The cytochrome *bd* quinol oxidase in *Escherichia coli* has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity *in vivo* by oxygen inhibition

Rita D'mello,¹ Susan Hill^{2†} and Robert K. Poole¹

Author for correspondence: Robert K. Poole. Tel: +44 171 333 4275. Fax: +44 171 333 4500.
e-mail: udbs065@bay.cc.kcl.ac.uk

¹ Division of Life Sciences,
King's College London,
Campden Hill Road,
London W8 7AH, UK

² Nitrogen Fixation
Laboratory, John Innes
Centre, University of
Sussex, Brighton BN1 9RQ,
UK

Cytochrome *bd* is a respiratory oxidase in *Escherichia coli* and many other bacteria. It contains cytochromes *b*₅₅₈, *b*₅₉₅ and *d* as redox centres, and is thus unrelated to the haem-copper super-family of terminal oxidases. The apparent affinities (*K*_m) for oxygen uptake by respiring cells and membranes from a mutant lacking the alternative oxidase cytochrome *bo'* were determined by deoxygenation of oxyleghaemoglobin as a sensitive reporter of dissolved oxygen concentration. Respiration rates were maximal at oxygen concentrations of 25–50 nM, but the kinetics were complex and indicative of substrate (i.e. oxygen) inhibition. *K*_m values were in the range 3–8 nM (the lowest recorded for a respiratory oxidase), and *K*_i values between 0.5 and 1.8 µM were obtained. Low temperature photodissociation of anoxic, CO-ligated membranes confirmed the absence of cytochrome *bo'* and revealed a high-spin *b*-type cytochrome identified as cytochrome *b*₅₉₅ of the cytochrome *bd* complex. Photodissociation in the presence of oxygen revealed binding of a ligand (presumably oxygen) to cytochrome *b*₅₉₅ at a rate much greater than that of CO binding, and formation of the oxygenated form of cytochrome *d*. The results confirm that both high-spin haems in the cytochrome *bd* complex bind CO and demonstrate that oxygen can also react with both haems. Substrate inhibition of oxidase activity, in addition to transcriptional regulation of oxidase synthesis, may play a crucial role in the regulation of partitioning of electron flux between the cytochrome *bd*- and *bo'*-terminated respiratory pathways.

Keywords: *Escherichia coli*, respiratory electron flux, quinol oxidases, oxygen affinity, cytochromes

INTRODUCTION

In bacteria, a single terminal respiratory oxidase catalysing the reduction of oxygen (as in mitochondria) is the exception, rather than the rule (Poole, 1983). Instead, the presence of branched respiratory chains, terminating in up to four different oxidases, has been claimed (e.g. Kitts & Ludwig, 1994). These multiple branched systems raise several important questions: in particular, what is the function of such apparent redundancy, how is electron

flux between the branches regulated, and are all the putative oxidases (frequently identified only on the basis of CO difference spectra or nucleotide sequence data) actually functional?

In *Escherichia coli*, two oxygen-reactive haems, cytochromes *o* and *d*, were clearly identified by photochemical action spectroscopy (Castor & Chance, 1959) and confirmed by the demonstration of oxygenated intermediates (Poole *et al.*, 1979, 1983a) and extensive studies of the solubilized and purified complexes (reviewed by Poole, 1994). The cytochrome *bo'* quinol oxidase (the term *bo'* is used to describe the haem-O- and haem-B-containing quinol oxidase of *E. coli*, the superscript referring to the

[†] Present address: Biology Building, University of Sussex, Brighton BN1 9QG, UK.

ligand-reactive haem in accordance with IUB nomenclature; Poole & Chance, 1995) is regarded as a proton-pumping oxidase, whereas cytochrome *bd*, which has a greater resistance to respiratory inhibitors, is not a proton pump and thereby is less effective at conserving the energy of respiratory electron transfer (Poole, 1983, 1994; Puustinen *et al.*, 1991). Studies of the regulation of synthesis of the two oxidases reveal that cytochrome *bo'* is synthesized maximally under conditions of high oxygen availability, whereas cytochrome *bd* is prominent under microaerobic conditions (Fu *et al.*, 1991), when the synthesis of cytochrome *bo'* is repressed (Cotter *et al.*, 1990).

The oxygen affinities of both oxidases are widely cited in relation to this differential synthesis, even though most data have been obtained using techniques that are incapable of measuring high affinities (e.g. Kolonay *et al.*, 1994). Rice & Hemphling (1978), however, used a modified polarographic technique with a large volume chamber and a probe covered with an ultra-thin membrane to measure oxygen affinities of cells grown in an unusual 'phauxostat' device and obtained a K_m for oxygen of 24 nM for cytochrome *bd*, in remarkable agreement with the classical studies of Longmuir (1954). However, all studies involving polarographic methods are limited at low oxygen tensions by the 'unstirred layer' at the electrode membrane (Lundsgaard *et al.*, 1978). Recognizing these limitations, we (D'mello *et al.*, 1994a, 1995) recently modified a highly sensitive spectrophotometric means of continuously monitoring oxygen depletion, as reported by the deoxygenation of oxyleghaemoglobin or oxymyoglobin (Appleby & Bergersen, 1980).

We report here the use of this method to demonstrate the exceptionally low K_m for oxygen for cytochrome *bd* in *E. coli* using a strain defective in cytochrome *bo'*. These measurements also reveal inhibition of oxidase activity at higher dissolved oxygen tensions, which may be important in regulating electron flux through branched respiratory chains in *E. coli* and other bacteria.

METHODS

Bacteria, growth, and preparation of cell and membrane suspensions. *E. coli* strain GL101 (F^- *cyo* *sdhC*; Lemieux *et al.*, 1992), which lacks the cytochrome *bo'* terminal oxidase, was a gift from Professor R. B. Gennis (University of Illinois, USA). Strain AN2342 ('wild-type'; Poole *et al.*, 1989) was a gift from Professor F. Gibson (Australian National University). Both strains were grown in Luria-Bertani (LB) medium, pH 7.0 (Maniatis *et al.*, 1982), supplemented with 0.2% (w/v) glucose and, for strain GL101, kanamycin (20 µg ml⁻¹) and tetracycline (20 µg ml⁻¹), in 2 l baffled flasks containing 400 ml medium at 37 °C with shaking at 200 r.p.m. Cultures were grown to mid-exponential phase (4 h) or, for strain AN2342, to late-stationary phase (20 h). Cells were harvested by centrifugation at 4000 g for 10 min; a portion (approximately 1 g) of the cell paste was resuspended in 25 mM potassium phosphate buffer (5 ml) and retained at 4 °C until used (within 6 h) for oxygen affinity determinations. The remaining cell paste was resuspended in 50 ml 50 mM PIPES buffer (pH 6.5), containing 8 mM magnesium acetate and a few grains of DNase (Sigma), and stored at -70 °C for approximately 24 h until membrane vesicles were

prepared using published procedures (D'mello *et al.*, 1994a, 1995). In brief, the thawed cell suspension was passed through a French pressure cell (Aminco) at 69 MPa. Centrifugation at 27000 g for 15 min removed cell debris; membrane vesicles were recovered from the supernatant fraction by centrifugation at 100000 g for 90 min. Membranes were suspended in the above buffer (4 ml, but lacking DNase) and stored at 4 °C until use (within 6 h). Protein in cells and membranes was assayed by the method of Markwell *et al.* (1978).

Determination of oxygen affinities. This was carried out as described previously by D'mello *et al.* (1994a, 1995). Oxygenated soybean leghaemoglobin (courtesy of Dr F. J. Bergersen, CSIRO Division of Plant Industry, Canberra, Australia) or sperm whale myoglobin (Sigma, no longer available) was diluted to 10–15 µM in phosphate buffer (25 mM, pH 7.0) containing 1 mM EDTA, which had been previously sparged with, and stored under, a gas mixture of 1% (v/v) oxygen in argon. Solutions were used within 6 h. Deoxygenation of the globins by respiration of cells or membranes was monitored by time-shared multi-wavelength spectrophotometry (Chance *et al.*, 1975) as described by D'mello *et al.* (1994a, 1995). The 1.3 ml capacity cuvette was filled with the globin solution and sealed with a finely perforated stopper, through which substrate solution (1 M succinate, 10 µl, or 1 M D-lactate, 10 µl) was injected. The stability of the oxygenated globin was checked by monitoring ΔA for 5–10 min. After addition of bacteria or membranes (5–50 µl), globin deoxygenation was continuously monitored by plotting $\Delta A_{575-560}$. Data were analysed as described previously by D'mello *et al.* (1994a). At least four separate determinations were carried out on each sample; means and standard deviations are presented.

Low temperature spectrophotometry. The apparatus and techniques used to record photodissociation spectra of samples in the presence or absence of oxygen have been described previously (Poole *et al.*, 1979). Suspensions of washed cells or membranes, containing 30% (v/v) ethylene glycol, were reduced with sodium succinate (5 mM, 30 min, 30 °C) or sodium lactate (5 mM, 30 min, 30 °C) in a 2 mm pathlength cuvette (1 ml capacity) and bubbled with CO for 2 min at room temperature. The cuvette was cooled to -23 °C and, when required, oxygen was introduced by vigorously stirring the contents with a coiled wire (20–30 vertical strokes), giving approximately 300 µM oxygen in the sample, which was then rapidly frozen at -78 °C (dry ice/ethanol). After equilibration in the sample compartment of the spectrophotometer, a baseline was recorded (CO-reduced *minus* CO-reduced) and the sample was photolysed for 2 min with the focused beam from a 150 W lamp. Subsequent difference spectra (post-photolysis *minus* pre-photolysis) reveal photodissociable haem proteins and their reactions with CO or oxygen.

RESULTS

Spectral characteristics of the strains used

The *Cyo*⁻ phenotype of strain GL101 was confirmed by difference spectroscopy (results not shown). In the CO difference spectrum, an α -band at 640 nm was identified as the CO-ligated form of cytochrome *d* and a trough at 620 nm was assigned to loss of absorbance of the reduced, unligated cytochrome on binding CO. In the γ (Soret)-region, the absorption maximum was at 420 nm and the trough was at 442 nm. These signals are quite distinct from those exhibited by cytochrome *o* in such spectra, namely a peak at 416 nm and a trough at 430 nm (Poole *et al.*

Fig. 1. Deoxygenation of oxyleghaemoglobin (a) during respiration of lactate by intact cells of *E. coli* GL101 (Cyo⁻ Cyd⁺). Oxygen consumption rates (V , nmol s⁻¹) and oxygen concentration (S , nM) in (b) were derived as described previously by D'mello *et al.* (1994a).

al., 1979). The Soret band of cytochrome *d* is weak (Poole *et al.*, 1982a) and so the 420 and 442 nm bands do not arise from this component either. Therefore, we conclude that, as in *Azotobacter vinelandii* (D'mello *et al.*, 1994b), these bands arise from CO binding to another haem in the cytochrome *bd* complex, namely cytochrome b_{595} , and confirm that strain GL101 (Lemieux *et al.*, 1992) contains cytochrome *bd* as sole terminal oxidase and lacks cytochrome *bo'*. The CO difference spectrum of the wild-type strain AN2342 (results not shown) grown to late-stationary phase was similar to that of GL101, but the Soret region revealed a low concentration of cytochrome *o*, evident from a peak at 415 nm, and a 'W'-shaped trough with minima at 430 and 443 nm, attributable to cytochromes *o* and b_{595} , respectively.

Measurements of the oxygen affinity of the cytochrome-*bd*-type oxidase

The basis and validity of the globin deoxygenation method have been described previously (D'mello *et al.*, 1994a, 1995). Several precautions were taken. First, we demonstrated that absorbance at the selected wavelengths was unaffected by the presence of membranes or cells in the cuvette. Second, doubling the cell or membrane concentration did not affect the measured K_m values.

Fig. 2. Deoxygenation of oxyleghaemoglobin (a) during endogenous respiration of intact cells of *E. coli* GL101 (Cyo⁻ Cyd⁺). Oxygen consumption rates (V , nmol s⁻¹) and oxygen concentration (S , nM) in (b) and the inset were derived (D'mello *et al.*, 1994a) using the data obtained between 3 and 700 nM oxygen [see part (a)], and the equations for substrate inhibition (Cleland, 1970). (c) Lineweaver-Burk plot.

Third, to ensure that the absorbance changes were attributable to deoxygenation, and not oxidation, of the globin, the cuvette contents were examined by wavelength-scanning before and after each deoxygenation experiment. In the calculation of results, full deoxygenation was taken as that level attained by respiration and not that achieved by dithionite; this avoids the problems caused by partial oxidation during the reaction or Fe(III) haem impurities in the globin sample used. Only data that were within the working range of each globin (D'mello *et al.*, 1994a) were examined.

Table 1. Oxygen affinities of cytochrome *bd* in *E. coli* cells and membranes

E. coli AN2342 is a wild-type strain and was grown to late-stationary phase to minimize the contribution of cytochrome *bo'* and maximize cytochrome *bd* (see text). GL101 is Cyo⁻. The values presented are means of four to six determinations with standard deviations in parentheses. The K_m values were calculated from the equations of Cleland (1970). ND, No component detected; –, not done.

Strain	Preparation, substrate	Myoglobin		Leghaemoglobin	
		K_m (μ M)	K_m (nM)	K_i (μ M)	V_{max} [nmol s ⁻¹ (mg protein) ⁻¹]
AN2342	Membranes, succinate	ND	3.4 (1.4)	0.94 (0.23)	0.49 (0.21)
GL101	Cells, lactate	–	8.4 (2.1)	0.83 (0.12)	1.13 (0.17)
	Cells, endogenous	ND	3.0 (2.7)	0.54 (0.07)	0.055 (0.001)
	Membranes, lactate	–	5.8 (0.6)	1.81 (0.47)	2.05 (0.39)

The deoxygenation kinetics of oxymyoglobin and oxyleg-haemoglobin were followed with both cells and membrane preparations from strain GL101. Data for intact cells respiring lactate (GL101 being defective in succinate dehydrogenase) are shown in Fig. 1(a, b). The progress of deoxygenation of oxyleg-haemoglobin (Fig. 1a) was used to compute the plot (Fig. 1b) of oxygen consumption rate (V) versus oxygen concentration (S). Respiration rates increased to a maximum at approximately 50 nM oxygen and declined at higher oxygen concentrations. Similar results were obtained with membranes (not shown). The most striking example of the decline in respiration at higher oxygen concentrations was afforded by experiments with whole cells respiring endogenous substrates (Fig. 2). The V versus S plot (Fig. 2b), calculated from the deoxygenation kinetics (Fig. 2a), showed that respiration rate increased sharply with increasing oxygen concentration. The inset in Fig. 2(b) shows an expanded abscissa and reveals more clearly the increasing respiration rate at oxygen concentrations up to about 25 nM. At oxygen concentrations above 25 nM, respiration decreased linearly as dissolved oxygen concentration increased. The Lineweaver–Burk plot (Fig. 2c) showed behaviour typical of substrate inhibition. Analyses using the equations of Cleland (1970) of several similar experiments using oxyleg-haemoglobin as supplier and monitor of dissolved oxygen are summarized in Table 1. The K_m values obtained with whole cells and membranes, using either lactate or endogenous substrates, were in the range 3–8.4 nM, markedly lower than any previously determined value. The K_i values obtained were in the range 0.54–1.81 μ M for strain GL101. The highest affinity was observed in cells respiring slowly [$V_{max} = 0.055$ nmol oxygen s⁻¹ (mg protein)⁻¹].

To determine whether the kinetics observed could be demonstrated in the wild-type strain, *E. coli* strain AN2342 was grown to late-stationary phase to minimize the contribution of cytochrome *bo'* and maximize that of cytochrome *bd*. Membranes from this strain, oxidizing succinate, gave a K_m for oxygen of about 3 nM (Table 1) and also exhibited substrate inhibition ($K_i = 0.94$ μ M).

These experiments exploited the low dissociation constant of oxyleg-haemoglobin to measure oxygen consumption kinetics in the range 0.003–0.3 μ M (Bergersen & Turner,

1985). To check for the presence of a component in these strains with an oxygen affinity in the 0.3–10 μ M range, oxymyoglobin was also used (Table 1). No component could be detected, consistent with the absence of cytochrome *bo'* in strain GL101 and the low levels of this oxidase in strain AN2342 grown under these conditions. We have previously shown that oxymyoglobin can be used to determine oxygen affinities for cytochrome *bo'* of *E. coli* (D'mello *et al.*, 1995) and *A. vinelandii* cytochrome *bd* (D'mello *et al.*, 1994a).

Ligand-binding reactions of cytochrome *bd*

The classical interpretation of substrate inhibition kinetics is that, at high (generally non-physiological) concentrations, the substrate acts as an inhibitor by binding to a second site in the enzyme, generating a 'dead-end' complex (Cleland, 1970). To date, evidence has not been presented that the *E. coli* cytochrome *bd* binds oxygen at two sites. However, this would be consistent with the proposal that CO and nitrite bind to both cytochrome *d* and cytochrome *b₅₉₅* (Hill *et al.*, 1993; Rothery *et al.*, 1987), that cyanide exhibits concerted binding to these haems – perhaps as a bridging ligand (Krasnoselskaya *et al.*, 1993), and that cytochrome *b₅₉₅* binds CO in a light-reversible manner (Poole & Chance, 1981). Furthermore, the *A. vinelandii* cytochrome *bd* binds both CO and oxygen at each high-spin haem (D'mello *et al.*, 1994b).

To extend these studies, photodissociation spectra of strain GL101, lacking cytochrome *bo'*, were recorded. Fig. 3 shows the photolysed (i.e. reduced, unligated) *minus* pre-photolysis (i.e. reduced, CO-ligated) difference spectrum recorded in the absence of oxygen at –100 °C. Although conventional CO difference spectra of this strain are dominated by the CO-ligated and reduced forms of cytochromes *d* and *b₅₉₅*, the characteristic features of cytochrome *d* in the 630–680 nm region are not detectable in this spectrum or after photolysis at other temperatures between –60 and –130 °C in the absence of oxygen (results not shown). This behaviour has been reported previously (Poole *et al.*, 1982a) and is the result of rapid rebinding of CO to cytochrome *d* after photolysis. Peaks in Fig. 3 at 439, 555 and 592 nm are attributed to the unligated Fe(II) form of haemoprotein(s); troughs at 421, about 532, and 570 nm result from loss of the CO-ligated

Fig. 3. Photodissociation spectrum of lactate-reduced, CO-ligated whole cells of *E. coli* GL101 (Cyo⁻ Cyd⁺) in the absence of oxygen at -130°C . The spectrum was recorded immediately after photolysis in the dual-wavelength scanning mode, with 575 nm as the reference wavelength, at 2.86 nm s^{-1} and a spectral band width of 4 nm. The pre-photolysis reduced plus CO minus reduced plus CO baseline is shown as a dashed line.

form. The signals are characteristic of a high-spin haem-B-containing protein, the spectrum resembling, for example, the (inverted) CO difference spectrum of myoglobin (Wood, 1984). The Soret: α/β ratio [$\Delta A_{439-421}/\Delta A_{555-570}$, as defined by Wood (1984)] is high, being 21 at -100°C (Fig. 3) and 28 at -130°C (not shown). However, these are minimum values, since the sample dilutions used, to maximize the resolution of the α and β bands, were not optimal for avoiding suppression of

the Soret bands (i.e. those regions of the spectrum where light scattering is most severe). Typical Soret: α/β ratio values for high-spin haem B proteins are around 32. Since strain GL101 does not express cytochrome *bo'*, and because cytochrome *d* is not detected under these conditions, the band at 592–595 nm is assigned to reduced, unligated cytochrome *b*₅₉₅, as is the 439 nm band (D'mello *et al.*, 1994a; Rothery *et al.*, 1987). We anticipated that maximal amplitude of the Soret signals of cytochrome *b*₅₉₅ (439 nm peak, 421 nm trough) immediately after photolysis would be observed at the lowest temperatures, because the lowest recombination rates are expected at these temperatures. However, the amplitude was maximal at -80°C , being higher than the amplitudes at -100°C and -130°C (results not shown). This might be the consequence of geminate recombination (Gibson, 1989) at the lower temperatures, i.e. the rapid return of a ligand molecule to the same site from which it has been photolysed.

The 439 nm peak and 421 nm trough decreased in amplitude after photolysis, approaching the baseline without change in band shape or position, indicating recombination of the cytochrome with CO. This is illustrated for -60°C in Fig. 4(a). The kinetics were pseudo-first order (Fig. 4c), with a half-time at -60°C of > 50 min. These changes were fully reversible by re-photolysis of the sample. At -100°C , negligible ($< \approx 4\%$) recombination was observed over a 60 min observation time (Fig. 4c).

To determine whether cytochrome *b*₅₉₅ binds oxygen, photodissociation spectra were recorded after introducing

Fig. 4. Kinetics of the reactions of oxygen and CO with cytochrome *b*₅₉₅ in *E. coli* GL101 (Cyo⁻ Cyd⁺). (a) Spectra recorded after photolysis of the lactate-reduced, CO-ligated sample in the absence of oxygen at -60°C . Scans were recorded at 0, 28 and 62 min after photolysis and approach the baseline (dashed). (b) Spectra recorded after photolysis in the presence of oxygen at -100°C at 0, 11 and 47.5 min. Absorbance changes (439–475 nm) in the absence of oxygen at -100°C (○) or -60°C [□, from part (a)] are shown in (c); closed symbols show ΔA after re-photolysis at the time indicated. (d) A similar plot for spectra obtained in the presence of oxygen at -100°C [see part (b)]. The bar in (c) and (d) shows a twofold change in ΔA .

Fig. 5. Photodissociation spectra of lactate-reduced, CO-ligated whole cells of *E. coli* GL101 (Cyo⁻ Cyd⁺) supplemented with oxygen at -130°C (a), -100°C (b), -80°C (c) and -60°C (d). The solid lines are the spectra recorded immediately after photolysis and the dotted lines in (b–d) are spectra recorded approximately 1 h after photolysis. Spectra were recorded in the dual-wavelength scanning mode, with 575 nm as the reference wavelength, at 2.86 nm s^{-1} and a spectral band width of 4 nm. Pre-photolysis reduced plus CO minus reduced plus CO baselines (not shown) were featureless.

oxygen at -23°C immediately before freeze-trapping. At -130°C (Fig. 5a), the dominant peaks were at 595, 555–557 and 438 nm, very similar to those observed in the absence of oxygen (Fig. 3) and therefore attributable to cytochrome b_{595} . However, a new signal at 650 nm was observed, due to oxygenated cytochrome d (Poole *et al.*, 1983a). This signal was most intense at -100°C (Fig. 5b). At -80°C (Fig. 5c) and -60°C (Fig. 5d), spectra recorded at frequent intervals after photolysis (not shown) showed development of a trough at 560 nm, due to cytochrome b oxidation and the approach of the 438 nm peak to the baseline. These photodissociation spectra are similar to those described previously in which a 632.8 nm He-Ne laser was used to photolyse selectively the cytochrome d -CO complex (Poole *et al.*, 1983b; Poole & Williams, 1987). In both that work and in the present, the first scan after photolysis at -100°C reveals the characteristic signals of cytochrome d between 631 and 650 nm. Cytochrome b_{595} is more prominent, relative to the cytochrome d signal, after photolysis with 'white' light (Fig. 5b) than after photolysis with the laser (Poole & Williams, 1987). This is expected, since the CO-ligated form of cytochrome d (with absorbance maximum near

636 nm) more effectively absorbs the photolysing radiation (632.8 nm) than does the CO-ligated form of cytochrome b_{595} (with absorbance maximum near 570 nm). Following photolysis with the laser, oxidation of cytochrome b_{595} was observed to accompany disappearance of oxy-cytochrome d (Poole & Williams, 1987). Further studies of the reaction progress following photolysis with white light will be necessary to understand the electron transfer events under these conditions.

Fig. 4(b) shows repetitive scanning of the reaction progress with oxygen at -100°C ; both the 438 nm peak and the 420 nm trough approached the baseline, without initial detectable shift in band positions and at a greater rate than in anoxic samples. Since the baseline is the spectrum of the reduced, CO-ligated sample, the spectral changes indicate the binding of a ligand (oxygen) to cytochrome b_{595} to give an adduct with spectral properties similar to those of the CO-ligated species. The kinetics were pseudo-first order (Fig. 4d), with a $t_{1/2}$ at -100°C of about 35 min. The rate of ligand binding to cytochrome b_{595} was thus much greater in the presence of oxygen. Indeed, in the absence of oxygen, no measurable ligand

binding occurred after photolysis of the CO compound until the temperature was raised to about -60°C (this work) or higher (Poole *et al.*, 1981). Interestingly, we noted that, even in the presence of oxygen, the absorbance changes in the Soret region attributed to cytochrome b_{595} were fully reversible by white light (Fig. 4d).

DISCUSSION

The deoxygenation of oxyleghaemoglobin provides a suitably sensitive assay for the determination of the oxygen affinity of cytochrome *bd*. The K_m (3–8 nM) determined by this method is about fourfold lower than that measured with a modified oxygen electrode with growing cells (Rice & Hempfling, 1978). Much higher values have been obtained using conventional Clark-type oxygen electrodes and cytochrome *bd* purified from *E. coli*. Kita *et al.* (1984) obtained a value of $0.38\text{ }\mu\text{M}$ using an electrode 'sensitive to low oxygen concentration', whilst Kolonay *et al.* (1994) drew 'tangents to the oxygen consumption curves at intervals of $0.5\text{ }\mu\text{M}$, resulting in approximately 20 separate rates' and reported a K_m value of $2\text{ }\mu\text{M}$, almost 1000-fold higher than the lowest values given in the present paper. The purified cytochrome-*bd*-type oxidase from *Klebsiella pneumoniae* has also been shown to have a very high affinity for oxygen (K_m 20 nM), measured using the spectral properties of leghaemoglobin (Smith *et al.*, 1990). The astonishingly high apparent affinity for oxygen of the *E. coli* cytochrome *bd* is in marked contrast to cytochrome *bd* in *A. vinelandii*, which has an apparent K_m for oxygen of about $4.5\text{ }\mu\text{M}$ (D'mello *et al.*, 1994a), measured using the same method. All three oxidases are structurally very similar and the disparity in the measured K_m values is surprising. It should be noted, however, that our values for *A. vinelandii* (D'mello *et al.*, 1994a) and *E. coli* (this paper) were determined *in situ* using either membranes or cells and the direct electron donor to the oxidase in these experiments is therefore the natural quinone pool. In contrast, the value for the *K. pneumoniae* oxidase, for example, was determined for the purified oxidase provided with ubiquinol-1, duroquinol or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine as substrates, and DTT or ascorbate to maintain the donors in their reduced states. It is probable that the nature and concentration of electron donor used in these various experiments is in part responsible for the disparate K_m values reported.

The physiological function of the *A. vinelandii* oxidase is rapid oxygen consumption and respiratory protection of nitrogenase (Kelly *et al.*, 1990; Poole, 1994) and the *K. pneumoniae* oxidase has also been proposed to lower the oxygen concentration and allow nitrogenase synthesis and function (Smith *et al.*, 1990). Similar roles for cytochrome *bd* in *E. coli* have been demonstrated (Hill *et al.*, 1990): mutants of *E. coli* defective in cytochrome *bd* show increased sensitivity to oxygen of fermentation and of microaerobic oxygen-dependent nitrogenase activity *in vivo* (in strains harbouring *K. pneumoniae nif* genes). The very high affinity of the *E. coli* oxidase reported here is consistent with the view that this oxidase serves to

scavenge oxygen under oxygen-limited growth conditions.

Oxygen concentrations higher than about 50 nM inhibit respiration via cytochrome *bd*. Substrate inhibition is particularly marked under conditions of low electron flux through the oxidase. These results have interesting physiological implications for the control of respiratory electron flux in *E. coli* and provide a possible solution to an enigma. Studies using *cydAB-lacZ* fusions have shown that cytochrome *bd* expression is not shut down during aerobic growth (Cotter *et al.*, 1990). Cytochrome *bo'* levels increase about 150-fold over anaerobic conditions, but cytochrome *bd* levels fall only 3-fold from 600 molecules per cell during anaerobic growth. We propose that inhibition of cytochrome *bd* activity at intracellular oxygen concentrations in the micromolar range might achieve appropriate control of electron flux through the proton pumping branch, i.e. that terminated by cytochrome *bo'*, rather than through the non-proton pumping cytochrome *bd* (Puustinen *et al.*, 1991). Rothery *et al.* (1987) also noted a slight increase in respiration rate as oxygen concentration fell from about $180\text{ }\mu\text{M}$ to $60\text{ }\mu\text{M}$, but did not attribute this to oxidase activity. Such a model for control of respiratory flux is applicable in principle to any branched respiratory system in which one oxidase is of the *bd* type, and might be extended to other branched chains for which models implicating the redox poise of the quinol pool (Guerin & Camougrand, 1994) are currently considered. Oxygen inhibition of respiration also occurs in cysts and trophozoites of the parasitic protozoon *Giardia muris* (Paget *et al.*, 1989), but in this case inhibition of the (unidentified) oxidase occurs at oxygen concentrations greater than $15\text{--}27\text{ }\mu\text{M}$ and is irreversible.

There has been much uncertainty regarding the number of ligand-reactive haems in cytochrome *bd* of *E. coli*. The evidence that cytochrome *d* binds both CO and oxygen is unequivocal; in addition to the photochemical action spectrum (Castor & Chance, 1959), evidence for oxygen binding has come from photolysis of CO-ligated cytochrome *d* in the presence of oxygen at low temperatures, resulting in the oxygenated form (Poole *et al.*, 1983a), and resonance Raman spectroscopy (Poole *et al.*, 1982b; Kahlow *et al.*, 1991). The early photochemical action spectra of stationary phase cells (Castor & Chance, 1959) did not reveal a band that could be attributed to the second high-spin haem of the complex (cytochrome b_{595} , then called a_1) in the α -region. However, subsequent photochemical action spectra using a liquid dye laser as actinic light source (Edwards *et al.*, 1981) revealed in *E. coli* (and *A. vinelandii*) a very clear band at 592 nm, which was attributed to 'cytochrome a_1 '. These measurements were not extended to $> 610\text{ nm}$ (because of the range of the dye used) and therefore did not reveal the anticipated band of cytochrome *d*. Nevertheless, in the belief that cytochrome b_{595} is not an oxidase in *E. coli*, these results have remained puzzling, unsubstantiated and largely ignored.

The present photodissociation spectra obtained with a Cyo⁻ strain confirm the CO-binding behaviour of cyto-

chrome b_{595} in *E. coli* and demonstrate for the first time oxygen binding by this haem. The spectral features of the *E. coli* cytochrome *bd*-type oxidase are indistinguishable from those described recently for *A. vinelandii* (D'mello *et al.*, 1994b). The clarity of the signals obtained in the cytochrome *bo'*-deficient mutant must lead us to question our previous assignment of a 436 nm peak in low-temperature photodissociation spectra to a form of cytochrome *o* (Poole & Chance, 1981); it seems likely that this component was cytochrome b_{595} . Comparison of the kinetics of ligand recombination after photolysis in the absence or presence of oxygen, as measured in the Soret region, strongly supports the view that cytochrome b_{595} binds oxygen and provides a molecular basis for the substrate inhibition phenomenon. We cannot rule out the possibility that oxygen binding to cytochrome *d* dramatically increases the rate of CO binding to cytochrome b_{595} (positive co-operativity). However, the view that cytochrome b_{595} binds oxygen is consistent with (i) the photochemical action spectrum of Edwards *et al.* (1981), (ii) the emerging view that the active site of this oxidase is a haem-haem 'binuclear' centre (D'mello *et al.*, 1994b; Hill *et al.*, 1993; Krasnoselskaya *et al.*, 1993) functionally analogous to haem-Cu_B, and (iii) the present measurements of oxygen uptake kinetics, which implicate a second site for oxygen binding. The present data do not allow us to identify the haem that binds oxygen at the lowest concentrations of oxygen. It is noteworthy, however, that the formation of oxy-cytochrome *d* is less extensive at the lowest temperatures used for photolysis, suggesting sluggish reactivity with the ligand under such conditions. Although little is known about the ligand reactivities of haems b_{595} and *d*, we note that the haem of cytochrome *d* is a chlorin, i.e. a porphyrin in which one ring (C in haem D) is reduced by saturation of one β -pyrrolic double bond (Timkovitch *et al.*, 1985). Another chlorin-type structure is sulphaemoglobin, in which H₂S adds across a β - β double bond of a pyrrole, disrupting the porphyrin conjugation and forming a chlorin (Berzofsky *et al.*, 1971). Sulphaemoglobin is one of the main non-functional forms of haemoglobin and, by extension, it might be supposed that oxygen co-ordination by cytochrome *d* will be sluggish. However, nitrite reacts with cytochrome b_{595} about 10-fold slower than with cytochrome *d* (Rothery *et al.*, 1987).

Proposals regarding co-operativity between two oxygen-binding sites in bacterial oxidases have been made before. Kinetics of oxygen reduction by *Pseudomonas aeruginosa* cytochrome *cd*₁ (a nitrite reductase that also reduces oxygen; Poole, 1983) led Ingledew & Saraste (1979) to conclude that this complex, containing two cytochromes *c* and two cytochromes *d*₁, can reduce oxygen to water in two ways. In the first, at high oxygen, the enzyme has a high apparent K_m and V_{max} . Oxygen is bound to each *d*₁ haem. At low oxygen concentrations, only a single oxygen is bound and is fully reduced to water. Migration of O₂²⁻ from one haem to the second may be necessary for full reduction. Based on the kinetics of inhibition by nitrite of cytochrome *bd* in *E. coli* membranes, Rothery *et al.* (1987) hypothesized the existence of two oxygen-binding sites in this oxidase. Whether the presence of two sites might

explain the ability to relieve CO inhibition of respiration by photolysis of either the CO-cytochrome *d* adduct (Castor & Chance, 1959) or the CO-cytochrome b_{595} adduct (Edwards *et al.*, 1981) can be answered only by further study.

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