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Formation of the 680 nm-absorbing form of the cytochrome *bd* oxidase complex of *Escherichia coli* by reaction of hydrogen peroxide with the ferric form

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Reduced minus aerated difference spectra of membranes from *Escherichia coli* (grown under oxygen-limited conditions) show, in addition to the 650 nm trough attributed to the oxygenated form of cytochrome *d*, a smaller trough centred at about 680 nm of unknown origin. When the reference spectrum is that of a sample oxidized with ferricyanide and to which hydrogen peroxide was added, the trough proportions changed, the 680 nm species being more dominant. Similarly, when 8.8 mM hydrogen peroxide is added to a persulphate-oxidized sample, a peak at 680 nm is immediately formed. No such compound is observed when peroxide is added to persulphate-oxidized membranes from a cytochrome *d*-deficient mutant. It is concluded that the 680 nm species represents a peroxy form of haem *d*, which is stable at room temperature and is probably an intermediate in the reaction mechanism of this oxidase.

Cytochrome *bd* oxidase complex; Spectroscopy; (*Escherichia coli*)

1. INTRODUCTION

Reduced minus aerated difference spectra of membranes from oxygen-limited *Escherichia coli* exhibit a number of features that have been assigned to components of the cytochrome *d*-containing oxidase complex [1,2]. The 630 nm band is due to haem *d* itself (a chlorin) [3]. The trough near 650 nm, although being described as the oxidized, i.e. ferric, form of cytochrome *d* for many years, is now attributed to the oxygenated or oxycytochrome *d* [4]. A broad trough at about 595 nm, previously ascribed to 'cytochrome a_1' is probably a high-spin *b*-type cytochrome, cytochrome *b*-595 [5], spectrally similar to haemoprotein *b*-590 purified from soluble fractions [6]. Only the 680 nm species remains enigmatic; it appears as a broad trough in reduced minus aerated difference spectra and has been studied in *Azotobacter vinelandii* [7], *Acetobacter pasteurianus* [8] and *E. coli* [9].

In the last two, it can be observed during the reaction sequence initiated at low temperatures by photolysis of the carbon monoxide-liganded ferrous form of cytochrome *d*. Decay of the first-formed oxy species is followed by the appearance (as a peak in difference spectra relative to the unphotolysed form) of the band at 680 nm [8,9]. Here we report that the 680 nm species can be formed in the reaction of peroxide with the oxidase complex and propose that the species represents a second, relatively stable intermediate in the reduction of 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 salts-Luria medium containing 35 mM glucose as carbon source [10]. Prior to harvest, the air

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supply to the fermenter was reduced 5-fold to 0.2 $l \cdot min^{-1} \cdot l$ culture^{-1} and the stirring speed to 150 rev/min to maximize induction of the cytochrome *d* complex. Membranes were prepared from Ribi-broken cells in the presence of 6-aminohexanoic acid and *p*-aminobenzamidine as protease inhibitors. These methods are reported fully elsewhere [10]. Strain AN2343 is an isogenic cytochrome *d*-deficient mutant (Downie, J.A., Gibson, F., Cox, G.B. and Poole, R.K., unpublished) which lacks signals due to cytochromes *d*, *b*-558 and *b*-595. The mutation maps close to the *cydC* locus [11].

2.2. *Spectrophotometric methods*

Membranes were suspended in 0.1 M K phosphate buffer (pH 7.0) and spectra recorded at 25°C in a Johnson Foundation dual-wavelength scanning spectrophotometer [10]. Protein concentrations were about 3 $mg \cdot ml^{-1}$.

3. RESULTS

Fig.1a is typical of reduced minus aerated ('oxidized') difference spectra of *E. coli* membranes in showing a trough at 650 nm due to the absorbance of oxycytochrome *d* in the reference sample. The shoulder at 680 nm has hitherto been unidentified, but is enhanced in difference spectra (fig.1b) where the reference is the spectrum of an H_2O_2-supplemented sample.

Titration with ammonium persulphate of native, i.e. untreated membranes from the wild-type strain elicited no distinctive signals (not shown), suggesting that cytochromes were largely in the oxidized form. Further addition of 8.8 mM hydrogen peroxide, however, immediately resulted in a distinctive difference spectrum (fig.2) with a prominent peak at 680 nm. A trough at 650 nm suggested the loss, on peroxide addition, of the oxygenated form. In a similar experiment, H_2O_2 was replaced with ethylhydrogen peroxide (donated by Dr Kurt Wahlund, Ferrosan, PO Box 839, Malmö 20180, Sweden) with similar results (not shown).

A dithionite-reduced minus ferricyanide-oxidized difference spectrum of membranes from the cytochrome *d*-deficient mutant (fig.3a) shows a cytochrome *b* (560 nm) but not the signals from the oxidase complex, at 595, 630, 650 and 680 nm (see fig.1). The addition of hydrogen peroxide did not alter the spectrum or reveal a 680 nm-absorbing species (fig.3b), which is thus attributed to a component of the cytochrome *d*-containing oxidase complex.

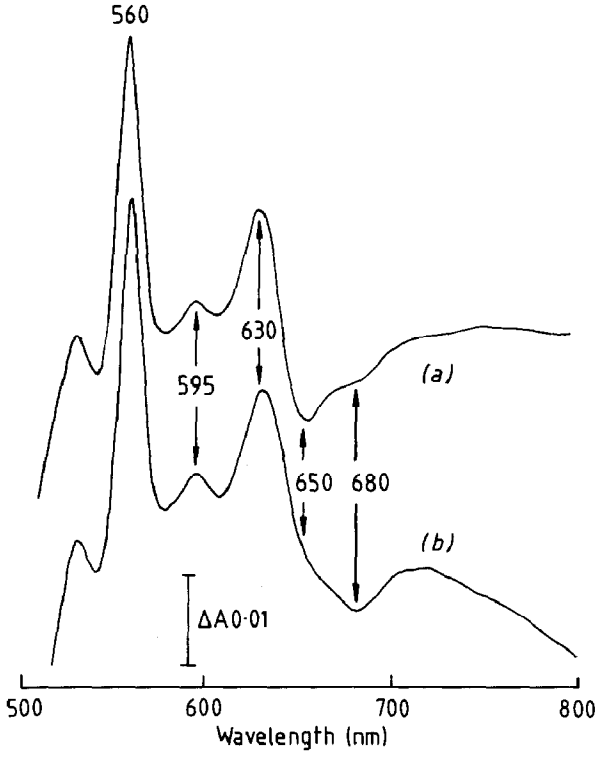

Fig.1. Spectrum a is a dithionite-reduced minus aerated difference spectrum of *E. coli* membranes, while in b the reference was a ferricyanide oxidized sample plus 8.8 mM H_2O_2. Spectra were recorded at 25°C in 1 cm cuvettes. The scan rate was 2.86 $nm \cdot s^{-1}$, the spectral band width 4 nm and the reference wavelength 575 nm.

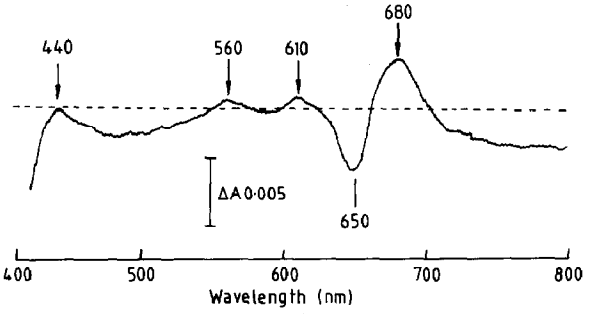

Fig.2. Reaction of H_2O_2 with persulphate-oxidized membranes. The spectrum of a membrane suspension oxidized with 12.5 mM ammonium persulphate was scanned, stored in the memory of the dual-wavelength spectrophotometer and subtracted from subsequent scans. Rescanning gave the dashed baseline. H_2O_2 was added to 8.8 mM to generate the difference spectrum shown. All other conditions were as in fig.1.

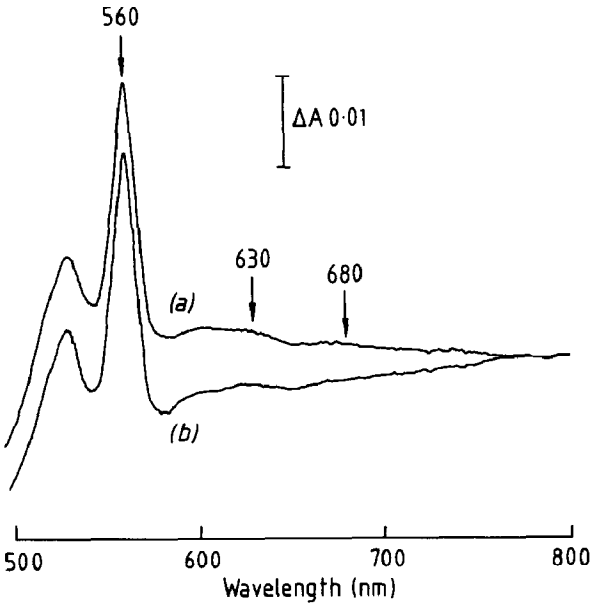

Fig.3. Effect of H_2O_2 on ferricyanide-oxidized membranes from a cytochrome *d*-deficient mutant. Spectrum a is the dithionite-reduced minus ferricyanide-oxidized difference spectrum. In b, 8.8 mM H_2O_2 was added to the oxidized sample. Conditions were as in fig.1.

4. DISCUSSION

A peroxy form or peroxide compound of any true cytochrome oxidase, other than aa_3 types, appears not to have been described before. The literature on peroxy intermediates of mitochondrial cytochrome *c* oxidase (aa_3 type) is confused, but it is clear [12] that the resting oxidase binds some H_2O_2 [13] whereas the pulsed oxidase (reduced, then reoxidized) binds H_2O_2 more effectively [14]. The unligated or pulsed species, which appears to absorb at 420 nm [15] may be described as a peroxidatic form, resembling peroxidases [16,17] and is presumed to be five coordinate at the Fe_{a3} site, accepting H_2O_2 as a sixth ligand to generate the (428 nm) peroxide compound.

Similarly, ferricyanide- or persulphate-oxidized *E. coli* cytochrome *d* reacts rapidly with hydrogen peroxide or ethyl hydrogen peroxide to give a compound with a distinctive absorbance maximum. The experiment in fig.2 suggests that peroxide addition to persulphate-oxidized membranes results in loss of some oxy form, as shown by the 650 nm trough. Indeed, the oxy form is remarkably stable and the result may be explicable by displacement of oxygen from the oxy form of cytochrome *d* by high peroxide concentrations.

There have been only a few studies on the 680 nm component observed in a number of bacterial species. Deconvolution of the absolute absorption spectrum of 'oxidized' cytochrome *d* in *A. vinelandii* [7] revealed three components with peaks at 635, 648 and 670 nm, the last of which probably corresponds to the form described here. On anoxia, the 648 nm band, now attributable to the oxy form, decreased before the 670 nm form. The latter was also diminished by reduction in the presence of cyanide [18], but was unaffected by CO [19]; these observations are consistent with the proposal that the haem is ferric in this form. A similar absorbance band is clearly seen in *E. coli* (fig.1) and *Acetobacter pasteurianus* [8], and particularly when a CO liganded sample is photolysed at sub-zero temperatures in the presence of oxygen. The first formed oxy compound at 650 nm decays coincident with the appearance of the 680 nm form. Although, in both organisms [8,9] the kinetics of loss of the oxy form and of formation of the 680 nm-absorbing species are quite different, suggesting that there is not a simple interconversion of the oxy compound to the presumptive peroxide compound, the simultaneous conversion of the latter to a third intermediate or product (the oxidized form?) might be compatible with the observed kinetics.

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