The Light-reversible Binding of Carbon Monoxide to Cytochrome a_1 in *Escherichia coli* K12

By ROBERT K. POOLE,^{1*} ROBERT I. SCOTT¹
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.*

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Difference spectra at 77 K of intact *Escherichia coli* K12 grown under oxygen-limited conditions revealed the presence of cytochrome a_1 . In the presence of CO, the band of the reduced form, observed in both the α and γ regions of the spectrum, was decreased. Dual-wavelength scanning spectrophotometry at sub-zero temperatures revealed a flash-dissociable CO-binding pigment with a broad band around 595 nm, identified as cytochrome a_1 . Photolysis in the presence of O_2 revealed no such band in difference spectra where the reference spectrum was that of the CO-liganded form, a result consistent with the binding of O_2 to cytochrome a_1 . Repeated cycles of photolysis and recombination of the cytochrome with CO were demonstrated at -46°C . The apparent energy of activation for the reaction with CO was $10.9\text{ kcal mol}^{-1}$ (45.6 kJ mol^{-1}). The results are discussed in relation to previous assumptions and results regarding ligand binding to cytochrome a_1 and the function of this cytochrome in bacterial respiration.

INTRODUCTION

Cytochrome a_1 was first described in bacteria about 50 years ago. The studies of Kubowitz & Haas (1932) and Warburg *et al.* (1933) showed that *Acetobacter pasteurianum* contained an autoxidizable, CO-binding respiratory pigment spectrally distinguishable by its absorption maximum in the reduced form (589 nm; Kamen & Horio, 1970) from the cytochromes now designated as b , c and c_1 . The introduction by Chance (1953*a, b*) of double-beam spectrophotometry enabled him to obtain refined CO difference spectra and photodissociation spectra of the CO complexes of various cytochrome oxidases: he showed that the CO complex of cytochrome a_1 differed from that of cytochrome a_3 and that the CO photodissociation spectrum of cytochrome a_1 agreed closely with the photoaction spectrum obtained by Kubowitz & Haas (1932) for the respiratory enzyme of *A. pasteurianum*. Confirmation of the role of cytochrome a_1 as a terminal oxidase in this system came from more-refined photochemical action spectra (Castor & Chance, 1955, 1959).

However, a similar role for cytochrome a_1 in other bacteria has not been established (Smith *et al.*, 1970; Weston & Knowles, 1973). In photochemical action spectra of CO-inhibited respiration of *Escherichia coli*, bands attributable to cytochromes o and d (a_2), but not a_1 , were observed (Castor & Chance, 1959). In the stopped-flow dual-wavelength spectrophotometric experiments of Haddock *et al.* (1976), the cytochrome a_1 of anaerobically (xylose) grown *E. coli* was shown to react with O_2 with a half-time of 25 ms, too slow for it to be considered kinetically competent to act as a terminal oxidase. Rice & Hempfling (1978) observed no differences in the K_m values of *E. coli* for O_2 when cells possessing

cytochromes *o*, *d* and a_1 were compared with those possessing only cytochromes *o* and *d*. The role of cytochrome a_1 in *E. coli* remains a mystery, but despite the paucity of knowledge on its composition, ligand-binding properties and the divergence of published values for its mid-point potential (e.g. Hendler & Shrager, 1979; Reid & Ingledew, 1979), it is frequently depicted in schemes of the respiratory chain (Hendler & Shrager, 1979; for review, see Bragg, 1979) and described as an oxidase (Jurtshuk *et al.*, 1975). It is widely assumed to bind CO (e.g. Trutko *et al.*, 1978) as does the corresponding cytochrome in certain other bacteria such as *Acetobacter* (Castor & Chance, 1959; Meyer & Jones, 1973). Haddock *et al.* (1976) were cautious in their interpretation of CO difference spectra in concluding that 'the concentration of cytochrome a_1 is low and it is difficult to determine if it binds CO'. However, a more recent investigation of cytochrome a_1 in *E. coli* by Reid & Ingledew (1979) described the ineffectiveness of CO in influencing the behaviour of this cytochrome in redox titrations and it was concluded that cytochrome a_1 does not bind CO, ruling out the possibility that this pigment is an oxidase in *E. coli*.

This paper presents results of low-temperature spectrophotometric studies which show that cytochrome a_1 of *E. coli* grown under conditions of oxygen limitation does bind CO and that the binding is reversible by white light. We do not, however, conclude that this necessarily identifies cytochrome a_1 as a terminal oxidase.

METHODS

Organism and growth conditions. *Escherichia coli* K12 (strain A1002) was grown under oxygen-limited conditions in batch culture. The defined medium contained 40 mM-sodium succinate as carbon source and was supplemented with Casamino acids (Poole *et al.*, 1979*a*). For each experiment, two starter cultures (each 20 ml in 250 ml Erlenmeyer flasks) were incubated unshaken for 24 h at 37 °C before inoculation into 10 l of medium. The culture was slowly stirred by a magnetic stirrer, but not sparged with air; stirring was sufficient only to create a stable vortex extending to about one-fifth of the depth of the culture and to prevent sedimentation of the cells. The rate of oxygen transfer to the medium was measured in the absence of biomass using Na_2SO_3 with Cu^{2+} 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 (Pirt, 1975). Residual sulphite in samples removed from the vessel at 5 to 30 min intervals was determined by iodometric titration with thiosulphate. Typically, the oxygen transfer rate was about $5 \text{ mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$.

Cells were harvested after 24 h growth when the cell count was about 8×10^8 bacteria ml^{-1} and the A_{420} (10 mm light path; Perkin-Elmer-Coleman 124 DB spectrophotometer) had reached 0.3 to 0.5. Such cultures were oxygen-limited (R. I. Scott & R. K. Poole, unpublished results). Cells were harvested, washed and resuspended as described by Poole *et al.* (1979*a*), except that the final concentration was 0.10 g (wet wt) cells ml^{-1} . Except for the 77 K difference spectra shown in Figs 1 and 2, the cell suspension contained 30% (v/v) ethylene glycol.

Recording of difference spectra by split-beam spectrophotometry. Difference spectra were obtained at 77 K using the apparatus described by Salmon & Poole (1980). Fourth-order finite difference analysis of the spectra was also performed as described by these authors.

Dual-wavelength scanning spectrophotometry. Cell suspensions containing ethylene glycol were reduced with sodium succinate, bubbled with CO and cooled using the protocol described by Poole *et al.* (1979*a*). The Johnson Foundation dual-wavelength scanning spectrophotometer (DBS-3) has been recently described by Scott *et al.* (1981) and Bashford *et al.* (1980). A full description of the earlier instrument on which it is based is given by Chance & Graham (1971).

RESULTS AND DISCUSSION

Identification of cytochrome a_1 in 77 K difference spectra

Figure 1 shows difference spectra of intact cells grown under conditions of oxygen limitation. At 77 K, the α absorption peak of the reduced *minus* oxidized spectrum (Fig. 1*a*) can be resolved into at least two components (556 and 558 nm). The β band is complex; two of the multiple components (R. I. Scott & R. K. Poole, unpublished results) are visible at 529 and 535 nm and the presence of a third is suggested by the shoulder at lower wave-

Fig. 1

Fig. 2

Fig. 1. Difference spectra recorded at 77 K of *E. coli* grown under conditions of oxygen limitation. In (a), the contents of the sample cuvette were reduced with dithionite before freezing, whilst the contents of the reference cuvette were oxidized by addition of a drop of '20-volume' H_2O_2 . In (b), a suspension of dithionite-reduced cells was split into two and one portion was bubbled with CO for 5 min before freezing; the difference spectrum is reduced + CO minus reduced. For both spectra, the scan rate was 2 nm s^{-1} and the bandwidth was 1 nm. The cell suspension contained $40 \text{ mg protein ml}^{-1}$. The vertical bar represents $0.1 A$ for (a) and $0.05 A$ for (b).

Fig. 2. Difference spectra recorded at 77 K and their numerical analyses. Spectra (a) and (c) are each the sum of six spectra obtained by oxidizing the contents of a reference cuvette with H_2O_2 and reducing the contents of the sample cuvette with dithionite and, in the case of (c), bubbling with CO for 5 min. Spectra (b) and (d) are the fourth-order finite difference analyses of spectra (a) and (c), respectively, and were computed using differencing intervals of 3.8, 3.4, 3.2 and 3.0 nm. Spectra (a) and (c) were scanned at 0.2 nm s^{-1} with a spectral bandwidth of 1.0 nm. The cell suspension contained $40 \text{ mg protein ml}^{-1}$. The vertical bar represents $0.2 A$ for (a) and (b) and $0.27 A$ for (c) and (d).

length. The characteristic absorption of reduced cytochrome d in the red region of the spectrum is seen at 626 nm; the deep trough at 649 nm is due to the absorption of the oxidized form (in the reference cuvette). The remaining feature of the α spectral region at 592 nm is attributed to cytochrome a_1 (Jurtshuk *et al.*, 1975). In the reduced + CO minus reduced spectrum (Fig. 1b), at least three clearly distinguishable CO-binding pigments are revealed. First, the cytochrome o -CO complex gives rise to the broad peaks at about 418, 539 and 574 nm, while the broad trough centred at 558 nm is attributed mainly to the disappearance of the reduced form of cytochrome o from the sample cuvette. The corresponding trough in the Soret region, expected at about 432 nm (Poole *et al.*, 1979a; Scott *et al.*, 1981), is probably masked by the intense bands from a (perhaps including d) type

Fig. 3. Dual-wavelength scanning spectrophotometry of cytochromes in *E. coli* grown under conditions of oxygen limitation. In (a), the spectrum of a suspension of anoxic CO-liganded, reduced cells was scanned and stored in a digital memory of the dual-wavelength spectrophotometer; the reference wavelength was 620 nm. Subsequent scans were difference spectra with the stored spectrum subtracted. The first scan, before photolysis, yielded the baseline indicated by the dashed line, whilst the continuous line is the first spectrum after flashing the sample 10 times with a 200 J lamp. In (b) the procedure was identical except that the cell suspension was supplemented with about 0.4 mM-O₂ before freezing. In (c), the fully oxidized *minus* reduced + CO spectrum is shown. The spectra of a CO-liganded, reduced sample and a ferricyanide-oxidized sample were individually recorded and stored in digital memories of the spectrophotometer and the resulting difference spectrum was plotted. The dashed line is the reduced + CO *minus* reduced + CO baseline. In (a), (b) and (c), the temperature was -58 °C and the scan rate was 1.24 nm s⁻¹. The cell suspensions contained about 0.1 g (wet wt) cells ml⁻¹. The vertical bar represents 0.002 A for (a) and (b) and 0.004 A for (c).

cytochromes. Secondly, cytochrome *d* is identified by the peak at 640 nm and the trough at 623 nm. Its contribution to the Soret region is very small (Chance, 1953*a*; Reid & Ingledew, 1980; R. K. Poole & B. Chance, unpublished results) so that the deep trough in Fig. 1 (b) at 445 nm is indicative of an additional *a*-type cytochrome. The trough at 596 nm is attributed to cytochrome *a*₁ and we propose that this component gives rise to much of the 445 nm trough. A trough at around 440 nm in CO difference spectra was attributed to cytochrome *a*₁ by Revsin & Brodie (1969) and Hendler & Nanninga (1970), but these authors did not demonstrate a corresponding α band to support this proposal. In Fig. 1 (b), no distinct band in the α region that could be attributed to the CO complex of cytochrome *a*₁ was observed, perhaps because, unlike cytochrome *a*₃, the spectrum of the reduced form of cytochrome *a*₁ is not substantially shifted by CO (Ingledew, 1977). The apparent band at 608 nm is due to the neighbouring troughs at 596 and 623 nm.

Further evidence for the binding of CO to reduced cytochrome a_1 is presented in Fig. 2. Fourth-order finite difference analysis was used to resolve more clearly the band of cytochrome a_1 at 592 nm in the reduced *minus* oxidized difference spectrum (Fig. 2*a, b*) and the lowered intensity of this band in the reduced + CO *minus* oxidized spectrum (Fig. 2*c, d*). The peak due to cytochrome d shifted 6 to 7 nm to the red on binding CO. Difference spectra presented by Pudek & Bragg (1974) indicate that reduced cytochrome a_1 does not react with 13.6 mM-KCN.

Flash photolysis of CO-liganded, reduced cells in the absence or presence of O₂

The reactions of cytochromes with O₂ and CO at sub-zero temperatures can be studied by the trapping methods devised by Chance *et al.* (1975*a*) for investigations of mitochondrial cytochrome c oxidase. The CO-liganded cytochrome is brought to the chosen experimental temperature within the spectrophotometer, whereupon its spectrum is scanned and stored in one of the digital memories of the spectrophotometer. Flash photolysis *in situ* dissociates the CO from the cytochrome and, at appropriate temperatures, the recombination of the cytochrome with CO can be monitored by dual-wavelength scanning spectrophotometry. A difference spectrum is plotted, the reference spectrum being that of the reduced + CO sample prior to photolysis (stored in the memory). Suspension of the cells in ethylene glycol allows O₂ to be introduced before freezing at temperatures (−20 to −30 °C) where it does not readily displace the bound CO (Chance *et al.*, 1975*a*; Poole *et al.*, 1979*a*). On photolysis, the reaction of the unliganded cytochrome with O₂ can be similarly studied. Such experiments are shown in Fig. 3.

Figure 3(*a*) shows the first spectrum after photolysis of the anoxic CO-liganded, reduced cells. The spectrum is a difference spectrum, the reference being the reduced + CO sample before photolysis. The broad troughs centred at about 535 and 570 nm are due to the CO complex of cytochrome o , as is the small peak above the baseline (reduced + CO *minus* reduced + CO; dashed line) at about 558 nm. We attribute the increased absorption, with respect to the baseline, centred at about 595 nm to photolysis of the CO complex of cytochrome a_1 . No such band was observed after photolysis of aerobically grown cells (Poole *et al.*, 1979*a*) which contain very low levels of a_1 , but a similar band was seen in cells grown anaerobically on glycerol/fumarate. Under these anoxic conditions, no bands attributable to cytochrome d were seen on the red side of the presumptive cytochrome a_1 peak. This observation has been consistently made, even at temperatures below −100 °C (Poole & Chance, 1980), and tentatively explained as reflecting the extremely rapid recombination of CO with cytochrome d . When O₂ was present (Fig. 3*b*), a trough centred at 640 nm was seen, attributed to an intermediate compound in the reaction of cytochrome d with O₂ (Poole & Chance, 1980), but no band assigned to cytochrome a_1 around 595 nm was seen. There are at least two explanations for this surprising observation. First, the oxygenation of the sample at about −25 °C during preparation may displace the bound CO, so that the spectra before and after photolysis are identical. Although mitochondrial cytochrome c oxidase remains tightly bound to CO during addition of O₂ under these conditions, preliminary data show that the occupancy of CO bound to cytochrome d in oxygen-limited *E. coli* is decreased on oxygenation (R. K. Poole, R. I. Scott & B. Chance, unpublished results). A second possibility is that the CO does remain bound to cytochrome a_1 during the oxygenation, is lost on photolysis, and that the reduced cytochrome binds O₂ to give a compound spectrally indistinguishable from the CO complex. The spectral similarity of the complexes that cytochrome o makes with O₂ and CO, respectively, has been demonstrated previously (Poole *et al.*, 1979*a, b*) and a comparison of the CO and O₂ complexes for various haemoproteins (e.g. haemoglobin, myoglobin, cytochrome c oxidase) shows that consistency in the position of the absorption bands is possible (Chance

Fig. 4. Light reversibility of the binding of CO to reduced cytochrome a_1 . The experiment was conducted as described in the legend to Fig. 3(a) except that the temperature was -46°C . The absorbance, measured at 595–585 nm, in successive scans recorded at 250 s intervals is shown plotted as a function of time. Each arrow shows the time of the last of 10 flashes of a 200 J lamp. The half-times of the apparent first-order recombinations are (from left to right) 10.3 min and 14.1 min. The cell suspension contained about $0.1\text{ g (wet wt) cells ml}^{-1}$. The dashed line represents the value of $\Delta A_{595-585}$ before photolysis.

et al., 1975*b*). The remaining features in Fig. 3(b) (troughs at 535 and 564 nm) are due to oxidation of *b*-type cytochromes at this temperature (Poole *et al.*, 1979*a*). Similar troughs were seen in the fully oxidized *minus* reduced + CO spectrum (Fig. 3*c*), which, in the region 570 to 670 nm, resembles the inverse spectrum shown in Fig. 2(c).

The progress of the reaction of reduced cytochrome a_1 with CO was studied by repetitive wavelength-scanning spectrophotometry (Fig. 4). After photolysis by 10 flashes of a 200 J lamp, CO recombined with cytochrome a_1 with apparent first-order kinetics. The half-time of the reaction calculated from a semilogarithmic plot of absorbance change versus time was 10.3 min. When approximately half of the cytochrome was recombined, reversibility of the binding was demonstrated by giving a further 10 flashes. The cycle of recombination and photolysis was again repeated, the half-times for the second and third recombinations being 12.3 min and 14.1 min, respectively. The amount of the a_1 -CO complex that was photolysed by the first 10 flashes was not markedly increased by the second set of flashes even though the latter was given when about half of the cytochrome was still uncombined with CO. The proportion of the total a_1 -CO complex that could be photolysed by giving 10 flashes was estimated at 24%. Under the same conditions (-100°C), 84% of the cytochrome *o*-CO complex was photolysed in cells grown under conditions of oxygen limitation (experiments not shown), whilst in aerobically grown cells 10 flashes gave over 95% photolysis (Scott *et al.*, 1981).

The apparent energy of activation for the reaction of cytochrome a_1 with O_2

The experiment shown in Fig. 4 was repeated over a range of temperatures to yield an Arrhenius plot (results not shown). In each experiment, only the first recombination reaction after photolysis was considered because of the increasing half-time of the reaction observed in successive cycles of photolysis and recombination (Fig. 4). The recombination was considered to be first-order, and half-times obtained from semilogarithmic plots of the reaction progress were used to calculate the velocity constants. Best fit to the absorbance changes at 595–585 nm gave an apparent energy of activation of $10.9\text{ kcal mol}^{-1}$ (45.6 kJ mol^{-1}), correlation coefficient $r = 0.96$. Extrapolation to room temperature (20°C) indicated a half-time for the reaction of 960 ms.

By extrapolating to other temperatures, it is possible to compare the measured rates of reaction of CO with cytochrome a_1 and the reaction of this ligand with other cytochromes. In such calculations, it is assumed that (a) the concentration of CO (about 1.2 mM ;

de Fonseca & Chance, 1979) greatly exceeds that of cytochrome a_1 , so that the reaction proceeds under pseudo-first-order conditions and (b) the binding of CO is essentially irreversible in the dark. At -91°C , the second-order rate constant for CO binding to cytochrome a_1 is $0.007\text{ M}^{-1}\text{ s}^{-1}$, compared with a value of about $0.3\text{ M}^{-1}\text{ s}^{-1}$ for cytochrome o in *E. coli* (data in Poole *et al.*, 1980); whilst at -95°C , the value for cytochrome a_1 ($0.003\text{ M}^{-1}\text{ s}^{-1}$) may be compared with $9.6\text{ M}^{-1}\text{ s}^{-1}$ for avian mitochondrial cytochrome a_3 (calculated from the data of Chance *et al.*, 1975*b*). Clearly, the rates of CO binding to cytochrome a_1 are very low compared with the rates of binding to cytochromes with established roles as terminal oxidases.

Conclusions

The data presented in this paper provide strong evidence for the binding of CO to cytochrome a_1 of *E. coli* grown under conditions of oxygen limitation and for the reversal of this binding by white light. Thus, if this cytochrome acts as an oxidase in *E. coli*, it should have been detectable in the photochemical action spectra of Castor & Chance (1959). We cannot rule out the possibility that CO is binding to inactive or denatured haemoprotein (Ben-Gershom, 1961).

It has been stated (Keilin, 1966) that there is no known example of a CO-binding pigment that does not also react with O_2 , and an attractive interpretation of the data in Fig. 3(b) is that cytochrome a_1 binds O_2 . However, the pigment can function as a terminal oxidase only if it is kinetically and thermodynamically competent to participate in electron flow to O_2 (Ingledeu, 1977).

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