EVIDENCE FOR A FUNCTIONAL OXYGEN-BOUND INTERMEDIATE IN THE REACTION OF *ESCHERICHIA COLI* CYTOCHROME *o* WITH OXYGEN

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1. Introduction

It is now well established that the primary events in the reaction with oxygen of reduced cytochrome oxidase aa_3 in higher eukaryotic cells involve the stepwise formation of three categories (A, B, C) of functional intermediates [1–3]. Compounds of type A are considered to be 'oxy' compounds of the ferrous haem, having the composition $a_3^{2+} \cdot O_2$. Compounds of type B are considered to be peroxide compounds ($Cu^{2+}\ a_3^{3+} \cdot O_2^{2-}$ or $\cdot O_2H_2$) or equivalent haem Fe–Cu peroxide bridge structures. Compounds of type C are formed from the mixed-valence oxidation.

The reactions with oxygen of bacterial cytochrome oxidases have been relatively poorly characterised. The remarkable stability of an oxygenated intermediate in the oxygen reaction of cytochrome *o* from the myxobacterium *Vitreoscilla* has been exploited, and has led to a description of its spectral properties [4], its role as the predominant form of the oxidase in intact cells in the aerobic steady state(s) [5] and, most recently, its formation from an earlier intermediate in which one molecule of oxygen is bound to a cytochrome *o* dimer [6].

This paper reports the application of the low-temperature trapping method [7], of greatest advantage in the study of the oxygen reaction of cytochrome aa_3 [1–3], to a bacterial terminal oxidase, namely cytochrome *o* in aerobically-grown *Escherichia coli*.

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2. Experimental

E. coli K12 (strain A1002) was grown at 37°C under conditions of vigorous aeration in a laboratory fermenter. The growth medium was a defined mineral salt medium, essentially that in [8], but supplemented with casamino acids (1 $g.l^{-1}$) and with 40 mM sodium succinate as carbon source.

Harvested cells were washed once with a buffer that contained 50 mM Tris–HCl, 2 mM $MgCl_2$ and 1 mM EGTA (pH 7.4) and resuspended in the same at 10 g wet wt cells/100 ml buffer, containing ethylene glycol (final conc. 30%, v/v). The suspension was reduced with 25 mM succinate; the ferrous cytochrome *o*–CO compound was formed by bubbling the anoxic suspension with CO for 5 min. After a further 5 min the cuvette was transferred to −20°C to −25°C at which temperature oxygen was stirred in. At this temperature, no ligand exchange with O_2 occurs [7]. The oxygen-supplemented, but CO-bound, cells were then cooled to −80°C and the reaction with O_2 initiated at the chosen temperature in the sample compartment of a dual-wavelength scanning spectrophotometer by flash photolysis. The spectrum of the reduced oxidase–CO complex could be stored in the digital memory of the spectrophotometer prior to photolysis and then subtracted from incoming data. In fig.1, spectra shown have subtracted from them the spectrum of the reduced oxidase–CO complex. Temperatures of observation used were appropriate to delineation of the profile of reactions of the oxidase with oxygen (−68°C to −121°C) and were maintained by a flow of cooled nitrogen gas over the cuvette and sample compartment.

Where indicated, the CO-ligand cells were imme-

diately trapped at −80°C without oxygenation so that subsequent photolysis afforded a study of the recombination of the oxidase with CO.

3. Results and discussion

Successive stages in the reaction of reduced cytochrome *o* with O_2 can be visualized when photolysis is performed over a range of temperatures. Each scan shown in fig.1 is the first recorded after flash photolysis at −121°C (a), −96°C (b), −88°C (c), −79°C (d) or −68°C (e). Scanning was from high to low wavelengths, each scan taking 85 s, so that the distinctive changes seen in the Soret region are those occurring ~70 s after initiation of the reaction at the indicated temperature. At −121°C the rate of reaction of the reduced oxidase with its oxidant (and especially with CO) is too slow to monitor. The difference spectrum (a) between the sample flash-photolysed in the presence of oxygen and the memory-stored spectrum of the reduced oxidase–CO compound is identical to that of the corresponding difference spectrum obtained in the absence of O_2. Troughs in this spectrum occur at 415 nm, 531 nm and 567 nm and are at wavelengths at which peaks are seen in conventional CO-reduced minus reduced difference spectra [9,10]. At each temperature between −115°C and −96°C, repetitive scans (not shown) recorded after photolysis show a successive decrease in the intensities of the peak at 432 nm and the trough at 415 nm so that the spectrum approached that of the baseline recorded before photolysis (i.e., the CO-reduced minus CO-reduced difference spectrum). These spectral changes are therefore indicative of ligand binding to the reduced oxidase. Following photolysis in the absence of oxygen, and thus during re-binding of CO, the spectral changes in the Soret region were qualitatively indistinguishable from those described above (decreases in the peak at 432 nm and the trough at 415 nm) but were much slower, half times at −105°C being 6.8 min in the presence of O_2 and 47 min in its absence. Further discrimination between the binding of O_2 and CO is afforded by their response to repeated flashes. In fig.2, the ligand binding that occurs in the absence of oxygen is fully reversible by a second flash given during the reaction. In contrast, the binding of

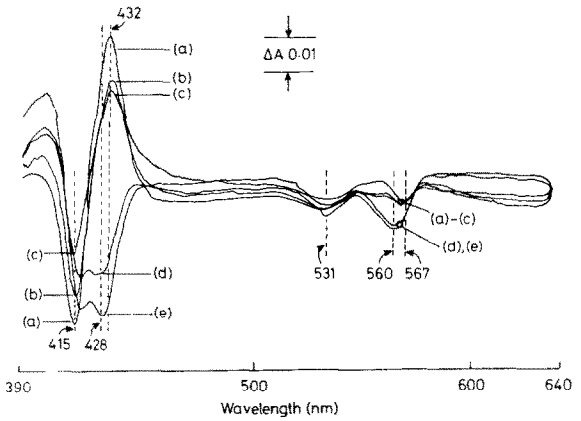

Fig.1. Difference spectra of the compounds formed following flash photolysis of the reduced cytochrome *o*–CO compound in intact *E. coli* in the presence of O_2 minus the CO-reduced sample. Five experiments are shown, in which the reaction was initiated and observed at: (a) −121°C; (b) −96°C; (c) −88°C; (d) −79°C; (e) −68°C. In each, flash photolysis occurs immediately before the start of the scan, which proceeds from right to left at a scanning speed of ~3.5 nm.s^{-1}. The pathlength was 4 mm in (a–c) and 5 mm in (d, e).

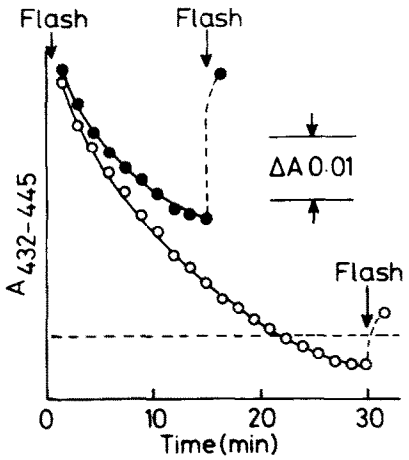

Fig.2. Kinetics of reaction of reduced cytochrome *o* with O_2 (○) or CO (●) and the effect of repeated flash photolysis. The reaction was initiated at the first flash (4 flashes of a 200 J lamp) by photolysis of the reduced oxidase–CO complex. Two further flashes were given at each of the two subsequent points shown. The temperature of reaction was −109°C ((○) O_2) or −79°C ((●) CO). The horizontal dashed line indicates the absorption of the CO-reduced complex prior to photolysis.

oxygen is characterised by only a small increase (~15% of the total change) in $A_{432-445}$. The phenomenon of the lower quantum yield for photolysis of oxygen compounds as compared with CO compounds is well known [11]. In summary, the reaction of oxygen with reduced cytochrome *o* yields a product with spectral properties similar to that of the CO compound, and is in this respect equivalent to cytochrome oxidase aa_3, myoglobin and haemoglobin; in all cases the CO and oxygen compounds exhibit similar absorbance bands. For this reason, a simple structure accepted for the oxy compounds of haemoproteins, $Fe^{2+} \cdot O_2$, appears plausible.

Scans subsequent to the first after photolysis (shown in fig.1b,c) indicate that the reaction progress results in the formation of a compound (not shown) at −96°C to −88°C in which the A_{432} max has disappeared, the trough at 415 nm has diminished in intensity, and a second trough has appeared at 428 nm. This compound, whose spectrum closely resembles that seen in the first scan at higher temperatures (e.g., −79°C; fig,1d), is stable at $\lesssim$ −90°C, having been observed unchanged in spectral character for >1 h at −96°C. Raising the temperature renders the compound unstable and the reaction sequence is reflected in the deepening of the 428 nm trough and the gradual disappearance of the 415 nm trough (fig.1d,e) to give ultimately the oxidized minus CO-reduced difference spectrum. Disappearance of the 432 nm peak and the appearance of the 428 nm trough is associated with a shift in the position of the trough in the α-region from 567 nm (characteristic of the reduced minus CO-reduced difference spectrum) to 560 nm (the position in oxidized minus CO-reduced difference spectrum). Thus, the intermediate formed from O_2 and the reduced oxidase, which is stable at < −98°C, is seen to be functional in electron transfer at higher temperatures.

The novel low-temperature trapping approach complements earlier studies of intermediates in the oxidase reactions in *Pseudomonas aeruginosa* [12,13] and the reaction of cytochrome *d* in *E. coli* that is presumed to occur via an intermediate *d** [14]. Detailed studies of the low-temperature kinetics of the reaction of *E. coli* cytochrome *o* with O_2 will be published elsewhere.

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