The Reaction of Cytochrome *o* in *Escherichia coli* with Oxygen

LOW-TEMPERATURE KINETIC AND SPECTRAL STUDIES

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1. The reactions of cytochrome *o* in intact cells of aerobically grown *Escherichia coli* with O₂ and CO have been studied at low temperature. 2. Flash photolysis of CO-ligated cells in the presence of O₂ and at temperatures between –79 and –102°C results in the oxidation of kinetically heterogeneous *b*-type cytochromes (including cytochrome *o*), but not of cytochrome *d*. 3. The reaction of reduced cytochrome *o* with O₂ involves O₂ binding to give intermediate(s) with spectral characteristics similar to that of the reduced oxidase–CO complex. Observation in the α -region suggests that unexplained ligand dissociation accompanies the initial O₂ binding. 4. At temperatures below –98°C, an ‘end point’ in the reaction is reached; further reaction and oxidation of cytochrome *o* occurs on raising the temperature. 5. There is a linear relationship between the rate of formation of the oxygen compound and the O₂ concentration up to 0.5 mM. The second-order constant for its formation (k_{+1}) is 0.91 M^{–1}·s^{–1} at –101°C. The reaction is not readily reversible, the value of k_{-1} being 1.4 × 10^{–5} s^{–1} and the k_d 1.5 × 10^{–5} M. 6. The energy of activation for this reaction at low temperatures is 29.9 kJ (7.1 kcal)/mol. 7. The reaction with O₂ is distinguished from that with CO by the markedly lower velocity and high photolytic reversibility of the latter. 8. Comparisons are drawn between the intermediate(s) in the O₂ reaction of cytochrome *o* in *E. coli* and those identified in other bacteria and in the reaction of cytochrome *aa*₃ with O₂.

At least nine different cytochromes have been identified in *Escherichia coli*: two type-*c* cytochromes (*c*₅₅₀ and *c*₅₄₈), five type-*b* cytochromes (cytochromes *b*₅₅₆, *b*₅₅₈, *b*₅₆₂, *b*₅₅₆^{NO₃–} and *o*), cytochrome *a*₁ and cytochrome *d* (*a*₂) (for references, see Haddock & Jones, 1977). Cytochrome *o* can be identified spectrally as a type-*b* cytochrome, absorbing maximally at 556 nm in reduced-minus-oxidized difference spectra (Poole & Haddock, 1975), with the ability to bind CO (Castor & Chance, 1959), and, in addition, exhibiting a high affinity for cyanide (Pudek & Bragg, 1974). Stopped-flow dual-wavelength spectrophotometry at room temperature has shown the *b*-type cytochromes of *E. coli* to be kinetically heterogeneous, with one species (presumably cytochrome *o*) oxidized so rapidly that it could fully support observed oxidation rates (Haddock *et al.*, 1976). Cytochrome *o* is thus considered to be a functional terminal oxidase in this organism, accepting electrons from a further *b*-type cytochrome (perhaps cytochrome *b*₅₅₆ or *b*₅₆₂; Poole & Haddock, 1975; Haddock & Jones, 1977) or from a quinone species (Downie & Cox, 1978), and catalysing the reduction of O₂ to water. Very little is known of the function or chemistry of this oxidase.

There appears to be no convincing evidence for the involvement of copper in cytochrome *o* or other electron-transfer components in *E. coli* (Haddock & Jones, 1977; Lund & Raynor, 1975). A CO-binding *b*₅₅₆ (77 K) with a mid-point oxidation–reduction potential at pH 7 of +80 mV has been identified as cytochrome *o* by Reid & Ingledew (1979). A partial purification of cytochrome *o* from *E. coli* has been reported by Ingledew (1977). In contrast, cytochrome *o* from the filamentous myxomycete *Vitreoscilla* has been extensively studied and characterized. The purified enzyme consists of two identical polypeptide chains and two molecules of protohaem IX, but no non-haem iron, copper or flavin (Tyree & Webster, 1978a). Co-operative binding of CO suggests sub-unit interaction in the reduced cytochrome. Oxidized cytochrome *o* has two identical non-interacting binding sites for CN[–]. The two haem groups may be separately reduced with dithionite, having mid-point potentials of +115 mV and –125 mV respectively (Tyree & Webster, 1978b).

The importance of identifying and characterizing functional oxygen compounds of cytochrome oxidases has been emphasized by Chance *et al.* (1975a). The development of multichannel spectroscopy (Chance *et al.*, 1975b) and low-temperature trapping methods (Chance *et al.*, 1975c) for flash photolysis of the membrane-bound cytochrome

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oxidase-CO complex in the presence of O_2 has enabled this aim to be realized. Two spectroscopically and kinetically distinct species, A₁ and B, have thus been identified as intermediates in the reaction of the fully reduced cytochrome oxidase of pigeon heart mitochondria with O_2 (Chance *et al.*, 1975a,d). Formation of 'oxy' compound A does not involve electron transfer to oxygen between -125 and -105°C. Compound A is distinct from the CO compounds in (i) small but significant differences in the positions and intensities of its absorbance bands, (ii) very large differences in kinetic and equilibrium constants and (iii) photosensitivity. It is recognized as a functional intermediate by its inherent instability and conversion into compound B, a reaction involving oxidation of the haem and copper moiety of the reduced oxidase to form a peroxy compound. Above -60°C, B-type compounds serve as effective electron acceptors from cytochromes *a*, *c* and *c*₁.

The present paper reports on the application of the low-temperature techniques that have been so successful in elucidating the reaction with O_2 of cytochrome oxidase *aa*₃ to the terminal reaction of cytochrome *o* in intact cells of *E. coli*. Previous studies aimed at studying the mechanism of reaction of oxidases with O_2 in bacteria have used rapid scanning (Shimada & Orii, 1976) or stopped-flow spectroscopy at ambient temperatures (Greenwood *et al.*, 1978), or have exploited the remarkable stability of intermediates in the reaction with O_2 of cytochrome *o* from *Vitreoscilla* (Webster & Liu, 1974; Webster & Orii, 1977).

Experimental

Organism, growth conditions and preparation of cells

Escherichia coli strain A1002 (K12Y *mel ilv*⁻ *lacI*⁻ *metE*⁻), kindly supplied by Dr. B. A. Haddock (Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K.), was used. The organism was grown in the basic medium described by Poole & Haddock (1974). The mineral-salts solution used, however, was a modification of that of Pirt (1967) and contained, per litre: EDTA, 5g; FeCl₃, 0.5g; ZnO, 0.05g; CuCl₂, 0.01g; Co(NO₃)₆H₂O, 0.01g; H₃BO₃, 0.01g; (NH₄)₆Mo₇O₂₄·4H₂O, 0.01g. The carbon source was 40mM-sodium succinate. The medium was further supplemented with casamino acids (1g/litre) and isoleucine, valine and methionine (each at 0.02g/litre). MgCl₂·6H₂O (final concn. 1mM) and these three amino acids were sterilized separately by autoclaving and membrane filtration respectively, and then added aseptically to the bulk of the cooled, sterile medium. Starter cultures (20ml) in 100ml Erlenmeyer flasks were inoculated with a few drops of a stock culture that contained glycerol (12.5%, w/v) and had been stored at -20°C. After it had been shaken at 37°C for 16-

24h, the starter culture was used to inoculate 5 litres of the same medium in a 6-litre-capacity New Brunswick Laboratory Fermenter (New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.). Forced aeration was at 5 litres of air/min and a stirring rate of 400rev./min; the growth temperature was 37°C.

Cells were harvested in the late-exponential phase of growth when *A*₄₂₀ (10mm light-path; 1:10 dilution) had reached 0.3-0.5. Absorbance was measured with a Perkin-Elmer Coleman 124 DB spectrophotometer.

Cells were harvested by centrifugation in the 6×250ml HB-4 rotor of a Sorvall Superspeed RC2-B centrifuge. The rotor was operated for 10min at 4°C and 10 444g (*r*_{av.} 13.8cm). Cells were washed once in a medium that contained 50mM-Tris, 2mM-MgCl₂·6H₂O and 1mM-EGTA, adjusted to pH7.4 with 1mM-HCl, by resuspension and centrifugation under the above conditions. Finally, cells were resuspended to a final concentration of 10g (wet wt.) per 100ml of buffer, containing ethylene glycol at a final concn. of 30% (v/v). Such suspensions were stored at -30°C for up to 24h before use.

Further preparation of suspensions for kinetic studies

Reduction of cell suspensions was achieved by adding sodium succinate (25mM final concn.) to the suspension in a cuvette of 4 or 5mm light-path and incubation for 5min. The reduced-oxidase-CO complex was formed by bubbling the anoxic suspension with CO for 5min followed by a further 5min incubation at room temperature. The cuvette was cooled in an ethanol/solid CO₂ bath (-20 to -25°C) and allowed to equilibrate for 5min in the dark. The suspension containing the CO-liganded cells was then vigorously stirred with a coiled stainless-steel wire, closely fitting the cuvette. Stirring served to introduce oxygen at a concentration up to about 1mM. Stirring twice per second for 30s gave 360μM-O₂ (Chance *et al.*, 1975c). Unless otherwise specified, this was the O₂ concentration used. Thereafter, the cuvette was immediately placed in a freezing mixture at -78°C and was stored for up to 15min before use.

After equilibration at -78°C for 5-15min, the cuvette was transferred to the sample chamber of a dual-wavelength scanning spectrophotometer or multichannel dual-wavelength spectrophotometer. The cuvette was maintained at the temperature at which reaction kinetics were to be observed; illumination by the measuring light was avoided. After thermal equilibration and, where appropriate, the storing of the spectrum of the reduced-oxidase-CO complex in the computer memory of the spectrophotometer (Chance & Graham, 1971; Chance *et al.*, 1975a), the reaction with oxygen was initiated by photolysis of the sample with a 200J flash lamp.

Physical methods

A wavelength-scanning spectrophotometer, referencing the spectra to a fixed wavelength (generally 575 nm), was used to record the results shown in Figs. 4, 5, 6 and 10 below. In a digital memory could be stored the characteristics of a baseline, and from which could be subsequently read out corrective signals to the measuring wavelength, effectively subtracting the stored baseline from the incoming data.

The kinetics of absorbance changes (Figs. 2 and 3 below) were recorded with a multi-channel dual-wavelength apparatus (Chance *et al.*, 1975b).

Results

Cytochrome content of intact cells

In view of the diversity of qualitative and quantitative contributions to the cytochrome spectrum of *E. coli* grown under different conditions, we first established the identity of cytochromes present under our growth conditions. In contrast to previous results with cells grown aerobically on glycerol (Haddock *et al.*, 1976), significant amounts of cytochromes *a*₁ and *d* were detectable in reduced-minus-oxidized spectra of cells from vigorously aerated cultures (peaks in Fig. 1a at 593 and 627 nm). The peak in the α -band of the *b*-type cytochromes is observed at 560 nm, being shifted by 2–4 nm from the maxima observed at 77 K (Haddock *et al.*, 1976). Splitting of the α -band was not observed at –80 to –90°C. β - and γ -bands showed maxima at 530 and 428 nm respectively. Although cytochromes *a*₁ and *d* are detectable, the spectrum of aerobically grown cells is clearly distinct from that of cells grown anaerobically with glycerol and fumarate, the amounts of cytochromes *a*₁ and *d* being greater in the latter (Fig. 1b). The peaks in the α - (550–570 nm) and γ -regions are also somewhat broader in anaerobically grown cells, owing to contributions from *c*-type and multiple *b*-type cytochromes in the α -region and from *a*-type cytochromes in the γ -region. Flash photolysis of the reduced CO-liganded cells in the absence of O₂ and at temperatures that do not favour recombination of CO results in the formation of the reduced sample. The spectrum in Fig. 1(c) is that of the differences between such flash-photolysed cells and CO-liganded reduced cells. Troughs at 415, 531 and 567 nm thus represent absorption maxima of the reduced CO-complex. Cytochrome *d* (and perhaps *a*₁) binds CO under these conditions, but their complexes are not readily photolysed (see below), and so they are not observed in this type of spectrum. Spectrum 1(d) is that of a persulphate-oxidized sample minus the CO-reduced state.

Fig. 1. Difference spectra of *E. coli* grown aerobically and anaerobically

Cells were grown and harvested as indicated in the Experimental section. Reference spectra were stored in the digital memory of a scanning dual-wavelength spectrophotometer and subsequent spectra were corrected for the stored spectrum with 575 nm as a reference wavelength. The cells used, the type of difference spectrum recorded, the temperature of recording were as follows: (a) aerobically grown, succinate-reduced-minus-oxidized (by shaking), –84°C; (b) anaerobically grown (glycerol with fumarate), dithionite-reduced-minus-persulphate-oxidized, –96°C; (c) aerobically grown, flash-photolysed CO-reduced (i.e. reduced)-minus-CO-reduced, –110°C and (d) aerobically grown, persulphate-oxidized-minus-CO-reduced, –92°C. In all cases the cell concentration was about 100 mg (wet. wt. of cells)/ml.

Contribution of cytochrome *d* to kinetic measurements

The possible interference from cytochrome *d* oxidation in kinetic studies of cytochrome *o* was assessed in the experiment shown in Fig. 2. Events after flash photolysis of CO-liganded cells in the presence of O₂ were monitored at wavelengths appropriate to cytochrome *d* (608–630 nm) and *b*-type cytochromes (566–540 nm). A downward deflection in traces B and D indicates oxidation of cytochrome *b*, presumably cytochrome *o*, at –79 and –102°C. At the lower temperature, the half-time was calculated to be 6.9 min by using a Guggenheim analysis (Gutfreund, 1972) of the digitized data. (The correlation coefficient for the Guggenheim plot was 0.95.) In contrast (traces A and C), $A_{608-630}$ changed little over the period of observation. The insensitivity of the

Fig. 2. Measurements of reactions of cytochromes *b* and *d* after flash photolysis of CO-liganded cells in the presence of oxygen

Two experiments are illustrated, one at -102°C (a) and the other at -79°C (b). Time proceeds from left to right. A downward deflection indicates absorbance decrease and oxidation of the cytochromes. Traces A and C show the absorbance changes due to cytochrome *d* at 608–630 nm and traces B and D the changes due to cytochrome(s) *b* at 566–540 nm. The pathlength was 4 mm.

cytochrome *d*-CO complex to photolytic dissociation compared with other CO complexes was noted by Castor & Chance (1959), although such complexes are photodissociable, and flash photolysis has been used to study the binding of CO to *Pseudomonas* cytochrome oxidase (Parr *et al.*, 1975). For the present purpose its importance is that studies of cytochrome *o* are not frustrated by cytochrome *d*, particularly in the Soret region where their absorption bands are not well separated.

Kinetic heterogeneity of *b*-type cytochromes

Reaction of cytochrome(s) *b*, measured at 427–460 nm after flash photolysis of CO-liganded cells, at various temperatures, is shown in Fig. 3. At temperatures of -88°C and below, change in $A_{427-460}$ proceeds monophasically with apparent first-order kinetics over the period of observation. Half-times of the reaction were 4.2 min at -94°C (correlation coefficient of Guggenheim plot is 0.99) and 1.05 min at -88°C (correlation coefficient 0.98). At -67°C and more clearly at -58.5°C , the reaction was biphasic, with a first phase complete within 1 min of photolysis. At -58.5°C , the half-time of the second slower phase was 9.0 min (correlation coefficient 0.98). Resolution of two phases of cytochrome *b* oxidation (with half-times of 3 and 23 ms at room temperature) has been described by Haddock *et al.* (1976). Their conclusion, that this heterogeneity results from two discrete pools of cytochrome rather than a result of slowing introduced by the time constant of their instrument, seems

Fig. 3. Kinetics of the reaction of *b*-type cytochromes with oxygen at various temperatures

The reaction with oxygen of *b*-type cytochromes in CO-liganded cells was followed by monitoring absorbance changes at 427–460 nm. The reaction was initiated by flash photolysis at the moment shown and the reaction observed at -58.5°C (trace a), -67°C (trace b), -88°C (trace c) or -94°C (trace d). Time proceeds from left to right. An upward deflection indicates absorbance and oxidation of the cytochrome(s). The path-length was 4 mm.

to gain support from measurements at low temperature. An alternative interpretation, however, of the biphasicity of absorption changes at lower temperatures is that it reveals, in Fig. 3, intermediate steps in the reaction sequence of a homogeneous pool of cytochrome *b*.

Dual-wavelength scanning spectroscopy

Repetitive difference spectra, with reference to the spectrum of the reduced cytochrome *o*-CO complex, obtained after flash photolysis of CO-liganded cells in the presence of O_2 , are shown in Figs. 4 and 5. At -105°C the predominant spectral change is an increase in A_{415} , relative to the reference spectrum stored in the instrument memory, and a decrease in A_{432} (Fig. 4). These absorption changes towards the baseline that was recorded before flash photolysis (and thus representing a CO-reduced-minus-CO-reduced spectrum) are indicative of ligand binding. That this ligand is O_2 and not CO is demonstrated by comparison with ligand binding after photolysis in the absence of O_2 and thus when CO is the available ligand. CO binding at these temperatures is extremely slow; at -105°C the half-times for the apparent first-order kinetics of binding of O_2 and CO, measured at 432–444 nm, are 6.8 and 47 min respectively. Furthermore, the compound formed in the presence of O_2 is not significantly photolysed by repeated flashes (results not shown), whereas that formed in the absence of O_2 can be photolysed several

Fig. 4. Repetitive wavelength scanning of the reaction of cytochrome *o* with oxygen at -105°C

The spectrum of a suspension of CO-liganded reduced cells was scanned and stored in a digital memory of a dual-wavelength spectrophotometer. The reference wavelength was 575 nm. Subsequent scans are difference spectra with the stored spectrum subtracted. The first scan, before photolysis, yielded the baseline indicated by the broken line. The reaction with oxygen was initiated by flash photolysis; the numbering of the successive spectra is with reference to the first scan after the flash. Scanning proceeds from right to left at about 3.5 nm/s. Absorbance increments and the wavelengths (in nm) of distinctive features of the spectra are shown.

Fig. 5. Repetitive wavelength scanning of the reaction of cytochrome *o* with oxygen at -98°C

The experimental conditions and presentation of the results are identical with those in Fig. 4, except that the reaction was observed at -98°C .

minutes after initial photolysis to give a spectrum indistinguishable from the original (see Fig. 10 below). In the α -region, the absorbance difference between the photolysed sample and the baseline increases, suggesting ligand dissociation. The absorption minimum of the difference spectrum is shifted from 567 to about 564 nm. Little change occurs in the β -region of the spectrum centred at about 530 nm.

Between about -95 and -100°C the reaction with O_2 appears to reach an 'end-point'. At these temperatures, the binding of O_2 , observed as the approach of the difference spectrum at 432 nm to the baseline, is largely complete within 6 min (i.e. the first four scans of Fig. 5). An absorption trough at 428 nm, at the same wavelength seen in the fully oxidized-minus-CO-reduced difference spectrum (Fig. 1d), appears. The spectrum recorded 1 h after the last scan shown in Fig. 5 was indistinguishable from it. A similar phenomenon can be observed at 560–567 nm; again, absorbance changes at about 530 nm were small.

Further reaction beyond the 'end-point' of Fig. 5 is afforded by raising the temperature. At -79°C (Fig. 6), the peak in the difference spectrum at 432 nm is not seen within the first scan (complete within 90 s of photolysis). Instead, the prominent spectral feature is a broad trough with clearly resolved components at about 415 and 428 nm. The α -band absorption is also broad; the major contribution is from a species absorbing at 567 nm.

Plots of the absorbance change at 432 nm relative to the isosbestic point, 444 nm, reveal that at temperatures between -91 and -105°C the reaction with O_2 proceeds monophasically (Fig. 7) and with apparent first-order kinetics (logarithmic analyses not shown). At -98°C the reaction is seen to have reached the 'end-point'. However, at -107°C , and less markedly at -109°C (results not shown), the reaction kinetics are well fitted by two lines. Similar biphasic kinetics were observed in three independent experiments after photolysis at temperatures between -106 and -108°C . At -113°C , the rate and extent of absorbance change is too small to allow discrimination between linear and non-linear kinetics.

Dependence of the oxygen reactions on temperature

Arrhenius plots for the observed absorbance changes at 432–444 nm (between -91 and -113°C ; obtained by dual-wavelength scanning spectroscopy) and at 427–460 nm (between -67 and -94°C ; data of Fig. 3) are shown in Fig. 8. Best fit to the data at 427–460 nm gives an apparent energy of activation of 33.2 kJ (7.7 kcal)/mol (correlation coefficient, $r = 0.974$), and the best fit to the data at 432–444 nm gives a value of 38.5 kJ (9.2 kcal)/mol ($r = 0.963$). However, a satisfactory fit ($r = 0.969$) can be made to the combined data, yielding an apparent energy of activation of 29.9 kJ (7.14 kcal)/mol. It therefore

Fig. 6. Repetitive wavelength scanning of the reaction of cytochrome *o* with oxygen at -79°C . The experimental conditions and presentation of the results are identical with those of Fig. 4, except that the reaction was observed at -79°C .

Fig. 7. Kinetics of reaction of reduced cytochrome *o* with oxygen

The spectrum of a suspension of CO-liganded reduced cells was scanned and stored in the digital memory of a dual-wavelength spectrophotometer (reference wavelength 575 nm). The reaction with oxygen was initiated by flash photolysis. Subsequent scans showing the course of the reaction have subtracted from them the stored spectrum. The absorbance, measured at 432–444 nm in successive scans, is shown plotted as a function of time after photolysis, and observed at the four temperatures shown.

seems appropriate to identify the faster phases of the reactions shown in Fig. 3 (and used in the Arrhenius plot) with the reactions of cytochrome *o* identified by scanning spectroscopy.

Dependence of the reaction on oxygen concentration

By adding various amounts of O_2 with the stirring technique, it is possible to obtain and analyse the kinetics of formation of the first compound formed with O_2 . The O_2 concentration is taken to be that at the moment of freeze-trapping. The reversible equilibrium for the combination of cytochrome *o* and O_2 at low temperatures can be written as:

where A represents an intermediate in which oxygen is bound to the oxidase, and where B represents a further product that may be a second intermediate or the oxidized enzyme. The second-order constant for the forward reaction and an approximation of the

Fig. 8. The apparent energy of activation for the reaction of cytochrome *o* with oxygen

The Arrhenius plot incorporates data from two types of experiments. Data indicated by the solid circles were obtained at 427–460 nm in the multichannel apparatus as described in the legend to Fig. 3; velocity constants were calculated from Guggenheim plots of the initial phase of the reaction with oxygen after flash photolysis. Open-circle data were obtained at 432–444 nm from repetitive wavelength scans as described in the legend to Fig. 4. In these cases, the biphasic kinetics observed at -107°C ($10^3/\text{temperature} = 6.02 \text{ K}^{-1}$; Fig. 7) precluded the use of Guggenheim analyses. Consequently, half-times were measured from semilogarithmic plots of the reaction progress and were used to calculate velocity constants, assuming first-order kinetics. The slope corresponds to 29.9 kJ (7.14 kcal)/mol.

first-order constant for the reverse reaction are given by the plot of Fig. 9 of the pseudo first-order velocity constant against the O_2 concentration on a linear scale. K_{+1} corresponds to $0.91 \text{ M}^{-1} \cdot \text{s}^{-1}$ at -101°C . The reaction is not readily reversible, the value of k_{-1} being $1.4 \times 10^{-5} \text{ s}^{-1}$ and k_d $1.5 \times 10^{-5} \text{ M}$.

Reaction of cytochrome *o* with CO

Photolysis of samples that were trapped at -78°C when anoxic allowed the recombination of the reduced oxidase with CO, rather than its reaction with O_2 , to be studied. The reaction with CO at -92°C is shown in Fig. 10. At temperatures below this, the reaction was so slow as to make detailed analysis of the recombination problematic. The reaction is distinguished from that with O_2 in at least three ways. First, the velocity of the reaction is greatly decreased by comparison with the O_2 reaction at a

Fig. 9. Dependence on oxygen of the velocity of the oxygen reaction of cytochrome *o*

The reaction of cytochrome *o* with oxygen was studied by using repetitive wavelength-scanning spectrophotometry as described in the legend to Fig. 4, but at -101°C . Velocity constants were calculated from half-times measured in semilogarithmic plots of absorbance changes in successive spectral scans. The O_2 concentration was varied by stirring in various amounts of O_2 . The kinetic plot allows the determination of k_{+1} (forward velocity constant/ O_2 concentration, the slope of the line), an estimate of k_{-1} (the velocity when the O_2 concentration is zero, the intercept) and the equilibrium constant $k_d (= k_{-1}/k_{+1})$. The line shown is that of best fit and the correlation coefficient (r) is 0.71.

given temperature [compare Fig. 10 (CO ; -92°C) with Fig. 5 (O_2 ; -98°C)]. Secondly, observation of the reaction with CO in the α -, β - or γ -regions of the spectrum illustrates ligand binding, i.e. a tendency for successive scans to approach the baseline spectrum of the liganded enzyme. Thirdly, absorbance changes in the γ -region are symmetrical with respect to the baseline, changes at 415–444 nm and at 432–444 nm being equal in magnitude but in opposite directions. In contrast, and as Fig. 4 shows, the reaction with O_2 is characterized by more marked changes at 432–444 nm (and 428–444 nm) than at 415–444 nm. The kinetics of recombination with CO are shown in Fig. 11 analysed in a semilogarithmic plot. At 415–444 nm the absorbance changes appear biphasic, whereas at 432–444 nm the data are well-fitted by a line indicating apparent-first-order kinetics.

Discussion

The 'CO-binding pigment', cytochrome *o*, was first described in a variety of bacteria, including *E. coli*, and was shown to account for the major portion of the light-sensitivity of respiration in exponentially growing cultures of this organism, maxima being observed at 416, 535 and 566 nm in photochemical action spectra (Castor & Chance, 1959). However,

Fig. 10. Repetitive wavelength scanning of the reaction of cytochrome *o* with CO at -92°C

The spectrum of a suspension of CO -liganded, reduced cells, frozen without stirring in O_2 , was scanned and stored in the digital memory of a dual-wavelength spectrophotometer. The reference wavelength was 575 nm. Subsequent scans are difference spectra, the stored spectrum having been subtracted. The first scan, before photolysis, yielded the baseline indicated by the broken line. In the absence of O_2 , trace 1, the first after photolysis, is essentially the reduced-minus- CO -reduced difference spectrum. Successive scans, examples of which are shown (3, 5, 7, 9, 22) were at 95 s intervals. Absorbance increments and the wavelengths (in nm) of distinctive features of the spectra are shown.

Fig. 11. Kinetics of reaction of reduced cytochrome *o* with CO at -92°C

The absorbance changes measured at 415–444 nm (○) and at 432–444 nm (●) after flash photolysis of a CO-liganded sample (Fig. 10) are shown.

cytochrome *d* is present together with cytochrome *o* in aerobically grown cells in somewhat variable amounts (Pudek & Bragg, 1974; Haddock *et al.*, 1976; the present paper) and may confound studies of the terminal reactions of the electron-transport chain with oxygen, since both oxidases are capable of supporting significant proportions of the respiration (Castor & Chance, 1959; Haddock *et al.*, 1976). The present work confirms the early observation that cells containing cytochrome *d* are relatively insensitive to light (Castor & Chance, 1959) and enables the method of flash photolysis to be exploited in a study of the reactions of cytochrome *o* (and no other oxidase) with oxygen.

The 'triple-trapping' (Chance *et al.*, 1975c) methodology that has unequivocally identified functional intermediates in the reaction of the membrane-bound (Chance *et al.*, 1975a,d) and solubilized (Chance *et al.*, 1978) cytochrome oxidase *aa₃* with O_2 has been successfully adapted to study the oxygen reaction of cytochrome *o* in *E. coli*. Thus flash photolysis of the CO-liganded oxidase in intact cells and in the frozen state results in CO dissociation, and the subsequent binding of O_2 , followed ultimately by valence changes and oxidation of the cytochrome.

An early intermediate in the reaction of the oxidase with O_2 is an oxygen compound with spectral properties similar to that of the CO-liganded species that may be described as compound A. This designation does not imply a structural, spectral or functional equivalence to the compound A formed by reaction of cytochrome *aa₃* with O_2 , although the two intermediates exhibit certain similarities. Evidence identifying compound A as the primary intermediate is afforded by kinetic data. There is a linear relationship between the O_2 concentration and the rate of formation of compound A up to a pseudo-first-order constant of $5 \times 10^{-4} \text{ s}^{-1}$ at -101°C .

Flash photolysis in the absence or presence of O_2 at the lowest temperatures used here results in identical difference spectra (relative to the spectrum of the reduced CO-liganded oxidase). The consequent combination with O_2 differs from that with CO in the following ways. First, the CO-liganded oxidase is readily photodissociable, whereas the O_2 compound is resistant to photolysis by the intensities of light used, in keeping with the lower quantum yield for photolysis of O_2 compounds (Gibson & Ainsworth, 1957). Secondly, the velocity of recombination of the reduced oxidase with O_2 is much higher than the recombination with CO (compare Figs. 7 and 11). Unlike the CO compound, the O_2 compound is capable of further reaction(s) to yield the oxidized cytochrome, and is thus functional in electron transport. A marked feature of the spectral studies is the asymmetry of changes in A_{415} and A_{432} relative to the isosbestic point at 444 nm. During the reaction with CO, absorbance changes at these wavelengths proceed at equal rates, whereas the reaction with O_2 is characterized by slow absorbance changes at 415–444 nm relative to the changes at 428–444 nm and 432–444 nm. The species contributing to these absorbance changes remain to be identified.

The spectral changes occurring in the α - and β -regions have not been entirely explained. Although in the γ -region the spectrum of the presumptive O_2 adduct approaches that of the baseline (representing the CO-liganded species), the accompanying α -absorption changes occur in the opposite sense, i.e. away from the baseline. A simple explanation may be that the spectrum of the O_2 adduct is similar to the CO complex in the γ -region, but resembles more closely the spectrum of the oxidized enzyme in the α -region. Alternatively, the α -changes may reflect simultaneous dissociation of O_2 (rapidly) or CO (slowly), perhaps at a second binding site of different affinity. Indeed, the binding to the reduced enzyme of CO (Fig. 11) and O_2 (Fig. 7) is biphasic when observed at appropriate wavelength pairs and temperature.

Complexity in the kinetics of ligand binding to haem proteins is well documented, multiphasic kinetics having been described for the reactions with

CO of *Pseudomonas aeruginosa* cytochrome oxidase (Wharton & Gibson, 1976), detergent-solubilized cytochrome oxidase aa_3 (Sharrock & Yonetani, 1977), myoglobin (Austin *et al.*, 1975) and carboxy-methylated cytochrome *c* and haem *c* octapeptide (Alberding *et al.*, 1978) and, of particular interest in the present study, protohaem (Alberding *et al.*, 1976). The different rebinding processes for ligands have been ascribed to the ligand encountering successive barriers during its passage to the binding site.

Comparison of the oxygen reaction of cytochrome *o* with that of cytochrome oxidase aa_3 shows the former to have a lower apparent energy of activation. Also, the product of the cytochrome *o* reaction, like that formed in *Pseudomonas* (Greenwood *et al.*, 1978) does not appear to be a highly dissociable compound. Since the temperature coefficients of first-order reactions (such as the dissociation of compound A to give O_2 and reduced oxidase) usually exceed the value of several kilojoules for diffusion-controlled reactions, at higher temperatures the dissociation constant would tend to increase above the measured value of $1.5 \times 10^{-5} M$ (at $-101^\circ C$). Nevertheless the value is likely to remain three orders of magnitude lower than that described for the oxygen reaction of cytochrome aa_3 . The rapid conversion of compound A to compound B in the latter has been proposed to act as a kinetically effective trap, so that the oxygen is rapidly converted into an undissociable product. In *E. coli* the requirement for trapping oxygen in an undissociable compound may be necessitated by the low velocity of reaction of the oxidase with O_2 , which is $0.91 M^{-1} \cdot s^{-1}$ at $-101^\circ C$; the corresponding velocity constant for the membrane-bound cytochrome aa_3 at a comparable temperature ($-94^\circ C$) is $685 M^{-1} \cdot s^{-1}$.

A variety of electronic structures may be written for the intermediate oxygenated compound formed in the reaction of cytochrome *o* with O_2 , but their description is not justified on the basis of results described in the present paper. A feature of the cytochrome *o* system in *E. coli* is its apparent simplicity, due in part to the absence of copper (at least in an e.p.r.-detectable form; Lund & Raynor, 1975) and the ability to obviate *a* and *c*-type cytochromes by restoring functional electron-transport activity in particles from a haem A^- -deficient mutant by incubation with haematin and ATP (Haddock & Jones, 1977). However, the experimental results are consistent with the initial formation of an oxygenated intermediate with spectral properties similar to the CO compound. A comparison of the CO and O_2 compounds for various haemoproteins (e.g. haemoglobin and myoglobin; Chance *et al.*, 1975a) shows that consistency in the position of the absorption bands is possible. In contrast, other compounds have been identified as oxygenated intermediates, on account of their distinctive spectra, resembling neither

the oxidized nor the reduced species. Such compounds have been described at the haem *d* moiety of nitrate reductase of *P. aeruginosa* (Shimada & Orii, 1976), in the reaction of *E. coli* cytochrome *d* with O_2 and cyanide [d^* (Pudek & Bragg, 1974, 1976)] and in the filamentous myxobacterium *Vitreoscilla*.

The oxygenated form of cytochrome *o* in *Vitreoscilla* is far more stable than that described in the present paper. Half-times for the disappearance of the oxygenated form of the solubilized oxidase are of the order of hours at room temperature (Webster & Liu, 1974). Observations of this intermediate as the predominant steady-state form of the cytochrome in respiring intact cells (Webster & Orii, 1977) and its replacement by the reduced form on anoxia (Webster & Orii, 1978) argue that the compound has a functional physiological role in cyclic changes between the fully oxidized and reduced forms. In this system, too, it has been proposed that the nature of the oxygen binding may be structurally similar to that in oxymyoglobin and oxyhaemoglobin, because of the similarities of the visible absorption spectra, the infrared oxygen-stretch band (Choc *et al.*, 1978) and resistance to autoxidation (Webster & Liu, 1974; Liu & Webster, 1974). Most recently, an earlier intermediate has been trapped at $0^\circ C$ in which 1 mol of cytochrome binds $\frac{1}{2}$ mol of oxygen; the haem remains in the ferrous state. Oxygen is proposed to catalyse the further conversion of this 'compound I' to the oxygenated compound (in which two haem groups bind one oxygen molecule) and thence to oxidized cytochrome and peroxide anion (Tyree & Webster, 1979).

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