The Reaction with Oxygen of Cytochrome Oxidase (Cytochrome *d*) in *Escherichia coli* K12: Optical Studies of Intermediate Species and Cytochrome *b* Oxidation at Sub-zero Temperatures

By ROBERT K. POOLE,¹* IAN SALMON¹† AND BRITTON CHANCE²

¹ Department of Microbiology, Queen Elizabeth College (University of London), Campden Hill, London W8 7AH, U.K.

² Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, U.S.A.

(Received 22 September 1982)

Optical changes in *d*- and *b*-type cytochromes, following initiation of the reaction of cytochrome oxidase *d* with O₂, have been studied in cells and derived membrane particles from oxygen-limited cultures of *Escherichia coli* K12. At successively higher temperatures between –132 and –88 °C, the first scan after photolysis of the CO-liganded, reduced oxidase in the presence of O₂ shows a diminution of cytochrome *d*₆₅₀ (believed to be an early intermediate in the O₂ reaction) and a slow increase in absorbance at 675 to 680 nm due to an unidentified chromophore. A similar sequence occurs when a single sample is scanned repetitively at –91 °C. At higher temperatures, oxidation of at least two spectrally distinct cytochromes *b* occurs. Selective photolysis of the cytochrome *d*–CO complex with a He-Ne laser shows that neither of these cytochromes is the CO-binding cytochrome *o*₄₃₆. In all oxidation states examined, no absorbance in the 720 to 860 nm region was observed; it is concluded that both cytochromes *d* and *o*₄₃₆ lack redox-active copper that has an environment similar to the copper(s) in mitochondrial cytochrome *c* oxidase.

The amount of cytochrome *d*₆₅₀ (but not the amount of reduced cytochrome *o*₄₃₆) formed after photolysis is directly proportional to the oxygen concentration in the sample at the time of freeze trapping. The results are discussed in relation to the composition and mechanism of action of cytochrome *d*.

INTRODUCTION

An unusual pigment with an absorption band well within the red region of the spectrum (at about 630 nm) was first observed in *Escherichia coli* and *Shigella dysenteriae* (Yaoi & Tamiya, 1928). The component, designated cytochrome *a*₂ (Keilin, 1933), was shown by several workers to be widespread among bacteria, autooxidizable, and capable of combining with CO and cyanide (for a review, see Lemberg & Barrett, 1973). Following the discovery that the prosthetic group is an iron-chlorin (Barrett, 1956), the enzyme was subsequently reclassified as cytochrome *d*.

Castor & Chance (1959) obtained photo-action spectra for the relief of CO inhibition in several bacteria and demonstrated the role of cytochrome *d* as an oxidase. More recently, Hoffman *et al.* (1980) have demonstrated the same function for cytochrome *d* in *Azotobacter vinelandii*. In *E. coli* and other bacteria, this oxidase is formed when the availability or utilization of oxygen is restricted (for references, see Poole & Chance, 1981), a finding consistent with the high ($K_m = 0.025 \mu\text{M}$) O₂ affinity of the *E. coli* enzyme (Rice & Hempfling, 1978). This value may be lower than the K_m for O₂ of cytochrome *aa*₃ (Jones, 1978; Poole *et al.*, 1979*a*).

† Present address: Biological Laboratory, The University, Canterbury, Kent CT2 7NJ, U.K.

The mechanisms by which cytochrome oxidases reduce oxygen (dioxxygen) are currently receiving much attention. The mode of action of the mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) has been most extensively studied (Chance, 1981; Wikström *et al.*, 1981). However, the diversity of structures exhibited by bacterial oxidases, having the same function as the mitochondrial enzyme, offers the opportunity for identifying the essential features of oxidase action in a comparative study. The redox-active metal centres in cytochrome *c* oxidase are two irons in haems *a* and *a*₃ and two coppers. In the reaction with O₂, the reactive centre consists of the coupled metal ion pair, cytochrome *a*₃ and the 'EPR invisible' Cu_a or Cu_B. However, with the exception of the structurally-simple *aa*₃-type oxidases recently identified in bacteria (Ludwig, 1980), there is little convincing evidence for the involvement of copper in bacterial cytochrome oxidases. In these systems, mechanisms for oxygen reduction must be sought that involve electron donation only from haems. The best-characterized bacterial oxidases are the cytochrome *o* of the myxobacterium *Vitreoscilla* and the cytochrome *cd*₁ oxidase (EC 1.9.3.2) of *Pseudomonas* (for a review, see Poole, 1982*a*), both of which lack copper.

Although the *Pseudomonas* system has been the subject of intensive biochemical and biophysical characterization, and intermediates in the reaction with O₂ have been identified (Greenwood *et al.*, 1978), this oxidase is somewhat atypical in being water-soluble and also having nitrite reductase activity. We are studying, therefore, the reactions between O₂ and reduced cytochrome *d* in *E. coli*, exploiting the low-temperature trapping techniques that have proved successful in identification of intermediates in the O₂ reactions of the mitochondrial oxidase (Chance *et al.*, 1975*a, b*) and of cytochrome *o* in *E. coli* (Poole *et al.*, 1979*b, c*; Poole & Chance, 1981; Poole, 1982*b*). The results presented here extend our recent finding (Poole *et al.*, 1982*a*) that reduced cytochrome *d* recombines with CO at temperatures lower than for any other oxidase studied. At temperatures above ~ -153 °C, photolysis of the CO complex is followed by ligand exchange for O₂ giving a compound (*d*₆₅₀) that we believe to be an early intermediate in the O₂ reaction (Poole *et al.*, 1983) and not the fully oxidized form. This paper reports on subsequent reactions of this compound and cytochrome *b* oxidation. Parts of this work have been presented in abstract form (Poole, 1982*b*; Poole *et al.*, 1982*b*).

METHODS

Organism, growth conditions and preparation of cells and membranes. These were exactly as described in the preceding paper (Poole *et al.*, 1983).

Low temperature spectral studies. These were performed as described by Poole *et al.* (1983). Oxygen was introduced to the CO-liganded, reduced cell suspension at -22 to -25 °C either by vigorously stirring with a wire (Chance *et al.*, 1975*a*) or by gently mixing with the suspension a volume of the buffer (containing 30% v/v, ethylene glycol) that was air saturated (2 mM-O₂; de Fonseca & Chance, 1980) at -22 °C. After photolysis, the extent of absorbance changes of cytochrome *d* in the near-infrared region was linearly dependent on oxygen concentration as adjusted by the addition of air-saturated buffer (see Results). This enabled a calibration of the stirring technique to be made: stirring for 30 s was found to give 410 μM-O₂, a value in reasonable agreement with 360 μM, the value given by Chance *et al.* (1975*a*) and assumed in our previous work on the *E. coli* oxidases. Aeration for 30 s of the CO-reduced sample at -25 °C (= 410 μM-O₂) typically resulted in a 5 to 30% loss of the (reduced + CO) form of cytochrome *d* (see also Poole *et al.*, 1983). After trapping the oxygenated sample at -78 °C and equilibrating at low temperatures in the dual-wavelength spectrophotometer, photolysis was achieved with the xenon lamp or laser described previously, or a more powerful 4.0 mW He-Ne laser (Spectra Physics, Mount View, Calif. 94042, U.S.A.). The laser beam was directed to the sample by light guides and positioned to give maximum illumination of the cuvette as judged from the photomultiplier voltage. To investigate the possibility that the multiple flashes from the xenon lamp might perturb the temperature of the sample, a thermocouple was inserted in a cell suspension in the 2 mm path-length cuvette at -25 °C and then frozen. Direct measurement of the temperature of the suspension equilibrated in the spectrophotometer at -80 °C showed it to be constant (-80 ± 1 °C) during 30 flashes of the xenon lamp over a period of 43 s.

Physical methods. The Johnson Foundation dual-wavelength scanning spectrophotometer was used (Poole & Chance, 1981; Poole *et al.*, 1983). Transmitted light was monitored with an S-10 type photomultiplier for the 390 to 700 nm range (EMI 9592B or 9214) or an S-1 type photomultiplier (RCA 7102) for the 550 to 860 nm range. Reference wavelengths are given in the Figure legends.

Fig. 1. Flash photolysis of CO-liganded, reduced cells at low temperatures and observation in the 550 to 860 nm range. In (a), the sample was freeze-trapped in the absence of O₂ and its spectrum scanned and stored in the digital memory of the dual-wavelength spectrophotometer at -132°C . This spectrum was subtracted from a subsequent prephotolysis spectrum to yield the reduced + CO minus reduced + CO baseline, or from the spectrum recorded immediately after photolysis (30 flashes of a 200 J xenon lamp). In (b) to (f), the procedure was identical except that O₂ (approx. 400 μM) was present and spectra were scanned at -132°C (b), -97°C (c), -88°C (d), -80°C (e) or -70°C (f). In each case, the reference wavelength was 720 nm and the scan speed approx. 1.4 nm s^{-1} . Protein concentration was 11.8 mg ml^{-1} .

RESULTS

Spectra of compounds formed between cytochrome d and oxygen at sub-zero temperatures after photolysis with white light

When the CO-liganded, reduced forms of mitochondrial cytochrome oxidase (cytochrome *aa*₃) or *E. coli* cytochrome *o* are flash photolysed in the absence of O₂ at low temperatures (below about -120°C), the spectrum obtained is that of the reduced form. This arises because the

Fig. 2. Repetitive wavelength scanning of the reactions with O_2 of cytochromes at $-91^\circ C$. The spectrum of a suspension of CO-liganded reduced cells was scanned and stored in the memory of the dual-wavelength spectrophotometer; the reference wavelength was 500 nm. Subsequent scans are difference spectra with the stored spectrum subtracted. The first scan, before photolysis, yielded the baseline indicated by the dashed line. The reaction was initiated by 30 flashes of a 200 J lamp. Subsequent scans were initiated approx. 4.1, 8.2, 12.3, 16.4, 25.5 and 40.6 nm after the start of the baseline. The vertical bar represents $\Delta A = 0.008$ above 500 nm and 0.016 below 500 nm. The scan speed was approx. 1.4 nm s^{-1} and the protein concentration 8 mg ml^{-1} . The directions of absorbance changes in successive scans are decreases at 430, 438, 560 and 652 nm and an increase at 675 nm.

Fig. 3. Absorbance changes during the reaction with oxygen at $-91^\circ C$. The absorbance changes shown in Fig. 2 and measured at 430–447 nm (○), 652–636 nm (●) and 675–695 nm (□) are plotted as a function of time. The vertical bar represents ΔA 0.008, 0.0016 and 0.0008, respectively. Zero time for each plot is the moment of traversing the first wavelength of each pair in the first scan after photolysis.

recombination of CO with the reduced oxidase is immeasurably slow at these temperatures. However, Poole & Chance (1980, 1981) showed that cytochrome *d* behaves anomalously in similar experiments, the only features observable in the 410 to 660 nm region being due to cytochrome o_{436} . Fig. 1(a) confirms and extends these results, the difference spectrum (with respect to the CO-liganded form) being featureless as far as 860 nm. The explanation (Poole *et al.*, 1982a) is that reduced cytochrome *d* binds CO at exceptionally low temperatures (-269 to $-153^\circ C$) so that recombination is complete within the first scan at $-132^\circ C$. In the presence of $400 \mu M O_2$ (Fig. 1b) the difference spectrum shows an intense peak at 650 to 652 nm (attributed

to the formation of an early intermediate, d_{650} ; Poole *et al.*, 1983) and a trough at 636 nm, resulting from disappearance of the CO-liganded form in the flash photolysis spectrum. Figs 1(c) to 1(f) show the first spectra, and their respective baselines, obtained after photolysis at successively higher temperatures. At -97°C (Fig. 1c) the major peak and trough remain but a shoulder to the 652 nm peak appears at 670 to 690 nm. Further warming to -88°C (Fig. 1d) or -80°C (Fig. 1e) causes this shoulder to intensify with concomitant loss of the peak. The shoulder is very broad but centred at 670 to 675 nm. At -80°C and higher temperatures (-70°C ; Fig. 1f), a trough at 562 nm indicates oxidation of *b*-type cytochrome(s). There were no absorbance changes between 700 and 860 nm, with respect to the CO-reduced baseline, over the temperature range studied.

The reactions of *b* and *d*-type cytochromes are also seen when a single sample is scanned repetitively at a constant temperature. At -91°C (Fig. 2), photolysis with white light resulted in the simultaneous decrease in absorbance at 652 nm and increase at 675 nm. In this single sample, the constancy of the 636 nm intensity is more clearly seen. Between 500 and 600 nm, the α and β bands of cytochrome(s) *b* at about 530 and 560 nm, respectively, are seen. In the Soret region, all scans but the first show a cytochrome *b* trough at 430 nm which deepens concomitantly with the α band. Changes at 652, 430 and 675 nm are shown in Fig. 3. Plots of the logarithm of the absorbance changes of the first two components were each bilinear, suggesting two pseudo-first-order processes for each component (results not shown). For cytochrome ' d_{650} ' (652 nm), the faster phase persisted for approx. 10 min after photolysis ($t_{\frac{1}{2}}$ approx. 30 min) and was followed by a much slower phase with $t_{\frac{1}{2}} > 200$ min. Similar kinetics were observed in five such experiments at this temperature. Multiphasic kinetics in haemoprotein reactions have been observed by others, particularly in ligand-binding studies (e.g. Poole *et al.*, 1979c; Denis & Richaud, 1982) and, although not fully understood, have been interpreted in terms of discrete energy barriers encountered by the approaching ligand (Austin *et al.*, 1975). Although continuous recording of the absorbance change at a fixed wavelength (with respect to a fixed reference wavelength) would have provided data more suitable for detailed kinetic analysis, the purpose of the present experiment was to demonstrate the kinetic distinction between the changes at 652 and 430 nm compared with the almost linear increase in absorbance of the unidentified chromophore at 675 nm. There are no distinct changes in the region between 440 and 450 nm (Fig. 2) where the Soret band of *a*-type cytochromes is expected. The weakness of the Soret bands of *d*-type cytochromes has been reported previously (Chance, 1953; Yamanaka & Okunuki, 1963; Poole *et al.*, 1981; Poole & Chance, 1981).

Reactions with ligands of cytochromes in membrane particles

Reactions similar to those described above for intact cells were also observed in membrane particles prepared by ultrasonic disruption and differential centrifugation (Poole *et al.*, 1983). Photolysis at -94°C of an anoxic sample that had been reduced with succinate and bubbled with CO revealed only cytochrome o_{436} (results not shown). In the presence of $400\text{ }\mu\text{M-O}_2$, however, additional signals at about 635 nm (trough) and 652 nm (peak) were seen at -94°C . Thus, the reactions attributed to both cytochrome o_{436} (Poole & Chance, 1981) and cytochrome *d* are those of oxidases tightly bound to the cytoplasmic membrane.

*Reactions of cytochromes *d* and *b* following photolysis with monochromatic light*

Cells from O₂-limited cultures contain, in addition to cytochrome *d*, two other cytochromes whose CO-complexes may be photolysed by white light, cytochromes a_1 and o_{436} . Cytochrome a_1 is present at low concentrations; its small absorbance at about 595 nm in difference spectra, with the CO-liganded form as reference (Poole *et al.*, 1981), does not confound the large spectral changes of cytochrome *d* seen in O₂-supplemented samples (Fig. 1b to f; Fig. 2). In contrast, cytochrome o_{436} has distinctive absorbances in the Soret, α and β regions (Poole & Chance, 1981) and cannot be easily distinguished from other *b*-type cytochromes that may be oxidized by cytochrome *d* (Figs 2 and 3). Monochromatic light from a He-Ne laser (633 nm), however, photolyses the cytochrome *d*-CO complex, but not the *o*-CO complex (Poole & Chance, 1981);

Fig. 4. Oxidation of cytochrome *b* during the O_2 reaction. The spectrum of a CO-liganded, reduced cell suspension was scanned at $-81^\circ C$ and stored in the memory of the dual-wavelength spectrophotometer (reference wavelength 500 nm) and subtracted from subsequent spectra. The CO-reduced *minus* CO-reduced baseline is shown by (a). Photolysis was by 10 min exposure to the beam of a 4 mW He-Ne laser and followed immediately by a further scan (b; dashed line) and another after 16 min (c). The sample temperature was then raised to $-70^\circ C$, returned to $-81^\circ C$ (results not shown) and recycled again, holding the temperature at $-70^\circ C$ for 5 min. Spectrum (d) is that of the twice-recycled sample *minus* the reduced + CO reference spectrum. The vertical bar represents $\Delta A = 0.016$ above 500 nm and 0.04 below 500 nm. The scan speed was approx. 2.8 nm min^{-1} and the protein concentration 11.5 mg ml^{-1} .

thus, further evidence for oxidation of *b*-type cytochromes is afforded by Fig. 4. The first scan after laser photolysis at $-81^\circ C$ is characterized by the 652 nm peak, a small shoulder at 675 nm and a trough at 437 nm. Repetitive scanning of the sample over 20 min (results not shown) revealed changes in the signals similar to those seen after photolysis with white light (Fig. 2). After transient warming of the sample (by disconnecting the flow of cooled N_2 to the cuvette), the troughs at 437 and 562 nm deepen. After warming, the temperature was returned to $-81^\circ C$, the temperature at which the reference spectrum was scanned, to obviate optical artefacts arising from light scattering and devitrification. A further warming cycle intensified the 675 nm component, deepened the 562 nm trough and shifted the Soret trough by 7 nm to 430 nm. This indicates successive oxidation of at least two cytochromes *b*. That the 437 nm component observed after laser photolysis in the presence of O_2 is not cytochrome o_{436} was confirmed by comparing directly the spectra of forms obtained in the presence and absence of O_2 . Thus at $-100^\circ C$, with $400 \mu M$ O_2 present, photolysis with the He-Ne laser gave the near-infrared signals of cytochrome *d* plus a prominent trough at 437 nm similar to that seen at $-81^\circ C$ (Fig. 4). Further irradiation of the sample with 30 flashes from a xenon lamp resulted in a broad Soret peak (maximum 439 nm) that is probably the sum of the intense peak of cytochrome o_{436} (Poole & Chance, 1981) and the trough extant prior to the xenon flashes (results not shown). In contrast, a repeat of the experiment with an anoxic suspension gave a featureless spectrum after laser photolysis (not shown) and the spectrum of cytochrome o_{436} after xenon flashing (see Fig. 2*b* of Poole & Chance, 1981). These experiments show that the cytochrome o_{436} -CO complex is

Fig. 5. Appearance of *b* and *d*-type cytochromes following photolysis with monochromatic light. The spectrum of a CO-liganded, reduced cell suspension was scanned and stored in the memory of the dual-wavelength spectrophotometer at -131°C (reference wavelength 500 nm). Subtraction of this from the subsequent scan of the unphotolysed sample gave the reduced + CO minus reduced + CO baseline (dashed line). The sample was exposed for 10 min to the beam of a 0.95 mW laser and the spectrum recorded (a) using the stored spectrum as reference. The sample was then warmed to -90°C and returned to -131°C where a further difference spectrum (b) was recorded. The scan speed was approx. 1.4 nm s^{-1} and the protein concentration 8 mg ml^{-1} .

Fig. 6. Dependence of the intensities of bands due to cytochromes o_{436} and *d* on O₂ concentration in the sample. Each CO-liganded, reduced cell suspension, to which the indicated concentration of oxygen had been added at -25°C , was scanned and stored in the dual-wavelength spectrophotometer and subtracted from subsequent scans of the same sample. Photolysis was achieved by 10 min exposure to a 4 mW He-Ne laser (○), and was followed in (●) and (□) with 10 flashes from a 200 J xenon lamp. The wavelength pairs measured are 652 minus 636 nm (○, ●) and 436 minus 415 nm (□). The temperature was -132°C , the scan range 410 to 720 nm, the reference wavelength 500 nm, and the scan speed approx. 1.4 nm s^{-1} . The protein concentration was 14.6 mg ml^{-1} .

photolysed by white light in the absence or presence of O₂ and that a trough observed following laser photolysis in the presence of O₂ at the same temperature is due instead to another *b*-type cytochrome.

Cytochrome *b* oxidation is a consequence of the reaction of cytochrome *d* with O₂. Laser photolysis of the oxidase at -131°C (Fig. 5) gives rise to the same spectral changes in the near-infrared as those seen after photolysis with white light (Fig. 1*b*). As shown previously (Poole &

Chance, 1981) the Soret changes are small when only cytochrome *d* is photolysed. However, warming the sample to -90°C and recycling to -131°C results in the oxidation of a *b*-type cytochrome seen at 437 nm.

Effect of oxygen concentration on the reactions of cytochrome d

The concentration of dissolved O_2 at the time of freeze-trapping was varied by gently mixing O_2 -saturated buffer with the CO-saturated cell suspension at -25°C . Photolysis of the sample at -132°C resulted in the appearance of cytochrome *d* signals, whose magnitude was linearly proportional to oxygen concentration, up to 0.6 mM- O_2 (Fig. 6). In the Soret region, a broad band at 440 to 450 nm of low intensity was observed, as shown in Fig. 5. At higher O_2 concentrations, the intensity of the *d* signals declined, probably due to displacement of CO from the oxidase and consequent O_2 binding. Irradiation with white light following each laser experiment gave approximately 15% enhancement of the *d* signals, but also elicited the appearance of cytochrome o_{436} in the Soret region. The intensity of the latter signal was relatively independent of O_2 concentration; the slight increase is presumably due to the influence of the increasing intensity of the broad Soret band of cytochrome *d*.

DISCUSSION

The remarkable functional properties of cytochrome *d* in *E. coli* are its ability to bind CO at temperatures between ~ -269 and -153°C (Poole *et al.*, 1982*a*) and the rapid ligand exchange that occurs when the CO-liganded form is photolysed in the presence of O_2 at higher temperatures (Poole *et al.*, 1983; this paper). Both these features are unique amongst cytochrome oxidases studied to date. An explanation of these phenomena in terms of the enzyme's composition and structure cannot be given at present. This oxidase has proved particularly refractory to purification, but studies of the solubilized and partially purified respiratory oxidase complex (Reid & Ingledew, 1980) have suggested the presence of two spectrally and potentiometrically different *b*-type cytochromes, and two haems *d*. Such a configuration is closely analogous to the cytochrome oxidase of *Pseudomonas aeruginosa* (Kuronen & Ellfolk, 1972). This enzyme contains two haems *c* and two haems d_1 , the latter being the binding site(s) for CO, cyanide and O_2 (Parr *et al.*, 1975; Greenwood *et al.*, 1978). It also exhibits rapid CO rebinding after photolysis at temperatures close to that of liquid He (Sivaram *et al.*, 1982). However, in marked contrast to the *E. coli* cytochrome *d*-containing oxidase, this enzyme is membrane-attached but water-soluble.

The function of copper

In the reaction of the mitochondrial cytochrome *c* oxidase with oxygen, the reactive centre consists of the coupled metal pair cytochrome a_3 and its associated copper (Cu_{a_3}), whereas cytochrome *a* and Cu_a serve an electron 'reservoir' or 'ballast' function (Chance, 1981), donating electrons to the bound peroxide intermediate in O_2 reduction. The only band in the visible spectrum of the enzyme attributable to copper is a weak absorption at 830 to 840 nm (for references, see Beinert *et al.*, 1980). There is no convincing evidence for the role of copper in the respiratory chain of *E. coli*. An EPR investigation by Lund & Raynor (1975) of membrane particles from nine bacterial species, including *E. coli*, failed to show a Cu^{2+} signal in either oxidized or reduced forms at 77 K.

The present study shows that if copper is present in the cytochrome *d* of *E. coli*, its environment must be quite different from that in mitochondrial cytochrome oxidase, since no significant absorbance between 720 and 860 nm was detectable over a wide temperature range, where all oxidation states of the oxidase are thought to be represented. Since these cells also contain cytochrome o_{436} (Poole & Chance, 1981), it is inferred that this oxidase too lacks copper in the form found in the mitochondrial enzyme.

The unidentified chromophore at 670 to 680 nm

Analysis of the absolute absorption spectrum of cytochrome *d* in *Azotobacter vinelandii* (in a state described as oxidized, although no details of sample preparation were given) has revealed three Gaussian components (Kauffman & van Gelder, 1973*a*). The peak positions (with band widths) were 635 (34), 648 (21) and 670 (38) nm. On exhaustion of oxygen, the 648 nm band decreased before the 670 nm component. The latter was also diminished by reduction in the presence of cyanide (Kauffman & van Gelder, 1973*b*) but was unaffected by CO (Kauffman *et al.*, 1980). A similar absorption band (675 to 680 nm) has also been described in *E. coli* (Pudek & Bragg, 1974; Hendler & Shrager, 1979) but not identified. In *Thiobacillus denitrificans*, intense bands at 675 to 680 nm were seen in reduced minus oxidized preparations when the reductant was Na₂S or in CO difference spectra, and were attributed to cytochrome *d* (Aminuddin & Nicholas, 1974). In the present paper, the appearance of a broad absorbance centred at about 675 to 680 nm is clearly seen when a CO liganded sample is photolysed at -91 °C (Fig. 2) or in the first scan after photolysis at higher temperatures (Fig. 1). Although its formation is coincident with the loss of the 650 nm compound and cytochrome *b* oxidation (Fig. 2), the kinetics of its formation are quite distinct (Fig. 3) and there is no clear isosbestic point between 650 and 680 nm during these transitions. Both observations suggest that the 650 and 680 nm components are only indirectly related. The haematin *d* from *P. aeruginosa* cytochrome oxidase has an absorption maximum at 684 nm (Yamanaka & Okunuki, 1963) but would not be expected to be functional in the reaction with oxygen.

Reductants of cytochrome d

'Although coordinate synthesis of cytochromes *b*₅₅₈ and *d* is observed under a variety of apparently unrelated growth conditions, the obligatory reduction of cytochrome *d* by cytochrome *b*₅₅₈ has yet to be established' (Haddock & Jones, 1977). Laser photolysis of the CO-liganded cytochrome *d* (which does not activate the reaction with oxygen of cytochrome *o*₄₃₆) and formation of cytochrome *d*₆₅₀ is followed by reduction of at least two *b*-type cytochromes that can be distinguished by their Soret absorption bands. Neither of the *b*-types is cytochrome *o* (which is still liganded to CO under these conditions), so that evidence is obtained here for two partially independent routes of electron transfer to oxygen:

The immediate reductant of cytochrome *o*₄₃₆ under these conditions has not been identified by selectively photolysing the *o*₄₃₆-CO complex but, in aerobically-grown cells, where the contribution of cytochrome *d*-mediated electron transfer is undetectable (Poole *et al.*, 1979*b*), cytochrome *o*₄₃₂ appears to be oxidized by a *b*-type cytochrome with a Soret absorbance maximum at 428 nm. The point of divergence of electron flow from a pool of quinones or *b*-type cytochromes remains to be identified. In the *d*₁-containing oxidase of *P. aeruginosa*, the electron donors to cytochrome *d*₁ are *c*-type cytochromes (Wharton & Gibson, 1976) whilst in *A. vinelandii* a cytochrome *b* → *d* branch apparently resembles that of *E. coli* (see Haddock & Jones, 1977).

Intermediates in the reaction with oxygen

The hypothesis that cytochrome *d*₆₅₀ is an early intermediate in the reaction of reduced cytochrome *d* with oxygen is advanced in the accompanying paper (Poole *et al.*, 1983). The present results, and Fig. 1 of Poole & Chance (1980), support this idea by showing that the reactions following the formation of the 650 nm absorbing form result in a spectrum whose only distinctive feature in the near-infrared region is a trough at 636 nm, attributable to the disappearance from the difference spectrum of the CO-liganded form. We propose that the product of the reaction is the fully oxidized enzyme, which has no distinctive spectrum in this region. The alternative explanation of the current data is that the 650 nm form, the immediate product of the reaction with O₂, is the fully oxidized enzyme. If this were the case, subsequent electron transfer from *b*-type cytochromes would be expected to re-form the reduced oxidase.

Since the spectrum of the latter (recorded with reference to the CO + reduced form) is characterized by an intense band at 628 nm and a trough shifted to 644 nm (Fig. 1 of Poole *et al.*, 1983), and this form is not seen in the present study, we do not favour this explanation.

Further characterization of the 'invisible' and d_{650} forms is required to establish the valence states of the individual redox centres, whilst more detailed kinetic studies may reveal the basis for the proposed stability of intermediates in the reaction with O₂ of cytochrome *d*.

R. K. P. thanks the S.E.R.C. for financial support (GR/B/8503.6), the Wellcome Trust for a Travel Grant and the Nuffield Foundation for a Science Research Fellowship. We thank A. Sivaram and C. Kumar for valuable discussions.

REFERENCES

- AMINUDDIN, M. & NICHOLAS, D. J. D. (1974). Electron transfer during sulphide and sulphite oxidation in *Thiobacillus denitrificans*. *Journal of General Microbiology* **82**, 115–123.
- AUSTIN, R. H., BEESON, K. W., EISENSTEIN, L., FRAUENFELDER, H. & GUNSALUS, I. C. (1975). Dynamics of ligand binding to myoglobin. *Biochemistry* **14**, 5355–5373.
- BARRETT, J. (1956). The prosthetic group of cytochrome *a*₂. *Biochemical Journal* **64**, 626–639.
- BEINERT, H., SHAW, R. W., HANSEN, R. E. & HARTZELL, C. R. (1980). Studies on the origin of the near-infrared (800–900 nm) absorption of cytochrome *c* oxidase. *Biochimica et biophysica acta* **591**, 458–470.
- CASTOR, L. N. & CHANCE, B. (1959). Photochemical determinations of the oxidases of bacteria. *Journal of Biological Chemistry* **234**, 1587–1592.
- CHANCE, B. (1953). The carbon monoxide compounds of the cytochrome oxidases. I. Difference spectra. *Journal of Biological Chemistry* **202**, 383–396.
- CHANCE, B. (1981). The cycling of oxygen through intermediates in the cytochrome oxidase–oxygen reaction. In *Current Topics in Cellular Regulation*, vol. 18, pp. 343–360. Edited by R. W. Estabrook & P. Srere. New York & London: Academic Press.
- CHANCE, B., GRAHAM, N. & LEGALLAIS, V. (1975*a*). Low temperature trapping method for cytochrome oxidase oxygen intermediates. *Analytical Biochemistry* **67**, 552–579.
- CHANCE, B., SARONIO, C. & LEIGH, J. S. (1975*b*). Functional intermediates in the reaction of membrane-bound cytochrome oxidase with oxygen. *Journal of Biological Chemistry* **250**, 9226–9237.
- DENIS, M. & RICHAUD, P. (1982). Dynamics of carbon monoxide recombination to fully reduced cytochrome *c* oxidase in plant mitochondria after low temperature flash photolysis. *Biochemical Journal* **206**, 379–385.
- DE FONSEKA, K. & CHANCE, B. (1980). Oxygen kinetics of frozen cytochrome oxidase. The capacity of the oxygen pocket. *Biochemical Journal* **185**, 527–530.
- GREENWOOD, C., BARBER, D., PARR, S. R., ANTONINI, E., BRUNORI, M. & COLOSIMO, A. (1978). The reaction of *Pseudomonas aeruginosa* cytochrome *c*-551 oxidase with oxygen. *Biochemical Journal* **173**, 11–17.
- HADDOCK, B. A. & JONES, C. W. (1977). Bacterial respiration. *Bacteriological Reviews* **41**, 47–99.
- HENDLER, R. W. & SHRAGER, R. I. (1979). Potentiometric analysis of *Escherichia coli* cytochromes in the optical absorbance range of 500 nm to 700 nm. *Journal of Biological Chemistry* **254**, 11288–11299.
- HOFFMAN, P. S., IRWIN, R. M., CARREIRA, L. A., MORGAN, T. V., ENSLEY, B. D. & DERVARTANIAN, D. V. (1980). Studies of photochemical action spectra on *N',N',N',N'*-tetramethyl-*p*-phenylenediamine-oxidase-negative mutants of *Azotobacter vinelandii*. *European Journal of Biochemistry* **105**, 177–185.
- JONES, C. W. (1978). Microbial oxidative phosphorylation. *Biochemical Society Transactions* **6**, 361–363.
- KAUFFMAN, H. F. & VAN GELDER, B. F. (1973*a*). The respiratory chain of *Azotobacter vinelandii*. I. Spectral properties of cytochrome *d*. *Biochimica et biophysica acta* **305**, 260–267.
- KAUFFMAN, H. F. & VAN GELDER, B. F. (1973*b*). The respiratory chain of *Azotobacter vinelandii*. II. The effect of cyanide on cytochrome *d*. *Biochimica et biophysica acta* **314**, 276–283.
- KAUFFMAN, H. F., VAN GELDER, B. F. & DERVARTANIAN, D. V. (1980). Effect of ligands on cytochrome *d* from *Azotobacter vinelandii*. *Journal of Bioenergetics and Biomembranes* **12**, 265–276.
- KEILIN, D. (1933). Supposed direct spectroscopic observation of the "oxygen-transporting ferment". *Nature, London* **132**, 783.
- KURONEN, T. & ELLFOLK, N. (1972). A new purification procedure and molecular properties of *Pseudomonas* cytochrome oxidase. *Biochimica et biophysica acta* **275**, 308–318.
- LEMBERG, R. & BARRETT, J. (1973). Bacterial cytochromes and cytochrome oxidases. In *Cytochromes*, pp. 217–236. London & New York: Academic Press.
- LUDWIG, B. (1980). Heme *aa*₃-type cytochrome *c* oxidases from bacteria. *Biochimica et biophysica acta* **594**, 177–189.
- LUND, T. & RAYNOR, J. B. (1975). Electron spin resonance of some bacterial respiratory membranes. *Bioenergetics* **7**, 161–166.
- PARR, S. R., WILSON, M. T. & GREENWOOD, C. (1975). The reaction of *Pseudomonas aeruginosa* cytochrome *c* oxidase with carbon monoxide. *Biochemical Journal* **151**, 51–59.
- POOLE, R. K. (1982*a*). The oxygen reactions of bacterial cytochrome oxidases. *Trends in Biochemical Sciences* **7**, 32–34.
- POOLE, R. K. (1982*b*). The reactions of terminal oxidases in *Escherichia coli* K12 with oxygen and carbon monoxide at sub-zero temperatures. *Biochemical Society Transactions* **10**, 485–488.

- POOLE, R. K. & CHANCE, B. (1980). Intermediates in the reaction with oxygen of cytochrome *d* in oxygen-limited *Escherichia coli*. *First European Bioenergetics Conference Short Reports*, pp. 107–108. Bologna: Pàtron Editore.
- POOLE, R. K. & CHANCE, B. (1981). The reaction of cytochrome *o* in *Escherichia coli* K12 with oxygen. Evidence for a spectrally and kinetically distinct cytochrome *o* in cells from oxygen-limited cultures. *Journal of General Microbiology* **126**, 277–287.
- POOLE, R. K., LLOYD, D. & CHANCE, B. (1979*a*). The reaction of cytochrome oxidase with oxygen in the fission yeast *Schizosaccharomyces pombe* 972h⁺. *Biochemical Journal* **184**, 555–563.
- POOLE, R. K., WARING, A. J. & CHANCE, B. (1979*b*). Evidence for a functional oxygen-bound intermediate in the reaction of *Escherichia coli* cytochrome *o* with oxygen. *FEBS Letters* **101**, 56–58.
- POOLE, R. K., WARING, A. J. & CHANCE, B. (1979*c*). The reaction of cytochrome *o* in *Escherichia coli* with oxygen. Low temperature kinetic and spectral studies. *Biochemical Journal* **184**, 379–389.
- POOLE, R. K., SCOTT, R. I. & CHANCE, B. (1981). The light-reversible binding of carbon monoxide to cytochrome *a₁* in *Escherichia coli* K12. *Journal of General Microbiology* **125**, 431–438.
- POOLE, R. K., SIVARAM, A., SALMON, I. & CHANCE, B. (1982*a*). Photolysis at very low temperatures of CO-liganded cytochrome oxidase (cytochrome *d*) in oxygen-limited *Escherichia coli*. *FEBS Letters* **141**, 237–241.
- POOLE, R. K., SALMON, I., SIVARAM, A., KUMAR, C. & CHANCE, B. (1982*b*). Ligand binding to cytochrome oxidase (cytochrome *d*) in *Escherichia coli*: low temperature optical and e.p.r. studies. *Second European Bioenergetics Conference Short Reports*, pp. 145–146. Villeurbanne: L.B.T.M.–C.N.R.S. éditeur.
- POOLE, R. K., SALMON, I., KUMAR, C. & CHANCE, B. (1983). The 650 nm chromophore in *Escherichia coli* is an 'oxy-' or oxygenated compound, not the oxidized form of cytochrome oxidase *d*: an hypothesis. *Journal of General Microbiology* **129**, 1335–1344.
- PUDEK, M. R. & BRAGG, P. D. (1974). Inhibition by cyanide of the respiratory chain oxidases of *Escherichia coli*. *Archives of Biochemistry and Biophysics* **164**, 682–693.
- REID, G. A. & INGLEDEW, W. J. (1980). The purification of a respiratory oxidase complex from *Escherichia coli*. *FEBS Letters* **109**, 1–4.
- RICE, C. W. & HEMPFLING, W. P. (1978). Oxygen-limited continuous culture and respiratory energy conservation in *Escherichia coli*. *Journal of Bacteriology* **134**, 115–124.
- SIVARAM, A., CHANCE, B. & CHING, Y. (1982). Low temperature photolysis and recombination kinetics of cytochrome *d* in *Pseudomonas* cytochrome oxidase. *Federation Proceedings* **41**, 1208.
- WHARTON, D. C. & GIBSON, Q. H. (1976). Cytochrome oxidase from *Pseudomonas aeruginosa*. IV. Reaction with oxygen and carbon monoxide. *Biochimica et biophysica acta* **430**, 445–453.
- WIKSTRÖM, M., KRAB, K. & SARASTE, M. (1981). *Cytochrome Oxidase: A Synthesis*, pp. 117–141. London: Academic Press.
- YAMANAKA, T. & OKUNUKI, K. (1963). Crystalline *Pseudomonas* cytochrome oxidase. III. Properties of the prosthetic groups. *Biochimica et biophysica acta* **67**, 407–416.
- YAOI, H. & TAMIYA, H. (1928). On the respiratory pigment, cytochrome, in bacteria. *Proceedings of the Imperial Academy (Japan)* **4**, 436–439.