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The cytochromes of anaerobically grown *Escherichia coli*

An electron-paramagnetic-resonance study of the cytochrome *bd* complex *in situ*

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The e.p.r. signals attributable to a cytochrome *bd*-type ubiquinol:O_2 oxidoreductase (cytochrome *b*-558–*b*-595–d) were studied in a cytoplasmic membrane preparation of *Escherichia coli* that had been grown on glycerol with fumarate as respiratory-chain oxidant. Two major high-spin ferric haem signals were resolved on the basis of their potentiometric behaviour: a rhombic high-spin species ($g_x = 6.25$, $g_y = 5.54$) was assigned to haem *b*-595, and an axial high-spin ($g_x = 5.97$, $g_y = 5.96$) species was assigned to the haem *d*. These signals titrated with $E_{m,7}$ values of 154 and 261 mV respectively, corresponding closely to optically determined values for haem *b*-595 and haem *d*. At high potentials (> 300 mV) the rhombic species attributable to haem *b*-595 underwent a partial transition to a second rhombic species with *g*-values of 6.24 (g_x) and 5.67 (g_y). The high-spin ferric haem spectra were affected by O_2, CO, cyanide and pH. A low-spin ferric haem signal was observed at $g = 3.3$ (g_z), which titrated with an $E_{m,7}$ of 226 mV, and this was assigned to haem *b*-558. The data support a model for cytochrome *bd* with two ligand-binding sites, a single haem *d* and a single haem *b*-595.

INTRODUCTION

Escherichia coli, grown anaerobically on the non-fermentable carbon source glycerol with fumarate as respiratory-chain oxidant, develops a respiratory chain that contains a cytochrome *bd* complex as ubiquinol:O_2 oxidoreductase (Reid & Ingledew, 1979). This oxidase has been isolated from both aerobically and anaerobically grown cells (Miller & Gennis, 1983; Finlayson & Ingledew, 1985), and its optical and enzymological properties from both sources appear similar. As isolated from aerobically grown cells it contains one or two haem *d* groups, one haem *b*-595, one haem *b*-558 and no copper (Lorence *et al.*, 1986; Kita *et al.*, 1984). Haem *d* is the O_2-binding site, and haem *b*-595 also appears to be involved in ligand binding (Poole *et al.*, 1981; Edwards *et al.*, 1981). Haem *b*-558 is the ubiquinol-reducible component of the oxidase (Green *et al.*, 1986). The components of the complex in membrane preparations from both aerobically and anaerobically grown cells have been resolved potentiometrically by using optical difference spectroscopy. In aerobically grown cells the haem *d*, haem *b*-595 and haem *b*-558 have midpoint potentials ($E_{m,7}$) of 247, 150 and 182 mV respectively (Lorence *et al.*, 1984*a*), whereas in anaerobically grown cells these components have midpoint potentials of 280, 150 and 250 mV respectively (Reid & Ingledew, 1979). Although much work has been done on the optical properties of this oxidase, comparatively little is known about its e.p.r. characteristics, and the work reported to date (Hata *et al.*, 1985; Kumar *et al.*, 1985) is in need of clarification. In particular, unequivocal assignments of the e.p.r. signals to the components of cytochrome *bd* are necessary.

Hata *et al.* (1985) studied the e.p.r. signals from cytochrome *bd* in a membrane preparation from aerobically grown cells. An axial high-spin ferric haem signal at $g = 6.0$ was assigned to haem *b*-558, and low-spin ferric haem signals at $g = 2.5$ and 2.3 were assigned to haem *d*. Under oxically oxidizing conditions, the haem *d* was reported to be present in a diamagnetic O_2-ligated ferrous form. However, Kumar *et al.* (1985) assigned a rhombic high-spin ferric haem species with *g*-values of 6.28 and 5.54 to haem *d* on the basis of triple trapping data, and the $g = 6.0$ axial signal was assigned to *b*-type cytochromes. In a similar study, Hata *et al.* (1987) assigned the rhombic high-spin ferric haem species to haem *b*-595. Kauffman *et al.* (1975) studied the e.p.r. properties of a membrane preparation from *Azotobacter vinelandii*, and resolved two high-spin ferric haem signals that were both attributed to the haem *d* of cytochrome *bd*. Haem *b*-595 (reported as cytochrome a_1) was assigned to a low-spin ferric haem signal at $g = 3.03$, and haem *b*-558 was assigned to low-spin ferric haem signals at $g = 3.69$ and 3.43. Both of the published studies on the e.p.r. characteristics of the purified cytochrome *bd* from aerobically grown (Hata *et al.*, 1985) and anaerobically grown *E. coli* (Finlayson & Ingledew, 1985) report both axial and rhombic high-spin ferric haem species. In both cases the axial species titrated with a midpoint potential of about 180 mV. Although no low-spin ferric haem signals were observed in the preparation examined by Finlayson & Ingledew (1985), that studied by Hata *et al.* (1985) had low-spin ferric haem signals at $g = 2.3$ and 2.5 that titrated with an $E_{m,7}$ comparable with the optically determined values for haem *d* in the purified enzyme (232 mV; Koland *et al.*, 1984).

In the present paper we report an e.p.r. study *in situ* of cytochrome *bd* from *E. coli* that had been grown anaerobically on glycerol with fumarate as respiratory-chain

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oxidant. We have studied the effects of redox potential, pH and the ligands O_2, CO and cyanide on the e.p.r.-detectable centres, and have assigned e.p.r. signals to the haem groups present in cytochrome *bd*.

METHODS

Growth of cells and preparation of electron-transport particles

E. coli strain EMG2 (prototroph, N.C.I.B. strain + 10124) was grown in 20-litre batches to late exponential phase on glycerol with fumarate as respiratory-chain oxidant (Reid & Ingledew, 1979). A cytoplasmic membrane preparation was prepared as described by Rothery *et al.* (1987). These membranes had oxidase activities of about 150 nmol of O_2/min per mg of protein with lactate as substrate. The haem *d* content of the membranes was determined from reduced-minus-oxidized optical difference spectra by using the wavelength pair 628 and 607 nm and a millimolar absorption coefficient of $8.0\,\text{mM}^{-1}\cdot\text{cm}^{-1}$ (Lorence *et al.*, 1986). Its concentration was found to be typically about 0.3 nmol/mg of protein.

Sample preparation, titrations and assays

Samples for e.p.r. spectroscopy were prepared in 3 mm-internal-diameter quartz e.p.r. tubes as described previously (Ingledew, 1983). E.p.r. spectra were obtained with a Bruker ER200D spectrometer (Bruker Spectrospin) equipped with a liquid-He cryostat (Oxford Instruments, Oxford, U.K.). Potentiometric redox titrations were performed as described by Dutton (1978). The oxidation–reduction mediators used, at concentrations of between 25 and 150 μM, were: 2-hydroxy-1,4-naphthoquinone ($E_{m,7} = -130$ mV); pyocyanine ($E_{m,7} = -30$ mV); duroquinone ($E_{m,7} = +10$ mV); 1,4-naphthoquinone ($E_{m,7} = +50$ mV); *N*-methylphenazonium methosulphate ($E_{m,7} = +80$ mV): 1,2-naphthoquinone ($E_{m,7} = +125$ mV); 2,6-dibromophenol-indophenol ($E_{m,7} = +190$ mV); 1,2-naphthoquinone-4-sulphonic acid ($E_{m,7} = +210$ mV); 2,6-dichlorophenol-indophenol ($E_{m,7} = +217$ mV); *NNN'N'*-tetramethyl-*p*-phenylenediamine ($E_{m,7} = +260$ mV); tetrachlorohydroquinone ($E_{m,7} = +340$ mV). Deletion of some of these mediators and/or substitution with others with appropriate $E_{m,7}$ values had no apparent effect on the observed redox and e.p.r. properties reported in the present paper. The ambient redox potential (E_h) was adjusted by using solutions of $Na_2S_2O_4$ or $K_3Fe(CN)_6$, and was measured with a combination platinum/reference (Ag/AgCl) electrode purchased from Russell pH Ltd. (Auchtermuchty, Fife, U.K.). The vessel was continuously flushed with N_2 (99.9%) that had been passed through a Nil-Ox O_2-scrubbing apparatus (Jencons Scientific, Hemel Hempstead, Herts., U.K.). Membranes were suspended in 100 mM-Tes/KOH buffer, pH 7.0, in the redox titration vessel (at 30 °C) and were poised at 0 mV to remove all traces of O_2. The potential was then adjusted to 450 mV and the titration was carried out in the reducing direction. Alternatively, after poising at 0 mV, the titration was done in the oxidizing direction. When titrations were carried out in the presence of CO, this gas was substituted for N_2 in the above procedure.

The effect of pH on the e.p.r. signals was investigated by suspending membranes in buffers of appropriate pH. For intermediate pH values appropriate mixtures of Mes/KOH buffer, pH 6, Tes/KOH buffer, pH 7, and Tricine/KOH buffer, pH 8, were used and the pH was adjusted with either 1 M-HCl or 1 M-KOH.

Protein was determined by the method of Lowry *et al.* (1951), modified by the inclusion of 1% (w/v) SDS in the incubation mixture to solubilize membrane-bound proteins. Bovine serum albumin was used as standard.

Computation

The program used to analyse the redox titration data was written by Professor A. R. Crofts (Department of Chemistry, University of Illinois, Urbana, IL, U.S.A.). E.p.r. spectral simulations were carried out by using a program simulating Gaussian lineshapes written by Professor J. C. Salerno (Department of Biology, Rensselaer Polytechnic Institute, Troy, NY, U.S.A.). Truncated double integrations of spectra were carried out by using an electronic spreadsheet.

Chemicals

Oxidation–reduction mediators were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) or Koch–Light Laboratories (Haverhill, Suffolk, U.K.). N_2 and CO were obtained from British Oxygen Co. (Guildford, Surrey, U.K.). All other chemicals were obtained from BDH Chemicals (Poole, Dorset, U.K.). or Sigma Chemical Co. (Poole, Dorset, U.K.).

RESULTS

E.p.r. resonances of oxidized cytochrome *bd*

Fig. 1 shows e.p.r. spectra of oxically oxidized membranes in both the $g = 6$ and $g = 3$ regions. There is an intense g_{xy} of an axial high-spin ferric haem at $g = 6$ (g_{xy}), and the g_z of a low-spin ferric haem is observed at about $g = 3.3$. The prominent signal at $g = 4.3$ is due to ferric iron of low symmetry that is not specifically associated with protein (Blumberg, 1967). The low-spin ferric chlorin signals observed by Hata *et al.* (1985) with *g*-values of 2.3 and 2.5 are observed in our membrane preparation (results not shown), but at very low intensity compared with those observed in their membranes from aerobically grown cells. However, membranes from aerobically grown *E. coli* FUN4/pNG2 (a gift from Professor R. B. Gennis, School of Chemical Sciences, University of Illinois, Urbana, IL, U.S.A.), which over-expresses cytochrome *bd* 8–10-fold compared with anaerobically grown *E. coli* EMG2, do exhibit readily observable low-spin ferric chlorin signals at about $g = 2.3$ and $g = 2.5$ (results not shown).

E.p.r. spectra of high-spin ferric haem groups

Of the three types of centre present in cytochrome *bd*, haem *d* and haem *b*-595 would be expected to have high-spin ferric haem signals on the basis of their putative role in ligand binding. The e.p.r. spectrum of the cytochrome *bd in situ* at about $g = 6$ was therefore further investigated by poising membranes anoxically at appropriate redox potentials. The published midpoint potentials, based on optical redox potentiometry, of haem *d* and haem *b*-595 are 280 and 150 mV respectively (Reid & Ingledew, 1979). Fig. 2 shows the e.p.r. spectra of membranes poised anoxically at 380 and 200 mV. Comparison of these spectra with that of the oxically oxidized membranes of Fig. 1(*a*) indicates that there is a major

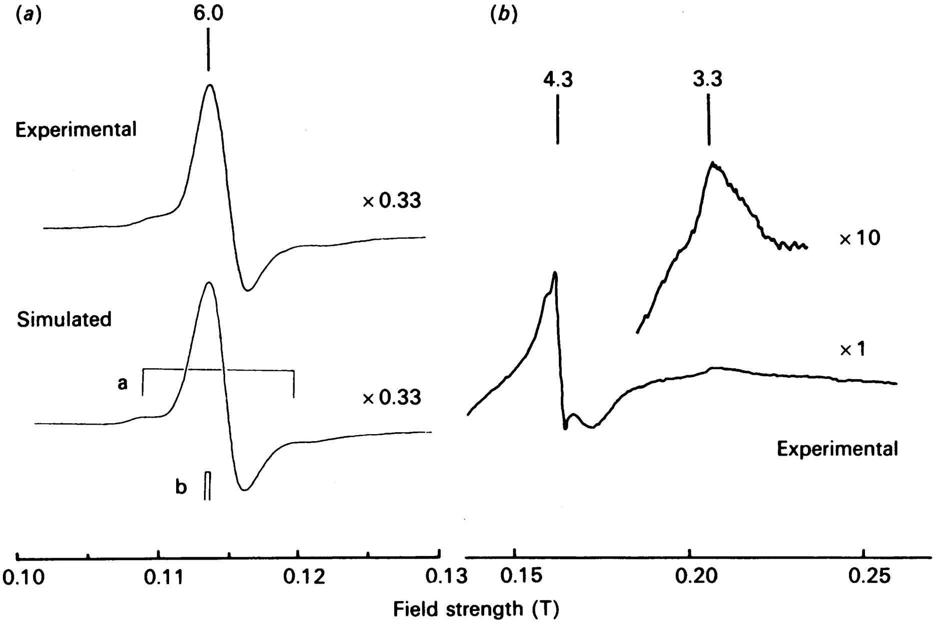

Fig. 1. E.p.r. spectra of air-oxidized membranes from fumarate-grown *E. coli* cells

Approx. 0.5 ml of *E. coli* membranes (protein concentration 25 mg/ml) was placed in a 3 mm-internal-diameter quartz e.p.r. tube and was vigorously aerated with a coiled stainless-steel wire. Membranes were suspended in 100 mM-Tes/KOH buffer, pH 7, containing 2 mM-$MgSO_4$. Spectrometer settings: temperature, 5 K; field modulation intensity, 1 mT_{pp}; microwave power, 20 mW; instrument gain, 2.5×10^4. g-values are indicated by numbers above the spectrum. (*a*) Experimental and stimulated spectra in the $g = 6$ region. The stimulation was obtained by adding two different components in suitable proportions. The g-values, linewidths and relative weights of these components are: component a, 6.24, 5.67 and 2.0, 0.1215, 0.1480 and 0.3000 mT, and 0.14; component b, 5.97, 5.96 and 2.0, 0.1800, 0.1200 and 0.3000 mT, and 0.86. The positions of the g-values are indicated by vertical bars. (*b*) Experimental spectrum showing the low-spin ferric haem signal at $g = 3.3$.

difference between the e.p.r. lineshapes of anoxically and oxically oxidized membranes (see below).

Noticeable in the high-potential (380 mV) spectrum of Fig. 2 are peaks at about $g = 6.2$, 6.0 and 5.6. This spectrum is consistent with the presence of both axial and rhombic ferric haem signals. In the low-potential (200 mV) spectrum the central axial haem signal (peak at $g = 6.0$) has almost titrated out, resulting in a spectrum dominated by the features of the rhombic haem signal at about $g = 6.2$ and 5.6. Truncated double integrations (Aasa *et al.*, 1976) of the two spectra in Fig. 2 indicate that the spin intensity of the 200 mV spectrum in this region is approximately half that of the 380 mV spectrum. The species responsible for the rhombic and axial ferric haem signals are therefore present in approximately equal concentrations in the cytoplasmic membranes containing cytochrome *bd*.

Noticeable in Fig. 2 is a significant difference in the lineshape of the rhombic haem signal between the 380 and 200 mV spectra. Its apparent g_y moves downfield and the intensity of its g_y peak–trough decreases when the axial haem is oxidized. Truncated double integrations indicate that the apparently smaller size of the rhombic g_y peak–trough in the high-potential spectrum is not due to loss of rhombic haem spin intensity. A haem–haem interaction between the axial and rhombic haem groups would explain this lineshape change, resulting in a subpopulation of the rhombic haem with an altered g_y when the axial haem becomes oxidized. This explanation of the lineshape change of the rhombic haem is supported by simulations of the e.p.r. lineshapes of the two high-spin ferric haems (see below).

The above results are consistent with an assignment of the axial and rhombic high-spin ferric haem signals to haem *d* and haem *b*-595 respectively. However, consideration of other results reported in the present paper is required to strengthen these assignments (see below and the Discussion section).

Redox potentiometry of the e.p.r. signals

The behaviour of the high-spin ferric haem signals was further probed by redox potentiometry (Fig. 3). The axial feature of the spectrum titrates with an $E_{m,7}$ of 261 mV, whereas the two rhombic haem features, the low-field peak and the high-field peak–trough, show apparently anomolous behaviour. With increasing E_h, the rhombic high-spin spectral features appear with an $E_{m,7}$ of 154 mV, but the high-field rhombic peak–trough gets smaller again at higher potentials. The analysis of these changes is complicated by spectral overlap with the putative second (minor) rhombic component and the broad peak–trough of the central axial component of the spectra.

The difference in the e.p.r. lineshape in the rhombic g_y region between samples poised at 380 mV and 200 mV (Fig. 2) and the behaviour of this feature in potentiometric redox titrations can be explained if there are two rhombic components in the high-potential spectrum. One of these would correspond to the rhombic component observed in the 200 mV spectrum (the low-

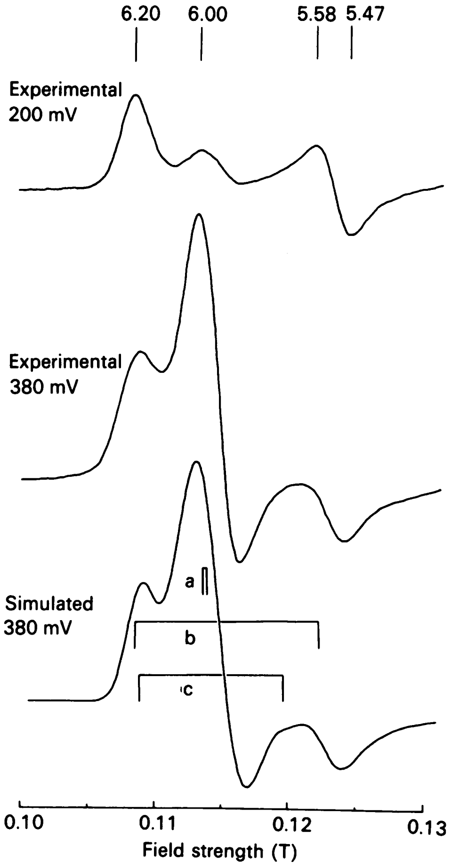

Fig. 2. E.p.r. spectra of redox-poised *E. coli* membranes

E.p.r. samples of *E. coli* membranes were withdrawn from an anaerobic redox titration vessel after being poised at 200 and 380 mV (see the Methods section). Membranes were suspended (protein concentration 27 mg/ml) in 100 mM-Tes/KOH buffer, pH 7, containing 2 mM-MgSO$_4$. Spectrometer settings: temperature, 6 K; field modulation intensity, 1 mT$_{pp}$; microwave power, 20 mW; instrument gain, 5×10^4. The simulation of the 380 mV spectrum (lowest trace) was obtained by adding three different components in suitable proportions. The *g*-values, linewidths and relative weights of these components are: a, 5.97, 5.96 and 2.0, 0.2400, 0.1800 and 0.3000 mT, and 0.50; b, 6.25, 5.54 and 2.0, 0.1215, 0.1480 and 0.3000 mT, and 0.33; c, 6.24, 5.67 and 2.0, 0.1215, 0.1480 and 0.3000 mT, and 0.17. The positions of the *g*-values are indicated by vertical bars.

potential component; Fig. 2), and the other would be derived from this component, appearing at higher redox potentials (the high-potential component) as a result of the interaction of the rhombic species with the axial species. A small difference in the position of the g_y features of these two rhombic components would result in the high-potential component cancelling out the low-potential component, leading to an apparently smaller rhombic g_y feature in the spectra recorded at high redox potentials. However, this lineshape transition of the rhombic signal is not complete upon full oxidation of the axial haem, and there is some variability in its extent between different batches of membranes. This explanation for the lineshape change of the rhombic species at high potentials is supported by simulation of the e.p.r. spectrum of fully anoxically oxidized membranes (see

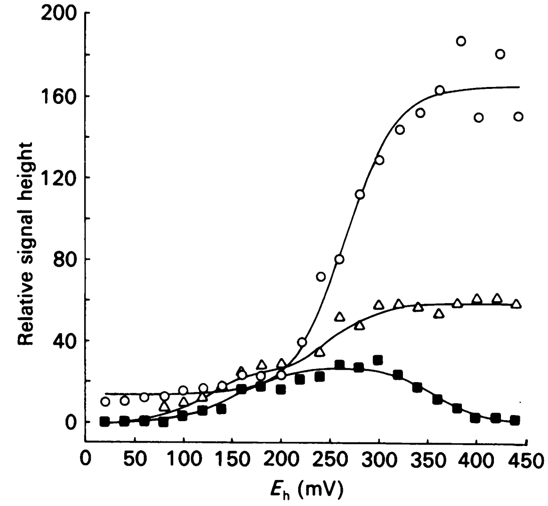

Fig. 3. Redox titration of the *g* = 6.0 region of the spectrum of *E. coli* membranes

Data from spectra recorded under the conditions of Fig. 2 were plotted. Continuous lines represent the *n* = 1 fits of the data to the Nernst equation with the use of single or multiple components (see the text). △, Low-field peak; ○, central peak–trough; ■, high-field peak–trough.

below). The redox potentiometry of the axial and rhombic high-spin ferric haem signals supports their assignment to haem *d* and haem *b*-595 respectively (see the Discussion section).

The other major haem signal in e.p.r. spectra of oxidized membranes containing cytochrome *bd* is the g_z of a low-spin ferric haem observed at *g* = 3.3. This signal titrates with an $E_{m,7}$ of about 226 mV (Fig. 4), a value intermediate between those of the two high-spin haem species, as would be expected for the haem *b*-558 of

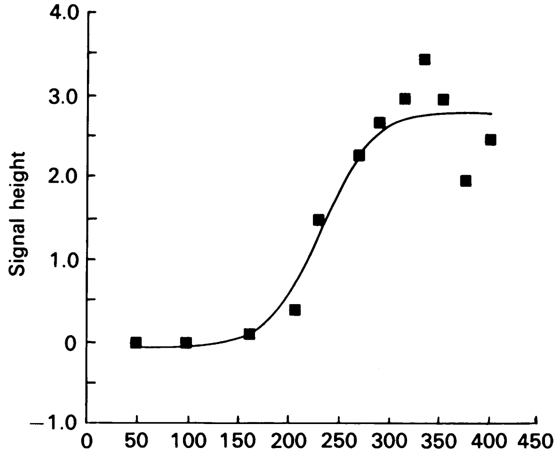

Fig. 4. Redox titration of the *g* = 3.3 low-spin ferric haem signal of *E. coli* membranes

The size of the *g* = 3.3 feature of the e.p.r. spectrum was plotted against redox potential. The data were obtained from spectra recorded at 5 K with samples under the conditions of Fig. 3. The continuous line represents an *n* = 1 fit of the data to the Nernst equation (see the text).

cytochrome *bd* on the basis of published potentiometric titrations (Lorence *et al.*, 1984*a*; Reid & Ingledew, 1979). We assign the $g = 3.3$ signal to haem *b*-558 (see the Discussion section).

Simulation of the anoxically oxidized spectrum around $g = 6$

A good fit to the experimental lineshape of fully oxidized cytochrome *bd* in the $g = 6$ region can be obtained by computer simulation (Fig. 2, lowest trace). This fit is obtained by using a total axial spin concentration that is equal to the total rhombic spin concentration, as indicated by the value of the double integrals in the high-spin region of the 380 mV and 200 mV spectra. The simulation is a composite of three components, one axial and two rhombic. Of the two rhombic components, one, a high-potential component, must be derived from the other, a low-potential component, to maintain a total rhombic-to-axial ratio of 1:1. We attribute the high-potential component to a subpopulation of haem *b*-595 that interacts with haem *d*.

Effect of O_2 on the high-spin ferric haem signal at about $g = 6$

O_2 affects the anoxically oxidized cytochrome *bd* and results in the spectral lineshape shown in Fig. 1(*a*) in the $g = 6$ region of the e.p.r. spectrum. No differences are observed between the spectra of air-oxidized membranes and those of anoxically oxidized membranes to which O_2 has been introduced or H_2O_2 added. This effect of O_2 on the fully ferric cytochrome is unusual. However, there is prior evidence showing that the optical spectrum of oxidized cytochrome *bd* is perturbed by O_2 (see the Discussion section).

Truncated double integrations of spectra of oxically and anoxically oxidized membranes in the $g = 6$ region indicate that their relative spin concentrations are approximately equal. This suggests that both the haem *d* and the haem *b*-595 are e.p.r.-visible under oxically oxidizing conditions. This contrasts with the data of Hata *et al.* (1985), who found that the low-spin ferric chlorin signals (which they attributed to haem *d*) were attenuated under oxic conditions, suggesting that haem *d* was largely e.p.r.-silent under these conditions. Our finding that there is no loss of spin intensity in the $g = 6$ region in oxically oxidized membranes suggests that O_2 perturbs the e.p.r. spectrum of haem *b*-595, resulting in a transition of its lineshape from rhombic to axial.

Only two components are necessary to simulate the lineshape of oxically oxidized membranes (Fig. 1*a*, lower trace). These correspond to a major axial component with narrower linewidths than the anoxic axial component and a minor rhombic component corresponding to the minor high-potential rhombic component in the anoxic simulation (component c in Fig. 2, lowest trace). Although only two components were used in the simulation, it is possible to simulate two major axial components attributable to haem *d* and haem *b*-595 with very similar or identical *g*-values.

Effects of CO and cyanide

CO causes diminution of the axial component of the e.p.r. spectrum at about $g = 6.0$. In redox titrations of membranes in the presence of CO, only a minor axial component is observed, and the species that titrates corresponds to the low-potential rhombic component reported in the present paper. This rhombic component titrates with an $E_{m,7}$ of 150 mV. CO therefore binds to haem *d* and raises its $E_{m,7}$ to greater than 400 mV (the highest potential of the titration). The effect of CO on the low-spin haem *b*-558 signal was not determined, as the $E_{m,7}$ of this haem is not perturbed by CO in optical redox titrations (Lorence *et al.*, 1984*a*).

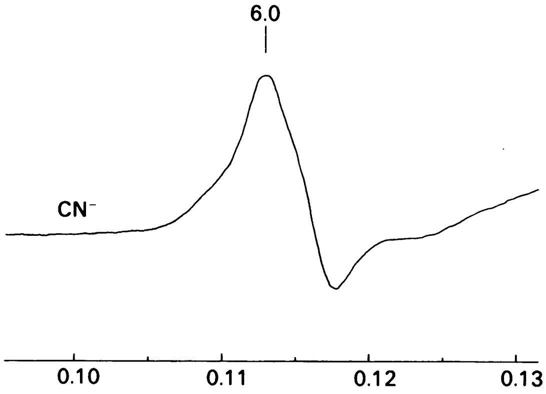

Fig. 5. Effect of cyanide on the spectrum in the $g = 6$ region of anoxically oxidized *E. coli* membranes

The cyanide adduct to the membrane-bound cytochrome *bd* was prepared as follows. Membranes (suspended in 100 mM-Bes/KOH buffer, pH 7.0; protein concentration 27 mg/ml) were poised anoxically at 0 mV in the presence of 10 mM-KCN and were then oxidized in 400 mV with $K_3Fe(CN)_6$ as oxidant. Samples were then withdrawn into e.p.r. tubes. Spectrometer settings: temperature, 10 K; field modulation intensity, 2 mT_{pp}; microwave power, 20 mW; instrument gain, 8×10^4.

Cyanide causes a major change in the spectral lineshape in the $g = 6.0$ region (Fig. 5). These changes were observed after oxidation of reduced ($E_h = 0$ mV) membranes in the presence of 10–25 mM-cyanide under anoxic conditions. The intensity of the high-spin signal is diminished compared with the intensities of spectra of oxically and anoxically oxidized membranes. The lineshapes of both the rhombic and axial components of the spectrum are altered. The central axial component clearly becomes slightly rhombic upon cyanide binding. An additional low-spin signal is observed in cyanide-treated membranes at $g = 2.96$, corresponding to a cyanide adduct to ferric haem *d*. This feature is very weak in spectra of cyanide-treated *E. coli* EMG2 membranes, but it is larger in membranes from *E. coli* FUN4/pNG2 (results not shown).

Effect of pH on the high-spin spectrum

pH affects the spectral lineshape of the e.p.r. spectrum of oxically oxidized membranes (Fig. 6). The relative size of the rhombic g_x component compared with the axial g_{xy} increases with increasing pH, and this lineshape change titrates with a pK_a of about 8.0. The data suggest that there is a pH-dependent equilibrium between the rhombic and axial components of the aerobic spectrum, and that this lineshape change is a result of changes in the geometry of the haem *b*-595.

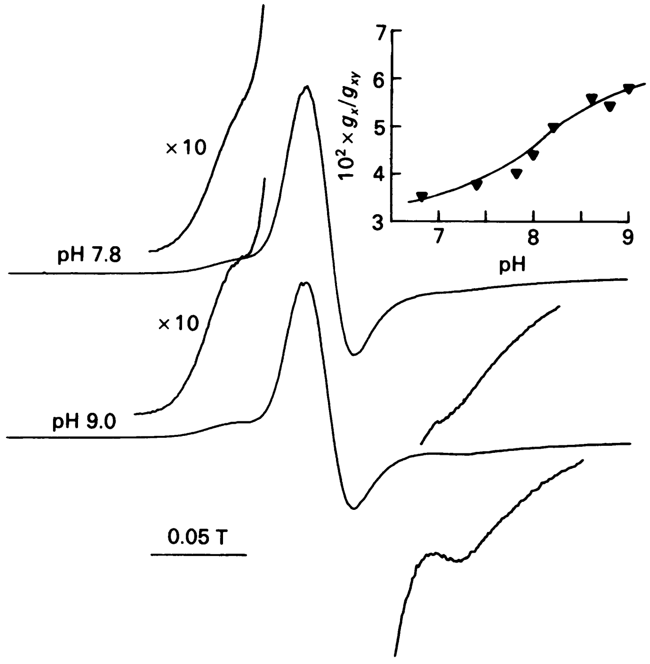

Fig. 6. Effect of pH on the spectral lineshape of oxically oxidized *E. coli* membranes

Membranes were suspended in buffers of appropriate pH (total buffer concentration 50 mM, protein concentration 30 mg/ml), samples were transferred to e.p.r. tubes and these were then vigorously aerated with a stainless-steel wire. Spectrometer settings were as for Fig. 2. Inset: a plot of the ratio of the intensity of the rhombic g_x to that of the axial g_{xy} versus pH.

DISCUSSION

Our assignment of the axial high-spin ferric haem signal to haem *d* is based on its potentiometric and ligand-binding behaviour. This signal has an $E_{m,7}$ of 261 mV, which is raised to above 400 mV in the presence of CO, as has been reported for haem *d* on the basis of optically monitored redox titrations (Lorence *et al.*, 1984*a*). The assignment is strengthened by comparison with the e.p.r. signals of other haemoproteins able to bind oxygen species. The ligand-binding haem of cytochrome aa_3 (Aasa *et al.*, 1976), cytochrome *b*-562–*o* (Hata *et al.*, 1985; J. C. Salerno, B. Bolgiano & W. J. Ingledew, unpublished work), thyroid peroxidase (Lukat *et al.*, 1988), horseradish peroxidase (Young & Siegel, 1987), spinach catalase (Hirasawa *et al.*, 1987), metmyoglobin (Young & Siegel, 1987) and methaemoglobin (Peisach *et al.*, 1969) all exhibit high-spin ferric haem signals. The only other haem *d*-containing haemoprotein to have been extensively studied by e.p.r. is *Pseudomonas* cytochrome cd_1, a dissimilatory nitrite reductase. This enzyme exhibits a rhombic low-spin ferric haem signal attributable to haem d_1 with *g*-values of 2.52, 2.42 and 1.73 (Muhoberac & Wharton, 1983). However, the structure of haem d_1 (Chang *et al.*, 1986) is quite distinct from that of haem *d* (Timkovich *et al.*, 1985). Cyanide binds to oxidized cytochrome *bd* and perturbs both its axial and rhombic high-spin e.p.r. signals.

We have assigned the rhombic high-spin ferric haem signal to haem *b*-595 on the basis of the similarity of its $E_{m,7}$ to the published values for this centre (Lorence *et al.*, 1984*a*), and this assignment is in agreement with that by Hata *et al.* (1987). The optical spectrum of haem *b*-595 is typical of a high-spin haem *b* (Lorence *et al.*, 1986), so that the observation of a high-spin e.p.r. signal attributable to this haem is much as expected. CO has been shown to bind to both haem *b*-595 and haem *d* on the basis of photochemical action spectra (Edwards *et al.*, 1981) and optically monitored CO-binding titrations (R. A. Rothery & W. J. Ingledew, unpublished work). However, in potentiometric redox titrations monitored optically (Lorence *et al.*, 1984*a*) and by e.p.r., CO has little effect on the $E_{m,7}$ of haem *b*-595, suggesting that it binds to the haem with low affinity. Alternatively, the tendency of CO to raise the $E_{m,7}$ of haem *b*-595 may be offset by an interaction energy with ferrous carbonmonoxyhaem *d*. Cyanide perturbs the rhombic high-spin signal, and this perturbation is probably due to cyanide binding to haem *b*-595.

On the basis of the double integrations of the spectra in the $g = 6.0$ region, we conclude that haem *b*-595 and haem *d* are present in the cytochrome *bd* in a 1:1 ratio. This contrasts with the data of Lorence *et al.* (1986), which suggest that the cytochrome contains two haem *d* groups per haem *b*-595.

Haem *b*-558 has an optical spectrum typical of a low-spin haem *b* (Lorence *et al.*, 1986), and its role in quinol oxidation (Green *et al.*, 1986) suggests that this haem is six-co-ordinate and low spin. The haem *b* groups of two other enzymes involved in the redox reactions of quinol species, mitochondrial complex III (Salerno, 1984) and chloroplast cytochrome b_6f (Salerno *et al.*, 1983), have low-spin e.p.r. spectra with a g_z greater than 3.3. Our assignment of the $g = 3.3$ signal to haem *b*-558 contrasts with that by Hata *et al.* (1985), who assigned the axial high-spin signal to this haem.

That the $E_{m,7}$ of the $g = 3.3$ signal does not correlate well with the published values for haem *b*-558 based on optical difference spectroscopy may be due to a freezing artifact. Alternatively, this may be due to differences in phospholipid composition between aerobically and anaerobically grown cells. Haem *b*-558 would be expected to be affected by such changes on the basis of the sensitivity of its $E_{m,7}$ to detergents in the purified enzyme (Lorence *et al.*, 1984*b*).

Our assignments of the high-spin signals attributable to cytochrome *bd* have to be reconciled with the effect of aerobiosis on the e.p.r. spectrum. The optical data of Poole *et al.* (1983) suggest that the oxically oxidized cytochrome has an O_2-ligated haem *d*, the haem *d*-650 species. These workers also proposed that the first spectrophotometrically detectable intermediate formed in the reaction of fully reduced cytochrome *bd* with O_2 is the haem *d*-650 species and that this species is O_2-ligated ferrous haem *d*, which is e.p.r.-silent. However, our data indicate that there is no loss of spin intensity in the $g = 6$ region of e.p.r. spectra upon aerobiosis. There is potentiometric evidence that O_2 affects fully oxidized cytochrome *bd*. Pudek & Bragg (1976) found that the haem *d*-650 'oxidized' species does not appear at high redox potentials under anoxic conditions unless portions of H_2O_2 solution or O_2-saturated water are added. Hendler & Schrager (1979) carried out redox titrations under oxic (aerobic) conditions and were able to study the potentiometric behaviour of the haem *d*-650 species.

At potentials above around 330 mV, the intensity of the 650 nm absorbance was found to be level at about one-third of its maximum. Its intensity fell sharply to zero at 330 mV, and then increased with decreasing potential until a maximum was reached at about 100 mV. Hence there appear to be two haem *d*-650 species, one ferrous and one ferric.

The effect of O_2 on haem *d* results in a change in the haem *b*-595 lineshape; this change is a manifestation of the interaction between the two high-spin haem groups. The effect of pH on the relative heights of the axial and rhombic components of the spectrum of the oxically oxidized high-spin haem groups indicates that within the active site of the cytochrome *bd* there exists an ionizable group with a pK_a of about 8. The presence of an ionizable group with a pK_a in this region has already been proposed on the basis of the optical changes elicited by nitrite at different pH values (Rothery *et al.*, 1987). This ionizable group is associated with haem *b*-595 and affects the relative concentrations of the axial and rhombic forms of this centre under oxic conditions.

We have assigned spectral features of the e.p.r. spectrum of membranes from anaerobically grown *E. coli* to the haem components of cytochrome *bd*, and have shown that this oxidase contains one haem *d* group and one haem *b*-595 group. The effects of O_2, E_h and pH on the e.p.r. signals are described. The data presented support a model for cytochrome *bd* with both haem *d* and haem *b*-595 involved in ligand binding.

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