The high-spin cytochrome *o*' component of the cytochrome *bo*-type quinol oxidase in membranes from *Escherichia coli*: formation of the primary oxygenated species at low temperatures is characterized by a slow 'on' rate and low dissociation constant

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Cytochromes *b* and *o* in membrane vesicles from aerobically grown *Escherichia coli* were readily reduced by succinate; one cytochrome, which we propose should be called cytochrome *o*', reacted with CO in the Fe(II) state to give a photodissociable CO adduct. The photodissociation spectrum (photolysed minus pre-photolysis) at sub-zero temperatures had a relatively high γ/α absorbance ratio, indicating a high-spin haem, which, in the reduced state, probably contributes little to the sharp α absorbance of the oxidase complex in membranes. Reaction with oxygen of the unliganded high-spin haem between $-132\text{ }^{\circ}\text{C}$ and $-95\text{ }^{\circ}\text{C}$ following photolytic activation gave a product that is identified as the oxygenated form, being spectrally similar to, but not identical with, the CO adduct. In membranes, the forward velocity constant at $-95\text{ }^{\circ}\text{C}$ was $61\text{ M}^{-1}\text{ s}^{-1}$, and the dissociation constant was $1.6 \times 10^{-5}\text{ M O}_2$, as it is in intact cells. These data clearly distinguish the oxygen-trapping strategy of the cytochrome *o*' in this oxidase from that of cytochrome *a*₃, and also suggest that the presence of the soluble flavohaemoglobin (Hmp) in intact cells is without effect on such measurements of the primary oxygen reaction. In view of recent findings that this oxidase complex contains predominantly one mole of haem O and one of haem B, a revised nomenclature for the oxidase complex is proposed, namely, cytochrome *bo*'.

Keywords: *Escherichia coli*, cytochrome *bo*, cytochrome *o*', electron transfer, quinol oxidase, oxygen reduction

INTRODUCTION

Escherichia coli contains two terminal oxidases (Poole, 1983, 1988; Poole & Ingledew, 1987). One of these is generally called cytochrome *o* ('*o*' for oxidase), following the classical nomenclature of Castor & Chance (1959), who first described the photochemical action spectrum of this oxidase. Increasing interest in the cytochrome *o*-containing quinol oxidase of *E. coli* may be attributed to the availability of the purified oxidase and the results of molecular genetic studies of the *cyo* genes, encoding the subunits of this oxidase, which have revealed significant structural similarities (Chepuri *et al.*, 1990; Saraste *et al.*,

1991) between this oxidase and the cytochrome *aa*₃-type oxidases of mitochondria and certain bacteria. The oxidase complex comprises four membrane-bound polypeptides (Kita *et al.*, 1984; Matsushita *et al.*, 1984), two moles of haem O (Puustinen & Wikstrom, 1991), or one of haem O and one of haem B (Puustinen *et al.*, 1992) and a single copper atom, which is coupled to the high-spin haem O to form a binuclear, ligand-binding site (Salerno *et al.*, 1990).

Little is known of the mechanism of oxygen reduction, which results ultimately in proton-pumping (Puustinen *et al.*, 1991). Early studies (Poole *et al.*, 1979a, b) used the triple-trapping procedure, devised by Chance *et al.* (1975a), to describe the formation of reaction intermediates and cytochrome oxidation following photolysis of the carbonmonoxy form of the enzyme in intact cells at

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sub-zero temperatures. Although clearly demonstrating the formation of an early intermediate, presumed to be the oxy complex, and the remarkably low dissociation constant (k_d) for the reaction of the enzyme with oxygen, these studies were limited by the relatively poor signal:noise ratios resulting from spectroscopy of frozen cell suspensions, particularly in the α and β spectral regions. This work relied on recording the difference spectra of the various states of the oxidase after photolysis, with the spectrum of the pre-photolysis state [i.e. the CO-ligated, Fe(II) form] subtracted. Furthermore, recent recognition of the existence of a haemoglobin-like molecule (Hmp) in *E. coli* (Vasudevan *et al.*, 1991) has raised the question of a permeability or diffusion barrier for oxygen imposed by the cell envelope, which might affect the observed oxygen kinetics in experiments utilizing intact cells.

Here we describe the reactions of cytochrome *o* in predominantly everted cytoplasmic vesicles at sub-zero temperatures and exploit recent studies of the purified oxidase to interpret more fully the early stages of oxygen-trapping and electron transfer. In particular, the recording at -130°C of difference spectra relative to the post-photolysis state facilitates description of the spectrum of the oxy form. Strains that over-express the *cyo* genes cloned on multi-copy plasmids (such as RG145; Au & Gennis, 1987), which we have used in recent work (Bolgiano *et al.*, 1991, 1993), synthesize a variant of the oxidase in which the low-spin haem B is replaced to variable extents by a second mole of haem O (Puustinen *et al.*, 1992). Therefore, a strain that is wild-type with respect to *cyo* has been used. A revised nomenclature for this oxidase, in line with international recommendations, is proposed.

METHODS

Cell growth and preparation of membranes *E. coli* strain A1002 (*mel ilv lacI metE*) was used. The growth medium contained (l^{-1}) K_2HPO_4 (4 g), KH_2PO_4 (1 g), NH_4Cl (1 g), K_2SO_4 (2.6 g), $CaCl_2$ (0.01 g), casamino acids (Difco, 1 g), sodium succinate (10.8 g) and a solution (10 ml) of trace elements (Poole *et al.*, 1979b). After autoclaving, the medium was supplemented with 1 M $MgCl_2$ (1 ml l^{-1}). Cells were grown in a 6 l New Brunswick fermenter, aerated by sparging with sterile air at $6 l min^{-1}$ and stirred at about 100 r.p.m. The fermenter was inoculated with 1.5 ml of a 21 h starter culture that had been grown in the same medium in an Erlenmeyer flask containing one-fifth its volume of culture. Cells were harvested in the late exponential phase of growth when oxygen was not limiting for growth; the yield was approx. 3 g wet wt l^{-1} . Harvested cells were washed in a buffer that contained 50 mM Tris (pH 7.4), 2 mM $MgCl_2$ and 1 mM EGTA, then disrupted by sonication. Membranes were prepared by differential centrifugation (Poole & Haddock, 1974).

Spectroscopy. To a 1 ml portion of membrane particles, in a 2 mm pathlength cuvette with plexiglass windows, ethylene glycol was added to a final concentration of 30% (v/v) (Chance *et al.*, 1975a). The sample was reduced by adding succinate to 12 mM and incubating for 30 min. The cuvette contents were bubbled with CO for 2.5 min and then equilibrated at about -25°C for 5 min, after which the cuvette was either plunged

into a freezing bath (ethanol/dry ice, -78°C) or else oxygen was introduced by stirring in small volumes of air-saturated buffer containing 30% (v/v) ethylene glycol; the O_2 concentration in this buffer was taken to be 1.0 mM at -23°C (Chance *et al.*, 1975a). Dual-wavelength scanning spectrophotometry (Poole *et al.*, 1979b) and multi-wavelength kinetic recordings (Chance *et al.*, 1975b) were made using the apparatus and general methods described elsewhere.

RESULTS

Spectral changes following low-temperature photolysis

Physiological substrates readily reduced cytochromes *b* and *o* in cytoplasmic membranes. Incubation with succinate for 30 min typically gave reduction to 86% (measured at the α -peak) of the level obtained with sodium dithionite; absorption maxima (in reduced minus oxidized difference spectra) were centred at 436 (γ) and 563 nm (α) at room temperature. Succinate reduced cytochrome *o* to 80–90% of the level given by dithionite, as assayed by CO difference spectra (430 nm trough minus 415 nm peak) at room temperature (results not shown).

When CO-ligated cytochrome *o* is exposed to actinic light, the bound CO is photodissociated, allowing recording of the photodissociation spectrum (photolysed minus pre-photolysis), providing the temperature is maintained sufficiently low to prevent recombination of the CO with the oxidase (Poole *et al.*, 1979a, b). Photolysis to 95% of the maximum attainable with this flash lamp was attained. The photodissociation difference spectrum, obtained by photolysing an anoxic sample at temperatures between -100°C and -132°C , and plotting the post-photolysis minus pre-photolysis difference (not shown), exhibited troughs (in descending order of magnitude) at 412–413, 533–535 (broad) and 566–567 nm. The only prominent peak was at 432 nm, but weak absorbance maxima were observed at 552 and 580 nm. When care was taken to keep the membrane concentration low enough to avoid underestimation of the intense Soret bands in the highly scattering frozen samples, the ratio of absorbance change in the Soret region (432 nm peak minus 413 nm trough) to that in the α -region (567 nm trough minus 580 nm, i.e. the ' α peak-trough' as defined by Wood, 1984) was 27–30. This value and the form of the photodissociation spectra were unaffected by the presence of oxygen in the sample at the time of freeze-trapping. These properties are unlikely to be influenced by interference from the low level of cytochrome *bd* complex present in the membranes, since (a) the Soret features of the ligand-binding cytochrome *d* are weak in the Fe(II) state (Poole, 1983, 1988; Poole *et al.*, 1982), (b) in the absence of oxygen, the CO adduct of cytochrome *d* reforms immediately after photolysis even at 77K (Poole *et al.*, 1982), and (c) similar results have been observed in membranes from a cytochrome *bd*-deficient, *bo*-overproducing strain (B. Bolgiano & R. K. Poole, unpublished data). Puustinen & Wikstrom (1991) presented a static difference spectrum (CO-reduced minus reduced) of the purified cytochrome *bo* complex and reported a γ/α ratio of 23.7, but the wavelengths used were not cited.

Fig. 1. Difference spectra recorded following photolysis, in the presence of oxygen, of the carbonmonoxy form of cytochrome *o'* in membranes of *E. coli*. The reference spectrum subtracted in each case is that of the pre-photolysis state at the same temperature. Experiments were performed at the temperatures shown. The dashed line drawn between the minima on either side of the α -trough for (a), as suggested by Wood (1984), provides a guide to the spin-state of the unligated haem (see Results). The gain on the absorbance scale > 500 nm is ten times that in the Soret region.

Fig. 1 shows spectra obtained after photolysis of the CO-ligated cytochrome *o* in the presence of oxygen. It is important to recognize that, in these experiments, the membrane concentration was increased to resolve better the absorbance changes in the α and β regions, with consequent diminution of the γ/α ratio. Photolysis at -132°C , in the presence of oxygen, introduced by stirring to give solution concentrations of $300\text{--}400\ \mu\text{M}$ (Chance *et al.*, 1975a), resulted in a photodissociation spectrum (Fig. 1a) with troughs (due to loss of the CO form) at 412 (γ), 533 (β) and 566 (α) nm. The minor trough at 633 nm arises from photolysis of the CO compound of small amounts of cytochrome *d* with concomitant form-

ation of the oxygenated form of cytochrome *d* (649 nm). The Soret peak at 432 nm is attributed to the Fe(II) form of cytochrome *o*. The peak at 552 nm is attributed mainly to the β -band of the uncomplexed Fe(II) form but is also influenced by the peak between the α and β bands of the CO complex, by comparison with spectra of myoglobin (Wood, 1984). We attribute the peak at 580 nm to the (weak) α -band of the uncomplexed protein. A shoulder at this wavelength is shown in the computed spectrum (total dithionite-reducible haem *minus* low-spin haem) of the high-spin haem in the purified enzyme (Puustinen *et al.*, 1992) consistent with this assignment. The observation that the 566 nm trough (α -band) does not extend appreci-

Fig. 2. Difference spectra recorded following photolysis, in the presence of oxygen, of the carbonmonoxy form of cytochrome *o'* in membranes of *E. coli*. The reference spectrum subtracted in each case is that of the post-photolysis state at the same temperature. Experiments were performed at the temperatures shown.

ably below the dashed line shown in Fig. 1(a) is indicative of high-spin haem (Wood, 1984). Thus, the photodissociation spectrum in this region, which has contributions from only the ligand-binding cytochrome *o* of the complex, supports the CO difference spectrum reported by Puustinen & Wikstrom (1991), who suggested, as did Scott & Poole (1982), that the CO-reactive high-spin haem contributes little, if at all, to the reduced minus oxidized difference spectrum. Note that the α -band of the CO complex (i.e. 566 nm trough in Fig. 1a) is sharpened (band width at half-peak height, 10 nm) relative to the β -band at 533 nm (corresponding band width 22 nm) or the α -band at 580 nm (band width 17 nm), exactly as shown for myoglobin (Wood, 1984).

Figs 1 and 2 show the results of cycling the sample used in Fig. 1(a) to successively higher temperatures after photolysis in the presence of oxygen. In each case, the sample was returned to -132 °C to obviate absorbance changes due solely to low temperature intensification (Vincent *et al.*, 1982). This temperature is close to the lowest

attainable with the liquid nitrogen transfer line cryostat. Spectra in Fig. 1 are plotted as differences with the pre-photolysis state (i.e. reduced, CO-liganded) subtracted, whereas spectra in Fig. 2 are plotted with the post-photolysis state (i.e. reduced, unliganded) subtracted. After cycling the sample to -115 °C (and returning to -132 °C), the spectrum (Fig. 2b) with the post-photolysis form subtracted showed a broad trough centred at 436 nm and a broad maximum centred at 558 nm. These features developed further after cycling to -105 °C (Fig. 2c) and -98 °C (Fig. 2d), with the additional appearance of a β -band centred at 530 nm, and a broad Soret trough extending to about 426 nm. The cytochrome *d* signals were unchanged. We attribute the absorbance changes between 400 and 600 nm to the reaction of reduced cytochrome *o* with dioxygen, generating a primary oxygenated form analogous to Compound A of cytochrome *a₃*, originally described by Chance *et al.* (1975a) at low temperature and described as structurally similar to oxyhaemoglobin and oxymyoglobin. Indeed, the iron-dioxygen stretching frequency (568 cm^{-1}) is the same as

Fig. 3. Kinetics of the reaction of oxygen with cytochrome *o*' in membranes from *E. coli*. Spectra in (a) are plotted with reference to the pre-photolysis state and show the changes at approximately 0.5 min intervals after photolysis at -106 °C. In (b) the dependence of the rate of the reaction between cytochrome *o*' and oxygen is shown. The absorbance changes measured as described in Methods were used to calculate the pseudo-first order velocity constant.

that found in these oxygen-binding proteins (Han *et al.*, 1990). The primary oxygenated intermediate of cytochrome *o* was first described by Poole *et al.* (1979a, b) and reported to have spectral properties similar to those of the CO complex. However, the improved spectral resolution obtained here with extensively washed, concentrated membranes from cells grown with vigorous aeration, and after subtracting the post-photolysis and pre-photolysis spectra indicate important differences. Thus, the approach of the Soret features toward the baseline in Fig. 1(a-d) was not symmetrical. Identity of the oxygenated and CO adducts would generate a null difference spectrum (oxygenated minus CO-liganded pre-photolysis). Instead, at temperatures between about -98 °C and -132 °C (Fig. 1a-d), the loss in absorbance at 430 nm was faster than changes at 415 nm, and the difference spectra in Fig. 2(b-d) showed that the presumptive oxy compound has an absorption coefficient lower than that of the reduced, unliganded form at 430-450 nm, and higher at 530 to about 570 nm.

After cycling the sample to -95 °C or -82 °C and back to -132 °C, the difference spectrum (Fig. 2e, f) revealed significant deepening and broadening of the γ trough centred at 430 nm and the development of troughs at 554 and 565 nm, as predicted for oxidation of cytochrome(s) *b*. In addition, the appearance of a peak at 630 nm (Fig. 2e) suggested some reduction of cytochrome *d* and the loss of oxygenated cytochrome *d*, relative to the post-flash (oxygenated) species. At -56 °C, (Fig. 1e, 2g), the trough due to cytochrome *b* oxidation was clearly split, suggesting the subsequent oxidation of a cytochrome absorbing maximally at shorter wavelengths.

Kinetics of CO and O₂ binding

Fig. 3(a) shows the kinetics of the reaction of cytochrome *o* with oxygen after photolysis at -106 °C. The baseline was the spectrum of the pre-photolysis (i.e. CO-liganded) state. The asymmetry in the approach of the 413 nm trough and the 431 nm peak to the baseline is marked and reflects (see above) the greater similarity of the oxygenated form to the CO adduct at 431 nm (where the latter has little absorbance) than at 413 nm (the absorbance maximum of the CO adduct). There were clear isosbestic points at 418.5 nm and 441 nm. Similar experiments performed over the temperature range -100 to -73 °C gave an Arrhenius plot with an activation energy of about 38 kJ mol⁻¹ (results not shown). Similar experiments in the absence of oxygen (not shown) gave symmetrical absorbance changes in the Soret region with respect to the baseline, and an activation energy in the temperature range -80 °C to 42 °C of 35 kJ mol⁻¹ (not shown).

We previously reported a surprisingly low dissociation constant and first-order rate constant for the reaction between cytochrome *o* and oxygen (Poole *et al.*, 1979a). To investigate the possibility that, in intact cells, the cell envelope presented a significant permeability barrier to oxygen diffusion to the oxidase, the experiments were repeated with cytoplasmic membrane particles, which are likely to exist largely as everted membrane vesicles after cell disruption by sonication (Poole, 1993). Recordings were made with the double dual-wavelength apparatus described by Chance *et al.* (1975b) at 430 minus 450 nm. The results in Fig. 3(b) show some scatter, reflecting the difficulty in controlling oxygen additions to the viscous ethylene glycol-containing sample in the dark, but are

representative of three such experiments. A line of best fit gives a K_{+1} of $61 \text{ M}^{-1} \text{ s}^{-1}$, significantly higher than that obtained with whole cells ($0.9 \text{ M}^{-1} \text{ s}^{-1}$ at -101°C ; Poole *et al.*, 1979a), but an order of magnitude slower than the K_{+1} value for membrane-bound cytochrome aa_3 ($685 \text{ M}^{-1} \text{ s}^{-1}$; Chance *et al.*, 1975a). Nevertheless, the dissociation constant is clearly very low. An estimate of K_{-1} (the velocity when the oxygen concentration is zero, the intercept) of $0.1 \times 10^{-2} \text{ s}^{-1}$ gives a k_d of $1.6 \times 10^{-5} \text{ M O}_2$, very close to the value obtained in whole cells (Poole *et al.*, 1979b). Despite some uncertainty in the precise values, the k_d is clearly lower than the value of $0.5 \times 10^{-3} \text{ M}$ reported for cytochrome aa_3 (Chance *et al.*, 1975a).

DISCUSSION

The results presented here demonstrate the predominantly high-spin character of the photodissociation spectrum of the ligand-binding haem in the *E. coli* cytochrome *o*-containing terminal oxidase complex in membranes. This high-spin assignment is consistent with the ESR studies of Hata *et al.* (1985), who used a purified oxidase preparation, and those of Salerno *et al.* (1990), who described a biphasic potentiometric titration of the $g = 6$ (i.e. high-spin) signal with components having $E_{m,7}$ values of $+180$ and $+280 \text{ mV}$. The high potential side of the bell-shaped titration curve (where signal height at $g = 6$ diminished at higher potentials) was attributed to oxidation of the copper centre which, when in the Cu(II) form is spin-coupled to a haem, presumably the ligand-binding haem at the active site of the enzyme.

The γ/α ratio in the photodissociation spectrum at low sample concentrations is 27–30, a value that is significantly higher than that anticipated for a low-spin haem, yet slightly less than typical high-spin haem B as in myoglobin ($\gamma/\alpha = 32$; Wood, 1984). Although the presence of a mixed spin state cannot be ruled out, the weak α -trough of the CO difference spectrum (Puustinen & Wikstrom, 1991), together with the photodissociation spectra (Fig. 1), and the low temperature narrowing of the α -band of the CO complex, are all suggestive of high-spin haem.

Reports of the atypical pyridine haemochrome spectrum of haems in membranes from cytochrome *o*-over-producing *E. coli* strains (Bolgiano *et al.*, 1991) and the subsequent demonstration of novel haem (O) in the oxidase purified from such a strain (Puustinen & Wikstrom, 1991) should prompt careful reconsideration of the nomenclature of this oxidase. 'On the basis of the strong homology between cytochrome *o* and cytochrome aa_3 -type oxidases', Puustinen and Wikstrom (1991) proposed the term 'cytochrome oo_3 ', which, they suggest, 'also appears historically pertinent'. We propose instead that the 1989 Recommendations of the Nomenclature Commission of the International Union of Biochemistry (Palmer & Reedijk, 1992) be adopted and that subsequent findings that the enzyme in wild-type cells contains one high-spin haem O at the binuclear site and one low-spin haem B (Puustinen *et al.*, 1992) be reflected in the name. The haem (O) analogous to that in cytochrome a_3 is in a

non-haemochrome linkage, being high-spin and ligand-binding, and should be called cytochrome o' . The resulting nomenclature, bo' , is in accord with the recommended name for cytochrome *c* oxidase aa_3 (EC 1.9.3.1), i.e. cytochrome ad' , although this name has not been generally adopted. In our view, cytochrome bo' is more appropriate than oo_3 or bo_3 , neither of which is 'historically pertinent'. The consecutive subscript numbering was intended to distinguish a cytochrome from previously named variants of the same type. Thus, Keilin (1966) named cytochromes a , a_1 , a_2 (now d , a chlorin) and a_3 , but Castor & Chance (1959) defined previously only one cytochrome *o* in this organism and no other types of haem O have been described subsequently; subscripts at this stage appear unnecessary and confusing.

The ability to record photodissociation spectra at the temperatures reported in this paper (-132°C or higher) has been reported only for cytochromes a_3 and o' . As in the case of cytochrome a_3 , this is attributed to the presence in the cytochrome bo' complex of a copper atom, which, in its Cu(I) state, binds photodissociated CO, effectively slowing geminate recombination of the ligand to the high-spin haem. This is supported by infrared spectroscopy (Chepuri *et al.*, 1990) and by the finding that in *E. coli* grown under conditions of copper deficiency in a chemostat (Ciccognani *et al.*, 1992) photodissociation spectroscopy grossly underestimates the quantification of cytochrome o' , by lowering cellular and oxidase copper levels and permitting CO recombination as in myoglobin (Wood, 1984) or cytochrome *d* (Poole *et al.*, 1982).

At -132°C , the photodissociation spectrum of cytochrome o' is unaffected by the presence (Fig. 1) or absence (not shown) of oxygen. This is attributed to the negligible reaction of oxygen with the Fe(II) haem at this temperature. The photodissociation spectrum observed in membranes is very similar to, but inverted with respect to, the CO difference spectra for intact cells (Bolgiano *et al.*, 1991) and for the purified oxidase (Puustinen *et al.*, 1991) (Table 1). Photochemical action spectra have been described for *E. coli* only twice; in such spectra, peaks are observed at wavelengths corresponding to the absorption maxima of the CO-inhibited form of the functional oxidase. The original action spectrum of Castor & Chance (1959) is almost identical, after inversion, to the photodissociation spectrum described here, but for the absence of the broad 580 nm signal. The action spectrum of Edwards *et al.* (1981) is also similar, but for their inability to record signals below about 540 nm, due to inadequate laser light intensity at these wavelengths. The data in Table 1 show that the oxidase whose properties are described here is spectrally similar to the ligand-binding haem o' of the purified oxidase (Puustinen & Wikstrom, 1991) and to the functional oxygen-reactive cytochrome observed in photochemical action spectra.

In this work, the spectral changes that follow reaction of the oxidase with oxygen at sub-zero temperatures have been followed by recording spectra with respect to both the pre-photolysis state (i.e. reduced + CO), as in previous work, and the post-photolysis state at -132°C (i.e. reduced, unliganded). The latter type of difference

Table 1. Comparison of absorption maxima in CO difference spectra for some haem O- and B-containing proteins

	β -bands (nm)		α -bands (nm)		Reference
	Fe(II) CO ($\lambda_{\max}$)	Fe(II) ($\lambda_{\min}$)	Fe(II) CO ($\lambda_{\max}$)	Fe(II) ($\lambda_{\min}$)	
<i>E. coli</i> cytochrome <i>o'</i> *	537	552	570	585	Puustinen & Wikstrom (1991)†
<i>E. coli</i> cells	535	550	567	—	Castor & Chance (1959)‡
<i>E. coli</i> cells	—	555	570	584	Edwards <i>et al.</i> (1981)‡
<i>E. coli</i> membranes (RG145)*	530	550	565	583	Bolgiano <i>et al.</i> (1991)†
<i>E. coli</i> membranes	535	552	567	580	This paper§
Myoglobin	538	558	579	594	Wood (1984)

* Over-producing strain; such strains synthesize variant forms of the oxidase with higher ratios of haem O:haem B than in the wild-type.

† CO-difference spectrum, room temperature.

‡ Photochemical action spectrum, room temperature.

§ Photodissociation spectrum, -130°C . Spectrum inverted with respect to CO-difference spectrum.

spectrum shows more clearly the spectral features of the initial ligand-binding reaction than hitherto. They show that the first optically detectable intermediate has absorbance bands that are more intense than those of the reduced form (the 'baseline spectrum') in the 530–570 nm region and less intense in the Soret region near 436 nm. These changes can be assigned to the formation of an oxygenated intermediate (Fe^{2+}O_2 or $\text{Fe}^{3+}\text{O}_2^-$) described previously in kinetic studies (Poole *et al.*, 1979a, b).

At the lowest temperatures studied, the spectrum (Fig. 2a) exhibits a 558 nm peak and a 436 nm trough. After photolysis at -105°C to 98°C , however, the α/β region has peaks at 530–565 nm and a trough is developing at 426–430 nm. At this point, the difference spectrum (post-photolysis minus reduced, unliganded) is similar to a photodissociation spectrum obtained in the absence of oxygen (see, for example Bolgiano *et al.*, 1993). This suggests that at about -98°C the species formed is an oxygenated form with a spectrum similar to that of the CO species. The spectral similarity of the oxygen and CO adducts of haem proteins is well established (Wood, 1984). The form of the enzyme observed after photolysis at lower temperatures (e.g. Fig. 2a) is unclear and must be the subject of future studies.

Puustinen & Wikstrom (1991) have drawn attention to the distinctive blue-shifted pyridine haemochrome spectrum of *o*-type cytochromes, compared to haem B proteins, as did Bolgiano *et al.* (1991). The comparative data in Table 1 also reveal significant differences between the CO difference spectra of the two haem types, taking myoglobin as a well characterized example of a high-spin protohaem protein. The β -bands of Fe(II) myoglobin are shifted about 5 nm to higher wavelengths (compared with the average values for cytochrome *o'* (Table 1), whilst the α -bands are shifted about 11 nm further to the red than the corresponding cytochrome *o'* bands. Both haems have broad, weak absorbances in the unliganded, reduced state and thus contribute little to reduced *minus* oxidized spectra in the α - and β -regions, as first suggested by Scott

& Poole (1982). This finding requires careful consideration in spectral studies of cytochrome *o*-containing oxidases.

Although the physiological function of the haemoglobin-like protein in *E. coli* (Vasudevan *et al.*, 1991) is not yet known, it does bind oxygen (Ioannidis *et al.*, 1992; Orii *et al.*, 1992) and its properties suggest that it could have oxygen transport properties similar to those postulated for the *Vitreoscilla* haemoglobin (Khosla & Bailey, 1988). Nevertheless, its presence (at wild-type levels) in the intact cells used previously (Poole *et al.*, 1979a, b) does not explain the remarkably low dissociation constant described in that paper, since extensively washed membranes (this paper) reveal similar kinetic parameters.

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