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The enantioselectivity of quinohaemoprotein alcohol dehydrogenases: mechanistic and structural aspects

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Dedicated to: Professor Hans Duine on the occasion of his retirement from the Enzymology chair of the Delft University of Technology, Delft

Abstract

Quinohaemoprotein alcohol dehydrogenases, QH-ADHs, isolated from *Acetobacter*, *Gluconobacter* and *Comamonas* species show appreciable enantioselectivity in the oxidation of chiral primary and secondary alcohols. Current views of the structural and mechanistic factors of importance for the understanding of the enantioselective performance of these enzymes are reviewed. Structural properties of QH-ADH, Type I, from *C. testosteroni*, and QH-ADH, Type II, from *A. pasteurianus* have been deduced from homology modeling studies based on the X-ray crystallographic data available for PQQ-containing quinoprotein methanol dehydrogenases, MDHs. Mechanisms that have been proposed for quino(haemo)protein alcohol dehydrogenase-catalyzed substrate oxidation are discussed in relation to the constraints set by the observed enantioselectivity. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Pyrroloquinoline quinone; Quinohaemoprotein; Alcohol dehydrogenase; Enantioselective oxidation; Chiral alcohols; Kinetics; Mechanism; Structure; Molecular modeling; Review

1. Introduction

The identification in 1979 of pyrroloquinoline quinone, PQQ, as the cofactor of certain dye-linked methanol dehydrogenases, MDHs [85,258], and glucose dehydrogenases, GDHs [84], provided a major stimulus for the search of other enzymes that might contain this cofactor. Shortly after the detection and structure elucidation of PQQ, a number of claims regarding its presence in dehydrogenases and oxidases of both bacterial and eukaryotic origin were made [81]. With the development of more accurate and reliable analytical procedures, however, it became clear that the distribution of PQQ is most probably limited to a relatively small group of bacterial dehydrogenases [80,185]. Eventually, other quinones including topa quinone, TPQ [166], its lysyl derivative, LTQ [316] and tryptophyl–tryptophan quinone, TTQ [219], could be identified as the organic cofactors of enzymes that were once suggested to contain PQQ. Today, enzymes possessing either one of the cofactors shown in Fig. 1 are collectively addressed as quinoproteins. An impressive number of comprehensive accounts of the current

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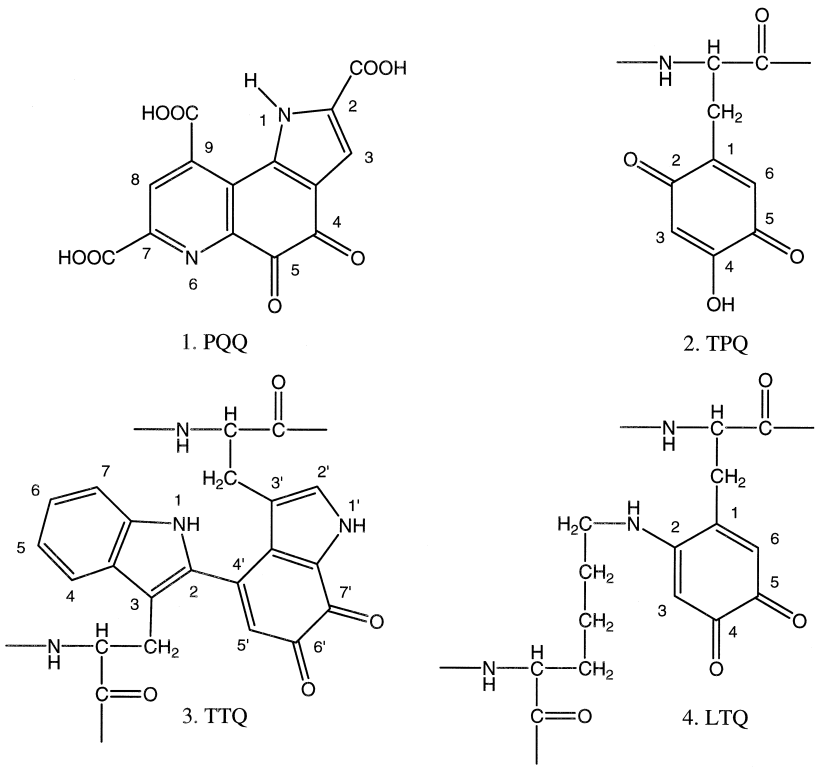


Fig. 1. Structures of quinoprotein cofactors. (1) PQQ [85,258]; (2) TPQ [166]; (3) TTQ [219]; (4) Lysine tyrosylquinone, LTQ [316]. TPQ, TTQ, and LTQ are derived from the side chains of amino acids that form an integral part of the polypeptide backbone; PQQ is non-covalently bound.

status of quinoprotein research covering various aspects of cofactor identification, enzyme characterization, and distribution have appeared [14, 17,18,63,80,120,184,218].

X-ray crystallographic investigations of PQQ-containing MDHs [118,328–330], TPQ-containing amine oxidases, AOs [191,324,326], and TTQ-containing methylamine dehydrogenases, MADHs [47–50,140,312,313], have provided insight into the structural aspects of quinoprotein enzymes. Information regarding the distribution and physiological role of quinoproteins has become available as well [213,218,276]. As a result, the novelty of quinone cofactors among the array of organic coenzymes and cofactors of which role and function in metabolism were already firmly established, is wearing off. Still, a number of important questions remain to be solved. In this respect, the catalytic mechanism of the PQQ-containing alcohol dehydrogenases is a key issue. Despite intensive efforts by a number of groups only limited insight into the events leading to the transfer of reducing equivalents from substrate to PQQ and from reduced PQQ to the electron acceptor has been gained. This situation is particularly frustrating when considering PQQ-containing quinohaemoprotein alcohol dehydrogenases. As shown in Table 1, representatives of this class display appreciable enantioselectivity in the oxidation of low-molecular chiral alcohols of which the pure enantiomers are of interest for synthetic applications. Their performance may well be compared to enantiomeric ratio values currently realized with non-biological catalysts [110,235,295] where $E = 40$ appears to be the rule [311], and $E = 200$ a remarkable exception [56]. Realisation of the full potential of quinoprotein alcohol

Table 1
Enantioselective properties of quino(haemo)protein alcohol dehydrogenases

Enzyme	Source	Substrate	*E*-value (preference)	Refs.
MDH	*Hyphomicrobium* X	solketal	1 (−)	[114]
QH-ADH (type II)	*G. oxydans*	glycidol	26 (*S*)	[115]
	G. oxydans	solketal	12 (*R*)	[114]
	G. oxydans	1,2-cyclo-hexanediol	> 50 (*R*)	[3]
	A. pasteurianus	glycidol	7–18 (*S*)	[200]
	A. pasteurianus	2-butanol	13–20 (*S*)	[200]
	A. aceti	glycidol	17 (*S*)	[114]
QH-ADH (type I)	*C. testosteroni*	solketal	> 100 (*R*)	[114]
	C. testosteroni	glycidol	1 (−)	[115]
	C. testosteroni	2-butanol	14 (*S*)	[281]
	C. testosteroni	2-hexanol	105 (*S*)	[281]
	C. testosteroni	2-heptanol	315 (*S*)	[281]
	C. testosteroni	2-octanol	> 800 (*S*)	[281]

dehydrogenases in industrial processes, however, requires the enzymes not only to be enantioselective, but also to be readily available, highly active and suitably stable [101,268]. Although such properties may eventually be attained through brute force screening, recombinant DNA techniques, or directed evolution protocols, proper understanding of the structural and mechanistic factors involved in enantioselective catalysis will be an important tool to guide such efforts.

In addition to the prospects for applications in the fields of fine chemicals synthesis [112, 114,115,280], biosensor devices [179,183,192, 259,279,336] and possibly combinatorial biocatalysis, PQQ-containing quino(haemo)protein dehydrogenases represent a class of fundamentally interesting enzymes in their own right. Although the substrate specificity fingerprints show appreciable overlap with those of NAD(P)-dependent alcohol dehydrogenases, their natural electron acceptors, ubiquinone [59,188, 189,214], cytochrome-*c* or blue copper proteins [67,68], are found towards the oxidative end of the biological redox scale. By consequence, dehydrogenation reactions catalyzed by quinohaemoproteins are strictly irreversible. Yet, the magnitude of the driving thermodynamic potential does not inspire particularly high catalytic reaction rates. Although it would appear rather naïve to expect the Gibbs free energy difference of reactants and products to be proportional to the (catalytic) reaction rate, such correlations have indeed been described for simple exothermic reactions [193,267], hypothized for enzymatic conversions [282], and on occasion pointed to interesting phenomena obstructing the free equilibration of chemical species, i.e., the spin-restrictions governing autoxidation reactions.

At present, comparative enzymology of quinoproteins has not provided convincing explanations for the relatively low catalytic rates of MDHs and QH-ADHs. In particular, considering the impressive turn-over numbers ($\approx 10^5$ s^{-1} [241]) measured for the related PQQ-containing quinoprotein GDH, it would seem that the exchange rate of reducing equivalents between substrate and cofactor in QH-ADHs ($\approx$ 10^2 s^{-1} [68,200]) could be further improved by several orders of magnitude. Again, knowledge of the 'enantioselectivity-controlling' step of the quinohaemoprotein alcohol dehydrogenase mechanism might provide clues for the interpretation of the rate-controlling step [194] that leads to this intriguing situation.

As indicated above, the enzymology of quinoproteins has been the subject of a fair number of reviews. Comprehensive accounts of the actual chemistry occurring in the active site, on the other hand, are less numerous. In order to emphasize both the fundamental and practical aspects of this highly interesting field of re-

search, the enantioselective properties of quinohaemoproteins provide an attractive directive. In addition to its practical consequences for applications in biocatalytic kinetic resolution processes, enantioselectivity can be a valuable tool in the elucidation of kinetic mechanisms. Accordingly, this paper has been organized as follows. In Section 2 we present a brief summary of the kinetic description of enzymatic enantioselectivity in terms of the ratio of specificity constants, the enantiomeric ratio, *E*. The formal identification of the kinetic constants collected in *E*, with the Gibbs free energy barriers along the reaction coordinate is discussed in the context of Eyring Transition State Theory, TST. Implications of the location of the 'enantioselectivity-determining' reaction step(s) on the reaction coordinate are emphasized. In Section 3 an account is given of the current knowledge of structural and kinetic properties of quino(haemo)protein alcohol dehydrogenases that have a bearing on the catalytic mechanism. Subsequently, an overview of reaction mechanisms that have been proposed over the years for this class of enzymes is given in Section 4. In Section 5 we discuss the possible relevance of alternative chemical and mechanistic schemes for the catalytic and enantioselective properties of quinohaemoprotein alcohol dehydrogenases. Recent results of molecular modeling experiments for representative QH-ADHs from *Acetobacter* and *Comamonas* species are proposed as a frame of reference. Perspectives for further developments in this area are addressed in Section 6.

2. Enantioselectivity as a mechanistic tool

Following its introduction by Sih et al. [45, 46], the enantiomeric ratio, $E = (k_{cat}/K_M)_R/(k_{cat}/K_M)_S$ has gained widespread acceptance as the parameter of choice to quantify the enantioselective properties of enzymes. Comprehensive reviews on the determination and the use of *E*-values in the field of biotechnological applications have appeared [174,284]. Meanwhile, it has become clear that the enantiomeric ratio can also be of fundamental importance as a valuable source of inspiration for the interpretation of kinetic properties in terms of mechanistic features. Of both practical and fundamental importance is the fact that the *E*-value reflects *relative* reaction rates. Since *E*-value determinations do not, in general, require specific knowledge of the amount of active enzyme present, experimental difficulties that are commonly encountered when measuring *absolute* reaction rates of enzyme-catalyzed reactions do not apply. Estimates of the rate-determining quality of the reaction steps involved in enantioselective catalysis under various conditions of, e.g., temperature, pressure, and medium composition, can thus be readily obtained. A price has to be paid, however. Whereas measurements of absolute rates may provide data on individual reaction steps, such information is not directly available from *E*. A brief introduction of the theoretical background is given below. Detailed discussions have appeared elsewhere [9,201,245].

Eq. (1) represents the generic case of an enzyme catalyzing the conversion of mutually competitive enantiomeric substrates, *R*, viz. *S*, following irreversible Michaelis Menten-type kinetics:

$$\frac{r_R}{r_S} = \frac{\left(k_{cat}^R/K_M^R\right) * [E] * [R]}{\left(k_{cat}^S/K_M^S\right) * [E] * [S]} = E * \frac{[R]}{[S]} \qquad (1)$$

The direct applicability of Eq. (1) for substrate and product inhibition schemes can be appreciated by noting the (redundant) presence of the concentration of free enzyme, $[E]$. When additional substrates and/or reversible reactions are involved, more elaborate expressions are required [113,285,310]. Also in these cases, the enantiomeric ratio can be formed from the kinetic parameters, k_{cat} and K_M, for the enantiomeric substrates, albeit that the number of microscopic rate constants lumped in *E* increases rapidly when the kinetic schemes become more realistic. This complexity tends to

obscure the extent to which individual microscopic rate constants contribute to the overall enantioselective properties. Expressing the *E*-value in terms of reciprocal rate constants (characteristic reaction times) is helpful. A formally equivalent and visually appealing picture is obtained when rate constants are expressed in the (thermodynamic) format of Eyring TST [98, 300,301]:

$$k = \kappa \frac{k_B T}{h} \exp\left(- \frac{\Delta G^{\#}}{RT} \right) \quad (2)$$

The procedure is summarized in Eqs. (3) and (4). Details of the derivation of Eq. (4) can be found elsewhere [244,245].

$$E = \frac{k^R_{spec}}{k^S_{spec}} = \frac{k^R_1 k^R_2 / \left(k^R_{-1} + k^R_2 \right)}{k^S_1 k^S_2 / \left(k^S_{-1} + k^S_2 \right)} \quad (3)$$

$$E = \frac{\exp\left(\dfrac{\Delta G^{\#}_{spec,S}}{RT} \right)}{\exp\left(\dfrac{\Delta G^{\#}_{spec,R}}{RT} \right)} = \frac{\exp\left(\dfrac{\Delta G^{\#}_{A^S}}{RT} \right) + \exp\left(\dfrac{\Delta G^{\#}_{B^S}}{RT} \right)}{\exp\left(\dfrac{\Delta G^{\#}_{A^R}}{RT} \right) + \exp\left(\dfrac{\Delta G^{\#}_{B^R}}{RT} \right)} \quad (4)$$

Diagrams of the full scheme, the presentation in terms of the specificity constants, $k_{spec} = k_{cat}/K_M$ [231] (but see discussion in Refs. [26,60,96]), and the identification of the *E*-value as $RT \ln E = \Delta G^{\#}_{spec,S} - \Delta G^{\#}_{spec,R} = \Delta\Delta G^{\#}$ are shown in Fig. 2, Panel A, for the enantioselective branches of a ping-pong kinetic scheme. Ternary complex kinetic schemes may require slightly different representations [285]. In addition to the assumptions underlying Eyring TST [70,136,142,301], transmission coefficients, κ, for the individual rate constants have been assumed identical. It must be emphasized that as a result of the numerical properties of sums of exponentials, the contribution of individual barriers to the overall enantioselectivity is governed by minor differences of the exponents ($\Delta G^{\#}$-values). Since $k_1 (= k_{on})$ is not normally rate controlling for dissolved enzymes in solution [30,216] (but see Refs. [167,290]), its contribution to the enantioselectivity will be negligible.

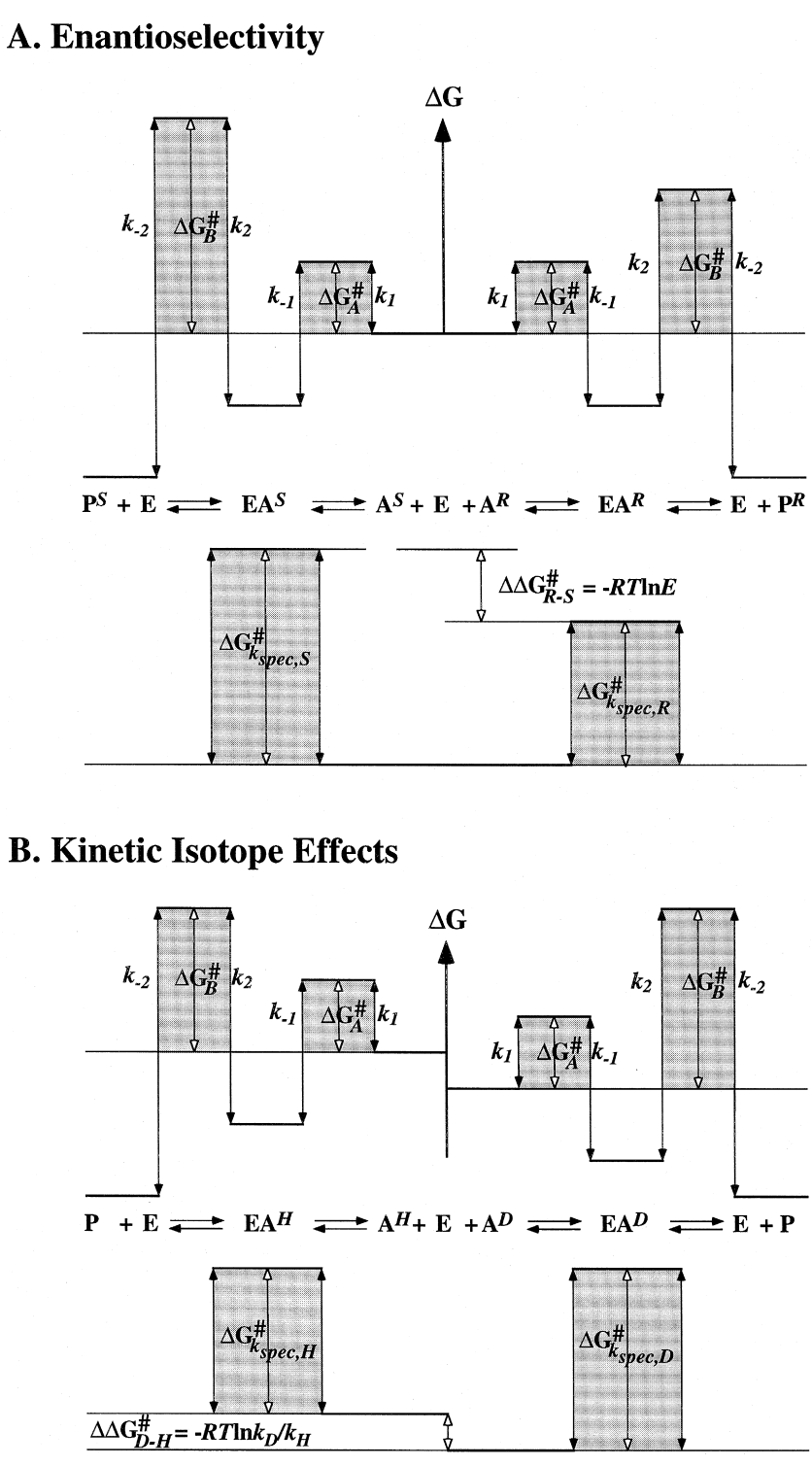
A great many different protocols for the representation of kinetic schemes by (free) energy profiles have been explored [40,41,91,102,186, 187,221,227,286,331,332]. The approach taken here appears to be unique in its clear recognition of the contribution of exponentially weighted activation energies of individual barriers to the specificity constant. Extending the thermodynamic formulation of Eyring TST by including thermodynamic activities of reactants and activated complexes [99,215] has been applied to hydrolytic enzymes [274,275,310]. Preliminary results for dehydrogenases have appeared [309].

It is instructive to apply the formal approach outlined above for the case of the enzymatic discrimination of enantiomers to the effects of isotopic substitutions on enzymatic reaction rates (Fig. 2, Panel B). Whereas the difference in rates observed for enantiomeric substrates has its origin in the Gibbs free energy difference of the 'enantioselectivity-determining' (diastereomeric) *transition states*, isotope effects according to the Bigeleisen–Wolfsberg formulation [35,141] reflect the perturbation of the vibrational frequencies contributing to the TS and *ground state* energy [261]. In this restricted sense, enantioselectivity can be considered to address the transition state more directly.

3. Characterization of quino(haemo)protein alcohol dehydrogenases

Dye-linked alcohol dehydrogenases containing PQQ (2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione, PQQ) and Ca^{2+} as cofactors have been isolated from various Gram-negative bacteria [17,80,217]. MDHs from

methylotrophic bacteria [11,19,119,212] and ethanol dehydrogenase from *Pseudomonas* sp. [120,262] are prominent members of this class. Quino *haemo* protein alcohol dehydrogenases,



QH-ADHs, form a second class containing haem *c* as an additional organometallic cofactor probably involved in the electron acceptor interaction [113]. Type I QH-ADHs are monomeric proteins (70 to 80 kDa) containing one molecule of PQQ, one Ca^{2+}, and a single *c*-type haem. Examples have been isolated from ethanol grown *C. testosteroni* [68,123], vanillyl alcohol grown *Rhodopseudomonas acidophila* [334,335] and *Pseudomonas putida* [299]. Type II QH-ADHs have been isolated from various acetic acid bacteria. They are membrane-associated enzymes composed of different subunits. A large subunit (72 to 80 kDa, subunit I) containing one molecule of non-covalently bound PQQ, one Ca^{2+}, and one haem *c*, is thought to possess the dehydrogenase activity [213]. A medium-sized subunit (43 to 53 kDa, subunit II), most likely involved in electron transport, contains three haem *c* moieties and shows a high degree of similarity to the CO-binding cytochrome c_{553} [214]. A small subunit (15 to 20 kDa, subunit III) has been speculated to promote the proper association of the subunits at the membrane interface [189]. In Type II QH-ADH from *A. pasteurianus* and *A. aceti* all three subunits appear to be essential for enzymatic activity. Note that the distinction between *classes* (haem/non-haem) and *types* (subunit composition) used here differs from that used by other authors [15,213].

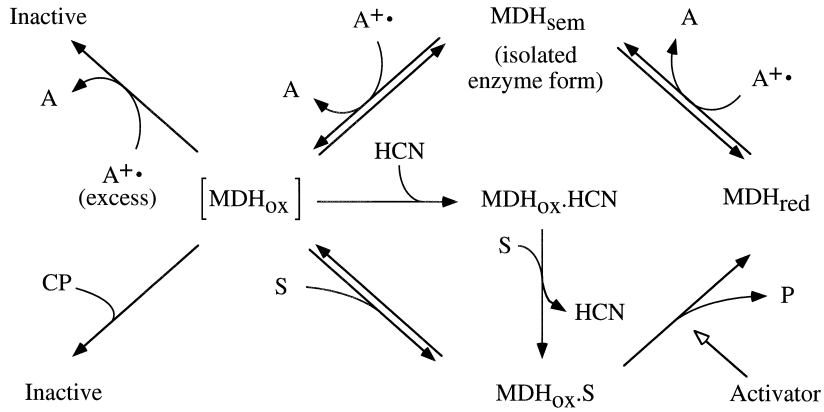
3.1. PQQ-containing MDHs

3.1.1. Kinetic properties

Before discussing the enzymological characterization of the quino*haemo*protein alcohol dehydrogenases, a short survey of the non-haem PQQ-containing alcohol dehydrogenases will be given. Soluble MDHs occurring in the periplasmic space of methylotrophic bacteria have played an important role in the development of quinoprotein enzymology. Although the presence of a cofactor of unknown structure, later to be identified as PQQ, was first reported for GDH in the sixties [132,133], MDHs from methylotrophic bacteria became the source for the actual isolation and structure elucidation of PQQ [61,85,258]. In addition, MDH (from *Methylophilus methylotrophus* and bacterium W3A1) was the first quinoprotein for which the three-dimensional structure was solved [328].

NAD(P)-independent MDH activity was originally described by Anthony and Zatman [16]. A large number of MDHs (EC 1.1.99.8) with similar properties have been characterized since [10]. As isolated, MDHs are dye-linked enzymes, requiring cationic electron acceptors, the presence of ammonia (which can sometimes be substituted with higher primary amines) as an activator, and pH ≅ 9 for in vitro activity. Most MDHs are dimeric proteins containing two molecules of PQQ [74]. Despite its ready availability from various methylotrophes, with *Hyphomicrobium* X as a particularly abundant source [83], MDH turned out not to be an easy target for the study of the catalytic mechanism. Several complications exist: (1) MDHs are usually isolated with their cofactor in a semiquinone form, a redox state that is unreactive with substrate [105]; (2) oxidation with artificial electron acceptors leads to inactivation unless substrate or carbonyl group reagents are present [10,12, 105]; (3) enzyme preparations appear to be inevitably contaminated with large amounts of endogenous substrate(s) of unknown origin [16,25,83,117]. By combining spectroscopic and

Fig. 2. Panel A: Identification of microscopic rate constants contributing to the enantiomeric ratio, *E*, with the Gibbs free energy profile along the reaction coordinate. Transition state free energies according to the Eyring TST formalism. Panel B: Idealized presentation of Gibbs free energies to be identified with microscopic rate constants for isotopically substituted substrates. It is assumed that the potential energy surface at the TS follows the Born–Oppenheimer approximation, while the isotopic mass does not contribute to the energy of the translational mode leading from reactant to product states.



Scheme 1. MDH_{red}, reduced MDH; MDH_{sem}, semiquinone form of MDH (EPR active); MDH_{ox}, fully oxidized form of MDH; $MDH_{ox} \cdot HCN$, the complex of MDH with cyanide; $MDH_{ox} \cdot S$, the complex of MDH with substrate alcohol or aldehyde; CP, cyclopropanol; activator, ammonia or low molecular primary amine; A and A^+, reduced and oxidized form of the electron acceptor (Wurster's Blue) (adapted from Ref. [105]).

kinetic techniques, a convincing kinetic scheme could nevertheless be devised [89,104–106] (Scheme 1). Key observations comprised the radical nature of the MDH semiquinone form [88], the acid extraction of stoichiometric amounts of PQQ and PQQH2 from this enzyme form [104], and the structure elucidation of the PQQ-adduct extracted from cyclopropanol-inactivated MDH [106] (Fig. 3).

The chemical mechanism that has been proposed on the basis of these findings will be discussed in Section 4.

3.1.2. The structure of PQQ-containing MDHs

X-ray crystallography of MDHs from *Me. methylotrophus* and *M*. W3A1 [328] showed the basic structure of the PQQ-containing α subunit to be a 'propeller fold' barrel consisting of eight 'blades,' also called 'tryptophan motifs' (Ws) since these residues hold together small stretches of β sheets around the superbarrel. Similar propeller motifs have been observed in a large family of proteins [111,277]. The small β subunit is nested in a zipper-like fashion along one side of the α subunit. The orientation originally assigned to PQQ in the active site has created confusion [158]. A detailed study of *M*. W3A1 (crystal form A) at 2.4 Å resolution [322,329] as well as the determination of the structure of MDH from *Methylobacterium extorquens* AM1 at 1.94 Å resolution [118] confirmed the position and orientation of PQQ as shown in Fig. 4. A detailed description

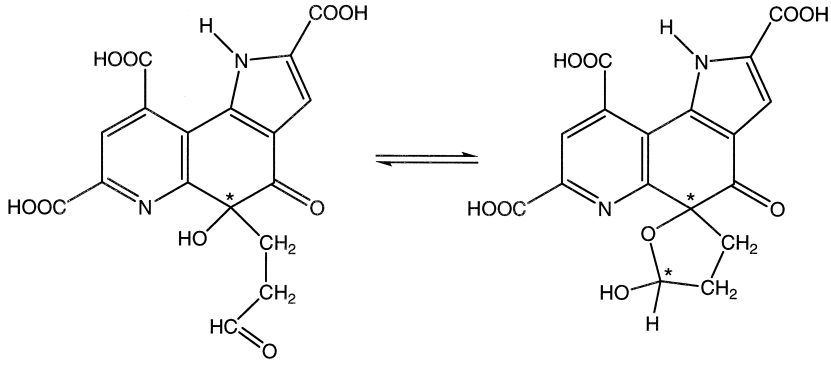


Fig. 3. Structures of the interconverting PQQ-adducts isolated from cyclopropyl alcohol-inactivated MDH [106]. Note presence of two chiral centers giving rise to diastereomeric mixtures.

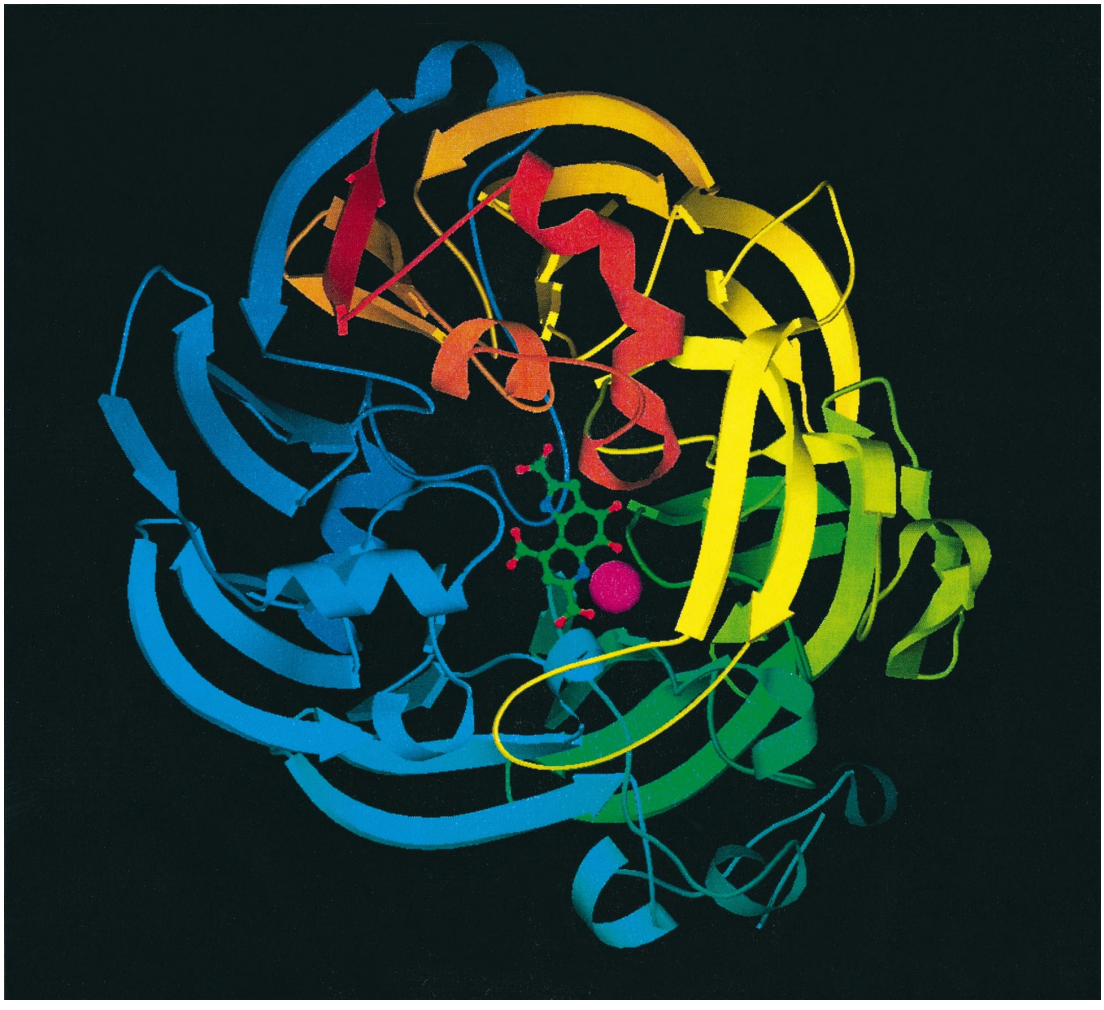


Fig. 4. Schematic representation of MDH from *Me. methylotrophus* W3A1 showing the eight-bladed β-propeller fold. PQQ shown in a ball and stick representation, calcium as pink sphere. This figure was generated using coordinates from pdb entry pdb1BN2 and MOLSCRIPT version 2.0.2 [190].

of the active site of MDH from *M*. W3A1 at 1.9 Å resolution revealed the presence of a substrate molecule in the vicinity of the cofactor [330].

Crystal structures have shown the presence of a rather unique non-planar eight-membered ring formed by two vicinal cysteines (residues 103 and 104). The disulfide-bridge was originally taken to be part of a *cis*-peptide bond [322,328], refined X-ray structures, however, have shown the cysteines to be in a *trans* configuration both in the MDH structures of *M.* W3A1 [329] and *M. extorquens* [118]. The disulfide bridge is positioned on top of the cofactor with its sulfur atoms at ± 3.5 Å parallel to the plane of the PQQ molecule. The bridge is solvent accessible forming one side of the active site funnel with the opposite wall made up of various hydrophobic residues. Tryptophan 243 (*M. extorquens*) forms the bottom of the active site cavity. PQQ is positioned parallel to this residue with the phenyl moiety of the indole group in what appears to be a π-stacking interaction with the pyridine ring of PQQ, thus sandwiching PQQ

between Trp 243 and the disulfide bridge. The calcium ion is liganded by the γ-carboxyl oxygens of Glu 177 (axial position), the amide oxygen of Asn 261, and the C(5)-carbonyl oxygen, the C(7)-carboxyl group and the pyridine nitrogen from PQQ occupying equatorial positions (residue numbering refers to MDH from *M. extorquens*). The remaining axial position is filled by an Ala 305. All distances of ligands to Ca^{2+} range between 2.4 and 2.8 Å. These values that are in fair agreement with data collected in the Cambridge database for, i.e., Ca^{2+}-carboxylates [43]. In the high resolution structures, water molecules are seen nearby the C(7)-carboxyl group and C(5)-carbonyl oxygen of PQQ. The proposed active site base, Asp 303, is located opposite the *o*-quinone moiety of PQQ. As a result of its limited dimensions, the funnel-shaped active site cavity places severe restrictions on the size of possible substrates. In accordance with experimental findings, it appears that only primary alcohols may enter the active site (Fig. 5).

The hydrophobic residues in the active-site funnel might play an important role in the docking of the naural electron acceptor of MDH, cytochrome c_L. Originally, it was thought that the small β-subunit was responsible for the interaction with this cytochrome due to the excess of lysines present (15 out of 74 residues) [12,236]. However, those lysines are not conserved in all MDHs. Further chemical studies suggested interaction of lysines at the surface of the α-subunit with carboxylates on the cytochrome. The importance of these ionic interaction was confirmed by the influence of the ionic strength on the rate of electron transfer [44,57]. For effective electron transfer, PQQ and the haem of cytochrome c_L need to be in close contact. The shortest possible pathway would be via the hydrophobic funnel. No lysines are present in the vicinity of the funnel entrance. Current ideas involve alignment and initial docking of the two proteins by electrostatic interactions via lysines at the surface of the α-subunit and carboxylates on the cytochrome followed by a change in docking position due to hydrophobic interactions between the active site funnel and the natural electron acceptor [62].

Determination of the three-dimensional structure of a macromolecule by X-ray crystallography is commonly regarded as the cornerstone of the structural characterization. While protein crystallographers feel confident that uncertainties in the structure are properly acknowledged by specifying the resolution and by giving reference to the protocols used, researchers that are less familiar with this discipline, on the other hand, tend to overlook the assumptions underlying the fitting and regression analyses and express too much faith in the description of the structure that is provided. On occasion, this widely held belief has been challenged (see Refs. [138,263] and comments in Ref. [169]). In this respect, the erroneous orientation of PQQ in the structure originally proposed for MDHs of *Me. methylotrophus* and *M*. W3A1 [328] may serve as an example of the fact that the information implied in X-ray structures may be biased by the underlying assumptions. Of course, this bias will be less severe when better resolutions are obtained. In the context of the present survey, two details stand out from the high-resolution structure reported for MDH from *M*. W3A1 [330]. Of these, the interpretation of the elongated electron density in the active site cavity in terms of the presence of a molecule of methanol is well supported by earlier findings of a single tightly bound methanol (or formaldehyde?) to MDH [117]. The out-of-plane position of the oxygen atom in the PQQ–C(5)-carbonyl group is more surprising, however, especially since a similar observation has been reported for the atom attached to the PQQ–C(4) position in the MDH from *M. extorquens* [118]. Both structures are shown in Fig. 6. Discussion of the possible relevance of these observations will be deferred until Section 5.

In addition to the remarks made above, the question may be raised whether the determination of good-quality structures has provided insight into the complications that have hampered

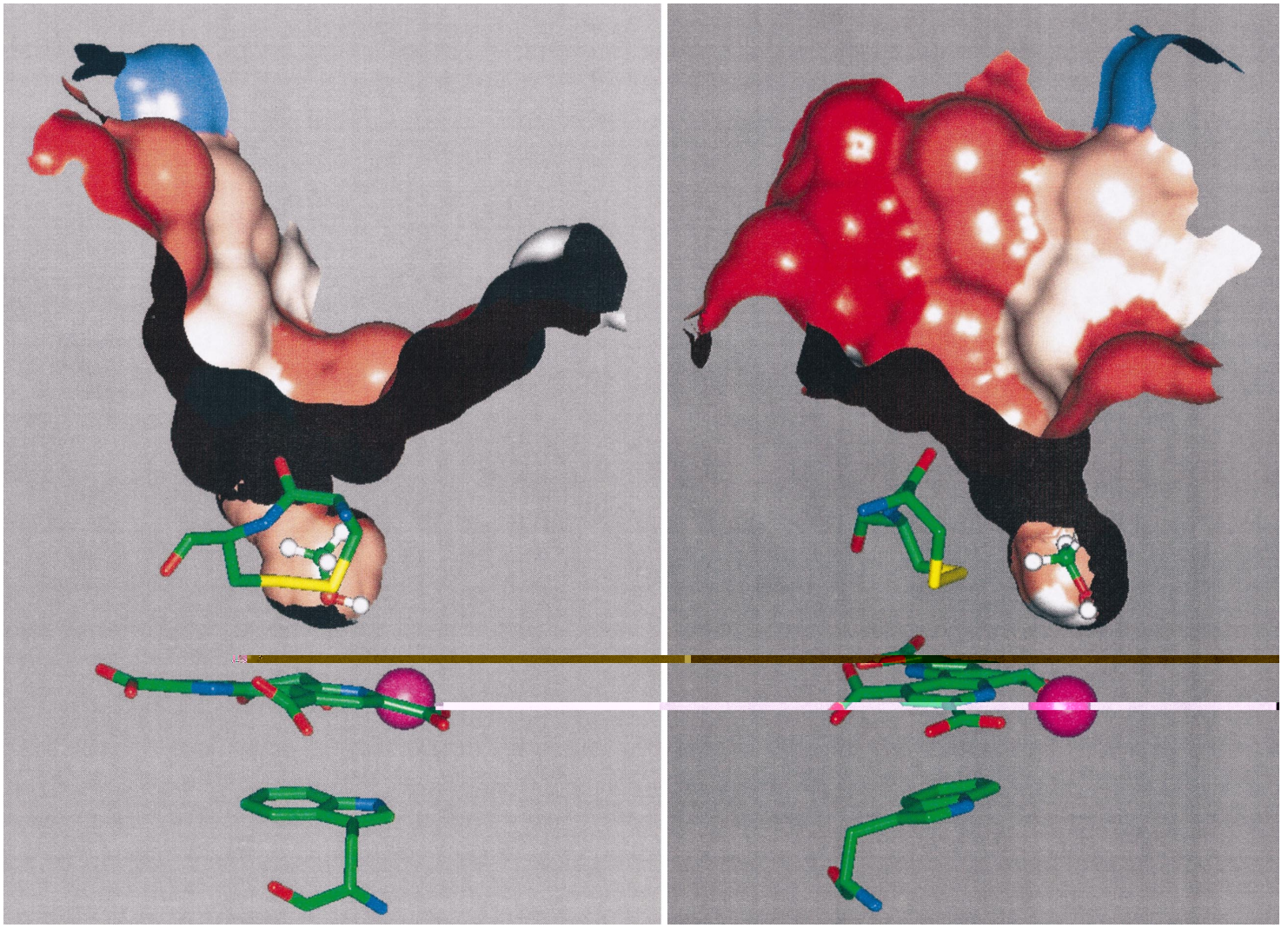


Fig. 5. View on the active site of MDH from *Me. methylotrophus* W3A1 showing the eight-membered disulfide bridge in stacking interaction with PQQ and underlying tryptophan residue (Trp 237). The molecule of methanol (or formaldehyde) present in the crystal structure is represented as a ball and stick model. The solvent accessible surfaces were generated while omitting the substrate molecule. Residues and PQQ shown as stick models, calcium as CPK model (radius divided by two). Figures generated using INSIGHT (Biosym).

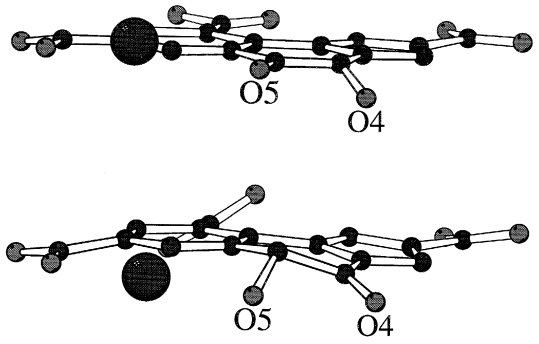


Fig. 6. Structures of PQQ in the active site of MDH. Panel A: Detailed view of the active site of MDH from *M. extorquens* (1.94 Å resolution, coordinates were kindly provided by the authors [118]). Panel B: Corresponding view of MDH from *M.* W3A1 (1.9 Å resolution, pdb entry 1B2N) [330]. Figures were generated using MOLSCRIPT version 2.0.2 [190].

kinetic investigations of MDHs (see Section 3.1.1). Clearly, the peculiar geometry of the PQQ–C(4) oxygen in MDH from *M. extorquens* and the PQQ–C(5) oxygen in MDH from *M.* W3A1 may pertain to the impaired interaction with substrate. There appears to be little reason, however, why fully oxidized PQQ would be unstable in the environment of the active site. The large amount of endogenous substrate equivalents (up to 90! [117]) likewise remains a mystery.

3.2. QH-ADHs from Acetobacter and Gluconobacter

3.2.1. Kinetic description

Type II QH-ADHs have been isolated from various acetic acid bacteria. They are membrane-associated enzymes composed of different subunits [1,188,189,200,213,214]. The QH-ADHs isolated from *A. aceti*, *A. pasteurianus*, and *G. oxydans* appear to follow regular ping-pong kinetics for a variety of substrates. Both natural (ubiquinone) and artificial (ferricyanide, Wurster's Blue) electron acceptors have been used. Ammonia or amines are not required for activity. pH optima are generally around pH 6. Substantial data sets have been reported for the conversion of non-chiral substrates, detailed analyses of the kinetic schemes in terms of Michaelis and inhibition constants, however, are not yet available [1,211,214]. The kinetic features of the conversion of chiral glycidol using whole cells and isolated QH-ADH from *A. pasteurianus* have been described [200]. The conspicuous presence of (chiral) glycidaldehyde as an intermediate in the reaction has been noted. While whole cells catalyze the conversion of glycidol to glycidic acid with concomitant release of only minute amounts of intermediate glycidaldehyde into the medium, conversions with isolated QH-ADH give rise to substantial accumulation of glycidaldehyde, eventually bringing the reaction to a complete halt. In analogy with the kinetic bi–bi ping-pong scheme including uncompetitive substrate inhibition reported for the mechanistically similar reaction catalyzed by the QH-ADH Type I of *Comamonas testosteroni* [113] (see below), the oxidation rate of a single enantiomer of the alcohol in the presence of aldehyde by the Type II enzyme has been expressed as:

$$r_{\mathrm{alc}} = \frac{\left(k_{\mathrm{cat(alc)}}/K_{\mathrm{M(alc)}}\right)[\mathrm{alc}][E]}{D_{\mathrm{Fe,alc,ald}}}$$

$$D_{\mathrm{Fe,alc,ald}} = 1 + \frac{[\mathrm{alc}]}{K_{\mathrm{M(alc)}}} + \frac{[\mathrm{alc}]/K_{\mathrm{M(alc)}}}{[\mathrm{Fe}]/K_{\mathrm{M(Fe,alc)}}} + \frac{[\mathrm{alc}]^2}{K_{\mathrm{M(alc)}}K_{\mathrm{i(alc)}}} + \frac{[\mathrm{ald}]}{K_{\mathrm{M(ald)}}} + \frac{[\mathrm{ald}]/K_{\mathrm{M(ald)}}}{[\mathrm{Fe}]/K_{\mathrm{M(Fe,ald)}}} + \frac{[\mathrm{ald}]^2}{K_{\mathrm{M(ald)}}K_{\mathrm{i(ald)}}}$$

with [alc], [ald], [E], and [Fe], the concentration of the alcohol, aldehyde, active enzyme, and ferricyanide, respectively. Experimental support for the consistency of this kinetic equation has been derived from the determination of the time course of the reaction of racemic glycidol catalyzed by purified QH-ADH [200]. Curiously,

dimerization of the intermediate aldehyde had to be included in the overall model as a reversible reaction. Inactivation of the enzyme by the aldehyde and inhibition by the dimer accounted for the abortion of the catalytic reaction that is observed to take place before glycidol is fully converted.

3.2.2. Structural characterization

All three subunits of Type II QH-ADH from *A. pasteurianus* and *A. aceti* appear to be essential for enzymatic activity. The indispensability of the small subunit (15–20 kDa, subunit III) in the QH-ADH from *G. suboxydans* has, however, recently been questioned [188,214]. The large subunit (72 to 80 kDa, subunit I) of Type II QH-ADHs, which possesses the dehydrogenase activity, contains one molecule of non-covalently bound PQQ and one haem *c* [1,213,293]. The genes of QH-ADH from *A. aceti* [144–146], *A. polyoxygenus* [292] *A. pasteurianus* [189,291]) and *G. suboxydans* [188] have been cloned and sequenced. X-ray structures of Type II QH-ADHs are still lacking. A working model has been obtained by molecular modeling. Cozier and et al. [59] constructed a homology model of the QH-ADH from *A. aceti* based the 31% sequence identity and 66% sequence similarity of the residues 1–590 of the QH-ADH of *A. aceti* [11,144,145] and residues 1–595 of MDH from *M. extorquens* [236]. Alignment was determined using a Needleman–Wunsch algorithm and a protein-sequence score matrix, followed by refinement by inspection and modification. Quanta/CHARMm was used to generate and relax side chain positions. Clashes were removed by hand and a force field-derived energy minimization was carried out with partial constraints on the backbone atom positions in order to preserve the basic structure as found in MDH. Thus, the resulting model of the N-terminal domain of *A. aceti* (QH-)ADH retains the characteristics of the MDH structure [59]. No attempts were made to model or position the C-terminal haem-containing domain.

Recently, we succeeded in constructing a model of the PQQ-containing domain of the large subunit of the Type II QH-ADH from *A. pasteurianus* [201] using the same approach as adopted to obtain a structural model for the QH-ADH from *C. testosteroni*. The amino acid sequence of QH-ADH from *A. pasteurianus* as reported by Takemura et al. [291] (accession number D13893) was used. Considering the extent of sequence identity (> 90%) between QH-ADH from *A. aceti* and QH-ADH from *A. pasteurianus* the alignment [13] of the former sequence with that of MDH from *M. extorquens* was used for the initial comparison. Alignments performed using ClustalW 1.6 [294] did not differ significantly. Coordinates were assigned going from the core to the surface residues of the protein. Configurations of loops were suggested by database searches and assigned. The unique cysteine-bridge was formed prior to refinement. PQQ and calcium were placed according to optimal superpositioning of the crude model and the MDH structure. Molecular dynamics, using CVFF, was used to remove sidechain clashes. Structures were checked using PROCHECK [195] and ProStat and although some deviation from normally observed values were found, the results were satisfactory. As a control the X-ray structure of MDH was run against ProStat which gave some unsatisfactory amino acid conformations as well. Because the model is built on the MDH structure, it is obvious that it will inherit the errors present. Since no obvious errors were observed for the active site residues, further refinement was not considered. No attempts were made to model the haem domain.

3.3. QH-ADH from C. testosteroni

Quinohaemoprotein alcohol dehydrogenase Type I was isolated from *C. testosteroni* (at that time known as *Pseudomonas testosteroni*) as the apoenzyme [122,123]. Only two other representatives of this single subunit type have been reported since: a PQQ-containing holoenzyme

from *P. putida* [207], and a holoenzyme from *R. acidophila* [334]. Like the Type II enzymes, QH-ADH from *C. testosteroni* does not require ammonia or amines for activity. Its pH optimum is around 7.5. PQQ-free QH-ADH apoenzyme is isolated from ethanol-grown *C. testosteroni* in the absence of extraneous PQQ. Purified apoenzyme, showing a single band on native gel electrophoresis (71 kDa) can be converted into active holoenzyme by addition of PQQ in the presence of calcium ions [68]. Incomplete reconstitution has been ascribed to the presence of 'nicked' apoprotein showing up as two bands of 51 and 25 kDa, respectively, on SDS/PAGE. Analysis of the N-terminal sequences of the fragments and comparison with the DNA sequence of the gene [283] suggests (proteolytic) scission to occur between two phenylalanine residues (450–451). Following reconstitution with PQQ, active holoenzyme and nicked, inactive, enzyme can be separated [68]. Binding of PQQ apparently leads to structural changes, as reflected by changes of spectral and chromatographic properties. Reconstitution of apoenzyme with PQQ analogues results in decreased activity [173] and enantioselectivity [79,174] for the oxidation of chiral alcohols.

The interaction between PQQ and haem *c* has been characterized by the shift of the maximum absorption of the haem moiety in reduced and oxidized QH-ADH as well as by a 60 mV increase in the haem midpoint redox potential upon reconstitution of apoenzyme with PQQ [67]. A shift of the ^{1}H NMR porphyrin methyl group resonances has been observed for the binding of PQQ in the oxidized form of the enzyme, while a shift of the methionine haem ligand accompanies binding in the reduced form [67]. The redox-dependent reorganization of surface residues at the haem edge of horse heart cytochrome *c* has similarly been related to the redox behaviour of the protein [252,253]. Spectroelectrochemical evidence for a conformational change triggered by haem reduction has been reported to accompany electron transfer in tetrahemic cytochromes c_3 [180]. Distance constraints on the intramolecular positioning of the haem *c* and PQQ redox centers have been deduced from ^{19}F NMR relaxation studies employing fluorine-labeled phenylhydrazones of PQQ [66].

3.3.1. Kinetics of QH-ADH Type I from C. testosteroni

The kinetic mechanism of QH-ADH Type I has been described in detail for the oxidation of primary (chiral) alcohols and aldehydes using ferricyanide as an electron acceptor [113]. Initial rate data could be described by hexa uni ping pong kinetic schemes in which the alcohols are oxidized to the corresponding carboxylic acids and the intermediate aldehydes are released from the enzyme. For some substrates uncompetitive inhibition had to be invoked. In general, oxidation of aldehydes is much faster than the oxidation of the alcohols from which they are derived. Progression curve analysis suggested reversible inactivation of the enzyme to take place in proportion to the concentration of ferricyanide present at the start of the conversion. Simpler kinetic schemes are expected for the oxidation of secondary alcohols [281].

3.3.2. Structural model of QH-ADH Type I

So far, X-ray crystallography of Type I QH-ADH from *C. testosteroni* has not been successful. A structural model has been constructed by homology modeling based on the sequence similarity of N-terminal residues 1–570 with the α subunit of MDHs from *M.* W3A1 and *M. extorquens* [175]. The amino acid sequence of QH-ADH of *C. testosteroni* has been compared to other QH-ADHs and related MDHs by Stoorvogel et al. [283]. Only 34% sequence identity was found with the sequences of MDHs. However, salient structural features appeared to be conserved on the primary sequence level. The repeated 'tryptophan motif' that constitutes the eight-bladed β-propeller fold is also present in QH-ADH. The active site residues, involved in PQQ and calcium binding, are completely conserved as well. The unique disulfide bridge and

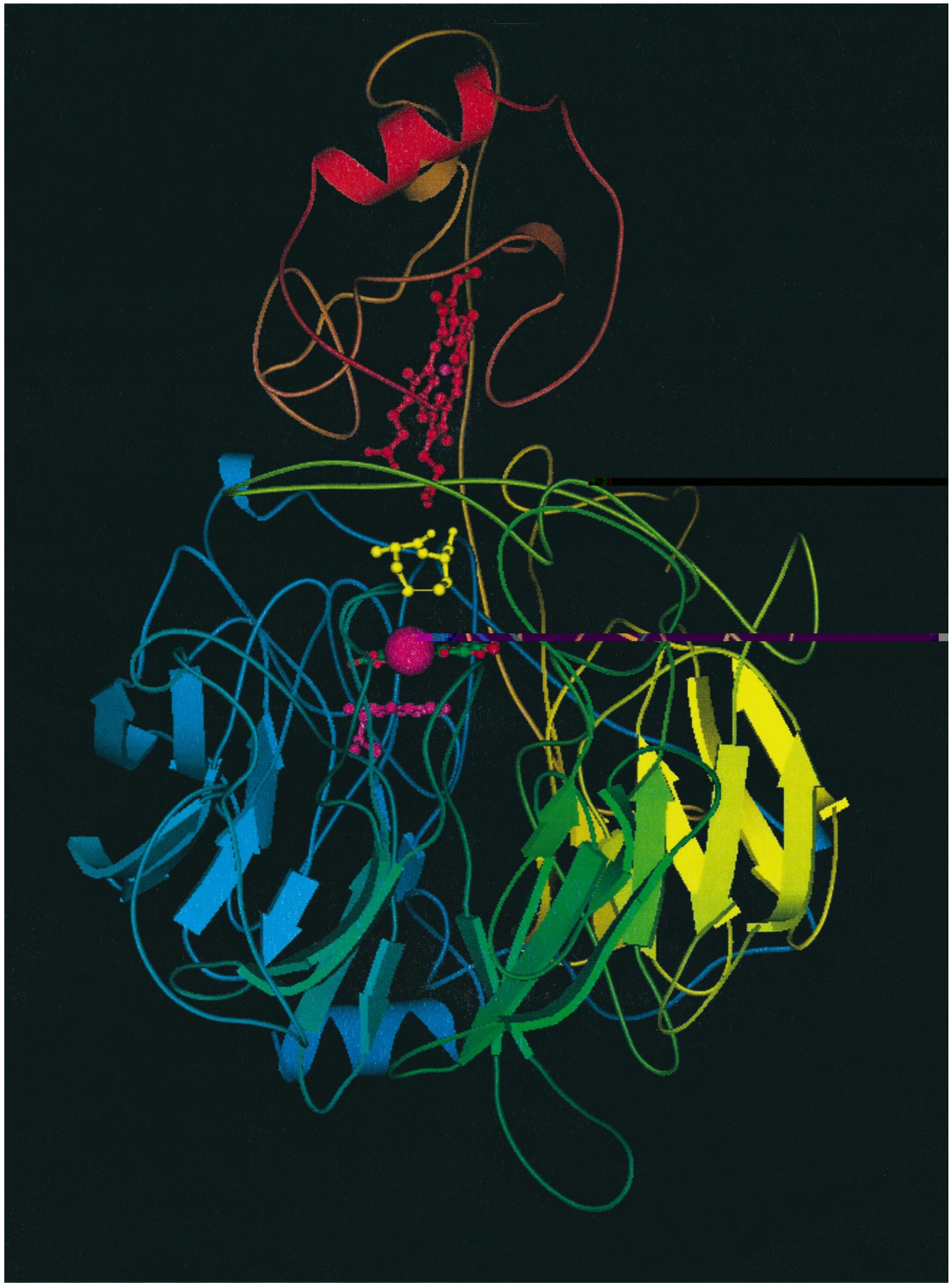


Fig. 7. Representation of homology model for QH-ADH from *C. testosteroni*. Haem shown in red, eight-membered cysteine bridge in yellow, PQQ in atom colours, calcium in pink and tryptophan in pink. Picture generated with MOLSCRIPT version 2.0.2 [190].

the tryptophan responsible for the stacking of PQQ are found at the primary sequence level of the N-terminal domain of QH-ADH in the same position. The conservation of these residues strongly suggested a similar arrangement of the active site structure for QH-ADH. The presence of the tryptophan motif served as a scaffold for the building of the overall folding of the PQQ-binding domain. The C-terminal haem-binding domain, residues 571–677, absent in MDHs, has been modeled using minimal sequence similarity with cytochrome c_5 from *Azotobacter vinelandii*, cytochrome *c* from *Plectonema boryanum*, and cytochrome c_{551} from *Ectothiorhodospira halophila* [33]. Using distant constraints inferred from ^{19}F NMR relaxation studies of trifluoromethylphenylhydrazine-derivatized PQQ bound to QH-ADH apoenzyme [66], as well as theoretical relations for optimal electron transfer [31,42], the relative positioning of the haem- and PQQ-binding domains was estimated (Fig. 7).

The model successfully accounted for the site of proteolytic attack (see Section 3.2), the substrate specificity, the change of spectral properties upon reconstitution of apoenzyme with PQQ [67], and the electronic interaction between haem *c* and PQQ. A discussion on the implications of the model for the enantioselectivity displayed by the enzyme in the oxidation of chiral alcohols and aldehydes is presented in Section 5.

4. Mechanism of action of quino(haemo)proteins

4.1. Structural and chemical properties of PQQ and derivatives

X-ray crystallography has played an important role in the detection and structure elucidation of PQQ. Kennard et al. [61,258] reported the structure of the PQQ–acetone adduct which led to the identification of PQQ as a novel cofactor. The structure of the dinitrophenylhydrazone of PQQ triester, an important derivative in early analytical protocols [303], was determined by van Koningsveld [307]. The structure of the penta-hydrated disodium salt of PQQ has been reported by Urakami et al. [148], who also described quantum-mechanical calculations on the stacking ability of PQQ important for the formation of charge-transfer complexes with aromatic amino acids. Although the reaction of PQQ with tryptophan has recently been shown to lead to a different product [149], the stacking interaction clearly plays a role in the accommodation of PQQ in quinoproteins. X-ray crystallographic data for free PQQ and PQQ monopotassium salt are available as well [308]. The X-ray structure determination of C(4) and C(5) adducts of PQQ triester and methanol and their possible relevance to quinoprotein catalysis have been reported [158]. An overview of X-ray structures that are currently known is given in Fig. 8.

The X-ray structures show the PQQ molecule to be almost completely flat with only a slight dihedral angle between the heterocyclic rings ($\pm 10°$). The C(2) and C(7)-carboxylic groups are in the plane of the tricyclic ring system. The C(9)-carboxylic group is tilted by $\pm 22°$. The non-planarity of this group has also been reproduced using high level ab initio quantum chemical calculations [7,8,337]. Adducts of PQQ show a slight twist in the ring system most probably to accommodate the substituted sp^3 carbon at the C(4) or C(5)-positions. In the mono-potassium complex [308] the K^+ atom is liganded by the C(7) carboxyl group, the carbonyl O(5) and two waters in diad symmetry with another PQQ molecule. The resulting structure shows a high resemblance with the Ca^{2+}-complexes found in the X-ray structures of the MDHs. Surprisingly, the positive charge of the potassium ion is balanced by complete ionization of the C(9) carboxyl group of the neighbouring PQQ molecule, leading to a reasonably strong hydrogen bond to the pyrrole N(1). Quantum chemical calculations show that this hydrogen bond may also be present in the non-ionized form of the C(9) carboxyl group.

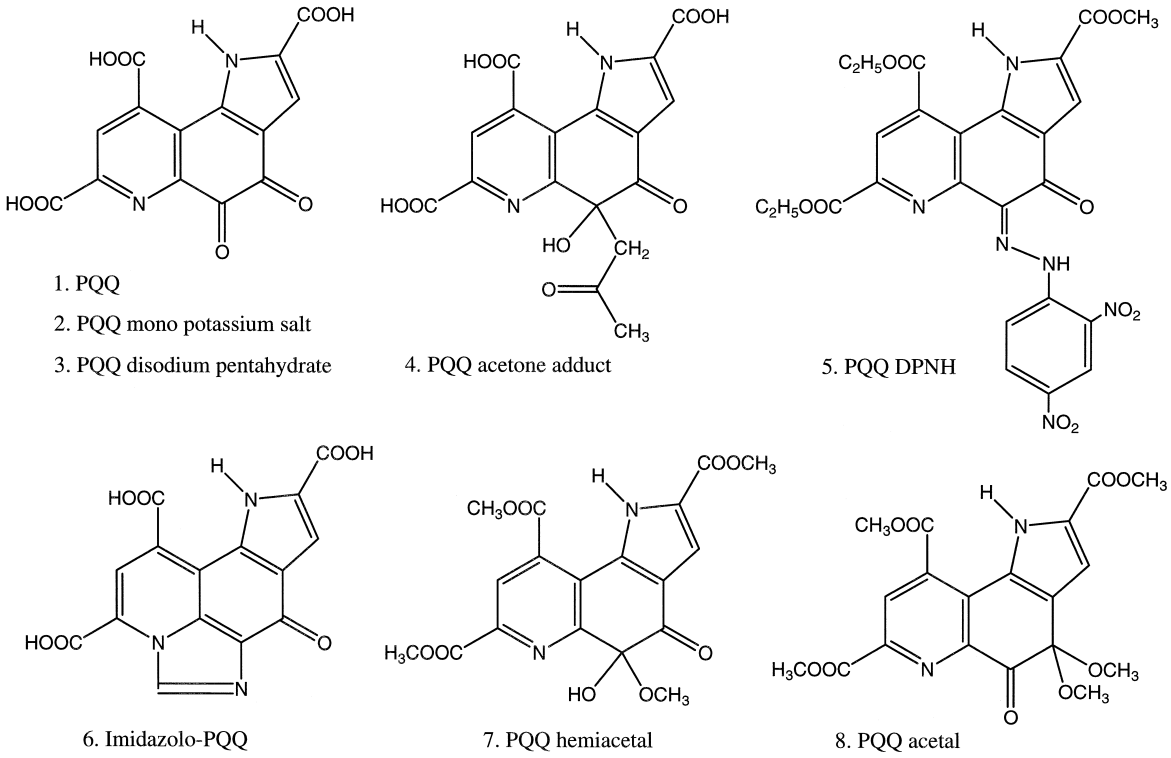


Fig. 8. PQQ and PQQ-related compounds for which structures have been determined by X-ray crystallography. (1) PQQ [308]; (2) PQQ mono potassium salt [308]; (3) PQQ disodium pentahydrate [148]; (4) PQQ acetone adduct [61,258]; (5) PQQ dinitrophenyl hydrazone [307]; (6) Imidazolo–PQQ [149]; (7) PQQ-C(5) hemiacetal [158]; (8) PQQ-C(4) acetal [158].

Studies of the chemical reactivity of PQQ were greatly stimulated when various routes for its chemical synthesis became available [38,55,107,108,134,135]. Although the original procedures suffered from low overall yields, synthetic bottle-necks could be relieved [171, 205,206,304] by appropriate adaptation of the procedure devised by Corey and Tramontano [55] or by novel strategies [202,203]. Methods for the microbiological production of PQQ have been described [6,164,302]. However, the commercial availability of PQQ (Fluka, Buchs) still relies on chemical synthesis [170].

The pivotal role of the *o*-quinone moiety of PQQ in quinoprotein catalysis was recognized even before its structure was fully known [321]. The semiquinone, $PQQH^{\cdot}$ (2), the quinol, $PQQH_2$3), and the tetrahydro derivative, $PQQH_4$ (5), can be obtained upon reduction (Fig. 9). The biological relevance of compounds 1–3 has been well established [104]. A possible role for the keto alcohol derivative (4), a likely intermediate during borohydride reduction of PQQ to $PQQH_4$ under aerobic conditions [87], has been suggested (see Section 4.2). The existence of a two-electron oxidized lactone of PQQ has been claimed on the basis of mass spectroscopic data [109]. PQQ can be considered a medium-potential biological oxidant distinct from the high-potential quinones that have been applied in organic synthesis [54]. The redox chemistry of PQQ has been investigated by several groups. From potentiometric titrations of $PQQH_2$ at several pHs Duine et al. [86,90] determined the redox potential of the PQQ/$PQQH_2$ couple. Bergethon [32] investigated the amperometric detection of PQQ in HPLC analysis. Redox behaviour at acid pHs was determined by cyclic voltammetry [178]. McWhirter and Klapper [220] obtained a value for the

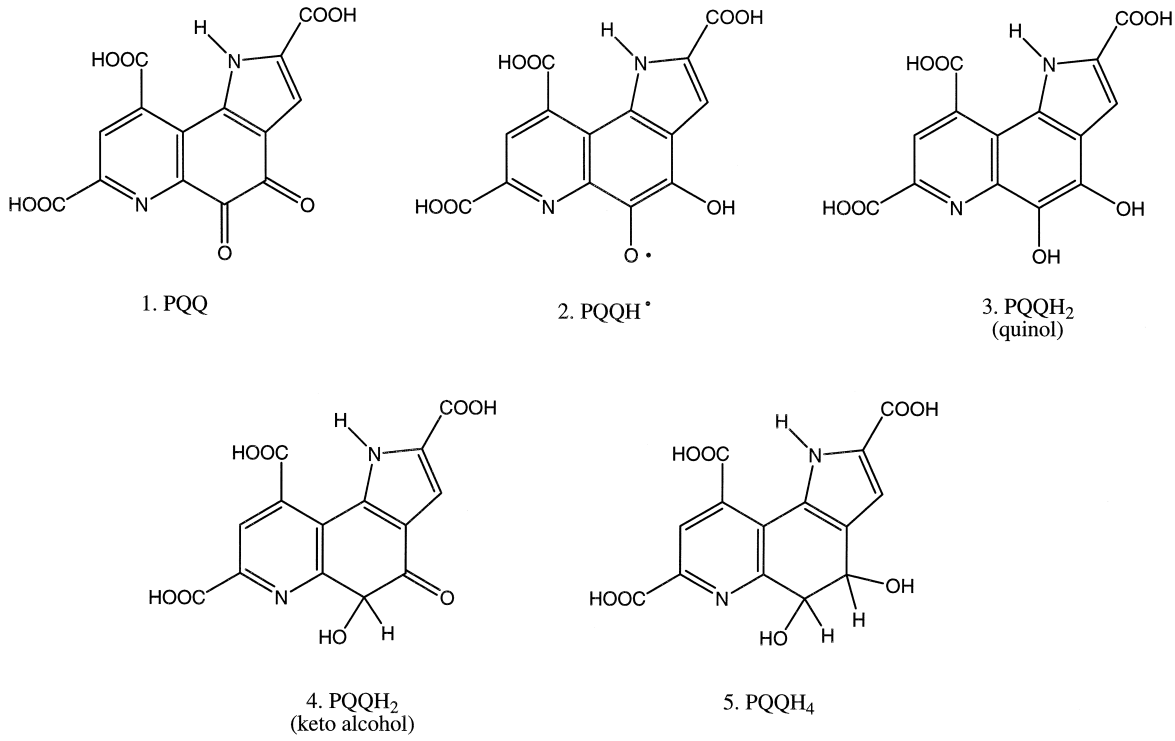


Fig. 9. Currently known redox forms of PQQ. (1) PQQ; (2) PQQ semiquinone; (3) $PQQH_2$ (quinol form); (4) $PQQH_2$ (keto alcohol form); (5) $PQQH_4$.

redox potential of the PQQ/PQQH$^{\cdot}$ couple at neutral pH from pulse radiolysis experiments. Eckert et al. [93,94] compared redox properties of PQQ with those of related *o*-phenanthroline quinones. The redox properties of a PQQ–ruthenium complex were determined by Schwederski et al. [265]. Itoh et al. [162] described the electrochemical behaviour of the trimethyl esters of PQQ and its N(1)-methylated analogue in aprotic organic solvents. Redox properties of PQQ are collected in Table 2. These numbers may be compared to the value of +80 mV (vs. NHE) of the haem *c* in *C. testosteroni* QH-ADH apoenzyme and +140 mV (NHE) in the holoenzyme [67].

The close resemblance of the structural features of PQQ and well-established chelating agents, e.g., α-picolinic acid [287] and 8-hydroxyquinoline [92], has been noticed [172,232]. Equilibrium complexing of 9-decarboxyPQQ with Cd(II) at pH 4.0 (ionic strength 1.0, $NaClO_4$) has been reported [232]. Formation of a 1:1 complex was deduced from titration plots [314]. Addition of Cu(II) apparently gave rise to 2:3 complexes. Both 1:1 and 1:2 complexes were observed in the titration of PQQ and several derivatives lacking oxygen substituents at C(4), and C(5), respectively, with Cu(II) [170]. In all cases, equilibrium constants were of order 10^6 M^{-1} [82]. Strong indications were found for the involvement of the C(5)-covalent hydrate in complex formation [170]. Complexation of PQQ and $PQQH_2$ with calcium requires higher concentrations. Equilibrium complexation (50 μM PQQ) was observed to occur at 100 mM Ca^{2+} in 20 mM MOPS/KOH, pH 7.5 suggesting an equilibrium constant of order 10^3 M^{-1} [66]. Changes of the UV/Vis-spectrum are in accordance with the formation of a PQQ–hydrate–Ca^{2+} complex. Similar changes have been observed upon addition of Ca^{2+} and Mg^{2+} to PQQ solutions [116]. Complex forma-

Table 2
Redox properties of PQQ and PQQ–metal complexes

Couple	Redox potential, V		Method	Refs.
	E_m (NHE)	E_m (SCE)		
$PQQ/PQQH_2$	0.090[a]		Potentiometric titration	[86]
	0.15[b]		Thin-layer cyclic voltammetry	[94]
+ Zn(II)	0.17/0.23[b]		Thin-layer cyclic voltammetry	[94]
	0.053[c]	1.75[d]	Cyclic voltammetry	[32,178]
$PQQ/PQQH^{\cdot}$	−0.122[e]		Pulse radiolysis	[220]
	−0.218[f]		Potentiometric titration	[86]
$PQQH^{\cdot}/PQQH_2$	0.22[e]		Pulse radiolysis	[220]
	−0.242[f]		Potentiometric titration	[86]
$PQQ/PQQH^{\cdot}$ − Ru		0.82[g]	Cyclic voltammetry	[265]
$PQQH^{\cdot}/PQQH_2$ − Ru		0.26[g]	Cyclic voltammetry	[265]

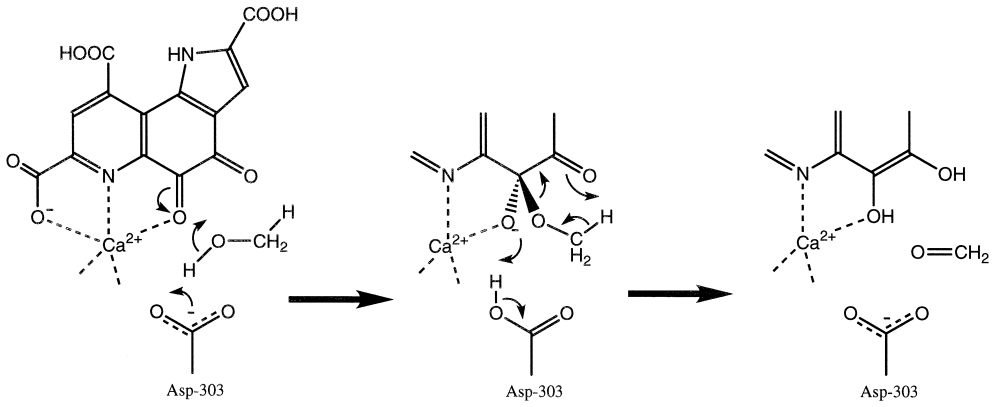
[a] 10^{-4} M PQQ, 0.1 M phosphate, pH 7.0, 5×10^{-6} M phenazine methosulfate, $K_3Fe(CN)_6$.
[b] 0.5 M sodium acetate, pH 5.6.
[c] 10^{-4} M PQQ, 0.016 M phosphate/0.1 M NaCl, pH 7.0.
[d] 3×10^{-4} M PQQ, 0.5 M KCl, pH 7.2.
[e] Pulse radiolysis-generated $CO_2^{\cdot}$ in O_2-saturated 0.1 M formate, 5 mM phosphate, mmolar quantities of PQQ, pH 7.2.
[f] 10^{-4} M PQQ, 2 M salicylate, pH 13, $K_3Fe(CN)_6$, from semiquinone formation constant of 2.54.
[g] Acetonitrile, 0.1 M $(Bu_4N)PF_6$. Adapted from [82].

tion of $PQQH_2$ with calcium in the presence of dithiotreitol (1 mM) required similar concentrations of Ca^{2+} [66].

The affinity of PQQ for nucleophiles added to the C(5) carbonyl group was discovered at an early stage [69,104,256,273]. Formation of C(4) adducts has been observed to occur when PQQ trimethyl ester is treated with methanol under acidic conditions [158]. Addition of amines as a first step in the oxidative deamination that was at one time believed to be a role of PQQ in quinoprotein amine oxidases and dehydrogenases that are now known to harbor different quinones, has been investigated by Itoh et al. [153,155–157]. The relevance of these model systems for the understanding of the mode of action of TPQ [223,224,315] and TTQ [152,159] containing quinoproteins, as well as of the reactivity of PQQ in biological samples [305,306] has been addressed [80]. Reactions of PQQ with various carbonyl group reagents, aimed at the formation of stable derivatives for the extraction and identification of PQQ in alleged quinoproteins have obtained due attention. Competing activities of hydrazones in the reduction [225] and derivatization of PQQ [226,308] have been reported.

4.2. Proposed reaction mechanisms

MDHs have been the prime subject of investigations into the chemical mechanism of quinoprotein alcohol dehydrogenase catalysis. Early suggestions on the participation of three-electron reduced enzyme species [222] could not be substantiated [65]. A revised reaction scheme [247] was rejected in favor of the kinetic scheme presented in Section 3.2.1 (Scheme 1). Conversion of the oxidized, substrate-containing, complex into the reduced enzyme and product is generally considered to be the rate-limiting step in the reductive half reaction [105,106] (for a review see Ref. [14]). This is also the only step requiring ammonia or primary amines as activators for the enzyme as isolated. A covalent PQQ-substrate adduct appears to be a prime candidate for this role (Scheme 2). Evidence for the actual participation of such an adduct is, however, largely circumstantial. An important motivation has been provided by the well-docu-

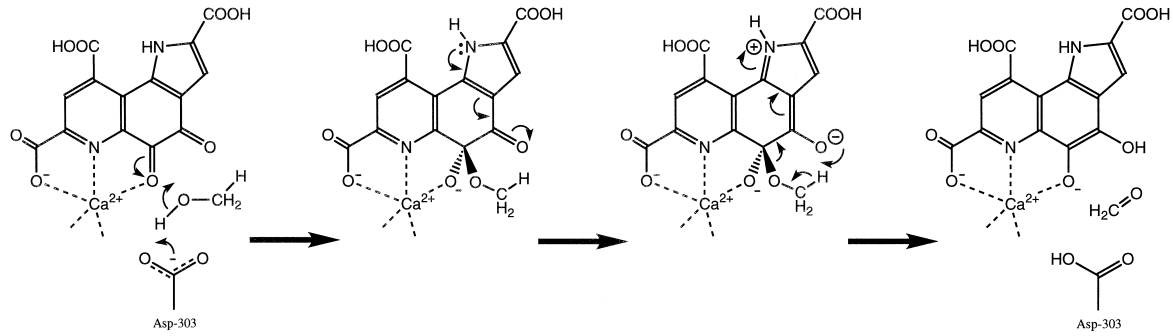


Scheme 2. Proposed reaction mechanism of MDH involving covalent adduct formation of PQQ and methanol followed by deprotonation and rearrangement.

mented reactivity of PQQ with a variety of nucleophiles [69,238], giving rise to the formation of C(5) carbonyl adducts [158]. The central position of such adducts in early mechanistic proposals is illustrated in Scheme 2.

The isolation of the corresponding PQQ-adducts (Fig. 3) from cyclopropanol- (and cyclopropanal-)inhibited MDH has been considered as a major indication for the involvement of an active site base setting up the alcohol for nucleophilic addition. This assumption was further supported when the X-ray structures revealed an aspartate residue (Asp 303 in *M. extorquens* MDH) situated in an appropriate position for proton abstraction. Curiously, changing this residue into a glutamate by site-directed mutagenesis resulted in a highly active enzyme with, however, a much lower substrate affinity [15]. This situation appears to be in agreement with observations on the flavoprotein-catalyzed oxidation of acyl-CoA [248] where replacement of a glutamate residue with very similar function by an aspartate leads to severe reduction of the reaction rate, suggesting a crucial dependence on the correct ''spacing''. Also, while hemiketal formation at C(5) would position the substrate α-hydrogen in a geometrically favorable configuration for transfer to PQQ via a six-membered transient, several objections can be raised. Of trivial importance is the notion that whereas free PQQ hardly requires catalysis for adduct formation, PQQ in MDH would require explicit base catalysis. This, of course, may well be caused by the specific environment of PQQ in MDH. An issue of more importance is the proton abstraction by the remaining carbonyl at C(4), an entity that is not normally known for its high basicity. Scheme 3 addresses a possible role of the pyrrole nitrogen in the promotion of the basic strength of this carbonyl group by conjugation [163] (Scheme 3).

It is not clear whether charge separation as shown in Scheme 3 will be an important contribution to the basic properties of the C(4)-carbonyl group. Comparison of the ^{13}C NMR chemical shifts of the C(5) and C(4) carbons indeed shows the C(4) atom to be slightly more shielded (DMSO-d_6, ppm, 173.3 (C-4); 179.3 (C-5); [238]). The (slight) difference of the electrophilic characteristics of the carbonyl groups that form the *o*-quinone moiety of PQQ has been investigated by Itoh et al. [158] in terms of the formation and relative stability of (hemi) ketals. A mechanistic proposal was designed that elaborated earlier suggestions [90,103] on the possible participation of a *p*-quinonoid tautomeric structure of PQQ (Scheme 4). It must be emphasized that N(1)-alkylated PQQ analogs show low but definite activity in the reconstitution of Type I QH-ADH apoenzyme from *C. testosteroni* [173]. In these experiments possible contamination of the derivative with authentic



Scheme 3. Proton abstraction facilitated by charge separation.

PQQ could be rigorously excluded by determining the different enantioselectivity of the resulting holoenzyme [79].

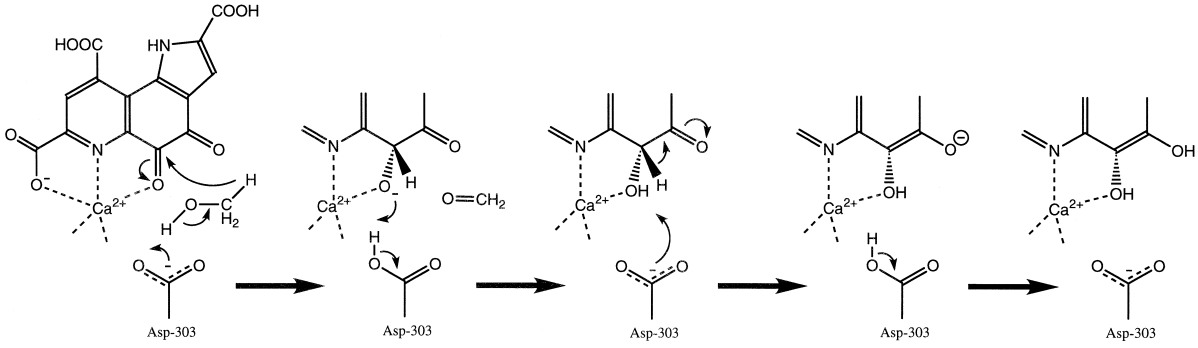
Although the possibility that PQQ might behave as a *p*-quinone cannot be rejected as yet, the physiological relevance of the proposed mechanism [158] depends strongly on the alleged presence of a suitably situated serine or threonine residue in the active site (see Section 3.1.2 for discussion of the erroneous assignment of the orientation of PQQ in early crystal structure). Currently available high-resolution X-ray data, however, do not support this view.

While these proposals considered covalent addition of the substrate alcohol to PQQ as a primary event, an interesting possibility was raised when the importance of Ca^{2+}, both as a structural and as a catalytic factor became evident (Table 3).

In the structures of MDH from *M. extorquens* and *M*. W3A1, Ca^{2+} is situated in the plane of the PQQ molecule with the C(7) carboxy group, the N(6) pyridine nitrogen and the C(5) oxygen as equatorial PQQ-derived ligands. Contributions from Asn 261, Glu 177, and a solvent water molecule complete the coordination. The role of Ca^{2+} in the catalytic mechanism is supported by a series of interesting observations, including a Ca^{2+}-lacking mutant enzyme devoid of activity [255], of which the activity could be restored (in part) by addition of Ca^{2+}, Ba^{2+} or Sr^{2+} [119,121,127,128]. In the mechanisms shown in Schemes 5 and 6, Ca^{2+} is portrayed as a Lewis acid promoting the polarization of the C(5) carbonyl for the transfer of a hydride [14,19].

A variation of the hydride transfer mechanism, based on high-level quantum mechanical calculations [337] is shown in Scheme 6.

Despite the great variety of mechanisms, some of the basic features of MDH catalysis are not covered by these proposals. Except, possi-

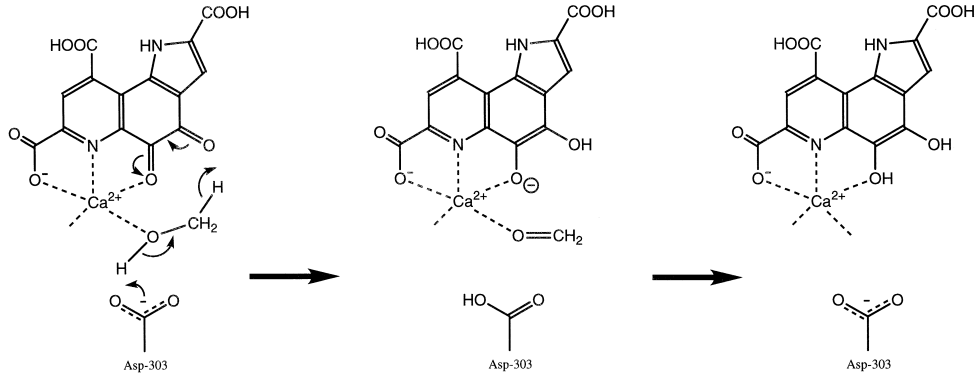


Scheme 4. Possible participation of enzyme-bound *p*-quinonoid vinylogous ester.

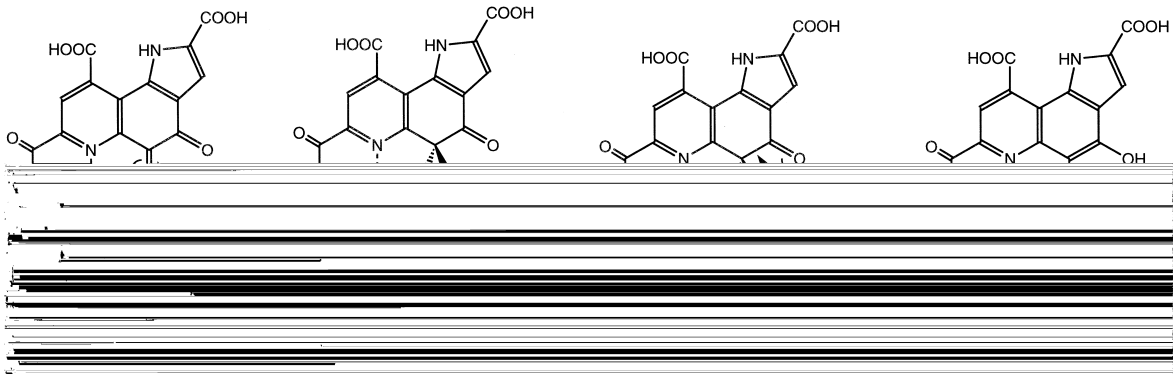
Table 3
PQQ-containing (haemo)quinoproteins for which the presence of Ca^{2+} (and closely related metal ions) has been established

Enzyme	Source	Metal ion	Metal ions capable of replacing Ca^{2+}	Refs.
MDH	*Hyphomicrobium X*	Ca^{2+}		[2]
	M. W3A1			[76,322]
	M. extorquens	Ca^{2+}	Sr^{2+}, Ba^{2+}	[119,255]
	P. denitrificans	Ca^{2+}	Sr^{2+}	[64,128,129]
QH-ADH (type I)	*C. testosteroni*	Ca^{2+}	Mg^{2+}, Sr^{2+}, Co^{2+}, Cd^{2+}, Ba^{2+}, Ni^{2+}, Fe^{2+}, VO^{2+}	[66,123]
QH-ADH (type II)	*Acetobacter* sp.	Ca^{2+}	Sr^{2+}	[66]
EDH	*P. aeruginosa*	Ca^{2+}	Sr^{2+}	[228]
GDH (soluble)	*A. calcoaceticus*	Ca^{2+}	Mn^{2+}, Cd^{2+}	[77,116]
GDH (membrane bound)	*E. coli*	Ca^{2+}/Mg^{2+}	Sr^{2+}, Co^{2+}, Cd^{2+}, Ba^{2+}, Fe^{2+}, Zn^{2+}	[72,208]
PVA-DH	*Pseudomonas* sp. VM15C	Ca^{2+}		[131,270,271]

bly, for some of the early suggestions [90,103], none of the schemes addresses the role of the activator ammonia. There may be some justification in the fact that ammonia (as the free base) is required as an activator only when the enzyme is assayed with artificial electron acceptors. With cytochrome as an electron acceptor, ammonia is not usually needed [12,75,104,127]. In this respect, both Type I and II QH-ADHs behave quite differently. No activator is required. It might be argued that a lysine residue, conspicuously present in the active site cavity of the QH-ADH homology models, assumes this role. An alternative function for this residue in the accommodation of product aldehydes has been proposed [173]. If one accepts the possibility that the natural electron acceptor, cytochrome-c_L of MDHs overcomes the need for an amine-type activator in the oxidized enzyme-substrate complex, i.e., by providing a lysine of suitable basicity under physiological conditions, it would appear that the kinetic scheme of a hexa-uni ping-pong reaction [12,105] is no longer valid and should be replaced by ternary complex kinetics involving oxidized enzyme, substrate/product, and cytochrome, most probably in a rate-controlling step. The ability of ammonia to restore enzyme activity when critical catalytic basic residues have been mutated has been described [147]. In this respect, restoration of activity by addition of ammonia for an inactive Lys258Ala mutant of *Escherichia coli* aspartate aminotransferase is of interest [296,297]. Not only was the degree of restoration found to be dependent on the type and concentration of the amine added, also, a correlation between the logarithm of the efficiency and the pK_a of the amine was observed.



Scheme 5. Hydride transfer by Lewis acid catalysis (I).



Scheme 6. Hydride transfer by Lewis acid catalysis (II).

These results suggest that exogenous amines would substitute the functional role of Lys 258. This approach has been applied to Lys 296-substituted rhodopsin [338], Lys 80-substituted leucine dehydrogenase [266], and Lys 329-substituted ribulose 1,5-bisphosphate carboxylase/oxygenase [126].

Suggestions for the oxidative half-reaction of MDHs feature the free-radical semiquinone form of PQQ with the unpaired electron localized in the *o*-quinone moiety (Scheme 7). There would appear to be little reason to speculate on the possible localization of the unpaired electron at the C(4) oxygen of MDH from *M. extorquens* [14] (or the C(5) oxygen in MDH from *M.* W3A1, for that matter [330]) in order to rationalize the out-of-plane positions of these atoms in the respective X-ray structures. Neither the extent of refinement, nor the hybridization state warrants such a proposal. Structures reported in the literature for cation-stabilized radicals derived from *o*-benzoquinones give no indication for out-of-plane positions of the oxygen atoms [176,257].

Although MDHs and QH-ADHs reported to date are dehydrogenases, with the possible exception of an extracellular quinoprotein oxidase that catalyzes conversion of enacyloxin IVa to enacyloxin IIa, for which the involvement of PQQ has not been unequivocally established [246], models for the reaction of $PQQH_2$ with dioxygen have been investigated. Indications for the role of Ca^{2+} in the oxidation of $PQQH_2$ trimethyl ester in acetonitrile have been found [160]. Ca^{2+} was shown to have a catalytic effect on this oxidation. An electron-transfer mechanism (SET) was proposed analogous to the reaction of 1,5-dihydroflavins with $^3O_2$. Schürer and Clark [264] used the results of semiempirical (PM3) MO calculations to propose that the

Scheme 7. Oxidative half-reaction of MDH.

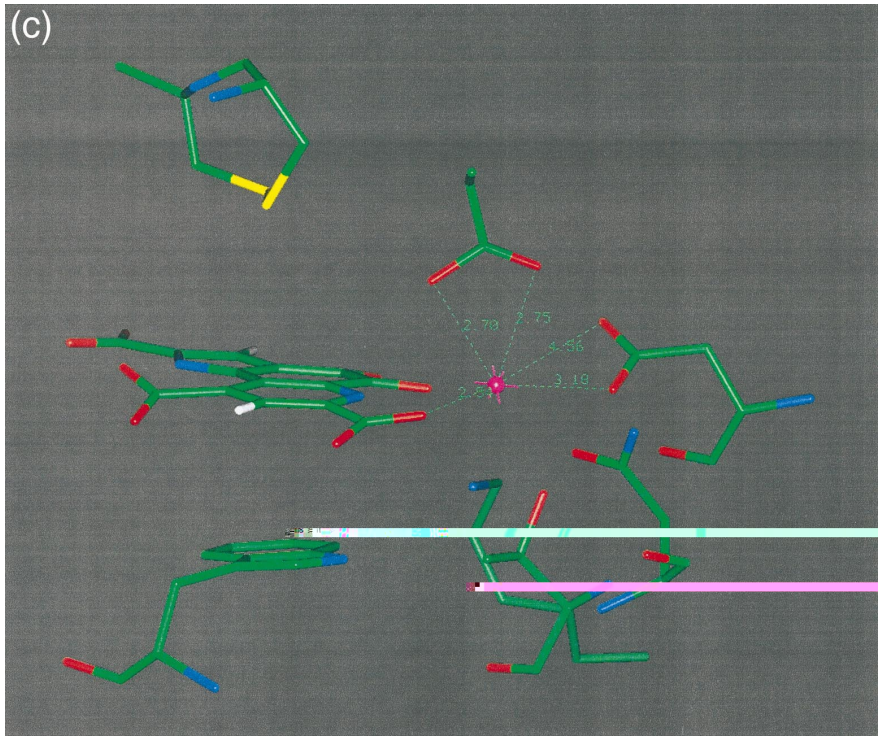


Fig. 10. Stick representation of active site residues from (a) MDH from *Me. methylotrophus* W3A1 (crystal structure at 1.9 Å resolution; (b) QH-ADH from *A. pasteurianus* (homology model); and (c) QH-ADH from *C. testosteroni* (homology model). The stacking of PQQ between the disulfide bridge and underlying tryptophan is a recurring theme in the pictures. A glutamate is conserved as a ligand for the calcium as is an asparagine residue. The active site base (aspartate) is present in all three structures. The apical position in the calcium ligation is taken up by an alanine in MDH (a) and a threonine in QH-ADH (b and c). The arginine that is in close contact to the O(4) position of PQQ in MDH (a) is replaced by a lysine in both QH-ADHs (b and c). Figures generated using INSIGHT (Biosym).

TI, protocols have been reported [234], as have the reasons for their failure [95]. Hybrid quantum mechanical and molecular mechanical, QM/MM, methods have been developed [319] and used [53,130] to obtain values for free energy differences. A computationally less expensive approach is to calculate force field potential energies and equate these to the corresponding $\Delta\Delta G^{\#}$-value. Clearly, the entropic contribution, which has been shown to be of substantial magnitude [244,245], will then be neglected. This may not be warranted for QH-ADH-catalyzed enantioselective conversions. Examples of QH-ADH enantioselectivity have been reported where the entropic contribution actually exceeds the enthalpic contribution [201]. A simple and computationally inexpensive method starts by constructing the TS model structure and searches the conformation space by suitable molecular dynamics algorithms [242]. In principle, a qualitative estimate of the relative stabilities of the TSs can be obtained when certain criteria are satisfied. Such criteria include the number of hydrogen bonds, absence of clashes, and, most importantly, limited root-mean-square, rms, deviations of the structures searched during the dynamics runs as compared to the starting structure. For this condition to hold, the starting structure has to be of good quality. This requirement is also commonly acknowledged from a slightly different perspective [52]: ‘‘In order to understand the mechanism of action of an enzyme, it is necessary to determine, as precisely as possible, the tertiary

structure of the protein . . . ''. Clearly then, when good quality structures (MDH) do not provide the answers, homology models (QH-ADH) will be even less meaningful. Still, computational efforts on QH-ADH homology models may not be completely wasted. Firstly, it is quite conceivable that the active site area of QH-ADHs will be as closely similar to that of MDHs as the models predict, while secondly, QH-ADH's active site chemistry may be more akin to the physiologically functional situation than that of the rather artefactually behaving MDHs. With respect to the active site residues, some differences occur (Fig. 10).

The cysteines involved in the disulfide bridge were linked in their final *trans*-position prior to the structural refinement. The conserved position of all other active site residues is inherent to the modeling procedure. The position of the non-conserved Trp 477 in MDH from *M. extorquens* is taken up by Val 479 in the model for QH-ADH from *C. testosteroni* and by Gly 479 in the model of QH-ADH from *A. pasteurianus*. These changes lead to a significant increase in the active site dimensions of the QH-ADHs, as compared to those of MDH. This might explain the observed difference in substrate specificity, i.e., the conversion of bulky alcohols (and even sterols) by QH-ADHs as opposed to the more restricted substrate specificity of MDHs. The non-conserved Arg 331 (*M. extorquens*) is replaced by Lys 335 in both QH-ADHs. It has been suggested that this arginine residue might play a role in the reaction

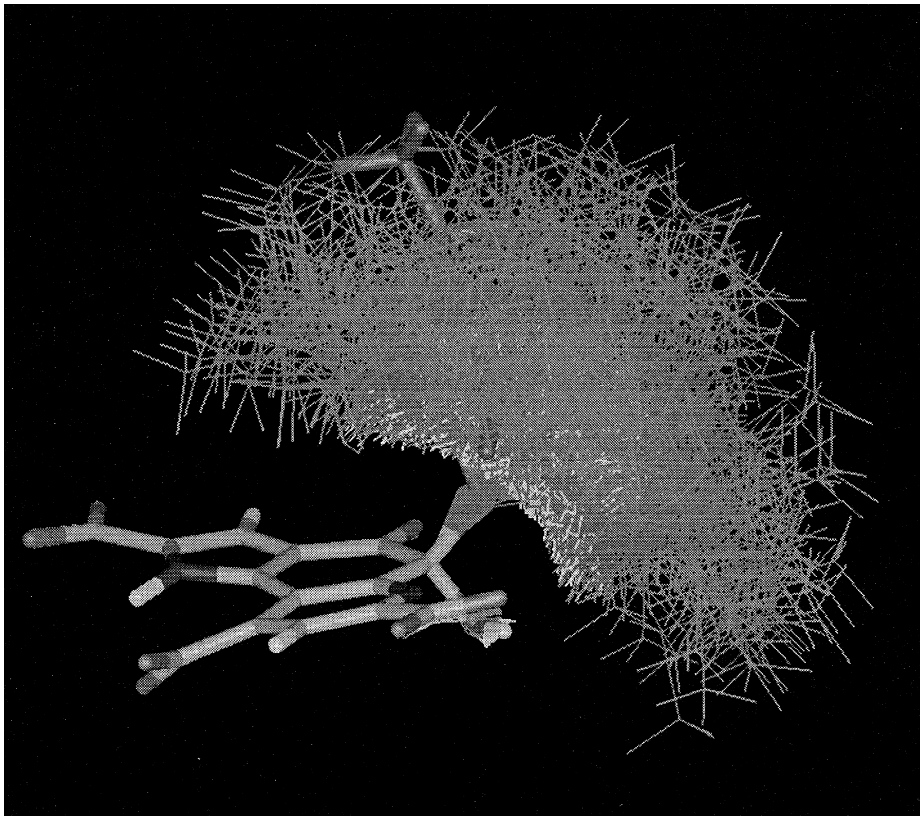


Fig. 11. Conformational search for the hemiketal intermediate formed by addition of (*S*)-2-hexanol to PQQ–C(5). Torsion angles around PQQ–C(5)–(O)–alcohol and alcohol–(O)–chiral C were rotated using steps of 10°. Four hundred conformations were generated using DISCOVER. Picture made using INSIGHT (Biosym).

mechanism of MDH by forming a hydrogen bond to the C(5)-carbonyl oxygen thus promoting build-up of positive charge on this atom [329]. Its replacement by an amino group might offer an explanation for the fact that QH-ADHs do not need to be activated by ammonia (Scheme 9).

As pointed out above, structural information for quinoprotein dehydrogenases has been deduced largely from homology modeling experiments. Of the currently existing models no coordinates are available for the PQQ-binding domain of QH-ADH from *A. aceti* [59]. 3D models of the Type I QH-ADH from *C. testosterni* [175] and the PQQ-containing domain of the large subunit of the Type II QH-ADH from *A. pasteurianus* [201] have been described (see Section 3 for details). In order to gain insight into the reaction mechanism and the enantioselectivity of this class of enzymes, preliminary molecular modeling experiments were performed aimed at the validation of proposed transition state structures with respect to the enantioselective features of these enzymes. Reaction intermediates leading to the respective transition states for the covalent PQQ-adduct reaction mechanism (the hemiketal intermediate, i.e., Schemes 2 and 3) and the hydride transfer mechanism (i.e., Schemes 5, 6 and 9) were subjected to closer examination. To evaluate the stability of the hemiketal intermediate, PQQ-substrate adducts were built and placed in the active site. To study the intermediates in which the alcoholate anion is complexed to the calcium ion, docking simulations were performed starting from outside the active site. Force field-derived energies for all complexes with substrates of opposite chirality were monitored and analyzed.

Conformational searching for the most stable PQQ-adduct structures was carried out by placing torsions on two dihedral angles. Rotations around the (PQQ)-C(5)-O(substrate) bond as well as the O(substrate)-C(α)-(substrate) bond were introduced. In case of the PQQ-(*R,S*)-2-hexanol adducts this was done for free PQQ as well as the for PQQ in the active site. The results for the (*S*)-2-hexanol PQQ-C(5)-adduct are shown in Fig. 11.

In order to fully search the active site, several different conformation of the PQQ-adduct were generated and placed in the active site. The dielectrical constant was set to 1 in all cases. MNDO runs were performed, within the GAMESS-US program [260], on the diastereomeric PQQ-(*R,S*)-2-hexanol adducts. Energies for the final structures were compared and found to give no significant difference between (*R*)- and (*S*)-substrate-PQQ-adduct (such differences have been reported to occur for hydrolase tetrahedral intermediate structures in which only the atoms of the active site serine were included [237]).

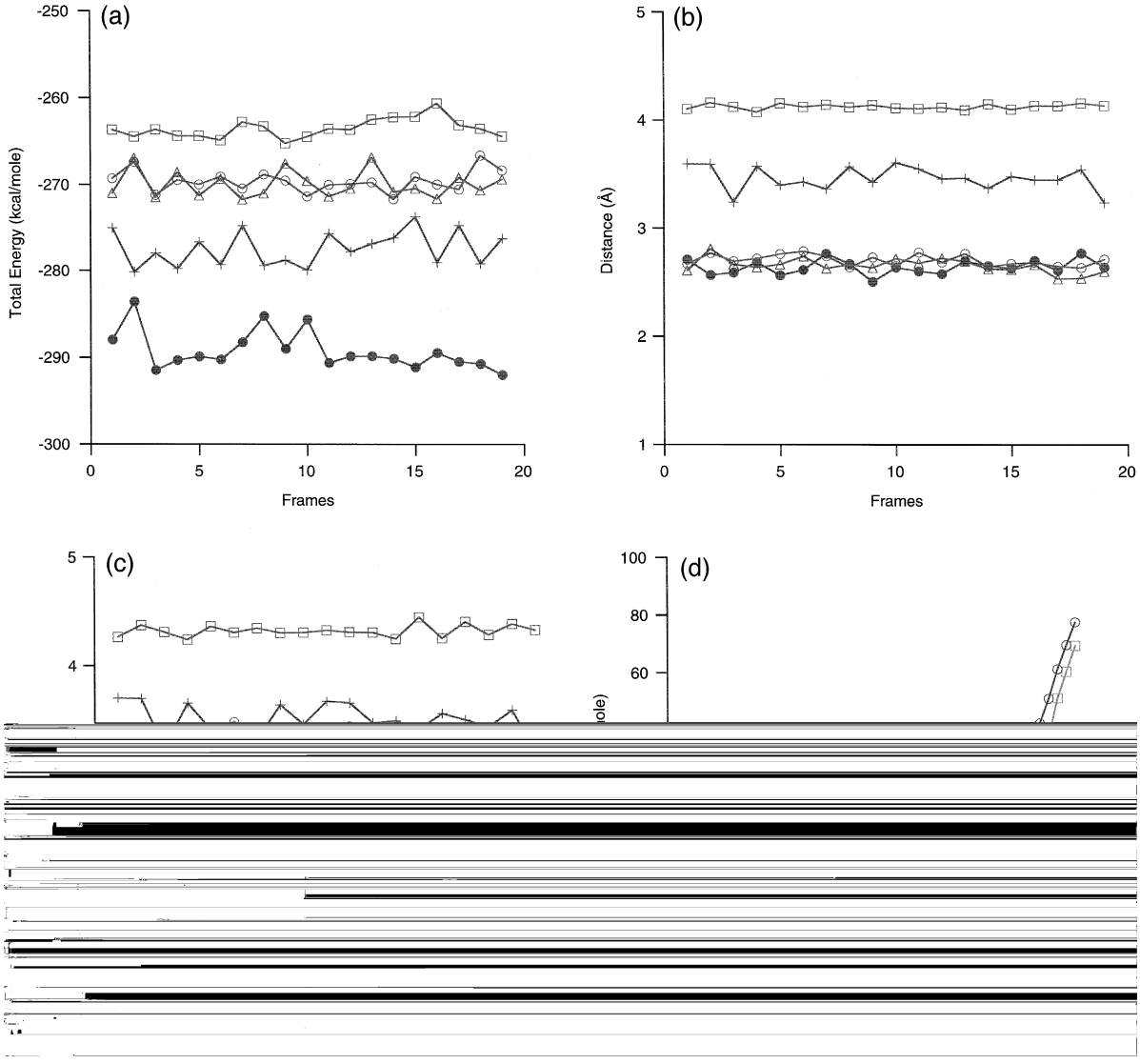
5.2.1. Enantioselectivity of QH-ADH from C. testosteroni

PQQ-C(5) adducts of (*R*)- and (*S*)-pentan-2-ol, hemiketal intermediates, were placed in the active site and subjected to minimization and molecular dynamics. Water molecules were added according to their coordinates in the reference structures. Waters showing substantial overlap with the PQQ-adducts were discarded. The whole structure was solvated during the dynamic runs. Although this is a very crude approximation of an enzyme performing a reaction, the idea holds that by calculating the energies for the different (enantiomeric) reaction intermediates, insight can be gained on the overall reaction mechanism. Similar force field energies were found for both adducts. The energy differences between the favorable conformations of the adducts with opposite chirality were sufficiently small to be in line with the observed enantiomeric ratio for this substrate [175].

5.2.2. Enantioselectivity of QH-ADH from A. pasteurianus

Both the hemiketal mechanism and the mechanism involving direct hydride transfer were

tested. For studying the intermediates resulting from covalent adduct formation the same approach was used as for the QH-ADH from *C. testosteroni*. From the resulting favorable conformations and their corresponding force field energies a clear preference for the (*S*)-enantiomer could not be inferred (Fig. 12a, b, c). This kind of simulation depends very much on the initial placement of the cofactor-substrate adduct, as was already known from the simulation performed on QH-ADH from *C. testosteroni*. To simulate the mechanism involving direct hydride transfer, substrate molecules were pulled into the active site, by applying a force at their alcohol oxygen. In order to use the coordinating power of the calcium ion, the opposite end of the pulling vector was located at this ion. Substrate molecules were docked into the active site and analyzed for their ability to transfer a hydride when in their lowest energy conformation. The results of this combined 'energy-distance' criterium are shown in Fig. 12d and e.



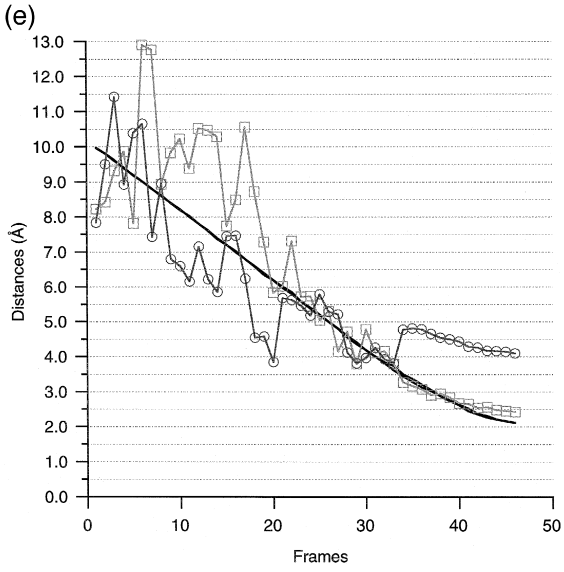


Fig. 12 (continued).

Preference for the (*S*)-enantiomer is in agreement with experimental data [200,201]. Considering the large number of assumptions made, it is hardly possible to discriminate between the mechanistic proposals. Yet, it appears that the intermediate calcium–alcohol complex that is invoked in Schemes 5, 6 and 9 as a precursor for hydride transfer is more likely to predict the correct enantioselectivity than the PQQ-C(5) adduct presented in Schemes 2 and 3. Diagrams of the preferred configurations involved in these mechanisms are given in Fig. 13.

6. Prospects

Unlike the situation in related areas of enantioselective biocatalysis, i.e., ''Kazlauskas rule'' for lipases [165,181], ''Prelog's rule'' for yeast-mediated reductions [100,251] and, of historical interest, ''Bertrand's rule'' for acetic acid bacteria in the conversion of vicinal diols [34], ''rules of thump'' for the prediction of the enantiopreference have not been formulated for the enantioselectivity of quino(haemo)protein

Fig. 12. Results of different approaches to simulate the reaction mechanism with respect to enantioselectivity determining transition states of PQQ containing quinoproteins. All calculations used the homology model of QH-ADH from *A. pasteurianus* and (*R*)- and (*S*)-2-butanol as substrate. Calculations were performed using DISCOVER. Net atomic charges for PQQ and PQQ-adducts and substrates were calculated using MOPAC. Parameters for calcium were adapted from Wavefunction. Docking was performed by pulling the oxygen of the substrates towards the calcium position with a maximum force of 100 kcal mol^{-1} $Å^{-1}$. Initially, substrates were placed well away from the protein surface ($\pm$10 Å). Following each pull the substrate was submitted to a short (1000 cycles) molecular dynamics run at 400 K with subsequent cooling to 50 K before energy minimization and archiving of the resulting structure. Each structure was analyzed for its force field energy components and for the distance between the hydrogen to be transferred and the C(5)-position of PQQ. Runs were also carried out allowing relaxation of residues in a 10 Å sphere around PQQ in which case the substrates were initially placed at a distance of 19.0 Å from PQQ. Up to 10 Å the substrates were pulled in steps of 1.0 Å, from 10–1 Å in steps of 0.5–0.2 Å. The same simulated annealing approach as described above was used. Runs were carried out for (*S*)-2-butanol and (*R*)-2-butanol. Interestingly, in these runs the disulfide bridge had to be constrained to its position. Due to non-balanced charges and van der Waals interactions, the disulfide bridge slowly swung away from its original position when no constraints were applied. Whether this points to a severe error in the model, or is a feature exhibited by the original MDH-crystal structure as well, is not clear at the moment. The importance of well balanced charge–charge interactions has been shown to be of major importance in protein simulations. Because of non-balanced charge–charge interactions or the absence of proper counter-charges (as in the case of MDH without its ß-subunit) protein unfolding is likely to occur. *Graphs a, b and c* show the results of calculations performed assuming the covalent adduct to be the enantioselectivity determining step in the oxidation of (*R*)- and (*S*)-2-butanol. *Graph a* displays the calculated force field energies of a subset of residues containing the active site and the hemiketal intermediate corresponding to the (*R*)- or (*S*)-substrate PQQ-adduct. As the calculation was only performed on a subset of residues contained in a sphere with a radius of 10 Å, no long-range electrostatic forces are incorporated. *Graphs b and c* show the distance of the hydrogen to be abstracted to the PQQ-C(4) and PQQ-O(4) position, respectively. –●– (*R*)-2-Butanol (run 1#); –|– (*R*)-2-Butanol (run 2#); –○– (*S*)-2-Butanol (run 1#); –□– (*S*)-2-Butanol (run 2#); –△– (*S*)-2-Butanol (run 3#). *Graphs d and e* shows the results of ''docking'' runs, performed by pulling a molecule of substrate into the active site and onto the calcium. *Graph d* shows the energies involved. No emphasis should be given to the absolute values of the energies, as residues have been fixed to their positions and only a subset of residues has been used. –□– (*S*)-2-Butanol; –○– (*R*)-2-Butanol. In *Graph e* the distance from the alcohol oxygen to the calcium and the hydrogen-to-be-transferred to the PQQ-C(5) position is displayed. Runs were performed on the alcohol, not on the alcoholate. –□– (*S*)-2-Butanol; –○– (*R*)-2-Butanol; — Distance to calcium atom.

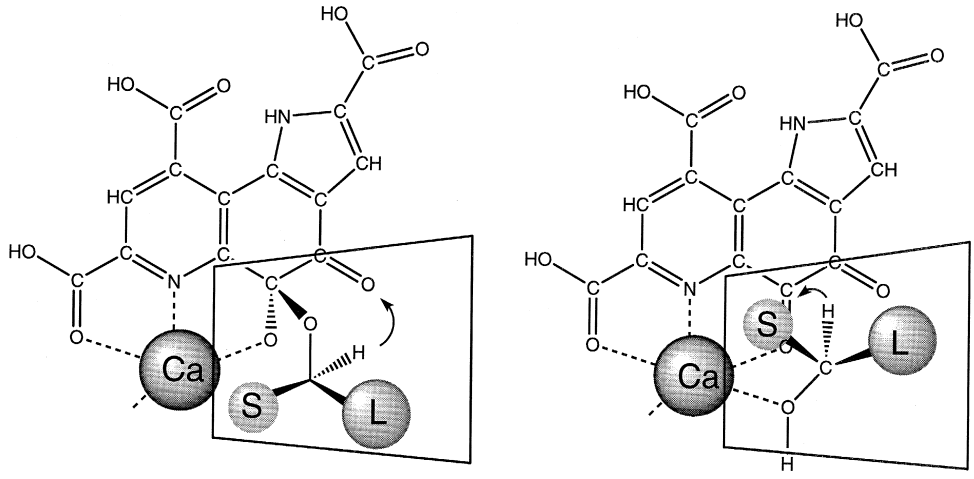


Fig. 13. Structures of intermediates preceding the enantioselectivity determining transition state. On the left: ''best fit'' of secondary alcohols for the covalent adduct mechanism as proposed in Schemes 2 and 3, leading to wrong prediction of QH-ADH enantiopreference; on the right: ''best fit'' for calcium-ligated secondary alcohols as proposed in Schemes 5, 6 and 9, leading to correct prediction of QH-ADH enantiopreference. S, small substituent, L, large substituent on chiral carbon. Boxes indicate active site limitations as deduced from homology models of QH-ADHs.

alcohol dehydrogenases. Qualitative correlations of enzyme enantioselectivity and substrate structure were originally proposed for liver alcohol dehydrogenase [168]. Similar approaches have been documented since [197,229,243,298]. An attempt to rationalize the observed enantiopreference for quinoproteins has been reported [201]. Fig. 14 summarizes the results in a ''substrate overlay'' diagram.

Clearly, such diagrams should be treated with due care. Not only because ''averaging'' the preferred structures does not lend credit to the fact that some of the ''preferred'' *enantiomers* may not be ''preferred'' substrates, and may actually perform worse than ''non-preferred'' enantiomers of good substrates. Also, one would ideally like to compare transition state structures and *not* Michaelis-type complexes (see Section 2 for analysis). Thirdly, there may be appreciable ambiguity with respect to the ordering of ligands. In this respect, the possible orientation of benzylalcohol is of interest. MDH has been reported to catalyze removal of the pro-*S* hydrogen (Ref. 16 in Ref. [12]). Whereas comparison with the enantiopreference for secondary alcohols would suggest pro-*R* specificity, comparison with glycidol and solketal suggests the observed pro-*S* specificity (Fig. 14). Finally then, the characteristics of the individual enzymes may defy correlations of this type.

An important aspect of quinohaemoprotein enantioselectivity is the location of the enantioselectivity determining reaction step on the reaction coordinate. Since this step may not be identical to the rate-controlling step, appropriate analysis of the mechanism is required. In this respect, the study of (deuterium) isotope effects on the reaction rate as well as on the enantioselectivity may be helpful. It must be emphasized that location of the rate controlling step following the enantioselectivity determining step has no influence on the enantiomeric ratio. This situation has been verified in the case of QH-ADH Type I from *C. testosteroni* [113], and QH-ADH Type II from *A. pasteurianus* [200] for the electron acceptors ferricyanide and decylubiquinone (U. Wandel, personal communication). Even though ferricyanide is rate controlling, no effect of ferricyanide concentrations of *E* were found. It will be interesting to investi-

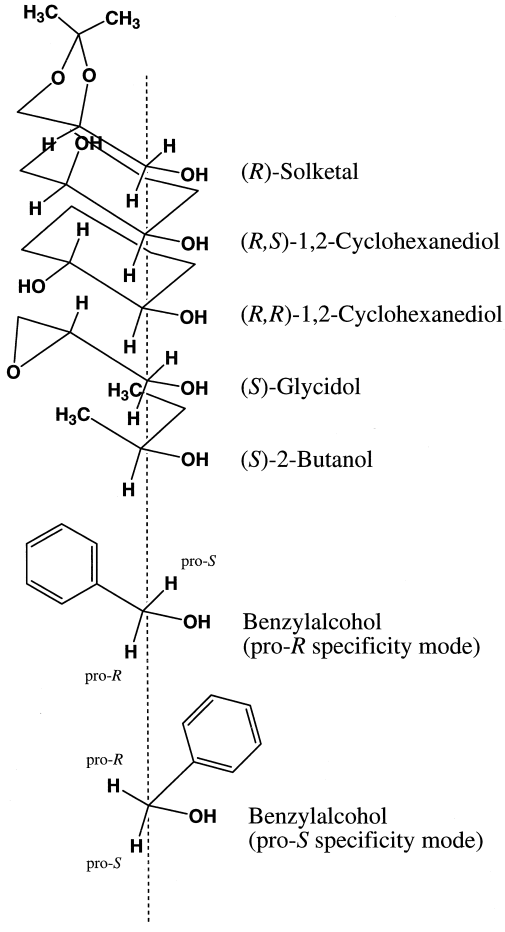


Fig. 14. Overlay of substrate enantiomers that are preferentially oxidized by quinohaemoprotein dehydrogenases of *Acetobacter*, *Gluconobacter* and *Comamonas* sp. as compared to the pro-chiral specificity for benzylalcohol reported for MDH (adapted from Ref. [201]).

gate possible effects of intramolecular, viz. intermolecular electron transfer as well. Kinetic studies of quinohaemoprotein D-fructose dehydrogenase from *Gluconobacter industrius*, a three-subunit enzyme containing PQQ and haem *c* [5,333] showed parallel reciprocal plots in the oxidation of D-fructose with varying concentrations of ferricyanide indicative of ping-pong kinetics [204]. A reciprocal plot of the reduction of ferricyanide at saturating concentrations of D-fructose gave a break which was considered to result from the involvement of two active centers, namely PQQ and haem *c*. The electron transfer rate between $PQQH_2$ and haem *c* was calculated as $2.2 \pm 0.4 \times 10^4$ M^{-1} s^{-1}, with k_{cat}'s of 93 ± 14 s^{-1} and 162 ± 7 s^{-1} for the slow (reduced haem at low ferricyanide concentrations) and fast (oxidized haem at high concentrations) branches of the pathway, respectively. Rate limitations of this kind for QH-ADH alcohol dehydrogenases should thus be considered.

Current knowledge of the structure of PQQ-containing dehydrogenases relies heavily on the X-ray crystallographic data obtained for MDHs. The structure elucidation of GDH from *Acinetobacter calcoaceticus* (soluble form, expressed in recombinant *E. coli*) has been announced (B.W. Dijkstra, personal communication) and will probably add to our understanding of the role of PQQ in quinoprotein-mediated redox reactions. Kinetic and mechanistic details of the reactions catalyzed by this enzyme have been reported [239–241]. Presumably even more interesting will be the eventual structure elucidation of the membrane-bound GDH from this and related organisms. This enzyme catalyzes the oxidation of D-glucose and other monosaccharides to the corresponding lactones. The electron acceptor is ubiquinone [209,210]. A low extent of sequence identity with MDHs has been found, based on which a model has been constructed [58]. In the model, a histidine residue is seen to replace the disulphide bridge present in MDHs, a finding consistent with earlier data on the involvement of a histidine residue in the binding of PQQ to this enzyme [143]. Recent isolation of a new quinoprotein dehydrogenase from *G. oxydans* DSM 4025 that effectively oxidizes L-sorbose as well as a broad variety of primary and secondary alcohols, aldehydes, aldoses, and ketoses, but not methanol or formaldehyde might bridge the gap between PQQ-containing GDHs and alcohol dehydrogenases [20]. Some of the structural constraints deduced to be of importance for the enantioselectivity of e.g., QH-ADHs may have to be reconsidered once new structures become available.

Acknowledgements

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