Stellingen behorende bij het proefschrift *Structural and mechanistic aspects of quinohaemoprotein ethanol dehydrogenase from Comamonas testosteroni* van G.A.H. de Jong.

1 De bereiding van het phenylhydrazon van PQQ zoals uitgevoerd door Buffoni *et al.* levert niet het door hen gewenste produkt op.

Buffoni. F., Cambi, S & Moneti, G. (1992) *Biochim. Biophys. Acta.* 1116, 297-304.

2 De argumenten van Itoh *et al.* voor een "*p*-chinon mechanisme" van PQQ in methanol dehydrogenase zijn gebaseerd op een ruimtelijke positie van PQQ in dit enzym (Xia *et al.*) die onjuist is.

Itoh, S., Ogino, M., Fukui, Y., Murao, H., Komatsu, M., Oshiro, Y., Inoue, T., Kai, Y & Kasai. (1993) *J. Am. Chem. Soc.* 115, 9960-9967.

Xia, Z.Y., Dai, W., Xiong, J., Hao, Z., Davidson, V.L., White, S. & Mathews, F.S. (1992) *J. Biol. Chem.* 267, 22289-22297.

3 De bewering door Oyama *et al.* dat het enacyloxine oxydase een PQQ-afhankelijk enzym is, zou veel geloofwaardiger zijn als men meer blanco experimenten had uitgevoerd.

Oyama, R., Watanabe, T., Hanzawa, H., Sano, T., Sugiyama, T. & Izaki, K. (1994) *Biosci. Biotech. Biochem.* 58, 1914-1917.

4 Dat het alcohol dehydrogenase uit *Rhodopseudomonas acidophila* een quinohaemoprotein zou zijn zoals beweerd door Matsushita en Adachi, valt niet op te maken uit de literatuur waarnaar door hen wordt verwezen.

Matsushita, K. & Adachi, O. (1993) Bacterial quinoproteins glucose dehydrogenase and alcohol dehydrogenase. In *Principles and Applications of Quinoproteins* (Davidson VL, editor) Marcel Dekker, New York, 47-63.

Yamanaka, K. & Tsuyuki, Y. (1983). *Agric. Biol. Chem.* 47, 2173-2183.

Yamanaka, K. (1991) *Agric. Biol. Chem.* 55, 837-844.

5 Gezien de gebruikte concentratie voor de opname van het UV/Vis spectrum van enacyloxine oxydase, is de bewering [Oyama *et al.*] dat dit enzym geen haem c bevat voorbarig.

Oyama, R., Watanabe, T., Hanzawa, H., Sano, T., Sugiyama, T. & Izaki, K. (1994) *Biosci. Biotech. Biochem.* 58, 1914-1917.

6 Het bepalen van de activiteit van sulfide dehydrogenase op de manier beschreven door Schneider en Friedrich is onbetrouwbaar.

Schneider, A. & Friedrich, C. (1994) *FEBS Letters* 350, 61-65.

7 Het valt te betwijfelen of de door Marín en Amaro aanbevolen methode om enzymen te detecteren, bruikbaar is voor de detectie van periplasmatische eiwitten in het geval dat *Thiobacillus cuprinus* overgaat van heterotrofe naar chemolithotrofe groei.

Marín, I. & Amaro, A.M. (1993) *Biohydrometallurgical technologies* pp 473-478.

8 Na het mislukken van de zogeheten carpool-strook als middel om de verkeersdrukte te verminderen zou de volgende maatregel kunnen zijn: het verplaatsen van het gaspedaal naar de passagierskant van de auto.

9 Promotieonderzoek met als onderwerp "alcoholdehydrogenase" is een stimulans om de maximale omzettingsnelheid van de alcoholdehydrogenasen van de betrokkene vast te stellen.

10 Zowel het niet als wel gebruiken van een kruiwagen kan ernstige gevolgen hebben.

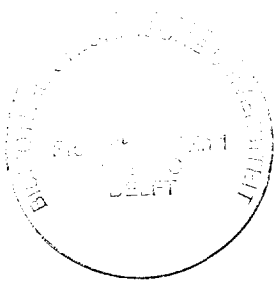
11 Het op de werkplek blokkeren van het verkrijgen van informatie via "06-nummers" geeft informatie over het personeelsbeleid van de betreffende instantie.

12 Dagen van congresbezoek zouden moeten worden bekort op het aantal vrije dagen, mits de persoon in kwestie zijn collega's een gedetaileerd verslag kan voorleggen van zijn bevindingen.

Delft, 12 september 1995.

Structural and mechanistic aspects of quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni*

Proefschrift



ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus Prof. ir. K.F. Wakker,
in het openbaar te verdedigen ten overstaan van een commissie,
door het College van Dekanen aangewezen,
op vrijdag 6 oktober 1995 te 16.00 uur

door

Govardus Adrianus Hubertus DE JONG

doctorandus chemie

geboren te Udenhout

Dit proefschrift is goedgekeurd door de promotor:
Prof. dr. ir. J.A. Duine

Samenstelling promotiecommissie:

Rector Magnificus, voorzitter
Prof. dr. ir. J.A. Duine, TU Delft, promotor
Prof. dr. B.W. Dijkstra, RUG
Prof. dr. B.A. Averill, UvA
Prof. dr. J.G. Kuenen, TU Delft
Dr. S.S. Wijmenga, KUN
Dr. ir. J.J. Jongejan, TU Delft
Dr. S. de Vries, TU Delft

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Errata

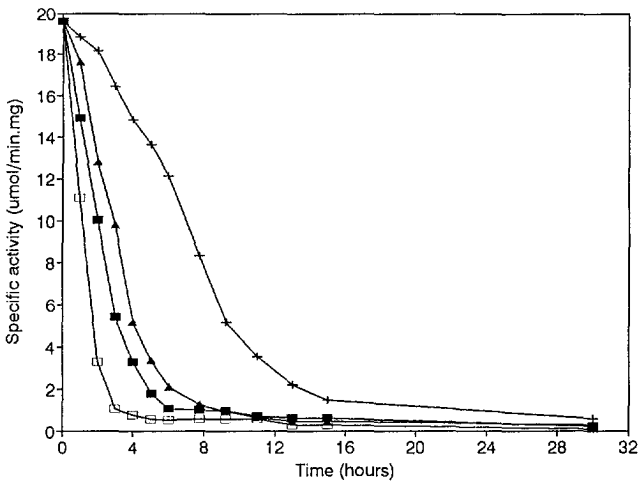
- References 13 and 14, page 43 should be interchanged

- Equations 4 and 5 on page 98 should read:

$$\frac{1}{T_{1,m}} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{\omega_S^2\tau_c^2}\right) + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2}\right)$$

$$\frac{1}{T_{2,m}} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{\omega_S^2\tau_c^2}\right) + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2} + \tau_e\right)$$

- Figure 1, page 120 should display:



Aan mijn ouders

Voorwoord

Uiteindelijk is alles dus nog op zijn pootjes terecht gekomen. Het bewijs daarvan heeft U nu in handen. De uiteindelijke inhoud van dit proefschrift is weliswaar redelijk verwijderd van de oorspronkelijke gedachte achter mijn onderzoek, maar het is me toch gelukt om er voor te zorgen dat ik de afgelopen jaren als een succes mag ervaren. Hoewel dit proefschrift op mijn naam staat zou het zonder de hulp van enkele andere mensen toch een heel stuk moeilijker zijn geweest. Mijn grote dank gaat als eerste uit naar mijn promotor Hans Duine. Hans, je was af en toe naar mijns inziens wat te nauwkeurig in het nakijken van mijn artikelen, maar gelukkig werden ze er wel duidelijk beter op. Uiteraard ook mijn begeleider in het onderzoek. Jaap, je was af en toe wat te positief over de uitkomsten van de experimenten, maar je positieve visie heeft wel geholpen om ook moeilijke tijden door te komen. Toen ik reeds enkele jaren bezig was, kwam uiteindelijk Simon op de proppen. Simon je was een erg grote steun en jou fanatisme en enthousiasme heeft er sterk toe bijgedragen dat het uiteindelijk toch gelukt is.

Graag wil ik ook de NMR faciliteit van de SON en met name Sybren Wijmenga bedanken voor het meten van mijn NMR spectra en de vruchtbare discussies die hieruit volgden. De mensen buiten Nederland die hebben meegeholpen aan dit onderzoek: Gorge Caldeira, Isabel Moura, José Moura, Thomas Loehr and Jie Sun, thanks for your big help!

Naast de begeleiding die ik zelf kreeg, heb ik ook hulp gehad van mensen die als opleiding hadden "gekozen" voor een stage bij mij. Rob Coster en Peter Rommens nog bedankt voor het vele werk, al zal het jullie mischien niet meteen opvallen wat jullie bijdrage aan dit proefschrift uiteindelijk is geweest.

Natuurlijk zal ik zeker niet mijn collega's en vrienden van de afdeling Enzymologie vergeten omdat zij hebben gezorgd voor een aangename sfeer welke noodzakelijk is om goed te kunnen functioneren. Ook buiten het werk, voor zover daar nog sprake van kon zijn, was het weleens goed om wat afwisseling te krijgen in de vorm van het zaalvoetbal of een enkel SWAK-feestje. Hoewel het erg gevaarlijk is om mensen een speciale pluim op te zetten, waardoor anderen zich vergeten voelen, vind ik het toch nodig om met name Peter, Arie, Bert, Dion, Vincent en Arjen te noemen omdat zij ook zowel tijdens het werk als daar buiten gestreeft hebben om de sfeer te verhogen. Ook wil ik Joke bedanken voor de begeleiding bij het kweken in de 100 liter fermentor, al was het af en toe niet duidelijk wie nou wie begeleidde. Ook de andere mensen in dit gebouw die altijd voor me klaar stonden bedank ik bij deze gelegenheid.

Tenslotte wil ik bij deze ook mijn ouders, familie en vrienden bedanken, voor wie het af en toe niet duidelijk was wat ik nu precies deed, maar het was "iets met alcohol en enzymen".

Aard

Contents

Chapter 1

General introduction

Abbreviations

ADH, alcohol dehydrogenase; CDTA, trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid; EDH, ethanol dehydrogenase; EDTA, Ethylenediamine-tetraacetic acid; E_M, Midpoint redox potential; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ET, electron transfer; FAD, flavine adenine nucleotide; ^{19}F-NMR, Fluorine nuclear magnetic resonance; HL-ADH, horse liver alcohol dehydrogenase; ^{1}H-NMR, Proton nuclear magnetic resonance; kDa, kilo Dalton; MADH, methylamine dehydrogenase; MDH, methanol dehydrogenase; m-GDH, membrane-bound glucose dehydrogenase; NAD, nicotineamide dinucleotide; NADP, nicotine amide dinucleotide phosphate; NDMA, 4-nitroso-*N*,*N*-dimetylaniline; NMR, nuclear magnetic resonance; PQQ, 2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione; PQQH$^{\cdot}$, semiquinone form of PQQ; $PQQH_2$, quinol form of PQQ; $PQQH_4$, four-electron-reduced PQQ; PsCCP, Pseudomonas cytochrome *c* peroxidase; QH-EDH_I, quinohaemoprotein ethanol dehydrogenase type I; QH-EDH_{II}, quinohaemoprotein ethanol dehydrogenase type II; RR, resonance Raman; s-GDH, soluble glucose dehydrogenase, TPQ, topaquinone; TTQ, tryptophyl-tryptophan quinone; UV/Vis, ultraviolet/visible; 1-D, one-dimensional; 2-D, two dimensional; 3-D, three-dimensional.

Aim of the thesis

The aim of this thesis was to investigate structural and mechanistic aspects of quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni* (QH-EDH). This enzyme was discovered in the past by our group, and the apo-form produced by this organism proven to require PQQ (pyrroloquinoline quinone, 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione) for reconstitution to activity. Studies on the enzyme seemed attractive for several reasons: the general interest and know-how of our research group on quinoproteins (enzymes having a quinone as a cofactor); the possible interplay of the two redox centres in the enzyme (PQQ and haem c), and the fact that PQQ can be replaced by analogues, enabling studies on the intramolecular electron transfer; the enantioselectivity of the enzyme towards certain C_3-alcohol synthons, providing knowledge on the structure of the active site is essential for optimization of this capability.

In initial studies on the apoenzyme, it appeared that part of the final preparation consisted of inactive molecules. Despite many attempts, active and inactive molecules could not be separated from each other. However, reconstitution with PQQ changed the situation because holo-enzyme and nicked, inactive apoenzyme showed different chromatographic behaviour (Chapter 2). Since the results from these studies already provided indications for an interplay between the haem c group and PQQ, QH-EDH (in apo- as well as holo-form) was investigated by several spectroscopic techniques (Chapter 2+3). Although it became clear from this that binding of PQQ induces rotation around the methionyl-haem bond and induces a more compact conformation of the enzyme, distances between the two redox centres could not be calculated from this. In principle, ^{19}F-NMR could make this possible since the distance between a ^{19}F-atom and a paramagnetic centre (Fe^{3+} in the oxidized form of the haem in this case) can be determined. This could indeed be achieved by introducing adducts of PQQ with hydrazines (in which the phenyl ring is substituted with trifluoromethyl groups) into the active site (Chapter 4). Since reconstitution requires the presence of Ca^{2+}, the role of this cation was further explored. It appeared (Chapter 5) that quite a number of other metal ions could replace Ca^{2+}, just as found for several other PQQ-linked dehydrogenases. The conclusions regarding the project and their relevance for the field of quinoprotein enzymology are discussed in the summary.

Quinoproteins

PQQ

The structural elucidation of PQQ (2,7,9-tricarboxy-1*H*-pyrrolo-[2,3-*f*]quinoline-4,5-dione) as the organic cofactor of methanol dehydrogenase (MDH) came in 1979 from two

independent research groups. EPR, ^{1}H-NMR and mass spectroscopy revealed that the cofactor of the enzyme from *Hyphomicrobium X* was not a pteridine, but a quinone structure that contained two nitrogen atoms (1). Subsequently, the structure of a crystalline acetone adduct was found by applying X-ray diffraction of a crystal of this derivative isolated from whole bacteria (2). Final proof of the structure was obtained by the chemical synthesis of PQQ (3).

PQQ is an orthoquinone, a property manifesting itself in three biologically-relevant redox-states: the quinone form PQQ, the semiquinone form PQQH$^{\cdot}$, and the quinol form $PQQH_2$ (Figure 1). One electron reduction of PQQ gives rise to PQQH$^{\cdot}$ formation, whereas two-electron reduction leads to the formation of $PQQH_2$. All three redox states have been proposed to occur in methanol dehydrogenase (4). A four electron reduced form presumed to have no biological relevance, $PQQH_4$, can be produced by the action of sodium borohydride on PQQ (3).

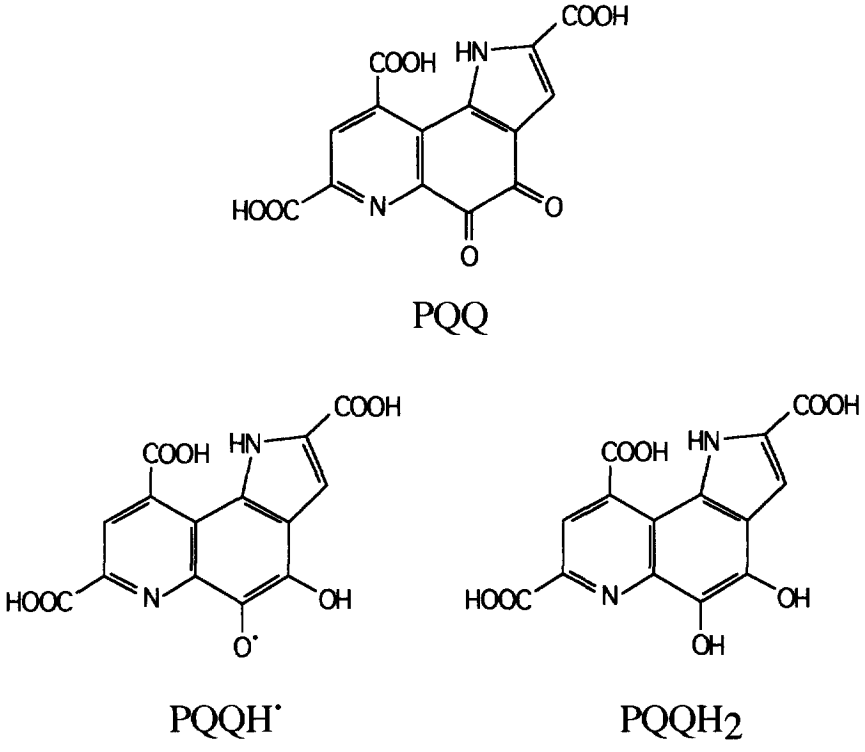


Figure 1. Redox states of PQQ.

Methods to reveal the presence of PQQ in a sample can be subdivided into four categories: 1. Biological assays, using an apoenzyme for which the specificity for PQQ as a cofactor has been established (5,6). 2. Chromatographic techniques combined with monitors which make use of the UV/Vis absorbance (7), fluorescence (8) or redox properties of the compound (9). 3. A chemical assay based on the fact that PQQ acts as a catalyst for the oxidative decarboxylation of certain amino acids. 4. A GC-MS technique which can be applied after PQQ has been transformed into a more volatile derivative (10). Despite this impressive number of available techniques, the presence of PQQ in eukaryotes, especially in mammalian organisms, is still a controversial matter. Large amounts of PQQ have been detected with the chemical assay method (9), but this could not be confirmed with a biological assay or with the GC-MS method (11). Since no PQQ-containing enzyme has yet been isolated from these organisms, it seems safe to regard PQQ as a cofactor restricted to dehydrogenases in Gram-negative bacteria. On the other hand, many reports claim that the application of PQQ to animals or plants can cure or prevent certain diseases (12). Therefore, although it seems that PQQ has no function as a cofactor in these organisms, a physiological role cannot be excluded.

In the past it has been claimed for several enzymes that they contain covalently bound PQQ, (e.g. for amine dehydrogenases and copper-containing amine oxidases). Nowadays it is generally accepted that these enzymes contain the protein-integrated quinone cofactors TTQ (13) and TPQ (14), (Figure 2).

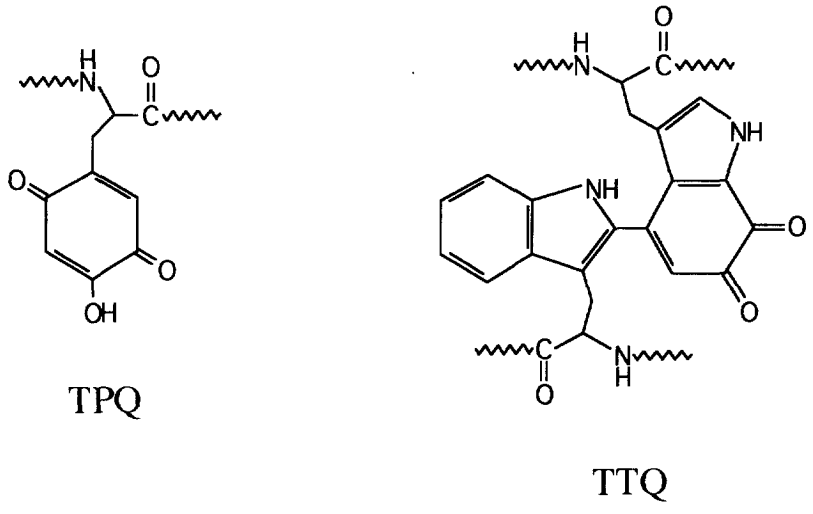


Figure 2. Structures of TPQ and TTQ.

PQQ-containing enzymes

Since the discovery of PQQ a number of bacterial dehydrogenases isolated from Gram-negative bacteria have been found to contain this cofactor. These enzymes, indicated as quinoproteins, are involved in the oxidation of alcohols (6,15-24) (methanol, ethanol, quinate, polyvinyl alcohol, lupanine and polyethylene glycol, the latter one is still speculative), and sugars (25-28) (aldose sugars and fructose).

For a number of these quinoproteins the primary structure has been deduced from the nucleotide sequences of the corresponding gene (29-36). It appears from this that the enzymes are strongly related, except soluble glucose dehydrogenase (s-GDH) (Figure 3). The highest similarity is found for a stretch of amino acids in the C-terminal part of the enzymes, including that for s-GDH. It has been proposed, therefore that this region is involved in binding of PQQ (29,30,37). However, the recently obtained 3-dimensional structure of MDH from *Methylophilus* W3A1 (38), shows that the amino acid residues involved in PQQ binding are scattered all over the protein chain, just as for Ca^{2+} binding (all the PQQ containing enzymes contain or require Ca^{2+} for activity, except membrane bound glucose dehydrogenase (m-GDH) which prefers Mg^{2+}). Since the amino acid residues presumed to be involved in PQQ and Ca^{2+} binding and the glycine and tryptophan residues thought to be responsible for the "propeller" structure in MDH are also present at the same positions in the protein chain of the other quinoproteins (Figure 4), similarity of the 3-D structure is likely too (39). On the other hand, since differences occur with respect to subunit composition and mechanistic properties, deviations will exist. Crystallization experiments with s-GDH (40) and QH-EDH_I (41) have been succesfull so that the answer may become soon available.

The role of the bivalent metal ion, either Ca^{2+} or Mg^{2+}, is still unclear. The diversity in the behaviour of quinoproteins towards treatment with chelating reagents, suggests that the binding is not uniform in this group of enzymes. For instance, removal of Ca^{2+} in m-GDH and ethanol dehydrogenase (EDH) from *Pseudomonas aeruginosa* occurs rather easily, as deduced from the liberation of PQQ from these enzymes upon treatment with EDTA or CDTA (42). On the other hand the calcium cannot be removed with EDTA or CDTA from MDH from *Methylobacterium extorquens* (43) and the quinohaemoprotein alcohol dehydrogenase (QH-EDH_{II}), from *Gluconobacter suboxydans* (44), as judged from activity measurements. The loss of PQQ observed upon calcium removal of some quinoproteins, suggests an important role for the calcium in PQQ binding. In the case of QH-EDH_I, described in this thesis, this contribution seems not essential since PQQ was not detached during preparation of the Ca^{2+}-depleted enzyme (45). A totally different role of Ca^{2+} has been observed for s-GDH, where it induces or is involved in the dimerization of the enzyme (A.J.J. Olsthoorn, personal communication).

Reconstitution experiments with Ca^{2+} (Chapter 5) suggest that two processes are involved: the first step in which the calcium becomes bound to the protein; a second slower step of unknown nature, leading to active enzyme. The specificity observed for QH-EDH_I upon

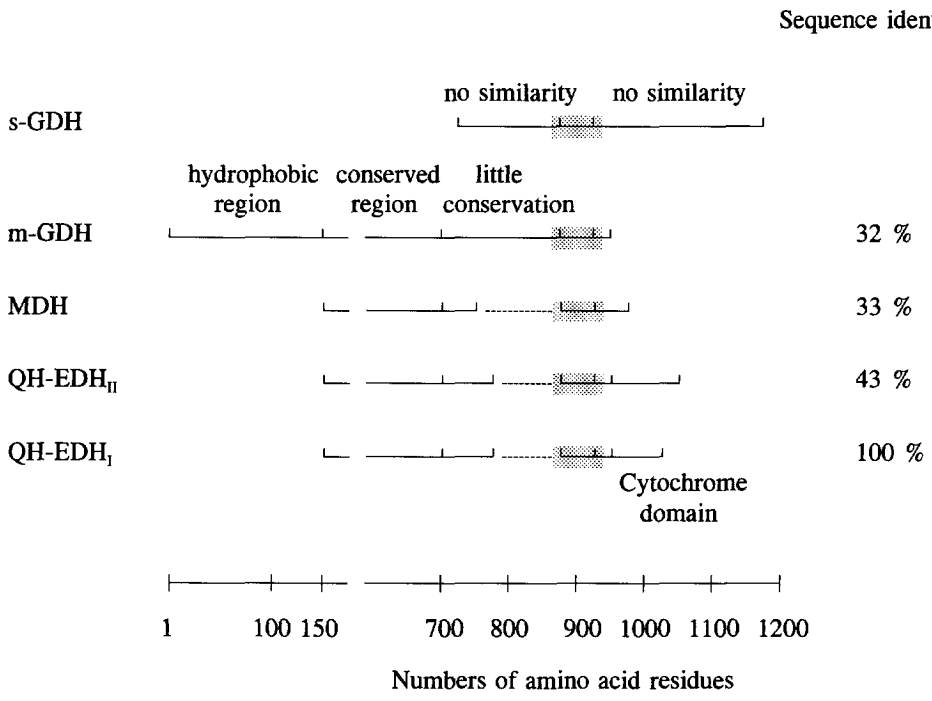


Figure 3. Alignment of the amino acid sequences of PQQ-containing dehydrogenases. ▓ is the part formerly thought to be the PQQ binding site. ------ is a break in the scale. Sequence identity is based on a comparison with QH-EDH$_{I}$. (taken from ref. 37)

substituting other bivalent cations for Ca^{2+} (Chapter 5) appears to be different from those observed for EDH (42), s-GDH (46), m-GDH (47) and QH-EDH$_{II}$ (44), suggesting differences in the topology of the active sites of these enzymes. A comparison with MDH is complicated by the fact that the Ca^{2+} can only be removed under denaturating conditions (48). However, substitution of Ca^{2+} in MDH can be achieved by replacing Ca^{2+} by other metals ions in the growth medium. Growth on these media is observed, suggesting the presence of the metal ions in MDH, which was confirmed indeed by atomic absorbtion spectroscopy (49,50).

Many bacteria are able to produce the protein part of the quinoprotein but not PQQ, as has been found for m-GDH (51), QH-EDH$_{I}$ (6) and quinate dehydrogenase (52). These apoenzymes can be reconstituted with PQQ *in vitro* and *in vivo*. The binding of PQQ leads to a conformational change of the QH-EDH$_{I}$ (53,54). This phenomena has also been observed

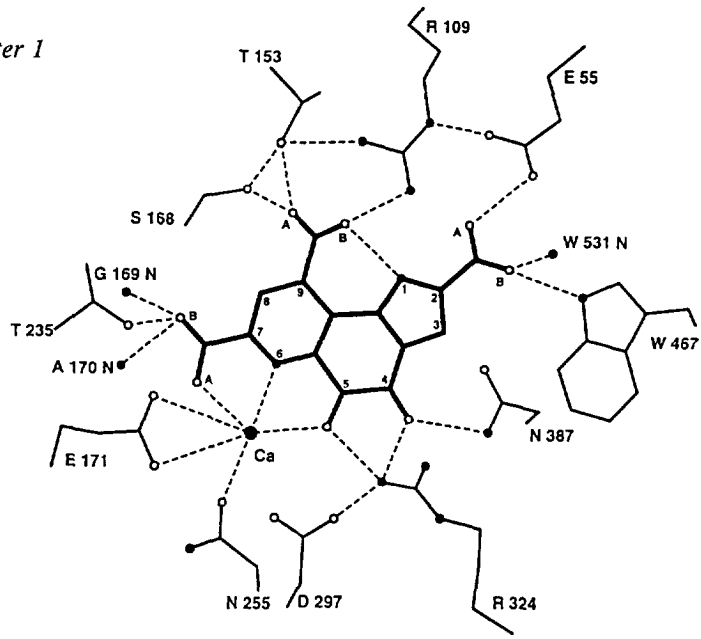


Figure 4. Bonds between PQQ, Ca^{2+}, and amino acid residues in MDH (taken from ref. 38).

for s-GDH and m-GDH (Adachi, paper in press). The conformational change is detected by linewidth changes in NMR experiments (Chapter 3) and slight changes in the g-values of the EPR spectra (Chapter 2). The conformational change allowed the apo-QH-EDH to be separated from holo-QH-EDH with column chromatography. The calcium ion seems to have no influence on this conformational change, as shown by the almost equal properties on high-resolution carboxy-methyl cellulose chromatography and similar NMR spectra of calcium-containing and calcium-depleted holo-QH-EDH (unpublished data). The availability of quinoprotein apoenzymes creates the opportunity to perform reconstitution experiments with PQQ-derivatives and analogues. Reconstitution experiments with substituted PQQ can be used to reveal the factors relevant for enantioselectivity of the enzyme (Chapter 2). Reconstitution with derivatized PQQ was used to introduce a special label, ^{19}F, in the active site (Chapter 4). The experiments performed with PQQ analogues (Chapter 2) showed that substituent groups as small as a methyl group, lowered the activity and enantioselectivity. Substituent groups larger than a methyl group also affect the binding affinity of the analogue enormously. The lack of a complete orthoquinone moiety, however, was not detrimental to holo-enzyme formation, as observed by the 100 % of reconstitution of $PQQH_4$ and PQQ-5OH with the apoenzyme, suggesting that this part of the molecule is essential for activity but not for binding.

Haemoproteins

Cytochromes

Haemoproteins are a class of macromolecules which has three distinct functions: myoglobin and haemoglobin serve as reversible oxygen transport proteins; the cytochromes b and c function as reversible one electron transfer agents; and the cytochromes P450 and peroxidases are involved in irreversible, covalent transformations of substrates. In spite of the diverse functions, all haem proteins have the unifying feature of a common active site composed of an iron-porphyrin complex. It is this "chromophore", which is the centre of all the diverse functions of haem proteins. The term cytochrome *c* is based on a spectral and structural classification, related more to the haem and its mode of attachment to the polypeptide chain than to the protein which surrounds it. Cytochromes *c* are haemproteins, with the haem covalently attached to the polypeptide chain by thioether bridges to two (or in exceptional cases, one) cysteine residues. The structure of haem c is given in Figure 5, with the conventional numbering of the pyrrole rings, showing the feature, unique to cytochromes c, of the thioether bridge. In contrast cytochromes a, b, d, myoglobin and haemoglobin all have a protohaem instead, with the cysteine bridges replaced by vinyl groups, and the haem not covalently attached to the protein. Cytochromes a, d and b differ in the site of attachment of other substituents around the haem ring. Cytochromes all have a characteristic three band absorption spectrum in the reduced state, shown in Figure 6 , with an α absorption band around 550-604 nm in the yellow, a β band around 520-546 nm in the green and a γ or Soret band around 400-450 nm in the far violet.

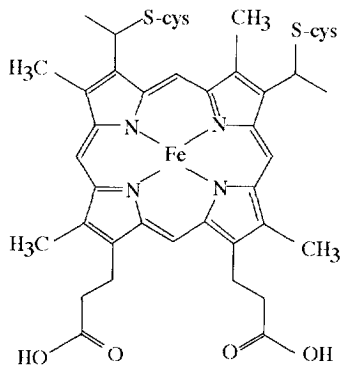


Figure 5. Structure of haem c.

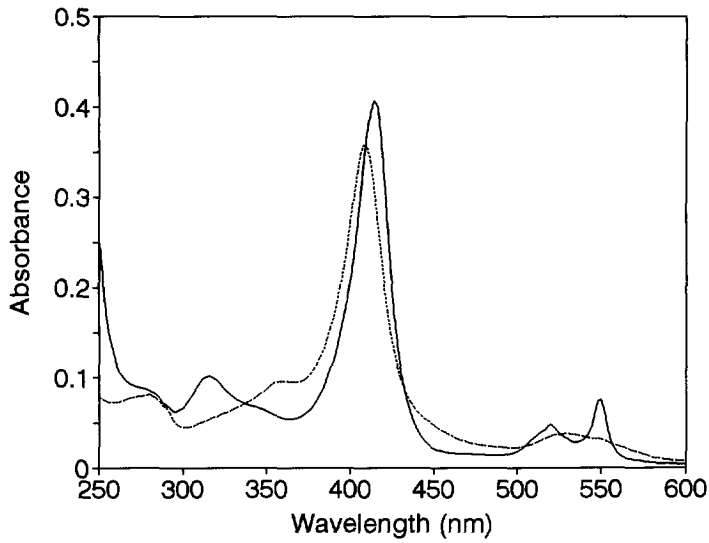


Figure 6. Absorption spectra of oxidized (- - - -) and reduced (———) horse heart cytochrome *c*.

In haemproteins iron exists in two common oxidation states: the ferrous state (Fe(II): $[Ar]3d^64s^0$); and the ferric state (Fe(III): $[Ar]3d^54s^0$. In addition there is the less common ferryl state (Fe(IV): $[Ar]3d^4s^0$), which occurs during the catalytic cycle of some enzymes, such as catalases (55) and peroxidases (56). The tendency of a redox couple to donate or accept electrons is given by the redoxpotential (E) and is measured as a midpoint redox potential with reference to the standard hydrogen electrode (0 V) The value of E exhibited by an individual couple is a reflection of the relative stability of the reduced and the oxidized state. The standard Fe(III)/Fe(II) midpoint redox potential for the metal ions in aqueous solutions is + 0.77 volts (57). The midpoint redox potential (E_m) of free protoheme IX in aqueous solution (pH 7.0) is -115 mV (58), while substituted protohaem is found in cytochromes *c* with E_m values as low as -400 mV and in others with E_m as high as +400 mV (59).The differences in mid-point redox potential can be caused by a change in bonding interactions at the redox-centre, differences in interaction of the redox-centre charge with all other charges and dipoles of the protein and solvent, or redox conformational differences (60).

Only histidine and methionine have been unambiguously identified as ligands in cytochromes *c* though lysine ligation has been suggested to occur at alkaline pH in some cases (61,62) and in a mutated cytochrome c, where the methionine is replaced by a lysine

(63). The haem group of cytochrome *c* is found to be structurally constant and the visible spectrum is influenced only by the extraplanar ligands provided by the protein and also by the surrounding protein. This can be illustrated by the existence of cytochromes *c* having a pentacoordinate haem giving rise to a high spin spectrum (64). Other cytochromes with a hexa-coordinate haem have low spin spectra. A major subdivision of the low-spin haem c proteins is based on the presence of a band at 695 nm in the spectrum of the ferricytochrome (59). The latter is present in those cytochromes with a histidinyl-methionyl-Fe coordination while those with a bishistidinyl coordination lack a band at this wavelength. Rather more subtle spectroscopic differences further distinguish the different low-spin cytochromes c. These are presumably based on variations in the general environment provided by the protein but the detailed structural basis for these minor spectroscopic changes is not known. The c-type cytochromes have been classified into three groups according to their amino acid sequence (65). An additional fourth group was proposed by Pettigrew and Moore (59). This classification is given in Table 1.

Haem c containing enzymes

The main role for cytochromes of the c group appears to be one of transferring electrons and since the electron is not usually considered to be a substrate, these proteins are not enzymes in the strict sense. There is however a class of proteins containing haem c, which are enzymes capable of reacting with and converting substrate molecules to products. Examples of these enzymes are cytochrome *c* peroxidase, nitrite reductase, quinohaemoprotein fructose dehydrogenase, quinohaemoprotein alcohol dehydrogenase and flavohaemoprotein dehydrogenases.

- *Cytochrome c peroxidase*

Cytochrome *c* peroxidases are involved in the removal of H_2O_2 which is formed during incomplete reduction of O_2. The two best-studied cytochrome *c* peroxidases are from *Ps. aeruginosa* and *Saccharomyces cerevisiae*. The cytochrome *c* peroxidase from *Ps. aeruginosa* (PsCCP) is a dihaem-containing monomer, located in the periplasm, capable of reducing hydrogen peroxide to water (66). The two haems from PsCCP have very different properties that make them well suited for their functions: a low potential low spin haem, which carries out the catalysis, and a high potential high spin haem, which feeds the electrons to the active site. There appears to be a complex interaction between the haems, preventing binding of substrate unless the enzyme has the reducing power to form water. Recently a second dihaem containing cytochrome *c* peroxidase has been isolated from *Nitrosomonas europaea* (67). In contrast the cytochrome *c* peroxidases isolated from *Saccharomyces cerevisiae* contain one non-covalently bound protohaem IX in a polypeptide (68). In addition, the cytochrome *c*

Table 1. Classification of c-type cytochromes: major structural divisions (taken from ref. 65).

Class	Features	Subdivision	Features	Examples	Ref.
I	Low spin, histidinyl-methionyl haem coordination, haem near N-terminus, size 80 - 120 amino acids	(a) large size	Loop of residues closes bottom of haem crevice	Mitochondrial cyt. *c*,	224
				cyt. c_2 *T. versutus*	225,226
				cyt. *c*-550	227
		(b) small size	Lacks loop of (a) Left side folds downward to close bottom of haem crevice	*P. aeruginosa* cyt. *c*-551	228
				M. extorquens cyt. c_H	82
II	Haem near C-terminus	(a) High-spin	Histidyl only haem coordination	Cyt. *c*'	105
		(b) Low-spin	Histidinyl-methionyl coordination	Cyt. *c*-556 QH-EDH$_I$	105 Chapter 2 and 3
III	Multi-haem, one haem 30-40 amino acids bis-histidyl coordination	on base of haem content		cyt. c_3 (3 haem)	229
				cyt. c_3 (4 haem)	230, 231
IV	Multi-haem, bis-histidyl and histidinyl-methionyl coordination	on base of haem content		photosynthetic reaction center cytochrome *c*	232

peroxidase from *S. cerevisiae* contains a tryptophan radical as a cofactor. The *S. cerevisiae* enzyme shows a strong preference for basic cytochrome *c* donors, whereas the *Ps. aeruginosa* enzyme reacts with the acidic cytochrome c-551 (69).

- *Nitrite reductase*

nitrite reductase (cytochrome cd_1) is a homodimeric enzyme (70). The subunits each contain a haem c and a haem d_1. The enzyme can accomplish the four electron reduction of oxygen to water in addition to performing its likely physiological role of catalyzing the single electron reduction of nitrite to nitric oxide. The site of nitrite binding is probably the haem d_1.

- *Quinohaemoprotein fructose dehydrogenase*

A quinohaemoprotein fructose dehydrogenase was first purified to homogeneity from *Gluconobacter* spp (71). It was found to consist of three subunits, one of which was reported to contain haem c. The enzyme has been isolated as a holoenzyme containing the cofactor PQQ. Kinetic studies, clearly indicated the involvement of both redoxcentra, PQQ and haem c, in catalysis (72). Recently the enzyme has been isolated without haem c, which raises doubts about the original preparations (73).

- *Flavohaemoprotein dehydrogenases*

Flavohaemoprotein dehydrogenases are enzymes containing a flavin- as well as a haem c-containing subunit. The most investigated enzyme of this group is p-cresol methylhydroxylase that has been isolated from a number of *Pseudomonads* (74). It converts p-cresol into p-hydroxybenzyl alcohol by succesive dehydrogenation and hydration reactions and can also oxidize the product to p-hydroxybenzaldehyde by subsequent dehydrogenation. The enzyme exists in a tetrameric form with two identical flavoprotein subunits and two identical c-type cytochrome subunits (75). The flavoprotein subunit contains a covalently bound flavin prosthetic group (an 8--O-tyrosyl-FAD) and the c-type cytochrome subunit is much smaller. Although strongly bound, surviving manipulations during purification, the subunits can be reversibly separated by isoelectric focusing (76). During catalysis, hydroxylation of p-cresol occurs by the abstraction of two hydrogens by the flavoprotein moiety after which the putative p-quinone methide intermediate is believed to be attacked by water to yield p-hydroxybenzylalcohol. The two electrons are then transferred one at the time to the haem on the cytochrome subunit and then to an accepter protein *in vivo* (77). The acceptor protein is believed to be azurin (78), which is supported by the high rates of electron transfer between these proteins (A.C.F. Gorren, personal communication). A flavocytochrome *c*, preferring 4-ethylphenol as substrate, has also been characterized (79). Different kinetic mechanism have been found for this enzyme dependent on the type of electron acceptor used. Phenazine methosulphate reacts with the flavin moiety whereas ferricyanide reacts with the haem c (A.C.F. Gorren, personal communication).

Classification of the above described enzymes into the 4 classes of cytochromes *c* in Table 1, is predominantly based on the amino acid sequence of the proteins. However, not all cytochrome c-containing enzymes have been sequenced. The flavocytochrome c, p-cresol methylhydroxylase, from *P. putida* contains a cytochrome *c* subunit of 96 amino acids that

shows some sequence similarity to class I c-type cytochromes (80). The QH-EDH falls into the class IIb (haem c near C-terminus, low spin, His-Met coordination). However, it should be realized that the size of the QH-EDH does not coincide with the examples of this class. The amino acid sequence of QH-EDH derived from the recently determined DNA sequence was compared with amino acid sequences of several cytochromes in a protein sequence data bank (81). No large similarities were detected, the highest homology being about 26%, compared to a cytochrome c_{553} form the algae *Plectonema boryanum*.
Because of the similarity in the amino acid sequence between MDH and the quinoprotein part of QH-EDH, it's tempting to speculate on the origin of the cytochrome *c* part of QH-EDH. When QH-EDH is considered to be a gen-fusion product of the quinoprotein part and its natural electron acceptor a cytochrome *c*, one would expect a similarity between the cytochrome *c* part of $QH\text{-}EDH_I$ and the natural electron accepter of MDH, cytochrome c_L. However, the amino acid sequence of cytochrome c_L does not show homology with cytochromes in any database, and constitutes a novel class of c-type cytochromes (82).
For the cytochrome c part $QH\text{-}EDH_{II}$, the biggest similarity, though very low, was found for a small Class I cytochrome *c* (cytochrome c_{555}) from the methanotroph *Methylococcus capsulatus* (83). The function of this cytochrome *c* is unknown. Comparison between the amino acid sequences of $QH\text{-}EDH_{II}$ and cytochrome c_L show an even smaller homology, but the methionine residue used as the sixth ligand is at the same position and there is a region close to this methionine residue of four identical amino acids (83).

Active site topology

The activity of an enzyme depends predominantly on the structure of the active site. Whenever a cofactor is involved in catalyses, the position of the cofactor in relation to the protein chain is very important. Knowledge of this position together with the identity of the cofactor provides information on the mechanism of the catalyzed reaction. In several enzymes, however, more than one cofactor is involved in catalyses. In these cases the question raises what the positions of these cofactors are and how the cofactors interact with each other. To obtain information on this, studies on the structure of the active site of proteins have to be carried out. NMR, EPR, and X-ray crystallography are commonly used for this.

X-ray crystallography

X-ray crystallography is able to provide the 3-dimensional structures of crystallized proteins. Studies have been performed on several quinoproteins: MDH (38,84), methylamine dehydrogenase (85) and s-GDH (40). It can also be used to reveal interaction between proteins as has been shown for the ternary protein complex of methylamine dehydrogenases, amicyanin and cytochrome c_{551i} (86). A partial structure of QH-EDH$_I$ was obtained by a method using molecular replacement (41). For these calculations the data from the X-ray structure of MDH were used. This method worked well for the PQQ-binding domain of the QH-EDH. However, because the haem c domain is lacking in MDH, only a provisional positioning of it could be made. The X-ray structure of the quinoproteins MDH and MADH clearly shows the $\alpha_2\beta_2$ structure. The larger α subunit has a structure of a disc with 7 (MADH) or 8 (MDH) repeated topologically identical units. Each of these units is composed of four antiparallel β strands (W-shaped). Because of the similarity of the structure of MDH to the blades of a propeller, it is called 'propeller' structure (87). A very interesting aspect seen in the crystal structure is the disulphide bridge of two adjacent cysteines. Reduction of this disulphide bridge blocks the electron transfer to cytochrome *c*. It has been suggested that the disulphide bridge might function in the stabilization or protection of the PQQ semiquinone from solvent at the entrance of the active site (88). The same adjacent cysteines are conserved in QH-EDH$_{II}$ (61) and QH-EDH$_I$ (64). A structure of QH-EDH$_{II}$ has been modelled on the base of the X-ray structure of methanol dehydrogenase (89).

Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) spectroscopy makes use of the fact that atomic nuclei oriented by a strong magnetic field absorb radiation at characteristic frequencies. The usefulness of NMR results largely from the fact that nuclei of the same element in different environments give rise to distinct spectral lines. This makes it possible to observe signals from individual atoms even from those present in complex biological macromolecules in solution. NMR spectroscopy has developed into a powerful method to study structures both of metal environments in proteins and of proteins in general. Nowadays one- (1D), two- (2D) and three-dimensional (3D) NMR spectroscopy techniques are indispensable tools in the study of structure and function of macromolecules (90). In recent years several 3-dimensional structures of small proteins have been elucidated with the aid of NMR using ^1H and ^{13}C-NMR on labelled proteins (91-93). However, its feasibility is restricted to proteins smaller than 40 kDa. Because of their too large size is, it is momentarily impossible to obtain the tertiary structure of quinoproteins with NMR. However, NMR spectroscopy can be used to investigate protein residues in close vicinity with metal sites in proteins with molecular

weights much larger than 40 KDa, whenever a paramagnetic metal ion is an integral part of the protein or the naturally occurring diamagnetic metal ions can be replaced by a paramagnetic metal ion (94). The presence of a paramagnetic centre in proteins causes complications on measuring 1D- and 2D-NMR (95). Chemical shift criteria can not always be used to assist assignment, and the quality of the spectra is in some cases diminished by paramagnetic broadening. Despite the problems, additional structure information can be obtained from resonance line widths and paramagnetic chemical shifts caused by the interaction between the resonating nuclei and the unpaired electron residing on the metal ion (90). The paramagnetic shift is defined as the difference between the actual chemical shift and the shift of an "analogous" diamagnetic compound (96). These paramagnetic shifts may be dipolar (pseudocontact) or scalar (Fermi contact) in nature. The Fermi contact describes the way in which spin density may be delocalized through covalent bonds into orbitals of a particular nucleus giving rise to an interaction between electronic spin and nucleus. In haem groups spin density of the paramagnetic Fe(III) ion is delocalized through π bonds. It is commonly assumed that Fermi contact shifts are limited to protons on the haem and its immediate ligands (97). The contact shift is independent of the magnetic field. The protons not directly associated with the haem, sense only the dipolar contact, which arises from a through space dipolar interaction between the nucleus and the unpaired electron. In contrast to the Fermi contact interaction, the dipole-dipole interaction is anisotropic, i.e. its magnitude and sign depend on the orientation of the vector connecting nucleus and metal with respect to the magnetic field. For nuclei that are more than a few bond lengths away from the paramagnetic center, their interaction with the unpaired electron is purely dipolar and depends on geometrical positions, thus providing important structural information (98). In the case of haem proteins much information already exists on the isotropic shifts of the protons on the porphyrin ring itself and of protons belonging to amino acids in the close vicinity of the iron (97, 99-101). In the reduced form, several resonances such as those of the haem meso protons or protons of an axial methionine ligand are shifted outside the main protein envelope due to the large ring currents of the porphyrin ring (102). In the oxidized, paramagnetic form, the signals of the protons close to the metal ion are shifted even further outside the region of the generally poorly resolved diamagnetic polypeptide envelope by Fermi contact and/or dipolar interactions. Because individual resonances can be assigned, it is possible to obtain information by the use of these resonances as markers for ligand binding (103, 104), spin state (105) and redox-linked spin-state changes (106,107). An interesting example of the use of NMR in analysis of the structure of a haem protein concerns p-cresol methylhydroxylase (108). Significant differences are observed with 1-D NMR between the spectra of the oxidized enzyme and the separated oxidized cytochrome *c* part of the enzyme. The observed shifts of the ^{1}H-NMR resonances were interpreted as a reorientation of the methionine ligand, resulting from a rotation. This rotation takes place when the cytochrome *c* part binds to the flavin unit. The same interpretation has also been used to explain the shifts in the ^{1}H-NMR spectrum of QH-EDH$_I$ upon PQQ-binding (Chapter 3). The resonances of

the protons belonging to the methyl groups of the porphyrin group, which are well resolved in the oxidized haem, undergo large shifts when PQQ is bound to the apoenzyme.
Although ^{1}H-NMR is dominating, NMR spectroscopy of other nuclei is possible nowadays. One of these is ^{19}F-NMR, the applicability based on the fact that ^{19}F is present in 100 % natural abundance and has a receptivity to NMR detection that is second only to that of the proton (109). Incorporation of fluorinated amino acids into proteins provides a valuable tool for the study of structural changes with ^{19}F-NMR in these molecules that result from ligand binding or interaction with other macromolecules (110-113). ^{19}F-NMR has been shown to be a practical help in the distance measurements between copper and topaquinone in pig plasma amine oxidase (114). In these experiments, the relaxation effects of the paramagnetic Cu(II) on ^{19}F of the fluorine-labelled-topaquinone cofactor hydrazone affect are measured. The same approach has been applied to determine the distance between the haem c and PQQ in QH-EDH. Derivatization of PQQ in the enzyme with fluorine substituted hydrazines did not succeed. However, reconstitution of the apoenzyme with chemically synthesized fluorine labelled PQQ-phenylhydrazine adducts appeared to be possible (Chapter 4). The multiple peaks observed in the spectrum are probably caused by a hindered rotation of the fluorine labelled trifluoromethyl groups, creating different environments for the fluorine atoms, leading to more than one peak. The most prominent peaks have been used for distance determinations, revealing that the o-quinone moiety of PQQ is at least 10 Å away from the haem iron (Chapter 4).

EPR

Electron paramagnetic resonance (EPR) and electron spin resonance are synonymous terms for describing the resonant absorption of microwave radiation by a paramagnetic substance in a static magnetic field. Paramagnetic metal ions can function in enzymatic catalysis and/or electron transfer. However, the paramagnetic species may also be an organic free radical cofactor. EPR is a powerful method for characterizing the structure of the paramagnetic cofactor in enzymes, enzyme-substrate intermediates, and protein complexes (115). Sometimes when diamagnetic metal ions are the natural metal ions in enzymes it is possible to obtain structural information by replacing this metal ion by a paramagnetic one (116). This approach has been used with QH-EDH$_1$ by replacing Ca^{2+} with Co^{2+} and the vanadyl cation VO^{2+} (Chapter 5). In the cases where an enzyme contains more than one paramagnetic centre, magnetic coupling between the paramagnetic centres can occur, with which it is sometimes possible to perform a distance determination (117-119).

EPR has also been applied in the study of haemoproteins. The paramagnetic ferric state (Fe(III): $[Ar]3d^5 4s^0$) contains an unpaired electron so that it can be detected by means of EPR. Also ligands provided by the protein, can be studied, revealing information on spin-

state, and ligand binding (120). EPR spectra were recorded of the apo and holo-QH-EDH (Chapter 2) clearly showing that the haem was low spin in both enzyme forms. From the differences in g-values and linewidths between the apo- and holoenzyme, the effect of the presence of PQQ on the properties of the haem c could be assessed.

The semiquinone form of methanol dehydrogenase has been studied with EPR and ENDOR (1,121). The g-value and top-to-top width of the radical signal was in agreement with the signal found in partially oxidized QH-EDH (Chapter 2). In addition EPR spectra were recorded of the free radical after release from methanol dehydrogenase and a simulation of the spectra were performed (1).

Resonance Raman

Raman spectroscopy is a form of vibrational spectroscopy in which a sample is interrogated with an intense light beam and the spectrum of Raman active vibrational modes is obtained through analysis of the inelastically scattered photons. Resonance Raman (RR) spectroscopy is a specialized form of Raman spectroscopy in which the wavelength of the interrogating light beam lies under an electronic absorption band of the sample. This produces a resonance enhancement in the scattering intensity of only those bands that are coupled to the exited electronic transition. This resonance effect minimizes, the interference from bands due to nonabsorbing portions of the sample, allowing one to zoom in on and selectively probe chromophoric sites. The metal centers of metalloproteins are quite often chromophoric, having electronic transitions that can be selectively excited with near-ultraviolet or visible radiation. Moreover, many of these centers are located at the active site responsible for the function of the metalloprotein. Because the frequencies and the intensities of resonance Raman bands are sensitive to the nature of the chemical bond and the molecular configuration of atoms participating in the vibrations, the resonance Raman technique can be used to monitor structural changes of a chromophore that are induced or modulated by its environment. Thus resonance Raman spectroscopy can provide important structural and mechanistic information about chromophoric proteins.

Resonance Raman has been used to prove the occurrence of TPQ in amine oxidases (122) and of TTQ in MADHs from three different bacterial sources (123). The set of conjugated double bonds in quinones leads to strong UV and visible absorption bands making quinone species excellent subjects for RR spectroscopy. Derivatization of quinones with phenyl hydrazines generates even stronger absorption bands (13,122,124). A disadvantage of derivatization with hydrazines is that the natural cofactor is difficult to distinguish in the RR spectra. Derivatization of PQQ with hydrazines have also shown to lead to stronger absorbtion in the visible range (Chapter 4), however no RR spectra of quinoproteins containing these derivatives have been recorded.

Haemoproteins have been extensively studied by RR (125,126). Since only vibrations associated with the selected electronic transition are enhanced (*ihe* α, β and Soret bands are normally used for excitation) the haem ring vibrations are observed in the spectrum. The frequencies of these and other vibrational modes of the haem group are sensitive to the electronic structure, ligation- and redox state of the central metal ion (127). The coordination number, spin, and oxidation state of the iron atom affect the core size of the porphyrin, which is defined by the distance between nonadjacent pyrrole nitrogen atoms (128,129). Through extensive studies of haem proteins and model compounds, marker bands of the porphyrin core size and of electron density in the porphyrin π and π^* orbitals have been discovered among high frequency (1350 - 1650 cm^{-1}) skeletal modes. These are very useful in establishing oxidation, spin, and ligation states of the haem (125). The Resonance Raman spectra of QH-EDH show that the haem is hexa-coordinated and low-spin (Chapter 3). Only small differences were observed between the spectra of the apo- and holo-QH-EDH, which indicates that the changes observed with ^{1}H-NMR and EPR of the haem upon PQQ binding (Chapter 2 & 3) are not caused by a change in the ring-current of the haem c.

Ultraviolet/visible spectroscopy

Molecules can exist in a variety of excited states and ultraviolet/visible spectroscopy monitors the transitions between ground- and exited states. The ultraviolet/visible absorption spectrum of cytochromes can reveal information on the nature of ligands (55,59,130,131), changes in ligand binding (132,133), the spin-state (51,55,131), redox-state of the iron, redox-linked spin-state changes (106,107), and the type of cytochrome (59). The UV/Vis spectra of apo-QH-EDH is similar to that of oxidized horse heart cytochrome *c* with an absorption band at 695 nm, indicating the presence of a methionine ligand (Chapter 2). A comparison of the spectra of apo- and holo-QH-EDH in the same redox state, revealed shifts in absorption maxima and an increase of absorbance intensity of the haem absorbance bands due to the presence of PQQ (Chapter 3).

UV/Vis spectroscopy can also be used to establish the redox state of the quinone cofactor in quinoproteins (26,134). However in the case of holo-QH-EDH this is complicated by the dominant haem absorbance in the region of PQQ absorbance (Chapter 2 and 3). To eliminate the contribution of the haem absorbance, a difference spectrum of the holo- minus the apoenzyme in the same oxidation state was taken. However, the result is complicated by the effects on haem absorbance caused by the binding of PQQ. The redox state of PQQ in the oxidized as well as the reduced QH-EDH is based on the relative molar absorbtion coefficient and the maxima of absorbance (Chapter 3). In addition to the analysis of the quinone cofactor, UV/vis spectroscopy can be used to study the derivatization of the quinonoid cofactor with nucleophilic reagents (135-138). Derivatization of PQQ in QH-EDH with phenylhydrazines was not succesfull, based on the fact that no changes were observed in the

UV/vis spectrum. Binding of different PQQ-derivatives to QH-EDH could be followed by changes in the UV/vis spectrum, which correlated more or less with the absorbance of the derivative. The UV/vis spectrum after chromatography of the reconstituted QH-EDHs could showed the presence of the derivative (Chapter 2 and 4).

UV/Vis spectroscopy has also been used to detect the changes in absorbance which are related to removal of calcium from holo-QH-EDH. This phenomena is ascribed to a loss of PQQ-Ca complexation. Reconstitution of calcium-depleted holo-QH-EDH with calcium showed the restoration of UV/Vis absorbance change. Reconstitution of calcium-depleted holo-QH-EDH with other divalent metal ions showed changes in the UV/Vis absorbance strongly related to that caused by Ca, however with differences in intensity increasements (Chapter 5).

Intramolecular electron transfer

Redox proteins contain one or more redox-active cofactors, which may be metal ions and/or an organic compound. Two types of electron transfer can be distinguished, intra- and intermolecular electron transfer. Intermolecular electron transfer occurs between one or more different proteins. Since the cofactors often are located in the protein interior, it is likely that intermolecular electron transfer (ET) in protein-protein complexes will take place over large molecular distances ($\geq$ 10 Å) (139,140). Although the electron donors and acceptors are expected to be weakly coupled, the ETs are remarkable fast and proceed with high specificity (141). Intramolecular electron transfer takes place in one single protein molecule, from one redox centre to another. This type of electron transfer has been investigated in detail by several research groups in recent years in order to understand the factors that control the rate of these non-adiabatic reactions.

It is known from semiclassical ET theory (142) that the rate for a nonadiabatic ET reaction depends on a nuclear factor, which in turn depends on the driving force of the reaction ($-\Delta G^0$) and the extent of nuclear reorganization (λ) accompanying the electron transfer, and an electronic factor, which depends on the distance and the medium separating the electron donor and the acceptor. The rate constant is dependent on these two factors. The distance dependence of the donor-acceptor interaction is clearly influenced by the electronic structure of the pathway. In proteins direct covalent pathways between redox centres may not exist or may be prohibitively long. Because bond mediated interactions are expected to dominate the direct "through space" interaction so severely in long distance charge transfer reactions, the proper picture for electron transfer in proteins must include a combination of covalent (through bond) and short distance through space interactions (143). The donor-acceptor separation distance, the relative orientation of donor and acceptor, and the nature of the intervening medium all affect the overlap of acceptor and donor orbitals, controlling

the magnitude of the electron tunnelling matrix element, and thus the rate of electron transfer. Simple square barrier tunnelling models yield an exponential decay of H_{DA}, the coupling of the interaction between the orbitals of the donor and the acceptor, with the distance separating the donor and the acceptor, when a homogeneous medium operates the two redox centres (1414). For more complex bridging systems, the electronic structure of the bridge influences coupling between the donor and acceptor.

Recently there has been keen interest in the mechanism of electron transfer in native and modified proteins. This interest has intensified because of new methods for attaching redox labels to proteins and site directed mutagenesis techniques for preparing electron transfer proteins with tailored tunnelling bridges (145).

The flavocytochrome b_2 (L-lactate: cytochrome *c* oxidoreductase) is an enzyme which has interesting properties for the study of intramolecular electron transfer. The crystal structure of the flavocytochrome shows an N-terminal domain containing protohaem IX and a C-terminal domain containing flavin mononucleotide. The two domains are connected by a single segment of polypeptide chain which constitutes the interdomain hinge (146). The hinge is crucial in mediating electron transfer between the flavin- and haem containing domain. The most likely role of the hinge is to confer domain mobility, allowing movement of the cytochrome domain with respect to the flavin domain, and thereby providing an optimal position of the amino acids in both subunits, involved in the electron transfer. Electron transfer was compared between the wild-type enzyme and a mutant enzyme, which lacks six amino acids in the interdomain hinge. The electron transfer in the mutant enzyme is some 300 fold lower than in the wild type enzyme. This large decrease in electron transfer is ascribed to the restricted binding of the interdomain hinge in the mutant enzyme, which does not allow a correct position for the two subunits (147). Regarding the fact that the flavocytochrome b_2 has two subunits, with one haem accepting the electrons produced in the reaction, a somewhat similar mechanism might be expected for QH-EDH_1.

Much less is known about electron transfer in QH-EDH. Kinetic evidence obtained so far suggests that substrate oxidation occurs at the PQQ site (148). The immediate reduction of the haem c after addition of substrate suggests that electrons are transferred from PQQ to the haem c. In view of this mechanism, the haem c might be responsible for transferring electrons to an as yet unknown physiological electron acceptor (probably the copper protein azurin, Kraayveld, Geerlof, Duine, unpublished results). The homology in amino acid sequence between QH-EDH and MDH which lacks a haem c, suggests a similar structure for the domain containing the PQQ and a separate domain containing the cytochrome *c* part, probably in analogy with flavocytochrome b_2. Preliminary investigations (unpublished data) using stopped flow kinetics showed the rapid reduction of the haem c upon reconstitution of the apoenzyme with PQQ in the presence of substrate.

Alcohol oxidoreductases

Redox conversions are important in metabolism and energy conservation of living cells. The enzymes catalyzing these steps require a cofactor which can be either a flavin, a nicotinamide, a pterin, a quinone cofactor, a haem or non-haem metal centre. The cofactors differ in their redox potential, affinity for the protein and the way in which the reduced form is reoxidized. Connected to the topic of this thesis, an overview will be given of the enzymes catalyzing oxidation of alcohols, the alcohol oxidoreductases, with special attention to the microbial ones. These enzymes can be subdivided into four groups: nicotinamide-nucleotide (NAD(P)-dependent alcohol dehydrogenases (ADHs); alcohol oxidases; quinoprotein ADHs and nicotinoprotein ADHs, which contain a NAD(P) as bound cofactor. Apart from the diversity in structures and cofactor identity, there is an important difference in the location of these enzymes. NAD(P)-dependent dehydrogenases are located in the cytoplasm but flavoprotein alcohol oxidases in the peroxisomes. Quinoprotein ADHs either occur in "soluble form" or in membrane-integrated form, corresponding with a location in the periplasmic space or on the outer surface of the cytoplasmic membrane.

Nicotinamide-nucleotide (NAD(P))-dependent ADHs

NAD and NADP are coenzymes used by alcohol dehydrogenases. In contrast to nicotinoprotein ADHs where NAD and NADP are bound cofactors, in NAD(P)-dependent ADHs they dissociate from the enzyme after substrate conversion. The formed NAD(P)H is reoxidized by the respiratory chain, monooxygenases or enzymes involved in reductive biosynthetic routes. The redox potential of the couple NAD^+/NADH (-320 mV) is very low compared to that of PQQ (+90 mV).

NAD(P)-dependent alcohol dehydrogenases have been found in many microorganisms, plant and animal tissues (149) They can be subdivided into at least four different types with respect to the primary protein structure (150-152). The first group consists of the zinc-containing medium-chain alcohol dehydrogenases, initially termed the long chain family, but now, after discovery of a still longer alcohol dehydrogenase (153) better termed medium chain family. The enzymes contain two or four subunits of 350-375 amino acid residues. They oxidize a wide range of aliphatic and aromatic alcohols to their corresponding aldehydes and ketones. In mammalian tissues at least six isoenzyme forms of this type of enzyme occur (154). The two most studied enzymes are those from horse liver and baker's yeast (155-157). The second group consists of short chain alcohol dehydrogenases without a metal ion. A typical example of this group is the *Drosophila melanogaster* alcohol dehydrogenase (158). This dimeric enzyme has a preference for secondary alcohols such as 2-propanol (159). The third group consists of the iron-dependent or "iron activated" alcohol

dehydrogenases (160,161). The "iron activated" alcohol dehydrogenases are not as their name suggests, all iron activated, but rather they may be activated by a range of divalent metal ions (162). The presence of iron has been unambiguously assessed only in an alcohol dehydrogenase from *Zymomonas mobilis* (160,161). The alcohol dehydrogenase from *Bacillus methanolica* binds one zinc and one to two magnesium ions per subunit but no iron (163). Whether the metal ions assist catalysis or have a structural function is still to be determined. Each enzyme within a group shares 35 to 54 % of amino acid sequence identity with any of the other enzymes in this group, but no significant similarity with enzymes from the other group. Three nicotinoprotein alcohol dehydrogenases, those from *Amycolatopsis methanolica, Mycobacterium gastri* and *Bacillus methanolica,* have been partially sequenced and shown to have a high degree of similarity with the "iron activated" alcohol dehydrogenases (section: Nicotinoprotein ADHs). This suggests that there may be a nicotinoprotein sub-group of the "iron activated" alcohol dehydrogenases. The last group of the NAD-dependent alcohol dehydrogenases is much less known and consists of alcohol dehydrogenases longer than the classical type (153).

Nicotinoprotein ADHs

Nicotinoprotein alcohol dehydrogenase contain NAD(P) as bound cofactor, which make the assay conditions different from that for the NAD(P)-dependent ADHs. Four types have been found until now, with large differences in structural as well as catalytic properties (164). The first enzyme which was discovered in this group is called formaldehyde dismutase, because it converts two molecules of formaldehyde in to methanol and formic acid. This enzyme is tetrameric with subunits of M_r 55 kDa (165). It was isolated from *Pseudomonas putida* F61 grown on formaldehyde, functioning as a formaldehyde detoxifying enzyme. The synthesis of the enzyme is induced at growth on formaldehyde. The enzyme oxidizes primary alcohols, except methanol, and some secondary alcohols *in vitro*, using 4-nitroso-N,N-dimethylaniline alcohol dehydrogenase (NDMA) as an electronacceptor.

Subsequently a nicotinoprotein alcohol dehydrogenase was found in thermotolerant *Bacilli* (166,167). This enzyme oxidizes short chain alcohols including methanol, but needs Mg^{2+} and requires an activator protein when it is assayed with NAD (168). It is a decameric enzyme with subunits of 43 kDa.

The third nicotinoprotein alcohol dehydrogenase was isolated from the Gram-positive bacteria *Amycolatopsis methanolica* and *Mycobacterium gastri* (169). They catalyze the oxidation of methanol with the concomitant reduction of the artificial electron acceptor NDMA and dismutate formaldehyde to methanol and formate. The enzymes are decameric proteins with subunit molecular masses of 49-50 kDa. Both enzymes contain Zn^{2+} and Mg^{2+}. The two nicotinoprotein alcohol dehydrogenases from *Amycolatopsis methanolica* and *Mycobacterium gastri* have been partially sequenced and showed a high degree of identity (63

% identity in the first 27 N-terminal amino acid residues). Alignment of these both enzymes with the alcohol dehydrogenase from *Bacillus methanolica* (170) revealed considerable similarity indicating the close relationship of both enzymes to the "iron dependent" alcohol dehydrogenases.

The fourth nicotinoprotein is 4-nitroso-N,N-dimethylaniline-dependent alcohol dehydrogenase (NDMA-ADH) also from *Amycolatopsis methanolica*, a trimeric enzyme consisting of subunits of 39 kDa and one firmly bound NAD as cofactor. The enzyme oxidizes alcohols, except methanol only in the presence of NDMA, which regenerates the internally bound NADH through oxidation (164). The enzyme has 56 % identity with the N-terminus of horse liver alcohol dehydrogenase (HLADH), which could indicate that it is a member of the group I ADHs, although it has a trimeric composition and belongs to the nicotinoproteins.

Alcohol oxidases

Alcohol oxidases are enzymes capable of oxidizing alcohols to the corresponding aldehyde with the formation of H_2O_2. The largest group consists of flavoprotein oxidases, enzymes containing a flavine adenine dinucleotide (FAD) as a tightly bound cofactor. In fungi a flavoprotein methanol oxidase without any other cofactor is responsible for the oxidation of methanol (171), but the enzyme does not occur in prokaryotes or higher eukaryotes (172). Methanol oxidase is present in methylotrophic yeasts like *Candida boidinii*, *Hansenula polymorpha* and *Pichia pastoris* (173) and molds like *Poria contigua* (174). They are located in the peroxisomes, membrane-enclosed organelles containing a matrix of proteins, including catalase (175). The enzyme consists of 8 identical subunits, each containing non-covalently bound FAD. Besides methanol, the methanol oxidase oxidizes other primary alcohols, however with a lower specific activity. Recent structural analysis of the coenzyme revealed the occurrence of two different forms of the flavine adenine dinucleotide (176). Besides the majority of flavoprotein oxidases able to convert methanol, other oxidases have been found to convert less familiar substrates: vanililyl-alcohol oxidase from *Pencillium simplicissium* (177) and veratryl oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju* (178). An aromatic alcohol oxidase found in the slug *Arion ater* is a peculiar example of an flavoprotein alcohol oxidase found in a higher eukaryote (179). However, the enzyme could possibly originate from yeasts inside the digestive system of the slug.

All flavoproteins capable of oxidizing primary alcohols seem to be oxidases, but since the DNA nucleotide sequence of a membrane bound alcohol dehydrogenase showed significant homology with four flavin proteins, this suggests that it is a flavoprotein too (180).

Oxidases have been found in *Pseudomonas* species which are able to oxidize secondary alcohols with carbon number greater then five and which seem to contain only iron as a

cofactor (181-183).

Quinoprotein alcohol dehydrogenases

Several different types of PQQ-dependent alcohol dehydrogenases have been characterized in oxidative bacteria such as pseudomonads and acetic acid bacteria. The enzymes catalyze the oxidation of a wide variety of alcohols and mostly also aldehydes. The quinoprotein alcohol dehydrogenases can be subdivided into two classes according to the presence or absence of a covalently bound haem. The quinoprotein alcohol dehydrogenases containing a haem c are called quinohaemoprotein alcohol dehydrogenases and can be subdivided on the base of their structure in type I: the monomeric soluble enzyme form and type II: the multi subunit, membrane-bound form.

Quinoprotein alcohol dehydrogenases

Quinoprotein alcohol dehydrogenases (QADHs) have been isolated from several Gram-negative bacteria (16,17,184-186). A great number of primary alcohols and aldehydes are oxidized with these enzymes. MDH, the first known PQQ-containing enzyme, differs from quinoprotein ethanol dehydrogenase (QEDH) because the K_m value for methanol of the QEDHs is 1000 times higher than that for ethanol. With respect to catalytic properties and subunit structure the QEDHs resemble MDH. QADHs have a $\alpha_2\beta_2$ structure (187), a high optimum pH of 9.0-9.5 use an artificial electron acceptor like phenazine methosulphate or Wurster's Blue whereas ammonia or a primary amine is required as activator. The natural electron acceptor for MDH is cytochrome c_L, for QEDH it is cytochrome c_{EDH}. Maximal activity of MDH and QEDH with their natural electron acceptors is obtained at pH 7, a condition in which the activators do not function.

Quinohaemoprotein alcohol dehydrogenases type I

A quinohaemoprotein alcohol dehydrogenases (QH-EDH) of type I was first isolated as apoenzyme (lacking the cofactor PQQ) from *Comamonas testosteroni* (6). Since then only two other examples of this type have been described: a holoenzyme from *P. putida* (186) and from *Rhodopseudomonas acidophila* (188). The latter enzyme is a monomer of 72 kDa and contains one haem c per enzyme molecule (186). However, doubts regarding the presence of a haem in this enzyme still exist. The QH-EDH from *C. testosteroni* does not require an activator for catalysis and has a pH optimum of 7.7. The conversion of steroids containing a primary alcohol function (Chapter 2) shows that large substrates can be converted. Experiments performed by A. Geerlof et al. showed the suitability of the QH-EDH in the enantioselective oxidations of the racemic glycerol-based, C_3-synthon solketal (2,2 dimethyl-4-(hydroxymethyl)1,3-dioxolane) (189). Solketal and the corresponding aldehyde can be used as building blocks in the preparation of enantiomerically pure biologically active compounds

such as phospholipids (190-192), platelet aggregation factor (193-195), (S)-β-blocking agents (196) and others (197-200). QH-EDH type I does not show any enantioselectivity towards the C_3-synthon glycidol. However, QH-EDH type II was found to be very suited in the enantioselective oxidation of this compound (201). Another attractive aspect of QH-EDH for application is that electrochemical cofactor regeneration can be achieved when immobilized on a electrode (202).

Quinohaemoprotein alcohol dehydrogenases type II

The type II quinohaemoprotein alcohol dehydrogenases have been isolated from *Acetobacter* and *Gluconobacter* species and they are responsible for the first step in the oxidation of ethanol to acetic acid in vinegar fermentation (18-20). They show a lower optimum pH of 4 - 6. The substrate specificity is more restricted than for QH-EDH_I. QH-EDH_{II} oxidizes only primary alcohols from ethanol to hexanol and shows a low activity for formaldehyde and acetaldehyde. In contrast to all other quinoprotein alcohol dehydrogenases, which occur in soluble form, most probably in the periplasm, the type II enzymes are integrated into the cytoplasmic membranes. They consist of three subunits, the largest one of 72-80 kDa probably containing the PQQ and a haem c, a cytochrome c_{553} subunit of 48-53 kDa containing 2 haems c, and a 14-17 kDa subunit of unknown function. The cytochrome c_{553} subunit seems to be essential in the formation of a functional complex in the respiratory chain (203). The gene coding for the largest subunit has been sequenced and exhibits close similarity to the amino acid sequence of quinoprotein methanol dehydrogenase (37). From this it seems that this subunit consists of a quinoprotein domain and a cytochrome *c* domain that is missing in MDH analogous to QH-EDH from *C. testosteroni*. Close similarity also exists between the quinoprotein part of QH-EDH_{II} and QH-EDH_I, however, the cytochrome *c* parts of both proteins shows no similarity.

Mechanistic aspects of alcohol oxidoreductases

Nicotinamide-nucleotide (NAD(P)-dependent ADH's

Nicotinamide-nucleotide-linked dehydrogenases were among the earliest two substrate enzymes to be subjected to a detailed kinetic study, and provided much of the original stimulus for the necessary extension of kinetic theory already developed for one-substrate and hydrolytic enzymes. The nicotinamide nucleotides can be considered as substrates from the kinetic point of view. Although they form a stable complex with the dehydrogenase, the dissociation constant of the complex is sufficiently high to make that these dehydrogenases

are always isolated without attached coenzyme. Catalysis by these enzymes starts with the formation of a ternary complex of enzyme, coenzyme, and substrate in a series of ordered steps (sequential ordered). The Theorell-Chance mechanism is an ordered mechanism in which the ternary complex of enzyme, NAD(H) and substrate/product does not accumulate under the reaction conditions. It describes the kinetics of alcohol oxidation by LADH (Scheme 1). The oxidation of primary alcohols is an ordered mechanism, with the coenzyme binding first and the dissociation of the enzyme-NADH complex being rate determining (204). For secondary alcohols and methanol, hydride transfer is sometimes rate limiting and the ordered binding of the coenzyme before substrate does not hold. The pH dependence observed for 2-butanol and 3-pentanol, suggests that the hydride transfer is not alone rate-limiting and that deprotonation of the alcohol is partly rate limiting (205).

$$E \rightleftharpoons EO \rightleftharpoons EOAlc \rightleftharpoons EOAld \rightleftharpoons ER \rightleftharpoons E + R \qquad (1)$$

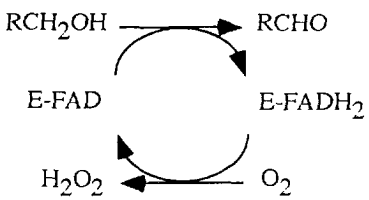
scheme 1: Simplified Theorell-Chance pathway of alcohol-oxidation catalyzed by HLADH. Abbreviations: O, NAD^+: R, NADH: Alc, alcohol; Ald, aldehyde. (taken from ref 206)

In the oxidation of alcohols, a hydride ion is abstracted from the substrate and directly transferred to the 4 position of the nicotinamide ring of the NAD^+, whilst a proton is released into the solvent. Proton transfer and hydride transfer are distinct steps, proton transfer preceding hydride transfer. The active site zinc ion plays an important role in this mechanism. Because of the impossibility of zinc to be oxidized or reduced in these enzymes, the zinc in alcohol dehydrogenases does not donate electrons: rather it serves as a Lewis acid (207). The catalytic Zn^{2+} ion is ligated by two sulphur atoms belonging to cysteines, and by a nitrogen atom of histidine. The fourth ligand is an ionizable water molecule. The nicotinamide ring of the NAD^+ is bound close to the zinc ion. The relevant oxygen atom of the substrate is directly coordinated to the zinc ion, displacing the bound water molecule so that the metal ion remains tetracoordinated. The hydride transfer takes place from the alcohol to the NAD^+ in a direct and stereospecific way. The rate constants for hydride transfer for aliphatic primary alcohols are similar, however, the rates of dissociation of alcohols and aldehydes decrease with increasing carbon chain length (208). This latter results from the difference in the interaction of water with the alcohols (209). In the catalytic cycle, four pH-dependent steps have been recognised, three which have been attributed to the ionization of the zinc bound water in the various complexes and a fourth pH dependent step for which two mechanisms have been proposed. [1] Brändén and Eklund suggest that the zinc bound water deprotonates after NAD^+ binding and the alcohol deprotonates on binding to the zinc ion: thus water dissociates from the zinc (210). [2] The mechanism of Cook and Cleland involves

alcohol binding to the $E.NAD^+$ complex and subsequent proton transfer via a hydrogen bonded network of amino acids to the solvent (211). The kinetic isotope effects are better described by this latter mechanism (212).

Flavoproteins

Flavoprotein alcohol oxidases catalyze the oxidation of alcohols under the formation of H_2O_2 via a ping-pong mechanism (scheme 2). During this process, reducing equivalents are transferred from substrate to the flavin coenzyme, which in turn can transfer them to oxygen. The electron acceptor O_2 becomes quantitively reduced to H_2O_2.



(2)

While the regulation of methanol oxidase in yeasts is fairly well understood and the gene has been cloned and its nucleotide sequence elucidated (213), the biochemical mechanism of the methanol conversion is not fully resolved. Because of the lack of the knowledge concerning the alcohol oxidase, comparisons are made with flavoprotein oxidases like D-amino acid oxidase and lactate oxidase. These enzymes operate according to a carbanion mechanism, i.e. a proton is first abstracted from the C-H bond to be oxidized. Subsequently a transient covalent adduct is formed between the carbanion and the N(5) of the flavin. The intermediate breaks down to give reduced flavin and the oxidized substrate. A carbanion mechanism for methanol is however difficult to envisage due to lack of stabilization of the negative charge in the alcoholic carbanion. Thus it was suggested that the mechanism of methanol oxidase involves a radical mechanism (214-216). Evidence for a radical mechanism comes from data obtained from inactivation studies of methanol oxidase with cyclopropanol (215). Stopped-flow kinetic experiments with (deuterated) methanol indicate that the oxidative step involves scission of the C-H bond of the substrate which is partly rate-limiting (217). In addition it could be concluded that the dissociation rate of formaldehyde from the enzyme-formaldehyde complex also contributes considerably to the overall rate. These data demonstrate that the mechanism of methanol oxidase is complicated by the strong interaction of the formaldehyde with both oxidized and reduced enzyme and by the fact that the formaldehyde is also a substrate of methanol oxidase.

Quinoprotein alcohol dehydrogenases

In the quinoprotein alcohol dehydrogenases the mechanism of the oxidation of alcohols is best studied for MDH. Work on the catalytic cycle suggests that MDH oxidizes its substrate and releases the product prior to the reaction with its electron acceptor. There are two types of mechanisms proposed consistent with this idea. In the first type, reducing equivalents are transferred directly from substrate to PQQ, generating $PQQH_2$ plus the oxidized product (Figure 7). No evidence for this mechanism is obtained. The second type of mechanism is based on the formation of an PQQ-substrate product at the active site prior to the reduction of PQQ to the $PQQH_2$ quinol form (Figure 8). Evidence for the formation and isolation of a PQQ-substrate complex comes from inhibition experiments with cyclopropanol (218-220).

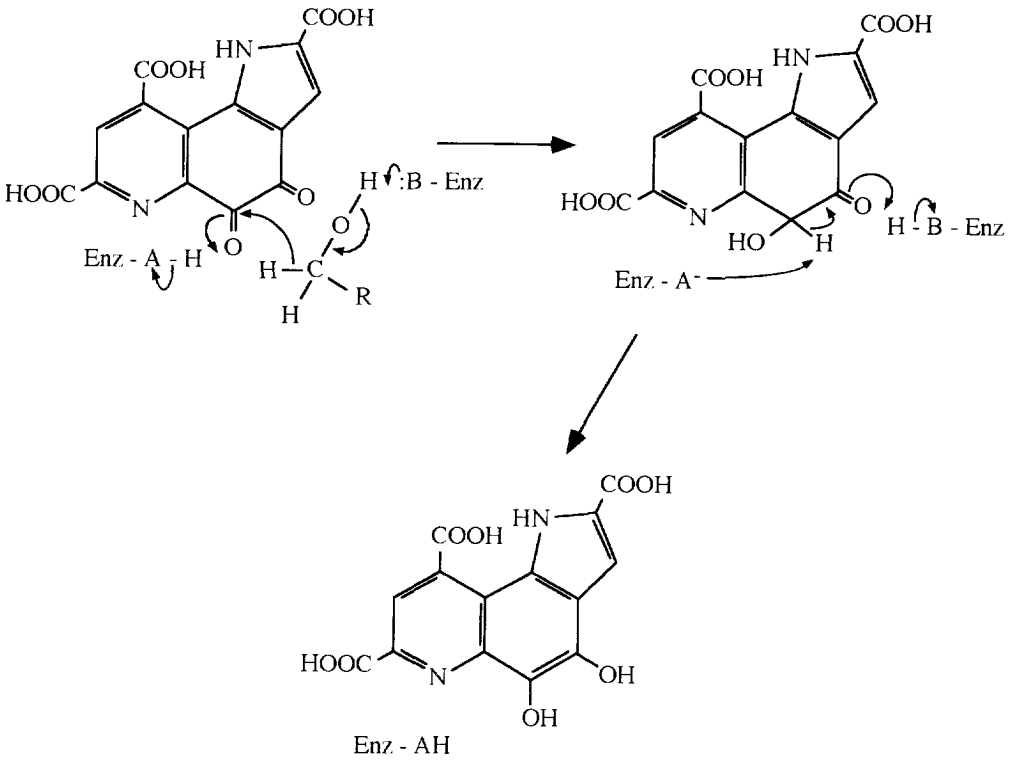


Figure 7. The "direct mechanism" I for alcohol oxidation by MDH_{ox} (taken from ref. 83).

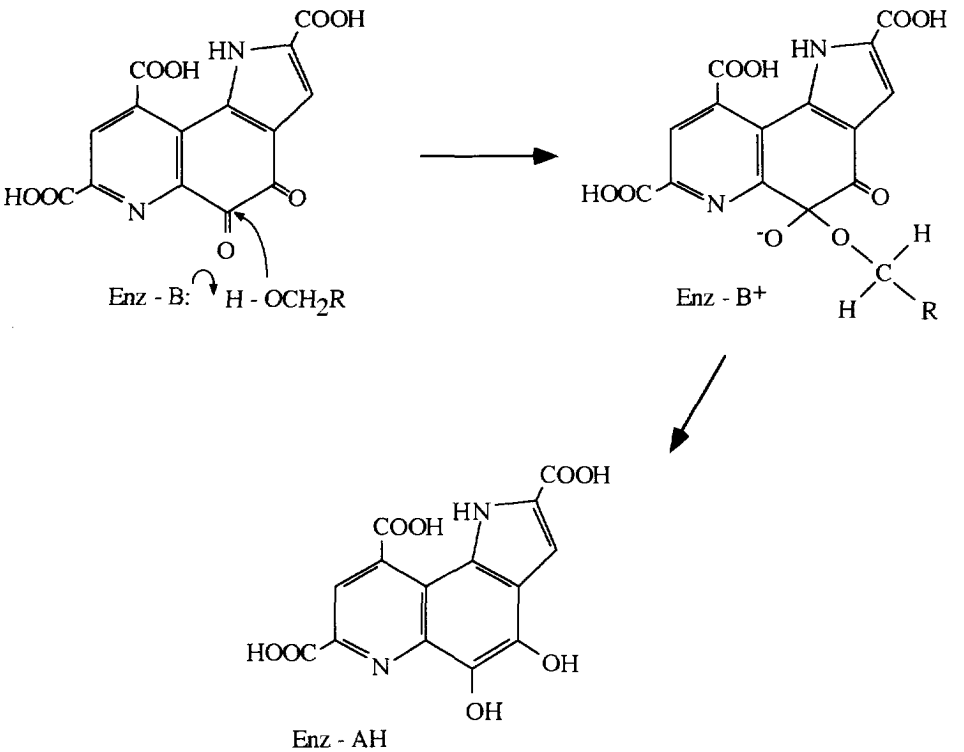


Figure 8. The "indirect mechanism" for oxidation of alcohols by MDH_{ox} (taken from ref. 83).

On the base of these findings Frank and Duine propose a mechanism involving a base catalyzed proton abstraction concerted with attack of the oxyanion on the C-5 of PQQ (219). This mechanism involves a hemiketal intermediate. The hemiktal intermediate has been proven to exist by fluorescence experiments (219). Slight modifications of this mechanism have been proposed by Anthony (83), Houck and Unkefer (83). Though evidence exists for the second mechanism, other more unusual mechanisms like the action of PQQ as a p-quinone instead of a o-quinone cannot be ruled out (Figure 9) (221). Recent studies performed by Itoh et al. (222) provide some additional evidence for the possibility of this mechanism. They isolated a dimethyl acetal adduct of methanol and the trimethylester of PQQ as the major product, after treatment of PQQ with methanol under acidic condition, where the methanol was added at the C(4) position of PQQ. However, the mechanism described by Itoh et al. in relation to the amino acid in the immediate surrounding of PQQ are based on a completely wrong x-ray structure (see ref 223). This means that the amino

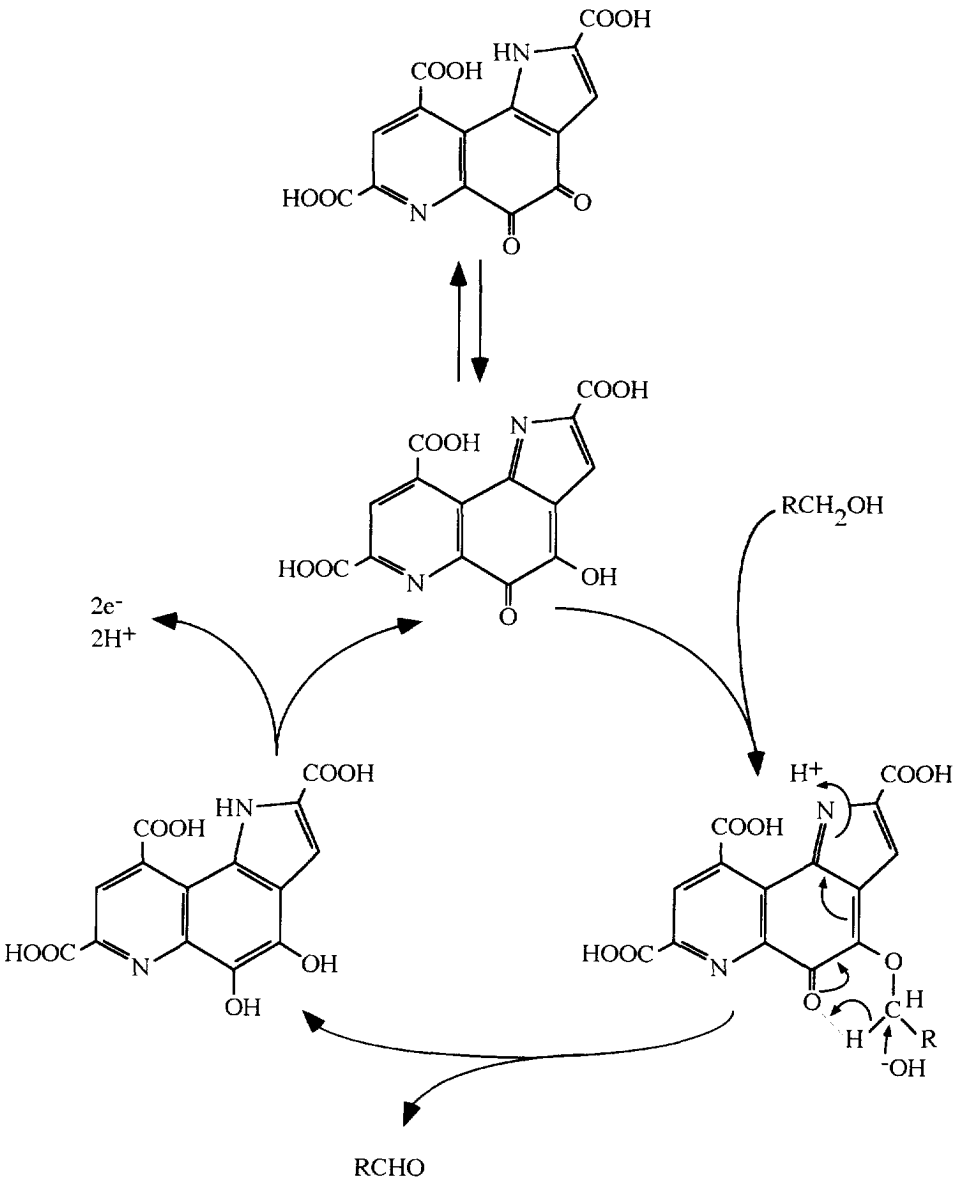


Figure 9. The p-quinone mechanism for alcohol oxidation by MDH_{ox} (taken from ref. 221).

position for their function in the catalytic process. The homology in amino acid sequence between MDH and QH-EDH would imply at least some relationship regarding the conversion of alcohols. Activity measurements performed with the QH-EDH using N_1-methyl-PQQ showed an activity > 5 % (Chapter 2). This analogue misses the pyrrole proton at N-1 which is necessary for a p-quinone mechanism. This results strongly suggest that the p-quinone mechanism is not right.

The mechanism of QH-EDH_I is well less studied. The most striking difference between MDH and QH-EDH_I is the presence of a haem in the latter enzyme. This has great implications in the use of electron acceptors. MDH needs an activator when assayed with an artificial electronacceptor and has a pH optimum $\geq$ 9. This in contrast to the pH optimum of 7 when QH-EDH is assayed with artificial electron acceptors, without the requirement of an activator. However when MDH is assayed with the natural electronacceptor cytochrome c_L, there is no need for an activator and the pH optimum changes to 7. On a closer look one might suggest that QH-EDH is a genfusion product of a quinoprotein, like MDH and the natural electron acceptor, a cytochrome *c*. This would imply that there might be a similarity in sequence between the cytochrome *c* part of QH-EDH and cytochrome c_L. However, comparison of both amino acid sequences show very little similarity.

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Chapter 2

Quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni*: purification, characterization, and reconstitution of the apoenzyme with PQQ-analogues

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Abbreviations

PQQ, 2,7,9-tricarboxy-1H-pyrrolo(2,3-f)quinoline-4,5-dione: $PQQH^{\cdot}$, semiquinone form of PQQ; $PQQH_2$, quinol form of PQQ; $PQQH_4$, four-electron-reduced PQQ; 5-OH-PQ, 5-Hydroxy-pyrroloquinoline-tricarboxylic acid; QH-EDH, quinohaemoprotein ethanol dehydrogenase.

Abstract

Pyrroloquinoline quinone (PQQ)-free quinohaemoprotein ethanol dehydrogenase (QH-EDH) apoenzyme was isolated from ethanol-grown *Comamonas testosteroni*. The purified apoenzyme, showing a single band of 71 kDa on native gel electrophoresis, could be only partially converted into active holoenzyme by addition of PQQ in the presence of calcium ions. Besides a band with a molecular mass of 71 kDa, additional bands of 51 and 25 kDa were observed with SDS/PAGE. Analysis of the N-terminal sequences of the bands and comparison with the DNA sequence of the gene, suggested that the latter two originate from the former one, due to scission occurring at a specific site between two vicinal residues in the protein chain. The extent of scission appeared to increase during growth of the organism. After addition of PQQ to apoenzyme, holoenzyme and nicked, inactive enzyme could be separated. Holoenzyme prepared in this way was found to contain equimolar amounts of PQQ, Ca^{2+} and covalently-bound haem. EPR spectra of fully oxidized apo- and holo-QH-EDH showed g-values typical for low-spin haem c proteins. In partially oxidized holo-QH-EDH an organic radical signal attributed to the semiquinone form of PQQ was observed. Binding of PQQ leads to conformational changes, as reflected by changes of spectral and chromatographic properties. Reconstitution of apoenzyme with PQQ analogues resulted in a decreased activity and enantioselectivity for the oxidation of chiral alcohols. Compared to PQQ, analogues with a large substituent had a lower affinity for the apoenzyme. Results with other analogues indicated that possession of the o-quinone/o-quinol moiety is not essential for binding but it is for activity.

Introduction

Quinohaemoprotein ethanol dehydrogenase, QH-EDH, from *Comamonas testosteroni* catalyses the NAD-independent oxidation of a broad range of primary alcohols to the corresponding aldehydes and the (subsequent) oxidation of aldehydes to carboxylic acids. The soluble enzyme can be routinely isolated as the inactive, PQQ-free, haem *c*-containing apoenzyme from ethanol-grown cells cultivated at ambient temperatures. In the absence of PQQ in the medium, growth on alcohol still occurs and is most probably catalyzed *via* a NAD dependent alcohol dehydrogenase [2]. Nevertheless it should be stressed that under this condition the QH-EDH apoenzyme is still produced [1]. Active holoenzyme is obtained by addition of equimolar amounts of PQQ (the structure of this cofactor is depicted in Fig. 7) in the presence of calcium ions. Alternatively, holoenzyme can be isolated directly from cells grown in the presence of PQQ. Small-scale isolation and preliminary characterization of apo- and holo-QH-EDH have been described in the past [1]. In the meantime, it was found that medium- and large-scale cultivation of *C. testosteroni* at different conditions of pH,

temperature and ionic strength, resulted in widely varying productivities of apo- and holoenzyme. Notably, cells grown in the presence of PQQ added to the culture media contained low amounts of holo-QH-EDH. In contrast, in the absence of PQQ, substantial amounts of apo-QH-EDH were produced. However, apoenzyme preparations that were purified to homogeneity, as judged by the appearance of a single band on gel electrophoresis under non-denaturing conditions, showed saturation of activity already upon addition of substoichiometric amounts of PQQ. These findings prompted us to reinvestigate the purification and characterization of this enzyme.

A preliminary investigation of the effect of chemical modifications of the cofactor PQQ on the catalytic properties of QH-EDH showed the o-quinone moiety of PQQ to be indispensable for catalytic activity [3]. Recently, attention has been drawn to the enantioselective properties of this enzyme in the oxidation of chiral alcohols [2,4,5]. Its application for the kinetic resolution of the racemic, glycerol-based, C_3-synthon solketal (2,2 dimethyl-4-(hydroxymethyl)1,3-dioxolane) has been patented [6]. In view of the prominent enantioselectivity in the conversion of racemic solketal that is displayed by QH-EDH carrying authentic PQQ, we investigated the effects of minor, mostly steric, changes in the PQQ molecule on the enantioselective properties of QH-EDH.

Materials and methods

Preparation of the PQQ-derivatives

PQQ was synthesized as described previously [7]. 3-Methyl-PQQ and 3-Propyl-PQQ were prepared by a similar procedure. N_1-alkylated derivatives were prepared as described [8]. 8-Methyl-PQQ was synthesized by a modification of the procedure developed for the preparation of C_8-deuterated PQQ [9]. $PQQH_4$ was prepared as described previously [10]. 5-Hydroxy-pyrroloquinoline-tricarboxylic acid (5-OH-PQ) was obtained by dehydration of $PQQH_4$ [11].

Growth and harvesting of Comamonas testosteroni

Comamonas testosteroni LMD 26.36 (ATCC 15667) was grown without PQQ on a mineral medium with 1% ethanol as carbon and energy source at 23°C [1]. Cells were harvested at the end of the exponential growth phase for the preparation of the holoenzyme. For apoenzyme isolation, cells were harvested at an early stage in the exponential growth phase.

Apoenzyme isolation

Cell paste (200 g) was suspended in 200 ml 20 mM Mops/KOH buffer, pH 7.5, and disrupted in a French pressure cell at 110 MPa. The suspension was centrifuged at $100.000 \times g$ at 4°C for 2 hours. The supernatant (200 ml) was diluted with 800 ml 20 mM Mops/KOH, pH 7.5, containing 5 mM $CaCl_2$ (buffer A) and applied to a CM-Sepharose

column (15×3 cm) equilibrated in buffer A, at a flow rate of 3 ml/min. The column was eluted with buffer A containing 0.5 M NaCl. The pooled active fractions were dialysed for 24 hours against buffer A and the dialyzate applied to a Mono-S column (Pharmacia, HR 16/10) previously equilibrated with buffer A. A gradient of 0 - 0.2 M NaCl in buffer A was applied at a flow rate of 1.5 ml/min during 1 hour. Pooled active fractions were further purified by gel filtration on a Pharmacia Superdex 75 column equilibrated with buffer A and the eluting active fractions stored at -80°C.

Reconstitution and holoenzyme isolation

Reconstitution occurred by incubating apoenzyme (10 μM) with PQQ or PQQ-analogue (10 μM) in buffer A. Activity and enantioselectivity were measured after 10 min. incubation at 20 °C. Unbound cofactor was removed by gelfiltration on a PD10 column in buffer A. To obtain pure holoenzyme (without nicked protein or enzyme without PQQ), the reconstituted enzyme was applied to a Mono-S column (HR16, Pharmacia) equilibrated with buffer A. The column was eluted with a NaCl gradient from 0-0.2 M in buffer A at a flow rate of 1.5 ml/min in 1 hour. Monitoring occurred with a Hewlett Packard photodiode array detector at 280 nm, taking ultraviolet/visible spectra at several positions in the eluting peaks. Whether the enzyme was reconstitutable with the PQQ-analogues was judged from the appearance of a peak with the same retention time as pure holo-QH-EDH (Fig. 3).

Enzyme assay

QH-EDH was assayed using n-butanol as a substrate and potassium ferricyanide as electron acceptor in buffer A, as described [1]. Measurements of activities on sterols were performed with substrate concentrations ranging from 0.1-5.0 mM. Stock solutions (1 M) were prepared in methanol (methanol is not a substrate and no inhibitor for the enzyme). Enantioselectivity for solketal was determined according to the procedure described by Geerlof et al. [2]. The specific enzyme activity is defined aq the reduction of one μmol of ferricyanide.mg^{-1} protein.min^{-1} under the specified assay conditions.

Spectroscopy

Ultraviolet/visible absorption spectra of PQQ derivatives and enzyme preparations were measured on a HP 8524A Diode array spectrometer at 20 °C. Specific absorption coefficients were calculated for the apo- and holoenzyme from the amino acid sequence according to the method described by Gill and Von Hippel [12]. The contribution of haem to this was taken into account by using a molar absorption coefficient of 13.85 $mM^{-1}.cm^{-1}$ at 280 nm [13]. A correction for the contribution of PQQ was made by comparing the absorbance of denatured holo-QH-EDH in 6 M guanidine/HCl at 280 nm, with the absorbance of holo-QH-EDH at that wavelength in buffer A.

EPR spectroscopy was performed on a Varian E-9 spectrometer operating at X-band frequency and equipped with a home-made He-flow cryostat. Samples were oxidized with

appropriate amounts of ferricyanide, subjected to gelfiltration on Sephadex G-25 to remove excess oxidant and subsequently flushed with Argon to remove oxygen (which disturbs the high-field part of the EPR spectrum around g=1.5). Owing to the time required for these manipulations, the oxidized holo-QH-EDH still became partly reduced (see below).

Polyacrylamide-gel electrophoresis

Subunit molecular masses were determined under denaturing conditions by SDS/PAGE according to Laemmli [14] using a 10-20% polyacrylamide gradient gel and Mini Protean equipment (Bio-Rad). Enzyme samples were denatured by incubation for 5 min. at 100 °C in 2% SDS and 10 % β-mercaptoethanol. A low molecular weight calibration kit (Pharmacia) was used to derive the molecular masses. Homogeneity was checked with native gel electrophorese on a Phast System (Pharmacia) using a homogenous 20% polyacrylamide gel and reverse polarity (QH-EDH is a basic protein).

Gels were stained for protein with Coomassie Brilliant Blue G250. Haem staining was performed with 3,3',5,5'-tetramethylbenzidine [15].

Amino acid sequence analysis of the N-terminal part

QH-EDH preparations were electrophorized on a 10% polyacrylamide gel and the proteins were electroblotted on a polyvinylidene difluoride membrane [16]. Proteins on the membrane were stained with 0.1% Coomassie Brilliant Blue R250. The stained bands were cut out and processed in a Protein Sequenator (Applied Biosystems model 470A) equipped with on-line phenylthiohydantoin amino acid analysis using a model 120A PTH-amino acid analyzer.

Determination of calcium, iron, haem, protein and PQQ content

Determination of haem was performed as described by Bartsch [17]. A molar absorbtion coefficient of 25400 $M^{-1}.cm^{-1}$ at 249 nm was used (at pH 4) for PQQ [7]. Iron contents were determined by neutron activation analysis [18]. Determination of the calcium content was performed with flame atomic absorption spectrometry. Protein content of the fractions obtained during purification were determined according to Bradford [19] with desalted bovine serum albumin as a standard.

Results

Production and purification of apo- and holo-QH-EDH

A typical cultivation of *C. testosteroni* at 23 °C without PQQ produces 8 gram cell paste per litre in one week, when the cells were harvested at the end of the exponential growth phase. The purification scheme for the isolation of apo-QH-EDH from such cells is given in Table 1.

Table 1. Purification of apo-QH-EDH.
An amount of 200 g cell paste was used for this purification scheme. Activities were determined after reconstituting the fractions with PQQ.

Purification step	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Cell-free extract	12000	0.4	100	1.0
CM-Sepharose	410	10.0	80	27.6
Mono-S	220	20.1	78	55.6
Superdex-75	200	20.5	76	56.7

Active fractions obtained after gel filtration on Superdex 75 appeared to be homogeneous, as judged by peak purity criteria as well as by native PAGE (one single band with a molecular mass of 71 kDa observed after protein staining). Titration with PQQ, however,

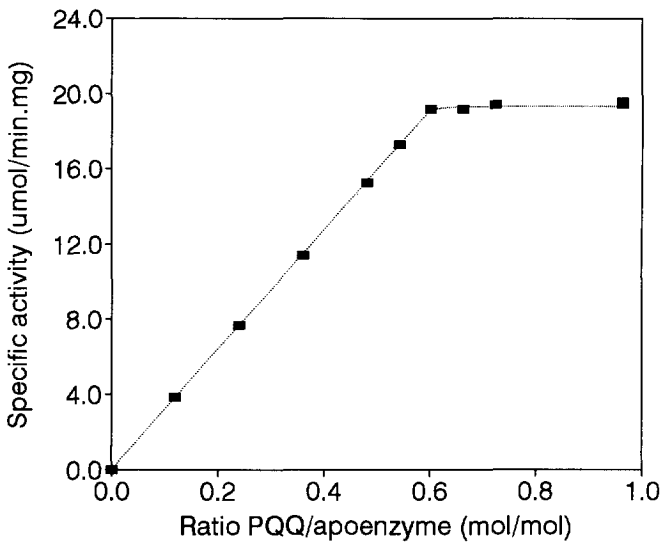

Figure 1. Titration of apo-QH-EDH with PQQ. A typical sample of apoenzyme, containing 40 % nicked protein, in 20 mM Mops/KOH, pH 7.5, containing 5 mM $CaCl_2$, was incubated with various amounts of PQQ and assayed according to the procedure described in the Materials and Methods section.

showed non-integral stoichiometry of the amount of apoenzyme and the amount of PQQ. For a typical preparation, a maximal specific activity of 20.5 μmol/mg/min was obtained already after addition of 0.6 equivalents of PQQ (Fig. 1). Extra addition appeared as unbound PQQ upon gel filtration of the mixture. On performing SDS/PAGE of the apoenzyme, bands with molecular masses of 51 and 25 kDa were detected in addition to a band of 71 kDa (previously calibrated as 67 kDa [1]) (Fig. 2). The bands with molecular masses of 71 and 25 kDa showed positive haem staining. After reconstitution with PQQ the preparation could be further separated now on a Mono-S column into an "active" and an "inactive" fraction (Fig. 3). The active fraction, considered to contain holo-QH-EDH, showed a specific activity of 31.3 μmol/mg/min, a single band with a molecular mass of 71 kDa on SDS/PAGE, and an absorption spectrum indicating the presence of reduced haem c. The inactive fraction showed no activity with alcohols or aldehydes, showed two bands with molecular masses of 51 and 25 kDa on SDS/PAGE, and an absorbtion spectrum indicating the presence of oxidized haem c but not of PQQ. All attempts to remove non-PQQ-binding apoenzyme prior to addition of PQQ failed, apparently due to the highly similar chromatographic and electrophoretic behaviour of apoenzyme able to bind PQQ and that unable to do so.

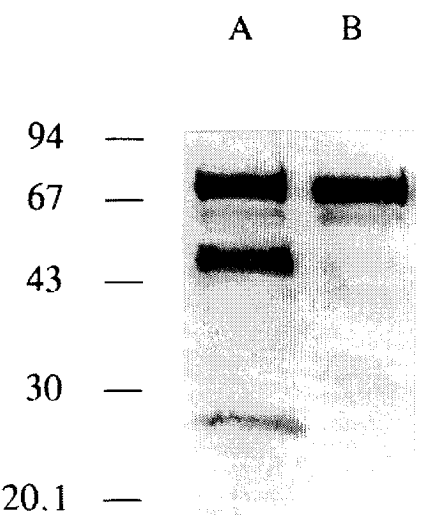

Figure 2. SDS/PAGE of apo- and holo-QH-EDH. Apo-QH-EDH with 40% nicked protein (according to the specific activity) was compared with the purified holo-QH-EDH, obtained by reconstitution of apo-QH-EDH with PQQ and subsequent removal of the nicked protein with Mono-S chromatography. Lane A: apo-QH-EDH, lane B: holo-QH-EDH.

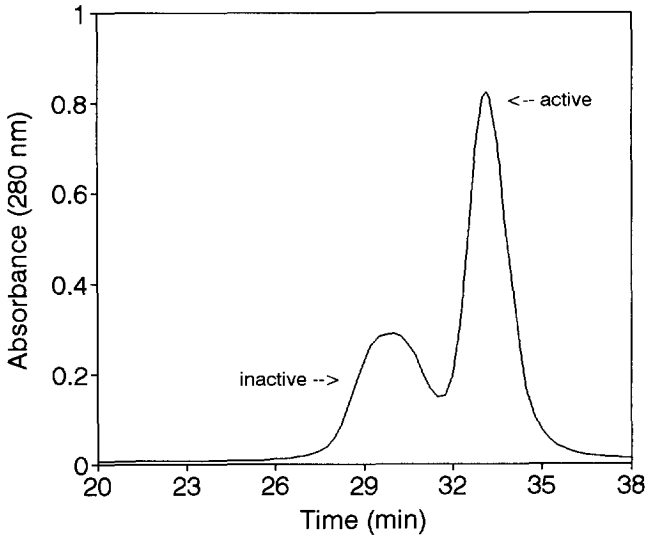

Figure 3. Mono-S chromatogram of PQQ-reconstituted apo-QH-EDH. 5 mg apo-QH-EDH containing approx. 40 % nicked protein (according to the specific activity), was reconstituted with PQQ and chromatographed on Mono-S, as described in the M&M section. Two peaks can be detected on Mono-S chromatography: the "inactive" peak consisting of the non-PQQ binding, nicked apoenzyme; the "active" peak consisting of holo-QH-EDH.

Harvesting the cells in an earlier stage of the exponential growth phase led to a decrease of the relative amount of the non-PQQ-binding protein fraction. However, the over-all yield per volume of culture medium decreased proportionally, yielding less than 2 mg apoenzyme per litre medium (4 gram wet cells) containing less than 5 % inactive apoenzyme. Addition of PQQ to the growth medium leads to an enormous enhancement of the specific growth rate [4]. Amounts of active enzyme per gram wet weight of cells grown in the presence of PQQ were, however, drastically reduced. Holo-QH-EDH isolated from these cells showed a single band with a molecular mass of 71 kDa on SDS/PAGE, and a specific activity of 31.3 μmol/mg/min. Raising the temperature from 23 °C to 30 °C caused enhancement of the growth rate [20], however, the total amount of recovered active apo-QH-EDH was again low.

Table 2. Amino acid sequence of the N-terminals of apo-QH-EDH and its fragments. Apo-QH-EDH was subjected to SDS/PAGE and the bands eluted and sequenced as indicated in the M&M section. Uncertainties in amino acid identification are given in brackets, unidentified ones by question-marks.

Protein (kDa)	N-terminal sequence
71	TGPAAQAAAAVQ R VDGDFI?ANA
51	TGPAAQAAAAVQ(R)VDGDF
25	F(N)AEPPK?KPFGRL

Amino acid sequence analysis

N-terminal amino acid sequence determinations were performed on the 71, 51 and 25 kDa bands obtained with SDS/PAGE of denatured apo-QH-EDH preparation. Results are shown in Table 2. Clearly the 71 and 51 kDa proteins have the same N-terminal sequence whereas the 25 kDa fragment has as different one. Comparismn of the amino acid sequences with the sequence deduced from the DNA of the gene encoding QH-EDH (J. Stoorvogel, unpublished results) revealed that the N-terminus of the 25 kDa fragment is located within the 71 kDa enzyme, and that this fragment is the C-terminal part of the protein. The molecular mass of the bare protein (without PQQ, Ca^{2+} and haem) calculated from the DNA sequence is 73200 Da. The calculated sizes for the fragments are 48663 and 24537 Da.

Haem and metal ion content

In order to establish the stoichiometry of the binding of non-proteinaceous compounds to apo-QH-EDH, quantification of the QH-EDH preparations was required. Calculation of the specific absorption coefficient gave an $A^{0.1\%}_{280nm} = 2.41$ for the apoenzyme and $A^{0.1\%}_{280nm} = 2.52$ for the holoenzyme. The molecular masses calculated from the DNA sequence are 73.7 kDa for the apoenzyme and 74.0 kDa for the holoenzyme. The number of metal ions and haem in the enzyme were calculated using these values for the specific absorbtion coefficients and molecular masses. The molar absorption coefficients for the α and γ band of the reduced apo-QH-EDH calculated from the pyridine haemochrome test were $\epsilon_{416} = 1.52\times10^5\ M^{-1}.cm^{-1}$ and $\epsilon_{550} = 2.49\times10^4\ M^{-1}.cm^{-1}$. For the holo-QH-EDH, the method provided values of $\epsilon_{416} = 1.60\times10^5\ M^{-1}.cm^{-1}$ and $\epsilon_{552} = 2.47\times10^4\ M^{-1}.cm^{-1}$. The haem content appeared to be 1.10 mol/mol for the apoenzyme and 1.09 mol/mol for the holoenzyme. The Fe content calculated from neutron activation analysis was 0.86 $\pm$ 0.27 mol iron/mol for the apoenzyme and 0.95 $\pm$ 0.17 mol Fe/mol for the holoenzyme. The Ca content calculated from flame atomic absorbtion spectrometry was 0.51 $\pm$ 0.05 mol Ca/mol for the apoenzyme and 0.82 $\pm$ 0.05 mol Ca/mol for the holoenzyme.

Ultraviolet/visible absorption spectra

The apoenzyme is isolated in a form with a spectrum characteristic for oxidized haem c (Fig. 4), also showing a 695 nm band. The following ratios were found: A_{280}/A_{410} = 1.64 for the apo-QH-EDH$_{ox}$ and A_{280}/A_{416} = 1.21 for the holo-QH-EDH$_{red}$ (previously, respectively 1.84 and 1.70 [1]). On reconstitution with PQQ in the absence of added substrate, nevertheless an absorption spectrum similar to that of reduced cytochrome c is observed (Fig. 4), which is probably caused by the presence of endogenous substrate. Oxidized holo-QH-EDH could be obtained by treatment with ferricyanide. In some cases over 100 equivalents of potassium ferricyanide were required to achieve this.

EPR spectra

The g-values derived from the spectrum (Fig. 5) for the oxidized apo-QH-EDH are typical for anisotropic low-spin haem proteins (Table 3) [21]. The spectra also show resonances due to minor (<1%) contaminants at g=6, originating from high-spin ferric haem, and at g=4.3 from high-spin tetrahedral Fe^{3+}. The spectrum of the apoenzyme, and to a much lesser extent of the holoenzyme, further shows a g_z-like resonance at g=3.47 from a highly anisotropic low-spin haem.

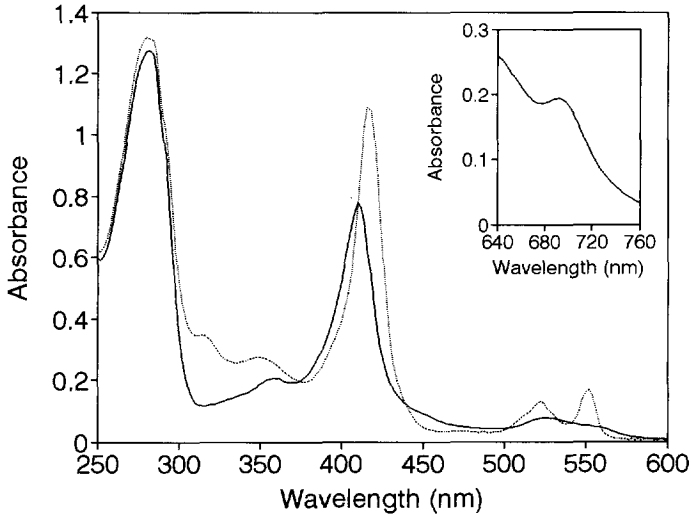

Figure 4. Ultraviolet/visible spectra of apo- and holo-QH-EDH. Samples of 7.3 μM enzyme in 20 mM Mops/KOH pH 7.5, containing 5 mM $CaCl_2$ were measured: (——) apo-QH-EDH containing 40 % nicked protein (according to the specific activity); (· · · · · ·) holo-QH-EDH (reduced form) after Mono-S chromatography. The insert shows the spectrum of apo-QH-EDH at a concentration of 292 μM.

Table 3. EPR parameters of haem c in QH-EDH.
W_x and W_z are the linewidths measured at half-height of the g_x and g_z-resonances, W_y is the peak to peak distance of the g_y-resonance, all expressed in mT.

	EPR parameter					
Enzyme	g_x	g_y	g_z	W_x	W_y	W_z
apo-QH-EDH	1.347	2.293	2.969	74.5	21.5	18.8
holo-QH-EDH	1.340	2.260	3.012	65.2	17.2	12.3

Quantification [22] revealed that this species represents about 15-20% of the major low-spin haem in the apoenzyme and 2-4% in the holoenzyme. Possibly, it originates from the non-reconstitutable fraction of the apo-QH-EDH. Quantification of the absolute spin concentrations of the major low-spin haem yielded values of 824 and 769 μM for the apo- and holo-QH-EDH, respectively.

Comparison of the spectra of the apo- and holoenzyme indicates small shifts in the three g-values and a significant sharpening of the resonances upon binding of PQQ (see Table 3). The linewidth in X-band EPR spectra of low-spin haems is determined mainly by g-strain and to a lesser extent by unresolved hyperfine interactions [23]. The decreases in linewidth (4-9 mT) observed are too large to be ascribed to changes in hyperfine interactions and are therefore due to a change in g-strain. The sharpening of the resonances may, therefore, originate from a decrease in flexibility of the peptide chain upon binding of PQQ, whilst the slight changes in the g-values may reflect a small conformational change, e.g. of the haem c ligands.

An intense signal around g=2 was observed in the holoenzyme (Fig. 6), but not in the apoenzyme. The signal is most likely due to the presence of the PQQ-semiquinone since its properties - the g-value, linewidth and relaxation behaviour - appeared to be very similar to those reported for PQQH$^{\cdot}$ in methanol dehydrogenase [24]. Quantification of the absolute spin concentration for the radical signal showed a value of 243 μM. The percentage of the absolute spin concentrations for PQQH$^{\cdot}$ compared to haem c in this holo-QH-EDH sample was thus 31,6 %.

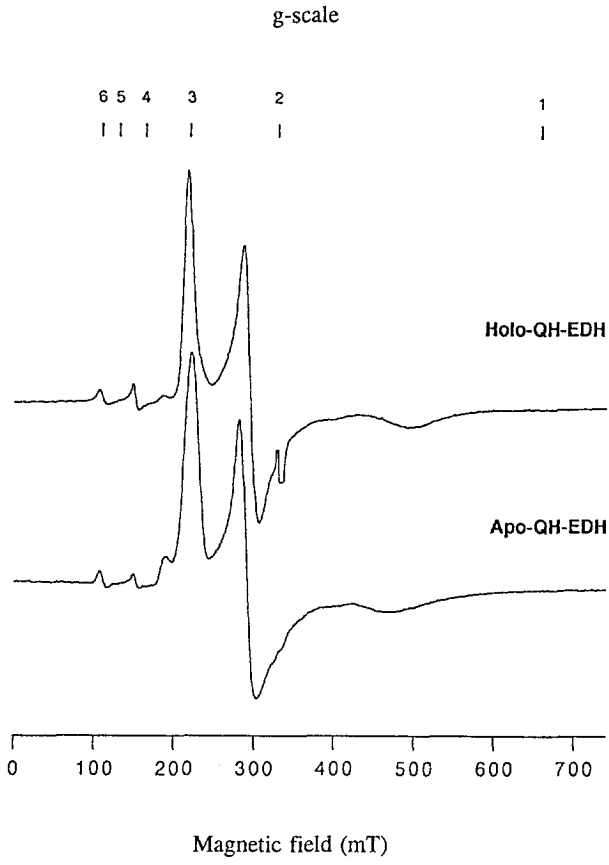

Figure 5. EPR spectra of oxidized holo- and apo-QH-EDH. Samples of 500 μM enzyme in 20 mM Mops, 5 mM $CaCl_2$ (pH 7.5), were prepared as described in the Materials and Methods section. EPR conditions: Microwave frequency 9.231 GHz; temperature 18 K; microwave power 2 mW; modulation amplitude 1.0 mT. The signal at g=2, which was out of scale under these measurement conditions, is removed from the trace of the holoenzyme and shown separately in Fig. 6.

Effects of PQQ modification on activity and enantioselectivity

QH-EDH catalyzes the dye linked oxidation of primary alcohols and aldehydes [1]. The enzyme also converted aldosterone and hydrocortisone with a specific activity of 1.3 and 2.3 μmol/mg/min, respectively, at concentrations of 5 and 1.5 mM respectively. No activity was observed with cholesterol, testosterone and β-estradiol, which are secondary alcohols. The low molecular weight secondary alcohols, 2-propanol and 2-butanol, also showed no activity. In order to investigate the effect of cofactor modification on the activity and

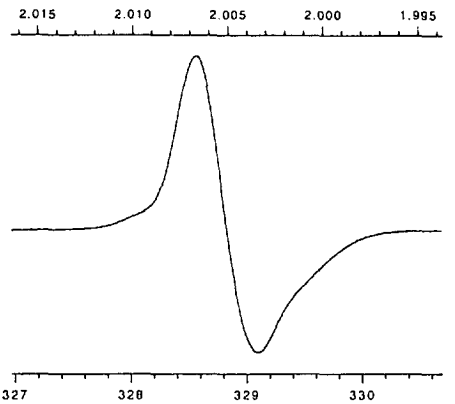

Figure 6. EPR spectrum of the organic free radical in holo-QH-EDH. The sample is the same as used as in Figure 5. EPR conditions: Microwave frequency 9.228 GHz; temperature 70 K; microwave power 2 μW; modulation amplitude 0.1 mT.

enantioselectivity, the apoenzyme was reconstituted with PQQ-analogues (Fig. 7), the results presented in Table 4. The activity and enantioselectivity were measured before chromatography on the Mono-S column. Compared to PQQ, the activity towards n-butanol and the enantioselectivity towards racemic solketal are lower with all the tested PQQ-analogues. Binding affinity of the analogues appeared to be variable as judged from the amount of "active" peak observed upon Mono-S chromatography after reconstitution (not shown). Some analogues (3-methyl-PQQ and N_1-methyl-PQQ) had similar affinity as PQQ, others (3-Propyl-PQQ and N_1-ethyl-PQQ) with a larger substituent hardly showed anybinding. Surprisingly, analogues with a modified o-quinone moiety ($PQQH_4$ and 5-hydroxy-pyrroloquinoline-tricarboxylic acid) had excellent affinity for the apoenzyme. Ultraviolet/visible spectra of the "active" peak obtained with some analogues, as well as apoenzyme reduced with dithiothreitol for comparison, were measured with the photodiode array detector (Fig. 8). From this it appeared that binding of PQQ and its analogues raised the absorbance in the 300-400 nm region and that variation occurs in the contributions around 315 and 350 nm.

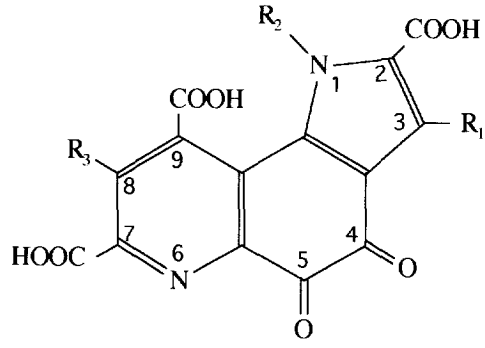

Figure 7. PQQ analogues. PQQ: R_1, R_2 and R_3 =H. 3-Methyl-PQQ: R_1 = $-CH_3$, R_2 and R_3 =H. 3-Propyl-PQQ: R_1 = $-CH_2CH_2CH_3$, R_2 and R_3 =H. N_1-methyl-PQQ: R_2 = $-CH_3$, R_1 and R_3 =H. N_1-ethyl-PQQ: R_2 = $-CH_2CH_3$, R_1 and R_3 =H. 8-Methyl-PQQ: R_3 = $-CH_3$, R_1 and R_2 =H.

Table 4. Activity, enantioselectivity and cofactor binding of holo-QH-EDH reconstituted with different PQQ-analogues.

QH-EDH has a preference for the R-enantiomer of solketal (3,5) . E (the enantiomeric ratio) is the parameter quantifying this preference. Assays were carried out with 1 mM butanol or 2 mM solketal. The relative amount of holoenzyme formation was determined by integration of the "active" peak obtained on Mono-S with reconstituted enzyme, as compared to that obtained with PQQ. n.d., is not determined.

PQQ-analogue	Activity (U/mg) with		Enantioselectivity towards solketal (E-value)	Holoenzyme formation (%)
	n-butanol	R-solketal		
PQQ	26.0	2.87	100	100
N_1-methyl-PQQ	0.8	0.11	5	100
N_1-ethyl-PQQ	0.2	n.d.	n.d.	5
3-Methyl-PQQ	3.1	0.34	10	100
3-Propyl-PQQ	0.3	0.03	7	0
8-methyl-PQQ	1.3	0.66	23	n.d.
$PQQH_4$	0	0	0	100
5-OH-PQ	0	0	0	100

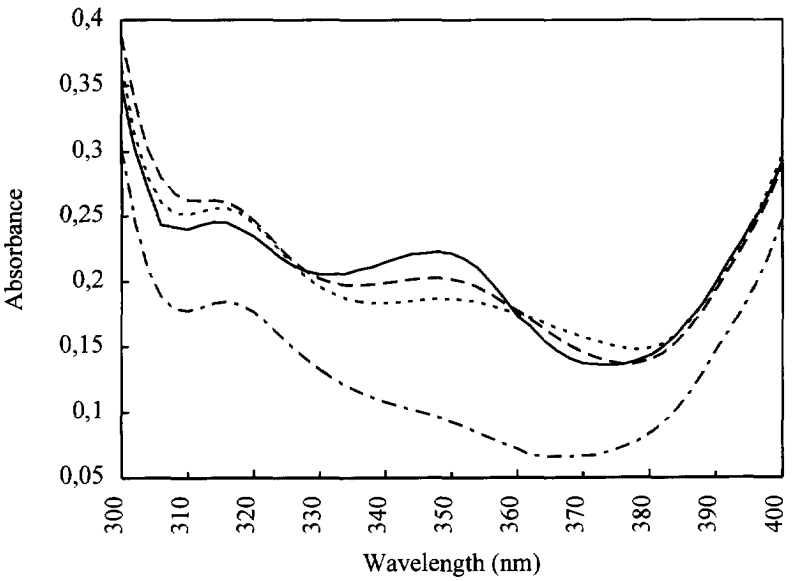

Figure 8. Ultraviolet/visible spectra of holo-QH-EDH with different PQQ-analogues. Spectra were taken with the photo-diode array detector at the "active" peak obtained with Mono-S chromatography of reconstituted enzyme. (——) N_1-methyl-PQQ; (· · · · · ·) 3-Methyl-PQQ; (------) PQQ. Spectra were normalized to $A_{280nm} = 1$. The lowest curve (- · - · -) represents the absorption spectrum of apo-QH-EDH reduced with excess of dithiotreitol.

Discussion

In the past few years, a number of difficulties were met in the isolation of QH-EDH. Although the apoenzyme could be routinely isolated in homogeneous form, as judged from the single band upon native PAGE, the yield (amount of protein) and quality (specific activity of reconstituted enzyme) varied considerably. Based on what is already known and the here described results, an explanation can be given for the variation and a strategy to circumvent it.

Production of enzyme is low at high specific growth rate, i.e. at growth above ambient temperature [1] or in the presence of PQQ, the latter activating alcohol oxidation via the QH-EDH respiratory chain. Although QH-EDH seems of no use to the organism for growth on alcohols in the absence of PQQ, the apoenzyme is synthesized in large amounts and the regulation of expression is very similar to that of PQQ-containing methanol dehydrogenase from methylotrophic bacteria [25]. It is clear, therefore, that reproducible yields require at

least a constant specific growth rate.

The apoenzyme appears to be vulnerable to scission at a specific site between two adjacent phenylalanines, resulting in two fragments of 25 and 51 kDa. Most probably, the cleavage occurs *in vivo* via a protease since the percentage of nicked protein increases during the exponential growth phase and spontaneous hydrolysis of the bond seems unlikely. Physically, nicked and normal apoenzyme appear to be very similar in view of their identical behaviour upon chromatography and native electrophoresis. Biologically, however, the two are dissimilar since the nicked enzyme is not reconstitutable to active holoenzyme with PQQ. *In vivo* reconstitution achieved by including PQQ in the growth medium prevents the proteolytic attack [5]. Most probably, the reason for this resistance is a more compact conformation of the holoenzyme in line with the less flexible protein structure. Conformational differences between the apo- and holoenzyme might also be responsible for the chromatographic separation occurring on the Mono-S column (Fig. 3).

From the insight obtained sofar, the following recommendations can be derived to obtain QH-EDH in adequate yield and quality in a reproducible way. Production of apo-QH-EDH without nicked enzyme requires the use of cells cultivated at low growth rate and harvested early in the exponential growth phase. On the other hand, production of holo-QH-EDH can be achieved in two ways: by cultivating the bacterium in the presence of PQQ or by *in vitro* reconstitution of apoenzyme with PQQ, the latter way requiring an additional purification step if nicked apoenzyme is present.

Based on the here derived specific absorption coefficients and molecular masses and the insight that inactive, not reconstitutable apoenzyme may be present, it is clear now that QH-EDH has a molecular mass of 74.0 kDa, and that it contains 1 haem c, 1 PQQ and 1 Ca^{2+} per enzyme molecule. The affinity for Ca^{2+} is not solely determined by the presence of PQQ since the apoenzyme also contains this metal ion. The presence of the same amino acids in similar positions in the sequence (J. Stoorvogel, unpublished) as those involved in Ca^{2+} binding (besides PQQ) in methanol dehydrogenase [26] could be responsible for this. Apparently, the binding is not so strong since substoichiometric amounts (0.82 and 0.51 Ca^{2+} per holo- and apoenzyme molecule, respectively) were detected. Most probably this is due to removal in Ca^{2+}-lacking buffers (required for the determination), as has also been observed for PQQ-containing ethanol dehydrogenases from *Pseudomonas aeruginosa* [27]. The small difference in Ca content between the apo- and holoenzyme indicates that the presence of PQQ hardly contributes to Ca^{2+} binding to the enzyme. (if it is assumed that Ca^{2+} does not bind to nicked protein in the apoenzyme preparations, apo- and holoenzyme behave similar).

As reflected by the ultraviolet/visible and EPR spectra of apo- and holo-QH-EDH, the haem c in QH-EDH is similar to that in low-spin haem c-containing cytochromes in which the haem iron is liganded by methionyl and histidinyl residues. Differences in absorption maxima of the haem *c* peaks and in EPR parameters indicate that the environment of the haem c in apo- and holoenzyme is not identical and that conformational differences exist.

Just as with other PQQ-containing ethanol/methanol dehydrogenases [27-29], the presence of endogenous substrate (alcohols present in buffer salts, alcohols/aldehydes in the atmosphere) is a handicap in obtaining fully oxidized enzyme. Addition of PQQ to the apoenzyme always results in a preparation in which the haem c is reduced. The redox status of PQQ in such preparations is unknown. Although formally seen it could be present, no semiquinone was observed in alcohol-reduced holo-QH-EDH with EPR. Oxidized holo-QH-EDH can be obtained by adding ferricyanide, but as soon as the excess electron acceptor is removed by gelfiltration, reduction takes place, as indicated by the presence of $PQQH^{\cdot}$ in such a preparation (Fig. 6). The redox form of QH-EDH responsible for this may have physiological relevance. Oxidation of substrate provides two reducing equivalents, formally converting PQQ into $PQQH_2$ after which redistribution occurs as indicated by the formation of reduced haem c. It is tempting to speculate that the latter is involved in subsequent transfer via one-electron-steps to the respiratory chain. Studies on the redox status of the cofactors of QH-EDH in the here observed redox forms are in progress.

The further investigation on the substrate specificity of QH-EDH shows that the enzyme only oxidizes primary alcohols and aldehydes, not secondary alcohols. Yet the accessibility of the substrate binding pocket must be high since steroids and substituted glycerol (solketal) are substrates. Despite the acceptance of these bulky substrates, discriminative power of the enzyme is very large, as follows from the excellent enantioselectivity (high E value) towards solketal. Apparently this is lost upon modification of the cofactor, as demonstrated by the lower E values of the PQQ-analogues. Decreasing activity and loss of specificity upon modifying the cofactor has also been observed for other types of enzymes, but the reverse case also occurs [30, 31]. Since a large difference in redox potential exists between the couples PQQ/$PQQH_2$ and alcohol/aldehydes and modification will not affect the former value so much, most probably inadequate fit of the analogue in the active site is responsible for the strong decrease in activity and selectivity. This view is supported by the observation that cofactor binding is also decreased when modifying PQQ with large substituents. Apparently, the o-quinone/o-quinol moiety is not essential for binding since $PQQH_4$ and 5-OH-PQ are able to bind, although it is for activity. It also appears from this that the Ca/o-quinone bond, proposed for methanol dehydrogenase [26], is not essential for cofactor binding in this enzyme. The absorption spectra of the different holo-QH-EDH containing PQQ-analogues, show only differences between 300 and 400 nm. In contrast to other PQQ-containing enzymes [32,33], instead of one, two maxima around 315 and 350 nm are observed (Fig. 8). This could be due to differences in the PQQ-environment or to different redox states of the enzymes. Since the same amino acids were found in the sequence of QH-EDH (J. Stoorvogel, unpublished) as those proposed to interact with PQQ in methanol dehydrogenase [26], the first possibility seems unlikely. The latter possibility is presently under investigation.

Acknowledgements

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Chapter 3

Characterization of the interaction between PQQ and haem c in the quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni*

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Abbreviations

EPR, electron paramagnetic resonance; MOPS, 2-(N-morpholino)-propanesulfonic acid; NMR, nuclear magnetic resonance; ppm, parts per million; PQQ, 2,7,9-tricarboxy-1*H*-pyrrolo(2,3-*f*)quinoline-4,5-dione: $PQQH^{\cdot}$, semiquinone form of PQQ; $PQQH_2$, quinol form of PQQ; QH-EDH, quinohaemoprotein ethanol dehydrogenase.

Abstract

Quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni* (QH-EDH) contains two cofactors, 2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione (PQQ) and haem *c*. Since previous studies on the kinetics of this enzyme suggested that both participate in electron transfer, spectroscopic investigations were performed of the oxidized and reduced holo- and apoenzyme (without PQQ but with haem *c*) to reveal the nature of the interaction between the two redox centres. From this it appears that the properties of the haem in the enzyme are affected by the presence of PQQ, as judged from the shift of the maxima in the ultraviolet/visible absorption spectra of the haem moiety in both reduced and oxidized QH-EDH and the 60 mV increase of the haem midpoint redox potential caused by PQQ addition. Also ^{1}H-NMR spectroscopy was indicative for interaction since binding of PQQ induced shifts in the resonances of the methyl groups of the porphyrin ring in the oxidized form of the apoenzyme and a shift in the methionine haem ligand resonance of the reduced form of the apoenzyme. On the other hand, resonance Raman spectra of the haem in the different enzyme forms were nearly similar. These results suggest that a major effect of PQQ binding to apo-QH-EDH is a rotation of the methionine ligand of haem c. Since no intermediate ^{1}H-NMR spectra were observed upon titration of apoenzyme with PQQ, apparently no exchange occurs of PQQ between (oxidized) holo- and apoenzyme at the NMR time scale and at that of the experiment. This is in agreement with the view that PQQ becomes tightly bound, the event leading to a compact enzyme conformation which is able to catalyze rapid intramolecular electron transfer.

Introduction

When grown on ethanol, *Comamonas testosteroni* produces quinohaemoprotein ethanol dehydrogenase (QH-EDH). Curiously, the enzyme is in its apo-form, that is it contains haem c but not PQQ because the latter compound is not produced by the organism. However, active holoenzyme can be obtained by adding PQQ to the apoenzyme in the presence of Ca^{2+} (Groen et al., 1986). QH-EDH oxidizes primary alcohols as well as aldehydes. Kinetic studies indicate that upon oxidation of an alcohol to its corresponding acid, the intermediate aldehyde formed is released from the enzyme after which it competes with alcohol for the enzyme (Geerlof et al., 1994a; Geerlof et al., 1994b). These studies also suggest that substrate oxidation occurs at the PQQ site after which the electrons are transferred one by one from $PQQH_2$ to the haem (Geerlof et al., 1994b). The intermediate required in this mechanism appears to be stable since the semiquinone form of PQQ ($PQQH^{\cdot}$) has been detected in this enzyme (de Jong et al., 1995). The question whether electron transfer from $PQQH_2$ or $PQQH^{\cdot}$ to ferri-haem *c* occurs *via* a long distance mechanism through the protein

or *via* a short one because the cofactors are close together, has not been answered yet. For that purpose, at least the nature of the interaction between the two cofactors should be known. Taking advantage of the fact that QH-EDH is obtained in its apo-form when cultivation is carried out in the absence of PQQ and reconstitution with PQQ to holoenzyme occurs easily, this should be feasible.

Recently some indications were obtained that the presence of PQQ indeed affects the properties of the haem since EPR spectroscopy revealed that a shift in the three g-values and a narrowing of the peaks of the low-spin haem *c* in the enzyme occurs upon PQQ binding (de Jong et al., 1995). To investigate the interaction further, several other spectroscopic techniques were applied to reduced and oxidized forms of the holo- and the apoenzyme.

Materials and methods

Apo-QH-EDH isolation

Apoenzyme was isolated as described (de Jong et al., 1995) from *Comamonas testosteroni* LMD 26.36 grown in a mineral medium on 1% (w/v) ethanol. Enzyme activities were measured using n-butanol as a substrate and potassium ferricyanide as an electron acceptor (Groen et al., 1986).

Reconstitution to holoenzyme

Reconstitution experiments were performed by adding 1 equivalent of PQQ to the apoenzyme in 20 mM MOPS/KOH, pH 7.5, containing 5 mM $CaCl_2$. After reconstitution, the preparation was chromatographed on a Mono-S column to separate holo-QH-EDH from non-reconstituted, inactive apo-QH-EDH (de Jong et al., 1995).

Ultraviolet/visible spectroscopy

Ultraviolet/visible absorption spectra were recorded on a Hewlett Packard HP 8452A, a SLM Aminco DW 2000 or a Shimadzu spectrophotometer (model 265FS). To obtain difference spectra of the oxidized or reduced enzyme, with and without PQQ, samples were oxidized by titration with potassium ferricyanide or reduced by addition of dithiothreitol or sodium ascorbate.

Midpoint redox potentials

Midpoint redox potentials were determined by means of potentiometric titrations, monitored optically, as described by Dutton et al (1971). The solution potential was measured with a Crison potentiometer equipped with Platinum (P1312 Radiometer) and Ag/AgCl (K8040 Radiometer) electrodes and is quoted relative to the normal hydrogen electrode. The following redox mediators were present at final concentrations of 5 μM: 1,4 naphtoquinone, methylene blue, triquat, phenosaphranine, benzylviologen, methylviologen, dichlorophenol-

indophenol, benzo-quinone, anthraquinone-2-sulfonic acid, phenazinemethosulfate, dimethyl-triquat, indigo tetrasulfonate, 2 hydroxy- 1,4-naphtoquinone, 5-hydroxy-1,4 naphtoquinone, duroquinone, phenazil and safranine. Solution redox potentials (in equilibrium) were varied by adding appropriate volumes of deaerated dithionite as reductant. All experiments were performed under an argon atmosphere (the gas passed through an Oxygen Trap from Chemical Research Supplies).

^{1}H-NMR spectroscopy

High-resolution ^{1}H-NMR spectra were recorded in the Fourier Transform mode on a Bruker AMX-300 spectrometer (300 MHz) equipped with a temperature control unit. The spectra were obtained by an exponential multiplication by 10 Hz line broadening of free induction decays prior to Fourier transformation to improve the signal to noise ratio. All chemical shift values are quoted in parts per million (ppm) from internal 3-trimethylsilyl-(2,2,3,3-$^2H_4$) propionate, positive values referring to low-field shifts. The samples in 20 mM MOPS/KOH, pH 7.5, containing 5 mM $CaCl_2$ were exchanged several times with the required buffer (prepared with 99.8% ^2H_2O) by centrifugation in a Centricon 10 microconcentrator (Amicon Co.). The enzyme concentrations varied from 0.5 to 1.7 mM. Oxidation of the enzyme was achieved by addition of potassium ferricyanide, reduction by addition of dithionite. Titration of apo-QH-EDH with PQQ was monitored at 293 K. The pH dependence of the haem resonances was studied over the range 5.6-9.4 at 293 K.

Resonance Raman spectroscopy

Resonance Raman spectra were recorded as described previously (Hurst et al., 1991; Loehr & Sanders-Loehr, 1993). The samples of the reduced apo- and holoenzyme were maintained near 0 °C in an ice water Dewar, whereas the oxidized samples were maintained at ~90 K in a liquid nitrogen Dewar during data collection (Loehr & Sanders-Loehr, 1993). The reduced samples were measured using 413.3-nm excitation, the oxidized samples using 514.5-nm excitation.

Results

Ultraviolet/visible absorption spectra

To study the effect of binding of PQQ on the optical spectrum of the haem c moiety in QH-EDH, the redox state of the haem must be the same in the absence as well as the presence of PQQ. This was achieved by addition of ferricyanide or dithiothreitol. Complete oxidation of the holo-QH-EDH with potassium ferricyanide required 20-100 equivalents depending on the preparation, caused by the presence of endogenous substrate (de Jong et al., 1995). Comparison of the reduced apo- and holoenzyme (Figure 1) revealed small shifts in the maxima and small increases in the intensity of all major (i.e. α, β, γ and δ)

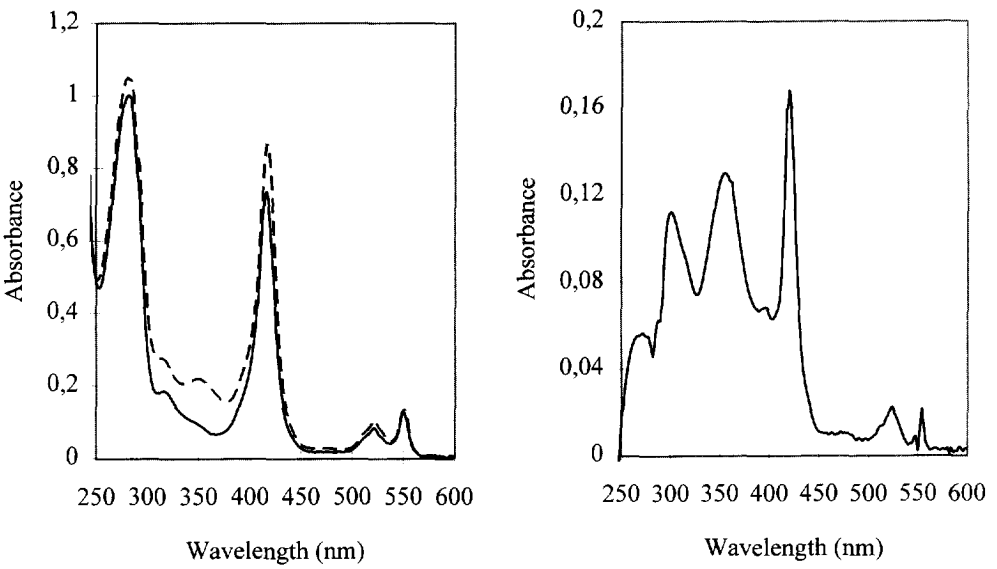

Figure 1. Ultraviolet/visible absorption spectra of reduced apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH 7.5, 5 mM $CaCl_2$. Reduction of apo-QH-EDH is performed by addition of 1 mM dl-dithiothreitol. Spectra were compared at equal concentrations using the specific absorption coefficients $A^{0.1\%}_{280nm}$ = 2.41 (apo-QH-EDH) and $A^{0.1\%}_{280nm}$ = 2.52 (holo-QH-EDH) (de Jong et al., 1995). (A) Ultraviolet/visible spectra of (——) apo-QH-EDH and (------) holo-QH-EDH. (B) Difference spectrum of the reduced holo-QH-EDH spectrum minus the reduced apo-QH-EDH spectrum.

cytochrome absorption bands upon binding of PQQ. In addition to changes in the peaks of the cytochrome, new broad absorbtion bands appear at 354 nm (Figure 1b) and 362 nm (Figure 2b) which are ascribed to bound $PQQH_2$ and bound PQQ, respectively. Evidence for these assignments is provided by the observation that further addition of dithionite, ascorbate, or dithiothreitol to the substrate-reduced holo-QH-EDH (Figure 1a), or further addition of ferricyanide to oxidized holo-QH-EDH (Figure 2a), did not change the optical spectra. The difference spectra of the holo- minus the apo-QH-EDH in the reduced and oxidized states were compared with the absorbance of free PQQ or $PQQH_2$ in the same buffer. No similarity was observed with either PQQ or $PQQH_2$, suggesting a change of the optical properties of the cofactor occurs upon binding to the enzyme. However, the larger molar absorption coefficient estimated for bound $PQQH_2$ as compared to that of bound PQQ and the blue shift occurring on reduction are properties similar to those of free PQQ (Duine & Frank, 1980). Significant differences between the optical spectrum of bound PQQ and free PQQ has also

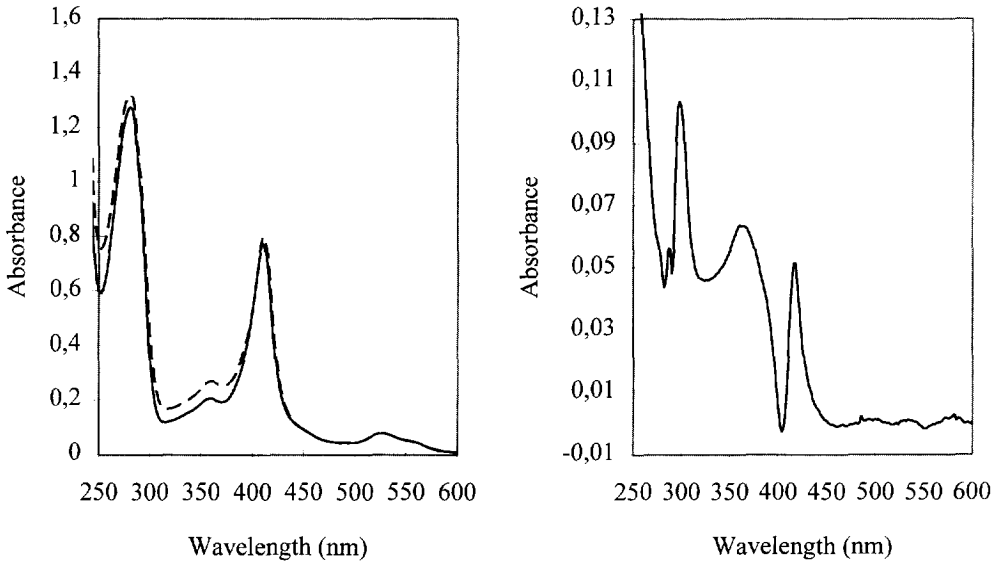

Figure 2. Ultraviolet/visible absorption spectra of oxidized apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH 7.5, 5 mM $CaCl_2$. Oxidation of holo-QH-EDH is performed by addition of ferricyanide. Spectra were compared at equal concentrations using the specific absorption coefficients $A^{0.1\%}_{280nm}$ = 2.41 (apo-QH-EDH) and $A^{0.1\%}_{280nm}$ = 2.52 (holo-QH-EDH) (de Jong et al., 1995). (A) Ultraviolet/visible spectra of (——) apo-QH-EDH and (-------) holo-QH-EDH. (B) Difference spectrum of the oxidized holo-QH-EDH spectrum minus the oxidized apo-QH-EDH spectrum.

been reported in the case of the PQQ-containing methanol dehydrogenases (Duine et al., 1981) and glucose dehydrogenases (Dokter et al., 1986).

The α band is shifted from 550.6 nm (apo) to 552.0 nm (holo), the γ band is shifted from 416.3 nm (apo) to 417.6 nm (holo) and the δ band from 316 nm (apo) to 314 nm (holo). Comparison of the cytochrome spectra in oxidized apo- and holoenzyme due to PQQ binding (Figure 2) mainly showed a shift in the γ band, from 410 nm (apo) to 412 nm (holo).

Midpoint redox potentials

The midpoint oxidation-reduction potential of the haem c in QH-EDH at pH 7.5 was obtained by following the absorbance of the α band during titration (Figure 3). A midpoint potential of +80 mV for the haem c in the apoenzyme and +140 mV for the holoenzyme was determined. In both cases haem c titrated with a value of n=1.

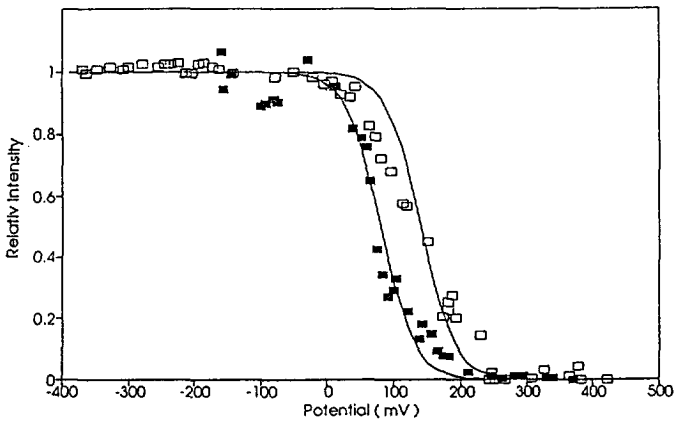

Figure 3. Determination of the midpoint redox potentials of apo- and holo-QH-EDH. Measurements were performed as described in the Materials and Methods section. (□) apoenzyme; (■) holoenzyme. The relative intensity projected on the y-axis is the increase in absorbance of the α band divided by the maximal increase of the α band upon total reduction of the haem c.

^{1}H-NMR spectroscopy of the reduced apo- and holo-QH-EDH at 293 K

^{1}H-NMR spectra were measured of reduced apo- and holo-QH-EDH at 293 K to investigate the nature of the axial ligands of the haem and the effect of PQQ binding on them. The high-field part of the spectra are shown in Figure 4. Apo-QH-EDH has resonances at -1.89 and -2.87 ppm and the holoenzyme at -1.52 and -2.72 ppm. The resonances observed outside the main absorption envelope in this region are assigned to the γ-methylene and ϵ-methyl protons of the haem-bound methionine (cf. Senn and Wütrich, 1983).

^{1}H-NMR spectroscopy of the oxidized apo- and holo-QH-EDH at 303 K

^{1}H-NMR spectra were measured of oxidized apo- and holo-QH-EDH at 303 K to investigate the effect of PQQ binding on the haem resonances (Figure 5). The low field part of the spectrum of oxidized apo-QH-EDH clearly shows all four resonances, each of three-proton intensity, from the protons belonging to the methyl groups on the porphyrin ring (cf. La Mar, 1979). The spectrum of oxidized holo-QH-EDH shows only 3 resonances due to overlap of two of the haem methyl resonances, leading to a single peak whose intensity accounts for 6 protons. All resonances shift substantially as a result of PQQ binding (Table 1), the shifts varying between 2.3 ppm upfield to 7.5 ppm downfield. Comparison of the spectra of apo- and holo-QH-EDH reveals much smaller linewidths for the latter. The resonance belonging to the three ϵ-methyl protons of the methionine ligand is located at -12.6 ppm for the apoenzyme and at -13.5 ppm for the holoenzyme.

Table 1. ^{1}H-NMR resonances from the haem methyl groups of oxidized apo- and holo-QH-EDH.

Samples of apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH* 7.5, 5 mM $CaCl_2$ were oxidized by titration with potassium ferricyanide. Chemical shift values are quoted in ppm from internal 3-trimethylsilyl-(2,2,3,3-$^2H_4$) propionate. Assignments of peaks 1 to 4 are based on Figure 5.

	apo-QH-EDH		holo-QH-EDH	
	293 K	303 K	293 K	303 K
	peak position (ppm)			
peak 1	37.0	34.1	43.7	41.6
peak 2	26.0	25.2	23.0	22.9
peak 3	23.0	23.1	22.7	22.9
peak 4	15.0	13.6	13.4	13.9

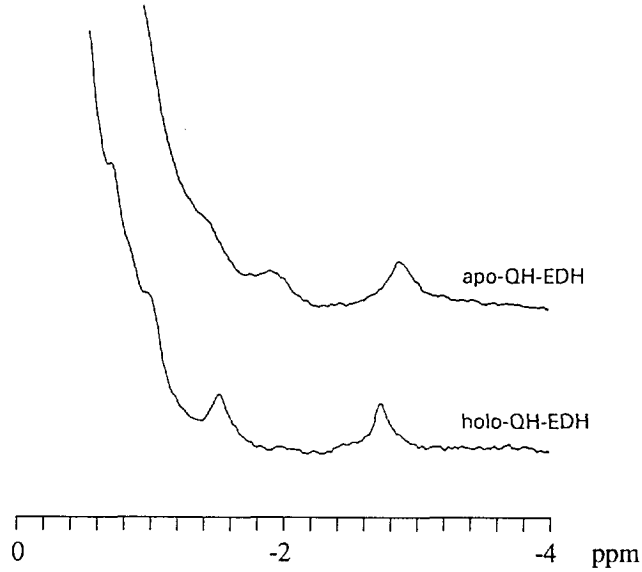

Figure 4. ^{1}H-NMR spectroscopy of the reduced apo- and holo-QH-EDH at 303 K. Samples of apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH* 7.5, 5 mM $CaCl_2$ were reduced by titration with dithionite. Holo-QH-EDH was separated from non-reconstitutable apo-QH-EDH by Mono-S column chromatography (de Jong et al., 1995). * Quoted pH values are meter readings uncorrected for the isotope effect.

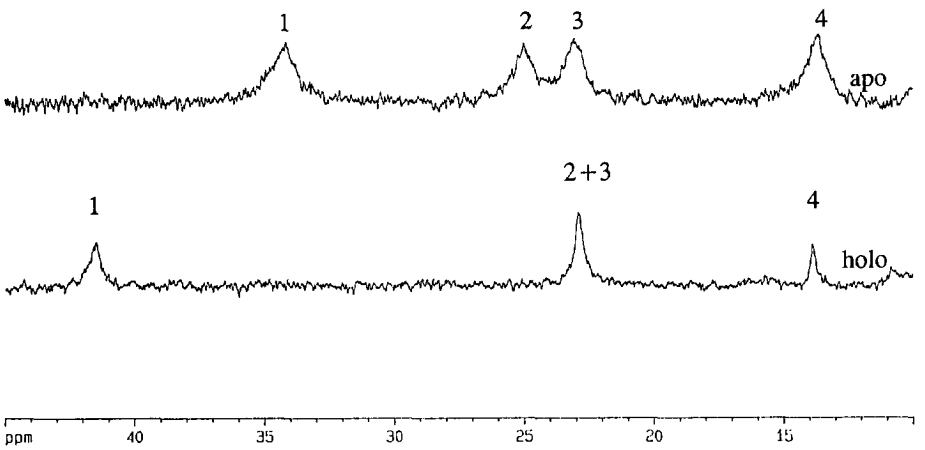

Figure 5. ^{1}H-NMR of the oxidized apo- and holo-QH-EDH at 303 K. Samples of apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH* 7.5, 5 mM $CaCl_2$ were oxidized by titration with potassium ferricyanide. Holo-QH-EDH was separated from non-reconstitutable apo-QH-EDH by Mono-S column chromatography (de Jong et al., 1995). * Quoted pH values are meter readings uncorrected for the isotope effect.

^{1}H-NMR measurement of a titration of oxidized apo-QH-EDH with PQQ

The effect of PQQ on the haem-methyl protons was also studied by titrating oxidized apo-QH-EDH with aliquots of PQQ. The enzyme was kept in the oxidized state during titration by the addition of excess potassium ferricyanide. ^{1}H-NMR measurements were performed at 293 K because the apo-QH-EDH slowly denatured at 303 K. During titration, the broad peaks of the oxidized apo-QH-EDH are converted into the sharper peaks of the oxidized holo-QH-EDH without the appearance of intermediate peaks, suggesting that there is no exchange of PQQ in the NMR time scale between the apo- and holoform, in agreement with column chromatography (de Jong et al., 1995). It was further observed that after addition of about 0.6-0.7 PQQ/apoenzyme, the NMR spectrum no longer changes, see e.g. the persistent presence of the residual peak at 37.0 ppm in Figure 6. This finding is in agreement with our previous results (de Jong et al., 1995) showing that the apoenzyme contains a non-reconstitutable fraction of about 30%. Indeed, the ^{1}H-NMR spectrum of holo-QH-EDH prepared by means of Mono-S chromatography to remove this fraction does not show the peak at 37.0 ppm (Figure 5). In contrast to the measurement at 303 K discussed above, the resonances of the four methyl groups of the porphyrin in the oxidized holo-QH-EDH are now all well resolved. The chemical shift values are summarized in Table 1.

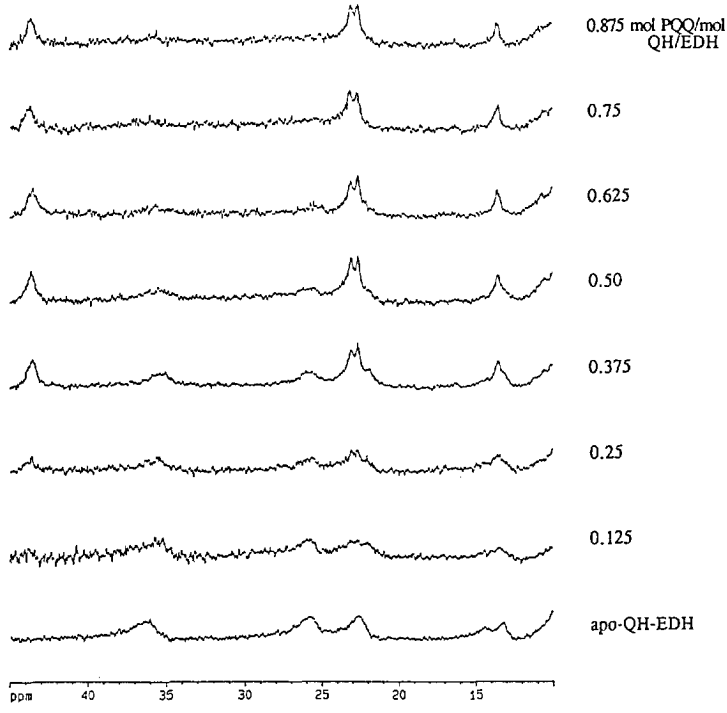

Figure 6. ^{1}H-NMR spectroscopy of the titration of the oxidized apoenzyme with PQQ at 293 K. Apo-QH-EDH in 20 mM MOPS/KOH, pH* 7.5, 5 mM CaCl2 was oxidized by titration with potassium ferricyanide. Every addition of PQQ corresponds to 0.125 equivalent based on the concentration of the apoenzyme. * Quoted pH values are meter readings uncorrected for the isotope effect.

^{1}H-NMR measurement of a pH titration of oxidized holo-QH-EDH

To exclude interference of pH effects of the comparisons (a slight drop in pH occurs upon removal of endogenous substrate when oxidized holo-QH-EDH is prepared), ^{1}H-NMR spectra were taken of oxidized holo-QH-EDH at various pH values. Examination of the ^{1}H-NMR spectra of the oxidized holo-QH-EDH at different pH values did not show the drastic changes in chemical shift values compared to the changes observed upon binding of PQQ to the apoenzyme. Chemical shifts as a function of the pH* are shown in Figure 7 for the two middle resonances. The changes of the chemical shifts of the haem methyl resonances were fitted by a one-proton titration curve. This yielded pK_a values of 7.9 ± 0.2 (resonance 2) and 8.2 ± 0.4 (resonance 3).

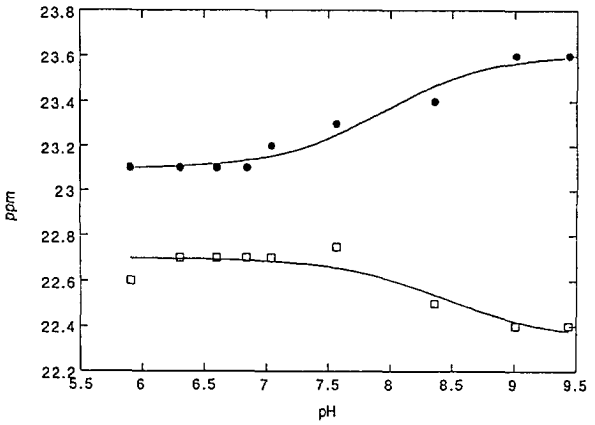

Figure 7. ^{1}H-NMR of oxidized holo-QH-EDH at varying pH and at 293 K. pH* titration of the haem methyl resonances (2(□) and 3(●), Figure 5). Data points are indicated by symbols; the solid lines correspond to theoretical one-proton titration curves with p*Ka*= 7.9 ± 0.2 (resonance 2), p*Ka*= 8.2 ± 0.4 (resonance 3). *Quoted pH values are meter readings uncorrected for the isotope effect.

Resonance Raman spectroscopy

Figures 8 and 9 show the resonance Raman spectra of the reduced forms of the apo- and holo-QH-EDH, respectively, using 413.1-nm excitation. The two spectra are very similar. The peak positions of various bands are all within 3 cm^{-1}, indicating little or no changes in bond lengths and/or force constants within the porphyrin system. The spectra are very similar to those of cytochrome *c* (cf. Hu et al., 1993). The finding that the 688 cm^{-1} (apo) or 689 cm^{-1} (holo) band which is predominantly a C_a-S stretching mode, differ by only 1 cm^{-1} indicates that the binding of PQQ does not affect the C_a-S bond strength. Figure 10 shows the resonance Raman spectra of the oxidized forms of the apo- and holo-QH-EDH in the high frequency region using 514.5 nm excitation. Measurement with Soret excitations (413.1 and 406.7 nm) was not possible since excitations in this region caused photoreduction of the oxidized samples, as was indicated by the oxidation state marker υ_4 at 1375 cm^{-1} (oxidized) and at 1360 cm^{-1} (reduced). Bands in the high frequency region are most characteristic of the haem group being in a low spin, hexa-coordinate state in both forms of the protein. The porphyrin skeletal modes observed with Q-excitation (υ_{10}, υ_{11}, υ_{19}), which reflect the bond strengths around the porphyrin ring, show no significant frequency shifts due to PQQ binding. All other porphyrin skeletal modes observed are typical of oxidized cytochromes *c*.

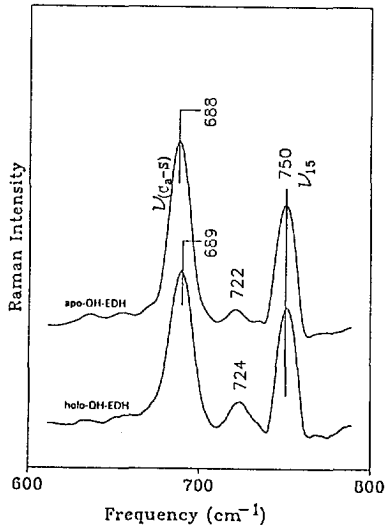

Figure 8. Resonance Raman spectra (600-800 cm^{-1}) of reduced apo- and holo-QH-EDH, with laser excitation at 413.1 nm, and at 273 K.

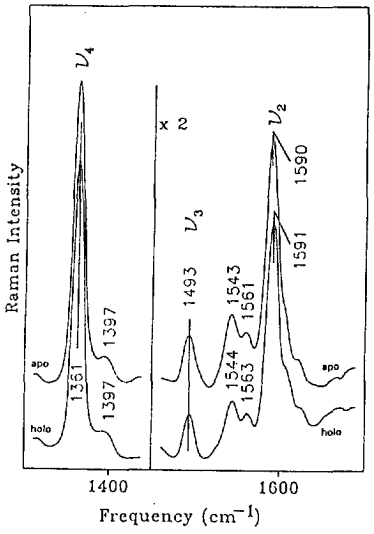

Figure 9. Resonance Raman spectra (1300-1700 cm^{-1}) of reduced apo- and holo-QH-EDH, with laser excitation at 413.1 nm, and at 273 K.

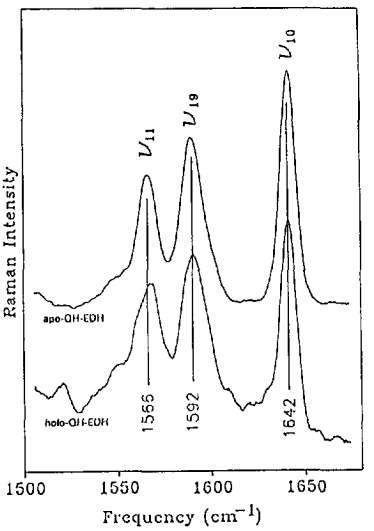

Figure 10. Resonance Raman spectra (1500-1700 cm^{-1}) of oxidized apo- and holo-QH-EDH, with laser excitation 514.5 nm, and at T= 90 K.

Discussion

As previously reported (de Jong et al., 1995), the 695 nm band observed in the ultraviolet/visible absorbtion spectrum of oxidized apo-QH-EDH suggests that the haem has a histidinyl-methionyl-Fe coordination. The ^{1}H-NMR spectra of reduced apo- and holo-QH-EDH (Figure 4) clearly confirm that the methionyl residue is the sixth ligand. Furthermore, the relatively sharp signals and narrow isotropic shifts in the spectra indicate that Fe^{3+} is in the low-spin state (Bertini & Luchinat, 1986). The resonance Raman spectra of reduced and oxidized enzyme also confirm the low-spin, hexacoordination state of the haem.

The broad signals between 15 and 37 ppm downfield of the internal standard in the ^{1}H-NMR spectra of the oxidized apoenzyme are attributed to the four haem methyl groups (La Mar, 1979). Upon titration with PQQ these are transformed into the relatively sharp resonances of the haem methyl groups in the oxidized holo-QH-EDH. Since no intermediate forms are observed, apparently no exchange of PQQ occurs at the NMR time scale and at that of the experiment.

The pK_a value observed for the methyl resonances in ^{1}H-NMR spectra of haem *c*-containing proteins is ascribed to that of the most buried propionic side chain of the porphyrin ring (Chao et al., 1979). Since from the spectra of holo-QH-EDH at varying pH

a pK_a value of 8 is deduced for the two middle haem methyl resonances, this extremely high value must be explained by assuming that the side chain is buried in a very hydrophobic environment.

Resonance peak 1 (Fig. 5 and 6) shifts substantially upon binding of PQQ, suggesting an increase of the paramagnetic contribution of the haem. However, this is in contradiction with the decrease in linewidth, suggesting a decrease of this contribution. Two possibilities are proposed to explain the discrepancy. First, binding of PQQ leads to additional binding interactions, resulting in a more compact structure of the enzyme molecule as a whole. Second, binding of PQQ makes the haem-containing domain more independent from other domains so that its rotation speed becomes faster. At present it is not possible to decide which hypothesis is correct. The tentative conclusion is that PQQ binding induces a conformational change, as was already suggested before from the differences between the chromatographic behaviour and the EPR signals of apo- and holoenzyme (de Jong et al., 1995).

Normally, the resonances of the four methyl groups in low-spin ferricytochromes are grouped together in two distinct pairs separated by 10 to 20 ppm (Turner, 1993). The most downfield pair is situated between 38 and 27 ppm and the relatively upfield pair between 17.5 and 7.2 ppm (Timkovich et al., 1984). It has been suggested that such a situation originates from two diagonally opposite pyrrole rings having similar distributions of the unpaired electron but dissimilar from the other pair. However, although several suggestions have been made (Timkovich et al., 1994) no clear answer has been given as to what causes the shifts of the methyl group resonances. The more dispersed pattern of the methyl resonances of QH-EDH suggest a different pattern of electron distribution. The high value of the methyl group (43.7 ppm) observed at 293 K in the holoenzyme is lower in the apoenzyme (37.0 ppm, assuming that the four methyl resonances are lined up in the same order for both enzyme forms). This indicates that the spin density on the pyrrole ring closest to that methyl group increases upon PQQ binding.

Substantial effects on the haem properties resulting from reconstitution have also been observed in the case of p-cresol methyl hydroxylase (McLendon et al., 1991). This enzyme consists of two identical flavoprotein subunits and two identical cytochrome *c* subunits which can be separated from each other and isolated in intact form (Shamala et al., 1986). Binding of the flavoprotein to the cytochrome subunit causes a shift of the methyl resonances up to 8 ppm and a strong increase of the midpoint potential of the cytochrome. Based on X-ray crystallographic data for the intact protein and the NOESY spectral data of the reduced cytochrome *c* subunit, it has been suggested that a reorientation of the axial methionyl residue occurs by a rotation of ~ 180 degrees around the $C\gamma$ -$S\delta$ bond upon binding of the flavoprotein subunit to the cytochrome subunit (McLendon et al., 1991). The reorientation is accompanied by a redistribution of the electron density in order to accommodate the new bonding, reflected by the shifts in the methyl resonances. Since similar phenomena occur upon binding of PQQ to apo-QH-EDH, rotation of the methionyl residue may also take place

in this enzyme.

The similar resonance Raman spectra of the different enzyme forms indicate that no significant changes in the porphyrin bonds and the C_a-S (cysteine) bonds occur (Hu et al., 1993). It also confirms that the shifts observed in the NMR spectra are not caused by ring current changes but possibly by a rotation of the methionine ligand. Resonance Raman spectroscopy does not readily detect changes of axial ligands to the haem, at least when they do not affect the spin state (Loehr & Loehr, 1973; Spiro, 1983). Furthermore, the axial ligand methionine makes no determinable contribution to the resonance Raman spectrum of cytochromes *c* in either oxidation state (Hu et al., 1993).

Our results indicate that binding of PQQ to apo-QH-EDH induces a conformational change of 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 of the 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 still unclear whether the interaction between the two cofactors is direct or indirect. Forthcoming information on the 3-dimensional structure of the enzyme may shed light on this.

Undoubtedly, the phenomena observed are related to the function of the enzyme, that is to catalyze efficient oxidation of alcohols and to transfer electrons rapidly, internally between PQQ and haem and externally from haem *c* to its unknown natural electron acceptor. Information on this may also be relevant for other quinoproteins, e.g. methanol dehydrogenase from methylotrophic Gram-negative bacteria, only containing PQQ and using cytochrome c_L as its (external) electron acceptor (Day & Anthony, 1990), and ethanol dehydrogenase from *Pseudomonas aeruginosa*, only containing PQQ and transferring its electrons to cytochrome c_{EDH} (Schrover et al., 1993). In this connection it should be mentioned that part of the amino acid sequence of QH-EDH is very similar to that of methanol dehydrogenase (including the two vicinal cysteines and the tryptophan sandwiched to PQQ, unpublished results) and that methanol dehydrogenase and ethanol dehydrogenase have similar properties. Thus, QH-EDH, probably being a fusion of the dehydrogenase and the electron-accepting cytochrome *c*, and obtainable in the apo- as well as the holo-form, seems an attractive model enzyme for the comparative studies on intramolecular and intermolecular electron transfer of similar systems.

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Chapter 4

Constraints on the intramolecular distance of the haem c and PQQ redox centres of the quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni*, as deduced from ^{19}F-NMR relaxation studies

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Abbreviations

EPR, electron paramagnetic resonance; ^{19}F-NMR, Fluorine nuclear magnetic resonance; ^{1}H-NMR, Proton nuclear magnetic resonance; HPLC, high pressure liquid chromatography; MDH, methanol dehydrogenase; PAO, pig plasma amine oxidase; PQQ, 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione; $PQQH_2$, quinol form of PQQ; QH-EDH, quinohaemoprotein ethanol dehydrogenase; TPQ, topaquinone; TFEH, trifluoroethylhydrazine; TFEH-PQQ, trifluoroethyl hydrazone of PQQ; TFMPH, trifluoromethylphenylhydrazine; TFMPH-PQQ, trifluoromethylphenyl hydrazone of PQQ; TTQ, tryptophyltryptophan quinone; T_1, longitudinal relaxation time; T_2, transversal relaxation time, 3D, 3-dimensional.

Abstract

Quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni* (QH-EDH) contains PQQ and haem c. Previous studies on the kinetics of this enzyme suggested that both cofactors participate in electron transfer. Spectroscopic investigations of the oxidized and reduced holo- and (PQQ-free) apoenzyme showed that the properties of the haem are affected by the presence of PQQ. In anticipation of the results of X-ray crystallography, the intramolecular separation of the cofactors was probed by studying the distance-dependent effects of the haem redox state on the NMR-properties of suitable reporter groups attached to PQQ. Following addition of an equal amount of trifluoromethyl-substituted alkyl- and phenyladducts of PQQ to QH-EDH apoenzyme, the reconstituted enzyme was subjected to ^{19}F-NMR spectroscopy. Oxidation of the low-spin diamagnetic ferro-haem to the paramagnetic ferric state did not lead to substantial broadening of the fluorine resonance signal in the spectra of these complexes. A minimal value on the intramolecular distance between the PQQ binding site and the haem c position was deduced from the Solomon-Bloembergen equations for distance-dependent paramagnetic relaxation processes. It is concluded that the fluorine and iron atoms in the haemoprotein-hydrazone complex are separated by at least 5Å. On the basis of these findings, close proximity of haem c and PQQ in QH-EDH holoenzyme cannot, however, be ruled out.

Introduction

Quinohaemoprotein ethanol dehydrogenase

Quinohaemoprotein ethanol dehydrogenase, QH-EDH, from *Comamonas testosteroni* catalyses the oxidation of primary alcohols to the corresponding aldehydes and the (subsequent) oxidation of aldehydes to carboxylic acids. The enzyme is routinely isolated as the inactive PQQ-free apoenzyme from cells grown on ethanol in the absence of added PQQ. Active holoenzyme can then be obtained by addition of equimolar amounts of PQQ in the presence of Ca^{2+} (Groen et al., 1986; de Jong et al., 1995a). Alternatively, holoenzyme can be isolated directly from cells grown on ethanol in the presence of PQQ.

Kinetic studies (Geerlof et al., 1994a; Geerlof et al., 1994b) suggest that substrate oxidation occurs at the PQQ site after which the electrons are transferred to the haem from which they are released to the electron acceptor. Turn-over rates for the reduction of ferricyanide during the QH-EDH-catalysed oxidation of ethanol are found to be 35 per second. Current theories of electron transfer processes between redox sites embedded in a protein matrix predict rates to be governed primarily by the difference in mid-point potentials, the reorganization of the sites on changing redox state, as well on the physical separation of the sites involved (Canters & van den Kamp, 1992). In the absence of pertinent

data on the contribution of the electron transfer steps to the rate of the over-all hexa-uni ping-pong kinetics of QH-EDH, a 20 Å intramolecular separation of haem c and PQQ could be considered an upper limit. Recent studies of the oxidized and reduced forms of QH-EDH apo- and holo-enzyme (de Jong et al, 1995b), on the other hand, showed that the properties of the haem in the enzyme are affected by the presence of PQQ. UV/visible spectra of both reduced and oxidized apo- and holoenzyme show a discrete shift of the haem absorption maxima. ^{1}H-NMR resonances of the methyl groups of the porphyrin ring are shifted upon introduction of PQQ into the oxidized form of the apoenzyme, while a shift in the methionine haem ligand resonance is observed for the reduced form. Also, a 60 mV increase of the haem mid-point redox potential occurs on addition of PQQ. Resonance Raman spectra of the haem in the different enzyme forms are seen to be hardly affected, however. These results have been interpreted to reflect a major effect of PQQ-binding to apo-QH-EDH on the rotation of the methionine ligand of haem c. It is concluded that PQQ becomes tightly bound, leading to a compact enzyme conformation that is capable of rapid intramolecular electron transfer. Pending the X-ray crystallographic determination of the three-dimensional structure of QH-EDH$_I$, homology modelling of QH-EDH$_I$ based on the 30% sequence homology of QH-EDH$_I$ and related quinoprotein methanol dehydrogenases has been attempted. Preliminary results suggest energy-minimized conformations to adopt a two-domain topology carrying the haem c and PQQ-binding domains at the opposite poles of a dumb-bell shaped protein. This topology is also suggested by a recent homology-based model of the QH-EDH$_{II}$ from *Acetobacter* (Cozier et al., 1995). This enzyme shows high sequence homology to QH-EDH$_I$. In order to reconcile the lack of conformational coupling that is suggested by the homology-based models, with the observed effect of PQQ addition on the conformation of the haem environment, it might be considered that the two domains are folded back onto each other. Since this would bring the haem and PQQ sites closer together, we decided to investigate the possibility to obtain an estimate of the spatial separation of the haem and PQQ-sites.

Effects of paramagnetic interactions on NMR relaxation rates

An elegant way to establish the distance and orientation of the organic cofactor and paramagnetic Cu(II) has been described by Williams & Falk (1986) for plasma amine oxidase, a copper containing quinoprotein. Labelling of the organic cofactor topaquinone was performed using fluorine substituted hydrazines. Measuring the effect of the paramagnetic Cu(II) on the relaxation time T_1 and T_2 by ^{19}F-NMR allowed the distance between the copper and the cofactor to be estimated. A similar approach might be applicable to the cofactors present in QH-EDH: PQQ and haem c.

Cytochromes *c* can exist in two physiologically important oxidation states. The reduced form is diamagnetic [Fe(II), low spin d^6, S=0], while the oxidized form is paramagnetic [Fe(III), low spin d^5, S=½] (Gao et al., 1991). The presence of a paramagnetic centre in

oxidized cytochrome *c* causes complications not found in other systems when measuring one-dimensional (1D) and two dimensional (2D) NMR (Feng et al., 1989). Chemical shift criteria can not always be used to assist assignment and the quality of the spectra is in some cases degraded by paramagnetic broadening. However, additional structure information can be obtained from resonance line widths and chemical shifts caused by the interaction between the resonating nuclei and the unpaired electrons residing on the metal ion (Canters et al.,1993). The paramagnetic shift is defined as the difference between the actual chemical shift and the shift of an "analogous" diamagnetic compound (Bertini et al.,1989). These paramagnetic shifts may be dipolar (pseudocontact) or scalar (Fermi contact) in nature. The Fermi contact describes the way in which spin density can be delocalized through covalent bonds into orbitals of a particular nucleus giving rise to an interaction between electronic spin and nucleus. As spindensity of the paramagnetic Fe(III) ion in haem can be delocalized through π bonds it is commonly assumed that Fermi contact shifts are limited to protons on the haem and its immediate ligands (Feng et al., 1990). The contact contribution in the ideal case of a single populated level for the iron is given by equation 1 (La Mar, 1979)

$$\frac{\Delta\nu}{\nu_0} = -A\frac{g\beta S(S+1)}{3(\gamma/2\pi)kT} \qquad (1)$$

where $\Delta v/v_0$ is the frequency shift relative to the spectrometer frequency, A is the hyperfine coupling constant, γ is the nuclear magnetogyric ratio, S is the electron spin quantum number, g is the spectroscopic splitting factor, β is the Bohr magneton, k is the Boltzmann constant, and T is the absolute temperature. The contact shift is independent of the magnetic field.

The protons that are not directly associated with the haem, sense only the dipolar contact, which arises from a through space dipolar interaction between the nucleus and the unpaired electron. In contrast to the Fc interaction, the dipole-dipole interaction is anisotropic, that is, its magnitude and sign depends on the orientation of the vector connecting nucleus and metal with respect to the magnetic field. For nuclei that are more then a few bonds away from the paramagnetic centre, their interaction with the unpaired electron is purely dipolar and depends on geometrical functions; thus providing important structural information (Xavier et al., 1993). The dipolar shifts are given in a simplified model by the following equation

$$\frac{\Delta V}{V_0} = \frac{1}{4\pi 3r^3}(3\cos^2\theta - 1)(\chi_{\parallel} - \chi_{\perp}) \qquad (2)$$

where $\chi_{\parallel} - \chi_{\perp}$ is the magnetic susceptibility anisotropy, r is the length of the vector joining

the metal and the resonating nucleus, and θ is the angle between the electron nuclear vector and the z-axis of the magnetic susceptibility tensor. In the S=1/2 spin state, the χ values are proportional to the square of the g values obtained from EPR to first order approximation. (Bertini et al., 1989). Equation 2 then becomes

$$\frac{\Delta V}{V_0} = \frac{\mu_0}{4\pi}\frac{\mu_b^2 S(S+1)}{9kTr^3}(3\cos^2\theta - 1)(g_{\parallel}^2 - g_{\perp}^2) \qquad (3)$$

where μ_0 is the permeability of vacuum, $g_\perp = g_z^2 - \frac{1}{2}(g_x^2 + g_y^2)$, $g_\parallel = g_x^2 - g_y^2$. The error in using g values rather then the χ values is estimated to range from 10 to 15 % (Bertini and Luchinat, 1986).

Protons in the vicinity of a paramagnetic metal ion experience large shifts which are of contact or pseudocontact origin, or both. The difficulty in separating the two shift mechanisms and the lack of a precise knowledge of the orientation of the electronic g tensor with respect to the molecular framework make it difficult to investigate distances between the centres under investigation and the metal ion (Dahlin et al., 1989) An alternative way of providing distance information is by measuring the longitudinal and transverse relaxation times. The Solomon-Bloembergen equations describe the relation of the longitudinal and transverse relaxation as a function of the distance (Villafranca, 1982). The Solomon Bloembergen equations are given in 4 and 5,

$$\frac{1}{T_{1,m}} = \frac{2}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{\omega_I^2\tau_c^2}\right) + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2}\right) \qquad (4)$$

$$\frac{1}{T_{2,m}} = \frac{2}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{\omega_I^2\tau_c^2}\right) + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2} + \tau_e\right) \qquad (5)$$

where γ_I is the magnetogyric ratio of the nuclear spin I, g is the Lande g factor of the electron, S is the electron spin, β is the Bohr magneton, r is the distance of separation of the nucleus and the paramagnetic centre, ω_s and ω_I are the electronic nuclear Larmor precession frequencies and A is the hyperfine coupling constant. The relaxation rate of the nucleus under observation is directly related to the correlation time of those processes which modulate the dipolar and scalar interaction (Mildvan, 1972). The first term in Equation 4 and 5 arises from dipolar interaction and are dependent on the correlation time τ_c, the second term arise from scalar hyperfine interaction, dependent on the electronic correlation time, τ_e. The linewidth $(\pi T_2)^{-1}$ is subject to an additional Curie spin term, this however, can be considered negligible for the low spin systems of interest here (Unger et al., 1985). In cases

were the scalar contribution can be neglected compared to dipolar interaction, calculation of the distance of the paramagnetic centre to the nuclei can be calculated from the linear dependence of T^{-1} to r^{-6}. In equation 6 and 7 the correlation times are expressed mathematically.

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_m} \tag{6}$$

$$\frac{1}{\tau_e} = \frac{1}{\tau_s} + \frac{1}{\tau_m} \tag{7}$$

where τ_r is the rotational correlation time, τ_m is the exchange lifetime and τ_s is the electronic correlation time. In the system under investigation the exchange of fluorine-labelled PQQ-derivative is very low, therefore $1/\tau_m \approx 0$. This reduces equation 7 to $1/\tau_e = 1/\tau_s$. Substituting into equation 6 yields $1/\tau_c = 1/\tau_e + 1/\tau_r$

Measurement of the paramagnetic contribution to $1/T_1$ (or $1/T_2$) is performed by subtracting the relaxation rate of a diamagnetic control from the total relaxation time (Villafranca, 1989)

$$\frac{1}{T_{1p}} = \frac{1}{T_{1(total)}} - \frac{1}{T_{1(diamagnetic)}} \tag{8}$$

Materials and Methods

Materials

All chemicals were from commercial suppliers, except for PQQ, prepared as described by Corey & Tramontano (1981), and the trifluoromethylphenylhydrazines (TFMPH), prepared as described by Forbes et al., (1960).

Preparation of the PQQ-derivatives

C(5)-hydrazones of PQQ and trifluoromethylphenylhydrazines (TFMPHs) were prepared by adding a slight excess of the hydrazine (0.1 M in methanol) to a saturated solution of PQQ in 1-propanol at 25 °C. The suspension was stirred for 24 hours at 25 °C. The solvent was removed by evaporation and the yellow solid was dissolved in 1 ml methanol. 50 ml of a solution of 10 mM K_2HPO_4, 10 mM NH_4Cl, pH 7 was added and the solution was passed

through a Sep-pak C_{18} cartridge equilibrated with 10 mM K_2HPO_4, 10 mM NH_4Cl, pH 7. After washing with 10 mM K_2HPO_4, 10 mM NH_4Cl, pH 7, the yellow coloured compound was eluted with methanol. The methanol was evaporated until 1 ml remained, 50 ml of 10 mM HCl was added and the solution was passed through a Sep-pak C_{18} cartridge equilibrated with 10 mM HCl. After washing with 10 mM HCl, the yellow coloured compound was eluted with methanol. The methanol was evaporated and the yellow solid was dissolved in 20 mM MOPS/KOH, pH 7.5. Purity of the compounds was checked by reversed phase HPLC, performed on a Waters HPLC system with a 5 μm C_{18} reversed phase cartridge. The eluent (flow rate 1.5 ml/min) consisted of a linear gradient (8 min) of 0-60 % acetonitrile in 0.2 % trifluoroacetic acid. The eluate was monitored with a Hewlett-Packard 1040A photodiode-array detector, taking absorption spectra of the eluted peaks, upslope, at the top, and downslope, to check homogeneity and to establish identity. The C(5)-hydrazone of PQQ and trifluoroethylhydrazine (TFEH) was prepared by adding a slight excess of the hydrazine (0.1 M in H_2O) to a 50 μM solution of PQQ in H_2O at 25 °C. The suspension was stirred for 24 hours at 25 °C. Purification was performed with a Sep-pak C_{18} cartridge in the same way as described above. PQQ-glycine was synthesized according to van Kleef et al. (1989).

Ultraviolet/visible spectra

Ultraviolet/visible spectra of PQQ derivatives and apoenzyme were measured on a Hewlett Packard 8524A Diode array spectrometer at 20 °C

Enzyme assay

Activities were measured according to the procedure described by Groen et al. (1986) using potassium ferricyanide (1mM) as electron acceptor in 20 mM MOPS/KOH, pH 7.5, containing 5 mM $CaCl_2$ (buffer A).

Inactivation experiments

Holo-QH-EDH was incubated with 1 equivalent of phenylhydrazine, semicarbazide and o-TFMPH in buffer A at 20 °C to derivatize the PQQ in the enzyme. After incubation for 1 hour activities were measured. After removal of the unbound reactants from the enzyme by a Pharmacia PD10 column, activities were measured again. To convert the PQQ in holo-QH-EDH into an oxazole, the holo-QH-EDH was diluted in 0.1 M glycine, pH 7.5, containing 5 mM $CaCl_2$ and kept for 24 hours at 20 °C. Spectra and activities were measured every hour.

Reconstitution experiments

Reconstitution of the apoenzyme with the PQQ-derivatives were performed in buffer A for 1 hour at 4 °C. Excess and unbound derivatives were removed with a Pharmacia PD10 column. To check binding and ultraviolet/visible properties, the reconstituted enzyme was applied to a Mono-S (Pharmacia, HR 16),of which the eluate was monitored with a Hewlett-

Packard 1040A photodiode-array detector. The protein was eluted with a linear gradient of 0-0.2 M NaCl in buffer A at a flow rate of 1.5 ml/min within 1 hour. Comparing the ultraviolet/visible spectra of the holo-QH-EDH with the apo-QH-EDH and the change in retention time can reveal if the derivative has bound (de Jong et al., 1995a). A second method used to check reconstitution, is to digest the formed holoenzyme with pronase E (Boehringer) at 40 °C for 6 hours. Subsequently, the solution is brought to pH 2.0 with 1 M HCl and passed through a sep-pak C_{18} cartridge equilibrated with 10 mM HCl. After washing with 10 mM HCl, the PQQ-derivative was eluted with methanol. The identity of the PQQ-derivative was checked by comparison of the retention time and ultraviolet/visible spectra with the authentic adduct on a HPLC reverse phase separation with photo-diode array detector as described (preparation of the PQQ-derivatives).

NMR measurements

^{19}F-NMR was performed on fluorine labelled PQQ-derivatives in buffer A on a Bruker spectrometer, operating at 400 MHz, using CCl_3F as an internal reference. These ^{19}F-NMR spectra were measured to see if the PQQ-derivatives made by reacting PQQ with the fluorine-substituted hydrazines still contained a fluorine group.

^{19}F-NMR was performed on holo-QH-EDH containing fluorine labelled PQQ derivatives in buffer A on a Bruker spectrometer, operating at 400 MHz, 4 °C. Spectra were recorded of reduced and oxidized samples of the QH-EDH. QH-EDH reduced automatically upon reconstitution, oxidation was achieved by addition of ferricyanide.

Results

Preparation of the PQQ-derivatives

Preparation of PQQ-derivatives by reaction of PQQ with TFMPH leads to formation of the PQQ adduct (Figure 1) which is either the hydrazone or the azo compound. Simultaneously, part of the PQQ is reduced as can be observed by UV/vis analysis. Depending on the nature of the hydrazine and the solvent used in the reaction, reduction of PQQ can completely dominate adduct formation (Mure et al.,1990). When the reaction of PQQ with phenylhydrazine is carried out in methanol, formation of the adduct is only 1 %, as compared to the 99% reduction of PQQ. The yield of adduct formation is slightly increased when methanol is replaced by other solvents in the following order: (water) < methanol < ethanol < hexanol < butanol < propanol. Substitutents on the phenylgroup can also stimulate adduct formation. Synthesis of the TFMPH-PQQ derivative in propanol gave yields up to 70%. When reduced PQQ was recovered, the yield could be raised to more than 95 %. Spectra of the TFMPH-PQQ derivatives are shown in Figure 2. In Table 1 the ultraviolet/visible absorbance maxima and the chemical shifts (δ) of the ^{19}F-NMR are displayed. The ortho-TFMPH-PQQ appears to be present in two tautomeric forms. Reaction

two tautomeric forms. Reaction of PQQ with TFEH in water results in the formation of two compounds, one is the hydrazone (Figure 3). One of the compounds formed appears to be an intermediate in the synthesis of TFEH-PQQ. When the reaction is carried out in 1-propanol this intermediate is the only product observed. The intermediate is stable in buffer A, however, it is transformed by the enzyme to PQQ with the loss of the fluorine label. The ultraviolet/visible spectra of the compounds are shown in Figure 4.

Table 1. Ultraviolet/visible absorption maxima and chemical shifts of the ^{19}F-NMR. UV/vis spectra were measured in 20 mM MOPS/KOH, pH 7 at 25 °C. ^{19}F-NMR spectra were recorded in 20 mM MOPS/KOH, pH 7 at 25 °C. Chemical shifts are quoted in parts per million (ppm) from internal CCl_3F standard.

compound	λmax(nm)	δ ^{19}F-NMR (ppm)
o-TFMPH-PQQ	272, 376, 418	- 60.0, - 58.7
m-TFMPH-PQQ	274, 430	- 62.6
p-TFMPH-PQQ	274, 430	- 61.9
TFEH-PQQ	250, 320, 364	- 61.6

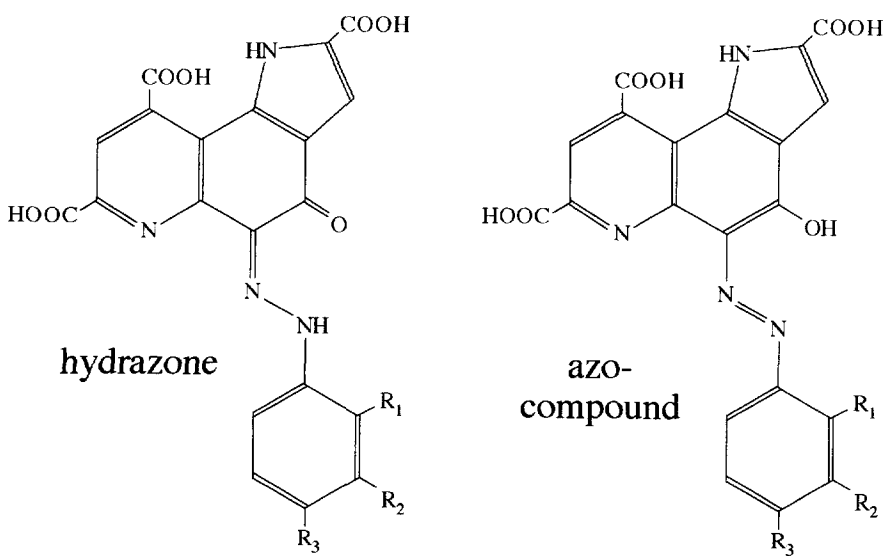

Figure 1. TFMPH-PQQ adducts

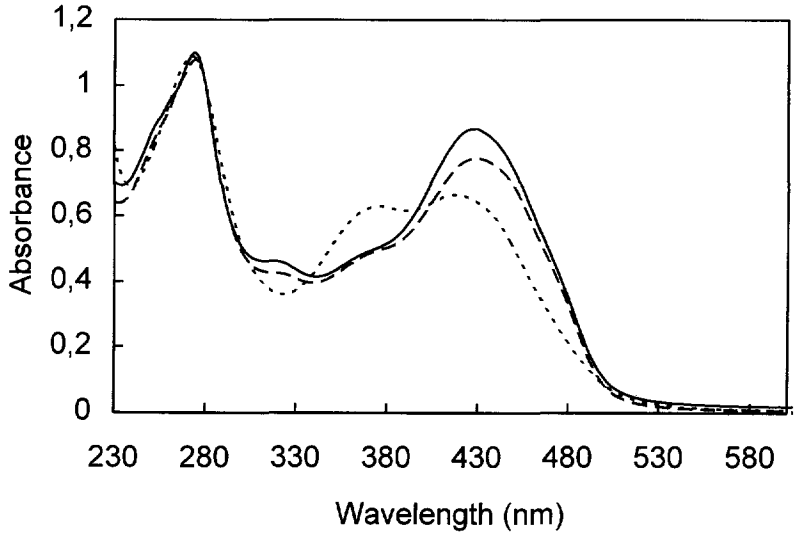

Figure 2. UV/Vis spectra TFMPH-PQQ, (· · · · ·) ortho- , (-----) meta- and (——) para-substituted TFMPH-PQQs. Spectra were taken in 20 mM MOPS/KOH, pH 7.5.

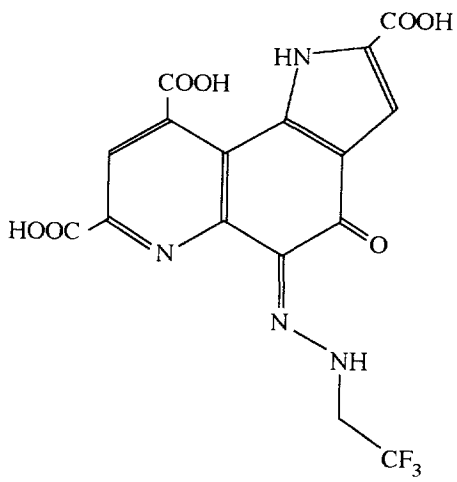

Figure 3. TFEH-PQQ hydrazone.

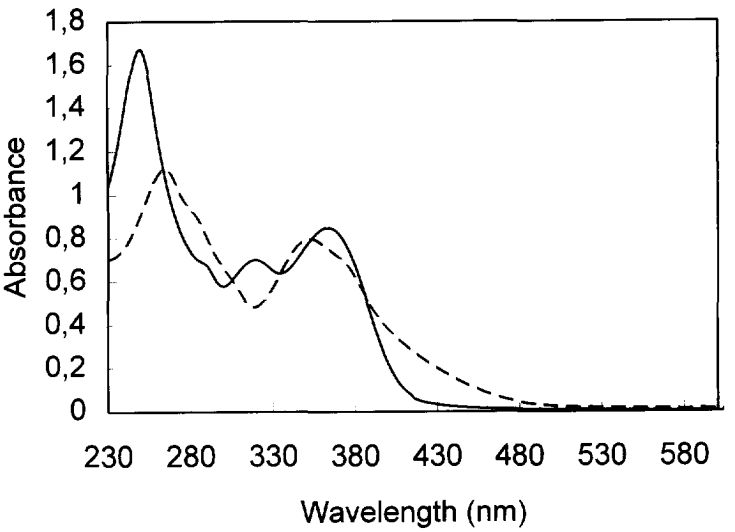

Figure 4. Ultraviolet/visible spectra of (——) TFEH-PQQ and (------) intermediate in 20 mM MOPS/KOH, pH 7.5.

Inactivation experiment

Reaction of holo-QH-EDH with phenylhydrazine, semicarbazide and o-TFMPH was attempted. After 1 hour incubation zero activity was measured. However, full activity was restored after removal of the hydrazines on a PD10 column, so no stable hydrazone formation had occurred. Incubating holo-QH-EDH in 0.1 M glycine, pH 7.5 over 24 hours showed no loss of activity and no change in ultraviolet/visible spectrum. Addition of ferricyanide to ensure that the PQQ is in the oxidized state also revealed no oxazole formation.

Reconstitution experiments introducing a fluorine label

Reconstitution of the apoenzyme with TFEH-PQQ resulted in the formation of almost 100% of holo-QH-EDH, as judged from the behaviour of the preparation on Mono-S chromatography. When the apoenzyme was incubated with stoichiometric amounts of TFMPH-PQQs, all of the derivative was bound as could be deduced from the broadening of the ^{19}F-NMR spectra. No signal of the unbound derivative was observed. However, part of the PQQ-derivative is both lost on the PD-10 column and during Mono-S chromatography. It is possible, however, to obtain about 25% of the enzyme in a holo-form after Mono-S

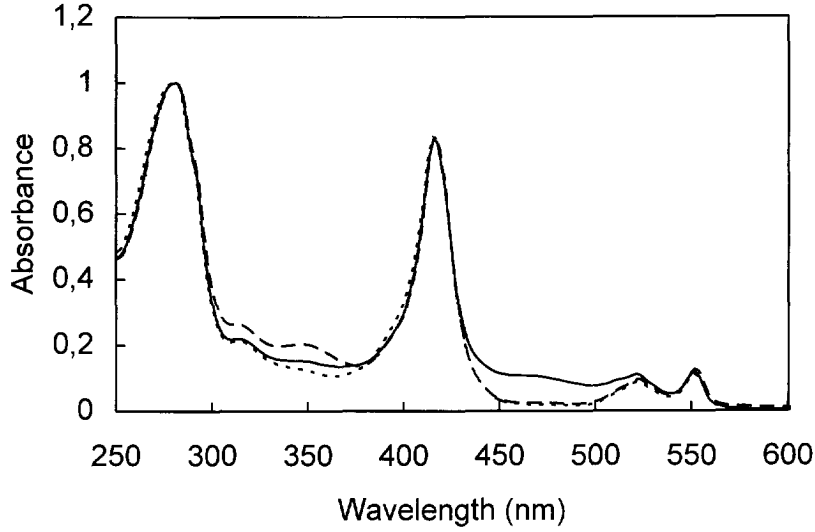

Figure 5. UV/Vis spectra of holo-QH-EDH containing PQQ, TFEH-PQQ or p-TFMPH-PQQ. Spectra were taken with a photo-diode array detector taken at the peak of the reconstituted enzyme chromatographed on Mono-S. Holo-enzyme with (------) PQQ, (——) p-TFMPH-PQQ, (· · · · ·) TFEH-PQQ.

separation. Because of the observation that TFMPH-PQQ adsorbs readily to all kinds of surfaces, it is not feasible to concentrate the enzymesamples without loss of the derivative. In Figure 5 the spectra of holo-QH-EDH containing TFEH-PQQ and p-TFMPH-PQQ are compared with holo-QH-EDH containing PQQ. To ensure that no change has occurred with the derivative upon binding, the holo-QH-EDH's were incubated with pronase and subjected to a reverse phase separation. The products obtained show identical spectra and retention times as the authentic PQQ-derivatives used originally for the reconstitution experiments

^{19}F-NMR of fluorine labelled holo-QH-EDH

^{19}F-NMR spectra of reduced holo-QH-EDH reconstituted with the fuorine substituted PQQ derivatives showed the presence of 4 fluorine labelled PQQ-derivatives. Measurements were performed at 279 K to prevent denaturation. For line width measurement ,the expression $T_2 = (\pi.\upsilon_{½})^{-1}$ was used, where $\upsilon_{½}$ is the linewidth at half-height expressed in Herz. A typical ^{19}F-NMR spectrum for reduced holo-QH-EDH reconstituted with with m-TFMPH-PQQ is shown in Figure 6. All spectra show one major peak and one or several extra peaks of unknown origin. The spectra of holo-QH-EDH containing o-TFMPH-PQQ contain 2 major

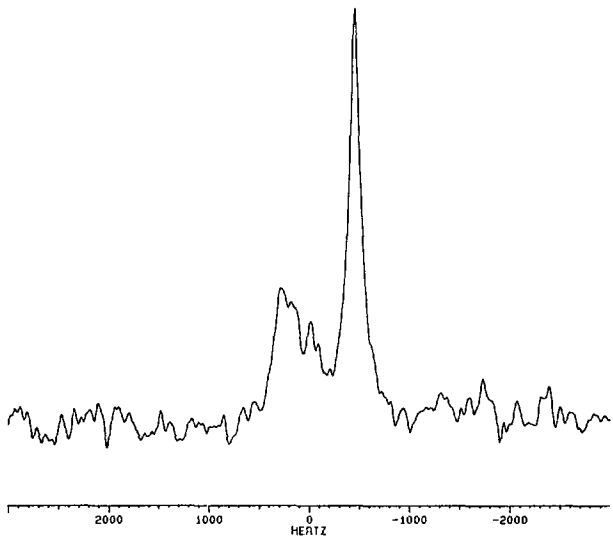

Figure 6. ^{19}F-NMR spectrum of reduced holo-QH-EDH containing m-TFMPH-PQQ at 279 K in buffer A.

peaks as the result of the two tautomeric forms which were already observed in the ^{19}F-NMR spectra of the free derivative. In Table 2 the linewidth and T_2 of the major peaks are displayed. Clearly all the PQQ derivatives bind to the enzyme as can be deduced from the linewidth broadening.

^{19}F-NMR spectra of oxidized holo-QH-EDH containing the 4 different fluorine labelled PQQ-derivatives were taken at 279 K. The samples were oxidized by adding excess ferricyanide. Spectra of the oxidized samples showed peaks with chemical shifts identical with the peaks from the reduced samples. In some cases several other peaks appeared. As a result of the complex spectra, interpretation was biased. It is assumed that the peaks arise from hindered rotation of the fluorine substituted phenylgroup in the active site of the enzyme, resulting in a different environment for the fluorine with respect to the haem and protein.

PQQ-TFEH is the only PQQ-derivative that binds sufficiently strong to survive chromatography of the reconstituted enzyme. In this case we could separate reconstituted enzyme from non-reconstitutable enzyme. The purified preparation showed ^{19}F-NMR spectra containing one main resonance and one small one in the reduced form and one peak in the

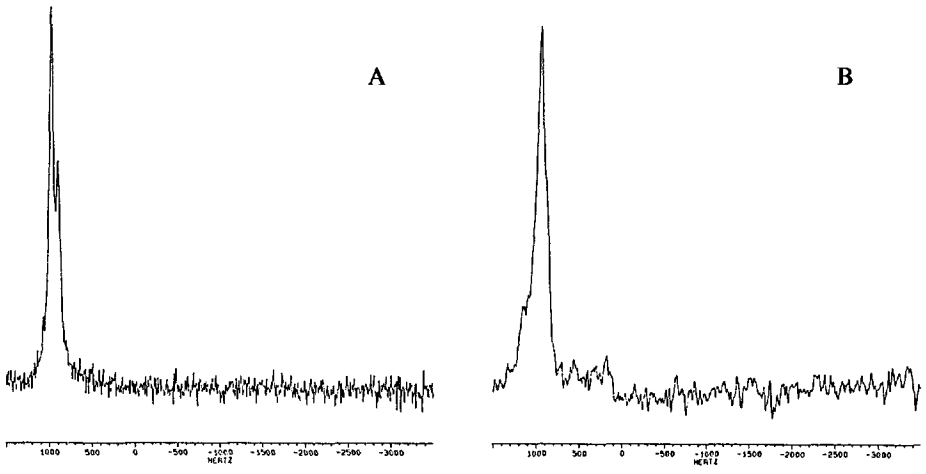

Figure 7. ^{19}F-NMR spectrum of reduced and oxidized holo-QH-EDH containing TFEH-PQQ. a) reduced holo-QH-EDH. b) oxidized holo-QH-EDH. Oxidation was performed by the addition of excess potassium ferricyanide.

oxidized form (Figure 7). The chemical shift difference before and after oxidation is almost zero suggesting the absence of large scalar or dipolar interactions. The linewidth measurements are still biased by the presence of overlapping peaks.

Neglecting the scalar contribution to the transverse relaxation gives an estimate of the minimal distance between the fluorine and Fe(III) centres. Calculations were performed using the major peaks observed for o- and m-TFMPH-PQQ, which did not show any broadening or shift differences. (The p-TFMPH-PQQ showed a complex spectrum with several peaks of almost equal intensity, making it almost impossible to draw conclusions regarding the distance of the PQQ derivative to the haem c). Broadening of signals less then 10 Hz as observed for the oxidized and reduced holo-QH-EDH containing the ortho- and meta-substituted TFMPH, implies that the distance between the haem c and the fluorine label is more than 4.7 Å. To estimate the distance from the orthoquinone moiety and the haem c, the size of the phenyl hydrazine has to be taken to account also. This adds a further uncertainty as to the position of the PQQ relative to the haem.

Table 2. Linewidths and T_2 of the ^{19}F-NMR signal for the reduced holo-QH-EDH's containing different fluorine substituted PQQ derivatives. Spectra were recorded at 279 K in 20 mM MOPS/KOH, pH 7.5.

PQQ-derivative	$\upsilon_{1/2}$ (Hz)	T_2 (msec)
o-TFMPH-PQQ	92, 100 *	3.5, 3.2
m-TFMPH-PQQ	136	2.3
p-TFMPH-PQQ	57	5.6
TFEH-PQQ	62	5.2

Discussion

The presence of quinohaemoprotein ethanol dehydrogenase apoenzyme, apo-QH-EDH, in *C. testosteroni* grown on ethanol in the absence of added PQQ, has stimulated discussion on the possible role of PQQ in the reconstitution of ethanol dehydrogenase activity. Meanwhile, several lines of evidence have been established in favour of a genuine vitamin character of PQQ for this organism when grown on short-chain primary alcohols (Groen et al., 1986; de Jong et al., 1995a). Important observations concern the high degree of homology between the primary sequence of QH-EDH and the so-called Type II QH-EDHs present in *Acetobacter* and *Gluconobacter* species (J. Stoorvogel, 1995). Lower, but highly significant homology has been established between both QH-EDH and Type II alcohol dehydrogenases and the quinoprotein methanol dehydrogenases from methylotrophic bacteria. Since excretion of PQQ into the growth medium appears to be restricted to a small number of species, the presence of PQQ in natural habitats is limited (van Kleef et al., 1989).
Thus, the physiological relevance of apo-quinoprotein production by *C. testosteroni*, can only be ascertained when definite information on the ecology of this organism becomes available. In this respect, it must be mentioned that the production of apo-quinoprotein is not restricted to QH-EDH. Several organisms, including *Escherichia coli*, have been found to produce quinoprotein glucose dehydrogenases in the PQQ-free apo-form (Dokter et al., 1986).
The high enantioselectivity of QH-EDH in the oxidation of chiral solketal has stimulated research on the possible application of either whole cells or the isolated enzyme in the manufacture of homochiral solketal by kinetic resolution(Geerlof et al., 1994a; Geerlof et al, 1994c). Kinetic studies of the isolated enzyme have shown that the over-all mechanism is in agreement with a hexa-uni ping-pong kinetic scheme (Geerlof et al.,1994b). With ferricyanide as the electron acceptor, primary alcohols or aldehydes reduce enzyme-bound PQQ in the first step, followed by successive reoxidation of PQQ by the one-electron

acceptor. Substantial evidence has been obtained in favour of PQQ as the primary site for alcohol and aldehyde addition and oxidation. In particular the effect of introducing small alkyl-substituents on PQQ on the enantioselectivity of the reconstituted QH-EDH points at the importance of the PQQ site for substrate addition (de Jong et al., 1995a). The homology of QH-EDH and quinoprotein MDHs lacking the haem prosthetic group, with respect to the residues that are expected to make up the PQQ-binding site in these enzymes supports this view. The primary site of interaction of the second substrate, ferricyanide, has not yet been established unequivocally. Again, the homology between QH-EDH and MDH structures and the inability of MDH to accept ferricyanide as an electron acceptor, suggests the haem group of QH-EDH as the primary site. Regarding the possible intramolecular electron transfer between the haem and PQQ-sites in QH-EDH, some caution must be expressed. Electron transfer between MDH and its natural electron acceptor, cytochrome c_L, has been well established.

From the available evidence, it must be concluded that intermolecular transfer of electrons between the PQQ-site of MDH and the haem of cytochrome c_L is not hampered by the relatively buried position of PQQ in MDH. Similarly, provided that the sequence homology of MDH and QH-EDH can be stretched to this account, intermolecular electron transfer between the PQQ-site of one molecule of QH-EDH and the haem group of another molecule would be feasible. Indeed, preliminary experimental results obtained by recording the reduction of the haems of partially reconstituted samples of QH-EDH apoenzyme show that upon addition of substrate, all haems become eventually reduced. Since migration of PQQ from one molecule of QH-EDH to another is obstructed by the tight binding of PQQ in holo-QH-EDH, intermolecular communication between PQQ and haem in QH-EDH preparations appears possible.

Intramolecular interaction between PQQ and haem in QH-EDH has been observed by spectroscopic techniques. In particular, substantial changes in the ultraviolet/visible and NMR spectrum of the haem are seen upon introduction of PQQ into apoenzyme preparations containing reduced and oxidized haem groups. Also, the redox-potential of the haem is seriously altered in the presence of PQQ. Since no effects are observed in the Resonance Raman spectra of reduced and oxidized apo- and holo-forms, it has been suggested that the introduction of PQQ results in the formation of a more compact conformation.

In order to obtain an estimate of the distance between the PQQ and haem-sites of QH-EDH we investigated the merits of a technique that has been applied to the quinoprotein copper-containing amine oxidase, PAO, from bovine plasma (Williams and Falk, 1986). By introducing a suitable NMR-active nucleus onto the topa-quinone cofactor of PAO, the distance between the position of the probe and the metal-ion was inferred from the distance dependent contribution to the NMR-relaxation of the probe signal by the paramagnetic copper(II)-ion. The suitability of ^{19}F-nuclei for this purpose was amply demonstrated. It remains to be seen, however, whether the limitations inherent to this technique will frustrate its reliability. Determination of the 3D-structure by X-ray crystallography (a preliminary

report has been presented, Huizinga, 1994) will be conclusive.
In the present case, additional limitations have been observed: a. contrary to Cu(II), low-spin Fe(III) shows short τ_s-values leading to small line-broadening that can only be accurately measured for distances below 5 Å; b. in order to relate the distance of the ^{19}F- and Fe(III)-positions to the topology of the PQQ- and haem-groups, fixed geometry of the entity carrying the ^{19}F-nuclei with respect to the PQQ, as well as to the haem must be assured; c. estimates on the relative position in three dimensions requires at least three independent measurements to be made.

Labelling PQQ with ^{19}F-substituted probes

From the arguments given above it will be clear that introduction of fluorine directly onto the pyrroloquinoline ring system of PQQ present in the QH-EDH holo-enzyme is the most desirable option. Sofar, PQQ derivatives in which one of the (three) non-exchangeable hydrogens is replaced by ^{19}F are not available. A second option is the introduction of fluorine-labelled groups using the innate reactivity of PQQ. Like all cofactors belonging to the quinoprotein group of enzymes, free PQQ is known to react with hydrazines leading to the formation of relatively stable hydrazones. Both topaquinone, TPQ, and tryptophyl-tryptophan quinone, TTQ, have been shown to react with hydrazines in their protein-bound form. As reported here, we were not able to derivatize PQQ bound to QH-EDH using various fluorine-labelled hydrazines. Three reasons for this failure were considered: a. the hydrazine is excluded from the active site for steric reasons; b. instead of addition to the ortho-quinone moiety of PQQ, the hydrazine favours reduction of this group; c. PQQ is present in QH-EDH predominantly in its reduced form, thus obstructing hydrazone formation.

In view of the experiments discussed below, we investigated the chemistry of hydrazone formation *in vitro*. It was found that depending on the hydrazine used, PQQ is reduced almost completely. Depending on the solvent employed only minor amounts of hydrazone could be obtained on a preparative scale. Since recycling of reduced PQQ to the reactive ortho-quinone form requires oxidation, which destroys the hydrazone that is already formed, in situ derivatization is not feasible unless the hydrazone initially formed is separated first. Application to enzyme-bound PQQ by separating hydrazone-containing and reduced holo-QH-EDH was not successful.

Investigation of the redox state of PQQ in holo-QH-EDH showed $PQQH_2$ to be the major species present. Although oxidation using ferricyanide was possible, reduced PQQ appeared to be the major product after addition of hydrazines. Reoxidation, again, destroyed both free hydrazine, as well as the hydrazone allegedly produced after the first addition. In order to circumvent the problems inherent to the use of highly reducing hydrazines, we investigated the possibility to obtain derivatives of enzyme-bound PQQ using the known reactivity of glycine towards PQQ. Under suitable conditions, glycine has been shown to react with PQQ leading to the formation of a stable oxazole. We found that a similar reaction did not occur

in the case of QH-EDH. This could mean that either enzyme-bound PQQ is not reactive, or that the oxazole of PQQ is unstable in the active site cavity. Direct reconstitution of QH-EDH apoenzyme with PQQ-oxazole, however, afforded completely inactive QH-EDH. Activity could be restored only after prolonged incubation with genuine PQQ, suggesting that PQQ-oxazole is indeed present in the PQQ-cavity, albeit with a lower affinity. These results stimulated us to investigate the possibility to introduce preformed fluorine-labelled PQQ-hydrazones into the PQQ-site of apo-QH-EDH.

Synthesis of PQQ-hydrazones employing trifluoromethyl-substituted phenylhydrazines afforded the required derivatives. Addition of hydrazones to the QH-EDH apoenzyme in suitable excess to ensure complete saturation, required removal of any free hydrazone remaining in solution. Due to the hydrophobic properties of the free hydrazones, complete separation was not feasible. Upon addition of equimolar quantities, this problem could be largely circumvented. Incomplete saturation of the PQQ-sites was taken for granted. Reaction of apo-QH-EDH with the hydrazone of PQQ and trifluoroethylhydrazine was performed by addition of excess hydrazone to ensure complete saturation, after which the remaining hydrazone could be well-separated due to its less hydrophobic nature. In each case, the presence of the hydrazone at the PQQ-site of QH-EDH was deduced from the time-dependent restoration of dehydrogenase activity upon exposure to genuine PQQ as opposed to the virtually instantaneous recovery of activity observed for untreated apo-QH-EDH.

^{19}F-NMR relaxation measurements

The presence of the derivative was verified further by the changes in UV/Vis absorption spectrum of the enzyme upon reconstitution with the derivatives and the changes in ^{19}F-NMR spectra of the reconstituted enzymes compared to the unbound derivatives. The ^{19}F-NMR spectra of the reduced holo-QH-EDHs showed resonances which were broader than the resonances of unbound PQQ-derivatives, indicating that the derivatives were bound. No resonances were observed that could be attributed to unbound derivative. The ^{19}F-NMR spectra showed more peaks than expected, especially in the oxidized form, which make it very difficult to interpret the spectra. Ideally only one resonance peak would be expected for the reduced as well as for the oxidized form. In that case broadening of the fluorine resonance caused by the paramagnetic ferric form of the haem c can be considered a clear estimate of the distance between fluorine and the haem c. In the present case, the spectra are more complex probably as a result of different orientations of the TFM-substituted phenylgroup, caused by hindered rotation in the active site. Even the ^{19}F-NMR of the holo-QH-EDH containing TFEH-PQQ gave two peaks in the reduced form. This sample should be the least affected by a hindered rotation in the active site because of the lack of a phenylgroup. Distance calculations could only be satisfactory performed using the ^{19}F-NMR spectra of holo-QH-EDH containing ortho- and meta-TFMPH-PQQ. A value of $\geq$ 5 Å as a minimal distance between the fluorine and haem was deduced, from these measurements. Larger distances can not be measured in systems with low-spin iron as the paramagnetic

species, because the short τ_s value of the low-spin Fe(III) ($2\text{-}8\times10^{-12}$ sec) gives only small broadenings compared to metal ions with long τ_s values like Cu(II), Mn(II) and VO(IV).

Clearly, determination of the 3D structure by x-ray crystallography will be very important to establish the distance and positions of the redox centres. The x-ray structure of the QH-EDH has not yet been resolved to detail, however, preliminary results have already allowed comparisons to be made with the α subunit of methanol dehydrogenase (MDH). MDH shows substantial homology of the amino acid sequence compared to QH-EDH (Stoorvogel et al., 1995). The similarity of part of the sequence suggests that the quinoprotein part of QH-EDH may have a similar structure as MDH. Using molecular displacement on the preliminary QH-EDH structure and the structure of MDH shows the haem domain to be rather independent from the quinoprotein part. The present results obtained from the ^{19}F-NMR relaxation experiments can be used to rule out a situation where the haem c takes up a position on the entrance of the active site of the quinoprotein part. Since the fluorine labelled PQQ derivatives all carry the hydrazines on the C(5) position of PQQ, meaning that the fluorine atoms will be located near the entrance of the active site funnel, a position of the haem near this entrance would implicate broadening of the ^{19}F-resonances which was not observed.

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Chapter 5

Effects of Ca^{2+} and its substitutes on quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni*.

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Abbreviations

CDTA, trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid; EDH, ethanol dehydrogenase; EDTA, Ethylenediamine-tetraacetic acid; EPR, electron paramagnetic resonance; MDH, methanol dehydrogenase; m-GDH, membrane-bound glucose dehydrogenase; PQQ, 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione; $PQQH^{\cdot}$, semiquinone form of PQQ; $PQQH_2$, quinol form of PQQ; $QH\text{-}EDH_I$, quinohaemoprotein ethanol dehydrogenase type I; $QH\text{-}EDH_{II}$, quinohaemoprotein ethanol dehydrogenase type II; s-GDH, soluble glucose dehydrogenase; UV/Vis, ultraviolet/visible.

Abstract

Active-site-bound Ca^{2+} in quinohaemoprotein ethanol dehydrogenase (QH-EDH) of *Comamonas testosteroni* could be removed with the chelators EDTA or CDTA. This did not lead to release of the cofactor, 2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione, abbreviated to pyrroloquinoline quinone (PQQ). However, Ca^{2+}-depleted QH-EDH was inactive and its reduced form showed lower absorbancies, especially in the 300 to 400 nm region of the optical absorbance spectrum, as compared to the normal holo-enzyme. Since the spectral difference was dissimilar from that induced by Ca^{2+} in free PQQ or $PQQH_2$, it is clear that the binding of PQQ to the protein can occur in the absence of Ca^{2+} but that the latter affects this. The optical absorbance spectrum and enzymatic activity were fully restored on adding Ca^{2+} to the depleted enzyme form. Reconstitution was also achieved with some other bivalent cations but the extent of activity recovery and spectral contribution to that of the quinone cofactor varied with the cation applied. EPR spectroscopy of enzyme reconstituted with paramagnetic cations confirmed that the cation binds to the enzyme. Surprisingly, EPR spectra of $PQQH^{\cdot}$ in QH-EDH reconstituted with a variety of metal ions, including paramagnetic ones, were very similar. Explanations for this finding are discussed in the light of the sequence homology found between PQQ-containing dehydrogenases and the recently elucidated 3-dimensional structure of one of them, methanol dehydrogenase.

Introduction

Quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni* (QH-EDH) is an enzyme which has haem *c* as well as PQQ (2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione, abbreviated to pyrroloquinoline quinone) as cofactors. It should be stressed, however, that this organism is unable to produce PQQ so that the enzyme is always isolated in the apo-form [1]. All strains of this organism available from the Delft Culture Collection Department appear to contain the enzyme [2] and a related enzyme has been isolated from *Comamonas acidovorans* (D. Kraayveld, A. Geerlof, J.A. Duine, unpublished results). Anther related enzyme has been found in *Pseudomonas putida* [3], but curiously in this case the enzyme occurs in the holo-form. A quite different type of quinohaemoprotein alcohol dehydrogenase has been purified (in holo-form) from *Acetobacter* and *Gluconobacter* species [4-8]. These hydrophobic, membrane-integrated enzymes, consisting of several subunits, are distinct from QH-EDH, being a soluble, monomeric enzyme [1].

Reconstitution of apo- to active holo-QH-EDH with PQQ occurs rapidly in the presence of Ca^{2+}, *in vitro* as well as *in vivo* [1], and holo-enzyme directly isolated from cells grown in a medium supplemented with PQQ contains Ca^{2+} [8]. The presence or the requirement of Ca^{2+} has been established for all other PQQ-containing enzymes investigated sofar, except

perhaps membrane-bound quinoprotein glucose dehydrogenase since it has a preference for Mg^{2+} in reconstitution [9]. No strict specificity exists with respect to the "natural metal ion" since successful replacement by others in the reconstitution assay has been reported in the case of quinoprotein glucose dehydrogenases [9,10]. To obtain Ca^{2+}-free enzyme for reconstitution experiments, depending on the enzyme concerned, in principle three possibilities exist: the use of the natural apo-enzyme, which is only feasible in cases where the organism does not synthesize PQQ (as applies to QH-EDH); growth of the organism in a medium depleted for Ca^{2+} but supplemented with the metal ion wanted, as applies to MDH [11,12]; removal of Ca^{2+} from the holo-enzyme. The latter possibility, for which the chelators CDTA and EDTA seem to be suited in the case of quinoprotein glucose dehydrogenases [9,13] and alcohol dehydrogenases [14], is attractive as it gives insight into: The ease of removal, that is the tightness with which Ca^{2+} is bound to the enzyme; the question whether concomitant release of PQQ occurs, as observed for some enzymes [13,14], indicating that Ca^{2+} could have at least a function in anchoring of the cofactor in such cases.

Although the 3-dimensional structure of a PQQ-linked methanol dehydrogenase (EC 1.1.99.8) [15,16] indicates which ligands may be involved in binding, the role of Ca^{2+} in structure and mechanism is far from established. Thus it could function as an anchor for PQQ, an essential structural factor for stabilizing the conformation of the holo-enzyme, or in a catalytic role, e.g. as Lewis acid in deprotonation of OH-group-containing substrates. In order to be able to make distinction between the possibilities, other aspects than effects on activity and release of PQQ should also be studied. QH-EDH seemed a good candidate for this since knowledge on the spectroscopic and kinetic properties of its apo- (without PQQ) and holo-form are available [17]. The work presented here concerns the effect of Ca^{2+} removal and replacement by other metal ions on activity, and on UV/Vis and EPR spectra.

Materials and methods

Enzyme isolation

Comamonas testosteroni LMD 26.36 was grown on a mineral medium (in the absence of PQQ) with 1% ethanol as carbon and energy source at 23°C [1]. Holoenzyme was prepared by reconstitution of apo-QH-EDH with Ca^{2+} and PQQ, followed by further purification, as described [8].

Enzyme assay

QH-EDH was assayed as described [1], with n-butanol as a substrate and potassium ferricyanide (1 mM) as electron acceptor, except that metal ion-depleted 20 mM MOPS/KOH, pH 7.5 (buffer A), was used as an assay buffer. Depletion occurred by incubating the buffer for 1 week with Amberlite IRC-718 (Serva).

Spectroscopy

Ultraviolet/visible absorbtion spectra were measured with a HP 8524A Diode array spectrometer at 20 °C.

EPR spectroscopy was performed on a Varian E-9 spectrometer operating at X-band frequency, and equipped with a home-built He-flow cryostat.

Calcium complexation with free PQQ and $PQQH_2$

Ultraviolet/visible absorbtion spectra of PQQ or $PQQH_2$ were taken in 20 mM MOPS/KOH buffer, pH 7.5 in the absence and in the presence of Ca^{2+} (100 mM). To prepare and stabilize $PQQH_2$, dithiotreitol (1mM) was added to a solution of PQQ (50 μM) in 20 mM MOPS/KOH buffer, pH 7.5.

Calcium removal from QH-EDH

To remove bound as well as adventitiously bound calcium, QH-EDH (in 20 mM MOPS/KOH buffer, pH 7.5, containing 5 mM $CaCl_2$) was chromatographed on a Pharmacia PD10 gel filtration column equilibrated with buffer A. Removal of functionally bound calcium from QH-EDH was performed at different temperatures by the addition of EDTA or CDTA (10 mM) to the QH-EDH eluted from the PD 10 column. The process was monitored by measuring the activity. Calcium-depleted QH-EDH was prepared by removing EDTA or CDTA on a Pharmacia PD10 gel filtration column equilibrated with buffer A, after incubating the enzyme with one of the chelators (10 mM) until no activity was found.

Reconstitution of calcium-depleted QH-EDH

Calcium-depleted QH-EDH (5 μM) was incubated with different amounts of calcium at 20 °C and the reconstitution process followed by measuring the activity. Reconstitution was also studied with several other metal ions (1mM). For the EPR experiments, the unbound metal ions were removed by buffer exchange. This occurred several times by applying centrifugation in a Centricon 10 microconcentrator (Amicon Co.), using buffer A.

Determination of the Ni content was performed with flameless atomic absorption spectrometry, that of the Ca content with flame atomic absorption spectrometry.

Results

Ca^{2+} removal from QH-EDH

QH-EDH was incubated with EDTA and CDTA (10 mM) at 15 and 25 °C and the effect on activity was followed in time (Figure 1). Inactivation due to calcium removal from the enzyme occurred faster with CDTA than with EDTA and the high temperature (25 °C) stimulated the process. It appeared that Ca^{2+}-depleted QH-EDH still contained 0.2 mol Ca^{2+} per mol of enzyme, although the preparations was completely inactive.

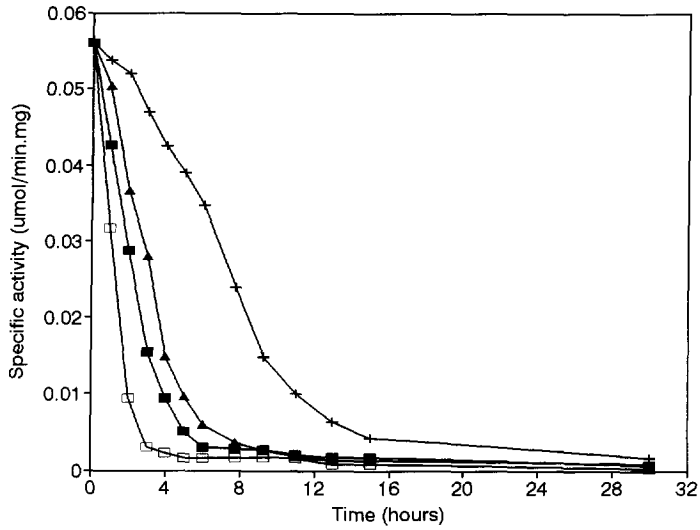

Figure 1. Inactivation of QH-EDH with EDTA and CDTA at different temperatures. Activities of QH-EDH after incubation with 10 mM EDTA or CDTA at 15 or 25 °C were measured as described in the M&M section. (▲) 10 mM CDTA, 15 °C; (□) 10 mM CDTA, 25 °C; (+) 10 mM EDTA, 15 °C; (■) 10 mM EDTA, 25 °C.

The UV/Vis spectra of several enzyme forms are depicted in Figure 2a, indicating that, especially in the 300 to 450 nm region, reduced QH-EDH has the highest, reduced apo-QH-EDH has the lowest absorbancies, whereas those of Ca^{2+}-depleted, reduced QH-EDH are in between. The difference spectrum of reduced QH-EDH minus Ca^{2+}-depleted, reduced QH-EDH (Figure 2b) shows that binding of Ca^{2+} to the latter gives rise to a general increase of the absorbancies in that region, especially at 306, 354, and 420 nm. In contrast, the binding of reduced PQQ (mainly $PQQH_2$, see below) not only induces similar increases in that region but also shifts of the haem c bands, as shown by the difference spectrum of reduced, Ca^{2+}-depleted QH-EDH minus reduced apo-QH-EDH (Figure 2c), as noted before [8].

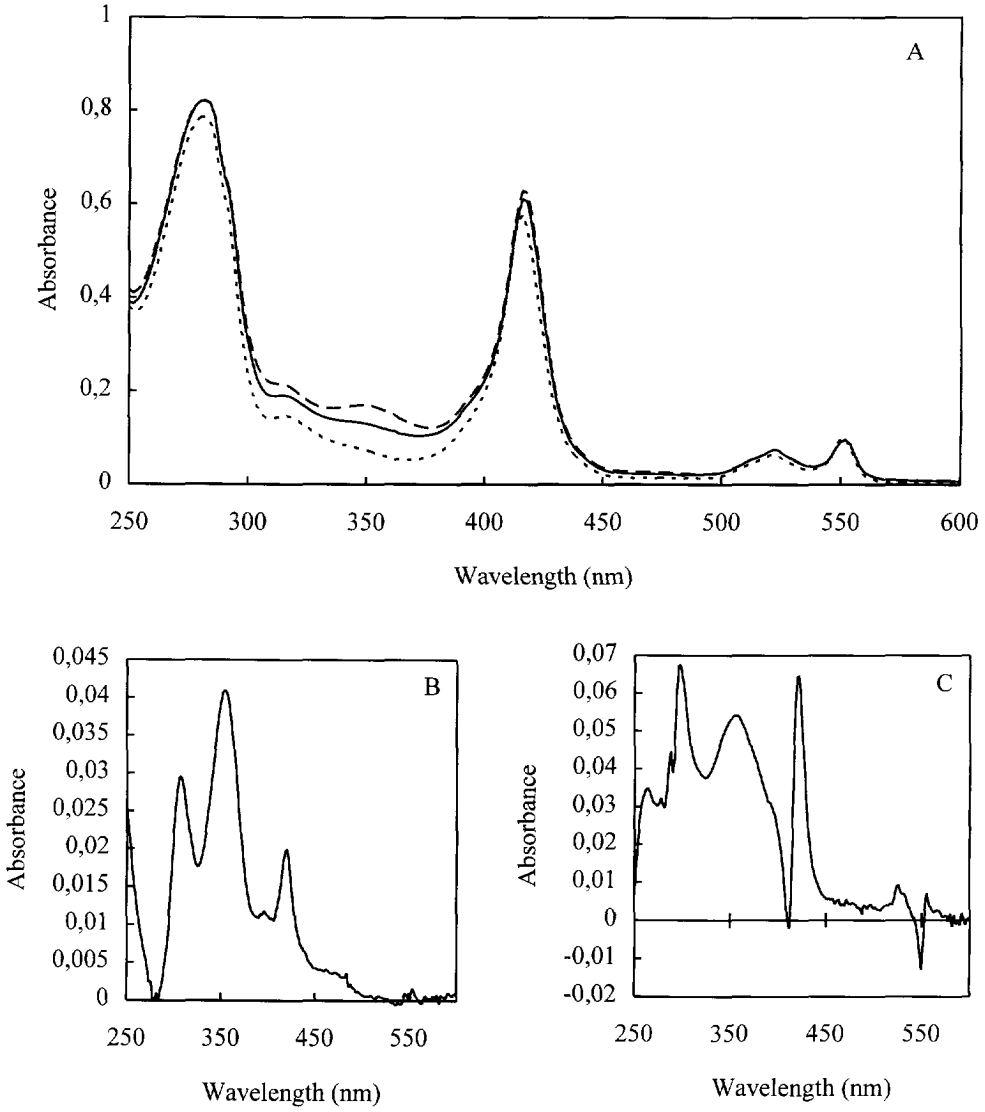

Figure 2. Ultraviolet/visible absorption spectra of reduced apo-QH-EDH, calcium depleted QH-EDH and QH-EDH. (a) Spectra of reduced apo- and QH-EDH in buffer A. (· · · · ·) apo-QH-EDH; (——) calcium-depleted QH-EDH; (------) QH-EDH in buffer A, containing 5 mM $CaCl_2$. The spectrum of reduced apo-QH-EDH was taken from ref. [9]. Spectra were scaled using the specific absorption coefficients $A^{0.1\%}_{280nm}$ = 2.41 (apo-QH-EDH) and 2.52 (QH-EDH) (ref. 9). (b) Difference spectrum of the reduced QH-EDH minus the calcium-depleted reduced QH-EDH spectrum. (c) Difference spectrum of the reduced calcium-depleted QH-EDH minus the reduced apo-QH-EDH spectrum.

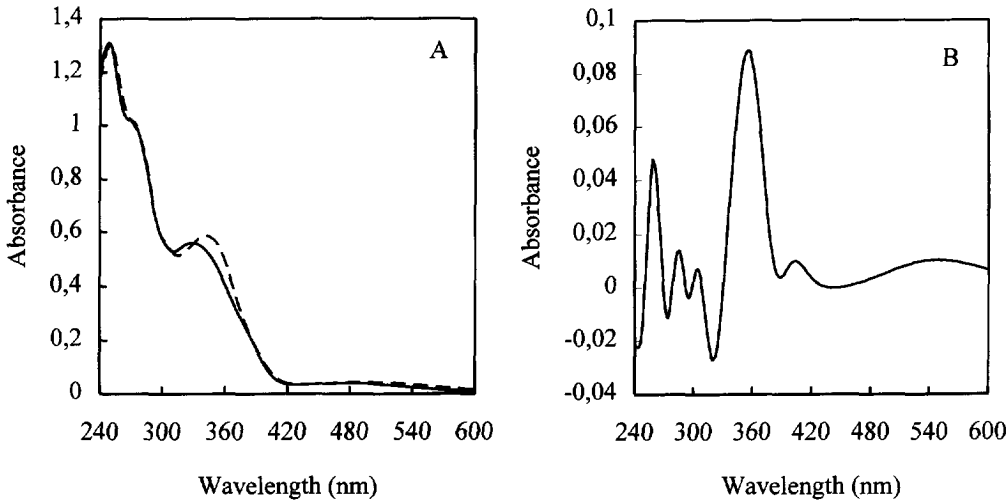

Figure 3. Ultraviolet/visible absorbtion spectra of PQQ in absence and presence of Ca^{2+}. (a) (——) 50 μM PQQ in 20 mM MOPS/KOH, pH 7.5; (------) 50 μM PQQ in 20 mM MOPS/KOH, pH 7.5, containing 100 mM $CaCl_2$. (b) Difference spectrum of that of 50 μM PQQ with 100 mM $CaCl_2$ minus the spectrum of 50 μM PQQ in the absence of calcium.

Calcium complexation with free PQQ and $PQQH_2$

Titration experiments monitored by taking UV/Vis spectra, indicated that high amounts of Ca^{2+} are required (0.1 M) to induce a change in the spectrum of PQQ. The effect of this metal ion on the spectrum of PQQ in metal ion-depleted 20 mM MOPS/KOH buffer, pH 7.5 is shown in Figure 3a and 3b. A similar effect has been found on using sodium acetate buffer, pH 6 (11). Ca^{2+} also affects the spectrum of $PQQH_2$ (Figure 4 a and b).

Reconstitution of Ca^{2+}-depleted QH-EDH with Ca^{2+}

Ca^{2+}-depleted, reduced QH-EDH (5 μM) was incubated with varying concentrations of Ca^{2+}, ranging from 5 μM to 5 mM. The reconstitution process was followed by taking samples at various times and measuring the activity. As is illustrated by the curves in Fig 5, the rate and final level of reconstitution are almost independent of the Ca^{2+} concentration (lower levels are only observed at Ca^{2+} concentrations of 5 and 10 μM). It was calculated from these data that the dissociation constant of the Ca^{2+}-QH-EDH complex is about 0.5 μM. In view of this value, those of the dissociation constants of the EDTA- and CDTA-Ca^{2+} complexes [23], and the concentration of chelator used, it is clear that these two chelators are able to remove the bound Ca^{2+} from QH-EDH.

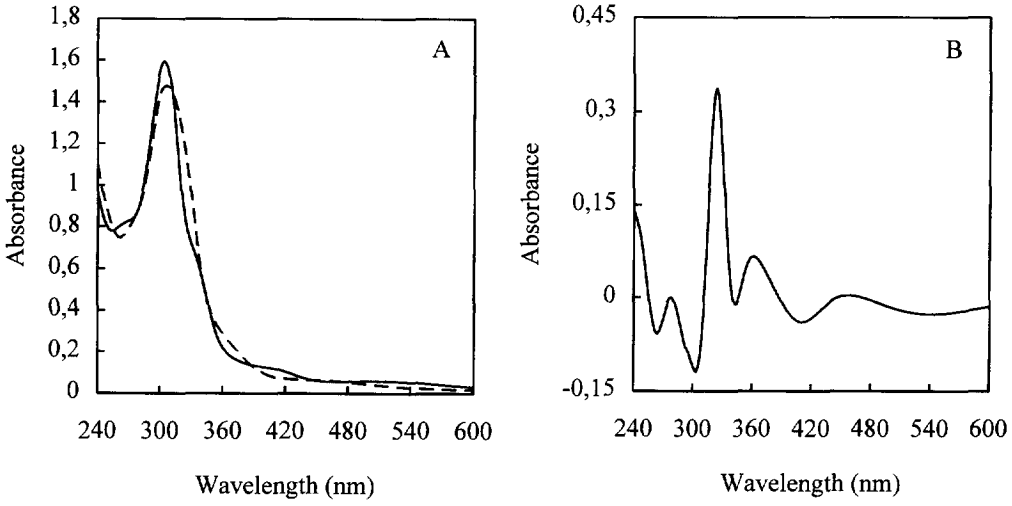

Figure 4 . Ultraviolet/visible absorbtion spectra of $PQQH_2$ in the absence and presence of Ca^{2+}. (a) (——) 50 μM $PQQH_2$ in 20 mM MOPS/KOH, pH 7.5, (------) 50 μM $PQQH_2$ in 20 mM MOPS/KOH, pH 7.5, containing 100 mM $CaCl_2$. (b) Difference spectrum of the spectra of 50 μM $PQQH_2$ with 100 mM $CaCl_2$ minus the spectra of 50 μM $PQQH_2$ in absence of calcium.

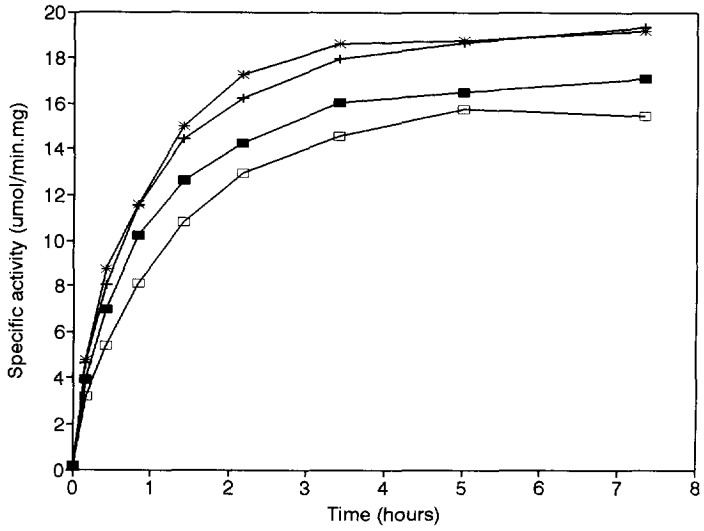

Figure 5. Reconstitution of calcium-depleted QH-EDH with various Ca^{2+} concentrations. 5 μM calcium-depleted QH-EDH was incubated at 25 °C with the following calcium concentrations: (□) 5 μM; (■) 10 μM; (+) 100 μM; (×) 1mM. The reconstitution with a calcium concentration of 5 mM is omitted because no differences could be detected with the experiment using 1 mM.

Reconstitution of Ca^{2+}-depleted QH-EDH with other metal ions

Figure 6 shows that several metal ions can substitute for Ca^{2+} in the reconstitution but that the final activity achieved is always lower than that with Ca^{2+}. Other cations tested gave negative results: Zn^{2+} and Cu^{2+} caused irreversible inactivation whereas Dy^{3+}, Eu^{3+}, Y^{3+}, and La^{3+} were ineffective. Enzyme reconstituted with Ba^{2+} or Sr^{2+} behaved unusual because the activity in the assay mixture steadily increased from zero to a constant value (the latter indicated in Figure 6) in a time span of about 6 min. This could be explained by assuming that these metal ions scarcely bind to reduced, Ca^{2+}-depleted QH-EDH but much better to an enzyme form which is slowly generated in the assay mixture.

The rates with which Ca^{2+}-depleted QH-EDH is reconstituted are shown for a number of bivalent metal ions in Figure 7. Although the final level values vary greatly, the time spans in which full reconstitution is achieved are more or less similar. This suggests that the low activity levels (as compared to that induced by Ca^{2+}) are not caused by weak binding of the metal ions but by their lower performance in catalysis. This view is supported by the observations that: reconstitution with Ni^{2+} provided an enzyme which contained 0.8 mol Ni^{2+} per enzyme molecule (after removal of excess Ni^{2+}) but which showed only 10 % of the activity achieved with Ca^{2+} reconstitution; increasing the concentration of metal ions in reconstitution did not lead to higheractivity plateau values.

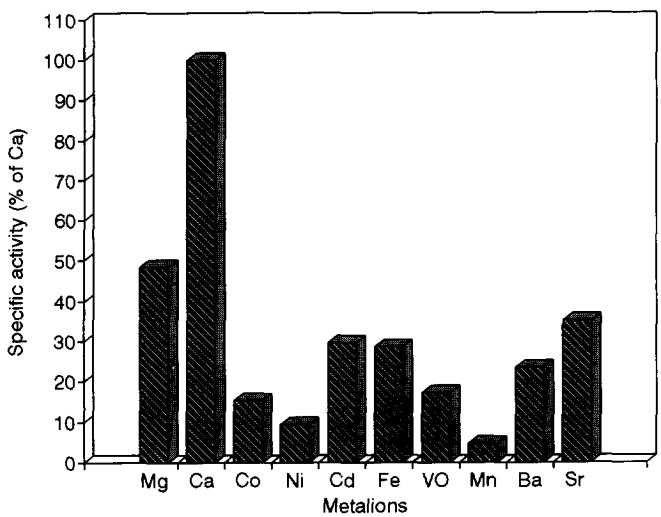

Figure 6. Activities of calcium-depleted QH-EDH reconstituted with various metal ions. Activities after reconstitution of 5 μM calcium-depleted QH-EDH with various metalions at a concentration of 1mM after 8 hours incubation at 25 °C.

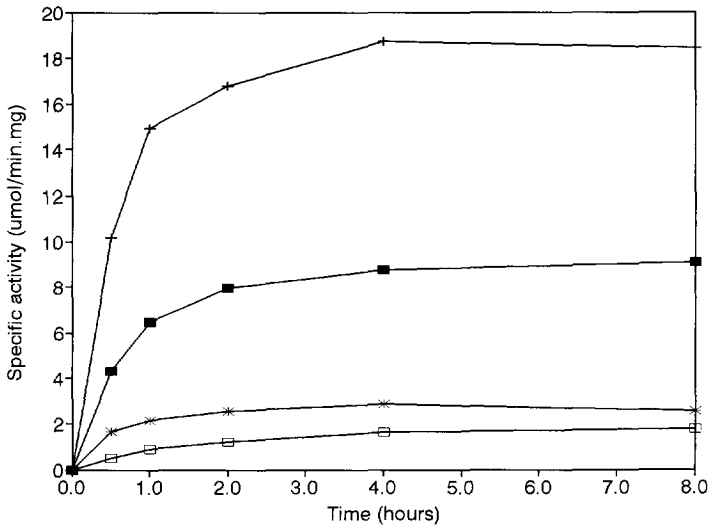

Figure 7. Reconstitution of calcium-depleted QH-EDH with various metalions. 5 μM calcium-depleted QH-EDH was incubated at 25 °C with 1mM: (□) Ni^{2+}; (■) Mg^{2+}; (+) Ca^{2+}; (×) Co^{2+}.

As shown in Figure 8, the metal ions investigated induce similar maxima in the UV/Vis spectrum upon binding to Ca^{2+}-depleted, reduced QH-EDH but the increase of absorbancies in the 300 to 450 nm region varies substantially. Sr^{2+} appears to be more effective in this respect than Ca^{2+}, although Sr^{2+}-reconstituted enzyme is much less active (Figure 6).

EPR spectroscopy

Ca^{2+}-depleted, reduced QH-EDH was reconstituted with Co^{2+} and VO^{2+} and the excess of metal ions removed. As shown in Figure 9 and 10, the EPR spectra of the reconstituted enzyme are quite different from that of the free metal ions in solution, also confirming that binding occurred to the enzyme.

The signal of Co^{2+} arises from a S=3/2 system. The g-value of 4.3 is characteristic for Co^{2+} in octahedral symmetry. The resonances are very broad, in part due to hyperfine structure from the I=7/2 nucleus. Quantitation of the signal showed that it accounts for 73% of the enzyme concentration.

The vanadium signal is axial with $g_{x,y}$= 1.98, g_z= 1.96 and $A_{x,y}$=6,6 mT, A_z= 19.5 mT, suggesting that the vanadyl cation, VO^{2+}, is bound. Quantitation of the signal indicated a substoichiometric presence of vanadium as VO^{2+} (7-10 % of the enzyme concentration).

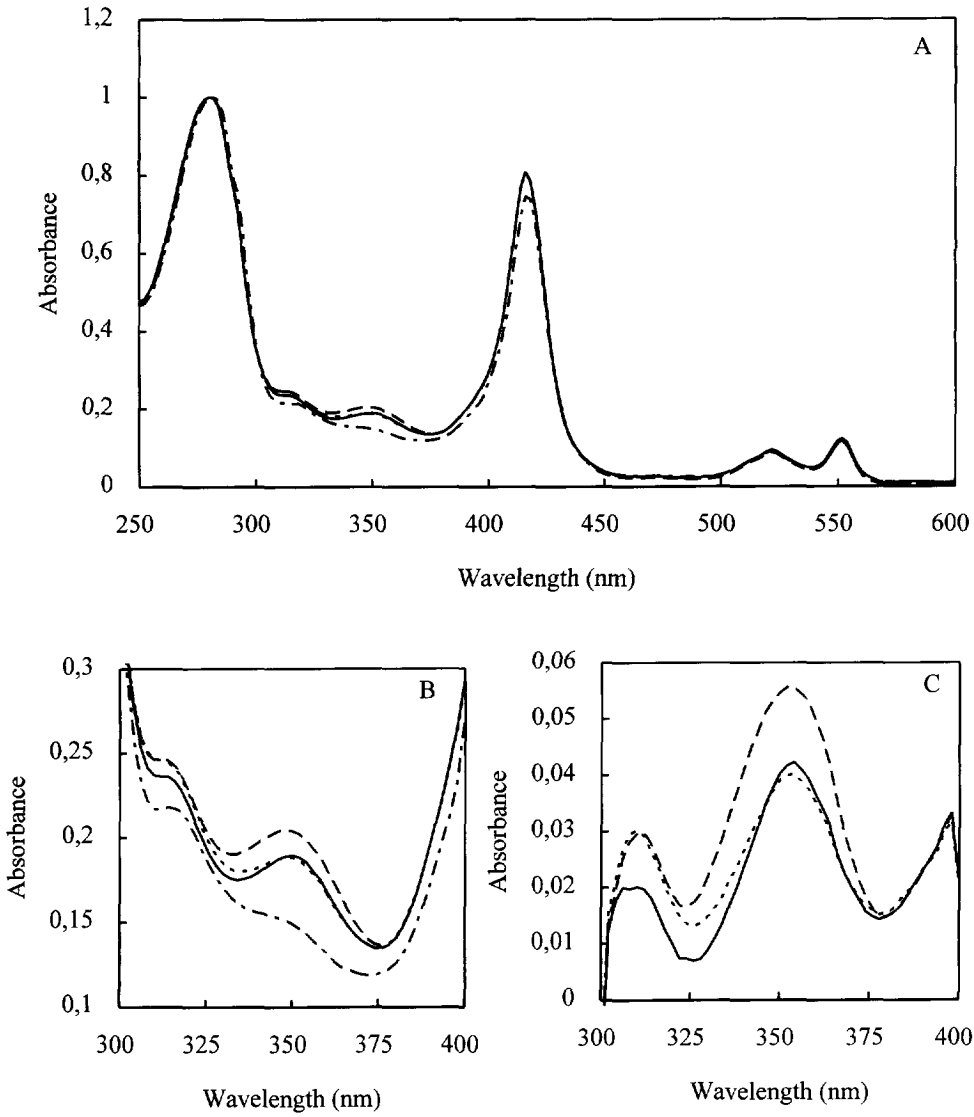

Figure 8. Ultraviolet/visible absorbtion spectra of Ca^{2+}-depleted QH-EDH reconstituted with various metalions. (a) Ultraviolet/visible absorbtion spectra of 5 μM QH-EDH reconstituted with 1 mM metalions at 25 °C. Buffer 20 mM MOPS/KOH, pH 7.5. (· · · · · ·) Mg^{2+}; (——) Ca^{2+}; (-----) Sr^{2+}. The spectrum of 5 μM Ca-depleted QH-EDH (- · - · -) is added for comparison. b) Enlargement of the ultraviolet/visible absorbtion spectra of calcium-depleted QH-EDH and QH-EDH reconstituted with different metalions between 300 and 400 nm. c) Difference spectra of QH-EDH reconstituted with different metalions minus the calcium-depleted QH-EDH spectrum.

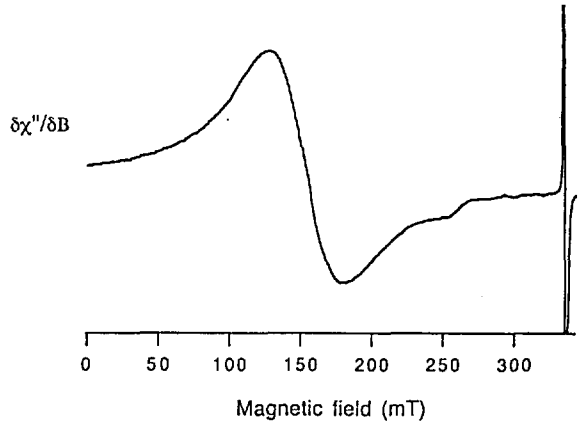

Figure 9. EPR spectrum of Ca^{2+}-depleted QH-EDH reconstituted with Co^{2+}. A sample of 250 μM Ca^{2+}-depleted QH-EDH in 20 mM MOPS/KOH (pH 7.5), was reconstituted with 1 mM Co^{2+} at 25 °C and excess metal ion was removed. EPR conditions: Microwave frequency 9.230 GHz; temperature 26 K; microwave power 2 mW; modulation amplitude 1.0 mT.

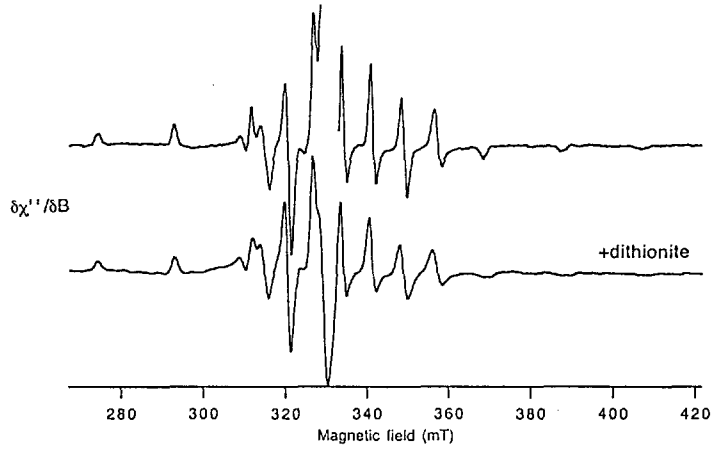

Figure 10. EPR spectrum of Ca^{2+}-depleted QH-EDH reconstituted with VO^{2+}. A Sample of 1 mM Ca^{2+}-depleted QH-EDH in 20 mM MOPS/KOH (pH 7.5), was reconstituted with 1 mM VO^{2+} at 25 °C, and excess metal ion was removed. EPR conditions: Microwave frequency 9.248 GHz; temperature 77 K; microwave power 2 mW; modulation amplitude 1.0 mT. A) Spectrum of VO^{2+} reconstituted QH-EDH. B) Spectrum of VO^{2+} reconstituted QH-EDH after addition of solid sodium dithionite.

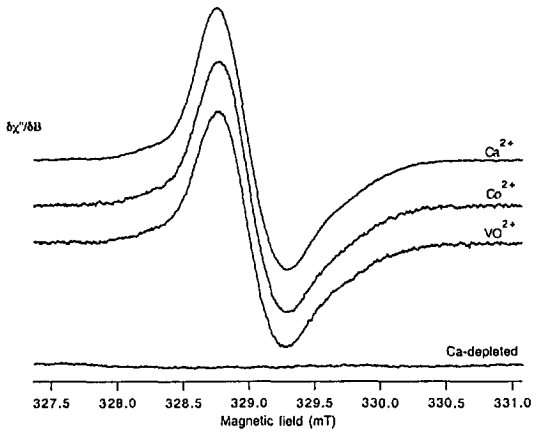

Figure 11. EPR spectra of PQQH$^{\cdot}$ in Ca^{2+}-depleted, reduced QH-EDH reconstituted with Ca^{2+}, Co^{2+} and VO^{2+}. Samples of 250 μM Ca-depleted QH-EDH in 20 mM MOPS/KOH (pH 7.5), were reconstituted with 1 mM $CaCl_2$, $CoCl_2$ and $VOSO_4$ at 25 °C. EPR conditions: Microwave frequency 9.232 GHz; temperature 65 K; microwave power 0.8 W; modulation amplitude 0.1 mT. The spectrum of a Ca^{2+}-depleted QH-EDH is added for comparison.

Reduction with excess dithionite approximately doubled the intensity of the VO^{2+}-signal, suggesting that part of the bound vanadium was in the EPR-silent V^{5+} state, and also led to a broadening of all resonances, which is particularly clear in the high-field portion of the spectrum (Figure 10b). Furthermore, the intense signal due to PQQH$^{\cdot}$ disappeared after reduction with dithionite.

QH-EDH containing Co^{2+}, VO^{2+} or Ca^{2+}, all three showed a signal belonging to PQQH$^{\cdot}$ (Figure 11). The amount of radical was, however, about two times lower in the presence of Co^{2+} or VO^{2+} (13%) than with Ca^{2+} (25%). The Ca^{2+}-depleted enzyme did not show a radical signal (Figure 11). The lineshape and the power saturation behaviour of the PQQH$^{\cdot}$ signal was the same, irrespective of whether Co^{2+}, VO^{2+} or Ca^{2+} was bound.

Discussion

The results show that QH-EDH belongs to the category of PQQ-containing enzymes where the metal ion can be rather easily removed with chelator without inducing a release of PQQ. Comparison with its closest relatives, the methanol dehydrogenases (MDHs) [18] and ethanol dehydrogenase (EDH) [14], reveals, however, some differences. Treatment of MDHs with chelators does not remove the bound Ca^{2+} (except under denaturating conditions [19]) whereas release of Ca^{2+} and PQQ occurs spontaneously in manipulating EDH [14]. Ca^{2+} is

not required for binding of PQQ to apo-MDH [19] but it is for EDH [14]. Thus despite similar capabilities to convert primary alcohols, high similarity of the amino acid sequence of the "quinoprotein part" of QH-EDH to that of the complete MDHs [20], and the fact that the amino acids thought to be responsible for Ca^{2+} binding and for catalytic activity of the active site of MDH are present in the same positions in the protein chain of QH-EDH [20], the tightness of Ca^{2+} binding and its contribution to PQQ binding seem to vary from enzyme to enzyme.

Different behaviour of the enzymes is also observed with respect to becoming reconstituted with a certain metal ion and the resulting level of activity achieved with this. As shown in Table 1, QH-EDH has a relatively broad specificity for the metal ions with which it can be reconstituted but the activity achieved with a certain metal ion varies from enzyme to enzyme. On the other hand, the effect of the metal ions on the reduced quinone cofactor in QH-EDH and MDH appears to be similar: intensification occurs of the effect on the reduced cofactor (most probably $PQQH_2$ with some contribution of $PQQH^{\cdot}$, as suggested by the EPR spectra) as demonstrated by the spectrum of reduced QH-EDH, Sr^{2+} being the most effective Figure 8c, whereas scarcely a shift in the maximum occurs; also intensification of the spectrum of the semiquinone form of MDH occurs, as judged from the comparison of Ca^{2+}- and Sr^{2+}-containing MDHs [12]. Based on this and the structural similarity, it seems that Ca^{2+} binds to QH-EDH in a similar way as to MDH, although differences exist with respect to effects exerted on PQQ binding and catalytic performance.

The fact that the reconstitution rates are independent of the Ca^{2+} concentration (Figure 5) suggests that this process occurs in two steps: a rapid binding step of the metal ion to the enzyme, leading to an intermediate enzyme form which is inactive in the assay; a slower step in which the inactive intermediate is converted into the active holo-enzyme. Although the rate of the first step will depend on the Ca^{2+} concentration, this is not observed in these experiments because this step is more rapid than the second, Ca^{2+}-independent one.

The difference spectrum between the reduced Ca-containing holo-QH-EDH and the reduced apo-QH-EDH ([8], and Figure 2) showed positive absorbances in addition to shifts in the α and γ band of the haem spectrum. The summation of the spectra shown in Figure 2b and 2c, results in the same difference spectrum. Thus the shifts in the maxima of the haem are caused by PQQ binding to the QH-EDH. Ca^{2+} has no contribution to this but just seems to enlarge the contribution of PQQ to the spectrum of QH-EDH. The difference spectra (3b and 4b) representing Ca^{2+} complexation with free PQQ and $PQQH_2$, respectively, are both dissimilar compared to the difference spectra of the Ca complexation in the reduced holoenzyme (Figure 2b), suggesting that either a large influence of the amino acid residues surrounding PQQ is exerted, preventing or overshadowing a contribution of Ca^{2+}, or that differences exist with respect to the binding site for the Ca^{2+} to the free and the bound cofactor.

Table 1. Activities of quinoprotein alcohol- and glucose dehydrogenases reconstituted with various metal ions.

Activities are related to the activity for Ca^{2+} which is set at 100. +, positive effect on the activity after reconstitution of the Ca-depleted enzyme with the metal ion of interest. -, no effect on the activity after reconstituting of the Ca-depleted enzyme with the metal ion of interest. n.d., is not determined.

	Ca^{2+}	Sr^{2+}	Mg^{2+}	Cd^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}	Ba^{2+}	Fe^{2+}	VO^{2+}	Zn^{2+}
MDH [12]	100	300	nd	nd	nd	nd	nd	nd	nd	nd	nd
EDH [14]	100	88	0	0	0	nd	nd	nd	nd	nd	nd
QH-EDH_I [this work]	100	35	48	30	4	15	10	25	28	18	0
QH-EDH_{II} [20]	+	+	nd	nd	-	-	-	nd	nd	nd	nd
s-GDH [23]	100	68	0	127	63	10	nd	7	0	nd	0
m-GDH [23]	100	70	114	18	0	33	nd	10	3	nd	100

The absence of $PQQH^{\cdot}$ in the EPR spectrum of Ca^{2+}-depleted QH-EDH and the presence of a substantial amount of $PQQH^{\cdot}$ in the enzyme forms containing Ca^{2+}, Co^{2+} or VO^{2+} clearly indicates that the metal ion affects the formation of $PQQH^{\cdot}$. Wether this is caused solely by the formation of an active enzyme, regardless of the metal ion used, or is caused by an alteration of the active site is not known.

At first sight, the conclusion that the metal ion in QH-EDH is similarly bound as in MDH, that is close to PQQ, is not in agreement with the EPR results since the spectra of $PQQH^{\cdot}$ observed for the QH-EDHs substituted with different metal ions do not vary and no coupling occurs between the paramagnetic metal ion and $PQQH^{\cdot}$ in Co^{2+}- and VO^{2+}-reconstituted enzymes. Five possibilities can be indicated to explain this.

Firstly, the Ca^{2+} removed from the enzyme is not bound to PQQ but to another site crucial for activity of the enzyme. This seems unlikely in view of the structural and spectral similarities between QH-EDH and MDH and the fact that only 1 Ca^{2+} is found per enzyme molecule [8,23].

Secondly, the chelators bind to the Ca^{2+} in the enzyme but do not remove it. The resulting inactive enzyme form can be reactivated by adding a metal ion which can remove the bound chelator. This possibility seems very unlikely since different extents of reconstitution and

different intensifications of spectral absorbancies were produced, depending on the metal ion used. Moreover, a similar pattern of activities was found when apoenzyme (without PQQ and isolated in the absence of chelator) was reconstituted with PQQ and the metal ions.

Thirdly, Ca^{2+} is situated in the vicinity of $PQQH_2$ (and perhaps PQQ) but not in that of $PQQH^{\cdot}$, e.g. due to the occurrence of large conformational changes in the redox cycle of the enzyme. However, the exclusion of the semiquinone state seems not justified since the structural information obtained for the bound Ca^{2+} just originates from an MDH crystal prepared from the semiquinone form of the enzyme [15].

Fourthly, reconstitution occurs with traces of Ca^{2+} present in the salts added or released from the wall of the vessel or from the protein upon addition of the salt. This possibility seems unlikely in view of: the different extents of reconstitution achieved and the variety in optical absorption spectra obtained with the metal ions tested; the unlikeliness that the chelators would not remove the adventitiously bound Ca^{2+}.

Fifthly, the $PQQH^{\cdot}$ signal observed derives from enzym containing Ca^{2+} and from enzyme containing the paramagnetic substituting metal ion, explaining why all spectra are the same. In this connection it should be recalled that Ca^{2+}-depletion was not complete (0.2 mol Ca^{2+}/per mol enzyme) and the substituting metal never exceeded a ratio of 0.8 metal ion/enzyme molecule. On the other hand, The $PQQH^{\cdot}$ signal was absent in Ca^{2+}-depleted enzyme and the enzyme preparations reconstituted with paramagnetic metalions always had a lower level of $PQQH^{\cdot}$ than the enzyme reconstituted with Ca^{2+}. This suggests that the $PQQH^{\cdot}$ signal really belonged to enzyme molecules reconstituted with the paramagnetic ion, implying that the supposition is incorrect.

Sixthly, the Ca^{2+} in QH-EDH (and probably in MDH) is positioned in such a way that it does not affect the distribution of the unpaired electron in $PQQH^{\cdot}$. This is in accordance with the observation that the main effect of the metal ions is on the intensity of absorbancies of the reduced cofactor, not on that of the maximum of the absorbance peak (whereas they do with respect to the optical and EPR spectra of the free form of the cofactor). It seems, therefore, that the metal ions exert secondary effects, e.g. by affecting the polarity of the active site and in this way affecting the cofactor. On the other hand, since metal-ion-lacking, PQQ-containing enzymes are inactive without exception, the metal ions are essential for catalytic activity. Furthermore, no proportional relationship exists between the effects exerted on PQQ with respect to its spectrum and its activity: Sr^{2+} gives the highest intensification of the spectrum for MDH as well as QH-EDH but the activity is 3 times higher for MDH [13] whereas it is lowered to 80% for EDH [15] and to 35% for QH-EDH, as compared to the Ca^{2+}-containing form. This could be interpreted that still another effect is caused by the metal ions which is not connected to the spectral effect. Apart from this, it is clear that either the metal ion directly participates in catalysis or brings PQQ in an orientation fitted for that. Discrimination between these two possibilities cannot be made at present.

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Summary

The goal of this study was to investigate the interaction and the relative positions of the two redox centra in the quinohaemoprotein ethanol dehydrogenase from *Comamonas testosteroni* and the role of the Ca^{2+} in this. In order to carry out these studies, a characterization of the enzyme was required. The enzyme is isolated in the apoform, lacking the cofactor PQQ but containing haem c (Chapter 2). Full activity for this enzyme is obtained after reconstitution with PQQ in the presence of calcium.The new value established for the molar absorption coefficient of PQQ raised questions on the PQQ content of QH-EDH. Titration of the apoenzyme with PQQ showed that the maximum activity was already obtained after 0.7 equivalents of PQQ had been added. Gel filtration and native electrophoresis of the apoenzyme showed one band of 70 kDa but on SDS/PAGE one band of the same molecular mass and two others of smaller size were seen.The reasons for this are as follows: The apoenzyme as isolated consists of a mixture of normal and nicked protein; nicked protein does not bind PQQ. A procedure was developed to separate holo-enzyme and nicked apoenzyme. This separation is based on the conformational change induced by the binding of PQQ to the enzyme. It suggests that PQQ binding affects the conformation of the enzyme, as was also established by spectroscopic methods (see below). The reconstituted, further purified holoenzyme contains one haem c, one PQQ and one Ca^{2+}.

In the past few years, the quinohaemoprotein alcohol dehydrogenase from *Comamonas testosteroni* has proven its suitability in the enantioselective oxidation of racemic solketal. Solketal is an attractive precursor in the synthesis of several pharmaceutical compounds. In principle enantioselectivity can be studied by applying protein as well as cofactor engineering of the enzyme. The isolation of an apoenzyme lacking the cofactor PQQ, raises the opportunity to reconstitute the apoenzyme with analogues of the natural cofactor. It is shown in Chapter 2 that modification of PQQ affects binding, activity and enantioselectivity in a negative way. Although the enantioselectivity is less with all analogues, these experiments clearly show the role of PQQ in the oxidation of the substrate. Modifying the o-quinone moiety of the cofactor abolishes activity but not binding of the cofactor. Substitution with alkyl groups at the C-3 and the N(1) position decreased binding when the alkyl group was larger than a methyl group.

All experimental evidence obtained sofar suggests that during catalysis, substrate, which can be a primary alcohol or a aldehyde, is oxidized by PQQ. PQQ becomes reduced in this step and the electrons from the reduced PQQ are subsequently donated to the second redox centre of the enzyme, the haem c. The reduced haem will transfer the electrons to the natural or an artificial electron acceptor. At present it cannot be ruled out that artificial electron acceptors, being active with other PQQ-containing dehydrogenases, might also react directly with the PQQ-site in QH-EDH. Possible interaction between the two redox centra was investigated using spectroscopic techniques which could provide information on the properties

of the haem c. The following techniques were applied: ultraviolet/visible absorbance spectroscopy, ^{1}H-NMR, resonance Raman and EPR (chapter 2+3). In addition the influence of PQQ binding on the midpoint redox potential of the haem c was determinated. Most techniques showed substantial differences between holo and apo-enzyme, but not resonance Raman spectroscopy. From these experiments we conclude that a binding of PQQ to the enzyme results in a conformational change, together with a rotation of the methionyl axial ligand of the haem. The changes induced by PQQ might be necessary to enable rapid intramolecular-electron transfer.

QH-EDH seems an attractive candidate for studying intramolecular electron transfer between two redox centra. An important aspect in suvh a study is the distance between the two redox centra. The best way to obtain this information is by X-ray analysis, however, these attempts have not been succesfull sofar. Therefore we tried to determine the distance between the PQQ and haem c by ^{19}F-NMR spectroscopy. This method is based on the broadening of the NMR signals of fluorine reporter molecules caused by a paramagnetic centre. In the measurements presented in Chapter 4, the paramagnetic centre is Fe^{3+} in the oxidized haem, and the reporter is a fluorine-hydrazine labelled PQQ molecule. Experiments were performed to insert PQQ-phenylhydrazine adducts into the apoenzyme in which the phenyl ring is substituted with a trifluoromethyl group at the ortho-, meta- or para-position. These derivatives were made by reacting the fluorine substituted hydrazines with PQQ in organic solvents and purifying them on reversed phase columns. The purified PQQ adducts appeared to contain the fluorine label and the apoenzyme could be reconstituted with them. The binding of some of the derivatives was less tightly than with PQQ (PQQ could replace the analogue from the active site), however, but stoichiometric binding of these derivatives did occur as evidenced by ^{19}F-NMR. The distance calculated from these spectra showed a minimal distance of 5 Å between the fluorine atom and the iron atom of the haem. The lack of knowledge on the orientation of the fluorine labels with respect to the haem c, renders this method unsuitable to determine the relative positions of the haem c and PQQ.

Another important aspect of the enzyme is the role of the calcium ion. Removal of the calcium from the active holo-enzyme resulted in inactive enzyme, which still contained PQQ (Chapter 5). Thus, the affinity of PQQ for this enzyme seemed not to be affected by the presence or absence of calcium in the way as it is in other PQQ-containing dehydrogenases where the removal of calcium leads to dissociation of PQQ. When Ca^{2+} was removed, a decrease in ultraviolet/visible absorbancies of the enzyme was observed. The enzymatic activity of Ca^{2+}-depleted enzyme could be reactivated by incubation with calcium, which also restored the ultraviolet/visible absorbancies. A great variety of other metal ions can be used to reactivate the Ca^{2+}-depleted enzyme. These metal ions showed a completely different pattern of reconstitution level compared to other PQQ-containing dehydrogenases. Reconstitution experiments with paramagnetic metal ions confirmed the binding of metal ions to the enzyme. These experiments showed that calcium is essential for the activity. However, whether calcium is directly involved in catalysis or only in the correct positioning of the PQQ, cannot be concluded from these experiments.

Samenvatting

Strukturele en mechanistische aspecten van quinohaemoproteïne ethanol dehydrogenase uit *Comamonas testosteroni*

Het doel van dit onderzoek was om de relatieve posities en de interaktie te bepalen tussen de twee redox-centra in quinohaemoproteïne ethanol dehydrogenase uit *Comamonas testosteroni* en de rol van calcium hierin. Om deze studies uit te kunnen voeren, is een karakterisering van het enzym nodig. Het enzym wordt geïsoleerd in de apo-vorm, zonder de cofaktor PQQ, maar met een haem c (Hoofdstuk 2). De volledige activiteit van het enzym wordt verkregen na reconstitutie met PQQ in aanwezigheid van calcium. De nieuwe waarde die werd bepaald voor de molaire absorptie coëfficient van PQQ, riep echter vragen op omtrent de hoeveelheid PQQ in het QH-EDH. Titratie van het apo-enzym met PQQ liet zien dat de maximale activiteit reeds bij toevoeging van 0.7 equivalent PQQ werd bereikt. Gelfiltratie en natieve electroforese van het apo-enzym lieten slechts één band van 70 kDa zien, maar op SDS/PAGE waren één band van dezelfde grootte en twee andere kleinere banden zichtbaar. De reden hiervoor is de volgende: het apo-enzym bestaat uit een mengsel van normaal en geknipt enzym; het geknipte enzym bindt geen PQQ. Een procedure werd ontwikkeld om holo-enzym en geknipt apo-enzym van elkaar te scheiden. Deze scheiding is gebaseerd op de conformatie verandering van het enzym geïnduceerd door PQQ binding aan het enzym. Dit laat zien dat PQQ binding de conformatie van het enzym verandert, hetgeen ook bevestigd zou worden met spectroscopische methoden (zie beneden). Het gereconstitueerde, verder gezuiverde holo-enzym bevat één haem c, één PQQ en één Ca^{2+}.

In de afgelopen jaren is duidelijk geworden dat QH-EDH uit *Comamonas testosteroni* bij uitstek geschikt is voor de enantioselektieve oxidatie van racemisch solketal. Solketal is een interessante bouwstof in de synthese van farmaceutische verbindingen. In principe kan men enantioselectiviteit bestuderen door zowel eiwit- als ook cofaktor-engineering toe te passen op het enzym. De isolatie van een apo-enzym zonder de cofaktor PQQ biedt de mogelijkheid om apo-enzym te reconstituteren met analoga van de natuurlijke cofaktor. In hoofdstuk 2 wordt aangetoond dat modificatie van PQQ de binding, activiteit en enantioselektiviteit in negatieve zin beinvloedt. Hoewel de enantioselektiviteit minder is met alle gebruikte analoga, laten deze experimenten duidelijk zien dat PQQ direct betrokken is in de oxidatie van het substraat. Het veranderen van de o-quinon structuur van de cofaktor leidt tot het verdwijnen van de activiteit, maar beïnvloedt de binding niet. Het aanbrengen van alkyl groepen, groter dan een methyl groep, op de C-3 en de N(1) positie resulteert in een slechtere binding.

Alle experimenten die tot nu toe zijn uitgevoerd, suggeren dat bij de katalyse, het substraat, een primair alcohol of een aldehyde, wordt geoxideerd door PQQ. PQQ wordt gereduceerd in deze stap en de elektronen van PQQ worden één voor één overgedragen aan het tweede redox centrum van het enzym, de haem c. De gereduceerde haem groep geeft

vervolgens de elektronen door aan de natuurlijke of een artificiële elektronenacceptor. Op dit ogenblik valt niet uit te sluiten dat artificiële elektronenacceptoren, die ook actief zijn met andere tot nu toe bekende PQQ-bevattende dehydrogenases, ook met de PQQ-site in QH-EDH reageren. De eventuele interactie tussen de twee redox centra is onderzocht met behulp van spectroscopische methoden, welke informatie verschaffen over de eigenschappen van de haem c. De volgende technieken werden gebruikt: ultraviolet/zichtbare absorptie spectroscopie, ^{1}H-NMR, resonantie Raman en EPR (Hoofdstuk 2+3). Daarnaast werd de invloed van PQQ op de midpoint redox potentiaal van de haem c gemeten. De meeste technieken lieten aanzienlijke verschillen zien tussen apo en holo-enzym, behalve resonantie Raman spectroscopie. Op basis van deze experimenten concludeerden wij dat de binding van PQQ leidt zowel tot een conformatie verandering van het enzym, als tot een rotatie van de methionine axiale ligand van de haem groep. De veranderingen welke door PQQ worden geïnduceerd kunnen nodig zijn om snelle intramolekulaire elektron overdracht mogelijk te maken.

QH-EDH lijkt een interessante kandidaat om intramolekulaire elektron overdracht tussen twee redox centra te bestuderen. Een belangrijk aspect in een dergelijke studie is de afstand tussen de twee centra. De beste manier om deze informatie te verkrijgen is m.b.v. X-ray analyse, echter de pogingen zijn tot nu nog zonder succes. Daarom hebben we geprobeerd om de afstand tussen PQQ en haem c te bepalen m.b.v. ^{19}F-NMR. Deze methode is gebaseerd op de verbreding van de NMR signalen van de fluor reporter molekulen, veroorzaakt door een paramagnetisch centrum. In de metingen van Hoofdstuk 4 is Fe^{3+} in geoxideerd haem het paramagnetisch centrum, and het reporter molekuul is een fluor-hydrazine gelabeld PQQ molekuul. Experimenten werden uitgevoerd om PQQ-phenylhydrazine addukten in het apo-enzym te zetten waarin de phenyl groep gesubstitueerd is met een trifluor-methyl groep op de ortho, meta of para-plaats. Deze derivaten werden gesynthetiseerd door fluor gesubstitueerde hydrazines te laten reageren met PQQ in organische oplosmiddelen en ze daarna op te zuiveren m.b.v. reversed phase kolommen. De gezuiverde PQQ addukten bevatten het fluor label en het apo-enzym kon er mee worden gereconstitueerd. De binding van sommige derivaten was zwakker in vergelijking met PQQ (PQQ verdingt het derivaat uit de active site), maar volgens de ^{19}F-NMR was de binding stoichiometrisch. De analyse van de spectra liet zien dat de afstand tussen het fluor atom en het ijzer atom in de haem minimaal 5Å moest zijn. Vanwege het gebrek aan kennis omtrent de orientatie van de fluor labels met betrekking tot de haem c, is deze methode niet geschikt om de relative posities van PQQ en haem c te bepalen.

Een ander belangrijk aspekt van het enzym is de rol van het calcium ion. Verwijdering van calcium van aktief holo-enzym resulteert in inaktief enzym, wat nog steeds PQQ bevatte (Hoofdstuk 5). De affiniteit van PQQ voor dit enzym lijkt dus niet te worden beinvloed door calcium, in tegenstelling tot andere PQQ-bevattende dehydrogenases, waar verwijdering van calcium leidt tot de dissociatie van PQQ. Wanneer calcium wordt verwijderd, neemt de ultraviolet/zichtbare absorptie van het enzym af. De enzymatische activiteit van het enzym

zonder calcium kon worden hersteld door incubatie met calcium waardoor ook de ultraviolet/zichtbare absorptie terugkomt. Een groot aantal andere metaalionen kan worden gebruikt om het calcium-vrije enzym te reactiveren. Deze metaalionen lieten een kompleet verschillend reconstitutie patroon zien, vergeleken met ander PQQ-bevattende dehydrogenases. Reconstitutie met paramagnetische metaalionen in combinatie met EPR spectroscopie bevestigde de binding van de metaalionen aan het enzym. Deze experimenten lieten zien dat calcium essentieël is voor de activiteit. Echter, of calcium direct is betrokken in de katalyse of alleen nodig is om PQQ in de juiste positie te brengen, kan uit deze experimenten niet worden geconcludeerd.

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Curriculum vitae

Govardus Adrianus Hubertus (Aard) de Jong werd geboren op 14 februari 1965 te Udenhout. Na een periode van 9 jaar in het Brabantse land verhuisde hij naar het Gelderse Druten waar hij in 1983 zijn diploma ongedeeld VWO behaalde. In hetzelfde jaar begon hij met de studie Chemie aan de Katholieke Universiteit te Nijmegen. In 1989 studeerde hij hier af met als hoofdvak Biochemie en de bijvakken Microbiologie en Bio-Organische Chemie. Na een klein half jaar als hovenier te hebben gewerkt, startte hij in 1989 een promotie-onderzoek bij de Vakgroep Microbiologie en Enzymologie onder de begeleiding van Hans Duine. De uiteindelijke resultaten van dit onderzoek zijn in dit proefschrift beschreven. Tijdens zijn promotietijd nam hij in decemder 1991 deel aan de biotechnologische studiereis naar India, waarvan hij samen met Arie Geerlof als samensteller en editor fungeerde voor het eindverslag.*

Vanaf september 1994 is hij werkzaam als post-doctoraal medewerker bij de Vakgroep Microbiologie en Enzymologie van de TU Delft.

* Geerlof, A. & de Jong, G.A.H. (editors) (1992) *Biotech study tour India 1991.*