The haem b_{558} component of the cytochrome *bd* quinol oxidase complex from *Escherichia coli* has histidine–methionine axial ligation

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The cytochrome *bd* ubiquinol oxidase from *Escherichia coli* is induced when the bacteria are cultured under microaerophilic or low-aeration conditions. This membrane-bound respiratory oxidase catalyses the two-electron oxidation of ubiquinol and the four-electron reduction of dioxygen to water. The oxidase contains three haem prosthetic groups: haem b_{558} , haem b_{595} and haem *d*. Haem *d* is the oxygen binding site, and it is likely that haem *d* and b_{595} form a bimetallic site in the enzyme. Haem b_{558} has been previously characterized spectroscopically as being low spin and has been shown to be located within subunit I (CydA) of this two-subunit enzyme. It is likely that haem b_{558} is associated with the quinol oxidation site, which has also been shown to be within subunit I. In a previous effort to locate the specific amino

acids axially ligated to haem b_{558} , all six histidines within subunit I were altered by site-directed mutagenesis. Only one, histidine-186, was identified as a likely ligand to haem b_{558} . Hence it was suggested that haem b_{558} could not have bis(histidine) ligation. In the current work, a combination of low-temperature near-infrared magnetic circular dichroism (NIR-MCD) and EPR spectroscopies have been employed to identify the nature of the haem b_{558} axial ligands. The NIR-MCD spectrum at cryogenic temperatures is dominated by the low-spin haem b_{558} component of the complex, and the low-energy band near 1800 nm is strong evidence for histidine–methionine ligation. It is concluded that haem b_{558} is ligated to histidine-186 plus one of the methionines located within subunit I of the oxidase.

INTRODUCTION

Most bacterial respiratory oxidases, as well as the eukaryotic mitochondrial aa_3 -type cytochrome *c* oxidases are members of the haem–copper oxidase superfamily [1–3]. The only well characterized prokaryotic oxidase which is clearly not a member of this superfamily of related enzymes is the cytochrome *bd* quinol oxidase (or cytochrome *bd* complex) [4,5]. The cytochrome *bd* complex is present in numerous bacterial species [6,7], but has been most thoroughly characterized in *Escherichia coli* [5–14]. This enzyme catalyses the oxidation of quinol in the bacterial cytoplasmic membrane and the reduction of molecular oxygen to water [15]. The redox reaction catalysed by the oxidase is coupled to the generation of a protonmotive force across the membrane [16,17]. The data indicate that quinol is oxidized at a site located near the periplasmic side of the membrane and that the protons liberated by this half-reaction are released in the periplasm [18–20]. The active site where oxygen is reduced to water appears to be physically separated from the quinol site, and the protons used to generate water must originate from the bacterial cytoplasm. By virtue of the separation of the sites where the two half-reactions are catalysed, the chemistry involving the substrate protons results in the net transfer of one proton per electron from the cytoplasm to the periplasm [4,17]. The work presented in this paper is part of a larger effort to obtain structural information about the enzyme. In particular, since the proposed mechanism suggests that the quinol oxidation occurs near the periplasmic side of the membrane, experimental data concerning the location of this site can be used to test this hypothesis.

The cytochrome *bd* complex is a heterodimer [21] with one copy each of subunit I (CydA; molecular mass 57 kDa) and subunit II (CydB; 43 kDa). Both subunits are transmembranous, and previous studies have indicated the presence of seven putative transmembrane helical spans in subunit I (A–G) and eight for subunit II (A–H) [22,23]. Affinity labelling studies with a photo-reactive ubiquinol analogue have indicated that the quinol binding site is located, at least in part, within subunit I [24]. Furthermore, it has been demonstrated that the ability of the enzyme to oxidize ubiquinol is selectively lost by proteolytic cleavage within a hydrophilic domain connecting putative transmembrane helices E and F in subunit I [19]. Similarly, monoclonal antibodies that selectively inhibit quinol oxidation also bind within this same region of the protein, which is called the ‘Q loop’ [20]. The Q loop is located on the periplasmic side of the membrane [19,20,22], consistent with the proposed mechanism.

Haem b_{558} (or cytochrome b_{558}) appears to be directly involved in the oxidation of quinol [10,25]. The optical spectrum of this component of the enzyme is typical of low-spin *b*-type cytochromes [8,26], and EPR studies have assigned a low-spin $g_z = 3.3$ signal to haem b_{558} [9,27]. This haem component of the oxidase has been located within subunit I by expression of the *cydA* gene, resulting in overproduction of haem b_{558} in the cytoplasmic membrane [28]. Site-directed mutagenesis was used to alter all six of the histidines in subunit I in a previous effort to locate the axial ligands to haem b_{558} [29]. Surprisingly, only substitutions for His-186 perturb the spectrum of haem b_{558} . This residue is predicted to be located at the periplasmic interface of transmembrane helix D and, thus, can easily be envisaged to be

Abbreviations used: NIR-MCD, near-infrared magnetic circular dichroism; MEGA-9, nonanyl-*N*-methylglucamide; SB12, *N*-dodecyl-*N,N*-dimethylammonio-3-propane sulphonate; HALS, highly anisotropic low spin.

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near the Q loop and a participant in quinol oxidation. The identification of the second axial ligand to haem b_{558} is particularly important to further the modelling of this subunit. The second ligand is expected to be an amino acid other than histidine and should be located near the periplasmic interface. Since it is clear that haem b_{558} is atypical for *b*-type cytochromes and does not have bis(histidine) ligation, the experimental approach of using site-directed mutagenesis to identify the second ligand becomes unwieldy since the number of potential targets is very large. Spectroscopic studies to identify the nature of the second ligand were motivated by the prospects of narrowing the search to a single type of amino acid. The EPR spectrum alone is insufficient to identify the nature of the haem ligation, but in combination with low-temperature near-infrared magnetic circular dichroism (NIR-MCD) the ligation can be determined [30,31]. The results strongly indicate that haem b_{558} has histidine-methionine ligation, thus providing a key piece of information and narrowing the search for the second ligand to methionine residues within subunit I.

EXPERIMENTAL

Studies were performed in parallel by the three laboratories participating in this work. Enzyme was prepared independently using two different preparative protocols and using different detergents. The data obtained from the parallel studies are entirely compatible.

The U.S. groups (R.B.G., T.K., Q.P. and J.P.) prepared enzyme essentially as has been described previously [5,14] using the detergent sarkosyl for the final samples. The samples for EPR spectroscopy were at a concentration of 0.49 mM protohaem in 18 mM Hepes buffer (pH 7.6), 2 mM EDTA, 0.025 % sarkosyl and 55 % (v/v) ethanediol (or 50 % glycerol). The samples used for NIR-MCD spectroscopy were at a concentration of 0.28 mM protohaem in $^2\text{H}_2\text{O}$ containing 20 mM Hepes buffer (p ^2H 7.8), 2 mM EDTA, 0.025 % sarkosyl and 50 % (v/v) [$^2\text{H}_3$]glycerol. The reported p ^2H value is simply the pH meter reading +0.4, in accordance with the suggestion of Glasoe and Long [32]. Deuterated reagents were obtained from Cambridge Isotope Laboratories.

The preparative protocol used by the U.K. group (A.J.T., F.S., M.R.C.) has not been described previously and is given in more detail. The final samples examined spectroscopically were in 50 mM Hepes in $^2\text{H}_2\text{O}$ containing 10 mM nonanyl-*N*-methylglucamide (MEGA-9) at p ^2H 7.9. An equal volume of [$^2\text{H}_3$]glycerol was added for the MCD samples. Samples in both MEGA-9 and sarkosyl detergents turn over the quinol decylbenzoquinone, as measured in a spectrophotometric assay.

Materials

$^2\text{H}_2\text{O}$ was obtained from Aldrich. MEGA-9, leupeptin, pepstatin, PMSF and Hepes were obtained from Sigma. *N*-dodecyl-*NN*-dimethylammonio-3-propane sulphonate (SB12) was obtained from Boehringer Mannheim.

E. coli strain and growth conditions

E. coli strain GO105/pTK1 was grown microaerobically at 37 °C in a 150 litre fermenter (Bioengineering AG, Wald, Switzerland) in 100 litres of minimal medium supplemented with 5 µg/ml $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 µg/ml nicotinic acid. Cells were harvested in the early stationary phase using a Millipore Pellicon tangential flow ultrafiltration system equipped with four 0.22 µm membranes. Cells were washed with 100 mM Tris/HCl, 10 mM EDTA, pH 8.4, using the same apparatus. Cells were broken

by two passes through a Manton-Gaulin press at a pressure of 55.2 MPa (8000 p.s.i.) in the presence of 1 µM leupeptin, 1 µM pepstatin and 200 µM PMSF.

Membrane preparation and protein purification procedure

Membranes were washed and pre-extracted according to the protocol of Smith et al. [33], adding one washing step with 20 mM Tris/HCl, pH 8.0, after pre-extraction with deoxycholate. The modified purification protocol was based on that published by Miller and Gennis [5]. The oxidase was solubilized from the membranes with 10 mM SB12 at a membrane protein concentration of 8 mg/ml. The mixture was stirred for 30 min and centrifuged at 150000 *g* for 30 min. The supernatant was chromatographed on a Protein Pak DEAE 15 HR column (Millipore) using an FPLC system (Waters 650E). The column was equilibrated and washed with 20 mM potassium phosphate, 1 mM EDTA, 3 mM SB12, pH 7.2, and a linear gradient to 300 mM potassium phosphate over 6 column volumes was used to elute the enzyme. Fractions with a 412 nm/280 nm absorbance ratio higher than 0.33 were pooled and concentrated 10-fold by ultracentrifugation (Amicon PM30 membrane). The solution was desalted with a Pharmacia PD10 column equilibrated with 20 mM potassium phosphate, 1 mM EDTA and 3 mM SB12. The solution was then rechromatographed on a DEAE column while, at the same time, changing the detergent to MEGA-9. The column was equilibrated with 20 mM potassium phosphate, 1 mM EDTA and 3 mM SB12, pH 7.2, and washed with 6 column volumes of 20 mM potassium phosphate, 1 mM EDTA and 15 mM MEGA-9, pH 7.2, after the enzyme was loaded on to the column. A gradient up to 300 mM potassium phosphate was applied for elution. Fractions with a 412 nm/280 nm absorbance ratio higher than 0.6 were pooled, concentrated and desalted. For storage, the buffer was changed to 50 mM Hepes, 1 mM EDTA and 15 mM MEGA-9, pH 7.5

Preparation of spectroscopic samples

For NIR-MCD experiments, the storage buffer had to be exchanged with the same buffer made with $^2\text{H}_2\text{O}$ at p ^2H 7.9. This was accomplished by using a Pharmacia PD10 column. To ensure that good optical glasses were obtained on freezing, [$^2\text{H}_3$]glycerol was added to 50 % (v/v) for low-temperature MCD experiments.

Protein determination

Protein concentration was determined with bicinchoninic acid solution using BSA as a standard [33]. The cytochrome *bd* concentration was estimated from reduced-minus-oxidized difference spectra using a molar absorption coefficient for ΔA (628 nm – 650 nm) of 18.8 mM $^{-1}$ ·cm $^{-1}$.

Spectroscopy

Electronic absorption spectra were recorded with a Hitachi U3000 spectrophotometer or an Aminco DW2000C spectrophotometer. EPR spectra were recorded on an X-band spectrometer (Bruker ER200D with an ESP1600 computer system) fitted with a liquid helium flow cryostat (Oxford Instruments; ESR9). MCD spectra were recorded on a Jasco J-500D spectropolarimeter for the wavelength range 300–1000 nm and a laboratory-built dichrograph for the range 800–2000 nm [30].

RESULTS AND DISCUSSION

Figure 1 shows the low-field X-band EPR spectrum of a preparation of cytochrome bd in the detergent MEGA-9. This spectrum is similar to that observed previously in the detergent Tween 80 [9] and also to the spectrum obtained in sarkosyl (results not shown). One difference, however, is the absence of the prominent signal at $g = 2.6$ previously reported [9]. This signal was observed in some samples in the current study and appears to be an artefact related to the glassing agent and conditions. The use of ethanediol as a glassing agent seems to be particularly deleterious, and its use results in loss of the signal at $g = 3.32$. All the results reported in this work, consequently, were obtained with glycerol as the glassing agent.

The main feature of interest for the current work is the signal at $g = 3.32$, which has been previously assigned as the g_z component of the haem b_{558} spectrum [9,27]. The g_x and g_y components are not observable. This is a HALS-type signal (highly anisotropic low spin), as indicated by the asymmetrical ramp or folded line shape [34].

The anisotropy referred to in the term HALS is that of the g -values in the observed spectrum. This actually arises from a low rhombicity at the haem ferric ion. As the symmetry approaches axial, the d_{zx} and d_{yz} orbitals become near-degenerate resulting in a sharing of the unpaired electron spin between them. Under these conditions, the g_z -value can approach a theoretical maximum of 4. Hence HALS should more properly refer to highly axial low-spin haem.

For b -type cytochromes with bis(histidine) co-ordination, the HALS signal correlates with the perpendicular orientation of the planes of the imidazole rings of the two axial ligands [34]. In such cases, the g_z value is typically greater than 3.5, however. The first demonstration that methionine–histidine haem ligation could generate a HALS signal was reported in the dihaem cytochrome c_4 from *Azotobacter vinelandii*, in which a ramp-shaped g_z value of 3.64 was identified as arising from methionine–histidine by NIR-MCD spectroscopy. It was proposed that the methionine lone pair orientation relative to the histidine plane can generate a pseudo- s_4 axis at the Fe(III) ion [35]. Recently a HALS-type signal with $g_z = 3.34$ was reported for cytochrome c_{552} from *Nitrosomonas europaea*, which was also shown to have histidine–methionine ligation [36].

Figure 2 shows the low-temperature NIR-MCD spectrum of

Figure 2 NIR-MCD spectrum of *Escherichia coli* cytochrome bd

The concentration was $175 \mu\text{M}$ protein in 50 mM Hepes, $^2\text{H}_2\text{O}$ buffer containing 10 mM MEGA-9 at $\text{p}^2\text{H} = 7.9/[^2\text{H}_3]\text{glycerol}$ (50%, v/v). Conditions: temperature, 4.2 K; magnetic field, 5 T.

pure cytochrome bd in MEGA-9. Under the cryogenic conditions used to obtain this spectrum [31], the signal from the low-spin ferric haem b_{558} is much greater than any contributions from the high-spin haem d and haem b_{595} components of the complex. The position of the low-spin charge transfer transition (E_{CT}) has been shown to be a reliable indicator of the haem axial ligation [30,31]. In the spectrum shown in Figure 2, the charge transfer transition has a peak position near 1800 nm, which is clearly in the range for haems with histidine–methionine ligation (1740–1860 nm) [30]. The spectrum is very similar to that of cytochrome c_{552} from *N. europaea* [36]. The EPR spectrum shows, besides the low-spin g_z value at 3.32, a low-spin haem at $g = 2.45$, 2.30, 1.84/1.81. However, the latter signal represents only about 10% of one haem, and is due to low-spin haem d . Hence the major low-spin component at $g = 3.32$ is associated with the NIR charge transfer band at ~ 1820 nm. Cytochromes with histidine–methionine ligation invariably exhibit a weak charge transfer absorbance near 695 nm [37]. This transition, however, can be difficult to

Figure 1 X-band EPR spectrum of *Escherichia coli* cytochrome bd

The concentration was $350 \mu\text{M}$ protein in 50 mM Hepes, $^2\text{H}_2\text{O}$ buffer containing 10 mM MEGA-9 at $\text{p}^2\text{H} = 7.9$. Conditions: temperature, 10 K; microwave frequency, 9.38 GHz; modulation amplitude, 1 mT; power, 2.0 mW.

Figure 3 UV-visible MCD spectrum of *Escherichia coli* cytochrome bd

The concentration was $31 \mu\text{M}$ protein in 50 mM Hepes, $^2\text{H}_2\text{O}$ buffer containing 10 mM MEGA-9 at $\text{p}^2\text{H} = 7.9/[^2\text{H}_3]\text{glycerol}$ (50%, v/v). Conditions: temperature, 4.2 K; magnetic field, 5 T.

resolve, and this is particularly so for cytochrome *bd* where transitions associated with haem *d* overlap in this region of the spectrum with the expected transition from haem b_{558} . In the region near 695 nm several weak transitions are apparent in the MCD spectrum (Figure 3). However, it is not possible at this point to resolve the origin of these transitions. All the features in the MCD spectrum in the region from 240 to 600 nm are typical for one low-spin ferric haem, showing that the MCD of cytochrome b_{558} dominates the spectrum.

The data firmly establish that the haem b_{558} has histidine-methionine axial ligation. This is unusual but not unprecedented for *b*-type cytochromes [30]. One of the ligands of haem b_{558} has been previously identified as histidine-186 [29]. It is therefore expected that site-directed mutagenesis of the methionine residues near the periplasmic surface of subunit I of the complex should eliminate or perturb the properties of haem b_{558} . Results demonstrating this will be presented in the near future.

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