Communication

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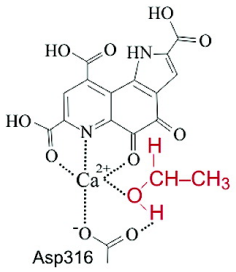

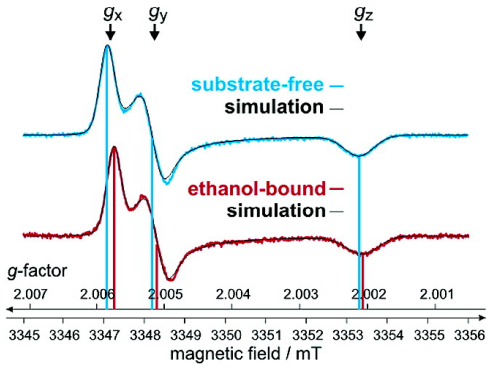

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Substrate-Binding in Quinoprotein Ethanol Dehydrogenase from *Pseudomonas aeruginosa* Studied by Electron Paramagnetic Resonance at 94 GHz

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Pyrroloquinoline quinone (2,7,9-tricarboxypyrroloquinoline quinone, PQQ, Figure 1) is one of several quinone cofactors that is utilized in a class of dehydrogenases known as quinoproteins.[1] Members of the class include methanol dehydrogenase (MDH), ethanol dehydrogenase (QEDH), quinohemoprotein alcohol dehydrogenase (QH-ADH), and glucose dehydrogenase (GDH).

Two mechanisms have been proposed for the reduction of substrate in quinoprotein alcohol dehydrogenases,[2] both of which commence with the PQQ fully oxidized. Both are initiated by amino acid (Asp) base-catalyzed proton abstraction of the hydroxyl proton of (m)ethanol, Figure 1. In an addition/elimination mechanism, the negatively charged substrate oxygen then performs a nucleophilic addition to the PQQ(C5) to form a covalent substrate−PQQ complex. This is followed by elimination of (m)ethanal, leaving the fully reduced $PQQH_2$. In a hydride transfer mechanism, a nucleophilic addition to the PQQ(C5) again occurs, but this time it is the hydride from C1 of the substrate that is transferred, completing the oxidization of the (m)ethanol to (m)ethanal. Subsequently, the PQQ enolizes to form $PQQH_2$.

In order to elucidate reaction mechanisms, emphasis is often given to obtaining protein structures by X-ray crystallography. Several have been reported for MDH,[3−9] QEDH,[10] QH-ADH,[11,12] and GDH.[13] All show that the active site consists of a PQQ−Ca^{2+} complex. Oubrie and co-workers have presented a structure of soluble GDH[13] in which a complex of reduced PQQ and glucose were resolved at 1.9 Å resolution. The positioning of the C1 atom of the glucose over the tetrahedral PQQ(C5) in the structure was used as a basis for arguing for a hydride ion transfer mechanism.

In alcohol dehydrogenases, however, obtaining substrate-bound structures has proven to be more difficult. In the structure of QEDH from *Pseudomonas aeruginosa*, Keitel and co-workers[10] observed no electron density from substrate but used docking software to model several alcohols into the active site region. In the QH-ADH from *Pseudomonas putida*, Chen and co-workers[11] observed acetone (the oxidation product of 2-propanol) bound in the active site, while in the same protein from *Comamonas testosteroni*, Oubrie and co-workers[12] reported tetrahydrofuran-2-carboxylic acid in the binding site.

For MDH, Xia and co-workers[5,6,8] reported structures at 1.9 Å resolution. Electron density in the active site was interpreted as either methanol[6] or water with a tetrahedral PQQ(C5).[5,8] These results have built-up support for a hydride transfer mechanism, which is also supported by molecular dynamics simulations;[14] however, without a substrate-bound structure or other evidence from spectroscopy, the conclusion that all PQQ-dependent alcohol dehydrogenases utilize the same hydride transfer mechanism now widely accepted for soluble GDH is clearly unsafe.

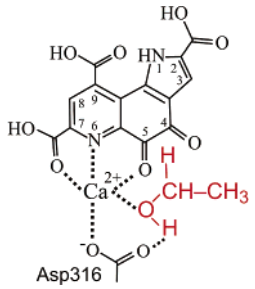

Figure 1. Putative model of ethanol binding to Asp316 and the PQQ−Ca^{2+} complex in QEDH. Nonbonded interactions are indicated by dotted lines.

The common aspects of both postulates are the initiation by proton abstraction from the (m)ethanol by the CO_2^- of Asp and that the (m)ethanol C1 must be relatively close to and above the PQQ(C5), Figure 1. These conditions imply quite tight geometric constraints on the position of the substrate, which have been supported by docking calculations.[10,14]

In this contribution, we have used continuous-wave (cw) high-field/high-frequency electron paramagnetic resonance (EPR) at 94 GHz (W-band) to study substrate binding in QEDH from *Pseudomonas aeruginosa*, taking advantage of the fact that in alcohol dehydrogenases a substantial proportion of the PQQ cofactor is found in the semiquinone form.[15]

In Figure 2, W-band cw-EPR spectra and simulations of the PQQ semiquinone radical-bound QEDH are displayed in the absence (a) and in the presence (b) of ethanol. QEDH enzyme was prepared as described previously.[16] To obtain the substrate-free sample, the protein was washed with freshly prepared buffer. Ethanol (1 mM) was then added to the buffer to yield the ethanol-bound sample. In the substrate-free enzyme, the principal values of the *g*-tensor, obtained by spectral simulation are $g_x = 2.00585(2)$, $g_y = 2.00518(2)$, and $g_z = 2.00212(2)$, giving $g_{iso} = 2.00438(2)$. All three principal values of the *g*-tensor decrease when ethanol is bound to the protein: $g_x = 2.00574(2)$, $g_y = 2.00511(2)$, and $g_z = 2.00207(2)$, giving $g_{iso} = 2.00431(2)$. The simulations included only the principal values of the *g*-tensor and an orientation-dependent line width. The latter did not significantly deviate between the different samples.

It is important to 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 addition of ethanol. Hence, the differences between the *g*-values represent the first spectral evidence for tight binding of ethanol to the PQQ−Ca^{2+} complex in alcohol dehydrogenases. The *g*-values

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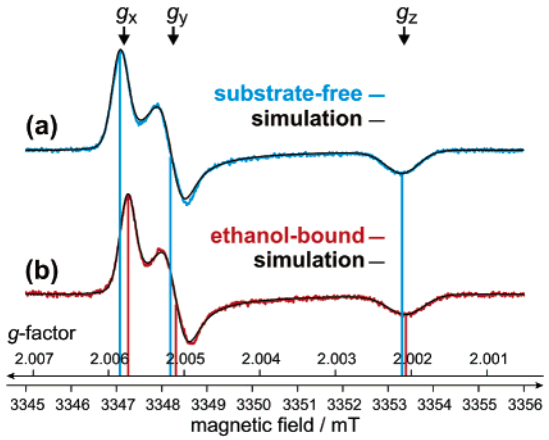

Figure 2. W-Band cw-EPR spectra and simulations of the PQQ radical bound in wild-type QEDH without (a) and in the presence of (b) substrate. For details of sample preparation, see the text. Spectra were recorded with a W-band spectrometer (Bruker BioSpin Elexsys E680). Conditions: temperature, 150 K; microwave frequency, 93.967 GHz (power, 50 nW); modulation amplitude, 0.2 mT (frequency, 100 kHz). A Li:LiF sample was used for *g*-factor calibration. Simulations were performed with the computer program SIMPOW6 obtained from Mark J. Nilges and the Illinois EPR research center (http://ierc.scs.uiuc.edu/nilges.html).

obtained for the substrate-bound sample are (within experimental error) identical to those that we have reported previously.[16] The samples used in that study were measured directly after reconstitution of the apoprotein with PQQ, implying that ethanol had become bound during this process, possibly due to the presence of traces of ethanol in the buffer or protein preparation. Our experience suggests that unless the enzyme is carefully washed with ethanol-free buffer, it is more than likely to have ethanol bound. This reflects a high binding affinity of the enzyme for ethanol, even when the PQQ cofactor is in the semiquinone form.

The shifts in the *g*-factor components of the radical are likely to be a consequence of a changed environment and/or geometry of the PQQ cofactor. From work on nitroxide spin-labels,[17] *p*-semiquinones,[18] and the tyrosyl amino acid radicals,[19] it is well-known that the g_x component of compounds with a high spin density on a carbonyl or NO group is particularly sensitive to changes in the immediate vicinity. Here, all three components became smaller in the presence of ethanol, although the g_x component shows the biggest shift of about 1×10^{-4}.

For the two ubiquinone-10 molecules Q_A and Q_B in the photosynthetic reaction center from *Rhodobacter sphaeroides*, a smaller g_x ($\Delta g_x = 2 \times 10^{-4}$) was observed for Q_B.[20] This was attributed to stronger hydrogen bonding and the more polar environment of Q_B. The difference in the g_x value discussed here for the PQQ radical in QEDH is of similar magnitude and raises the question of how ethanol binding to the PQQ−Ca^{2+} complex should lead to an apparent increase in polarity.

Analysis of electron density in MDH suggested the presence of a water molecule above the PQQ(C5),[5,8] which forms hydrogen bonds with both PQQ(O5) and the Asp-CO_2^- and a coordination bond with Ca^{2+} ion. In the QEDH, no water molecule was modeled into the structure at this position, but given the similarity of the enzymes its presence in the coordination sphere of the Ca^{2+} ion is nevertheless likely. Hence, the difference in binding between solvent water and substrate could simply be that ethanol lacks the hydrogen bond to PQQ(O5). Alternatively, if there is no water molecule bound at this position, then the formation of an additional coordinate bond to the Ca^{2+} ion from the alcohol oxygen would be expected to change the geometry of the PQQ−Ca^{2+} complex. In either case, if the result were a shorter distance between the PQQ(O5) and Ca^{2+}, then the effective polarity would increase, thus causing the decrease in *g*-tensor components.

To conclude, we have used the increased spectral resolution of high-field EPR to gain the first direct evidence for the tight binding of ethanol to a PQQ-dependent alcohol dehydrogenase by using the anisotropy of the *g*-tensor as a probe of the electronic structure of the PQQ radical. The results are consistent with either proposed mechanism. Nevertheless, if the substrate−PQQ−Ca^{2+} complex does have the structure discussed here, then in an addition/elimination mechanism, the substrate would have to break its coordinate bond to the Ca^{2+} before it could perform a nucleophilic attack on the PQQ(C5), which we consider unlikely.

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