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Proposal that the function of the membrane-bound cytochrome a_1-like haemoprotein (cytochrome *b*-595) in *Escherichia coli* is a direct electron donation to cytochrome *d*

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The cytochrome *d*-containing oxidase of oxygen-limited *Escherichia coli* comprises cytochromes *d*, cytochrome *b*-558 and cytochrome *b*-595, previously called cytochrome a_1. The reaction of the fully reduced complex with oxygen involves ligand binding to the ferrous haem *d* to form an oxygenated species, followed by oxidation of two *b*-type cytochromes, whose identity is unclear. Here we report kinetic studies on cytochrome *b*-595 oxidation and suggest that these results, together with optical and EPR data on the oxidase complex and its reaction with oxygen, are consistent with the hypothesis that the role of cytochrome *b*-595 is further reduction of the oxygen bound to cytochrome *d*.

Cytochrome oxidase; Cytochrome *d* complex; Cytochrome a_1; Cytochrome *b*-595; Cytochrome *b*-590; (*Escherichia coli*)

1. INTRODUCTION

Escherichia coli synthesizes two haemoproteins [1] that superficially resemble 'cytochrome a_1' [2]. One of these (haemoprotein *b*-590) is soluble and has been purified from anaerobically grown cells. It does not contain haem *A*; its optical spectrum, including the distinctive α-band of the reduced form at 590 nm, is instead attributable to high-spin haem *B* [3]. It exhibits high catalase and peroxidase activities and is probably equivalent to HPI [4]. The second (cytochrome *b*-595) is a component of the purified cytochrome *d*-containing oxidase complex. Its optical spectrum is very similar [5] to that of haemoprotein *b*-590 and it too appears to lack haem *A* [6]. Its function is unknown.

In this paper, we describe the kinetics of cytochrome oxidation following formation of the oxygenated compound of cytochrome *d* in membranes at sub-zero temperatures [7]. We propose that cytochrome *b*-595 is an electron donor to cytochrome *d* and that this component contributes to the high-spin haem signals detected in EPR studies of the oxidase complex and its reaction with oxygen.

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2. MATERIALS AND METHODS

2.1. *Organism, growth conditions and membrane preparation*

E. coli K12, strain AN2342 (F^-, kindly provided by Professor F. Gibson), was grown in a medium containing per litre: K_2HPO_4 (10.6 g); NaH_2PO_4 (6.1 g); $MgSO_4$ (0.2 g); $(NH_4)_2SO_4$ (2.0 g); Luria broth [8] (50 ml); trace elements solution [9] (10 ml); glucose (35 mM). Starter cultures (1 l), inoculated from rich agar plates, were grown in baffled shake flasks for 9 h to provide a 10% in-

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oculum for a New Brunswick 14 l fermenter containing 10 l of the same medium. The air flow rate was 10 l/min and the stirring speed 300 rev/min. At 14 h, the air supply was reduced 5-fold and the stirring speed 2-fold to maximize induction of the cytochrome *d* complex. Cultures were harvested 3.5 h later using a Sharples continuous flow centrifuge. Membranes were prepared as described [10], using a buffer that contained 0.1 M Tes, 20 mM Mg^{2+}, 0.25 M sucrose and 0.25 mM EGTA (pH 7), supplemented with the protease inhibitors 6-aminohexanoic acid (40 mM) and *p*-aminobenzamidine (6 mM) [11]. Membranes were exhaustively washed [12] and stored at −20°C.

2.2. *Low temperature methods*

Membranes (approx. 5.6 mg protein/ml of buffer), supplemented with 30% (v/v) ethylene glycol were reduced in a cuvette of 2 mm pathlength by adding Na succinate (10 mM) and incubating for 25 min at 20°C. The suspension was bubbled with CO for 2 min and after 5 min cooled to −23°C in dry ice-ethanol for a further 5 min. Oxygen was introduced in the dark by 30 s stirring with a coiled wire that snugly fitted the cuvette and the sample was then plunged into dry ice-ethanol at −78°C. After temperature equilibration in the cryogenic sample compartment of the Johnson Foundation dual-wavelength scanning spectrophotometer [13], during which time the cuvette was shielded from reference and measuring beams, the reaction was initiated by photolysis using 2 min exposure to a 30 mW HeNe laser (model 3235H-PC; Hughes Aircraft Co., Carlsbad, CA).

3. RESULTS

Fig.1 shows optical spectra recorded after irradiation of the CO-liganded, ferrous cytochrome *d* with a monochromatic actinic source (632.8 nm), which photolyses selectively the carbon monoxy form of this oxidase (absorption maximum 636 nm) and not that of cytochrome oxidase *o* (nearest absorption maximum about 570 nm). As reported [7,14], the first scan after photolysis (fig.1a) shows a prominent peak near 650 nm, which has been attributed to the 'oxy' form of cytochrome *d* and probably corresponds to a species stable at room temperature, long held to be the oxidized (ferric) form. As the oxy form decays (decrease in absorbance at 647 nm, relative to the CO-liganded, reduced form), troughs develop at 592 and 558 nm, attributable to oxidation of cytochromes *b*-595 and *b*-558, respectively. These changes are accompanied by deepening of a trough at 430 nm (not shown), also due to cytochrome *b* oxidation. In these membranes, in contrast to previous results [7,14], there was little absorbance increase at 670 to 680 nm.

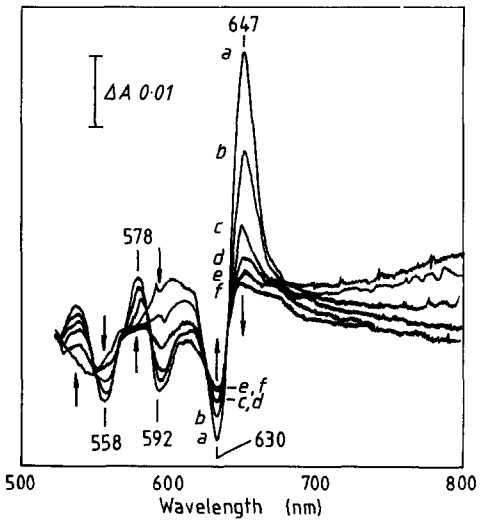

Fig.1. Repetitive wavelength scanning of the reactions at −100°C following formation of the oxy compound of cytochrome *d* in *E. coli* membranes. The spectrum of a suspension of reduced membranes, bubbled with CO, and to which O_2 was added at −25°C, was stored in the memory of the dual-wavelength spectrophotometer, with 570 nm as the reference wavelength. All subsequent scans were plotted with the stored scan subtracted. The reaction with oxygen was initiated by laser photolysis. Of 15 subsequent scans, those shown were commenced 0, 3, 19, 54, 79, and 126 min after the end of photolysis. The directions of absorbance changes with respect to the reference spectrum at wavelengths of interest are shown. The scan speed was 2.9 nm/s, the spectra band width 4 nm and the pathlength 2 mm.

4. DISCUSSION AND HYPOTHESIS

In a previous paper [15], EPR changes following photolysis in experiments similar to those described here were tentatively interpreted as revealing the sequential oxidation of cytochrome *d* and

at least one *b*-type cytochrome. These measurements were confined to high-spin species and the assignment of the axial and rhombic signals to components of the cytochrome *d* complex were based on previous assignments in *Azotobacter vinelandii* (see [2]) and compatible with other studies on *E. coli* where high-spin signals from the purified oxidase were detected [16,17]. Subsequently, Hata et al. [18] have shown that ferric cytochrome *d* is low-spin and suggested that a high-spin axial signal (*g* near 6) is attributable to cytochrome *b*-558, on the basis of its midpoint potential. The rhombic signals flanking the prominent axial signal were not identified, but it was suggested that they might arise from conformational changes in cytochrome *b*-558 or from solubilization. It is highly significant that these rhombic signals clearly develop before the axial signal (*b*-558) in low temperature kinetic experiments and that fig.1 shows that oxidation of cytochrome *b*-595 is an early event following laser photolysis of the carbonmonoxy cytochrome *d* complex. The EPR characteristics of the oxidase-associated or soluble cytochrome a_1-like haemoproteins have not been described in isolation, but there seems little doubt, based on the optical spectra and haem extractions, that both are high-spin *b*-type haemoproteins. The EPR signals from cytochrome *b*-595 have not been identified, but the rhombic signals at $g = 6.3$ and $g = 5.5$ are possible candidates.

The hypothesis that the rhombic high-spin EPR signals are from cytochrome *b*-595 and that this component is an immediate electron donor to cytochrome *d* is supported by, or consistent with, the following.

(i) By analogy with the reaction mechanisms of other terminal oxidases, it is likely that oxygen reduction will involve a bound peroxide intermediate and that this is further reduced by peroxidatic activity of the oxidase. Significantly, the soluble haemoprotein *b*-590 is indeed a hydroperoxidase [3] and although catalase activity of extensively washed membranes is negligible (Poole, R.K., unpublished), this may indicate that the membrane-bound cytochrome *b*-595 is reactive only towards bound peroxide.

(ii) The rhombic high-spin EPR signals described earlier resemble those of catalase [19] and peroxidase [20].

(iii) The cytochrome *b*-595 of the cytochrome *d* complex is not a contaminant or minor component of the preparation [21] but is in equistoichiometric amounts with *b*-558 [6] and is reproducibly observed in solubilized preparations [18].

(iv) Although values for the midpoint potentials of the rhombic signals have not been precisely determined, they are observable between 40 and 200 mV [18], consistent with the midpoint potential of *b*-595 (113 mV [5]).

(v) Similar behaviour of a cytochrome a_1-like component, being oxidized concomitantly with the decay of the oxy compound of cytochrome *d*, has been observed in *Acetobacter pasteurianus* [22].

(vi) The hypothesis provides a possible explanation of a band at 592 nm in the photochemical action spectrum of *E. coli,* attributed to 'cytochrome a_1' [23]. If cytochrome *d* were relatively CO-insensitive (as it is to cyanide [1,2]), or if its CO compound were more photosensitive than that of the a_1-like component, photolysis of the latter would be observed to stimulate respiration. A similar explanation has been advanced [24] to explain the appearance of a *c*-type cytochrome in the photochemical action spectrum of *Beneckea natriegens.* Although at low CO concentrations, cytochrome *d* has been claimed to be the only component of the oxidase complex that binds CO [6], there are unexpected complexities in the CO difference spectrum [6], namely a trough at 430 nm and a depression at 560 nm that remain unexplained.

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