Characterisation of the PQQ cofactor radical in quinoprotein ethanol dehydrogenase of *Pseudomonas aeruginosa* by electron paramagnetic resonance spectroscopy

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Received 29 January 2004; revised 9 March 2004; accepted 12 March 2004

First published online 26 March 2004

Edited by Peter Brzezinski

Abstract The binding pocket of the pyrroloquinoline quinone (PQQ) cofactor in quinoprotein alcohol dehydrogenases contains a characteristic disulphide ring formed by two adjacent cysteine residues. To analyse the function of this unusual structural motif we have investigated the wild-type and a double cysteine:alanine mutant of the quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* by electron paramagnetic resonance (EPR) spectroscopy. Thus, we have obtained the principal values for the full rhombic *g*-tensor of the PQQ semiquinone radical by high-field (94 GHz) EPR necessary for a discrimination of radical species in dehydrogenases containing PQQ together with other redox-active cofactors. Our results show that the characteristic disulphide ring is no prerequisite for the formation of the functionally important semiquinone form of PQQ.

Key words: Quinoprotein ethanol dehydrogenase; Electron paramagnetic resonance; Pyrroloquinoline quinone; Cofactor binding

1. Introduction

Pyrroloquinoline quinone (2,7,9-tricarboxypyrroloquinoline quinone, PQQ; Fig. 1) is one of several quinone cofactors utilised in a class of dye-dependent dehydrogenases, known as quinoproteins, which are distinct from the flavin- and nicotinamide-dependent oxidoreductases [1–6].

The quinoprotein methanol dehydrogenase (MDH) is among the best-characterised PQQ-dependent enzymes thus far. MDH has an $\alpha_2\beta_2$ tetrameric structure with each β-subunit folded around the surface of an α-subunit [7–11]. The latter is a super-barrel composed of eight radially arranged β-sheets, the so-called propeller fold. The PQQ cofactor bound to a Ca^{2+} ion is buried in the interior of the super-barrel and is sandwiched within van der Waals contact between the indole ring of a tryptophan residue and an unusual eight-membered disulphide ring structure formed from adjacent cysteine residues.

This remarkable ring structure occurs only in PQQ-containing quinoprotein alcohol dehydrogenases. Its function is unknown, although it has been postulated that it could play a role in protecting the intermediate free-radical form of the cofactor [12].

In site-directed mutagenesis experiments on quinoprotein MDH from *Methylobacterium extorquens* both cysteines were separately and simultaneously mutated to serine. All three enzymes were completely inactive and the mutant organisms were unable to oxidise methanol or use it as their sole source of carbon and energy [13]. Recent work on the quinohaemoprotein (type II) alcohol dehydrogenase suggests, however, that the disulphide ring is essential for intra-protein electron transfer in all alcohol dehydrogenases [14].

The purpose of this study is twofold. First, we address the question whether the characteristic disulphide ring of the PQQ binding pocket in alcohol dehydrogenases is necessary for the formation of the semiquinone form of PQQ. Second, we present fingerprint information on the high-field electron paramagnetic resonance (EPR) properties of the PQQ semiquinone. For the experiments we used the quinoprotein ethanol dehydrogenase (QEDH) from *Pseudomonas aeruginosa* [15]. The folding and the active site structure of this enzyme are very similar to quinoprotein MDH [16]. With QEDH, in contrast to quinoprotein MDH, the cofactor PQQ can be removed reversibly [17].

In the wild-type QEDH and in a double cysteine:alanine mutant the PQQ was found to be in its semiquinone state, which we have characterised by continuous-wave (cw) EPR spectroscopy. By using high field/high frequencies (94 GHz) we could resolve the rhombic nature of the *g*-tensor in frozen aqueous solution. As we show, there are only minor differences between the electronic structures of PQQ bound in the wild-type and mutant proteins, implying that differences in enzymatic activity between them are caused by secondary effects due to the absence of the disulphide ring, rather than by the absence of the semiquinone form or by modifications to the electronic structure of the cofactor itself.

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Abbreviations: DCPIP, dichlorophenol indophenol; DFT, density functional theory; ENDOR, electron-nuclear double resonance; EPR, electron paramagnetic resonance; GDH, glucose dehydrogenase; MADH, methylamine dehydrogenase; MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; QEDH, quinoprotein ethanol dehydrogenase

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00317-5

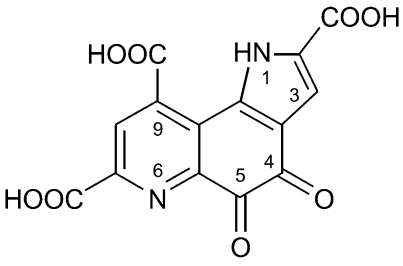

Fig. 1. Molecular structure of the PQQ cofactor.

2. Materials and methods

2.1. Construction of a QEDH expression vector and site-directed mutagenesis

A 1.8 kb DNA fragment encoding QEDH was amplified by polymerase chain reaction from pTB4003 [18] using the oligonucleotides 5′-GGAATACATATGACAACAAGAACCTCACCCGCC-3′ and 5′-AAAGGATCCTCAGCGGCTGGCGGTACGGTTGTC-3′. The fragment was digested with *Nde*I and *Bam*HI and cloned into *Nde*I-*Bam*HI sites of pET3a (Novagen, Darmstadt, Germany) obtaining plasmid pTB7501. Site-directed mutagenesis of the disulphide bridge resulting in a cysteine:alanine double mutant (Cys105Ala/Cys106Ala) was carried out with a Gene Editor Site-Directed Mutagenesis kit from Promega (Mannheim, Germany) using the oligonucleotide 5′-CGACATCCGCCCGGCCGCCGACGTGGTCAAC-3′ and pTB7501 as template, resulting in plasmid pTB7503.

2.2. Protein purification

For expression of apo-enzymes BL21(DE3)pLysS cells containing pTB7501 or pTB7503 were grown at room temperature. Expression was induced by isopropyl-β-D-thiogalactose. Apo-enzymes were purified to homogeneity as described previously for holo-QEDH [15].

2.3. Reconstitution of the holoforms of QEDH and the mutant enzyme

The formation of a enzymatically active holo-enzyme was achieved essentially as described previously [17]. A minimum of a fivefold excess of PQQ (Sigma, Taufkirchen, Germany) with respect to the apoprotein was used for reconstitution. The reconstitution mixture for the mutant enzyme in addition contained 100 μM ethanethiol. After reconstitution PQQ and ethanethiol were removed by rapid gel filtration on PD-10 columns (Pharmacia Biosciences, Uppsala, Sweden) and the samples were concentrated to a subunit concentration of 40 μM by ultrafiltration using Centricon centrifugal filter units with a 30 kDa cut-off (Millipore, Billerica, MA, USA).

2.4. Enzyme assay

Enzymatic activity was determined spectrophotometrically at 600 nm, following the reduction of dichlorophenol indophenol (DCPIP) in time as described earlier [15]. The enzyme reaction was started by adding 100 mM ethanol.

2.5. EPR sample preparation

The enzyme preparations were transferred into 3 mm (i.d.) quartz tubes for X-band (9–10 GHz) EPR and 0.6 mm (i.d.) quartz tubes for W-band (93–95 GHz) EPR and frozen in liquid N_2.

2.6. EPR instrumentation

Cw-EPR spectra at X-band frequencies (9–10 GHz) were obtained using a laboratory-built spectrometer. It consists of a Bruker ER041MR microwave bridge (Bruker, Rheinstetten, Germany) and an AEG-20 electromagnet. Samples were placed in a Bruker ER-4118X-MS-5W1 dielectric resonator, which was immersed in a laboratory-built helium-gas flow cryostat controlled by a LakeShore 321 temperature controller. EPR parameters were as follows: microwave frequency 9.68 GHz; microwave power 6 μW; 0.1 mT magnetic field modulation amplitude (100 kHz); temperature 100 K.

W-band cw-EPR spectra were recorded using a commercial W-band EPR spectrometer (Bruker Elexsys E680). A Li:LiF sample was used for subsequent *g*-factor calibration. EPR parameters were as follows: microwave frequency 94.0 GHz; microwave power 50 nW; 0.25 mT magnetic field modulation amplitude (100 kHz); temperature 80 K.

2.7. EPR spectral simulations

These were performed using the computer program SIMPOW6 obtained from Mark J. Nilges and the Illinois EPR research center (http://iercs.scs.uiuc.edu/nilges.html).

3. Results and discussion

Both wild-type and double cysteine:alanine mutant QEDH were active in the assay with DCPIP, implying that the disulphide bridge does not play a role in the general mechanism of the enzyme with the artificial electron acceptors. The K_M value of the mutant enzyme was increased by a factor of 5000 compared to the wild-type enzyme with a K_M of 22 μM. Therefore high concentrations of ethanol were used in the enzyme assay. Specific activity of the mutant enzyme was 180% compared to that of the wild-type.

The X-Band cw-EPR spectrum of the PQQ semiquinone radical in wild-type QEDH enzyme is shown in Fig. 2 (continuous line). The spectrum shows a slightly asymmetric line shape centred at $g_{iso} = 2.0043$ and a peak-to-peak line width of 0.5 mT and is similar to that observed in glucose dehydrogenase (GDH) [19], in quinohaemoprotein ethanol dehydrogenase [20], methylamine dehydrogenase (MADH) [21] and in MDH [22]. A slightly larger peak-to-peak line width of 0.7 mT has been reported for MDH [23,24]. Typical of all the EPR spectra reported so far is the shoulder at low field, and ease of microwave saturation. While the resemblance of the PQQ semiquinone spectrum for wild-type QEDH presented here to those of other PQQ-containing dehydrogenases is not unexpected, we surprisingly found a virtually indistinguishable semiquinone radical EPR spectrum for the double cysteine:alanine mutant (Fig. 2, dashed line). The signal intensities of the wild-type and the mutant sample were almost identical, indicating a similar radical concentration in both sample types.

To further characterise the PQQ semiquinone radical in wild-type and mutant, we performed high-field (94 GHz, W-band) EPR spectroscopy. As in the case of the X-band spectra no significant difference in the signal intensity between the wild-type and the mutant was observed. From previous Q-band (35 GHz) cw-EPR, the PQQ *g*-tensor could be resolved into an axial symmetric pattern with $g_{\parallel} = 2.0024$ and $g_{\perp} = 2.0056$ for MDH [23,22] and $g_{\parallel} = 2.0024$ and $g_{\perp} = 2.0048$

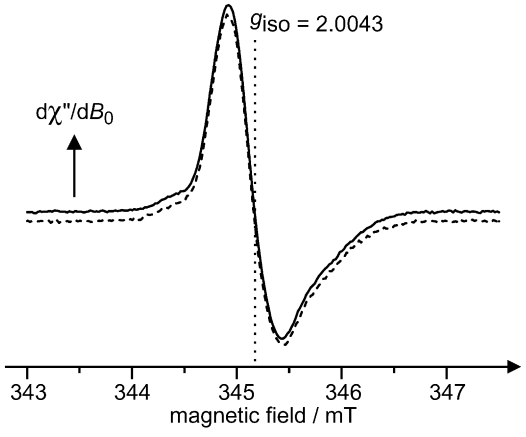

Fig. 2. Cw-EPR spectra at X-band of the PQQ cofactor bound in QEDH in the wild-type (continuous line) and mutant (dashed line) protein.

for MADH [21]. In the W-band cw-EPR spectra of QEDH wild-type (a) and the double mutant (b), presented in Fig. 3, the full rhombic symmetry of the g-tensor is resolved. By spectral simulation, also shown in Fig. 3, including only the principal values of the g-tensor and additional line width, reasonable fits of the experimental data could be obtained. For the wild-type, the principal values of the g-tensor are: $g_x = 2.00575(2)$, $g_y = 2.00512(2)$ and $g_z = 2.00209(2)$, giving $g_{iso} = 2.00432(2)$. For the double mutant, the principal values of the g-tensor are: $g_x = 2.00571(2)$, $g_y = 2.00513(2)$ and $g_z = 2.00207(2)$, giving $g_{iso} = 2.00430(2)$. The very similar principal values of wild-type and mutant indicate a virtually identical electronic structure of the PQQ semiquinone radical in the wild-type and the mutant protein.

Nevertheless, the fits are not wholly satisfying, particularly close to g_x and g_y. In the wild-type spectrum, this could be due to slight maladjustment of the microwave phase. This was difficult to adjust as the experiments had to be conducted at extremely low microwave power due to ease of saturation of the sample. In the double mutant spectrum, however, at g_y the fit is good, rather at g_x there is a low-field shoulder that is not apparent in the wild-type spectrum.

There are several reasons why the spectra may show differences. It might be that the conformation of the PQQ cofactor in the mutant protein is disturbed, so that it has completely different geometrical and electronic structure, and hence altered hyperfine couplings (hfcs). PQQ contains two nitrogens and has three α-protons, all of which could in principle be expected to have hfcs to the unpaired electron. From cw-EPR studies of the PQQ analogue 2,7-dicarboxypyrroloquinoline quinone (also known as 9-decarboxymethoxatin) in liquid solution, four proton and two nitrogen isotropic hfcs could be determined [25]. 9-Decarboxymethoxatin has an extra α-proton when compared to PQQ. An hfc of 2.6 MHz was assigned

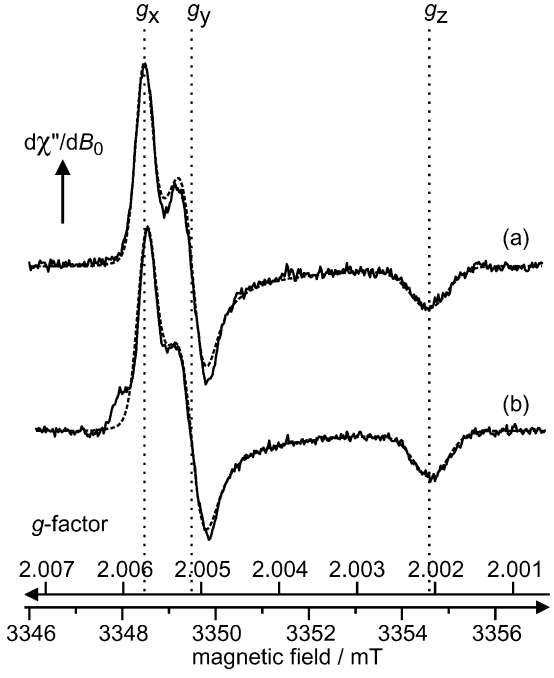

Fig. 3. Cw-EPR spectra at W-band of the PQQ cofactor bound in QEDH in the (a) wild-type and (b) mutant protein. Also shown (dashed lines) are spectral simulations performed using the program SIMPOW6.

to the proton at position 1 (see Fig. 1). Hfcs of 3.5 MHz and 5.2 MHz were also determined although they could not be specifically assigned to the remaining protons 3 and 8. Using Q-band electron-nuclear double resonance (ENDOR) on MDH, two proton hfc tensor components could be determined [26]. However, the hfcs could not be assigned, so although the authors concluded that the electronic structure is similar in vivo and in vitro, this conclusion is not very safe. On the other hand, all the hfcs are small compared with the EPR linewidth. So it is very unlikely that the low-field shoulder could stem from a hfc. Furthermore, differences in hfcs between wild-type and mutant should be clearly visible in the X-band spectra. Therefore, altered hfcs can be excluded as the origin of the low-field shoulder in the mutant protein.

Another possible consequence of a disturbed geometry of the PQQ cofactor in the mutant protein is a change in the g-factor of the radical. From work on nitroxide spin labels [27], para-semiquinones (benzo- and naphthoquinones) [28] and tyrosyl amino acid radicals [29], it is well known that the g_x component of compounds with a high spin density on a CO or NO group is particularly sensitive to changes in the local environment, such as polarity, and hydrogen bonding. If we assign the low-field shoulder to a species with changed g_x component we can estimate the proportion of this species to about 15% of the total amount of radicals. The g_x value of the species is increased by about 3×10^{-4} compared to the majority species and the g_x value of the wild-type.

For the two ubiquinone-10 molecules Q_A and Q_B in the photosynthetic reaction centre from *Rhodobacter sphaeroides* a difference of 2×10^{-4} in g_x with the larger g_x value for Q_A has been observed and attributed to weaker hydrogen bonding and/or the less polar environment of Q_A [30]. This has to be compared with a difference of 6×10^{-4} for duroquinone in the presence or absence of hydrogen bonds [27]. A much more pronounced effect on the g_x component has been observed for the tyrosyl radical in ribonucleotide reductase (RNR) from different species. In mouse RNR a hydrogen bond between the protein and the tyrosyl radical is formed [31] while this bond is absent in RNR from *Escherichia coli*. This change results in a difference of 15×10^{-4} in the g_x components with the larger g_x value for the tyrosyl radical in RNR from *E. coli* [32]. The difference in the g_x value discussed here for the PQQ radical in QEDH is similar to that observed for the gradual difference in the hydrogen bonding pattern and environment polarity of Q_A versus Q_B and is much less than for the case of a complete removal of strong hydrogen bonds. Therefore, the weakening of a hydrogen bond in a fraction of the sample might be responsible for the observed low-field shoulder in the mutant W-band spectrum. However, no firm conclusion about the change in the PQQ environment causing a corresponding g_x shift can be drawn at present since neither experimental data nor theoretical estimates are available regarding the sensitivity of the PQQ radical g-tensor to its environment.

The results presented here call for further examination of enzyme-bound PQQ cofactor in its semiquinone form by, for example, ENDOR in order to map the electronic structure of the cofactor and its protein surrounding. These experiments should be done in conjunction with density functional theory (DFT) calculations of the PQQ cofactor including nearby amino acids and the Ca^{2+} with which it interacts. Although some work along these lines has been presented, calculation of

hfcs was not included [10,33]. DFT methods in conjunction with the orientation selectivity achievable by W-band ENDOR experiments should allow an assignment of hfcs to molecular positions based on their characteristic anisotropy. Furthermore, these methods allow investigation of the dependence of the *g*-factor on the cofactor environment, e.g. hydrogen bonding.

Even though a thorough characterisation of the electronic structure of the PQQ semiquinone radical in QEDH is still required, we have provided two important pieces of information. First, we have shown that even in the double cysteine: alanine mutant without the characteristic disulphide ring the PQQ occurs in its semiquinone form with similar radical concentrations in the wild-type and the mutant. This shows that the disulphide ring is not essential for the generation of the PQQ semiquinone form. Second, we have resolved the full anisotropy of the *g*-tensor for the PQQ semiquinone radical and given the principal values. Knowledge of these fingerprint parameters is of particular importance for the interpretation of EPR data from species with more than one redox-active cofactor, e.g. membrane-bound GDH containing PQQ and ubiquinone, where a superposition of EPR spectra from different species might occur [34,35].

Acknowledgements: This work was supported in part by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 498, Teilprojekt C5 and Go 242/12-1).

References

[1] Duine, J.A. (1999) J. Biosci. Bioeng. 88, 231–236.
[2] Anthony, C. and Williams, P. (2003) Biochim. Biophys. Acta 1647, 18–23.
[3] Görisch, H. (2003) Biochim. Biophys. Acta 1647, 98–102.
[4] Kasahara, T. and Kato, T. (2003) Nature 422, 832.
[5] Oubrie, A. (2003) Biochim. Biophys. Acta 1647, 143–151.
[6] Yamada, M., Elias, M.D., Matsushita, K., Migita, C.T. and Adachi, O. (2003) Biochim. Biophys. Acta 1647, 185–192.
[7] Xia, Z.-X., Dai, W.-W., Zhang, Y., White, S.A., Boyd, G.D. and Mathews, F.S. (1996) J. Mol. Biol. 259, 480–501.
[8] Anthony, C. and Ghosh, M. (1998) Prog. Biophys. Mol. Biol. 69, 1–21.
[9] Xia, Z.X., He, Y.N., Dai, W.W., White, S.A., Boyd, G.D. and Mathews, F.S. (1999) Biochemistry 38, 1214–1220.
[10] Zheng, Y.-J., Xia, Z.-X., Chen, Z.-W., Mathews, F.S. and Bruice, T.C. (2001) Proc. Natl. Acad. Sci. USA 98, 432–434.
[11] Xia, Z.-X., Dai, W.-W., He, Y.-N., White, S.A., Mathews, F.S. and Davidson, V.L. (2003) J. Biol. Inorg. Chem. 8, 843–854.
[12] Avezoux, A., Goodwin, M.G. and Anthony, C. (1995) Biochem. J. 307, 735–741.
[13] Afolabi, P.R., Mohammed, F., Amaratunga, K., Majekodunmi, O., Dales, S.L., Gill, R., Thompson, D., Cooper, J.B., Wood, S.P., Goodwin, P.M. and Anthony, C. (2001) Biochemistry 40, 9799–9809.
[14] Oubrie, A., Rozeboom, H.J., Kalk, K.H., Huizinga, E.G. and Dijkstra, B.W. (2002) J. Biol. Chem. 277, 3727–3732.
[15] Rupp, M. and Görisch, H. (1988) Biol. Chem. Hoppe Seyler 369, 431–439.
[16] Keitel, T., Diehl, A., Knaute, T., Stezowski, J.J., Hohne, W. and Görisch, H. (2000) J. Mol. Biol. 297, 961–974.
[17] Mutzel, A. and Görisch, H. (1991) Agric. Biol. Chem. 55, 1721–1726.
[18] Diehl, A., von Wintzingerode, F. and Görisch, H. (1998) Eur. J. Biochem. 257, 409–419.
[19] Sato, A., Takagi, K., Kano, K., Kato, N., Duine, J.A. and Ikeda, T. (2001) Biochem. J. 357, 893–898.
[20] de Jong, G.A., Geerlof, A., Stoorvogel, J., Jongejan, J.A., de Vries, S. and Duine, J.A. (1995) Eur. J. Biochem. 230, 899–905.
[21] de Beer, R., Duine, J.A., Frank, J. and Large, P.J. (1980) Biochim. Biophys. Acta 622, 370–374.
[22] de Beer, R.D., van Ormondt, D., van Ast, M.A., Banen, R., Duine, J.A. and Frank, J. (1979) J. Chem. Phys. 70, 4491–4495.
[23] Duine, J.A., Frank, J. and Westerling, J. (1978) Biochim. Biophys. Acta 524, 277–287.
[24] Westerling, J., Frank, J. and Duine, J.A. (1979) Biochem. Biophys. Res. Commun. 87, 719–724.
[25] Rodriguez, E.J., Bruice, T.C. and Edmondson, D.E. (1987) J. Am. Chem. Soc. 109, 532–537.
[26] Duine, J.A., Frank, J., De Beer, R. and Westerling, J. (1984) Arch. Biochem. Biophys. 233, 708–711.
[27] Kawamura, T., Matsunami, S. and Yonezawa, T. (1967) Bull. Chem. Soc. Jpn. 40, 1111–1115.
[28] Burghaus, O., Plato, M., Rohrer, M., Möbius, K., Macmillan, F. and Lubitz, W. (1993) J. Phys. Chem. 97, 7639–7647.
[29] Un, S., Atta, M., Fontecave, M. and Rutherford, A.W. (1995) J. Am. Chem. Soc. 117, 10713–10719.
[30] Isaacson, R.A., Lendzian, F., Abresch, E.C., Lubitz, W. and Feher, G. (1995) Biophys. J. 69, 311–322.
[31] van Dam, P.J., Willems, J.P., Schmidt, P.P., Pötsch, S., Barra, A.L., Hagen, W.R., Hoffman, B.M., Andersson, K.K. and Gräslund, A. (1998) J. Am. Chem. Soc. 120, 5080–5085.
[32] Bleifuss, G., Kolberg, M., Pötsch, S., Hofbauer, W., Bittl, R., Lubitz, W., Gräslund, A., Lassmann, G. and Lendzian, F. (2001) Biochemistry 40, 15362–15368.
[33] Zheng, Y.-J. and Bruice, T.C. (1997) Proc. Natl. Acad. Sci. USA 94, 11881–11886.
[34] Miyoshi, H., Niitome, Y., Matsushita, K., Yamada, M. and Iwamura, H. (1999) Biochim. Biophys. Acta 1412, 29–36.
[35] Elias, M.D., Nakamura, S., Migita, C.T., Miyoshi, H., Toyama, H., Matsushita, K., Adachi, O. and Yamada, M. (2003) J. Biol. Chem. 279, 3078–3083.