appropriate cytochrome oxidase for their oxidation.

3.3. Glucose Dehydrogenases

The first description of glucose dehydrogenase and its prosthetic group was by Hauge (1964), PQQ being subsequently identified 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 and the other an integral membrane protein.

3.3.1. *The Membrane-bound Glucose Dehydrogenase*

This glucose dehydrogenase has been described in a wide range of bacteria including *A. calcoaceticus*, enteric bacteria, pseudomonads and acetic acid

bacteria (Table 2). Although the enzymes differ slightly in some properties such as substrate specificity and stability, they are similar in most essential respects (for reviews see Duine *et al.*, 1987; Matsushita and Adachi, 1993a; Matsushita *et al.*, 1994; Anthony, 1996, 1998). Glucose dehydrogenase catalyses the oxidation, to the lactone, of the pyranose form of D-glucose and other monosaccharides, usually including mannose, galactose, rhamnose and xylose. It differs from the soluble enzyme in that it is unable to oxidize disaccharides but, remarkably, a mutation in a single amino acid (histidine to asparagine) was sufficient to confer on the enzyme from *G. suboxydans* the ability to oxidize maltose (Cleton-Jansen *et al.*, 1991); modelling 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 (Beardmore-Gray and Anthony, 1986; Matsushita *et al.*, 1982a, 1987, 1989a,b). After solubilization from the membrane, the enzyme is isolated as a monomer of about 87 kDa, containing one PQQ molecule. The structural gene has been sequenced from four different bacteria; the predicted protein sequences are similar to each other and also have important similarities to other quinoproteins (Cleton-Jansen *et al.*, 1988a, 1989, 1990; Anthony, 1992a, 1996; Cha *et al.*, 1997). Topological and sequence analysis of the protein reveals that it is likely to have five membrane-spanning regions in the N-terminal region and this region is likely to contain the ubiquinone binding site (Yamada *et al.*, 1993b) (Fig. 3).

The enzymes from different bacteria differ 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 with respect to EDTA (Dokter *et al.*, 1986; Sode *et al.*, 1995a,b). The type I enzyme is easily denatured and occurs in *E. coli* and *Pseudomonas* sp., whereas the more stable type II enzyme occurs in *Acinetobacter* and *Gluconobacter*. The stability of the *E. 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 *E. coli* and *A. calcoaceticus* (Sode *et al.*, 1995a,b).

3.3.2. *The Soluble Glucose Dehydrogenase of* Acinetobacter calcoaceticus

Besides the typical membrane-bound glucose dehydrogenase, *A. calcoaceticus* also contains a completely different soluble enzyme which, to date, has only been described in this organism (Dokter *et al.*, 1986; Geiger and Gorisch, 1986, 1989; Gorisch *et al.*, 1989). It is a dimer of identical subunits of about 50 kDa, each containing one molecule of PQQ, and the sequence of the structural gene indicates that it is a periplasmic protein (Cleton-Jansen

Table 2 PQQ-containing quinoproteins that oxidize glucose.*

Enzyme	Location	Organism	References
Glucose dehydrogenase (soluble)	periplasm	*Acinetobacter calcoaceticus*	Hauge (1964); Cleton-Jansen *et al.* (1989); Dokter *et al.* (1986, 1987); Geiger and Gorisch (1986, 1989); Matsushita *et al.* (1989b, 1995a); Schlunegger *et al.* (1993)
Glucose dehydrogenase (membrane)	membrane	*Acinetobacter calcoaceticus*	Cleton-Jansen *et al.* (1988a,b); Duine *et al.* (1979); Matsushita *et al.* (1989b, 1995a)
		Pseudomonas	Imanaga (1989); Matsushita *et al.* (1980, 1982a); van Schie *et al.* (1984)
		Gluconobacter suboxydans	Ameyama *et al.* (1981); Cleton-Jansen *et al.* (1991); Matsushita *et al.* (1989c)
		Escherichia coli	Ameyama *et al.* (1986); Beardmore-Gray and Anthony (1986); Cleton-Jansen *et al.* (1990); Hommes *et al.* (1984); Matsushita *et al.* (1986, 1987); Shinagawa *et al.* (1986); Yamada *et al.* (1993a,b)
		Klebsiella pneumoniae	Neijssel *et al.* (1983, 1989); Buurman *et al.* (1990, 1994)

*The references given are to the enzymes about which most information is available; other bacteria with membrane glucose dehydrogenase are mentioned in the text. No glucose dehydrogenase has been described in which there is any prosthetic group in addition to the PQQ.

et al., 1989). It catalyses the oxidation of D-glucose, arabinose, galactose, xylose and also the disaccharides lactose, cellobiose and maltose. It is active with Wurster's blue (pH optimum 9) and 2,6-dichlorophenolindophenol (optimum pH 6), but not with ferricyanide. The soluble enzyme does not react with ubiquinone (Matsushita *et al.*, 1989b), 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.*, 1988). Although its predicted amino acid sequence has a small degree of similarity to the other quinoprotein dehydrogenases (Cleton-Jansen *et al.*, 1989; Anthony, 1992a), it lacks all their characteristic features including the eight 11-residue tryptophan docking motifs which are typical of all the other PQQ-containing quinoprotein dehydrogenases (Anthony, 1996). Its structure has not yet been determined although it has been crystallized (Geiger and Gorisch, 1986; Schlunegger *et al.*, 1993).

3.4. Aldehyde Dehydrogenases

At first glance there appear to be almost as many PQQ-dependent aldehyde dehydrogenases as there are alcohol dehydrogenases, but many of the first descriptions are not sufficient for their definitive identification, and considerable doubt has now been cast on their status as PQQ-containing dehydrogenases. There is a further confusion in that many alcohol dehydrogenases are also able to oxidize aldehydes and it is not certain to what extent this indicates a physiological function. The enzymes for which most information is available are the aldehyde dehydrogenases isolated with detergent from the membranes of acetic acid bacteria (Matsushita and Adachi, 1993c). These usually have a low pH optimum (about pH 4) and oxidize aldehydes of carbon chain length C_2–C_4. Their function is to catalyse the oxidation, in the periplasm, of aldehydes produced by the action of the membrane-bound quinohaemoprotein alcohol dehydrogenase. Free aldehydes have not been detected during the oxidation of ethanol to acetic acid and these two enzymes are responsible for the production of acetic acid from ethanol which is characteristic of acetic acid bacteria. The aldehyde dehydrogenase has two or three subunits, one being a cytochrome *c* component and the other originally thought to contain PQQ. However, mutants of one strain of *Acetobacter*, which were unable to produce PQQ and therefore produced inactive alcohol and glucose dehydrogenases, had the same level of aldehyde dehydrogenase activity as the parent strain, suggesting that this enzyme cannot be a PQQ-containing quinoprotein (Takemura *et al.*, 1994). Furthermore, the gene encoding this enzyme has been isolated and its predicted amino acid sequence does not show similarity to other quinoprotein dehydrogenases (Tamaki *et al.*, 1989). The

electron acceptor for these membrane aldehyde dehydrogenases has not been identified, but as there is usually no cytochrome *c* oxidase in these bacteria it is probable that electrons are passed to the cytochrome subunit and thence to ubiquinone in the membrane, as shown in the alcohol dehydrogenases of these bacteria.

4. THE IMPORTANCE OF DIVALENT METAL IONS IN THE STRUCTURE AND FUNCTION OF PQQ-CONTAINING QUINOPROTEINS

That divalent metal ions are important in the structure or function of these quinoproteins was first indicated by the early work on the membrane-bound enzymes (Duine *et al.*, 1983; Ameyama *et al.*, 1985) (Tables 3 and 4). PQQ can be removed from these enzymes by treatment with EDTA, heat, low pH or high salt concentrations and in some conditions they are produced as the apo-form, lacking PQQ. Reconstitution with PQQ then requires the presence of a divalent metal ion, which is most commonly Ca^{2+} or Mg^{2+}, but other divalent ions are sometimes as good or better (Table 4). Although these observations demonstrated a requirement for divalent metal ions for insertion of PQQ into the enzymes, they provided no indication of whether or not the metal ions had been incorporated as structural or functional components of the active enzymes.

4.1. Calcium in Methanol Dehydrogenase and Other Alcohol Dehydrogenases

The only enzyme for which a structure is available is methanol dehydrogenase, which has a Ca^{2+} ion tightly coordinated to the PQQ in the active site (Fig. 4; Section 5). The presence of Ca^{2+} in methanol dehydrogenase was first demonstrated in *Methylobacillus glycogenes* by Adachi *et al.* (1990a), and this was subsequently confirmed in the enzymes from other methylotrophs (Richardson and Anthony, 1992) (Table 3). It is not usually possible to remove the Ca^{2+} from methanol dehydrogenase by any treatment, including dialysis against chelating agents, but this has been achieved using the enzyme from *Methylophaga marina*, a methylotroph that is able to grow at high salt concentrations (Chan and Anthony, 1992).

That Ca^{2+} might play some catalytic role in methanol dehydrogenase was indicated by work using some unusual mutants of *Methylobacterium extorquens*. These mutants (defective in the *mxaA*, *K* or *L* genes) synthesize normal α and β subunits, and PQQ, but they produce an inactive enzyme

Table 3 Divalent metal ions in PQQ-containing quinoproteins that oxidize alcohols.

Enzyme	Organism	Metal determined	Metal for reconstitution	References
Methanol dehydrogenase	*Methylobacillus*	Ca	Ca Sr (during growth)	Adachi *et al.* (1990a)
	Methylobacterium	Ca	–	Richardson and Anthony (1992)
	Methylobacterium (*mxaA* mutant)	None	Ca = Sr = Ba (not Mg)	Goodwin *et al.* (1996); Goodwin and Anthony (1996)
	Methylophilus	Ca	–	Richardson and Anthony (1992)
	Paracoccus	Ca (Sr)	Ca Sr (during growth)	Harris and Davidson (1994a,b); Richardson and Anthony (1992);
	Hyphomicrobium	Ca	–	Richardson and Anthony (1992)
	Methylophaga marina	Ca	Ca	Chan and Anthony (1992)
Alcohol dehydrogenase (type I); quinoprotein	*Pseudomonas aeruginosa*	Ca	Ca Sr (not Mg Mn Cd)	Mutzel and Gorisch (1991); Schrover *et al.* (1993)
Alcohol dehydrogenase (type II) (soluble quinohaemoprotein)	*Comamonas testosteroni*		Ca (not Mg)	Groen *et al.* (1986); de Jong *et al.* (1995a,b)
Alcohol dehydrogenase (type III) (membrane quinohaemoprotein)	*Gluconobacter suboxydans*		Ca (not Mg)	Shinagawa *et al.* (1989)

Table 4 Divalent metal ions in relation to glucose dehydrogenase.

Enzyme	Organism	Metal active in reconstitution	References
Glucose dehydrogenase (soluble)	*Acinetobacter calcoaceticus*	Ca(100) Mn(67) Cd(60) (not Mg)	Geiger and Gorisch (1989); Olsthoorn and Duine (1996)
	Acinetobacter calcoaceticus (mutant)	Cd(127) Ca(100) Sr(68) Mn(63) Co(10) Ba(7) (not Mg)	Matsushita *et al.* (1995a)
Glucose dehydrogenase (membrane)	*Acinetobacter calcoaceticus*	Mg > Ca	Duine *et al.* (1983); Ameyama *et al.* (1985)
	Acinetobacter calcoaceticus (mutant)	Mg(115) Ca(100) Zn(100) Sr(70) Co(33) Cd(27) Ba(10)	Matsushita *et al.* (1995a)
	Escherichia coli	Mg > Ca > Co	Ameyama *et al.* (1985, 1986); Shinagawa *et al.* (1986)
	Klebsiella pneumoniae	Mg, Ca	Buurman *et al.* (1990); Neijssel *et al.* (1983)
	Pseudomonas fluorescens	Mg > Ca	van Schie *et al.* (1984); Ameyama *et al.* (1985); Imanaga (1989)

The presence of a metal ion (Ca^{2+}) has only been demonstrated in the soluble enzyme from *Acinetobacter calcoaceticus*. The values in parentheses refer to relative activities (%) of activity measured with calcium determined after reconstitution for a fixed length of time; they do not necessarily indicate the most active form of the enzyme.

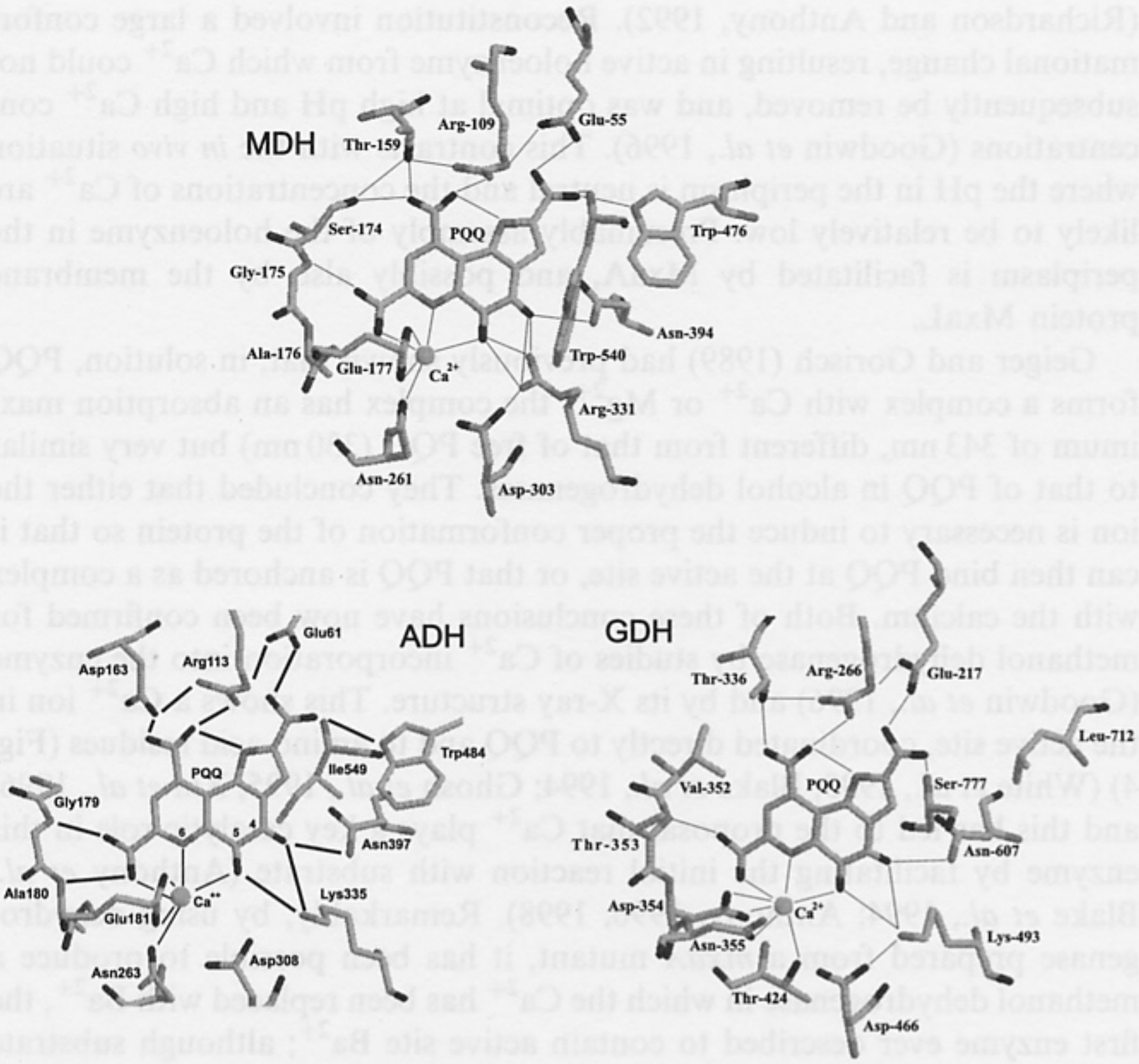


Figure 4 The equatorial interactions of PQQ and the coordination of Ca^{2+} in the active site of methanol dehydrogenase (MDH). This was determined by X-ray crystallography; the structures of alcohol dehydrogenase (type III) (ADH) and membrane glucose dehydrogenase (GDH) are based on molecular modelling studies (see Section 5). This figure also shows Asp303 which is likely to act as a catalytic base, and Arg331 which may also be involved in the mechanism. Figure 9 shows the axial interactions that are also involved in holding PQQ in place in the active site.

with an abnormal absorption spectrum in the PQQ region (Nunn and Lidstrom, 1986a). The DNA sequences of these genes indicate that MxaA is a periplasmic protein, MxaK is cytoplasmic and MxaL is an integral membrane protein. It is possible that two other proteins, MxaC (cytoplasmic) and MxaD (periplasmic), coded by genes in the same cluster as *mxaA*, are also involved in Ca^{2+} incorporation (Morris *et al.*, 1995). Reconstitution of active enzyme having the normal absorption spectrum was achieved by incubation of the purified enzyme with a high concentration of Ca^{2+}

(Richardson and Anthony, 1992). Reconstitution involved a large conformational change, resulting in active holoenzyme from which Ca^{2+} could not subsequently be removed, and was optimal at high pH and high Ca^{2+} concentrations (Goodwin *et al.*, 1996). 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 MxaA, and possibly also by the membrane protein MxaL.

Geiger and Gorisch (1989) had previously shown that, in solution, PQQ forms a complex with Ca^{2+} or Mg^{2+}; the complex has an absorption maximum of 343 nm, different from that of free PQQ (330 nm) but very similar to that of PQQ in alcohol dehydrogenases. They concluded that either the ion is necessary to induce the proper conformation of the protein so that it can then bind PQQ at the active site, or that PQQ is anchored as a complex with the calcium. Both of these conclusions have now been confirmed for methanol dehydrogenase by studies of Ca^{2+} incorporation into the enzyme (Goodwin *et al.*, 1996) and by its X-ray structure. This shows a Ca^{2+} ion in the active site, coordinated directly to PQQ and to amino acid residues (Fig. 4) (White *et al.*, 1993; Blake *et al.*, 1994; Ghosh *et al.*, 1995; Xia *et al.*, 1996) and this has led to the proposal that Ca^{2+} plays a key catalytic role in this enzyme by facilitating the initial reaction with substrate (Anthony *et al.*, Blake *et al.*, 1994; Anthony, 1996, 1998). Remarkably, by using dehydrogenase prepared from a *mxaA* mutant, it has been possible to produce a methanol dehydrogenase in which the Ca^{2+} has been replaced with Ba^{2+}, the first enzyme ever described to contain active site Ba^{2+}; although substrate binding is much poorer in the barium enzyme the activation energy is lower and hence the maximum catalytic rate is higher (Goodwin and Anthony, 1996).

The first demonstration of a metal ion in an alcohol dehydrogenase other than methanol dehydrogenase was in the (type I) quinoprotein ethanol dehydrogenase of *P. aeruginosa*, which has one Ca^{2+} per molecule of PQQ (Mutzel and Gorisch, 1991). This could be removed by treatment with CDTA (not EDTA) and reconstitution achieved by incubation with PQQ and Ca^{2+} or Sr^{2+}, but not with Mg^{2+}, Mn^{2+} or Cd^{2+}. Ca^{2+} (but not Mg^{2+}) is required for formation of active enzyme from the apoenzyme of the type II and type III alcohol dehydrogenases (the quinohaemoproteins), but the presence of calcium has not yet been demonstrated in the isolated enzymes (Groen *et al.*, 1986; Shinagawa *et al.*, 1989). However, sequencing and modelling studies of their structures, including the coordination sites for Ca^{2+} (Fig. 4, Section 5), are sufficiently similar to methanol dehydrogenase for it to be concluded that Ca^{2+} fulfils a similar role in the type II (Stoorvogel *et al.*, 1996) and type III (Cozier *et al.*, 1995) alcohol dehydrogenases.

4.2. The Role of Divalent Metal Ions in Glucose Dehydrogenase

The presence of a metal ion (Ca^{2+}) in a glucose dehydrogenase was first demonstrated in the soluble enzyme from *A. calcoaceticus* (Geiger and Gorisch, 1989). 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 *A. calcoaceticus* that is unable to synthesize PQQ and so only produces the apoenzyme (Matsushita *et al.*, 1995a). 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+} (Table 4). It should be noted that the percentage values in Table 4 were taken from experiments in which a standard assay was used and reconstitution was for a fixed length of time. These results do not necessarily show that the catalytic activity of the enzyme is highest with the metal ion giving the highest rate in this type of experiment. The K_m value for PQQ was 1.3 nM. A second approach to the study of the metal ion in the soluble glucose dehydrogenase was developed by Olsthoorn and Duine (1996) who expressed the *A. calcoaceticus* gene in *E. coli*, which does not produce PQQ. The soluble apoenzyme was isolated in the dimeric form, monomerization 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. The ions that could support reconstitution of the wild-type enzyme were also effective in this system (Table 4). After reconstitution, the holoenzyme resembled reconstituted methanol dehydrogenase (Goodwin *et al.*, 1996) in that Ca^{2+} could no longer be removed by chelating agents.

It is difficult to come to a firm conclusion about the role of the divalent metal ion in membrane glucose dehydrogenases. They are completely different from the soluble enzyme in their sequence, structure and location and the metal ion content of the membrane glucose dehydrogenase has never been determined. The only evidence available is from studies of reconstitution of inactive apoenzyme with PQQ to form the active holoenzyme. For this process Mg^{2+} often is better than Ca^{2+} and many other metals can be used instead of Ca^{2+} (Matsushita *et al.*, 1995a) (Table 4). For example, using the same mutant of *A. calcoaceticus* as was used in the study of the soluble enzyme (see above), it was shown that Mg^{2+} and Zn^{2+} were as effective as Ca^{2+} for reconstitution of active enzyme; in this case the K_m value for PQQ (40 nM) was much higher than for the soluble enzyme (1.3 nM) (Matsushita *et al.*, 1995a). The metal content of the reconstituted holoen-

In *P. aeruginosa*, the oxidation of ubiquinol is by way of the cytochrome bc_1 complex which is subsequently oxidized by a typical periplasmic cytochrome *c*, the terminal oxidase being a cytochrome *co* (Matsushita *et al.*, 1982b). In all the other bacteria the ubiquinol is oxidized directly by a ubiquinol oxidase; in *Acinetobacter*, *Escherichia* and *Klebsiella* the oxidase is cytochrome *bo* in oxygen-sufficient conditions but cytochrome *bd* in oxygen-deficient conditions (van Schie *et al.*, 1985; Beardmore-Gray and Anthony, 1986; Ameyama *et al.*, 1987; Matsushita *et al.*, 1987; Smith *et al.*, 1990; Juty *et al.*, 1997). As is often the case, the situation with the acetic acid bacteria is complex. In *Gluconobacter*, which only grows in conditions of high aeration, there is usually a typical cytochrome *bo* (Matsushita *et al.*, 1987). At low pH, however, a second 'cyanide-insensitive' and non-energy-generating pathway appears to operate. An intermediate in this pathway may be the cytochrome *c* subunit (subunit II) of the membrane alcohol dehydrogenase (Matsushita *et al.*, 1989c, 1994).

Many of the systems for glucose oxidation shown in Fig. 15 operate in reconstituted membrane systems and can generate a protonmotive force; this is consistent with observations that glucose oxidation by way of membrane glucose dehydrogenase leads to increased growth yields (van Schie *et al.*, 1985, 1987a,b,c; Mueller and Babel, 1986; Neijssel *et al.*, 1989; Adamowicz *et al.*, 1991; Neijssel and Demattos, 1994).

7. THE PHYSIOLOGICAL FUNCTIONS OF THE QUINOPROTEIN DEHYDROGENASES

It might be expected that the physiological function of enzymes that oxidize common growth substrates would not merit much discussion, but for the PQQ-containing quinoprotein dehydrogenases this is not the case. To summarize the following discussion in advance, it can be concluded that during growth on methanol and other alcohols as the sole source of carbon and energy the relevant periplasmic dehydrogenases usually play a key role in energy production, catalysing oxidation of the alcohol to the corresponding aldehyde, which is then either further oxidized or assimilated into cell material. In the case of the acetic acid bacteria, however, the PQQ-dependent alcohol and glucose dehydrogenases catalyse 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. The roles of the glucose dehydrogenases in other bacteria are varied and often a matter of debate, particularly in the case of the enteric bacteria.

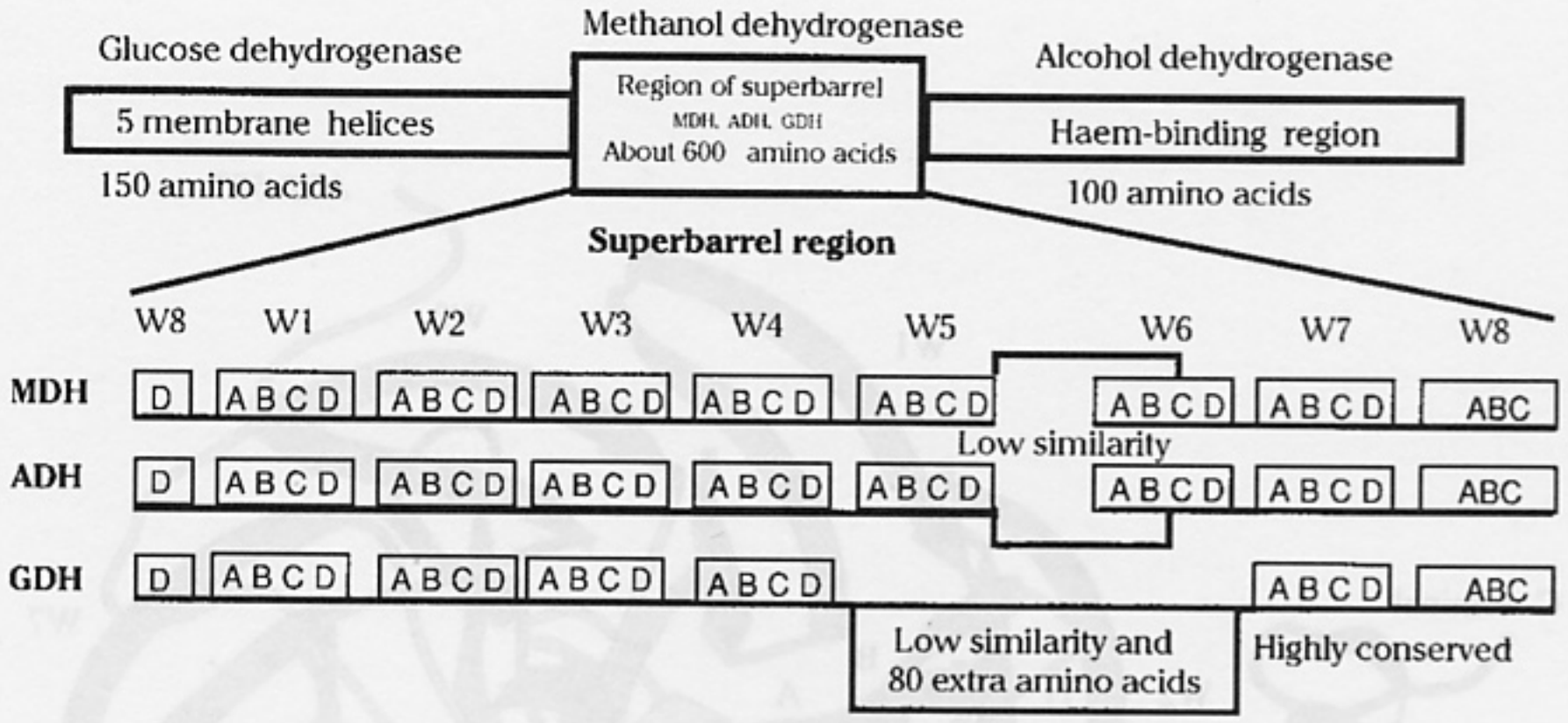


Figure 5 Amino acid sequence alignment of quinoprotein dehydrogenases. Each 'W' is a four-stranded β sheet (or propeller blade); the letters ABCD correspond to the four strands of each 'W' motif (see Fig. 6). These are the regions showing greatest similarity of sequence between the quinoproteins. There are many loops between, and within, the β sheets which show least similarity. For example, there is a long region with little conservation of sequence (including a large loop) between the end of the D-strand in W5 and the end of the D-strand of W6. The highly conserved region between strand-A in W7 and the end of strand-B in W8 was originally proposed to be a PQQ-binding domain; this is not the case (see Fig. 12).

and its implications with respect to enzyme mechanisms have been reviewed elsewhere (Anthony *et al.*, 1994; Anthony, 1996, 1998). The basic structure of the α subunit is a 'propeller fold' superbarrel made up of eight β sheet 'propeller blades' ('W' motifs) which are held together by novel tryptophan-docking motifs (Figs. 6 and 7). The β subunit is most unusual as it has no hydrophobic core and forms a very extended structure which wraps around the α subunit (Figs. 6 and 8). In the absence of any other obvious function for this subunit, it has been suggested that it acts to stabilize the folded form of the large chain. The absence of β subunits in most other PQQ-containing quinoproteins, however, indicates that it may have a specific (unknown) function in methanol dehydrogenase. The PQQ is in the centre of the α subunit, coordinated to a Ca^{2+} ion (Fig. 4) and is maintained in position by a stacked tryptophan and a novel eight-membered ring structure made up of a disulphide bridge between adjacent cysteine residues (Fig. 9).

The methanol oxidation reaction is initiated by abstraction of a proton from the alcohol by a base (Asp303) followed by attack, on the electrophilic C-5 of PQQ, of the resulting oxyanion to form a hemiketal intermediate; or attack by a hydride from the methyl group of the methanol (Anthony, 1996, 1998). It has been proposed that the Ca^{2+} acts as a Lewis acid through coordination to the C-5 carbonyl oxygen, thus facilitating formation of

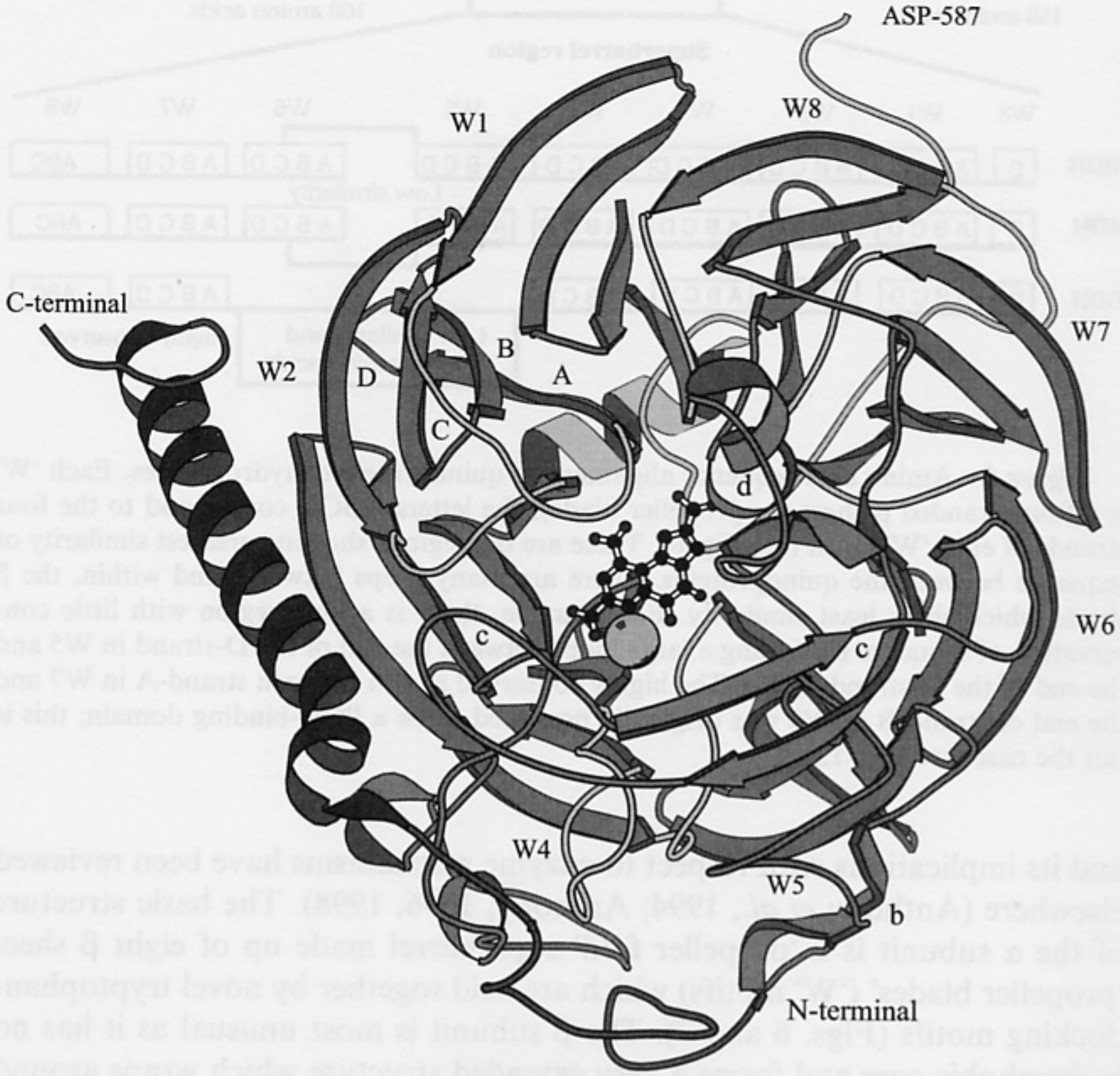


Figure 6 A drawing of an αβ unit of MDH looking down the pseudo 8-fold axis, simplified to show only the 'W' motifs of the α chain, and the long α helix of the β chain, but excluding other limited β structures and short α helices. The PQQ prosthetic group is in skeletal form and the calcium ion is shown 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. This figure is based on the structure in Ghosh *et al.* (1995).

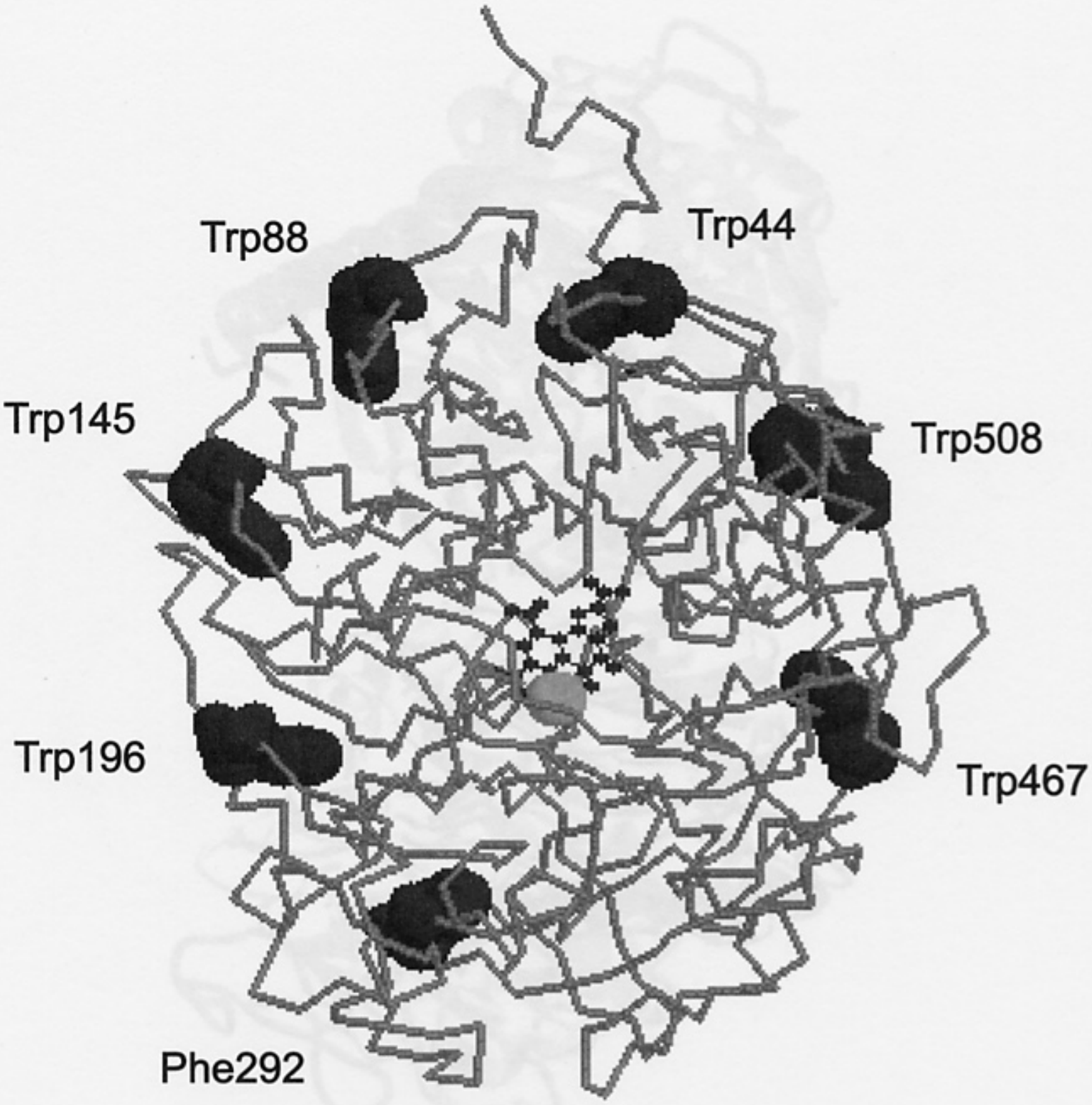


Figure 7 The girdle of tryptophan residues involved in docking the β sheets together. The tryptophan residues involved in docking are shown in spacefill mode and the rest of the chain as backbone. The PQQ prosthetic group is in skeletal form and the calcium ion is shown as a small sphere.

the electrophilic C-5 of PQQ. An alternative possibility is that Arg331 plays this role (Anthony, 1996; Xia *et al.*, 1996).

A key question in relation to this enzyme, and to the other PQQ-dependent dehydrogenases, is how electrons pass from the quinol form of PQQ ($PQQH_2$) to the outside of the protein and thence to the electron acceptor. The novel disulphide bridge structure is in close contact with the PQQ in the active site and, because reduction of this disulphide bond leads to loss of electron transfer to the cytochrome, it was thought at one time that it must play some direct role in this process; this has subsequently been shown to be unlikely (Avezoux *et al.*, 1995). Electron transfer to the cytochrome must



Figure 8 The $\alpha_2\beta_2$ tetrameric structure of methanol dehydrogenase.

occur one electron at a time; this leads to formation of the free radical semiquinone form, and the novel disulphide ring structure may play a role in stabilizing this semiquinone form of PQQ (Avezoux *et al.*, 1995; Anthony, 1996).

5.2. The Structures and Mechanisms of Alcohol and Glucose Dehydrogenases

The model alcohol dehydrogenase being considered here is the N-terminal region of subunit I of the type III alcohol dehydrogenase of acetic acid

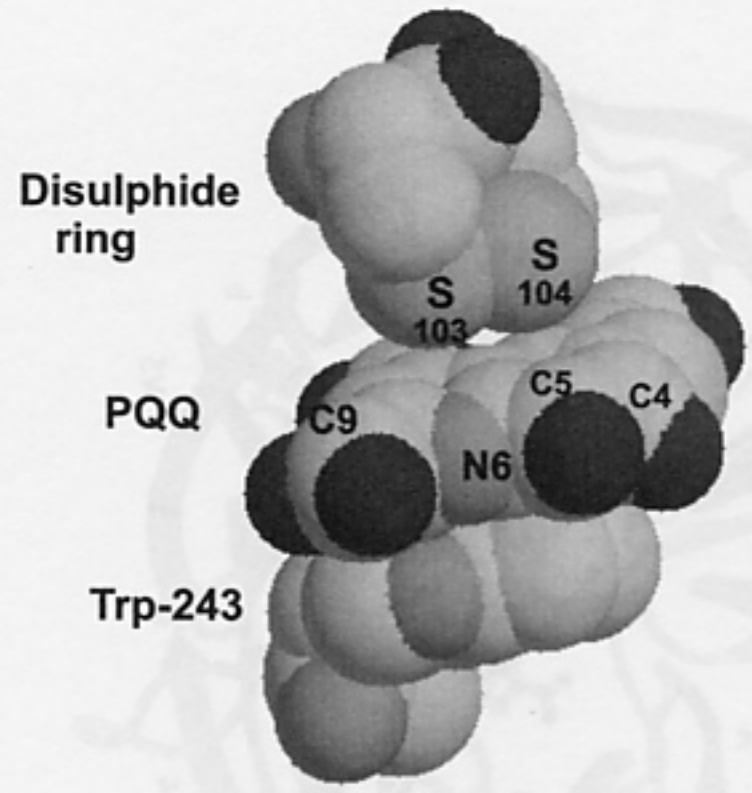


Figure 9 The novel disulphide ring in the active site of methanol dehydrogenase. The ring is formed by disulphide bond formation between adjacent cysteine residues. The PQQ is 'sandwiched' between this ring and the tryptophan that forms the floor of the active site chamber. The calcium ion is coordinated between the C-9 carboxylate, the N-6 of the PQQ ring and the carbonyl oxygen at C-5.

bacteria (Fig. 10) (Cozier *et al.*, 1995). 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 methanol dehydrogenase. 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 coordinations to the PQQ (Fig. 4). Especially important with respect to the mechanism is the conservation of the active site base (Asp303 in methanol dehydrogenase) and all the coordinations to the calcium ion. This suggests that the mechanism of this alcohol dehydrogenase is essentially similar to that of the methanol dehydrogenase. Comparison of the protein sequence of the soluble quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni* leads to a similar conclusion for that enzyme (Stoorvogel *et al.*, 1996).

In the model structure of the periplasmic portion of the membrane glucose dehydrogenase of *E. coli* (Fig. 11) (Cozier and Anthony, 1995), there is a sequence of about 80 amino acids where there is little similarity to the methanol dehydrogenase sequence and so this region cannot be modelled. The novel disulphide ring is replaced by a histidine residue which maintains the position of PQQ in the active site, consistent with the previous demonstration that a histidine residue is essential for binding PQQ (Imanaga, 1989) (Fig. 12). There are fewer equatorial interactions between the protein and PQQ (Fig. 4), perhaps explaining why it is possible to effect the reversible dissociation of PQQ from glucose dehydrogenase but not from methanol

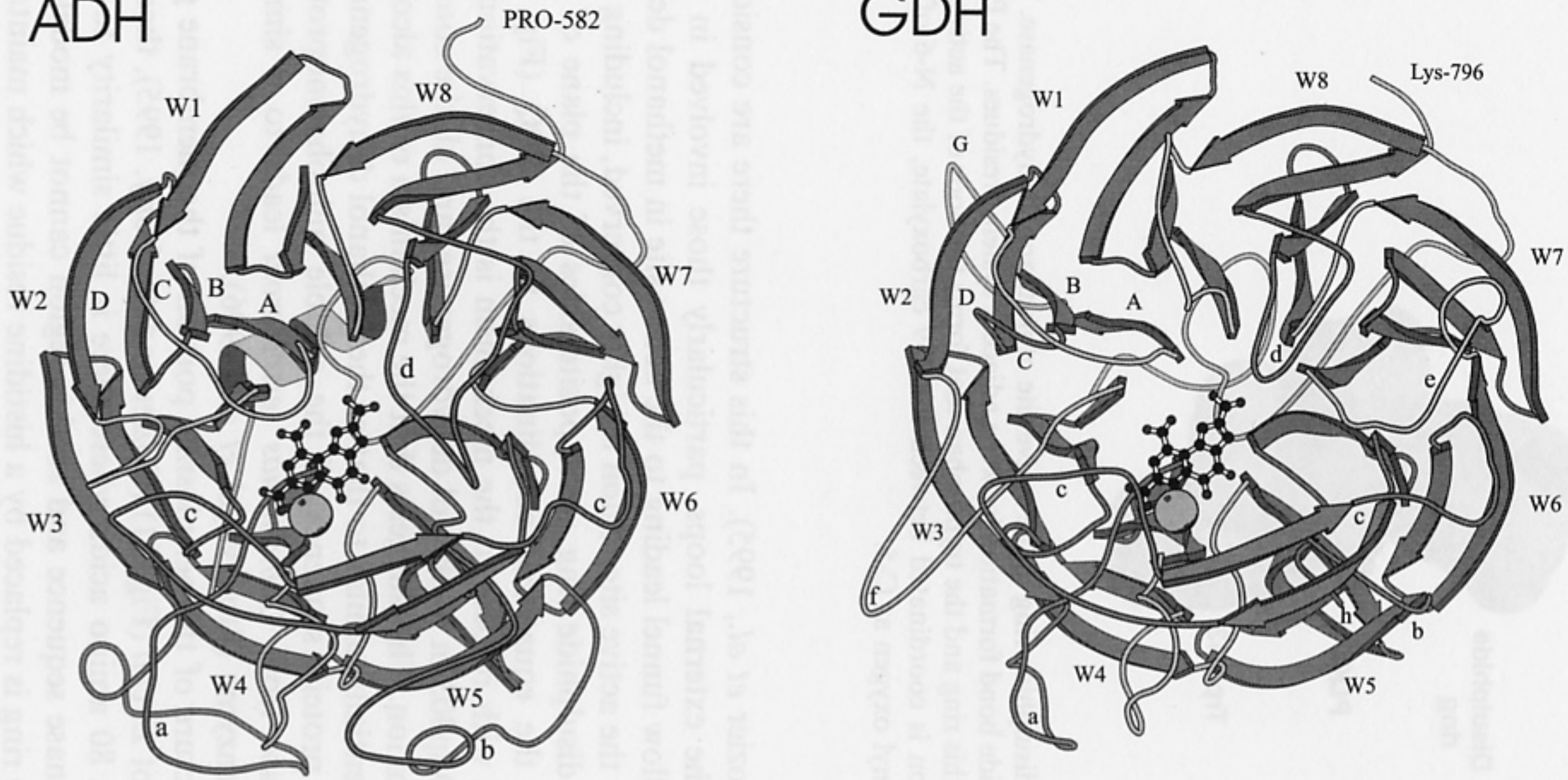


Figure 10 Schematic representation of the backbones of the quinohaemoprotein alcohol dehydrogenase (ADH) and glucose dehydrogenase (GDH), showing their major secondary structure. These model structures are based on that of methanol dehydrogenase which was determined by X-ray diffraction (see Fig. 6) (Ghosh *et al.*, 1995). The model ADH structure is of the N-terminal region of the quinohaemoprotein subunit I of the membrane complex (residues 1–590), omitting the C-terminal haem domain (Cozier *et al.*, 1995). The model GDH structure is of the C-terminal section of the membrane-bound GDH (residues 155–796), omitting the N-terminal membrane region (Cozier and Anthony, 1995). These residues are not present in MDH or ADH and the sequences are too long to model. The prosthetic group is shown as a ball and stick structure, and the Ca^{2+} as a van der Waal's sphere. The major loops are in black.

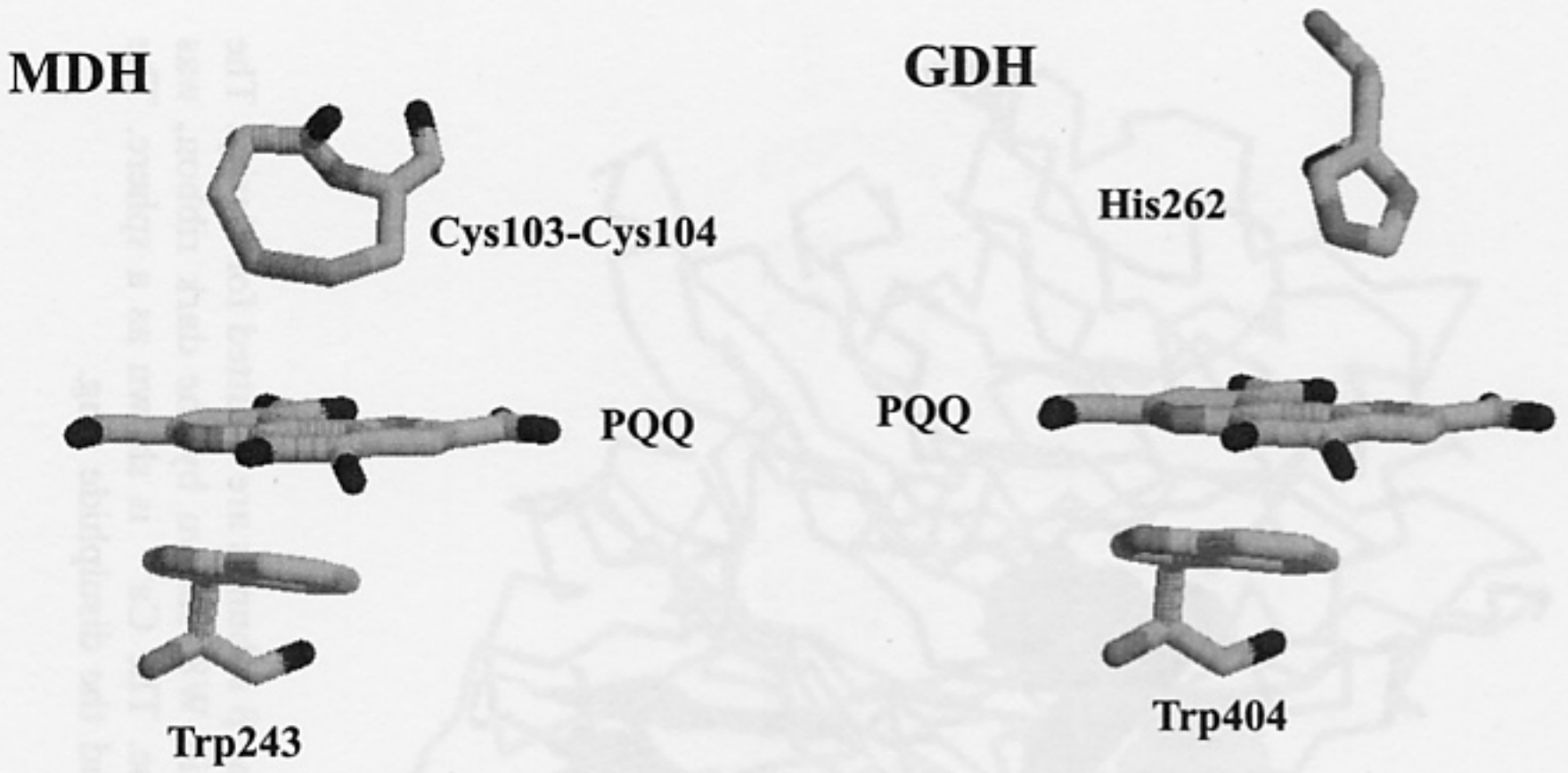


Figure 11 Comparison of the stacking interactions of the PQQ in methanol dehydrogenase (MDH) and the model glucose dehydrogenase (GDH). In MDH the PQQ is stacked between the coplanar Trp243 and the disulphide ring system of Cys103 and Cys104. In GDH the coplanar tryptophan is retained (Trp404) but the disulphide is not conserved. Instead, His262 may perform a similar role in helping to bind the PQQ into the active site region.

dehydrogenase. One clear difference between these proteins is that there is more 'space' in the glucose dehydrogenase active site, perhaps to accommodate the larger substrate. By analogy with the methanol dehydrogenase structure, Asp466 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. As discussed in Section 4, it is possible that the active site of glucose dehydrogenase may sometimes contain Mg^{2+} instead of Ca^{2+}, although many of the groups that are involved in coordination to Ca^{2+} in methanol dehydrogenase are conserved in the model glucose dehydrogenase. It has been suggested that Arg331 and not Ca^{2+} may act as a Lewis acid in the mechanism of methanol dehydrogenase; the equivalent residue in glucose dehydrogenase is Lys494 which might facilitate formation of the electrophilic C-5 of PQQ if Mg^{2+} is present in the active site and unable to fulfil the function proposed for Ca^{2+}.

Relatively little is known about the mechanism of glucose dehydrogenase, but some information is available from chemical modification studies of Imanaga (1989), and modelling studies have suggested that many features of its mechanism are likely to be similar to that of methanol dehydrogenase (Cozier and Anthony, 1995; Anthony, 1996, 1998). One key difference is that electron transfer from the reduced PQQ does not occur in two stages to a cytochrome *c* as in methanol dehydrogenase – electrons

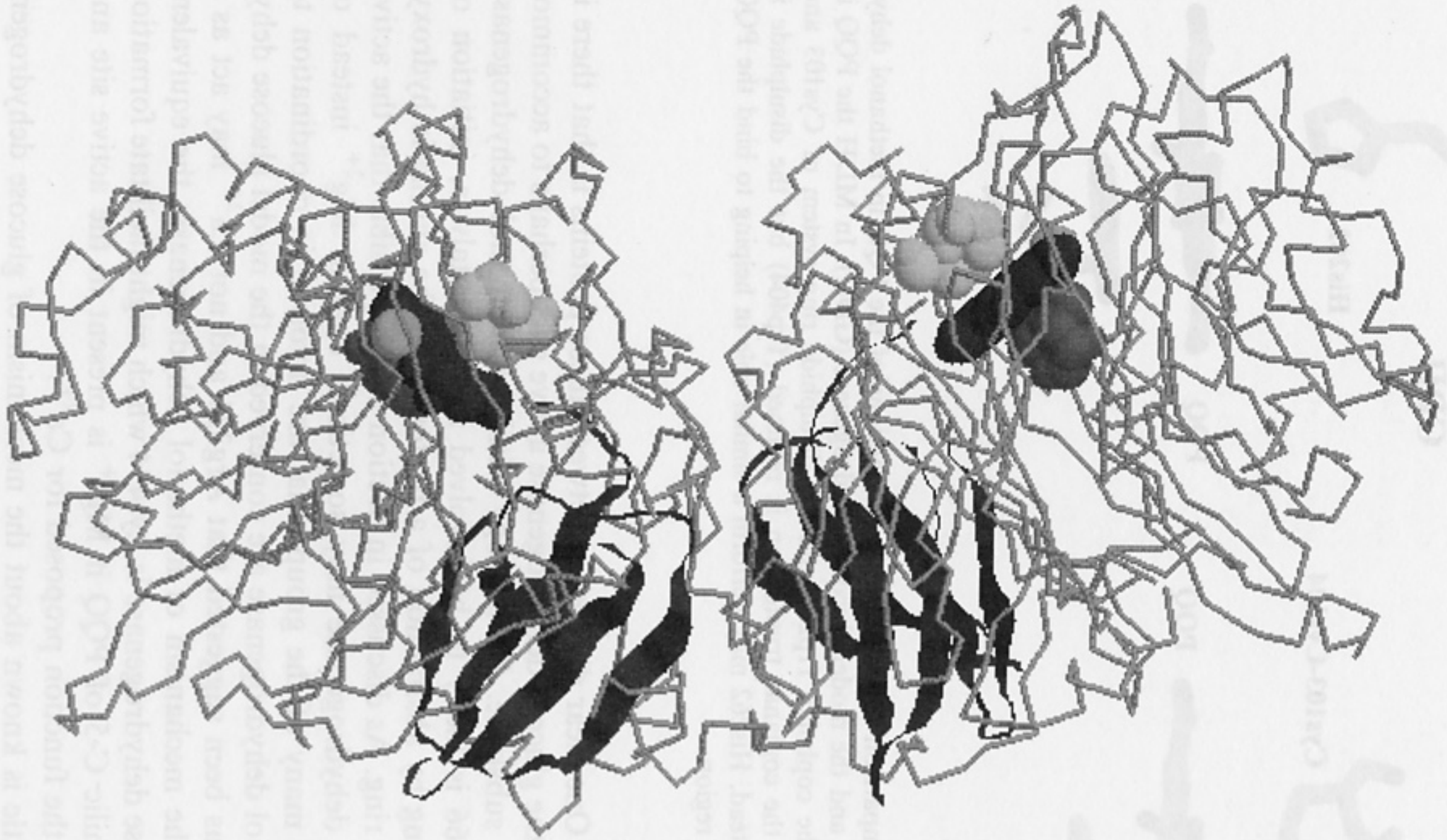


Figure 12 The α_2 dimer of MDH showing the highly conserved region. The β subunits are omitted for clarity. The highly conserved region, between strand-A in W7 and the end of strand-B in W8, shown by the dark ribbon, was originally proposed to be a PQQ-binding domain; this is clearly not the case. The Ca^{2+} is shown as a sphere. The PQQ is shown in the active site stacked between the hydrophobic Trp243 and the disulphide ring.

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 glucose dehydrogenase. This is perhaps consistent with the absence of the novel disulphide ring structure present in the alcohol dehydrogenases. There is no suggestion from the model structure or from the primary sequence that there is any hydrophobic region of the protein that could interact with the membrane other than the N-terminal transmembrane segments.

5.3. The Conserved Sequence that was wrongly identified as the PQQ-binding Domain in Quinoproteins

When the primary sequences of these 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 (for review see Anthony, 1992a). Remarkably, this sequence constitutes part of the main propeller structure and is not in any way directly involved in PQQ binding (Fig. 12). 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 genes coding for PQQ-dependent quinoproteins.

6. QUINOPROTEINS IN ENERGY TRANSDUCTION

The PQQ-dependent dehydrogenases all function in respiration and usually also in energy transduction; in all cases so far investigated, this involves the oxidation of substrates in the periplasm of Gram-negative bacteria (Table 1). This contrasts with oxidation systems involving 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 methanol dehydrogenase, electrons are passed directly to high potential *c*-type cytochromes which are only found in the periplasm and so the respiration system is similar to that of bacteria oxidizing inorganic substrates. In this case the product of substrate oxidation must then be transported into the bacteria and this is also necessary with some of the alcohol- and glucose-oxidizing systems. In other cases, however, the product is not used by the bacteria and is released into the medium. Clearly in these systems it is more appropriate to have the substrate oxidized in the periplasm of the bacteria.

Whatever the role of the quinoprotein (see Section 7), it 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 (Anthony, 1988, 1993a). The type of electron transport system for the quinoprotein dehydrogenases depends on the type of enzyme and on the type of terminal oxidase present. These fall into two main functional categories: those that oxidize periplasmic cytochrome *c* (cytochrome aa_3 and cytochrome *co*); and those that oxidize ubiquinol without the mediation of a cytochrome bc_1 complex (cytochromes *bo*, *bd* and *ba*) (Poole, 1988); most bacteria are able to synthesize only one of these two general types of oxidase, although more than one of the specific type may be produced. The specific oxidase that is in operation at any one time depends upon the growth conditions, the most usual determinant being the oxygen concentration. For example, enteric bacteria only produce quinol oxidases; cytochrome *bo* has a low affinity for oxygen and is usually produced when oxygen is plentiful whereas cytochrome *bd*, which has an extraordinarily high affinity for oxygen, is produced when oxygen is scarce or when it must be removed to a very low level in order to protect oxygen-sensitive enzymes. During methanol oxidation in some methylotrophs there is the less usual situation in which the carbon status of the cell determines the oxidase; in carbon excess conditions cytochrome *co* is synthesized, whereas only the cytochrome aa_3 is produced in carbon-limited culture (Cross and Anthony, 1980). Although the number of molecules of ATP produced per pair of electrons passing down the electron transport chain is not as clearly defined as previously thought, the potential yield of ATP will be higher in systems in which ubiquinone is oxidized by way of cytochrome bc_1 complex and periplasmic cytochrome *c* than when the terminal oxidase is a ubiquinol oxidase. The yield will also depend on whether or not the terminal oxidase has a proton-pumping function. When the electron transport chain bypasses the low redox potential ubiquinone/cytochrome *b* part of the chain (as in methanol oxidation) no more than one ATP per methanol oxidized is likely to be achieved.

6.1. Electron Transport Chains involving Soluble Alcohol Dehydrogenases

The electron transport systems from methanol dehydrogenase are summarized in Fig. 13 (for reviews see Anthony, 1988, 1992b, 1993a). They have in common the first step, which is electron transfer from the dehydrogenase to the specific cytochrome c_L in the periplasm (called cytochrome c_{551i} in *Paracoccus*). The interaction of these proteins depends on electrostatic interactions and so methanol oxidation is strongly inhibited by high ionic strength. During this first step in the oxidation of methanol, electrons pass from the dehydrogenase by way of cytochromes to the oxidase, and protons are liberated from the reduced PQQ into the periplasm, thus contributing to the protonmotive force. The cytochrome c_L is subsequently oxidized by cytochrome c_H, which is similar in all respects to the other small *c*-type cytochromes that mediate electron transfer between cytochrome bc_1 complexes and oxidases (cytochrome aa_3 or cytochrome *co*). The oxidase consumes protons on the inside face of the membrane and it may also act as a direct proton pump. A critical point with respect to energy transduction is that all these 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. The result of this is that the first step in the oxidation of methanol is likely to yield only one molecule of ATP (or less). *Hyphomicrobium* sp. and *Paracoccus denitrificans* are both able to act as denitrifying bacteria during anaerobic growth on methanol with nitrate, and in these conditions the cytochrome *c* is oxidized by a nitrite reductase.

The electron transport systems for periplasmic type I alcohol dehydrogenases (Fig. 14) are likely to be essentially similar to those for methanol oxidation (Fig. 13). The soluble quinoprotein alcohol dehydrogenase from *P. aeruginosa* reacts rapidly with a small *c*-type cytochrome called cytochrome c_{EDH} which might be assumed to be related to the cytochrome c_L of methylotrophs. It is, however, rather smaller (14.5 kDa compared with about 20 kDa) and there is no similarity between the 19 N-terminal residues of cytochrome c_{EDH} and cytochrome c_L (Schrover *et al.*, 1993). Although *P. aeruginosa* usually also contains a typical small *c*-type cytochrome which is the substrate for its oxidase, during growth on ethanol by way of the quinoprotein alcohol dehydrogenase there appears to be no cytochrome *c* able to mediate electron transport between cytochrome c_{EDH} and the oxidase; its electron transport chain (Fig. 14) might therefore be similar to that in the methylotrophic organism 4025 during growth on methanol in media containing a high concentration of copper (Auton and Anthony, 1989a,b; Anthony, 1992b) (Fig. 13). Thus, in *P. aeruginosa* a blue copper protein like azurin and an 'azurin oxidase' may be involved in electron transport

Methylobacterium extorquens

MDH ⟶ Cyt. c_L ⟶ Cyt. c_H ⟶ Cyt. aa_3 ⟶ O_2

Methylophilus methylotrophus carbon-limited conditions

MDH ⟶ Cyt. c_L ⟶ Cyt. c_H ⟶ Cyt. aa_3 ⟶ O_2

Methylophilus methylotrophus carbon-excess conditions

MDH ⟶ Cyt. c_L ⟶ Cyt. c_H ⟶ Cyt. *co* ⟶ O_2

Acetobacter methanolicus

MDH ⟶ Cyt. c_L ⟶ Cyt. c_H ⟶ Cyt. *co* ⟶ O_2

Organism 4025 low copper

MDH ⟶ Cyt. c_L ⟶ Cyt. c_H ⟶ Cyt. *co* ⟶ O_2

Organism 4025 high copper

MDH ⟶ Cyt. c_L ⟶ Azurin ⟶ Cyt. *co* ⟶ O_2

Paracoccus denitrificans

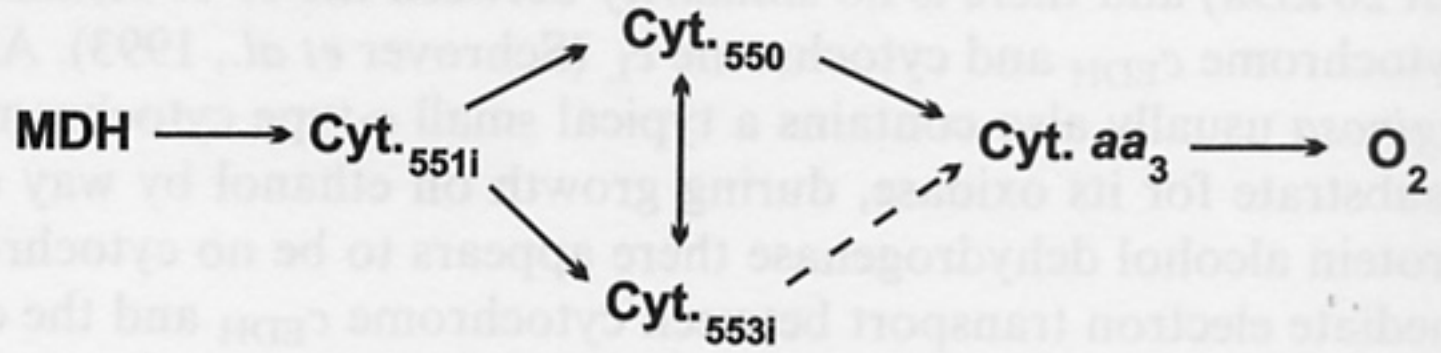


Figure 13 Electron transport chains of methylotrophs. The details of these chains are discussed in Anthony (1992b).

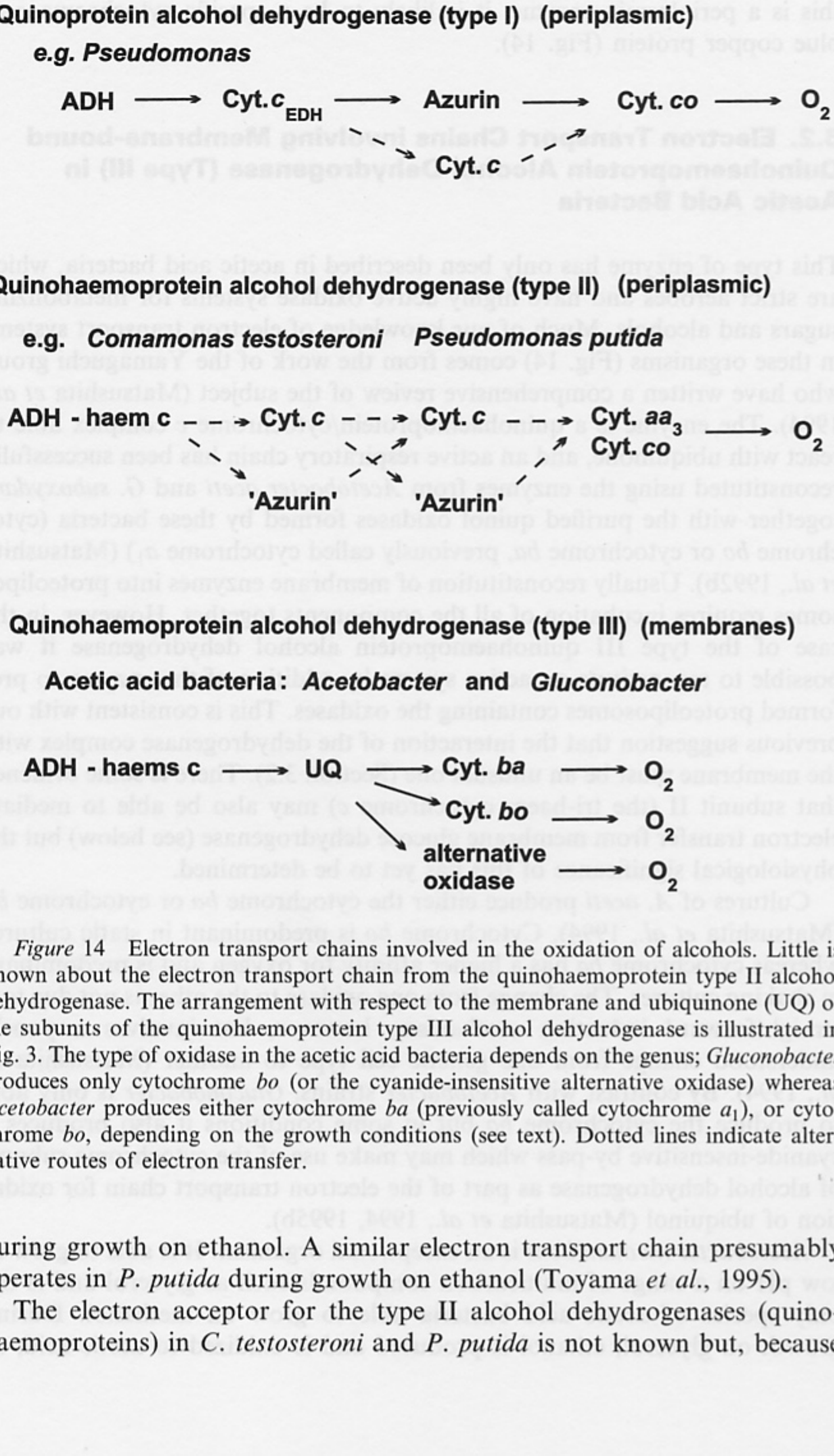


Figure 14 Electron transport chains involved in the oxidation of alcohols. Little is known about the electron transport chain from the quinohaemoprotein type II alcohol dehydrogenase. The arrangement with respect to the membrane and ubiquinone (UQ) of the subunits of the quinohaemoprotein type III alcohol dehydrogenase is illustrated in Fig. 3. The type of oxidase in the acetic acid bacteria depends on the genus; *Gluconobacter* produces only cytochrome *bo* (or the cyanide-insensitive alternative oxidase) whereas *Acetobacter* produces either cytochrome *ba* (previously called cytochrome a_1), or cytochrome *bo*, depending on the growth conditions (see text). Dotted lines indicate alternative routes of electron transfer.

during growth on ethanol. A similar electron transport chain presumably operates in *P. putida* during growth on ethanol (Toyama *et al.*, 1995).

The electron acceptor for the type II alcohol dehydrogenases (quinohaemoproteins) in *C. testosteroni* and *P. putida* is not known but, because

this is a periplasmic enzyme, it is likely to be a specific cytochrome *c* or blue copper protein (Fig. 14).

6.2. Electron Transport Chains involving Membrane-bound Quinohaemoprotein Alcohol Dehydrogenase (Type III) in Acetic Acid Bacteria

This type of enzyme has only been described in acetic acid bacteria, which are strict aerobes and have highly active oxidase systems for metabolizing sugars and alcohols. Much of our knowledge of electron transport systems in these organisms (Fig. 14) comes from the work of the Yamaguchi group who have written a comprehensive review of the subject (Matsushita *et al.*, 1994). The enzyme is a quinohaemoprotein/cytochrome *c* complex able to react with ubiquinone, and an active respiratory chain has been successfully reconstituted using the enzymes from *Acetobacter aceti* and *G. suboxydans* together with the purified quinol oxidases formed by these bacteria (cytochrome *bo* or cytochrome *ba*, previously called cytochrome a_1) (Matsushita *et al.*, 1992b). Usually reconstitution of membrane enzymes into proteoliposomes requires incubation of all the components together. However, in the case of the type III quinohaemoprotein alcohol dehydrogenase it was possible to reconstitute an active system by addition of the enzyme to preformed proteoliposomes containing the oxidases. This is consistent with our previous suggestion that the interaction of the dehydrogenase complex with the membrane must be an unusual one (Section 3.2). There is some evidence that subunit II (the tri-haem cytochrome *c*) may also be able to mediate electron transfer from membrane glucose dehydrogenase (see below) but the physiological significance of this has yet to be determined.

Cultures of *A. aceti* produce either the cytochrome *bo* or cytochrome *ba* (Matsushita *et al.*, 1994). Cytochrome *bo* is predominant in static cultures whereas cytochrome *ba* has a higher affinity for oxygen and is predominant in shaking cultures. The change from one oxidase to the other is not due to a straightforward induction mechanism, however, but involves a poorly understood change from one genetic cell type to another (Matsushita *et al.*, 1994). By contrast with *Acetobacter* strains, *Gluconobacter* is only able to produce the cytochrome *bo* but in some conditions it also produces a cyanide-insensitive by-pass which may make use of the cytochrome subunit of alcohol dehydrogenase as part of the electron transport chain for oxidation of ubiquinol (Matsushita *et al.*, 1994, 1995b).

Acetobacter methanolicus is an exceptional organism. It is able to grow at low pH on a range of multicarbon compounds such as glycerol and is the only species of acetic acid bacteria able to grow on methanol. During growth on glycerol, ethanol is produced and is oxidized to acetic acid; as

in other acetic acid bacteria this involves the ubiquinol oxidase cytochrome *bo*; cytochrome *ba* is not produced. By contrast, during growth on methanol the soluble periplasmic *c*-type cytochromes must be synthesized for reaction with methanol dehydrogenase together with an appropriate terminal oxidase (cytochrome *co*) able to oxidize cytochrome *c* (Fig. 13) (Elliott and Anthony, 1988; Chan and Anthony, 1991a,b; Matsushita *et al.*, 1992a).

6.3. Electron Transport Chains involving Membrane-bound Glucose Dehydrogenase

Electron transport from glucose dehydrogenase, which interacts directly with membrane ubiquinone, varies from organism to organism, depending on the nature of the oxidase(s) produced and their substrates (Fig. 15).

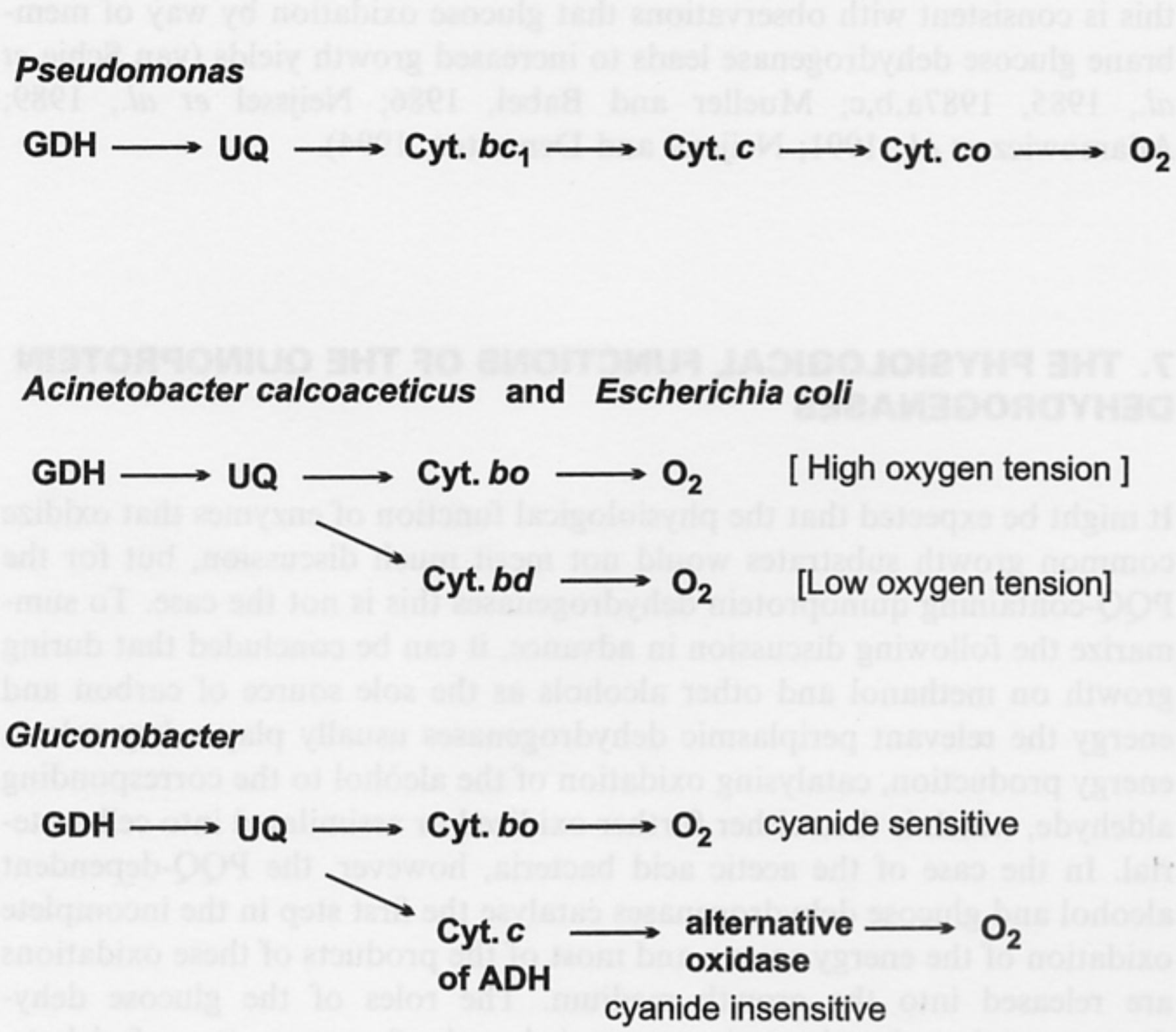


Figure 15 Electron transport chains involved in the oxidation of glucose.

In *P. aeruginosa*, the oxidation of ubiquinol is by way of the cytochrome bc_1 complex which is subsequently oxidized by a typical periplasmic cytochrome *c*, the terminal oxidase being a cytochrome *co* (Matsushita *et al.*, 1982b). In all the other bacteria the ubiquinol is oxidized directly by a ubiquinol oxidase; in *Acinetobacter*, *Escherichia* and *Klebsiella* the oxidase is cytochrome *bo* in oxygen-sufficient conditions but cytochrome *bd* in oxygen-deficient conditions (van Schie *et al.*, 1985; Beardmore-Gray and Anthony, 1986; Ameyama *et al.*, 1987; Matsushita *et al.*, 1987; Smith *et al.*, 1990; Juty *et al.*, 1997). As is often the case, the situation with the acetic acid bacteria is complex. In *Gluconobacter*, which only grows in conditions of high aeration, there is usually a typical cytochrome *bo* (Matsushita *et al.*, 1987). At low pH, however, a second 'cyanide-insensitive' and non-energy-generating pathway appears to operate. An intermediate in this pathway may be the cytochrome *c* subunit (subunit II) of the membrane alcohol dehydrogenase (Matsushita *et al.*, 1989c, 1994).

Many of the systems for glucose oxidation shown in Fig. 15 operate in reconstituted membrane systems and can generate a protonmotive force; this is consistent with observations that glucose oxidation by way of membrane glucose dehydrogenase leads to increased growth yields (van Schie *et al.*, 1985, 1987a,b,c; Mueller and Babel, 1986; Neijssel *et al.*, 1989; Adamowicz *et al.*, 1991; Neijssel and Dematttos, 1994).

7. THE PHYSIOLOGICAL FUNCTIONS OF THE QUINOPROTEIN DEHYDROGENASES

It might be expected that the physiological function of enzymes that oxidize common growth substrates would not merit much discussion, but for the PQQ-containing quinoprotein dehydrogenases this is not the case. To summarize the following discussion in advance, it can be concluded that during growth on methanol and other alcohols as the sole source of carbon and energy the relevant periplasmic dehydrogenases usually play a key role in energy production, catalysing oxidation of the alcohol to the corresponding aldehyde, which is then either further oxidized or assimilated into cell material. In the case of the acetic acid bacteria, however, the PQQ-dependent alcohol and glucose dehydrogenases catalyse 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. The roles of the glucose dehydrogenases in other bacteria are varied and often a matter of debate, particularly in the case of the enteric bacteria.

7.1. The Role of Periplasmic Quinoproteins that oxidize Alcohols

7.1.1. Methanol dehydrogenase

Methylotrophic bacteria are able to grow on reduced carbon compounds such as methane or methanol. Methane is produced in anaerobic environments by methanogenic bacteria and this provides a substrate for growth of methanotrophs at the aerobic surface. As well as being a product of methane oxidation, methanol arises in nature by hydrolysis of methyl ethers and esters present in pectin and lignin which are structural components of plants; methylotrophs growing on methanol are therefore abundant on the surfaces of leaves and in soil and water (Anthony, 1982). Methanol dehydrogenase is the only enzyme present in methylotrophic bacteria which is able to catalyse the oxidation of methanol to formaldehyde and it is therefore essential for growth on methanol or on methane. Although methanol dehydrogenase can also oxidize formaldehyde to formate this is unlikely to be important *in vivo*; indeed, some bacteria contain a regulatory modifier protein, the M-protein, which prevents this from happening by decreasing the affinity of the enzyme for formaldehyde (Long and Anthony, 1991). Every molecule of methanol that is used by the bacteria during growth on methane or methanol is oxidized to formaldehyde by methanol dehydrogenase and this step results in ATP production but not in production of NADH. Many methylotrophs are therefore unusual in having growth that is limited by reductant (NADH) rather than by ATP availability, a conclusion of considerable importance when predicting growth yields on methanol and methane (Anthony, 1986). During growth of methylotrophs on ethanol, methanol dehydrogenase may also be responsible for oxidation of this growth substrate to acetaldehyde (Dunstan *et al.*, 1972a; Anthony, 1982).

7.1.2. *Type I and Type II Periplasmic Alcohol Dehydrogenases*

The role of the type I quinoprotein alcohol dehydrogenase in *P. aeruginosa* and *P. putida* is straightforward; it is induced during growth on ethanol or other short-chain alcohols when it is the key enzyme for production of energy and precursors for carbon assimilation (Gorisch and Rupp, 1989; Schrover *et al*, 1993; Toyama *et al.*, 1995). Although the affinity of this enzyme for methanol is low, in *Rhodopseudomonas acidophila* it can also function in energy production during anaerobic growth on methanol in the light; in this case it has a role in the production of reducing power by reverse electron transfer (Anthony, 1982).

Comamonas testosteroni grows slowly on ethanol or butanol, oxidizing them by means of an NAD-linked ethanol dehydrogenase. In these conditions the apoenzyme of the type II alcohol dehydrogenase (a quinohaemoprotein) is induced; this contains haem but not PQQ, which cannot be synthesized by this organism. However, when PQQ is included in the growth medium the active holoenzyme is produced and the growth rate increases, implying that the PQQ-dependent alcohol dehydrogenase takes over the main role of ethanol oxidation (Groen *et al.*, 1986). By contrast with *C. testosteroni*, in *P. putida* the type II dehydrogenases, which are induced during growth on butanol or glycerol, are produced in the fully active form containing PQQ (Toyama *et al.*, 1995).

7.2. The Roles of Glucose Dehydrogenase in *Acinetobacter*, Pseudomonads and Enteric Bacteria

The first step in glucose metabolism by bacteria usually involves uptake across the cytoplasmic membrane. This occurs either by the phosphotransferase system, when transport is coupled with phosphorylation of the glucose to glucose 6-phosphate, or by a glucose transporter, the glucose then being phosphorylated in the cytoplasm. There are three main pathways for subsequent metabolism – glycolysis, the Entner–Doudoroff pathway or the hexose monophosphate pathway (Fig. 16). In some bacteria all three pathways can operate and in these cases glycolysis is the major route for glucose catabolism whereas the Entner–Doudoroff pathway is used mainly for gluconate metabolism. Although the hexose monophosphate pathway can effect the complete oxidation of glucose, its main function is usually to provide C_5 sugars and NADPH for biosynthesis. In organisms which contain an active glucose dehydrogenase, there is a fourth variant for glucose metabolism sometimes referred to as the direct (non-phosphorylating) oxidation pathway, glucose being oxidized in the periplasm (Fig. 16). This route is widely distributed among Gram-negative bacteria but a major bioenergetic advantage has not been obvious for this pathway and it was often referred to as a 'dissimilatory by-pass', expressing the apparent inefficient use of glucose. Operation of this pathway is evident by excretion of gluconate or 2-ketogluconate into the medium, but whereas the functional significance of this in some bacteria is clear (e.g. acetic acid bacteria, Section 7.3), in other cases it is debatable. Indeed, a mutant of *Pseudomonas cepacia* lacking glucose dehydrogenase grew as well as the wild-type on glucose as the sole carbon source (Lessie *et al.*, 1979). In the natural environment, however, the presence of glucose dehydrogenase may give bacteria an advantage over competitors for a variety of reasons and this is discussed below.

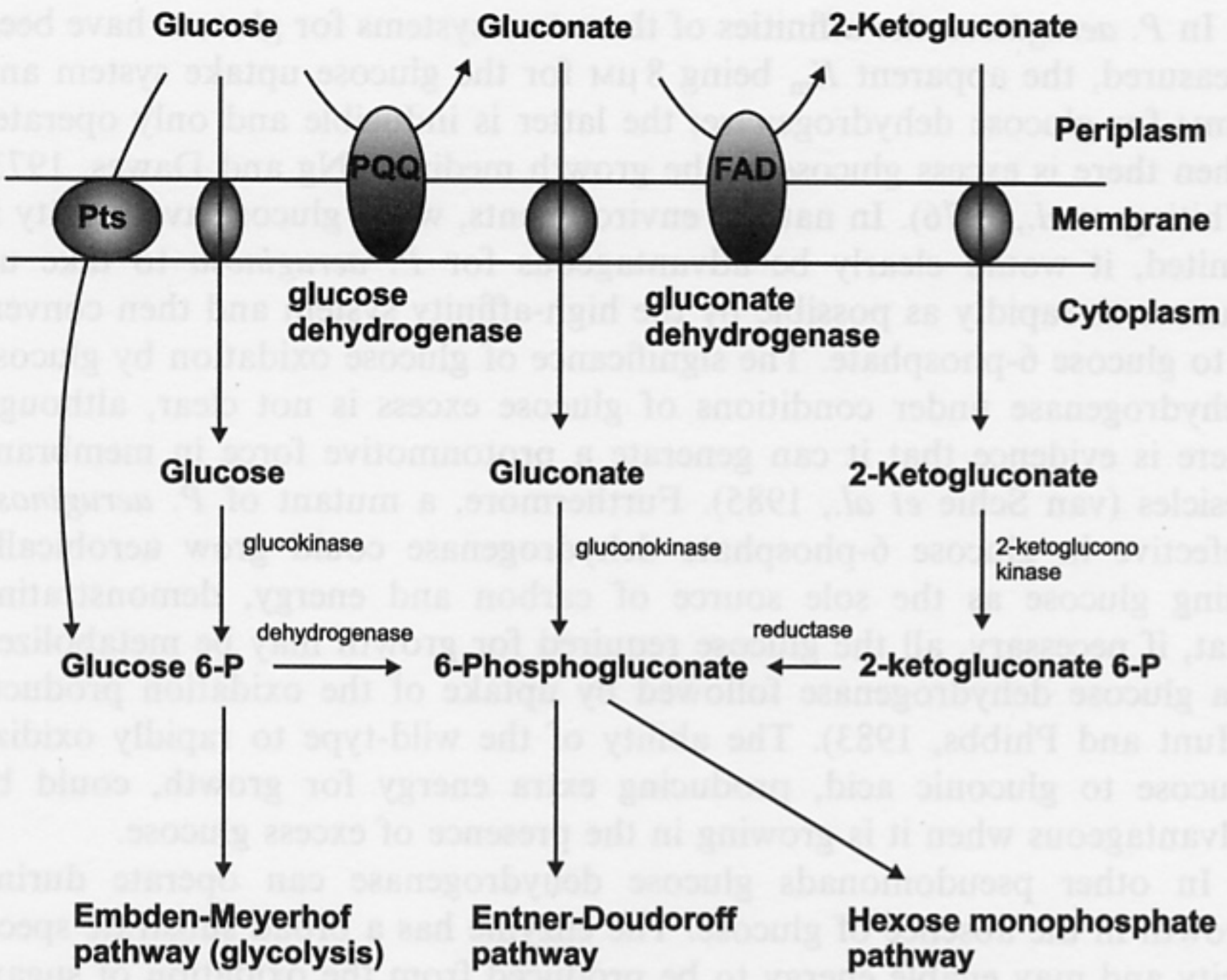


Figure 16 Alternative pathways for glucose metabolism. The abbreviation Pts refers to the PEP-linked phosphotransferase system.

7.2.1. *Glucose Dehydrogenase in Pseudomonads*

Members of the genus *Pseudomonas* are typically found in soil and water and are characterized by their ability to use a large variety of substrates as carbon and energy sources. They are aerobes, although some can grow in the absence of oxygen, using nitrate as the terminal electron acceptor. Most lack a complete glycolytic pathway and the Entner–Doudoroff pathway is the main route for glucose metabolism. The initial step in glucose metabolism usually involves uptake of glucose into the cell, followed by phosphorylation to glucose 6-phosphate (Fig. 16). Alternatively, glucose can be directly oxidized by glucose dehydrogenase to gluconic acid, which may then be further oxidized to 2-ketogluconic acid. As a result, acid accumulates in the growth medium and this is a diagnostic feature of the pseudomonads. The gluconate and 2-ketogluconate may be subsequently taken up by the bacteria and metabolized by the Entner–Doudoroff pathway.

In *P. aeruginosa* the affinities of these two systems for glucose have been measured, the apparent K_m being 8 μM for the glucose uptake system and 1 mM for glucose dehydrogenase; the latter is inducible and only operates when there is excess glucose in the growth medium (Ng and Dawes, 1973; Whiting *et al.*, 1976). In natural environments, when glucose availability is limited, it would clearly be advantageous for *P. aeruginosa* to take up glucose as rapidly as possible by the high-affinity system and then convert it to glucose 6-phosphate. The significance of glucose oxidation by glucose dehydrogenase under conditions of glucose excess is not clear, although there is evidence that it can generate a protonmotive force in membrane vesicles (van Schie *et al.*, 1985). Furthermore, a mutant of *P. aeruginosa* defective in glucose 6-phosphate dehydrogenase could grow aerobically using glucose as the sole source of carbon and energy, demonstrating that, if necessary, all the glucose required for growth may be metabolized via glucose dehydrogenase followed by uptake of the oxidation products (Hunt and Phibbs, 1983). The ability of the wild-type to rapidly oxidize glucose to gluconic acid, producing extra energy for growth, could be advantageous when it is growing in the presence of excess glucose.

In other pseudomonads glucose dehydrogenase can operate during growth in the absence of glucose. The enzyme has a broad substrate specificity and may enable energy to be produced from the oxidation of sugars which cannot be used as carbon sources. This has been demonstrated in *P. putida*, which can oxidize xylose, but cannot use it as a carbon source. Thus, when xylose was added to a chemostat culture growing under conditions of limiting carbon source (glucose or lactose), xylonolactone and xylonate formed and the growth yields, $Y_{glucose}$ and $Y_{lactate}$, increased, indicating that additional energy for growth can be provided by the oxidation of xylose (Hardy *et al.*, 1993). In the natural environment this versatility could give an advantage over competitors.

A role for glucose dehydrogenase in some bacteria, including *P. cepacia* and other soil pseudomonads, has recently been convincingly demonstrated by Goldstein, who has shown that such bacteria can make phosphate available from rock phosphate ore (Goldstein, 1995). This is particularly important in the region immediately surrounding the roots of plants (the rhizosphere) in arid and semi-arid soils where calcium phosphates provide a significant source of phosphate. There is evidence that this 'mineral phosphate-solubilizing' phenotype is a result of acidification of the periplasmic space and surrounding medium by the direct oxidation of glucose or other aldose sugars (produced by plant roots), the first step of which is catalysed by the membrane glucose dehydrogenase; the key acidic products are gluconic acid and 2-ketogluconic acid, which is particularly important in this context because it has the lowest pK of any organic acid produced by bacteria.

7.2.2. *Glucose Dehydrogenase in* Acinetobacter calcoaceticus

Acinetobacter species are aerobic chemoheterotrophs and, like the pseudomonads, they are found in soil and water and can use a wide range of substrates as carbon and energy sources. Some species can grow on glucose, metabolizing it by way of the Entner–Doudoroff pathway; others cannot do so, most strains of *Acinetobacter calcoaceticus* falling into this category. These strains usually synthesize an active glucose dehydrogenase and under certain growth conditions (for example carbon-limited growth on a mixture of glucose and acetate) they oxidize glucose to gluconate, which accumulates in the growth medium. The oxidation of glucose is associated with the formation of a protonmotive force and ATP synthesis and it has therefore been suggested that it functions as an auxiliary energy-generating system (van Schie *et al.*, 1987c). This is consistent with the observation that when glucose was added to a chemostat culture of *A. calcoaceticus* growing on acetate, the yield, $Y_{acetate}$, increased (Mueller and Babel, 1986).

In addition to the membrane-bound enzyme, *A. calcoaceticus* contains a soluble periplasmic glucose dehydrogenase, the only known example of such an enzyme (Section 3.3.2). There is no evidence that it is involved in electron transport 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 (Matsushita *et al.*, 1995a).

7.2.3. *Glucose Dehydrogenase in* Klebsiella pneumoniae

Klebsiella pneumoniae is a facultative anaerobe capable of nitrogen fixation. It is found in soil and water and metabolizes glucose mainly via the phosphotransferase system and the glycolytic pathway. However, during aerobic growth in the presence of excess glucose, gluconate and 2-ketogluconate are sometimes excreted into the growth medium (Hommes *et al.*, 1985; Buurman *et al.*, 1994). This occurs when there is a high energy demand on the cell and it has been suggested that the function of glucose dehydrogenase is to provide an additional contribution to the protonmotive force and ATP synthesis (Hommes *et al.*, 1985). However, there is evidence that in some situations (for example, when flux through glyceraldehyde 3-phosphate is limited by low phosphate availability), it provides an alternative source of energy, replacing glycolysis and the TCA cycle (Buurman *et al.*, 1994). It is possible that sometimes oxidation of glucose by way of glucose dehydrogenase could provide extra energy, whereas at other times it could be an alternative energy-producing system; further work is needed to clarify

this situation. It would also be of interest to ascertain if the enzymes of the Entner–Doudoroff pathway are induced when *K. pneumoniae* is grown under conditions of glucose excess. This would be comparable with growth of *E. coli* in excess glucose in the presence of PQQ, which results in the formation of active glucose dehydrogenase and induction of the enzymes of the Entner–Doudoroff pathway (Fliege *et al.*, 1992).

We speculate that an additional role for the direct oxidation of glucose in enteric bacteria might be to help provide protection against oxygen inactivation during transfer from aerobic growth to anaerobic fermentative growth. Many of the fermentative enzymes (e.g. pyruvate–formate lyase) are oxygen-sensitive, but are induced before conditions become completely anaerobic; they must, therefore, be protected from the remaining oxygen. To achieve this it is essential to have a source of electrons for rapid electron transfer to consume oxygen by way of the high-affinity oxidase (cytochrome *bd*) which is also induced during these conditions. Glucose dehydrogenase, which supports a high rate of respiration, could provide this. There is some evidence for such a role for glucose dehydrogenase in protecting the oxygen-sensitive pyruvate metabolism and nitrogenase in conditions supporting microaerobic nitrogen fixation in *K. pneumoniae*. In these conditions the yield of dinitrogen fixed per mole of glucose consumed is greater than in strictly anaerobic conditions due to the induction of cytochrome *bd* which has a very high affinity for oxygen. The electron transport chain involving this oxidase fulfils two functions; one is to provide ATP for nitrogenase function and the other is to remove trace amounts of inhibitory oxygen (Smith *et al.*, 1990; Hill *et al.*, 1990; Juty *et al.*, 1997). In whole cells glucose is an excellent substrate for electron transport by way of the cytochrome *bd* oxidase during microaerobic respiration and for nitrogen fixation in these conditions (Juty *et al.*, 1997). This respiration is likely to be by way of glucose dehydrogenase and unlikely to involve the glycolytic enzymes, but some rigorous experiments with appropriate mutants are needed to confirm this. It would also be of interest to determine if synthesis of the glucose dehydrogenase in *K. pneumoniae* is regulated in the same way as that of *E. coli*, where the glucose dehydrogenase structural gene has two promoters, one regulated negatively by cyclic AMP and the other regulated positively by oxygen (Yamada *et al.*, 1993a).

7.2.4. *Glucose Dehydrogenase in* E. coli

Escherichia coli is a facultative anaerobe found in the intestines of mammals and also in water, particularly sewage effluent. It usually metabolizes glucose by way of the phosphotransferase transport system followed by glycolysis and, in aerobic conditions, the TCA cycle. It cannot synthesize

PQQ but does make the membrane-bound glucose dehydrogenase apoenzyme and in the presence of PQQ this can be converted to the active holoenzyme both *in vivo* and *in vitro*. PQQ can act as a chemoattractant for *E. coli* (de Jonge *et al.*, 1996) and under conditions of low phosphate availability the PhoE porin, which is thought to be involved in PQQ uptake, is induced. Active enzyme may, therefore, form when *E. coli* is growing naturally in aquatic environments, alongside bacteria which excrete PQQ (Adamowicz *et al.*, 1991; Nickerson and Aspedon, 1992).

PQQ-dependent oxidation of glucose to gluconate by membrane vesicles of *E. coli* can generate a protonmotive force (van Schie *et al.*, 1985). In this organism the enzymes of the Entner–Doudoroff pathway are not subject to catabolite repression, so if active glucose dehydrogenase is available, glucose could be metabolized by the glycolytic pathway and the Entner–Doudoroff pathway at the same time (Fliege *et al.*, 1992). Metabolism by way of glucose dehydrogenase and the Entner–Doudoroff pathway is sufficient to support growth of *E. coli*, as shown by the ability of mutants lacking glycolytic enzymes to grow on glucose (Adamowicz *et al.*, 1991; Fliege *et al.*, 1992).

Wild-type *E. coli* does not normally oxidize glucose to gluconate but when grown in continuous culture under conditions of glucose excess and, for example, phosphate limitation, gluconate accumulated if PQQ was supplied in the growth medium (Hommes *et al.*, 1991). The biomass produced was similar to that of cultures grown in the absence of PQQ and the $Y_{glucose}$ decreased because of increased utilization of glucose. In contrast, when the bacteria were grown in the presence of PQQ under glucose limitation there was no accumulation of gluconate; this is because the affinity of glucose for the phosphotransferase system is much greater than that for glucose dehydrogenase. These results indicate that in cells grown in the absence of PQQ, under conditions of glucose excess, the respiratory chain was not working to full capacity; in the cells grown in the presence of PQQ, additional electron flow must have resulted from the oxidation of glucose to gluconate and presumably extra molecules of ATP were synthesized. In the natural situation in low-phosphate conditions where PQQ is available, the direct oxidation system and PhoE porin will be induced, and the Entner–Doudoroff pathway will operate (Fliege *et al.*, 1992; Yamada *et al.*, 1993a). The resulting extra electron flow may provide a bioenergetic advantage.

In addition to these possible roles in energy metabolism in *E. coli*, the respiratory chain involving glucose dehydrogenase 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 enzymes occurs, as suggested for *K. pneumoniae* (Section 7.2.3).

7.3. The Roles of Alcohol and Glucose Dehydrogenases in the Membranes of Acetic Acid Bacteria

We have found the literature on these bacteria rather confusing and contradictory, and here we attempt to summarize some of the key points of the relevant physiology (for reviews of growth and metabolism of acetic acid bacteria, see Asai, 1968; Swings, 1991; Matsushita *et al.*, 1994). Acetic acid bacteria grow in sugary, alcoholic environments and they are typically found on the surfaces of leaves, fruits and alcoholic beverages. Most of the earlier work has concentrated on their use in vinegar production and as spoilers in beers and wines (Swings, 1991). They have subsequently been exploited for microbial transformations that depend on their characteristic of catalysing incomplete oxidations, many of which are catalysed by quinoproteins (Asai, 1968; Matsushita *et al.*, 1994). Examples of industrial uses include the production of acetic acid from ethanol, gluconic acid from glucose, and sorbose from sorbitol. In the absence of added energy sources acetic acid bacteria are unable to grow on complex media such as peptone, nutrient broth or yeast extract, but they grow well at low to neutral pH when the medium includes an energy source such as ethanol, glucose or glycerol. Some strains are also able to use these substrates as a sole source of carbon and energy.

The acetic acid bacteria obtain their energy from the incomplete oxidation of ethanol or glucose. All acetic acid bacteria (*Acetobacter* and *Gluconobacter*) have a type III alcohol dehydrogenase (quinohaemoprotein) which oxidizes ethanol to acetaldehyde. A membrane aldehyde dehydrogenase (Section 4) then oxidizes the acetaldehyde to acetic acid, which is excreted into the growth medium. Sometimes the acetic acid can then be transported back into the cell and further metabolized.

Most *Gluconobacter* strains can also obtain energy from the incomplete oxidation of glucose to gluconate, catalysed by the membrane-bound glucose dehydrogenase. The gluconic acid is sometimes further oxidized in the periplasm to 2-ketogluconic acid by a membrane-bound flavoprotein dehydrogenase. *Gluconobacter* do not have a TCA cycle, and metabolize sugars (including gluconate) by way of the pentose phosphate pathway (Fig. 16).

A rather unusual situation is found in *Acetobacter diazotrophicus*, which is remarkable in being able to fix atmospheric dinitrogen while using glucose or ethanol as sole source of carbon and energy (Stephan *et al.*, 1991; Galar and Boiardi, 1995). Nitrogen fixation imposes a heavy demand for energy on the cell and there is evidence that in *A. diazotrophicus* synthesis of glucose dehydrogenase is three- to four-fold higher when cells are grown in batch culture under nitrogen-fixing conditions than when an alternative nitrogen source is present in excess (Galar and Boiardi, 1995). Gluconate accumu-

lated in the growth medium during the lag and early exponential phases, demonstrating that glucose dehydrogenase was responsible for oxidizing glucose. It was suggested that this oxidation provides an ancillary energy-generating system during nitrogen fixation and also that it might play a role in protecting nitrogenase from oxygen (Stephan *et al.*, 1991; Galar and Boiardi, 1995), as suggested for *K. pneumoniae* (Section 7.2.3).

8. SYNTHESIS OF PQQ

8.1. Origin of PQQ Backbone

The most important work on the origin of the PQQ backbone has come from analysis, using NMR spectrometry, of the PQQ produced when *Hyphomicrobium* X and *M. extorquens* AM1 were grown on labelled substrates. These results demonstrated that PQQ is derived from glutamate and tyrosine, both amino acids being incorporated intact (Houck *et al.*, 1988, 1989, 1991; van Kleef and Duine, 1988; Unkefer, 1993; Unkefer *et al.*, 1995). Thus, the tyrosyl side chain provides the six carbon atoms of the orthoquinone ring of PQQ and the pyrrole-2 carboxylic acid moiety is derived from internal cyclization of the amino acid backbone of tyrosine; the remaining five carbon atoms are from glutamate (Fig. 17).

These observations led to proposals of routes for conversion of tyrosine and glutamate to PQQ (see Unkefer, 1993 for review), but there is no direct chemical or biochemical evidence to support them. In the meantime the genetics of PQQ biosynthesis was under investigation, and a small polypeptide of 23–39 amino acids was shown to be involved in PQQ production in a number of bacteria. These polypeptides have a high identity and contain a conserved motif with glutamate and tyrosine residues separated by three other residues (Fig. 18). Site-directed mutagenesis was used to construct

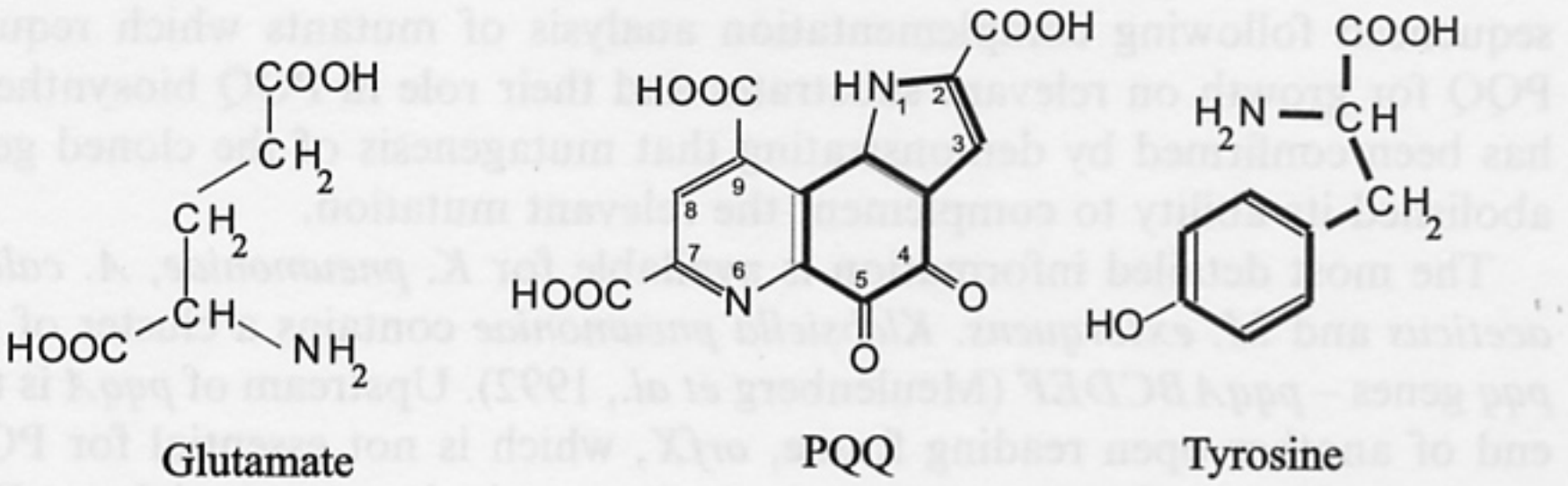


Figure 17 The origin of the carbon atoms in PQQ.

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                    MQWTKPAFTDLRIGFEVTMYFEAR    A. calcoaceticus PqqIV
                    |  |  |       |  |||  |          24 amino acids
                    M - WKKPAFIDLRLGLEVTLYISNR  K. pneumoniae  PqqA
                    |  |  |       |  |||  |          23 amino acids
                    MKWAAPIVSE ICVGMEVTSYESAEIDTFN  M. extorquens PqqA
                    |  |  |       |  |||  |          29 amino acids
MYRQHPSHPPQRSNFMTWSKPAYTDLRI GFEVTMYFASR    P. fluorescens PqqA
                    |  |  |       |  |||  |          39 amino acids
                    MMWTKPEVTEMRFGFEVTMYVCNR    M. flagellatum PqqD
                                                    24 amino acids
```

Figure 18 Alignment of the amino acid sequences of the proposed polypeptide precursor of PQQ in different bacteria. Data are taken from Goosen *et al.* (1989), Meulenberg *et al.* (1992), Morris *et al.* (1994), Schnider *et al.* (1995) and Gomelsky *et al.* (1996).

mutants of *A. calcoaceticus* containing either aspartate in place of the conserved glutamate residue (Glu16) or phenylalanine in place of the conserved tyrosine residue (Tyr20); in both cases PQQ synthesis was abolished (Goosen *et al.*, 1992). However, replacement of a nearby glutamate (Glu22) with aspartate had no effect on PQQ synthesis. Although this does not rule out the possibility that the small polypeptide has, for example, a regulatory function, the finding that genes with similarity to those coding for peptidases are also essential for PQQ synthesis (Section 8.2) lends support to the hypothesis that this cofactor is derived from a peptide precursor.

8.2. The Genetics of PQQ Biosynthesis

Genes involved in PQQ synthesis have been identified in a number of bacteria (Table 5; Fig. 19). In most cases these genes have been isolated and sequenced following complementation analysis of mutants which require PQQ for growth on relevant substrates and their role in PQQ biosynthesis has been confirmed by demonstrating that mutagenesis of the cloned gene abolished its ability to complement the relevant mutation.

The most detailed information is available for *K. pneumoniae*, *A. calcoaceticus* and *M. extorquens*. *Klebsiella pneumoniae* contains a cluster of six *pqq* genes – *pqqABCDEF* (Meulenberg *et al.*, 1992). Upstream of *pqqA* is the end of another open reading frame, *orfX*, which is not essential for PQQ synthesis. In *A. calcoaceticus* there are genes equivalent to *pqqA* (gene IV), *pqqB* (gene V), *pqqC* (gene I), *pqqD* (gene II) and *pqqE* (gene III) (Goosen *et al.*, 1989), arranged in the same order as in *K. pneumoniae*. No equivalent of

Table 5 Genes required for PQQ synthesis.

Gene symbol for strain shown (number of amino acids in predicted protein if known)							
K. pneumoniae	*A. calcoaceticus*	*M. extorquens* (old symbol in brackets)	*M. organophilum*	*M. flagellatum*	*P. fluorescens*	*E. herbicola*	Proposed function
A (23 aa)	IV (24 aa)	*A* (*pqqD*) (29 aa)	*D*	*D* (24 aa)	*A* (39 aa)		PQQ precursor
B (308 aa)	V (303 aa)	*B* (*pqqG*, *moxO*) (299 aa)	*G*	*G* (305 aa)	*B* (303 aa)		Transport
C (251 aa)	I (252 aa)	*C/D* (*pqqC*, *moxT*) (372 aa)	*C*	*C*	*C*		Oxygenase
D (92 aa)	II (94 aa)	*C/D* (*pqqB*, *moxV*) (372 aa)	*B*				
E (380 aa)	III (331 or 384 aa)	*E* (*pqqA*, *moxC/P*) (384 aa)	*A*			(378 aa)	Synthesis of metal-containing cofactor
F (761 aa)		*F* (*pqqE*, *moxH*) (709 aa)	*E*				Peptidase
		G (*pqqF*, *moxU*) (241 aa)	*F*				Peptidase non-catalytic subunit
orfX	*orf R*						Dipeptidase

Data are taken from Goosen *et al.* (1987), Mazodier *et al.* (1988), Biville *et al.* (1989), Meulenberg *et al.* (1990, 1992), Liu *et al.* (1992), Morris *et al.* (1994), Schnider *et al.* (1995), Gomelsky *et al.* (1996), Springer *et al.* (1996), Turlin *et al.* (1996), Toyama *et al.* (1997).

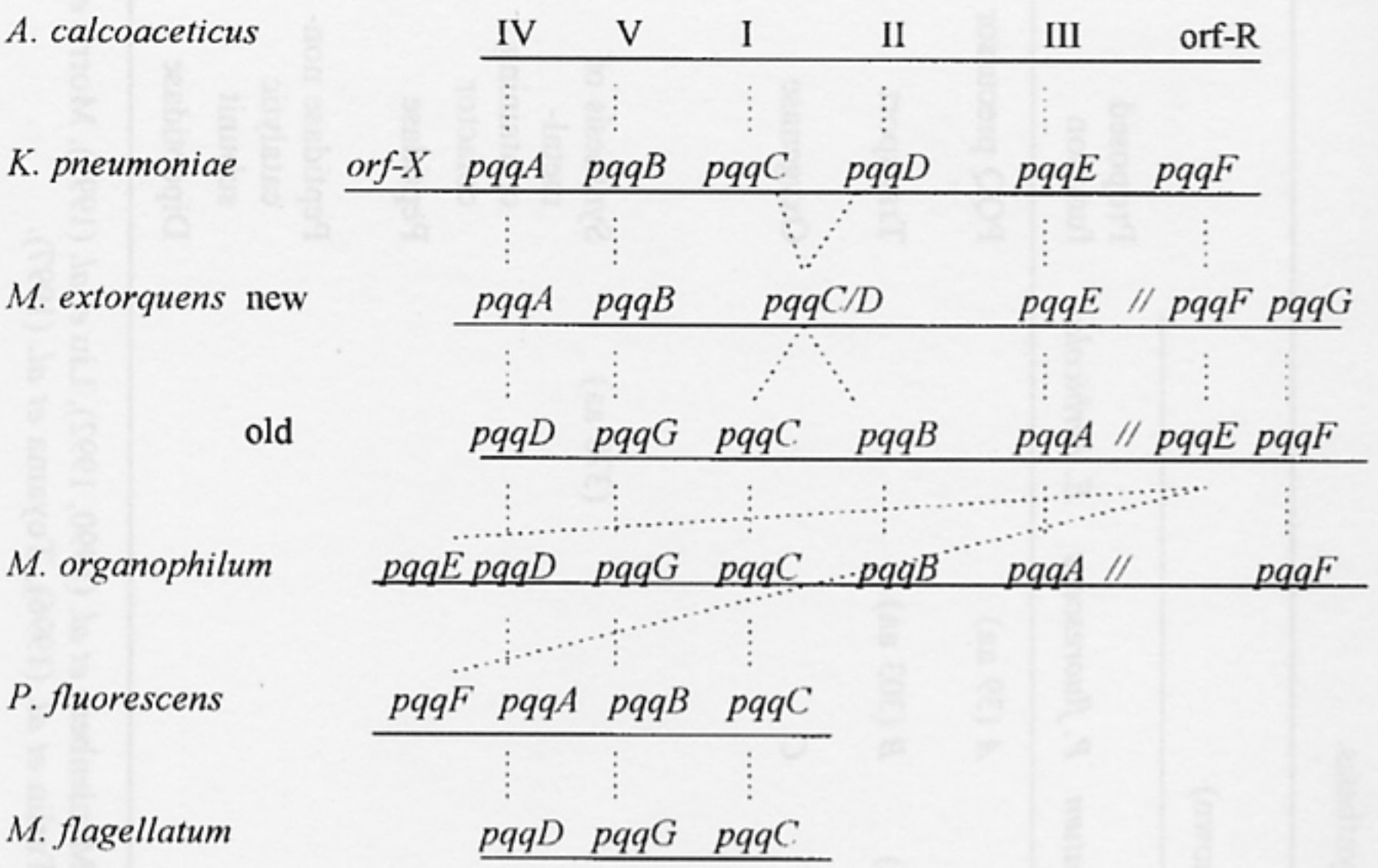


Figure 19 Organization of PQQ genes in different bacteria. Data are taken from Goosen *et al.* (1987), Mazodier *et al.* (1988), Biville *et al.* (1989), Turlin *et al.* (1996), Meulenberg *et al.* (1990; 1992), Morris *et al.* (1994), Springer *et al.* (1996), Toyama *et al.* (1997), Schnider *et al.* (1995), Gomelsky *et al.* (1996). The dotted lines indicate genes which are functionally equivalent.

pqqF has been reported, but downstream of gene III is an open reading frame, *orfR*, which is probably not essential for PQQ synthesis.

Methylobacterium extorquens also contains genes equivalent to the *pqqABCDEF* genes of *K. pneumoniae*, but in this case they are situated in two separate clusters (Morris *et al.*, 1994; Springer *et al.*, 1996; Toyama *et al.*, 1997). The nomenclature of the *M. extorquens pqq* genes is confusing; when they were first identified they were designated *mox* (methanol oxidation) genes; later, when their function in PQQ synthesis was demonstrated, they were called *pqq* genes and the labelling system used for the *pqq* genes of another methylotroph, *Methylobacterium organophilum*, was followed. However, recently it has been proposed that they should be renamed, to correspond to their functional equivalents in *K. pneumoniae* (Toyama *et al.*, 1997). The new and old nomenclature is shown in Table 5 and Fig. 19, but in the text only the new system is used. One cluster of *M. extorquens pqq* genes is adjacent to the *mxb* genes which are involved in the regulation of methanol dehydrogenase synthesis. It contains *pqqAB* and *E*. Between *pqqB* and *pqqE* is a gene encoding a protein which is similar at the N-terminal end to PqqC of *K. pneumoniae* (a polypeptide of 29.7 kDa) and at the C-terminal

end to PqqD of *K. pneumoniae* (a polypeptide of 10.4 kDa). Expression of the *M. extorquens* DNA in *E. coli* indicated that this region encodes a single protein of 42 kDa and it appears that in *M. extorquens* the *pqqC* and *D* genes have fused to give *pqqC/D* and the resulting protein can carry out the functions of both PqqC and PqqD. In *M. extorquens* there are another two *pqq* genes – *pqqF* and *pqqG*, which are linked; an equivalent of *pqqG* has not yet been described in *K. pneumoniae* or *A. calcoaceticus*.

A model for PQQ synthesis is shown in Fig. 20. The putative polypeptide precursor of PQQ is encoded by the *pqqA* gene of *K. pneumoniae* and its functional equivalents in other bacteria. Processing of this precursor must involve several steps, including cleavage by a specific protease or proteases, formation of the PQQ backbone from the relevant glutamate and tyrosine residues, and formation of the quinone groups. Analysis of those *pqq* genes which have been sequenced indicates that PqqF of *K. pneumoniae* and PqqF and PqqG of *M. extorquens* have similarity to a family of divalent cation-containing endopeptidases which are involved in processing small peptides (Meulenberg *et al.*, 1992; Springer *et al.*, 1996). The two PqqF proteins seem to be members of different subfamilies – the *K. pneumoniae* analogue is most closely related to the subfamily which contains pitrilysin, a periplasmic oligopeptidase found in *E. coli*, while the *M. extorquens* protein belongs to the subfamily containing mitochondrial processing peptidases. Members of this subfamily form heterodimers containing two similar subunits, one of which lacks the catalytic site. The predicted PqqG protein shows some identity to the C-terminal half of two members of the mitochondrial processing peptidase family, and it has been suggested that PqqF (which is predicted to contain the catalytic site) and PqqG (which does not appear to contain the catalytic site) may associate to form a heterodimer (Springer *et al.*, 1996). Interestingly, the *orfX* located upstream of the *K. pneumoniae pqq* operon and the *orfR* located downstream of the *A. calcoaceticus pqq* gene cluster both encode proteins similar to a human dipeptidase. However, mutations in these genes do not abolish the ability to synthesise PQQ, indicating that they are not essential for PQQ biosynthesis (Goosen *et al.*, 1989; Meulenberg *et al.*, 1990, 1992).

The predicted PqqE proteins of *K. pneumoniae* and *M. extorquens* contain a CxxxCxYC motif similar to that found in MoaA, which is involved in the biosynthesis of the molybdopterin cofactor of *E. coli* and NifB, which is involved in the biosynthesis of the iron-containing cofactors of nitrogenases (Toyama *et al.*, 1997). These proteins probably function in the donation of metal atoms to the relevant cofactor and the conserved CxxxCxYC sequence may be a metal-binding site (Menendez *et al.*, 1995). This suggests that an unknown metal-binding cofactor may be involved in PQQ synthesis.

PqqC probably catalyses the last step in PQQ biosynthesis; *pqqC* mutants of *K. pneumoniae* and *M. extorquens* accumulated an intermediate which

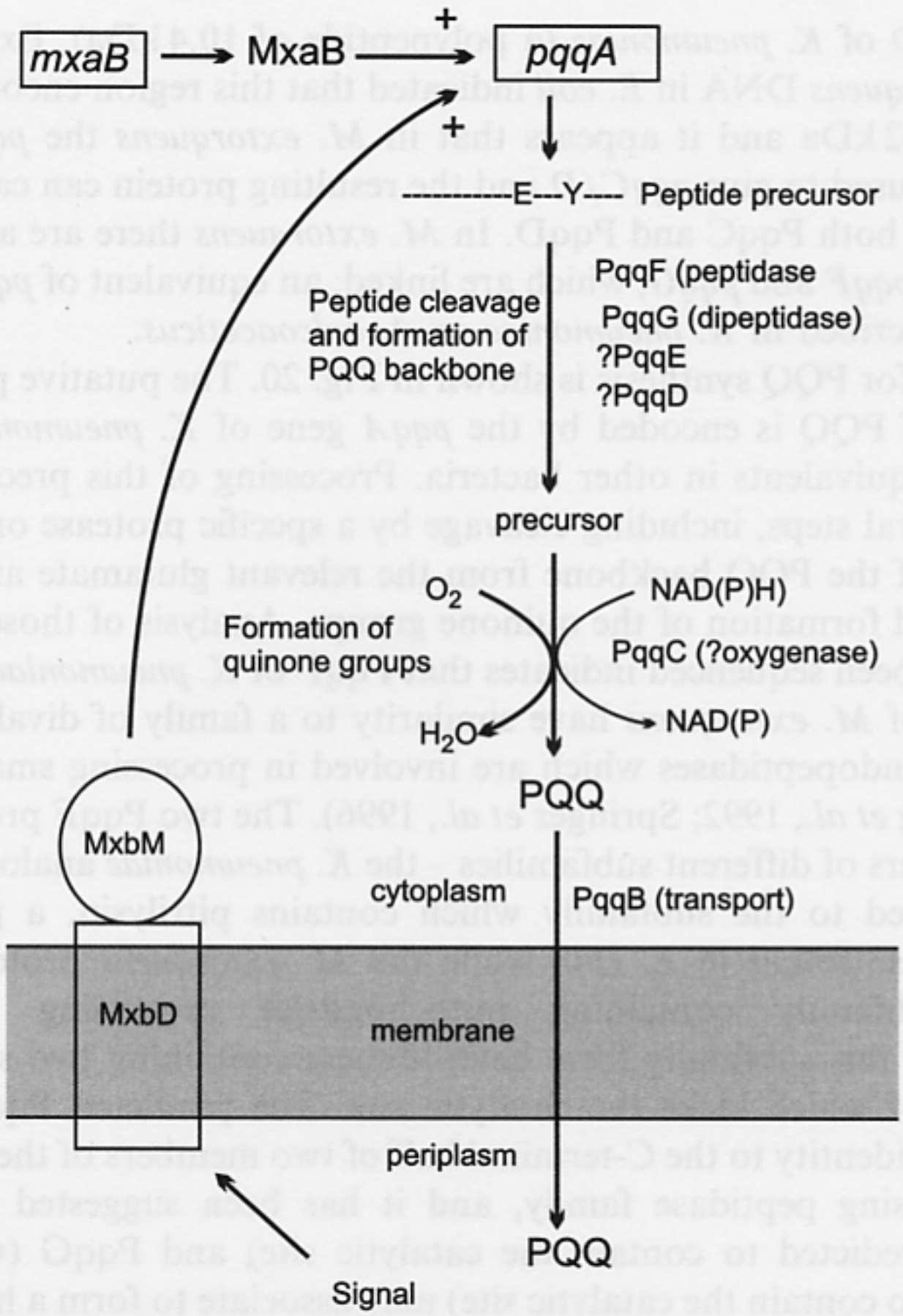


Figure 20 A model for PQQ synthesis in *Methylobacterium extorquens*. A signal (which could, for example, be formaldehyde) is transmitted by way of MxbD and MxbM, leading to the activation of the *pqqA* promoter; this is also regulated by MxaB. The polypeptide product of *pqqA* is processed in the cytoplasm to form PQQ and this probably involves the PqqCDEFG proteins. PQQ is then transported into the periplasm; PqqB, which is predicted to be a cytoplasmic protein, is necessary for this.

could be converted to PQQ *in vitro* on addition of an extract containing PqqC or PqqC/D respectively (Velterop *et al.*, 1995; Toyama *et al.*, 1997). Attempts to purify the intermediate were unsuccessful. Aerobic conditions and NAD(P)H were both essential for its conversion to PQQ, suggesting involvement of an oxygenase in the formation of the quinone groups of PQQ. However, if *pqqC* does encode an oxygenase it is not similar at the amino acid sequence level to any known monooxygenase or dioxygenase, and it is not obvious how this step would be catalysed in *Paracoccus* or *Hyphomicrobium* growing anaerobically on methanol with nitrate.

PqqB may be involved in transport of PQQ into the periplasm. This was suggested by Velterop *et al.* (1995) and Gomelsky *et al.* (1996), who demonstrated that *K. pneumoniae pqqB* mutants and mutants of *Methylobacillus flagellatum* defective in the equivalent gene, *pqqG*, accumulated PQQ intracellularly but did not excrete it into the growth medium. The PqqB protein does not contain any hydrophobic regions so it is unlikely to be a membrane protein directly involved in transport, but it might be required as a PQQ-binding protein or for modification of a membrane protein involved in PQQ transport.

8.3. Does *Escherichia coli* contain *pqq* Genes?

Escherichia coli is unable to synthesize PQQ, and produces glucose dehydrogenase as an apoenzyme, the holoenzyme only forming on addition of exogenous PQQ. There have been several reports that *E. coli* strains carrying heterologous *pqq* genes can synthesize PQQ and can therefore make active glucose dehydrogenase. However, the number of genes needed to obtain PQQ synthesis varies, depending on the source of the heterologous DNA. When *K. pneumoniae* DNA was used all six *pqq* genes seemed to be needed for PQQ production by *E. coli* (Meulenberg *et al.*, 1990, 1992) although later work indicated that small amounts of PQQ could be made in the absence of *pqqB* (which is possibly involved in PQQ transport) or *pqqF* (which encodes a peptidase) (Velterop *et al.*, 1995). This is consistent with the report that four *A. calcoaceticus pqq* genes (IV, I, II and III, equivalent to *pqqA*, *C*, *D* and *E* of *K. pneumoniae*) were required for PQQ synthesis by *E. coli* (Goosen *et al.*, 1989).

Escherichia coli does contain a gene which is functionally equivalent to the *pqqF* genes of *K. pneumoniae* and *M. extorquens*. Evidence for this was obtained by the demonstration that a 7.3 kb fragment of *E. coli* DNA containing an ORF with low identity to the *pqqF* gene of *M. extorquens* (Springer *et al.*, 1996) can complement a mutant of *Methylobacterium organophilum* which is defective in the equivalent gene (Turlin *et al.*, 1991, 1996). This might explain why it was not essential to provide the *pqqF* gene of *K. pneumoniae* in order to obtain PQQ synthesis in *E. coli* carrying the *K. pneumoniae pqq* genes. The *E. coli* fragment also contained an ORF with low identity to the *pqqG* gene of *M. extorquens* (Springer *et al.*, 1996) and complemented a *M. organophilum* mutant defective in the equivalent gene. Thus, *E. coli* contains genes which can function in the same way as the PqqF and PqqG proteins of *M. extorquens*. There is no evidence from sequence analysis that *E. coli* contains a gene with similarity to *pqqB*, but it is possible that another *E. coli* protein may be able to substitute for it in functional terms.

The work described above indicates that *E. coli* can synthesize PQQ, but only if provided with at least four heterologous PQQ genes. It is difficult to reconcile this with reports suggesting that *E. coli* strains carrying single genes from *Erwinia herbicola* (Liu *et al.*, 1992) and *Pseudomonas cepacia* (Babu-khan *et al.*, 1995) produced PQQ. However, in neither case was PQQ measured directly – synthesis was inferred because the *E. coli* strains carrying the heterologous DNA produced acid from glucose. The gene from *Erwinia herbicola* had a high degree of identity with *pqqE* of *K. pneumoniae* but the gene from *P. cepacia* (*gabY*) was not similar to any previously described *pqq* genes. It is predicted to encode a 14.2 kDa protein which has some regions similar to parts of the membrane bound components of periplasmic permeases such as HisQ and GlnP. It was suggested that GabY might catalyse PQQ production by a novel pathway, cause production of a cofactor which could replace PQQ in the apo-glucose dehydrogenase, or activate cryptic *pqq* genes.

Biville and colleagues (1991) also suggested that *E. coli* contains cryptic *pqq* genes. They were working with an *E. coli* strain lacking the phosphotransferase system (PTS), and therefore unable to metabolize glucose via the glycolytic pathway. This mutant could, however, grow on glucose if PQQ was added to the medium – the holoform of glucose dehydrogenase was produced and thus glucose could be oxidized to gluconic acid and catabolized via the Entner–Doudoroff pathway. When these PTS^- strains were plated onto glucose minimal medium (lacking PQQ) spontaneous 'revertants' arose, which could oxidize glucose to gluconic acid, and PQQ and low glucose dehydrogenase activities were detected in membrane preparations. The ability to grow on glucose could not, therefore, be explained by reversion of the original PTS mutations. The authors concluded that *E. coli* contains all the genetic information required for PQQ synthesis and that the spontaneous mutation resulted in expression of these genes. However, the data supporting this conclusion have recently been challenged (Matsushita *et al.*, 1997).

Analysis of the *E. coli* genome indicates that it does not contain any genes with high similarity to *pqqBCDE* of *M. extorquens*, suggesting that there is no cryptic *pqq* operon comparable with those described in the well-characterized PQQ-producing bacteria. However, proteins which do not share similarity at the amino acid level may still share functions; for example, ActA of *Listeria monocytogenes* and IcsA of *Shigella flexneri* have virtually no sequence similarity, but carry out similar functions in initiating actin assembly (Strauss and Falkow, 1997). The possibility that *E. coli* contains genes which have no sequence similarity to the known *pqq* genes, but can carry out the same functions, needs to be explored, as does the suggestion that there might be a novel pathway for PQQ synthesis in *E. coli*. This may

clarify the conflicting results concerning the ability of this organism to produce PQQ.

9. REGULATION OF SYNTHESIS OF PQQ AND QUINOPROTEIN DEHYDROGENASES

9.1. Synthesis of PQQ and Apoenzymes is not Coordinated

Synthesis of PQQ and the apoenzyme dehydrogenases is not necessarily coordinated and sometimes enzyme activity *in vitro* is several fold higher when PQQ is added to the assay mixture than in its absence (van Schie *et al.*, 1984; Hommes *et al.*, 1989). An investigation of PQQ synthesis and production of the apo- or holoenzymes of glucose dehydrogenase, methanol dehydrogenase and quinoprotein alcohol dehydrogenase in a variety of bacteria indicated that PQQ synthesis is not essential for apoenzyme production (van Kleef and Duine, 1989). By contrast, PQQ is only made when the quinoprotein is also being synthesized.

9.2. Regulation of PQQ Synthesis

It has been suggested, on the basis of DNA sequence analysis, that the *pqqA* and *B* genes of *K. pneumoniae* and *M. extorquens* and the equivalent genes in *A. calcoaceticus* are co-transcribed. Prediction of the mRNA structures suggests that a hairpin can form between the sequence for *pqqA* and *pqqB*, which might cause transcription termination. Analysis of the mRNA transcripts of *M. extorquens* confirmed that the major mRNA formed from transcription from the *pqqA* promoter was approximately 240 bases, and encoded only PqqA; a second, less abundant transcript was also detected, which encoded PqqA and PqqB (Ramamoorthi and Lidstrom, 1995). Furthermore, in *K. pneumoniae* expression of *pqqA* is 20-fold higher than that of *pqqC* or *pqqE* (Velterop *et al.*, 1995). These observations are consistent with the suggestion that *pqqA* encodes the precursor of PQQ – it would be needed in larger amounts than the products of other *pqq* genes, which presumably have catalytic roles in processing the precursor.

The promoters of the *pqqA* genes have not been conclusively defined, but possible −10 and −35 sequences have been identified in *K. pneumoniae* (CAATAT and TTGATC) and *M. extorquens* (CGATAT and TTGCAG) (Ramamoorthi and Lidstrom, 1995). The latter differs from the postulated promoter sequence (−10 TAGAA, −35 AAGACA) upstream of the *mxaF*

operon, which encodes the methanol dehydrogenase and cytochrome c_L structural proteins (Barta and Hanson, 1993; Chistoserdova *et al.*, 1994). However, upstream of the putative *pqqA* promoter, at bases −54 to −48, is the sequence AGAAACG, which is similar to the septanucleotide sequence AGAAATG found upstream of *mxaF* and other methanol-regulated promoters in *Methylobacterium organophilum* (Xu *et al.*, 1993) and it has therefore been suggested that *mxaF* and *pqqA* share some common regulators. This is supported by the observation that expression of *pqqA* is not regulated normally in mutants of *M. extorquens* defective in three genes essential for expression of the *mxaF* operon – *mxbM* and *mxbD*, which encode a two-component regulatory system, and *mxaB*, which encodes a positive regulator (Section 9.3.3). However, as noted above, synthesis of the apoenzyme and PQQ is not always coordinated and comparison of the level of expression of *pqqA* and the amounts of PQQ produced in these mutants indicated that regulation of *pqqA* transcription is not the only step at which synthesis is controlled (Ramamoorthi and Lidstrom, 1995). Whether the other factor(s) which regulate PQQ synthesis act at the transcriptional or post-transcriptional level has still to be determined. However, there is preliminary evidence that there is a weak C_1-inducible promoter upstream of *pqqF* and that expression from this promoter is not under the control of the regulatory genes thought to control methanol dehydrogenase production (Springer *et al.*, 1996).

9.3. Factors Affecting the Synthesis of the Quinoprotein Dehydrogenases

There is evidence from several studies that active methanol dehydrogenase, alcohol dehydrogenase and glucose dehydrogenase can be synthesized to quite high levels in the absence of their substrates, although a number of factors do affect their synthesis; for example, growth rate, the nature of the growth substrate or oxygen availability (Dunstan *et al.*, 1972b; Ng and Dawes, 1973; O'Connor and Hanson, 1977; Roitsch and Stolp, 1985, 1986; de Vries *et al.*, 1988; van Kleef and Duine, 1989; Frebortova *et al.*, 1997). Much of the early work is difficult to interpret because it was done using batch cultures and growth conditions were not well defined. The conclusions of some of the more detailed studies, using chemostat cultures, are summarized below.

9.3.1. *Synthesis of Glucose Dehydrogenase*

In *K. pneumoniae* several factors affect expression of glucose dehydrogenase. During growth in chemostat cultures in the presence of excess glucose under

potassium or phosphate limitation, glucose dehydrogenase activities were high; however, they were relatively low in glucose-limited cultures and in sulphate- or ammonia-limited cultures, despite the presence of excess glucose, indicating that synthesis was not necessarily regulated in response to the level of glucose in the medium (Hommes *et al.*, 1985). Growth rate also influenced glucose dehydrogenase production, although the effect depended on the growth conditions – activity in potassium-limited cultures increased with increasing dilution rate, whereas in phosphate-limited cultures activity decreased with increasing growth rate. It has been suggested that glucose dehydrogenase plays a role in the generation of reducing power when there is a high energy demand on the cell (Hommes *et al.*, 1985; Section 7.2.3) and it may be synthesized in response to this. However, the situation is clearly complex and further work is required to define precisely how changes in enzyme synthesis relate to the energy demand of the cell under different growth conditions.

Most strains of *A. calcoaceticus* cannot grow on glucose, but they can make glucose dehydrogenase and oxidize glucose to gluconic acid, and this may provide an additional energy source during growth on other substrates (Section 7.2.2). Synthesis appears to be regulated by derepression rather than induction because active enzyme is made during growth on a variety of substrates, independent of the presence of glucose in the medium. However, activity does vary with growth rate, being high at low growth rates, when there is a high requirement for maintenance energy. It has therefore been suggested that, as with *K. pneumoniae*, glucose dehydrogenase synthesis is regulated in response to the energy status of the cells (van Schie *et al.*, 1988).

By contrast, in *P. aeruginosa* glucose dehydrogenase is induced by glucose. Gluconate, the product of glucose oxidation, and glycerol are also inducers. However, during growth on a mixture of glucose and citrate, it is also regulated by catabolite repression (Midgley and Dawes, 1973; Ng and Dawes, 1973). Active enzyme is not produced when *P. aeruginosa* is grown anaerobically on glucose with nitrate as the terminal electron acceptor, but this is because PQQ is not made. The apoenzyme is induced, and active enzyme can be formed on addition of PQQ (van Schie *et al.*, 1984).

Escherichia coli only produces apo-glucose dehydrogenase, but there is evidence that it is induced by glucose. Levels are high in glucose-grown cells which are potassium-, phosphate- or sulphate-limited, and low in cultures grown on limiting glucose (Hommes *et al.*, 1991). The glucose dehydrogenase structural gene, *gcd*, has been sequenced (Cleton-Jansen *et al.*, 1990) and analysis of the upstream region indicates that there are two promoters. Studies using transcription fusions demonstrated that glucose induces transcription from the first promoter, which is also regulated negatively

by cyclic AMP; the second promoter is regulated positively by oxygen (Yamada *et al.*, 1993a).

9.3.2. *Synthesis of Methanol Dehydrogenase*

In *Methylophilus methylotrophus*, which is an obligate methylotroph able to grow only on methanol and methylated amines, methanol dehydrogenase activity is strictly controlled in response to the growth conditions. It is maximally repressed during growth in oxygen-limited cultures and, when cells are grown at different dilution rates under methanol limitation, the activity decreases as the standing concentration of methanol in the growth medium increases (Greenwood and Jones, 1986; Jones *et al.*, 1987; Southgate and Goodwin, 1989). However, methanol dehydrogenase does not catalyse the rate-limiting step in methanol oxidation, and the activity of the overall methanol oxidase system was high at all dilution rates. It has therefore been suggested that, when there are high levels of methanol in the medium, methanol dehydrogenase is repressed in order to prevent accumulation of formaldehyde, the toxic product of methanol oxidation. The methanol oxidase system is regulated by oxygen and methanol such that the energy demands imposed by the growth rate can be met. Repression of methanol dehydrogenase was also observed under similar conditions in *Hyphomicrobium* X (Duchars and Attwood, 1989).

In *Paracoccus denitrificans*, an autotrophic methylotroph which assimilates methanol by the ribulose bisphosphate pathway after oxidation to carbon dioxide, there is evidence for two mechanisms of regulation of methanol dehydrogenase synthesis (de Vries *et al.*, 1988). There is a basal level of synthesis during growth on high levels of multicarbon compounds which are good growth substrates, whereas under conditions of carbon limitation or at low growth rates it is derepressed. It is also regulated by induction but the inducer is formaldehyde, the product of methanol oxidation, and not methanol itself. Autotrophic growth on methanol is not energetically favourable compared with growth on multicarbon substrates. The combination of repression/derepression of methanol dehydrogenase in response to the availability of other growth substrates, together with product induction (which requires that the substrate is present at high enough levels for a long enough time period for the concentration of the product to be high enough to initiate induction), would therefore ensure that, in the natural environment, the methanol dehydrogenase system is only synthesized to high levels when there is no preferred substrate available. Similar patterns of regulation, i.e. derepression at low growth rates and induction by methanol, have been observed in another autotrophic methylotroph, a *Xanthobacter* sp., and in *Methylobacterium* sp., but

further work is needed to determine if formaldehyde rather than methanol is the inducer (O'Connor and Hanson, 1977; Roitsch and Stolp, 1986; Croes *et al.*, 1991).

9.3.3. *Molecular Mechanism of Regulation of Methanol Dehydrogenase Synthesis*

More than 25 genes (*mox* genes) are required for methanol dehydrogenase synthesis (Tables 5 and 6). Seven are *pqq* genes (Section 8.2), three (*mxaFGI*) are structural genes and three (*mxaAKL*) are required for insertion of calcium into methanol dehydrogenase. Of the remainder, seven are thought to be regulatory genes and the rest are of unknown function. The structural genes *mxaFGI* are located in an operon, and in the *Methylobacterium* strains *mxaF* and *G* are separated by *mxaJ*, which encodes a polypeptide of unknown function (Fig. 21).

Using transcriptional fusions it has been shown that the *mxaF* promoter of the *Methylobacterium* strains is expressed at a high level during growth on methanol compared with growth on succinate and that it is positively regulated by *mxaB* (Morris and Lidstrom, 1992; Xu *et al.*, 1993). The *mxaB* gene also regulates *pqqA* (Section 9.2). The promoters of two other *mox* genes – *mxaW* and *mxcU*, both of unknown function – are activated during growth on methanol, but this is not dependent on *mxaB*.

Transcription of the *mxaF* operon of the *Methylobacterium* strains is also regulated by two pairs of sensor kinase–response regulator proteins, encoded by *mxbDM* and *mxcEQ* (Xu *et al.*, 1993, 1995; Springer *et al.*, 1997). Two-component regulatory systems are common in bacteria and involve sensing of a signal by the membrane protein kinase which is then autophosphorylated. It can then interact with the response regulator, resulting in activation of transcription at a specific promoter(s). Sequence analysis indicates that MxbD and MxcQ are membrane proteins belonging to the histidine kinase superfamily, although their putative periplasmic loops are quite different, suggesting that they respond to different signals. MxbM and MxcE are DNA-binding proteins of the response regulator family. The *mxbDM* genes are probably co-transcribed and studies using *mxbD* transcriptional fusions showed that expression was considerably reduced in *mxcE* and *mxcQ* mutants, suggesting a hierarchical regulatory system. Thus, the *mxcQE* proteins would respond to a specific signal and switch on expression of *mxbDM*. The MxbD protein then presumably responds to a second signal, resulting in enhanced transcription of *mxaF*. The *mxbD* and *M* genes are also essential for expression of *pqqA* (Section 9.2), *mxaW* and *mxcU*; however, *mxcQ* and *mxcE* are not, indicating that there must be a

Table 6 Genes involved in methanol oxidation in *Methylobacterium* and *P. denitrificans*.

	Proposed function and location of gene product	Organism		
		M. extorquens	*M. organophilum*	*P. dentrificans*
Structural genes	α subunit of MDH (P)	*mxaF*	*mxaF*	*mxaF*
	cytochrome C_L (P)	*mxaG*	*mxaG*	*mxaG*
	β subunit of MDH (P)	*mxaI*	*mxaI*	*mxaI*
Regulatory genes	sensor kinase – response regulator (M/C)	*mxcQE* *mxbDM*	*mxcQE* *mxbDM*	*mxaYX*
	(C)	*mxaB*	*mxaB*	
	(M)			*mxaZ*
		mxbN	*mxbN*	
Insertion of Ca^{2+} into MDH	? Ca^{2+} binding (P)	*mxaA*		
	(C)	*mxaK*		
	(M)	*mxaL*		
Other	Third subunit of MDH or molecular chaperone (P)	*mxaJ*	*mxaJ*	
	Unknown (C)	*mxaR*		*mxaR*
	Unknown	*mxaS*		*mxaS*
	Unknown		*mxaW*	
	Unknown (C)	*mxaC*		
	Unknown (P)	*mxaD*		
	Unknown		*mxcU*	
	Unknown	*mxdR*		
	Unknown	*mxdS*		

(C), cytoplasm; (M), membrane; (P) periplasm; MDH, methanol dehydrogenase.
Key references for *M. extorquens*: Nunn and Anthony, 1988; Nunn *et al.*, 1989; Anderson *et al.*, 1990; Morris and Lidstrom, 1992; Lidstrom *et al.*, 1994; Morris *et al.*, 1995; Amaratunga *et al.*, 1997a,b; Springer *et al.*, 1995, 1997, *M. organophilum*: Machlin and Hanson, 1988; Xu *et al.*, 1993, 1995; *P. denitrificans*: Harms *et al.*, 1987; van Spanning *et al.*, 1991; Harms *et al.*, 1993.

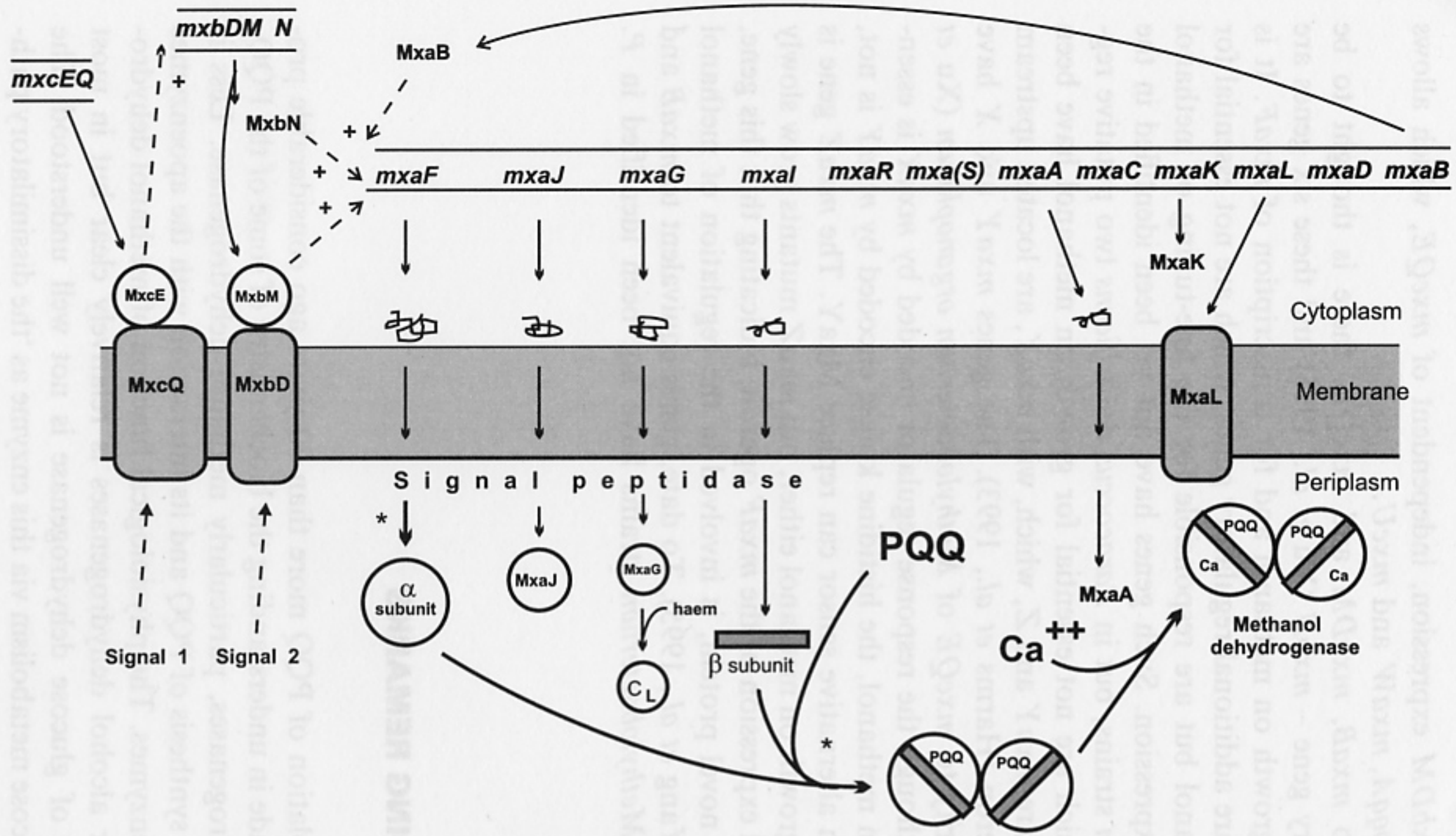


Figure 21 A model for the expression of methanol dehydrogenase in *Methylobacterium extorquens*; it is probably similar in *Paracoccus denitrificans* and related bacteria. Signal 1 is transmitted by way of MxcQ and MxcE, leading to transcription of *mxbDM*. These genes encode a second signal transduction system which responds to signal 2, resulting in activation of the *mxaFJGI* operon. The signals have not been identified, but one is likely to be methanol or formaldehyde. The *mxaF* promoter is also controlled by MxaB and MxbN. The *mxaFJGI* preproteins are transported into the periplasm where they are assembled into the proteins that are specifically involved in methanol oxidation – methanol dehydrogenase and cytochrome c_L. The * indicates two possible steps at which PQQ may be inserted. At least three proteins (MxaA, MxaK and MxaL) are involved in incorporation of Ca^{2+} into methanol dehydrogenase and MxaC and MxaD may also be involved in this.

basal level of *mxbDM* expression, independent of *mxcQE*, which allows transcription of *pqqA*, *mxaW* and *mxcU*.

In addition to *mxaB*, *mxbDM* and *mxcQE*, there is thought to be another regulatory gene – *mxbN* (Xu *et al.*, 1995) and these six genes are all required for growth on methanol and for transcription of *mxaF*. It is likely that there are additional regulatory genes, which are not essential for growth on methanol but are responsible for the fine-tuning of methanol dehydrogenase expression. Such genes have not yet been identified in the *Methylobacterium* strains, but in *Paracoccus denitrificans* two putative regulatory genes which are not essential for growth on methanol have been described. These are *mxaY* and *Z*, which, with *mxaX*, are located upstream of the *mxaF* operon (Harms *et al.*, 1993). The genes *mxaY* and *X* have significant similarity to *mxcQE* of *Methylobacterium organophilum* (Xu *et al.*, 1995) but, although the response regulator encoded by *mxaX* is essential for growth on methanol, the histidine kinase encoded by *mxaY* is not, indicating that an alternative sensor can replace MxaY. The *mxaZ* gene is not essential for growth on methanol either, but *mxaZ* mutants grow slowly and have reduced expression of the *mxaF* operon, indicating that this gene, which encodes a novel protein, is involved in the regulation of methanol dehydrogenase (Yang *et al.*, 1995). To date, genes equivalent to *mxaB* and *mxbDM* of the *Methylobacterium* strains have not been identified in *P. denitrificans*.

10. CONCLUDING REMARKS

Since the first isolation of PQQ more than 30 years ago considerable progress has been made in understanding the biochemistry of some of the PQQ-dependent dehydrogenases, particularly methanol dehydrogenase. Less is known about the synthesis of PQQ and its interaction with the apoenzymes to form the holoenzymes. The physiological function of methanol dehydrogenase and other alcohol dehydrogenases is relatively clear but in most bacteria the role of glucose dehydrogenase is not well understood. The description of glucose metabolism via this enzyme as 'the dissimilatory pathway' indicates inefficient use of glucose and, although we have speculated on its role in Section 7.2, we finish with a final, perhaps heretical, suggestion; that this enzyme had an important role to play in an ancestor of the present-day bacteria, providing a rapid means of metabolizing glucose, but that in many bacteria it may now be merely an 'evolutionary relic' which is occasionally useful.

ACKNOWLEDGEMENTS

Work in the authors' laboratory has been supported by the BBSRC and The Wellcome Trust.

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