Cytochrome *bo* from *Escherichia coli*: reaction of the oxidized enzyme with hydrogen peroxide

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Oxidized cytochrome *bo* reacts rapidly with micromolar concentrations of H_2O_2 to form a single derivative. The electronic absorption spectrum of this compound differs from that of the oxidized form of the enzyme reported by this laboratory [Watmough, Cheesman, Gennis, Greenwood and Thomson (1993) FEBS Lett. **319**, 151–154]. It is characterized by a Soret maximum at 411 nm, increased absorbance at 555 nm, and reduced intensity at 624 nm. The apparent dissociation constant for this process is of the order of 4×10^{-6} M, and the bimolecular rate constant for the formation of the new compound is $(1.25–1.7) \times 10^3$ $M^{-1} \cdot s^{-1}$. Electronic absorption difference spectroscopy shows this product to be identical with the compound formed from the reaction of the mixed-valence form of the enzyme with dioxygen. Investigation of this compound by room-temperature magnetic c.d. spectroscopy shows haem *o* to be neither high-spin nor low-spin ferric, but to have a spectrum characteristic of an oxyferryl species. There is no evidence for oxidation of the porphyrin ring. Therefore the binuclear centre of this species must consist of an oxyferryl haem ($S = 1$) coupled to a Cu(II) ion ($S = 1/2$) to form a new paramagnetic centre. The reaction was also followed by X-band e.p.r. spectroscopy, and this showed the disappearance in parallel with the formation of the oxyferryl species, of the broad $g = 3.7$, signal which arises from the weakly coupled binuclear centre in the oxidized enzyme. Since no new e.p.r.-detectable paramagnetic species were observed, the Cu(II) ion is presumed to be coupled to another paramagnet, possibly an organic radical. There is no evidence in the electronic absorption spectrum to indicate further reaction of cytochrome *bo* with H_2O_2 to form a second species. We argue that the circumstances of formation of this oxyferryl species are the same as those for the P form of cytochrome *c* oxidase, a species often regarded as containing a bound peroxide ion. The implications of these observations for the reaction mechanism of haem–copper terminal oxidases are discussed.

INTRODUCTION

Cytochrome *bo*, a quinol oxidase from *Esherichia coli*, is a member of a conserved superfamily of protonmotive haem–copper terminal oxidases which includes mitochondrial cytochrome *c* oxidase. Much recent effort has been directed at the biophysical characterization of purified cytochrome *bo* (e.g. Cheesman et al., 1994; Tsubaki et al., 1993; Watmough et al., 1993). Such studies will underpin future work on site-directed mutants of the enzyme, which, in the absence of a three-dimensional structure, provides the best way to test existing structural and mechanistic models of this class of enzymes (Gennis, 1992; Brown et al., 1993). In particular, these studies will aid our understanding of the structure and environment of the haem *o*:Cu(II) binuclear centre, which is the site of dioxygen reduction and may also be the site of proton pumping (Mitchell, 1987).

The oxidized form of cytochrome *bo* prepared in this laboratory is defined by characteristic electronic absorption bands at 406.5 nm (Soret) and 624 nm (ligand–metal charge-transfer band), by a broad complex e.p.r. signal with prominent features at $g = 9.1$, 3.7 and 2.68, and by a room-temperature magnetic c.d. (m.c.d.) spectra that reveals two charge-transfer bands characteristic of high-spin ferric haem *o* at 650 nm and 1100 nm (Watmough et al., 1993; Cheesman et al., 1993b). This form of the enzyme binds low concentrations (0.05–1.2 mM) of cyanide rapidly (K_{on} 80–95 $M^{-1} \cdot s^{-1}$), although this rapid phase of cyanide binding is abolished after preincubation of the enzyme with formate (N. J. Watmough, unpublished work). Reaction of oxidized cytochrome *bo* with 20 mM formate also leads to the disappearance of the $g = 3.7$ signal and its replacement with another broad signal centred on $g = 13$ (Calhoun et al., 1992; Watmough et al., 1993). This is attributed to a formate–ferric haem *o*/Cu(II) pair and is similar to the $g = 12/2.95$ signal that characterizes the so called 'slow' (sluggish cyanide binding) form of cytochrome aa_3 (Baker et al., 1987; Moody et al., 1991). Cytochrome *bo* exhibits a fast-to-slow transition in response to formate binding, as judged by the parameters of e.p.r. spectroscopy and cyanide-binding rates. However, unlike bovine cytochrome aa_3, this switch appears not to be effected by incubation of the enzyme at low pH (Moody et al., 1993a; Watmough et al., 1993). We refer to this form of cytochrome *bo* prepared in our laboratory as 'fast'.

The catalytic cycle of cytochrome *bo* has not yet been well studied. However, considering the close structural relationships between cytochrome *bo* and cytochrome aa_3, it might be expected to follow a similar reaction mechanism to the one proposed for that enzyme (Wikström and Babcock 1992; Laureus et al., 1993). This scheme proposes the formation of at least two stable intermediates which are spectroscopically distinct and are one oxidation level apart. One intermediate, generated from the reaction of dioxygen with the reduced binuclear centre, has been assigned to a peroxy or P species, formally Fe(III)–O^{2-}::Cu(II), which may have protonated states. The other is assigned to an oxyferryl or F species, namely Fe(IV)=O::H_2O–Cu(II). These intermediates have also been observed in cytochrome *aa*$_3$*-600*, a quinol oxidase from *Bacillus subtilis* (Laureus et al., 1993).

Another way to generate these intermediates is by the reaction

Abbreviations used: m.c.d., magnetic c.d.; pH*, apparent pH.

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of oxidized bovine cytochrome aa_3 with H_2O_2 (Wrigglesworth, 1984). The extent of this reaction and ratio between the two forms is dependent upon several factors, including the concentration of peroxide used (Wrigglesworth, 1984), the method of preparation of the enzyme (Weng and Baker, 1991) and pH at which the reaction takes place (Vygodina and Konstantinov, 1989).

The P form of bovine cytochrome aa_3 is characterized by a red-shifted Soret maximum (428 nm) and increased absorption in the region of 607 nm. It appears to be formed by treatment of the enzyme with low (micromolar) concentrations of either H_2O_2 or alkyl peroxides. This reaction can be reversed by the action of catalase (Vygodina and Konstantinov, 1987; Ksenzenko et al., 1992). A species with a similar electronic absorption spectrum is formed by the reaction of dioxygen with the 'mixed-valence' form of cytochrome aa_3 (Greenwood et al., 1974; Chance et al., 1979; Clore et al., 1980), by the reaction of oxidized cytochrome aa_3 with CO under aerobic conditions (Nicholls, 1978; Nicholls and Chanady, 1981) and by reverse electron transfer in intact mitochondria (Wikström, 1981; Wikström and Morgan, 1992). The fact that both H_2O_2 and alkyl peroxides form this species has been taken as evidence that peroxide ion or hydroperoxide ion forms an adduct with ferricytochrome a_3, but does not bridge to Cu_B (Vygodina et al., 1993). There is, however, a lack of detailed biophysical evidence of the nature of P.

The F form of cytochrome aa_3, formed by the reaction of the oxidized enzyme with higher (millimolar) concentrations of H_2O_2, also has a Soret maximum at 428 nm, but differs from the peroxy form in the visible region with a maximum at 580 nm. Evidence for the participation of this species in the catalytic cycle came from the reverse-electron-transfer experiments performed by Wikstrom (1981). Evidence for the participation of an oxyferryl species in the forward reaction comes from time-resolved resonance Raman studies (Han et al., 1990; Varotsis and Babcock, 1990).

The concentration of peroxide needed to generate P and F is dependent on the way in which cytochrome aa_3 is prepared. For this reason, Weng and Baker (1991), using a modification of the method of Hartzell and Beinert (1974), studied the reaction of H_2O_2 with enzyme that was completely in a form characterized by a red-shifted Soret maximum (424 nm), absence of a broad e.p.r. signal centred around $g = 12$, and an ability to bind cyanide with rapid monophasic kinetics (Baker et al., 1987; Moody et al., 1991). This showed that lower concentrations of peroxide than previously reported were needed to convert the enzyme quantitatively into the P form. However, even at these low (micromolar) concentrations of peroxide, there was a slow decay to the F form, the rate of both processes being greater at higher peroxide concentrations (Weng and Baker, 1991).

The purpose of the present paper is to describe the nature of the reaction of 'fast' cytochrome *bo* with H_2O_2 using optical, m.c.d. and e.p.r. spectroscopy and to demonstrate that the single product of this reaction contains an oxyferryl haem. We discuss how these results fit into current views of the reaction cycle of this family of haem–copper oxidases.

EXPERIMENTAL

Reagents

^2H_2O and H_2O_2 (30% w/v) were obtained from Aldrich. DEAE-Sepharose CL-6B was obtained from Pharmacia. Catalase (EC 1.11.1.6), ω-aminohexyl-agarose, dodecyl β-D-maltoside, Hepes, octyl β-D-glucopyranoside, superoxide dismutase (EC 1.15.1.1) and Taps were obtained from Sigma.

Bacterial strain and growth conditions

The *E. coli* strain RG145 which is deficient in cytochrome *bd* and overexpresses cytochrome *bo* has been described previously (Au and Gennis, 1987). The cells were grown aerobically in 100 litre batch culture on minimal medium and harvested as previously described (Cheesman et al., 1993a).

Purification of cytochrome *bo*

Cells were broken, washed cytoplasmic membranes isolated and cytochrome *bo* released from those membranes according to the prescription of Cheesman et al. (1993a). Purification of cytochrome *bo* was essentially as described by those authors, except that, after chromatography on DEAE-Sepharose CL-6B, the enzyme was further purified by chromatography on ω-aminohexyl-agarose prior to exchange into 50 mM Hepes/0.2% (w/v) octyl β-D-glucopyranoside, pH 7.5, and storage at −70 °C. Two different batches of cytochrome *bo* were used for these experiments and are designated 'NW8' and 'NW9', both of which contained 'fast' enzyme as judged by optical and e.p.r. spectroscopy.

Spectroscopy

Electronic absorption spectra were recorded using an SLM-Aminco DW2000 spectrophotometer. E.p.r. spectra were recorded using an ER200D X-band spectrometer (Bruker Spectrospin) interfaced to an ESP1600 computer and fitted with a liquid-helium-flow cryostat (ESR-9; Oxford Instruments). M.c.d. spectra were recorded on a circular dichrograph (Jasco J500D) interfaced with a superconducting solenoid with a 25 mm room temperature bore (Oxford Instruments), capable of generating magnetic fields up to 6 T.

Kinetics of the reaction of cytochrome *bo* with H_2O_2

Incubations were made in a thermostatically controlled (23 °C) 2.5 ml (1 cm pathlength) curvette containing a small magnetic follower. Reactions were started by the addition of H_2O_2 with a microsyringe. Absorbance changes were recorded using an SLM-Aminco DW2000 spectrophotometer operating in dual-wavelength mode interfaced to a Viglen-2 personal computer running dedicated software. For subsequent analysis, data files were converted into ASCII format and exported into a spreadsheet Excel v4.0 (Microsoft) which allowed the files to be edited and manipulated. The same data were replotted and fitted to the sum of two independent exponentials using the non-linear least-squares regression routine found in Grafit v3.0 (Erithicus Software).

Preparation of the mixed-valence CO adduct of cytochrome *bo*

Cytrochrome *bo* (55 μM) and buffer (50 mM Taps/1 mM EDTA/0.1% (w/v) dodecyl maltoside, pH 8.0) that had previously been sparged with oxygen-free nitrogen were stirred overnight in an anaerobic glovebox (Faircrest Engineering) in order to remove any traces of oxygen. Typical oxygen levels were < 1 p.p.m. in a nitrogen atmosphere. The enzyme was diluted 1:10 in buffer in a 3 ml anaerobic quartz cuvette that had been fitted with a septum. The anaerobic sample was removed from the glovebox and the electronic absorption spectrum recorded. The atmosphere in the cuvette was replaced anaerobically by CO, the cell incubated at 23 °C and the spectrum re-recorded periodically. Conversion into the mixed-valence CO adduct was complete after 45 min (Figure 5 below).

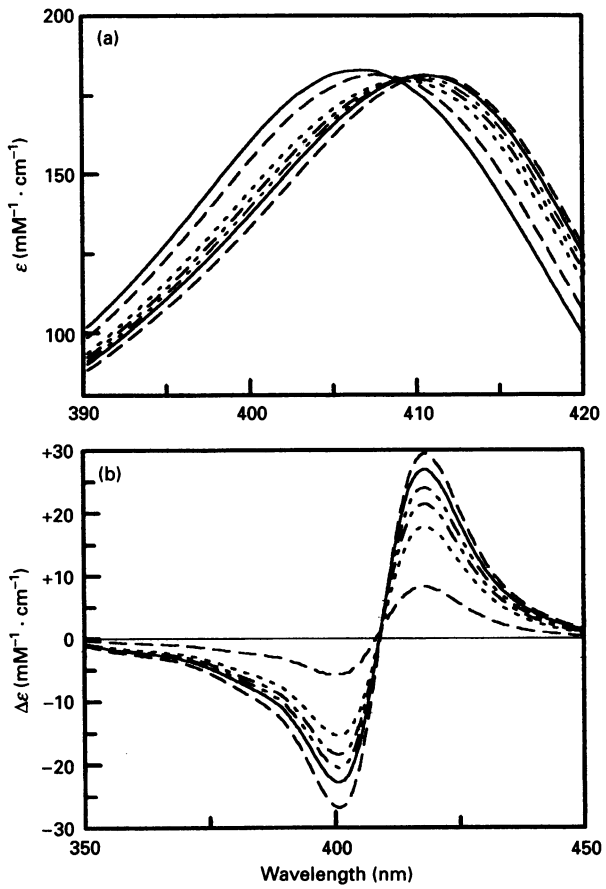

Figure 1 Changes in the Soret region of the electronic absorption spectrum of *E. coli* cytochrome *bo* upon the addition of H_2O_2

Cytochrome *bo* (NW8) was made 11.05 μM in a final volume of 0.5 ml of 50 mM Taps/1 mM EDTA/0.01% dodecyl β-D-maltoside, pH 8.0, containing 6 units of superoxide dismutase H_2O_2 was added from a stock solution of 0.5 mM to give the following final concentrations (from top to bottom on the left-hand side): ——, no addition; – – – –, 1.05 μM; ·····, 2.1 μM; – · – ·, 3.15 μM; – – –, 4.19 μM; ——, 6.26 μM; – – – –, 11.37 μM. (**a**) Shows the absolute spectra. (**b**) Shows the peroxide-minus-oxidized difference spectra. Spectra have been corrected for dilution and normalized to the isosbestic point at 409 nm to compensate for shifts in the baseline that occurred after each addition of H_2O_2.

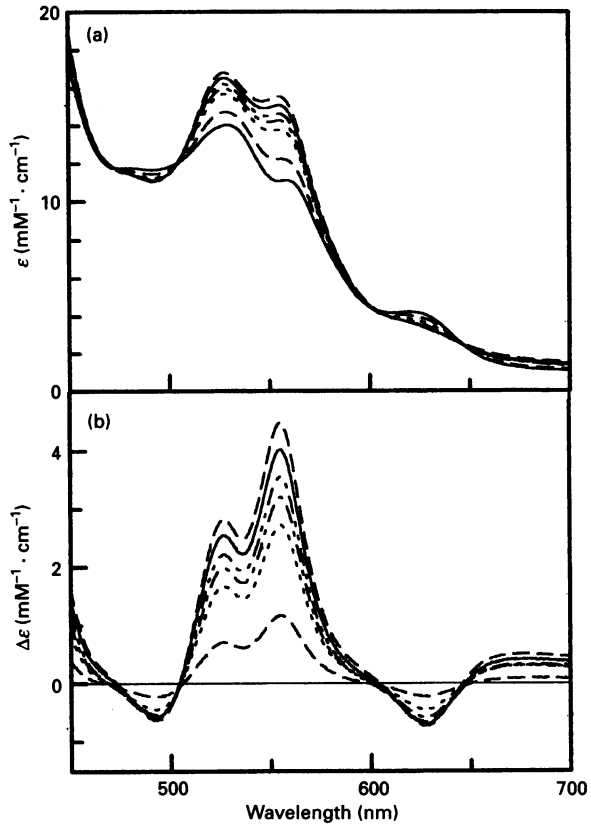

Figure 2 Changes in the visible region of the electronic absorption spectrum of *E. coli* cytochrome *bo* upon the addition of H_2O_2

Cytochrome *bo* (NW8) was made 11.05 μM in a final volume of 0.5 ml of 50 mM Taps/1 mM EDTA/0.01% dedecyl β-D-maltoside, pH 8.0, containing 6 units of superoxide dismutase. H_2O_2 was added from a stock solution of 0.5 mM to give the following final concentrations (bottom to top in central region): ——, no addition; – – – –, 1.05 μM; ·····, 2.1 μM; – · – ·, 3.15 μM; – – –, 4.19 μM; ——, 6.26 μM; – – – –, 11.37 μM. (**a**) Shows the absolute spectra. (**b**) Shows the peroxide-minus-oxidized difference spectra. Spectra have been corrected for dilution and normalized to the isosbestic point at 600 nm to compensate for shifts in the baseline that occurred after each addition of H_2O_2.

RESULTS

Effect of H_2O_2 on electronic absorption spectrum of fast cytochrome *bo*

The addition of stoichiometric amounts of H_2O_2 to fast cytochrome *bo* causes a slight decrease in intensity of the Soret band and its wavelength maximum to shift from 406.5 nm to 411 nm (Figure 1a). The difference spectrum (peroxide-minus-oxidized; Figure 1b) shows a peak at 418 nm and a trough at 400 nm ($\Delta\epsilon$ 27 × 10^3 $M^{-1} \cdot cm^{-1}$). The changes in the visible region of the spectrum are shown in Figure 2. Addition of peroxide causes the disappearance of the 624 nm charge-transfer band, suggesting a loss of high-spin ferric haem and a concomitant increase in the absorbance at 555 nm ($\Delta\epsilon$ 4 × 10^3 $M^{-1} \cdot cm^{-1}$). Increasing the concentration of H_2O_2 beyond 16 μM led to no further change in either region of the spectrum. These spectral changes could be reversed over a period of several hours by the addition of 50 units of catalase to the sample. The rate of this reverse reaction is faster at pH 6 than at pH 8, although the extent of the spectral changes induced by peroxide is independent of pH over the range 6.0–8.5.

Kinetics of the reaction of fast cytochrome *bo* with H_2O_2

To determine the rate of reaction of fast cytochrome *bo* with H_2O_2 and to ascertain that it proceeded directly to a single product, the kinetics of the process were measured. The rate of change of absorbance in the Soret region (420–411 nm) after addition of H_2O_2 (Figure 3a) could be analysed in terms of two independent exponential processes. One of these accounted for over 85% of the absorption changes observed. The observed pseudo-first-order rate constants for this fast process are linearly dependent on the concentration of H_2O_2 over the range (10–120) × 10^{-6} M (Figure 3b). The rate constants for the forward reaction (K_{on}) and reverse reaction (K_{off}) were calculated from the slope and intercept respectively of these second-order plots and are summarized in Table 1.

In order to determine whether the same process was causing the changes in the visible region of the spectrum, the experiment was repeated by monitoring the rate of change of absorbance using the wavelength pair 555–509 nm. The progress of the reaction and its rate dependence upon H_2O_2 concentrations were

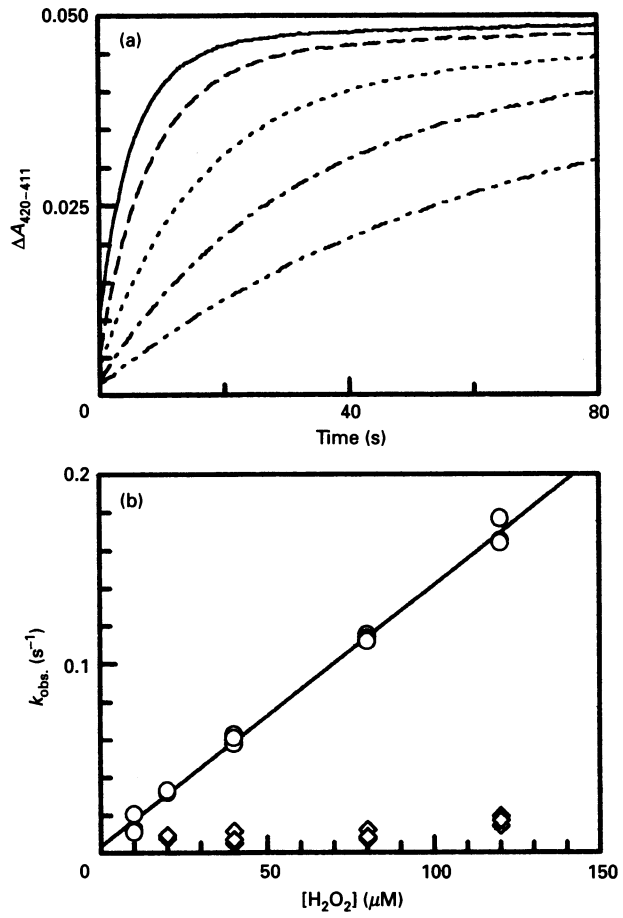

Figure 3 Kinetics of the reaction of H_2O_2 with *E. coli* cytochrome *bo* monitored in the Soret region

Cytochrome *bo* was made 1.83 μM in a final volume of 2.5 ml of 50 mM Taps/1 mM EDTA/0.01% (w/v) dodecyl β-D-maltoside, pH 8.0, containing 5 units of superoxide dismutase. H_2O_2 was added from a stock solution of 10 mM to give the following final concentrations (μM): A, 10; B, 20; C, 40; D, 80; E, 120. (**a**) Shows the relative rates of reaction over the first 80s. (**b**) Shows the dependence of the pseudo first order rate constants of the fast (○) and slow (◇) phases of the reaction as a function of H_2O_2 concentration.

Table 1 Kinetic H_2O_2 and thermodynamic parameters for the fast phase of the reaction of with cytochrome *bo* and fast cytochrome aa_3

Preparation	Monitoring wavelength (nm)	K_{on} ($M^{-1} \cdot s^{-1}$)	K_{off} (s^{-1})	K_d (M^{-1})
NW8	420–411	1.4×10^3	3.4×10^{-3}	2.43×10^{-6}
NW8	555–509	1.25×10^3	1.14×10^{-2}	9.12×10^{-6}
NW9	420–411	1.7×10^3	4.6×10^{-3}	2.71×10^{-6}
Cytochrome aa_3*	435, 606 and 655	3.9×10^{-2}	2×10^{-3}	5.1×10^{-6}

*Data taken from Baker and Weng (1992).

identical with those observed in the Soret region (results not shown). A plot of the observed pseudo-first-order rate constants for the fast (majority) phase of the reaction as a function of H_2O_2 concentration yielded rate constants ($K_{on}1.25 \times 10^3$ $M^{-1} \cdot s^{-1}$; K_{off} 1.14×10^{-2} s^{-1}). These are similar to those obtained when the reaction was monitored in the Soret region of the spectrum (Table 1) and close to the values obtained by Moody et al.

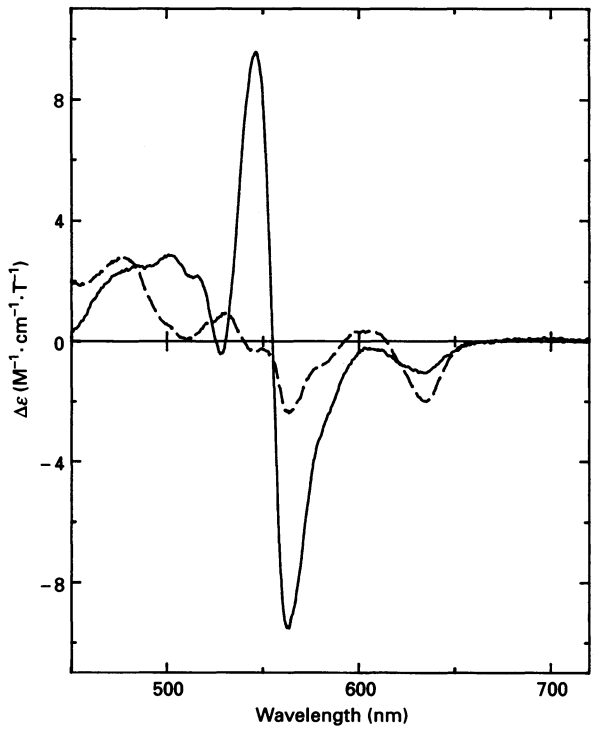

Figure 4 Room temperature m.c.d. spectrum of the product of the reaction of cytochrome *o* with H_2O_2

The m.c.d. spectrum of fast cytochrome *bo* (325 μM) in ^{2}H-labelled 50 mM Hepes/0.2% (w/v) octyl β-D-glucopyranoside, pH* 7.4, was recorded at 295 K and 6 T before (– – – –) and after (——) the addition of H_2O_2. In the interests of clarity, the spectral contribution of (the unchanged) low-spin haem *b* has been electronically subtracted.

(1993b) for the reaction of H_2O_2 with the membrane-bound enzyme.

Table 1 shows that the kinetics of the process is independent of both preparation and of the region of the spectrum in which the process is measured. From the calculated second-order rate constants it is possible to derive an apparent dissociation constant for the reaction. The values obtained ($K_d = 2.5 - 9.5 \times 10^{-6}$ M; Table 1) are consistent with those obtained from static titrations [$(4–13) \times 10^{-6}$ M].

Room-temperature m.c.d. spectroscopy of fast and peroxide-treated forms of cytochrome *bo*

The changes observed in the electronic absorption spectrum of cytochrome *bo* on the addition of H_2O_2 are similar to those observed when a strong ligand, such as cyanide, which induces a high-spin to low-spin transition of haem *o* (Cheesman et al., 1994), is added. In order to investigate whether the product of the reaction of cytochrome *bo* with peroxide does contain low-spin haem *o* with histidine and peroxide as the axial ligands, the m.c.d. spectrum of this species was measured. The spectrum showed that there was no doubling of the intensity of the Soret region of the spectrum expected to be associated with the formation of a second low-spin haem (Thomson et al., 1977). However, charge-transfer bands at 624 nm and 1100 nm, characteristic of high-spin haem *o* with histidine and water as the axial ligands (Cheesman et al., 1994) are absent, confirming that haem *o* is not high-spin.

In order to examine the form of the m.c.d. spectrum of haem *o* without interference from that of haem *b*, the contribution of the unchanged low-spin haem *b* was subtracted. Figure 4 shows

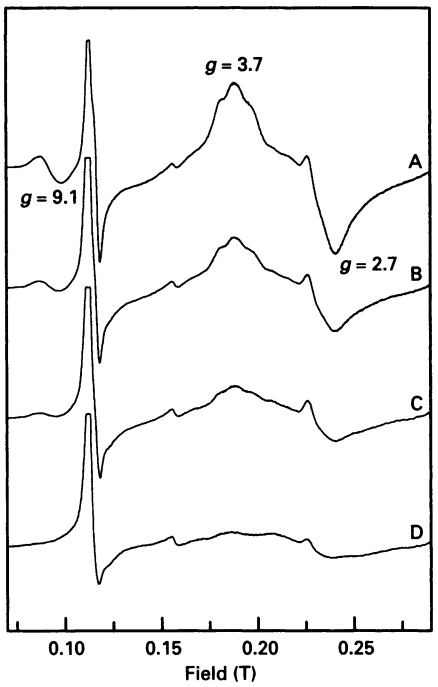

Figure 5 Effect of H_2O_2 on the X-band e.p.r. spectrum of *E. coli* cytochrome *bo*

Cytochrome *bo* was made 233 μM in 50 mM Hepes/0.2% (w/v) octyl β-D-glucopyranoside, pH 7.5, and the e.p.r. spectrum recorded. Sequential additions of 10 mM H_2O_2 were made so that the amount in the oxyferryl species (based on the absorbance at 555 nm), expressed as a percentage, is: in spectrum A, 0%; in B, 33%; in C, 52%; and in D, 100%. Conditions: temperature, 4 K; microwave frequency, 9.38 GHz; modulation amplitude, 1 mT; power, 20 mW.

the m.c.d. spectrum of isolated haem *o* both before and after reaction with H_2O_2. The spectrum of haem *o* in the fast enzyme is similar in lineshape and intensity to that of a number of histidine-ligated high-spin ferric haems with water as a sixth ligand (Cheesman et al., 1994). However, after reaction with H_2O_2 the spectrum shows a derivative shape with cross-over at 555 nm. The form and intensity of this spectrum is characteristic of oxyferryl haem found in compound II of peroxidases and compound ES of yeast cytochrome *c* peroxidase (Cheesman et al., 1991).

X-band e.p.r. spectroscopy of fast and peroxide-treated forms of cytochrome *bo*

These results suggest that the reaction product contains the redox centres $Fe_b(III)Fe_o(IV)$=O and $Cu_B(II)$. This should not possess the broad e.p.r. signature characteristic of the coupling between ferric haem *o* and $Cu_B(II)$ (Watmough et al., 1993). Figure 5 shows the effect on the X-band e.p.r. spectrum of the addition of H_2O_2 to cytochrome *bo*. Although the $g = 3.7$ signal disappears as the formation of the oxyferryl haem *o* forms, no new signals appear in the spectrum. This suggests that Cu(II) ion is not magnetically isolated when oxyferryl haem *o* ($S = 1$) is formed. It therefore must either be coupled to the haem to form an e.p.r.-silent species ($S = 3/2, 1/2$) or be coupled to an as yet unidentified paramagnet.

Reaction of mixed-valence cytochrome *bo* with oxygen

When fast cytochrome *bo* is incubated anaerobically under an atmosphere of CO, it forms the mixed-valence CO compound with redox centres: $Fe_b(III)Fe_o(II)$–CO::$Cu_B(I)$ (Greenwood et al., 1974; Puustinen et al., 1992). This species, which is completely formed within 45 min at 25 °C (Figure 6a), is characterized by an absorption spectrum with maxima at 415 nm (not shown), 536 nm and 562 nm (Figure 6a), that shows no evidence of the reduction of cytochrome *b*. Exposing this species to oxygen in the presence of light causes photolysis of CO and permits the reduced binuclear centre to react with dioxygen to give a species whose absolute spectrum is similar to that of the oxyferryl

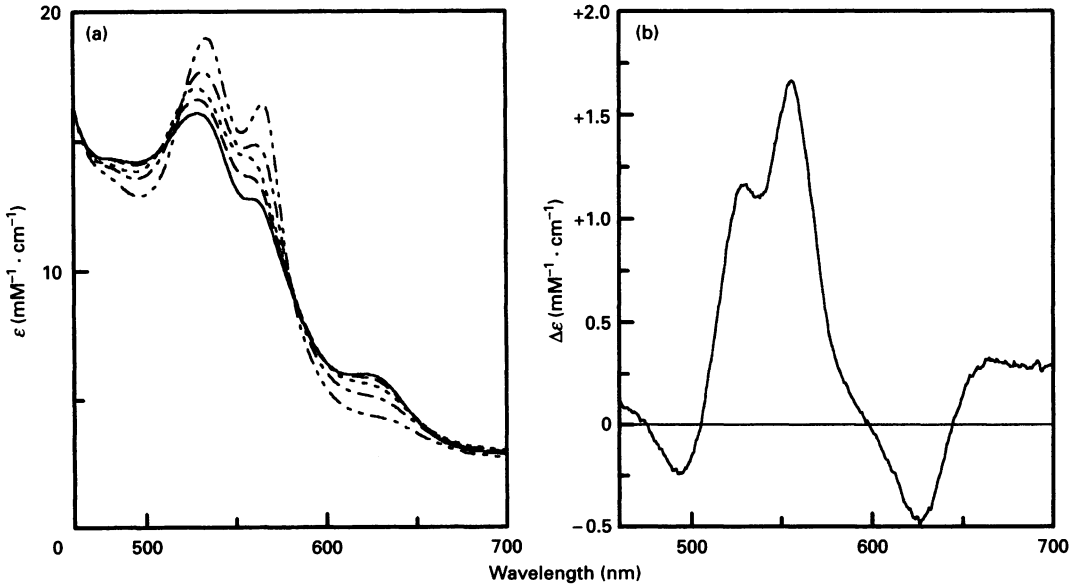

Figure 6 Reaction of dioxygen with CO-mixed-valence derivative of *E. coli* cytochrome *bo*

(**a**) Time course of formation of the CO mixed-valence cytochrome *bo* (conditions were as in the Materials and methods section): ——, 0 min; – – –, 5 min; -----, 15 min; —·—, 30 min; —··—, 45 min. (**b**) Difference spectrum of the product of the reaction of the mixed-valence oxidase with dioxygen minus the spectrum of the fast form of the enzyme.

cytochrome *bo* described above. The difference spectrum in the visible region of this species of the fast form of the enzyme (Figure 6b) has peaks at 555 nm and 529 nm and troughs at 493 nm and 624 nm, giving a form identical with the difference spectrum (peroxide-minus-oxidized) shown in Figure 2(b).

DISCUSSION

The present results show that fast oxidized cytochrome *bo* can be converted into a stable oxyferryl form by the addition of low (micromolar) concentrations of H_2O_2. The electronic absorption spectrum of the oxyferryl species is characterized by a Soret maximum at 411 nm, increased absorbance at 555 nm and a weak feature at around 630 nm. The room-temperature m.c.d. spectrum of this compound identifies unambiguously the presence of oxyferryl haem *o*. The form of the m.c.d. spectrum is typical of compound II of horseradish peroxidases, bovine liver catalase, myoglobin (low-pH form), chloroperoxidase (Dawson and Dooley, 1989; Stillman et al., 1976; Nozawa et al., 1980) and compound ES of yeast cytochrome *c* peroxidase. Hence the porphyrin ring is not oxidized in this derivative, but the haem is in the oxyferryl state [Fe(IV)=O].

The e.p.r. spectrum of this compound lacks the broad signals characteristic of high-spin haem *o* coupled to Cu_B(II) and has no features other than those arising from low-spin cytochrome *b*. Magnetically isolated Fe(IV) haems are e.p.r.-silent, owing to the large zero-field splitting of the $S = 1$ ground state. Exchange coupling with the unpaired electron of Cu_B(II) would lead to an odd total spin of the haem–Cu pair and could be formally analogous to the situation found in compound I of horseradish peroxidase. Here the unpaired electrons of the porphyrin radical, $S = 1/2$, and those of Fe(IV)=O are coupled to generate two states of total spin $S = 1/2$ and $S = 3/2$, the former being lower in energy. The e.p.r. signal arising from this state can only be detected using rapid-passage methods because the highly anisotropic nature of the exchange coupling causes the *g*-values to be anisotropic and the e.p.r. transitions to be spread across a wide field range. It could be, therefore, that the weak exchange coupling between Fe(IV)=O and Cu_B(II) will lead to difficulties in detecting an e.p.r. signal.

It is proposed that the binuclear centre formed by reaction with H_2O_2 is Fe(IV)=O::Cu(II). The same derivative appears to have been generated, albeit at lower occupancy, by the reaction of the mixed-valence cytochrome *bo* with dioxygen, by the reaction of fast cytochrome *bo* with CO under aerobic conditions (N. J. Watmough unpublished work) and after a short burst of turnover had been initiated by a two-electron donor (Moody et al., 1993b). Cytochrome *b* remains in the low-spin Fe(III) state in both compounds. The oxyferryl form of cytochrome *bo* identified here is most like compound F of cytochrome *c* oxidase (Chan et al., 1988; Larsen et al., 1990) in terms of the state of the binuclear centre, although the circumstances of formation are identical with those of compound P.

The kinetics of formation of oxyferryl cytochrome *bo* by reaction with H_2O_2 are similar to those observed for the formation of the P form of cytochrome aa_3 during the reaction of the fast conformer of that enzyme with H_2O_2 (Weng and Baker, 1991; Baker and Weng, 1992; Table 1). The unique properties of the haem *a* prosthetic group make direct comparison of the spectral changes induced by H_2O_2 in the two enzymes very difficult. However, it does appear that the 655 nm charge-transfer band of cytochrome aa_3 is equivalent to the 624 nm band of cytochrome *bo*, i.e. a marker of high-spin ferric haem. Disappearance of this 655 nm band in the reaction of cytochrome aa_3 with H_2O_2 and the concomitant increase in A_{607} that characterizes the P form of cytochrome aa_3 occurs at the same rate (Weng and Baker, 1991; Baker and Weng, 1992). In cytochrome *bo* the disappearance of the 624 nm band is paralleled by an increase in A_{555} that we have shown to be due to an oxyferryl haem *o*.

The P form of cytochrome aa_3 is identical with those forms of the enzyme known as 'Compound C' (Chance et al., 1979) and 'Compound III_m' (Clore et al., 1980). It may be formed by the reaction of the mixed valence form of the enzyme with dioxygen (Greenwood et al., 1974; Chance et al., 1979; Clore et al., 1980), by the aerobic reaction of the enzyme with CO (Nicholls, 1978; Nicholls and Chanadry, 1981), by reverse electron transfer in intact mitochondria (Wikström, 1981) and by the reaction of the enzyme with micromolar concentrations of H_2O_2 (Wrigglesworth, 1984; Vygodina and Konstantinov, 1989; Weng and Baker, 1991). The established similarity of the active sites of these two haem–copper oxidases (Gennis, 1992; Brown et al., 1993), combined with the circumstances of formation of oxyferryl cytochrome *bo* and the P form of cytochrome aa_3, strongly suggests that they are analogous species.

This is not to say that peroxide-bound haem a_3 does not participate in the catalytic cycle of cytochrome aa_3. Time-resolved resonance Raman spectroscopy demonstrates that such a species appears transiently after reaction of dioxygen with fully reduced cytochrome aa_3 (Varotsis et al., 1993). Whilst it has been argued that the short life time of this intermediate is an artefact of the experimental design and that in reality it corresponds to compound P (Wikström and Babcock, 1992), such a relationship appears not to have been established experimentally.

Our unexpected observation leaves some unanswered questions about the oxidation processes which form the oxyferryl species, either from the fast form by reaction with H_2O_2 or from the mixed valence form by reaction with dioxygen. Since H_2O_2 is a two-electron oxidizing agent, the product of the reaction with the binuclear centre is expected to lead to a derivative analogous to peroxidase Compound I. The data show that one electron is removed from the Fe(III) to generate the oxyferryl centre Fe(IV)=O, but the source of the second electron is not clear. There are several possibilities. An electron could be supplied either by the oxidation of Cu(II) to Cu(III) or by the formation of a free radical from an amino acid side chain. Cu(III) would be low-spin d^8 and therefore diamagnetic, which would leave the oxyferryl species undetectable by e.p.r. spectroscopy. If a free radical were to be generated close to the haem or to Cu_B, it would not necessarily be detectable by e.p.r. spectroscopy. The reaction of the reduced binuclear centre in the mixed-valence compound with dioxygen also requires an additional electron to yield oxyferryl haem *o* according to the stoichiometry:

$$\text{Fe(II)::Cu(I)} + O_2 + 2H^+ + 1e^- \rightarrow \text{Fe(IV)=O::Cu(II)} + H_2O$$

This again suggests a requirement either for an exogenous electron donor or for an electron to be abstracted from the protein itself. Of the two, the involvement of an amino acid radical would seem more plausible.

This argument has previously been put forward by Clore and co-workers to account for the e.p.r. spectrum of Compound III_m (Clore et al., 1980). Fee and co-workers, studying the major species formed by the reaction of *Thermus thermophilus* cytochrome caa_3 with H_2O_2 using Mössbauer spectroscopy, concluded that 70% of the haem a_3 was in the oxyferryl form (Fee et al., 1988). These authors also could not detect an e.p.r. signal from magnetically isolated Cu_B(II) and argued for the existence of an oxyferryl/radical species in which the radical is magnetically coupled to the cupric ion. This line of argument was extended by Weng and Baker (1991), who proposed both the P and F forms of bovine cytochrome aa_3 to be oxyferryl species in which P and

F are structural analogues of yeast cytochrome *c* peroxidase compound ES and compound II respectively. They argued that the slow transition from P to F they observed could be due to quenching of a putative tryptophan radical by excess peroxide.

Our observation of a single stable oxyferryl intermediate of cytochrome *bo* points to the possibility, previously suggested for cytochrome aa_3, that there may be a further electron-storage site within the protein. This work again raises doubts about the physical character of P and F, suggesting that they are related oxyferryl forms.

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REFERENCES

Au, D. C. and Gennis, R. B. (1987) J. Bacteriol. **169**, 3237–3242
Baker, G. M. and Weng, L. (1992) J. Theor. Biol. **158**, 221–229
Baker, G. M., Noguchi, M. and Palmer G. (1987) J. Biol. Chem. **262**, 595–604
Brown, S., Moody, A. J., Mitchell, R. and Rich, P. R. (1993) FEBS Lett. **316**, 216–223
Calhoun, M. W., Gennis, R. B. and Salerno, J. C. (1992) FEBS Lett. **309**, 127–129
Chan, S. I., Witt, S. N. and Blair D. F. (1988) Chem. Scr. **28A**, 51–56
Chance, B., Saronio, C. and Leigh, J. S. (1979) Biochem. J. **177**, 931–941
Cheesman, M. R., Greenwood C. and Thomson A. J. (1991) Adv. Inorg. Chem. **36**, 201–255
Cheesman, M. R., Watmough, N. J., Pires, C. A., Turner, R., Brittain, T., Gennis R. B., Greenwood C. and Thomson A. J. (1993a) Biochem. J. **289**, 709–718
Cheesman, M. R., Watmough, N. J., Greenwood C. and Thomson A. J. (1994) Eur. J. Biochem. **219**, 595–602
Clore, G. M., Andréasson, L.-E., Karlsson, B., Aasa, R. and Malström, B. G. (1980) Biochem. J. **185**, 155–167
Dawson, J. H. and Dooley, D. M. (1989) in Physical Bioinorganic Chemistry Series: Iron Porphyrins, Part III (Lever, A. B. P. and Gray, H. B. eds.), pp. 3–93, VCH Publishers, New York
Fee, J. A., Zimmerman, B. H., Nitsche, C. I., Rusnak, F. and Munck, E. (1988) Chem. Scr. **28A**, 75–78
Gennis, R. B. (1992) Biochim. Biophys. Acta **1101**, 184–187
Greenwood, C., Wilson, M. T. and Brunori, M. (1974) Biochem. J. **137**, 205–215
Han, S., Ching, Y. and Rousseau, D. L. (1990) Nature (London) **348**, 89–90
Hartzell, C. R. and Beinert, H. (1974) Biochim. Biophys. Acta **368**, 318–338
Ksenzenko, M. Y., Vygodina, T. V. Berka, Van Ruuge E. K. and Konstantinov, A. A. (1992) FEBS Lett. **297**, 63–66
Larsen, R. W., Li, W., Copeland, R. A., Witt, S. N., Lou, B., Chan, S. I and Ondrias, M. R. (1990) Biochemistry **29**, 10135–10140
Laureus, M., Morgan, J. and Wikstrom, M. (1993) Biochemistry **32**, 2664–2670
Mitchell, P. (1987) FEBS Lett. **222**, 235–245
Moody, A. J., Cooper, C. E. and Rich, P. R. (1991) Biochim. Biophys. Acta **1059**, 189–207
Moody, A. J., Rumbley, J. N., Gennis R. B., Ingledew, W. J. and Rich, P. R. (1993a) Biochim. Biophys. Acta **1141**, 321–329
Moody, A. J., Rumbley, J. N., Ingledew, W. J., Gennis, R. B. and Rich, P. R. (1993b) Biochem. Soc. Trans. **21**, 255S
Nicholls, P. (1978) Biochem. J. **175**, 1147–1150
Nicholls, P. and Chanady, G. A. (1981) Biochim. Biophys. Acta **634**, 256–265
Nozawa, T., Kobayashi, N., Hatano, M., Ueda, M. and Sogami, M. (1980) Biochim. Biophys. Acta **626**, 282–290
Puustinen, A., Morgan, J. E., Verkhovsky, M., Thomas, J. W., Gennis, R. B. and Wikström, M. (1992) Biochemistry **31**, 10363–10369
Stillman, M. J., Hollebone, B. R. and Stillman, J. S (1976) Biochem. Biophys. Res. Commun. **72**, 554–559
Thomson, A. J., Brittain, T., Greenwood, C. and Springall, J. P. (1977) Biochem. J. **165**, 327–336
Tsubaki, M., Mogi, T., Anraku, Y. and Hori, H. (1993) Biochemistry **32**, 6065–6072
Varotsis, C. and Babcock, G. T. (1990) Biochemistry **29**, 7357–7362
Varotsis, C., Zhang, Y., Appelman, E. H. and Babcock, G. T. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 237–241
Vygodina, T. and Konstantinov, A. A. (1987) FEBS Lett. **219**, 387–392
Vygodina, T. and Konstantinov, A. A. (1989) Biochim. Biophys. Acta **973**, 390–398
Vygodina, T., Schmidmaier, K. and Konstantinov, A. A. (1993) Biol. Membr. (Engl. Transl.) **6**, 883–906
Watmough, N. J., Cheesman, M. R., Gennis, R. B., Greenwood, C. and Thomson, A. J. (1993) FEBS Lett. **319**, 151–154
Weng, L. and Baker, G. M. (1991) Biochemistry **30**, 5727–5733
Wikström, M. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 4051–4054
Wikström, M. and Babcock, G. T. (1992) Nature (London) **356**, 301–309
Wikström, M. and Morgan, J. E. (1992) J. Biol. Chem. **267**, 10266–10273
Wrigglesworth, J. M. (1984) Biochem. J. **217**, 715–719

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