Substrate binding in quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* studied by electron-nuclear double resonance

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Binding of methanol to the quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* has been studied by pulsed electron-nuclear double resonance at 9 GHz. Shifts in the hyperfine couplings of the pyrroloquinoline quinone radical provide direct evidence for a change in the environment of the cofactor when substrate is present. By performing experiments with deuteriated methanol, we confirmed that methanol was the cause of the effect. Density functional theory calculations show that these shifts can be understood if a water molecule, which is often found in x-ray structures of the active site of quinoprotein alcohol dehydrogenases, is displaced by the substrate. The difference between the binding of water and methanol is that the water molecule forms a hydrogen bond to O5 of pyrroloquinoline quinone, which the methanol, by virtue of its methyl group, does not. The results support the proposal that aspartate rather than glutamate is the catalytically active base for a hydride transfer mechanism in quinoprotein alcohol dehydrogenases.

Pyrroloquinoline quinone (PQQ, Fig. 1) is one of several quinone derivatives that function as essential cofactors in a class of enzymes known as quinoproteins (1–3). X-ray crystallographic studies have allowed great progress to be made in the understanding of the structure and function of the PQQ-dependent enzymes (for reviews see refs. 4–6). There is special interest in the properties of the PQQ cofactor because its possible role as a vitamin in mammals is currently under debate (7–10).

The common structural feature of methanol dehydrogenase (MDH) is an αβ2 tetrameric structure (11–17), in contrast to quinoprotein ethanol dehydrogenase (QEDH), which is a homodimeric protein (α2) (18, 19). In both enzymes, the α subunit is a superbarrel composed of eight radially arranged β-sheets. The PQQ cofactor forms a complex with a Ca2⁺ ion buried in the interior of the superbarrel and is sandwiched between the indole ring of a tryptophan and an unusual eight-membered disulfide ring structure formed from adjacent cysteines (see Fig. 2).

Two alternatives have been proposed for the reaction mechanism in quinoprotein dehydrogenases (20). Initially, an addition/elimination mechanism was favored, a suggestion that is now considered unlikely (21), because a hydride transfer mechanism is preferred (22) (see Fig. 1). The PQQ is initially oxidized, and after substrate binding, reaction is initiated by a base-catalyzed proton abstraction of the hydroxyl proton of the alcohol. The characteristics of an Asp-303–Glu mutant protein (numbering scheme of MDH from *Methylobacterium extorquens*) were considered consistent with Asp-303 acting as the catalytically active base in MDH (16). Recently, however, this widely accepted view has been challenged with the results of a molecular dynamics study presented by Reddy and Bruice (23), who suggested that Glu-177 is the catalytic base. In either case, nucleophilic addition of the hydride from the substrate to the C5 position of PQQ is then expected to occur. Subsequently, the PQQ enolizes to form the quinol. Reoxidation proceeds in two sequential one-electron transfer steps to cytochrome via the PQQ radical, which is deprotonated, formally POQ⁺⁺ (24, 25).

Oubrie and coworkers (26) have presented a structure of PQQ-dependent soluble glucose dehydrogenase in which a complex of reduced PQQ and glucose was resolved at 1.9-Å

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Abbreviations: ENDOR, electron-nuclear double resonance; DFT, density functional theory; QEDH, quinoprotein ethanol dehydrogenase; hfc, hyperfine coupling; MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone.

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Results and Discussion

The active site of MDH (17). Shown is how the PQQ cofactor bound to Ca²⁺ is sandwiched between Trp-243 and the disulfide bridge formed by Cys-103 and Cys-104. Also shown are the amino acid residues (Asp-303 or Glu-177) that have been suggested to play a catalytic role in alcohol oxidation and a water molecule (Water362) that forms hydrogen bonds to OS of PQQ and Asp-303.

The positioning of the C1 atom of the glucose over the tetrahedral C5 atom of PQQ is one of the most convincing pieces of evidence for a hydride ion transfer mechanism. Thus far, however, a substrate-bound structure for a PQQ-dependent alcohol dehydrogenase has remained elusive. Recently, we provided spectroscopic evidence for substrate binding in QEDH, using continuous-wave EPR at 94 GHz (W-band) (27). Changes in the principal components of the g-tensor that could be detected after the addition of ethanol implied an increase in polarity near to the PQQ radical. This result could be qualitatively understood in terms of a change in the geometry or environment induced by presence of substrate. Although significant advances have been made in the calculation of g-tensors of small molecules and how they depend on hydrogen bonding (28), it is not generally possible to use them to provide quantitative structural information.

On the other hand, proton hyperfine couplings (hfcs) that can be determined by electron-nuclear double resonance (ENDOR) together with density functional theory (DFT) can provide reliable structural information (24, 29, 30). Furthermore, the catalytically inactive PQQ radical is a good model for the catalytically active oxidized PQQ because they differ only by one electron, but have the same protonation state and very similar geometries (24). Indeed, our previous DFT calculations have shown that the singly occupied molecular orbital of the PQQ radical in which the unpaired electron resides is almost identical to the lowest unoccupied molecular orbital in the oxidized PQQ, which is expected to act as hydride acceptor in alcohol oxidation. Hence, in this work we have studied in detail the binding of methanol to QEDH from Pseudomonas aeruginosa by using pulsed ENDOR at 9.7 GHz (X-band). Significant shifts in the hfcs of the PQQ cofactor between substrate-free and methanol-bound samples were observed. By performing DFT calculations on the PQQ radical including a truncated environment, these changes could be explained by the displacement of a water molecule found in the active site by methanol. The results support the proposal that Asp-303 is the catalytic base rather than Glu-177.

**EPR.** We had previously reported the X-band continuous-wave EPR spectrum of the PQQ radical bound in QEDH (31) and do not repeat it here. The spectrum shows a slightly asymmetric line shape centered at $g_{\text{iso}} = 2.0043$ and a peak-to-peak line width of 0.5 mT and is similar to that observed in MDH (3, 32, 33). No change in the X-band EPR spectrum could be detected when methanol was added. Hence, we turned to the higher resolution afforded by ENDOR.

**ENDOR.** X-band pulsed ENDOR spectra of the PQQ radical bound in QEDH in the absence and the presence of methanol are displayed in Fig. 3. The ENDOR spectra are centered at the nuclear Zeeman frequency ($\nu_1 = 14.75$ MHz) and, in the weak coupling limit, which is the case here, each set of equivalent protons gives rise to a pair of tensorial patterns, whereby the splitting is equal to the hfc tensor component. The ENDOR spectra were recorded at the maximum intensity of the two-pulse electron-spin echo EPR spectrum (data not shown), which is at the zero-crossing point of the continuous-wave EPR spectrum.

The ENDOR spectra exhibited very rich and well resolved hyperfine structure with hfcs ranging from 0 to 10 MHz. Prominent features were observed with hfc components at 9, 6, 5, and 4 MHz, which we have previously assigned to the hfc tensor components of the three PQQ protons bound at N1, C3, and C8 (24). Upon binding of methanol almost all of the lines in the ENDOR spectrum shifted by $\pm 3\%$. Note that the observed changes are completely reversible. Either spectrum could be generated sequentially from the same sample (or from different protein preparations) by washing or the addition of methanol.

The features at 9.30 and 6.20 MHz, which had been assigned to two hfc tensor components of the proton at C8, decreased to 9.06 and 6.03 MHz, respectively. The hfc tensor component at 5.00 MHz, which had been assigned to the proton at C1, increased to 5.15 MHz, and the hfc tensor component at 4.00 MHz, which had been assigned to the proton at C3, increased to 4.15 MHz. We conclude that in the presence of methanol the unpaired electron spin density (as reflected in the hfcs) increases on the pyrrolo ring (ring I) and decreases on the pyridine ring (ring III) (Fig. 1). To a first approximation, the unpaired electron spin density...
should correspond to the electron density within the singly occupied molecular orbital. Hence the changes imply that the electronic structure of the PQQ radical is directly affected by the presence or absence of methanol. One possible cause of this effect that is well documented in p-semiquinones is a change in the hydrogen bonding to the C–O groups (34, 35).

To confirm that the effect derives from the presence of methanol, we performed X-band pulsed ENDOR spectra on the PQQ radical bound in QEDH in the presence of deuteriated methanol. In Fig. 4 the spectrum is compared with that obtained with protiated methanol. The two spectra are almost identical (they have been scaled to have the same intensity at the peaks at 4.15 MHz). The spectra show that, as expected, the replacement of the protons in the substrate by deuterons did not affect the binding. However, in the middle of the spectra close to the nuclear Zeeman frequency, there is a clear reduction of intensity when deuteriated methanol is bound. The difference between the two spectra (scaled by a factor four by black line) and a simulation thereof (orange line). Note that the simulation included hfc tensor components A₁ and A₃ having opposite signs. To illustrate this point, the dashed vertical lines marking the principal hfc values are connected horizontally crossing the nuclear Zeeman frequency.

![Fig. 4. Pulsed-ENDOR spectra of the PQQ radical bound in QEDH. Shown are spectra with CH₃OH bound (red line) and CD₃OH bound (blue line). Also shown is the difference between the two spectra scaled by a factor of four (black line) and a simulation thereof (orange line). Note that the simulation included hfc tensor components A₁ and A₃ having opposite signs. To illustrate this point, the dashed vertical lines marking the principal hfc values are connected horizontally crossing the nuclear Zeeman frequency.](image)
its oxygen could form a coordinate bond with the Ca\textsuperscript{2+}. Such an interaction would not only increase the polarization of the methanol, thus assisting in the abstraction of the hydroxy proton on reconstitution or isolation of the native enzyme substrate was often bound. Therefore, after reconstitution the preparations were washed by repeated dilution concentration cycles in freshly prepared alcohol-free buffer (100 mM Tris-HCl, pH 8/100 mM NaCl/10 mM CaCl\textsubscript{2}) to obtain the substrate-free sample. Methanol was modeled into the active site according to ref. 23 and hydrogen-bonded to Glu-177, Asp-303, and Water362. Heavy atoms were taken from the x-ray structure of MDH (17), and protons were added and energy-optimized by using DFT methods. (\(c\) Glu model. Methanol was modeled into the active site by replacing one of the protons of Water362 by a methyl group. Methanol maintains a hydrogen bond with Asp-303, but there is no direct interaction with PQQ. (\(c\) Glu model. Methanol was modeled into the active site according to ref. 23 and hydrogen-bonded to Glu-177, while Water362 remained in the position given in the x-ray structure. Hfcs for the PQQ radical were calculated for the three structures and are presented in Table 1.

Fig. 5. Models of the active site of quinoprotein alcohol dehydrogenases showing the PQQ cofactor, the Ca\textsuperscript{2+} ion, Glu-177, Asp-303, Asn-261, Arg-331, and Water362. Heavy atoms were taken from the x-ray structure of MDH (17), and protons were added and energy-optimized by using DFT methods. (\(a\) Substrate-free. Water362 forms hydrogen bonds with Asp-303 and OS of PQQ. (\(b\) Asp model. Methanol was modeled into the active site by replacing one of the protons of Water362 by a methyl group. Methanol maintains a hydrogen bond with Asp-303, but there is no direct interaction with PQQ. (\(c\) Glu model. Methanol was modeled into the active site according to ref. 23 and hydrogen-bonded to Glu-177, while Water362 remained in the position given in the x-ray structure. Hfcs for the PQQ radical were calculated for the three structures and are presented in Table 1.

the methyl group can, in principle, be obtained from our ENDOR data. The dipolar part of the hfc directly depends on the distance between a nucleus and the unpaired electron spin, according to:

\[
v_{\text{hfc}}(\theta) = \frac{\mu_0}{4\pi h} \frac{g_e g_N \mu_B \mu_N}{r^3} \left(3 \cos^2\theta - 1\right)\rho,
\]

where \(g_e\) and \(g_N\) are the electron and nuclear \(\gamma\)-factors, \(\mu_0\) is the vacuum permeability, \(\mu_B\) and \(\mu_N\) are the Bohr and nuclear magnetons, respectively, \(h\) is Planck’s constant, \(\theta\) is the angle between the principal axes and \(r\) the distance between them, and \(\rho\) is the electron spin density that causes the interaction. For a rather localized electron, for example, a paramagnetic ion or a nitroxide spin-label, \(\rho\) can be set to unity. In the PQQ radical, however, the unpaired electron is delocalized over the \(\pi\)-system. The good agreement between the experimental and theoretical proton hfcs gives us confidence that the predicted electronic distribution is largely correct and we can use the Muliken spin densities for the heavy atoms given by the DFT calculations. For C5, \(\rho \approx 0.45\). Hence, almost 50% of the electron spin density is localized at this position. The situation here is slightly more complicated because we assigned the hfc to the three protons of a freely rotating methyl group. Nevertheless, if applied with caution, this approach is still instructive. For example, taking \(\nu = 1\) MHz from our experiment and setting \(\rho = 0.45\), we obtain an average \(r = 3.3\) Å, and a closest approach of 2.6 Å, which is close to the distances obtained from molecular modeling studies of hydride transfer to C5 of PQQ (37).

To conclude, we used pulsed ENDOR to examine the binding of methanol to QEDH. Although with a \(K_m\) value of 94 mM methanol is not the primary substrate (\(K_m = 0.014\) mM for ethanol) (38), we still observed significant changes in the hfcs of the PQQ radical. We confirmed that the changes arise from methanol by modifications to the spectra when using deuteriated methanol. This approach allowed us to give an estimate of the distance between the methyl group of methanol and the PQQ cofactor. Using DFT, it could be shown that the changes are consistent with the role of Asp as the catalytically active base, but contradict the proposal that Glu is the base. The latter proposal is solely based on in silico molecular dynamics (MD) simulations (23). That our experiments yielded a different conclusion indicates that although MD simulations are in many cases very useful they ought to be used with caution when no experimental data are available. We have shown that ENDOR and DFT are a powerful combination that can be used to obtain high-quality structural information, and in this case we have provided direct evidence for the location of a primary alcohol bound in the active site of a quinoprotein. The results support the proposal that a hydride transfer mechanism, which is accepted for soluble glucose dehydrogenase, is also active in quinoprotein alcohol dehydrogenases.

Materials and Methods

Enzyme Isolation. The apo-form of QEDH enzyme was heterologously overexpressed in Escherichia coli and purified to homogeneity with Tris-HCl buffer, in which the enzyme was active and appeared to be completely stable, as described for holo-QEDH (31, 38).

Reconstitution of the Holo-Form of QEDH. The formation of an enzymatically active holo-enzyme was achieved as described (39). In a previous study, we (27) had observed that after reconstitution or isolation of the native enzyme substrate was often bound. Therefore, after reconstitution the preparations were washed by repeated dilution/concentration cycles in freshly prepared alcohol-free buffer (100 mM Tris-HCl, pH 8/100 mM NaCl/10 mM CaCl\textsubscript{2}) to obtain the substrate-free sample. Meth-
Theoretical values were calculated by using DFT and a truncated structure based on the x-ray data for MDH (17).

Simulations. Orientation-selected ENDOR spectral simulations were performed by using the computer program SPLEEN (40).

Computational Process. To model the binding pocket of the PQQ cofactor in a quinoprotein, the high-resolution (1.2 Å) x-ray structure of MDH from M. extorquens (17) was used, rather than the lower-resolution (2.6 Å) x-ray structure of OEDH (19). This choice is justified because most amino acids found in close contact with the Ca$^{2+}$PQQ$^{3-}$ complex are conserved between the structures. We included the PQQ cofactor, the Ca$^{2+}$ ion, Glu-177, Asn-261, Asp-303, Arg-331, and Water362. The heavy atoms were fixed, while protons were added as appropriate and energy-optimized by using DFT at the unrestricted B3LYP/6–31G(d,p) level of theory, as implemented in the GAUSSIAN 03 (41) program package. Hfs for the PQQ radical (PQQ$^{4-}$) were calculated for the optimized structure at the same level of theory. To model binding of methanol, Water362 was converted into methanol, by replacing one proton with a methyl group but leaving the oxygen atom fixed. The methyl group of the methanol and all protons were then energy-optimized, and hfs were calculated again. Graphical representation of the structures was achieved with VMD (43) followed by rendering with POV-RAY (www.povray.org).

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Table 1. Theoretical and experimental hfc's of the protein-bound PQQ radical (PQQ$^{4-}$) with water or methanol occupying the active site

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<th>z</th>
<th>x</th>
<th>C3H</th>
<th>y</th>
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<th>x</th>
<th>C8H</th>
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<td>A$_{iso}$</td>
<td>A$_{dip}$</td>
<td>A$_{ii}$</td>
<td></td>
<td>A$_{iso}$</td>
<td>A$_{dip}$</td>
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<td>A$_{dip}$</td>
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The hfc tensor components, A$_{iso}$, are given by the sum of the isotropic part, A$_{iso}$, and dipolar part, A$_{dip}$, i.e., A$_{ii}$ = A$_{iso}$ + A$_{dip}$. All values are given in megahertz. Theoretical values were calculated by using x-ray data for MDH (17).