Partial purification and characterization of a soluble haemoprotein, having spectral properties similar to cytochrome a_1, from anaerobically grown *Escherichia coli*

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'Soluble' fractions obtained after high-speed differential centrifugation of extracts from ultrasonically disrupted *Escherichia coli*, grown anaerobically with glycerol and fumarate, contain at least two haemoproteins, distinguishable by their CO-binding characteristics. Reduced minus oxidized spectra show a maximum at 598 nm and a shoulder to the Soret region near 440 nm, features generally attributed to 'cytochrome a_1'. CO-reduced minus reduced difference spectra show the more rapidly CO-binding component to have a trough at 444 nm, also generally attributable to an *a*-type cytochrome. The partially purified a_1-like component has catalase and peroxidase activities, and lacks copper. An appropriate nomenclature for the a_1-like haemoprotein and its similarity to catalase are discussed.

Cytochrome a_1 *Catalase* *Peroxidase* Escherichia coli *Bacterial electron transport* *Anaerobic respiration*

1. INTRODUCTION

Cytochrome a_1 has remained the least well understood of the various cytochromes implicated as putative terminal oxidases in bacteria (review [1]). The confusion arises in part from the difficulties in defining cytochrome a_1. The original proposal [2] for a uniform terminology has gradually been expanded to include those cytochromes having an α-band in the reduced form between 585 and 598 nm [3,4]. In other respects, the spectral properties of cytochrome a_1 resemble those of mitochondrial and bacterial cytochromes aa_3 [1]. Thus, the CO-liganded, reduced form absorbs at 590–592 nm and 424–436 nm in the α- and Soret regions, respectively, the consequence being that cytochromes a_1 and a_3 cannot be distinguished by photochemical action spectroscopy alone [3]. The reduced form has been reported to absorb at 436–446 nm, again reminiscent of an *a*-type cytochrome.

Although cytochrome a_1 is a terminal oxidase in some bacteria [1,5–7] its role in *E. coli* is unknown. Authors in [5] found no evidence for cytochrome a_1 as an oxidase using photochemical action spectroscopy, whilst the use of a liquid dye laser as the actinic source in later experiments [6] led to the opposite conclusion. Stopped-flow experiments fail to identify cytochrome a_1 as a kinetically competent oxidase [8]. A haemoprotein in intact cells, having spectral properties normally assigned to cytochrome a_1, does bind CO, however [9].

Here, we report the solubilization, partial purification and characterization of a CO-binding haemoprotein from anaerobically grown *E. coli*, whose spectral properties resemble cytochrome a_1.

2. MATERIALS AND METHODS

2.1. *Organism and growth conditions*

E. coli K12, strain A1002 (NCIB 11825), was

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grown anaerobically with 0.5% (w/v) glycerol and 50 mM sodium fumarate in a medium [10] supplemented with molybdate and selenite [8] and sparged with O_2-free N_2. Cells were harvested when the apparent A_{600} was 0.4–0.6 [11].

2.2. *Cell disruption and partial purification of haemoprotein*

Cells were harvested by continuous flow centrifugation, washed once and then sonicated and fractionated by differential centrifugation in 50 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM EGTA (pH 7.4) [11]. The first supernatant [12] obtained by centrifugation at 225000 × *g* for 60 min (fraction 'S_1') was brought to 40% saturation with solid $(NH_4)_2SO_4$ and stirred for 30 min at 4°C before centrifugation at 23000 × *g* for 30 min. The pellet was resuspended in 50 mM Tris–HCl (pH 8) and dialysed at 4°C overnight against the same buffer. The dialysate was clarified by centrifugation as above, then loaded onto a column of DEAE–Sephadex A-50 pre-equilibrated with the same buffer. Elution with buffer was continued until A_{280} and $A_{408} \leqslant 0.03$ and followed by a linear gradient (total 500 ml) of 0–1.0 M NaCl in buffer. Fractions (10 ml) rich in catalatic and peroxidatic activity and cytochrome a_1 (as assessed in reduced minus oxidized difference spectra) were pooled, concentrated by ultrafiltration, dialysed for 12 h against 100 mM phosphate buffer (pH 7.0) and stored at −20°C until required.

2.3. *Analytical methods*

Metal contents were assayed on samples that had been extensively dialysed against 1 mM EDTA. A measured volume was placed in a lyophilising tube and dried in a stream of N_2. Constant boiling point 6 M HCl was added and the tube evacuated and sealed, followed by hydrolysis at 120°C for 18 h. Blank values were obtained by treating the dialysis buffer in the same way. Hydrolysates were analyzed by electrothermal atomic absorption spectroscopy with a Perkin-Elmer model 2380 instrument having deuterium arc background correction and HGA-400 graphite furnace. The programs used for copper [13] and iron [14] were based on published methods.

Optical absorbance spectra were recorded on a Pye-Unicam SP1700 split-beam spectrophotometer or a Johnson Foundation dual-wavelength scanning spectrophotometer [10,15]. NO gas was passed through a solution of NaOH (to remove N_2O) and then over silica prior to bubbling into

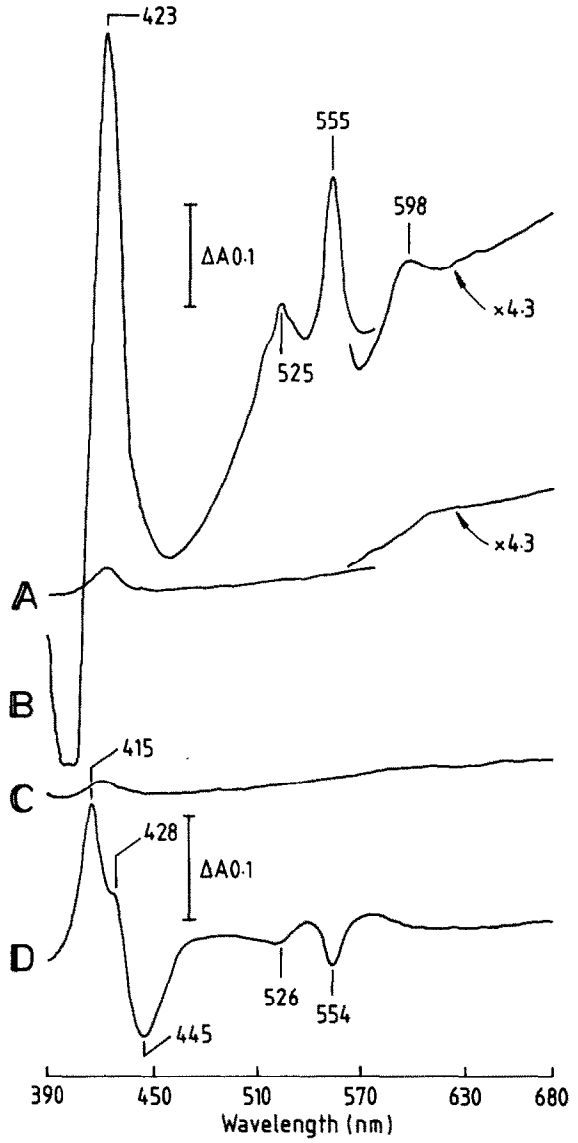

Fig.1. Difference spectra of a high-speed supernatant from ultrasonically disrupted *E. coli*, grown anaerobically on glycerol and fumarate. (A) A baseline (untreated sample minus untreated sample). (B) The reduced ($Na_2S_2O_4$) minus oxidized (persulphate) difference spectrum. (C) The reduced minus reduced baseline. (D) The effect of bubbling the sample cuvette with CO for 1 min. The scan rate was 2 nm·s^{-1}, path length 10 mm, spectral band width 1 nm, and protein concentration 46.8 mg·ml^{-1}.

water. The solution was assumed to be saturated and the NO concentration 2 mM at 20°C by interpolation from literature values for solubilities at 0 and 60°C. Cytochrome *c* peroxidase activity was assayed as in [16] except that equine cytochrome *c* was reduced with $Na_2S_2O_4$, excess being subsequently removed by passage through a Sephadex G-25 column. The final concentration of cytochrome *c* (assuming $\epsilon_{mM} = 19.5$) was 50 μM. Ethyl hydrogen peroxide (generously donated by Dr Kurt Wahlund, Ferrosan, PO Box 839, Malmö 20180, Sweden) was present at 90 mM. Catalase was determined by the UV-assay in [17], following the reaction for 15 s. Protein was determined as in [18].

3. RESULTS

Reduced minus oxidized spectra of membranes revealed a haemoprotein at approx. 595 nm (customarily referred to as cytochrome a_1) [11,19] and the more intense band of cytochrome *d* (not shown). However, the supernatant fraction (material non-sedimentable at an integrated force time $\approx 9 \times 10^{10}$ rad$^2 \cdot$s^{-1}) retains most of the ~595 nm component but undetectable amounts of cytochrome *d* (fig.1). The Soret band, peaking at 423 nm, is asymmetrical, having a shoulder on the longer wavelength side. These features are consistent with the presence of cytochrome a_1 [1]. Cytochromes of the *b*- and/or *c*-type are

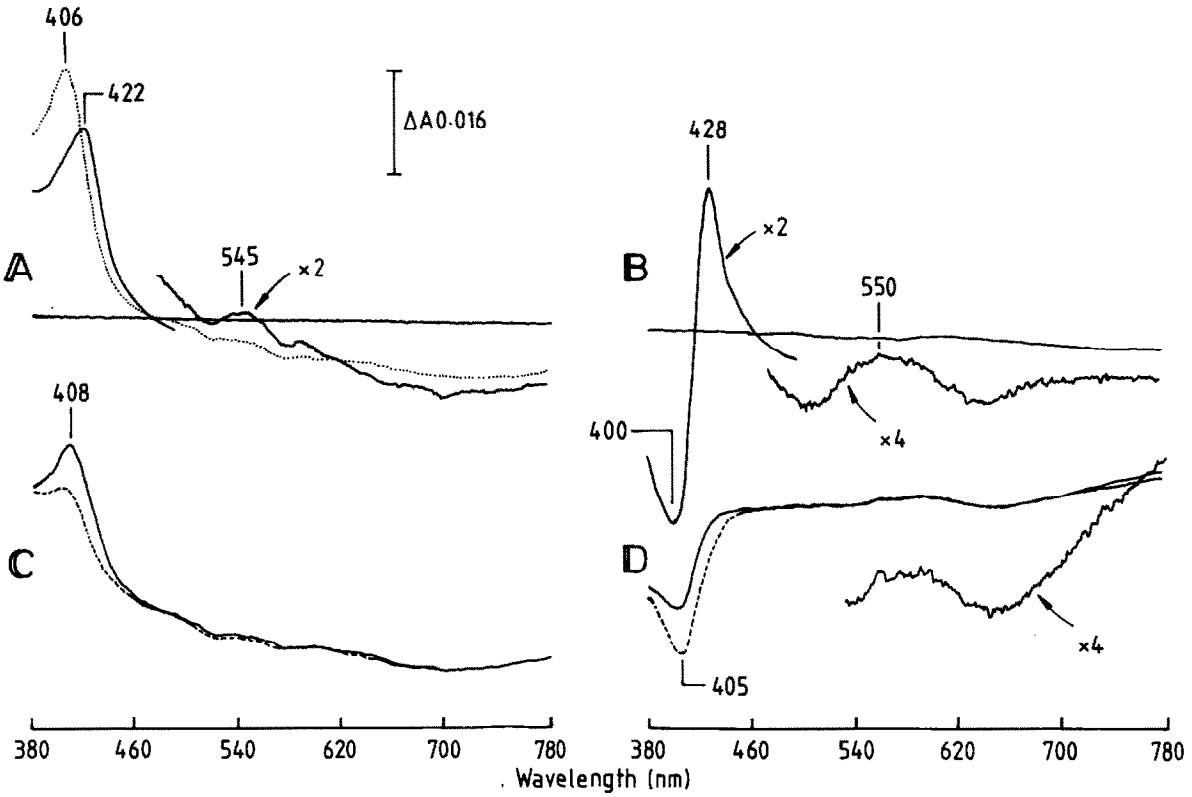

Fig.2. Absolute and difference spectra of the native haemoprotein complex and ligand-bound forms. Spectra were obtained using a dual-wavelength scanning spectrophotometer at 2.86 nm$\cdot$s^{-1} and with 475 nm as the reference wavelength. A and C show absolute spectra (with buffer as the reference spectrum). (A) The native enzyme (dotted line), a sample plus 1 mM KCN and buffer minus buffer baseline (unlabelled). (B) The native + cyanide minus cyanide difference spectrum and a native minus native baseline (unlabelled). (C) Spectra 20 min (solid line) and 1.5 h (dashed line) after adding ethylhydrogen peroxide (1 mM) to the native sample. (D) The corresponding spectra with the spectrum of the native form as reference. The absorbance calibration applies to all spectra, except where changes in the output gain are indicated. Path length, 10 mm; spectral band width, 4 nm; protein concentration, 0.41 mg$\cdot$ml^{-1}.

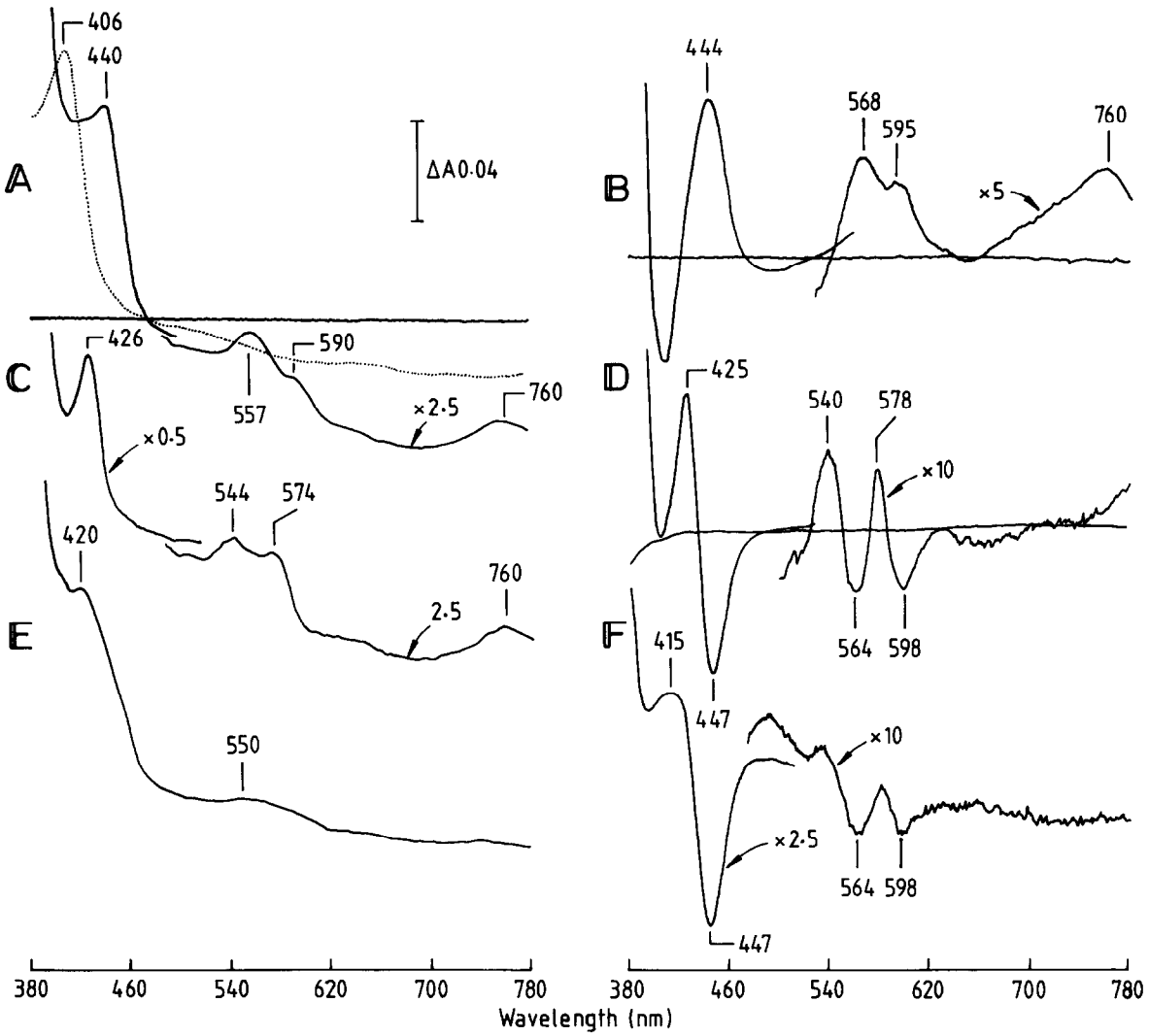

Fig.3. Absolute and difference spectra of the reduced haemoprotein complex and ligand-bound forms. Spectral conditions were as in fig.2. (A,C,E) Absolute spectra (with buffer as the reference spectrum). (B,D,F) Difference spectra, with the oxidized (B) or dithionite-reduced spectrum (D,F) as reference. (A) The native enzyme (dotted line), a sample reduced with approx. 2 mg $Na_2S_2O_4$ (solid line) and a buffer minus buffer baseline (unlabelled). (B) The reduced minus oxidized difference spectrum and an oxidized minus oxidized baseline. (C) The spectrum of the reduced form bubbled with CO for 3 min. (D) The CO-reduced minus reduced spectrum. (E) The reduced form plus 15 μl of NO-saturated buffer in a volume of 1 ml (final concentration, 0.03 mM). (F) The NO-reduced minus reduced spectrum. The absorbance calibration applies to all spectra except where changes in the output gain are indicated. Path length, 10 mm; spectral band width, 4 nm; protein concentration, 1.3 mg·ml^{-1}.

presumably responsible for the α-(555 nm) and β-(525 nm) bands (fig.1). The CO-difference spectrum reveals at least two CO-binding components. The trough at 444–448 nm is fully formed within 30 s of bubbling with CO, whereas 3–5 min of bubbling are necessary for complete development of the 415 nm peak and the troughs at about 526, 554 and 434 nm (not shown). Quantification of the 445 nm CO-binding haemoprotein in analytical subcellular fractionations shows that up to 90% is recovered in the high-speed supernatant fraction (unpublished). The absence of marked perturba-

tions by CO in the 590–600 nm region is consistent with the failure of this ligand to displace the α-absorption band of a_1-type cytochromes to shorter wavelengths [1,3].

The native form of the partially purified haemoprotein has an intense Soret absorbance at 406 nm which is red-shifted by cyanide and partially bleached by ethyl hydrogen peroxide with relatively minor changes in the 460–780 nm region (fig.2). The preparation is reducible by dithionite (fig.3A), the reduced minus oxidized difference spectrum showing maxima at 444, 568 and 595 nm (fig.3B) in marked contrast to the supernatant fraction (fig.1B). The 760 nm absorbance is attributable to dithionite. Complex formation with CO shifts the absorbance maxima of the reduced form to the blue (fig.3C) with the consequent formation of a striking CO difference spectrum (fig.3D). The 540, 564 and 578 nm absorbances are reminiscent of a CO-binding, *b*-type cytochrome [9], but the Soret region is distinctive. NO results in similar trough positions (fig.4F) to those given by CO, but the presumptive haem–NO^- complex is not characterized by well developed absorbance maxima.

The iron content of the preparation was high, 16.4 nmol·mg protein^{-1}, whilst copper was below the limits of the assay, <0.65 nmol·mg protein^{-1}. The estimated precision of each metal determination was $\pm$ 0.8–0.9 nmol for a solution containing 1 mg protein·ml^{-1}. Catalase and peroxidase activities of the partially purified preparation were high, being 321 s^{-1}·mg protein^{-1} and 89.8 nmol cytochrome *c* oxidized·min^{-1}·mg protein^{-1}, respectively.

4. DISCUSSION

There appears to be no convincing evidence for the presence of haem A in *E. coli* except for the report of traces of cryptoporphyrin a [20]. Nevertheless, by analogy with other bacteria, the 585–598 nm band is generally attributed to cytochrome a_1. Other compounds that spectrally resemble cytochrome a_1 and could be wrongly identified as such in *E. coli* include free protohaem, sirohaem, coproporphyrin or its zinc complex, and cytochrome *c* peroxidase (for references, see [1]). It is noteworthy that the catalase purified from *Comamonas compransoris* [21] is reported to have spectral properties that 'resemble the corresponding spectra of bovine liver catalase'. However, a CO difference spectrum of the *Comamonas* haemoprotein [7] shows features, especially the 444 nm trough, similar to the component described here, but not closely resembling the catalase from animal sources. The spectra of the partially purified complex are similar in shape, but not in detail, to those of authentic catalase. Thus the values for the troughs in CO difference spectra of the bovine liver enzyme (Sigma) obtained under the conditions of fig.3D, and with which they may be compared, are 432, 553 and 590 nm. The peaks are at 417, 532 and 570 nm (not shown). Further studies of the purified complex from *E. coli* are required to allow direct comparison with other catalases, which may be more diverse in microbes [21] than once thought. *E. coli* can synthesize two electrophoretically distinct catalases or hydroperoxidases (HP-I and HP-II) and one of these is constitutive and occurs in anaerobic conditions [22]. Only HP-I has been purified (from aerobically grown cells) [23]; optical properties of the reduced enzyme and its reaction with ligands have not been reported. The enzyme has peroxidatic activity with cytochrome *c* as substrate, albeit weakly in comparison with the catalatic activity [23]. High peroxidase activity (but with 2,3,6-trichloroindophenol) was also reported in an 'a_1b' preparation from *E. coli* [24]; unfortunately this work has not been reported fully.

Clearly, improved nomenclature of cytochrome a_1 is required, yet the distinguishing features suggested in [2] are now inadequate. In particular, the suggestion that cytochrome oxidases are necessarily membrane-bound and catalases soluble requires reassessment, since readily solubilized oxidases have been extensively characterized from, for example, *Vitreoscilla* (cytochrome *o*) and *Pseudomonas aeruginosa* (cytochrome cd_1) [1]. Unlike cytochrome *o* [5], the present definition of cytochrome a_1 does not require that the cytochrome be an oxidase.

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