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LOW-TEMPERATURE SPECTRAL AND KINETIC PROPERTIES OF CYTOCHROMES IN *ESCHERICHIA COLI* K-12 GROWN AT LOWERED OXYGEN TENSION

ROBERT K. POOLE [a,*], ROBERT I. SCOTT [a] and BRITTON CHANCE [b]

[a] *Department of Microbiology, Queen Elizabeth College (University of London), Campden Hill, London W8 7AH (U.K.) and* [b] *Johnson Research Foundation, Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104 (U.S.A.)*

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Summary

Escherichia coli K-12 was grown in batch culture in a medium containing succinate as carbon source, supplemented with casein hydrolysate, and with a rate of oxygen supply that resulted in dissolved O_2 tension falling to 10% of saturation in the latter stages of growth. Cytochromes in such cells were qualitatively indistinguishable from those present in cells grown under conditions of vigorous aeration where dissolved O_2 tension remained greater than 80% saturation. Spectra recorded at 77 K and their fourth-order finite difference analyses revealed the absence of cytochrome *b*-558 and only low concentrations of cytochromes a_1 and $d(a_2)$. At low temperatures, the reaction of cytochrome *o* with O_2 in intact cells, grown under lowered O_2 tension, proceeds through the same stages as observed previously in cells grown with vigorous aeration (Poole, R.K., Waring, A.J. and Chance, B. (1979) Biochem. J. 184, 379—389). However, much higher temperatures are required for comparable progress of the reaction in cells grown at lowered O_2 tensions. At −91°C, the reaction with O_2 involves ligand binding to give intermediate(s) with spectral characteristics similar to those of the reduced oxidase-CO complex. Temperatures of approx. −79°C are required for the observation of biphasic kinetics and the attainment of an 'end point' in the reaction, features that are seen at temperatures below −98°C in cells from vigorously-aerated cultures. At −32.5°C, oxidation of cytochrome *o* is observed. The energy of activation for this reaction at low temperatures is 29.9 $kJ \cdot mol^{-1}$. Binding with CO, in

* To whom correspondence should be addressed.

contrast to binding with O_2, is characterized by high photolytic reversibility and appears to be less affected by the degree of aeration of cells during growth.

Introduction

The adaptive nature of the cytochrome system of microorganisms was first reported in batch culture as long ago as 1950 [1—3]. However, such changes were first quantified with respect to dissolved oxygen concentrations by Moss [4,5] who, using continuous culture techniques in conjunction with the rotating platinum electrode, showed that in both *Escherichia coli* and *Klebsiella aerogenes* maximal amounts of cytochrome a_1 and *d* were synthesized at low O_2 tensions.

Subsequently, the synthesis of cytochrome *d* in *E. coli* and other bacteria has been shown to be stimulated when the availability or utilization of O_2 is restricted [6—9]. These results have been confirmed where growth conditions and O_2 availability have been rigorously controlled in continuous culture [10,11]. Similarly-elevated levels of cytochrome *d* occur when cells enter the stationary phase of growth [12—14] and it is tempting to speculate that, under these poorly-defined growth conditions, low oxygen availability, rather than cessation of growth per se, leads to the quantitative changes in cytochrome content. In *E. coli*, the synthesis of cytochrome *o*, a terminal oxidase, also able to support most of the observed respiration, does not appear to be so dramatically regulated and has been described as constitutive [15]. In *K. aerogenes* also, the cytochrome *o* level is relatively invariant, being independent of the O_2 supply during growth [10].

The above studies have been directed to describing quantitative changes in the redox carriers under different growth conditions but little attention has been paid so far to describing cytochrome function under conditions of low aeration. Using low-temperarure trapping and flash photolysis techniques, we [16,17] have recently studied in detail the reaction of cytochrome *o* with O_2 and CO in *E. coli* grown aerobically under conditions of vigorous aeration.

In the present paper, we describe the growth of *E. coli* under conditions of low aeration, poorly characterized before now. We show that such cells have slightly elevated levels of cytochrome *d* and contain cytochrome *o* that is spectrally indistinguishable from that in vigorously-aerated cells. However, in low-temperature kinetic studies of the reaction with O_2 of cytochrome *o*, the temperature at which formation of an early intermediate occurs and at which cytochrome oxidation occurs are shifted by over 20° C to higher temperatures. We conclude that the effects of varying growth conditions are complex, influencing not only the stoichiometries of respiratory chain carriers, but also the functional characteristics of individual components.

Methods

Organism, growth conditions and low temperature kinetic studies

E. coli K-12 (strain A1002) was grown exactly as described by Poole et al. [17], except that the efficiency of aeration of the culture was decreased by

stirring the fermenter contents at 700 rev./min and by passing air only into the air space above the culture in the fermenter at 4 l/min rather than sparging the medium. For low-temperature kinetic studies, cells were harvested in the early stationary phase of growth when A_{420nm} (10 mm light path; 1/10 dilution) had reached 0.4. Cells from 5 l (approx. 14 g wet wt.) were harvested, resuspended in buffer plus 30% (v/v) ethylene glycol and otherwise treated exactly as described previously [17].

Difference spectra at 77 K

Difference spectra [18] were obtained at liquid N_2 temperatures using a Pye Unicam SP 1700 spectrophotometer fitted with an accessory constructed at Queen Elizabeth College. This consisted of a small Dewar flask of which the lower 3 cm was silvered (Day-Impex Ltd., Station Works, Station Road, Earls Colne, Essex). A brass cuvette holder, the lower end of which was immersed in liquid N_2 in the Dewar, held two small (0.5 ml capacity, 2 mm path length) cuvettes about 3 cm from the photomultiplier. The cuvettes were individually removable from the holder. Cell suspensions were pipetted into the cuvettes which were then frozen by immersion in liquid N_2 and inserted into the cuvette holder. Condensation in the Dewar was eliminated by a rapid stream of air blowing over its outer surfaces. Reduction of samples was with succinate and oxidation by H_2O_2 as described in the legend to Fig. 3. Reduced + CO minus reduced spectra were obtained by bubbling a succinate-reduced cell suspension with CO for 5 min and recording the spectrum after a further 2 min. Molar absorption coefficients used for converting absorbance measurements in such spectra to cytochrome concentrations were those collected from several published values and given by Haddock et al. [19]. The concentration of cytochrome *o* was estimated from the absorbance at 568—551 nm in reduced + CO minus reduced spectra, in which region there was no interference from cytochrome *d*. The absorbance coefficient was that given by Daniel [20] for $A_{417-432nm}$, divided by the ratio of the Soret peak: α peak heights. This value, 13, was measured from the first spectrum after flash photolysis (e.g. Fig. 6) but at −90°C, at which temperature rates of CO recombination are negligible.

Numerical analysis of 77 K spectra. The analogue output from the spectrophotometer to the chart recorder was interrogated by means of a digital voltmeter (Solartron LM 1420-2) under the control of a data logger unit (Solartron Schlumberger) which transferred absorbance values to a Facit paper tape punch (Solartron Schlumberger 3245) at 0.2 nm intervals. To improve the signal-to-noise ratio, four successive scans were summed for each spectrum. No baseline corrections were made.

The paper tape was analysed by a Modus Modular One computer (Computer Technology Ltd., Hants.) programmed to prepare finite order difference spectra by using the algorithm of Butler and Hopkins [21]. The fourth-order finite difference spectrum was plotted on an X-Y recorder under computer control.

Noise levels in the method were estimated by computing a fourth-order finite difference spectrum of two cuvettes containing fully-reduced cell suspensions. The peak-to-peak noise levels are indicated in Figs. 2B and 3B by vertical bars at either end of the finite difference spectra. Wavelengths are quoted to the nearest 0.5 nm to indicate reproducibility but a precision of 1 nm is a more

realistic estimate of that obtainable with this instrumentation.

Analytical methods. Cell numbers and volumes were determined using a Coulter Counter Model Z_{B1} and Channelyzer C1000 [22].

Respiration rates of culture samples (used either undiluted or diluted with fresh growth medium) were made in a Clark-type O_2 electrode (Rank Brothers, Bottisham, U.K.) at 37°C. Cyanide was added (final conc. 150 μM) to cell suspensions adjusted by dilution to respire at 14 to 29 nmol $O_2 \cdot min^{-1} \cdot ml^{-1}$. The progress of inhibition was analysed by plotting the logarithm of respiration rate against time [14] and expressing inhibition as the time required for 50% inhibition of the initial rate.

Oxygen tension in the growth vessel was continuously monitored using an ethylene oxide-sterilized Mackereth electrode (silver cathode, lead anode; EIL, Hanworth Lane, Chertsey, Surrey) inserted in the culture.

The rate of oxygen transfer to the medium was measured in the absence of biomass using sodium sulphite with cupric ions as catalyst. The principle of this method is that the rate of oxidation of the sulphite is limited only by the rate of oxygen transfer (K_L a) from gas to liquid [23]. Residual sulphite in samples removed at 5 to 30 min intervals from the vessel was determined by iodometric titrations with thiosulphate [24].

The concentration of succinate in the growth medium was assayed enzymically [25] after removal of the cells by centrifugation of a culture sample for 5 min at room temperature in a bench centrifuge. The pH of the same supernatants were measured using a long-reach combination electrode (Activion Glass Ltd., Fife, U.K.).

Results

Comparison of growth of E. coli under conditions of lowered oxygen tension and of vigorous aeration

Fig. 1 shows the growth of *E. coli* in batch culture with 40 mM sodium succinate as carbon source and with an oxygen transfer rate of 12.3 mmol $\cdot l^{-1} \cdot$

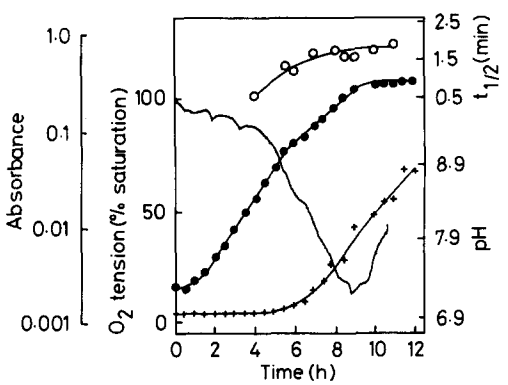

Fig. 1. Growth of *E. coli* in succinate-containing medium under conditions of lowered O_2 tension. The variables shown are: absorbance at 420 nm, after dilution of culture 1 : 10 (•———•); inhibition of O_2 uptake by 150 μM KCN, expressed as the time required (in min) for 50% inhibition of respiration rate (○———○); pH of the culture supernatant (+———+); dissolved O_2 tension (———).

h^{-1}, obtained by stirring at 700 rev./min. Dissolved oxygen tension drops from 100% saturation at the time of inoculation to about 85% in the first 4 h of growth and then decreases more rapidly to reach a minimum value of 10% saturation after 9 h. During this period, culture turbidity (A_{420nm}) increases in a biphasic fashion. The mean doubling time is 60 min between 1.5 and 5.5 h after inoculation and increases to 105 min before the onset of the stationary phase. Cell numbers (not shown) closely follow the increase in turbidity: a gradual decrease in mean cell volume (not shown) occurs between 5.5 and 9 h after inoculation. The rate of O_2 uptake increases continuously during growth and reaches a maximum as growth of the culture ceases. This respiration becomes progressively more resistant to inhibition by 150 μM KCN. The identity of the growth-limiting nutrient or environmental condition that prevents further growth is not known; at the onset of the stationary phase, the succinate concentration is in excess of 12.5 mM (results not shown). In the absence of pH control, the rapid rise in culture pH that starts after 5 h growth may contribute to inhibition of further growth. These growth conditions are referred to as conditions of 'lowered O_2 tension'. Cells used in subsequent experiments were harvested after 9 to 11 h in the early stationary phase of growth when the cell population had reached 3 to $4 \cdot 10^9$ bacteria per ml. When the O_2 transfer rate is increased to 54.2 mmol $\cdot l^{-1} \cdot h^{-1}$ by increasing the stirring speed to 1100 rev./min and other culture parameters held constant, the growth of the culture is indistinguishable from that shown in Fig. 1 except for two variables. Under these conditions, O_2 tension remains above 80% saturation throughout the period of growth, falling gradually to this level during the first 9 h of growth and then rising to 90% as the stationary phase is reached. The increase in resistance of respiration to inhibition by 150 μM CN^- occurs earlier and faster than when the O_2 tension is allowed to fall more rapidly (Fig. 1). These growth conditions are those used in our previous work [16,17] and are referred to subsequently as conditions of 'vigorous aeration'.

Cytochromes detectable in difference spectra recorded at 77 K

The succinate-reduced minus oxidized difference spectrum (α and β regions) of intact cells harvested after growth under conditions of lowered O_2 tension is shown in Fig. 2A. Absorption bands in the α region due to cytochrome a_1 (590 nm) and cytochrome *d* (628 nm) were visible but are not shown in Fig. 2. These cells also contain a small component absorbing at about 503 nm [26] that has been tentatively identified as coprotetrahydroporphyrin [27]. The only peaks clearly resolved are at 528 and 556 nm representing the β and α bands, respectively, of *b* and *c*-type cytochromes. The shape of the spectrum suggests considerable heterogeneity, the complexity of which is revealed by fouth-order finite difference analysis (Fig. 2B). In the α region, major peaks are observed at 542 nm (not labelled), 545.5 to 548, 553.5, 555.5 and 563 nm. Peaks at these or very similar wavelengths have also been reported by Shipp [13] who, in addition, described a major component at 560 nm. A minor peak present in our spectra (Fig. 2B) may correspond to this. The β band is resolved into major components at 520.5, 528, 535 and 537 nm (not labelled).

The bands observed in the α region have not been rigorously identified although several workers [13,28,29] have proposed that three *b*-type cyto-

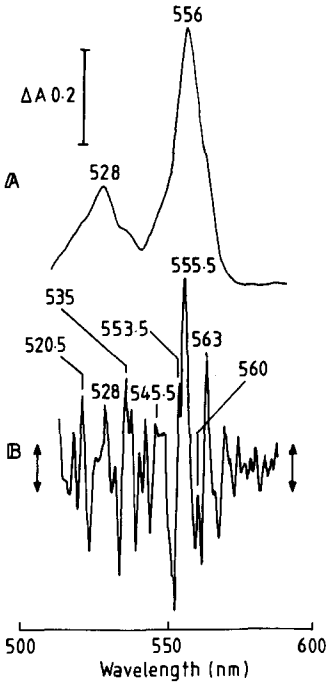

Fig. 2. Reduced minus oxidized difference spectrum (A) of *E. coli* grown at lowered oxygen tensions and its numerical analysis (B). The spectrum in A is the sum of four spectra, each obtained by oxidizing the contents of the reference cuvette with H_2O_2 and reducing that of the sample cuvette with 2.5 mM succinate for 2 min, before freezing the cuvettes and recording the spectrum at 77 K. The fourth-order finite difference spectrum (B) was computed using differencing intervals of 1.8, 1.4, 1.2 and 1.0 nm. Spectra were scanned at 0.2 nm · s^{-1} at a spectral bandwidth of 1.0 nm. The cell suspension contained 45 mg total protein · ml^{-1}.

chromes contribute to this region. The important point is that numerical analysis of spectra of cells grown under lowered O_2 tension does not reveal cytochrome *b*-558, which has been regarded as characteristic of an 'anaerobic-type' respiratory chain [7,15] and which is clearly resolved in numerical analysis of spectra of succinate-grown cells maintained anaerobically in the absence of an alternative electron acceptor (Scott, R.I. and Poole, R.K., unpublished data).

Higher derivative analysis of the Soret absorption bands (Fig. 3A) of *E. coli* have not been reported previously. Fig. 3B shows the presence of several components with absorption maxima at 418, 425 and 430.5 nm and a broad band extending from 438, through 441 to about 443 nm. The latter band is indicative of *a*-type cytochromes, and is perhaps composed of contributions from cytochromes a_1 and *d*. The latter, however, has little absorption in the Soret region ([12], Poole, R.K. and Chance, B., unpublished data).

Spectra corresponding to those in Figs. 2 and 3 but obtained with cells grown with vigorous aeration are not presented, being qualitatively identical to those shown. Concentrations of all cytochromes were somewhat higher in cells grown under lowered O_2 tension, the values, expressed as nmol per mg of total cell protein, being 0.041 (*d*), 0.253 ('b_1') and 0.014 (*o*). Corresponding values

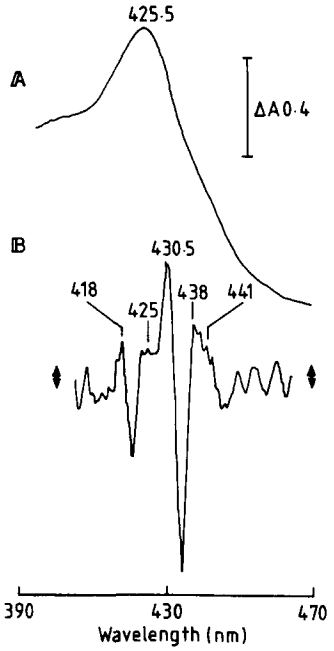

Fig. 3. Reduced minus oxidized difference spectrum (A) of *E. coli* grown at lowered oxygen tension and its numerical analysis (B). The conditions were those described in the legend to Fig. 3 except that the Soret band was scanned and the differencing intervals used were 3.0, 2.6, 2.4 and 2.2 nm.

for cells grown with vigorous aeration were 0.019, 0.157 and 0.010. Cytochrome a_1 was detectable in both cell types but at a concentration too low to quantitate.

Dual-wavelength scanning spectroscopy

Succinate-reduced cells in 30% (v/v) ethylene glycol were bubbled with CO for 5 min and after a further 5 min cooled to −25°C, at which temperature O_2 was stirred in to give a concentration of about 360 μM [30]. Thereafter, the cuvette was immediately placed in a freezing mixture at −78°C and, after equilibration, transferred to the sample chamber of the dual-wavelength spectrophotometer and maintained at the temperature of observation. The spectrum of the reduced oxidase-CO complex was recorded (reference wavelength 575 nm), stored in the digital memory of the instrument and effectively subtracted from all subsequent scans. Thus, a second scan of the spectrum (before flash photolysis) gave a baseline (the CO-reduced minus CO-reduced difference spectrum) shown by the dashed line in Fig. 4. The reaction with oxygen was initiated by photolysis of the sample using a 200-J flash lamp. In Fig. 4, the reaction progress at −91°C is shown. The first scan after photolysis approximates to the reduced minus CO-reduced difference spectrum and shows a peak at 432 nm and troughs at 415 and 567 nm. The β band (trough at about 431 nm) is not shown. Successive scans at about 90-s intervals reveal decreases in the intensities of bands at 415 and 432 nm and a deepening of the trough at

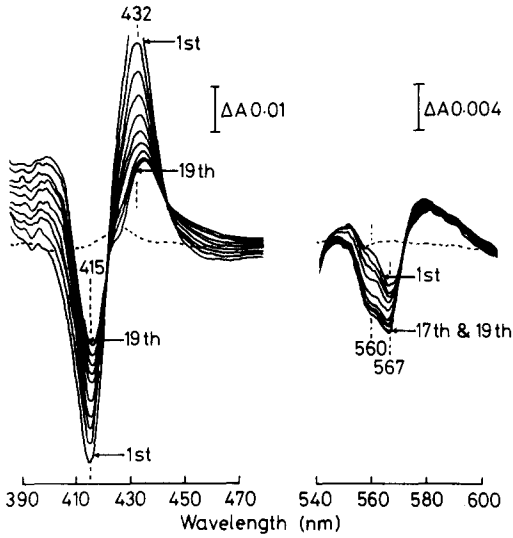

Fig. 4. Repetitive wavelength scanning of the reaction of cytochrome *o* with oxygen at —91°C. The spectrum of a suspension of CO-liganded reduced cells was scanned and stored in the digital memory of a dual-wavelength spectrophotometer. The reference wavelength was 575 nm. Subsequent scans are difference spectra with the stored spectrum subtracted. Before photolysis, a scan yielded the baseline, indicated by the dashed lines. The reaction with oxygen was initiated by flash photolysis; numbering of the successive scans (only alternate ones being shown) is with reference to the first scan after the flash. Scanning proceeds from right to left at about 3.5 nm · s^{-1}. Absorbance increments and the wavelengths (in nm) of distinctive features of the spectra are shown.

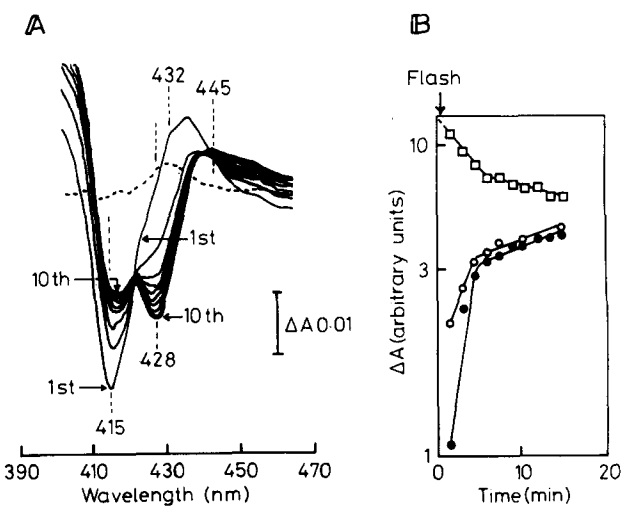

Fig. 5. The reaction of cytochrome *o* with oxygen at —79°C. In A, the repetitive wavelength scanning experiment was performed exactly as described for Fig. 4 but the temperature was —70°C. B shows the absorbance changes plotted as a function of time (plotted on a logarithmic scale) measured at 415—445 nm (□———□), 432—445 nm (○———○) and 428—445 nm (●———●). The reaction was initiated by flash photolysis at the time shown.

567 nm with the formation of a shoulder at 560 nm. These spectral changes appear to be identical with those observed at temperatures between −105°C and −110°C in cells from vigorously-aerated cultures [16,17]. Similarly, at −79°C (Fig. 5A), the reaction progress is similar to that seen at −98°C in cells from vigorously-aerated cultures. Semi-logarithmic analysis of the reaction measured at three appropriate wavelength pairs shows the reaction with O_2 to be biphasic (Fig. 5B). The slower phase (5—16 min) leads to the formation of a compound whose spectrum remains for about 1 h indistinguishable from spectrum 10 in Fig. 5A. A similar phenomenon is observed at −98°C in cells from vigorously-aerated cultures (see Figs. 5 and 7 in Ref. 17).

Photolysis of samples that were trapped at −78°C when anoxic allowed the recombination of the reduced oxidase with CO, rather than with O_2, to be studied. The reaction with CO shown in Fig. 6 is at −79°C and is thus directly comparable with the data of Fig. 5. In contrast to previous results [17], the rate of CO binding (half-time measured from semi-logarithmic plot in Fig. 6B; 10.5 min) is not markedly different from that of the faster phase of the O_2 reaction (half-time measured at 415—444 nm from Fig. 5B; 9.8 min). In agreement with previous results, however, the absorbance changes at 415—444 nm and 432—444 nm are symmetrical with respect to the baseline and are thus distinguished from the reaction with O_2 (Figs. 4 and 5). Furthermore,

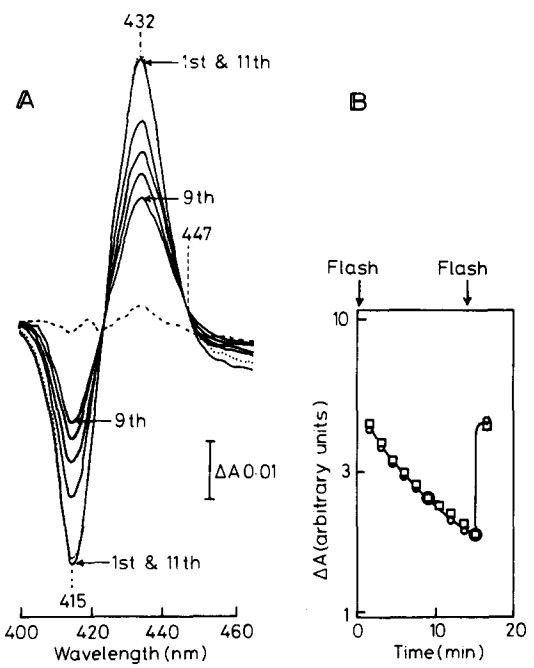

Fig. 6. The reaction of cytochrome *o* with CO at −79°C. In A, the repetitive wavelength scanning experiment was performed exactly as described for Fig. 5 but the temperature was −79°C and no O_2 was stirred into the CO-liganded cell suspension before freezing. B shows the absorbance changes plotted as a function of time (plotted on a logarithmic scale) measured at 415—447 nm (□———□) and 432—447 nm (○———○). The reaction was initiated by flash photolysis at the time shown by the first flash. Between the 10th and 11th scans (labelled, and shown by a dotted line in A), a second flash was given.

the reaction with CO is readily reversible by light. In Fig. 6, the reaction was initiated by six flashes of a 200-J lamp before the first scan. A further six flashes after the 10th scan resulted in the appearance of a spectrum in scan 11 that was qualitatively (Fig. 6A) and quantitatively (Fig. 6B) similar to the first.

The progress of the reaction with O_2 at −51°C (not shown) is similar to that observed at −79°C in cells from vigorously-aerated cultures [17]. Raising the temperature to −32.5°C is required for formation of the 428 nm and 561 nm troughs (characteristic of the oxidized minus CO-reduced difference spectrum) within the first scan (results not shown). It is of interest that, in these cells, but not in cells from vigorously-aerated cultures, the formation of an additional distinct trough at 503 nm is seen.

Dependence of the oxygen reactions on temperature

An Arrhenius plot (not shown) for the observed absorbance changes at 415—445 nm during the reaction of cytochrome *o* with O_2 at −99°C to −79°C gave an apparent energy of activation of 23.1 kJ/mol (correlation coefficient, $r = 0.995$). The Arrhenius plot for this reaction in cells from vigorously-aerated cultures, observed at 432—444 nm and at 427—460 nm [17], and at 415—444 nm (Poole, R.K., unpublished data), gives an apparent energy of activation of 29.9 kJ/mol. Both values are large and thus may represent the sum of more than one reaction.

Discussion

When *E. coli* is grown under conditions of vigorous aeration in the presence of a non-fermentable carbon source like succinate, the membrane-bound redox carriers include ubiquinone-8 and cytochromes *o*, *b*-556 and *b*-562 [15,31,32]. The absorption maxima given refer to spectra at 77 K, the 562 nm component being usually observed as a shoulder. Our previous studies of the low temperature reactions of cytochrome *o* with O_2 and CO were performed with such cells.

A major alteration to the aerobic electron transport chain involves the additional synthesis of cytochrome *b*-558 and *d*. Co-ordinate synthesis of these two components has been observed under the following growth conditions: during the late exponential or stationary phases of aerobic batch cultures growing on non-fermentable carbon sources [13,14], during aerobic growth on succinate in the presence of low concentrations of CN^- [7] and during sulphate-limited growth in continuous culture [34]. Although the growth conditions used here have some features in common with the above (growth to stationary phase, limited availability of oxygen), we have found no evidence for cytochrome *b*-558 in these cells. Thus, the cytochrome *o*, the kinetics of which are presented in Figs. 4, 5 and 6, is a component of a respiratory chain bearing more resemblance to the 'aerobic type' [15] rather than to the chain found anaerobically or under the associated conditions described above. The results of the present work, therefore, demonstrate that growth conditions greatly modify not only the qualitative and quantitative cytochrome content of the respiratory chain, as has been amply demonstrated before [4—11,35—37], but

also the functional and kinetic properties of these components, in this instance cytochrome *o*. While the effects described here were observed under conditions of lowered aeration, a more detailed study in a chemostat poised at different dissolved oxygen tensions is required to identify oxygen as the causal factor [10,11]. We have not measured the redox potentials of the growing cultures [10]. Recently, the *b*-type cytochromes present in *E. coli* grown aerobically or anaerobically have been shown to be potentiometrically distinct [29].

The cells grown under conditions of lowered O_2 tension described here contain significant amounts of cytochrome *d*. Its concentration is increased 2.2-fold over that found in vigorously-aerated cells, whilst the concentration of 'cytochrome b_1' (the α bands of *b* and *c*-type cytochromes) is increased 1.5-fold and cytochrome *o* 1.4-fold. However, we cannot comment on the reactions of cytochrome *d* in the two cell types, since we have been unable to detect in the absence of O_2 dissociation of its CO complex by the intensities of lights used in either cell type [16,17]. This relative insensitivity to photolytic dissociation was noted by Castor and Chance [12] although *d*-CO complexes are photodissociable and flash photolysis has been used to study the binding of CO to *Pseudomonas* cytochrome oxidase [38]. The sequence of spectral changes observed during the reaction of reduced cytochrome *o* with O_2 is similar to that observed previously in vigorously-aerated cells and will not be further discussed here. Further evidence for one or more functional intermediates in the reaction of bacterial cytochrome *o* with O_2 comes from studies of cytochrome *o* from the myxobacterium *Vitreoscilla* [39], the early work on *Acetobacter* by Iwasaki [40] and, most recently, the studies of Yang and Jurtschuk on *Azotobacter* [41].

The outstanding feature of the reactions of cytochrome *o* in cells grown at lowered oxygen tension is the higher temperature ranges required for the sequential stages of its reaction with O_2. In general, temperatures approx. 20°C higher are required than those used for cells grown under conditions of vigorous aearation [16,17]. In contrast, the characteristics of CO binding appear to be altered little (Table I). It is possible that the cytochrome(s) *o* synthesized under the two growth condition studies are separate haemoproteins with similar spectral properties but different ligand-binding properties. Interestingly, evidence for two forms of cytochrome *o* in *Acetobacter* was obtained also by

TABLE I

RATES OF LIGAND BINDING BY CYTOCHROME *o* IN *E. COLI*

Reaction of cytochrome *o* in intact cells with either CO or added O_2 (360 μM) was initiated by flash photolysis. Rates of binding, expressed as half-times in min were obtained from semi-logarthmic analyses of the absorbance change at 415 nm relative to 444 nm at —91°C.

Ligand binding	Cells grown under conditions of	
	Vigorous aeration	Lowered O_2 tension
O_2	7.9, 15.2 *	26.9
CO	17.4, 29.2	34.0

* Logarithmic analysis of absorbance changes at this wavelength reveal biphasic kinetics [17]. Biphasic kinetics in cells grown at lowered O_2 tension were observed only at higher temperatures (—79°C).

Daniel [20], who identified two species of *o* (absorbing at 558 and 565 nm CO difference spectra at 77 K) with O_2 affinities differing 10-fold but C affinities differing only 2-fold.

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