SHORT COMMUNICATION

Haemoprotein *b*-590 (*Escherichia coli*); Redesignation of a Bacterial 'Cytochrome a_1 '

By ROBERT K. POOLE,¹* BALDEV S. BAINES,¹† SALLY J. CURTIS,¹
HUW D. WILLIAMS¹ AND PAUL M. WOOD²

¹ Department of Microbiology, Queen Elizabeth College (University of London), Campden Hill,
London W8 7AH, UK

² Department of Biochemistry, University of Bristol, Medical School, University Walk,
Bristol BS8 1TD, UK

(Received 9 July 1984)

A 'soluble' fraction from anaerobically grown *Escherichia coli* contains a haemoprotein with spectral properties, notably an α -band in the reduced form at 585 to 595 nm, similar to cytochrome a_1 . Haem extraction of either the soluble preparation or whole cells yields haem *b*, but not haem *a*. In view of this, and the spectral similarities of the a_1 -like component to well-known high-spin haem *b* proteins, we propose that the name 'haemoprotein *b*-590' be used to describe this substance and that consideration be given to the applicability of the name to certain other cytochrome a_1 -like pigments in bacteria.

INTRODUCTION

In a few bacteria (e.g. *Acetobacter pasteurianus*), a terminal oxidase, cytochrome a_1 , has been identified whose absorbance maximum (reduced form) in the α -region lies at shorter wavelengths (585 to 596 nm) than the mitochondrial cytochrome oxidase aa_3 (Poole, 1983). Difference spectra of *Escherichia coli* frequently show such a band at about 595 nm. It has been called 'cytochrome a_1 ', solely by analogy with the cytochromes a_1 of other bacteria. There are conflicting views on whether *E. coli* 'cytochrome a_1 ' is an oxidase (Poole, 1983), but in intact cells it does bind CO (Poole *et al.*, 1981).

We have recently shown that a high-speed supernatant fraction from ultrasonically disrupted, anaerobically grown *E. coli* contains most of the 'cytochrome a_1 ' and have described the spectral and ligand-binding properties of the partially purified enzyme (Baines *et al.*, 1984). Here, we (1) report the absence of haem *a*, but presence of haem *b*, in preparations containing the a_1 -like haemoprotein, (2) suggest the origin of the α -absorbance band and (3) propose an improved nomenclature for this enigmatic haemoprotein.

METHODS

Organism and growth. *Escherichia coli* K12 (NCIB 11825) was grown anaerobically on glycerol and fumarate as described by Baines *et al.* (1984).

Haem extraction from a soluble cell-free extract. Washed cells were disrupted by sonication and fractionated by differential centrifugation. The high-speed supernatant was brought to 40% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and

† Present address: Biotechnology Department, Microbiology Division, Glaxo Group Research Ltd, Greenford, Middlesex UB6 0HE, UK.

the precipitated material collected (Baines *et al.*, 1984). The pellet was resuspended in a buffer (pH 7.7) that contained 50 mM-potassium phosphate and 0.01 mM-EDTA, and dialysed overnight at 4 °C against the same buffer. To the dialysate (1 ml) was added 9 ml ice-cold acetone/0.015 M-HCl (Murphy *et al.*, 1973). After stirring at 0 °C for 5 min, the mixture was filtered through a cellulose nitrate membrane filter (0.4 µm pore size; Nucleopore Corp., 7035 Commerce Cir, Pleasanton, CA 94566, USA). Pyridine was added to the filtrate to a final concentration of 10% (v/v) and the mixture rotary-evaporated to dryness under vacuum at <40 °C. The solid yellow-green haem extract was stored at -20 °C.

Haem extraction from whole cells. The method of Smith & Caughey (1978) was followed with minor modifications. Cells (25 g wet weight) were washed twice with water. The wet pellet was stirred vigorously for 15 min in 25 ml ice-cold acetone/water (4:1, v/v). The cells were collected on Whatman no. 1 filters and then stirred in 37.5 ml chloroform for 20 min at room temperature. The chloroform was decanted off and haems extracted from the cell residue by stirring for 1.5 h in 25 ml chloroform/pyridine (2:1, v/v) at room temperature. The yellow extract was separated by filtration and the extraction of the residue repeated twice. The filtrates were clarified using Nucleopore filters as above, evaporated to dryness (<40 °C) and the extracts stored at -20 °C.

Preparation and spectral analysis of pyridine haemochromes. The haem-containing extracts were dissolved in 12.4 M-pyridine and, immediately before recording the spectra, an equal volume of 0.15 M-NaOH was added (Fuhrhop & Smith, 1975). Spectra were recorded with a Johnson Foundation dual-wavelength scanning spectrophotometer. Sample reduction was with $\text{Na}_2\text{S}_2\text{O}_4$ (<3 mg ml⁻¹) and, where indicated, oxidation was with a few grains of ammonium persulphate.

RESULTS

Fig. 1(a) shows a reduced minus oxidized difference spectrum of the cell-free supernatant from *E. coli* after $(\text{NH}_4)_2\text{SO}_4$ fractionation. In addition to cytochrome c_{552} , a peak at 596 nm and a shoulder at 440 nm are seen, attributable to the soluble 'cytochrome a_1 '. The material used for Fig. 1(a) was treated with acid/acetone, which should solubilize *a*-, *b*- and *d*-type haem, leaving *c*-type haem covalently bound to protein. The resulting pyridine haemochrome spectrum (Fig. 1b) shows a peak at 558 nm characteristic of *b*-type haem (Fuhrhop & Smith, 1975); no haem *a*, which would absorb at 587 nm, was seen.

Difference spectra of whole cells and membranes also reveal 'cytochrome a_1 ' and so an extraction method specifically recommended for haem *a* (Smith & Caughey, 1978) was applied to cells. The solubilized material was treated with alkaline pyridine, and reduced with dithionite. Fig. 1(c) shows features attributable to *b*- and *d*-type haem (bands at 558 and 610 nm, respectively; Barrett, 1956), but again no 587 nm peak of haem *a*. Nonetheless, application of this method to cytochrome *caa*₃ from the thermophilic bacterium PS3 gave the expected yield of haem *a* (B. S. Baines & R. K. Poole, unpublished).

DISCUSSION

The majority of haem proteins can be classified as high-spin or low-spin, depending on the number of unpaired electrons associated with the iron atom (see Wood, 1984). All proteins with high-spin *b*-type haem show, in their uncomplexed, reduced state, an absorption band centred near 560 nm, examples being deoxymyoglobin and horseradish peroxidase (Eaton & Hofrichter, 1981). This, however, is the β -band, not the α -band; the latter is sometimes indistinct but lies near 590 nm. For myoglobin, the α -band can be resolved only in single-crystal spectra but in reduced + CO minus reduced difference spectra it forms a distinct trough, very like that reported for *E. coli* 'cytochrome a_1 ' by Baines *et al.* (1984). In view of the spectral similarities of the '*a*₁-like' protein to the above high-spin haemoproteins and the failure to detect *a*-type haem in either soluble fractions (this paper) or a purified oxidase complex (Miller & Gennis, 1983), we propose that *E. coli* 'cytochrome a_1 ' contains *b*-type haem in an environment that leads to a distinct α -band, as it does in tryptophan 2,3-dioxygenase.

The recommended procedure for nomenclature (Enzyme Nomenclature, 1978), having identified the haem prosthetic group (*b*-type) is to include the source in parentheses (*Escherichia coli*) and a name based on the α -band wavelength (in nm) determined at room temperature from absolute spectra of the purified protein. The peak is at about 590 nm (but broad), as measured with a Johnson Foundation instrument, calibrated with holmium and didymium filters (Baines

Fig. 1. Spectra of the soluble haemoprotein preparation from *E. coli* and of pyridine haemochromes of the extracted haems. Spectrum (a) is a reduced minus oxidized difference spectrum of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated fraction before haem extraction; (b) shows the reduced minus oxidized difference spectrum of the pyridine haemochromogen, after extraction with acid acetone. Spectrum (c) is the absolute spectrum in pyridine of the reduced haems extracted by the method of Smith & Caughey (1978). The absorbance bar represents 0.02 in (a), 0.02 (left) and 0.008 (right) in (b) and 0.08 (left) and 0.02 (right) in (c). The scan rate was 5.7 nm s^{-1} , the spectral band width 4 nm, and the reference wavelength 475 nm.

et al., 1984). We therefore recommend 'haemoprotein *b*-590 (*Escherichia coli*)'. 'Haemoprotein' is preferable to 'cytochrome' until the function is defined, since the long-wavelength α -band implies a high-spin configuration and such proteins seem invariably to have functions that involve ligand-binding as opposed to electron transfer (Wood, 1984). For a cytochrome, 'the characteristic mode of action involves a single-electron reversible equilibrium between the Fe(II) and Fe(III) states' (Enzyme Nomenclature, 1978). The soluble *E. coli* protein, however, has catalase and peroxidase activities (Baines *et al.*, 1984).

Of the many 'cytochromes a_1 ' listed by Poole (1983), only a few have been shown to contain a -type haem and so the present results and proposal have wide implications. Soluble 'cytochromes a_1 ', like the *E. coli b*-590, have been described in *Citrobacter* (Keevil *et al.*, 1979) and *Corynebacterium* (Jurtshuk *et al.*, 1975). It is notable that hydrocarbon oxygenase activity has been tentatively associated with the latter preparation and that certain oxygenases are high-spin b -type haem proteins (Wood, 1984). However, in other cases (e.g. *Acetobacter pasteurianus*), cytochrome a_1 is an oxidase and exhibits the expected low-temperature ligand exchange and O_2 -binding reactions (H. D. Williams & R. K. Poole, unpublished). The membrane-bound cytochrome a_1 of *Nitrobacter agilis* is part of an a_1c_1 complex involved in nitrite oxidation (Tanaka *et al.*, 1983). Both these cytochromes a_1 contain a -type haem. A cytochrome a_1 -like haemoprotein, but which lacks a -type haem, co-purifies with the *E. coli* cytochrome *bd* oxidase com-

plex (Miller & Gennis, 1983) and potentiometric resolution of the a_1 -like component gives a spectrum similar to that of our purified enzyme (Koland *et al.*, 1984). Further study is required to identify similarities and differences in the two preparations and to test the wider validity of the nomenclature proposed here.

This work was supported by grants from SERC (R.K.P. and P.M.W.) and the Royal Society (R.K.P.).

REFERENCES

- BAINES, B. S., WILLIAMS, H. D., HUBBARD, J. A. M. & POOLE, R. K. (1984). Partial purification and characterization of a soluble haemoprotein, having spectral properties similar to 'cytochrome a_1 ', from anaerobically grown *Escherichia coli*. *FEBS Letters* **171**, 309–314.
- BARRETT, J. (1956). The prosthetic group of cytochrome a_2 . *Biochemical Journal* **64**, 626–639.
- EATON, W. A. & HOFRICHTER, J. (1981). Polarized absorption and linear dichroism spectroscopy of hemoglobin. *Methods in Enzymology* **76**, 175–261.
- ENZYME NOMENCLATURE (1978). *Recommendations of the Nomenclature Committee of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes*, pp. 594–601. London: Academic Press.
- FUHRHOP, J.-H. & SMITH, K. M. (1975). Laboratory Methods. In *Porphyrins and Metalloporphyrins*, pp. 757–869. Edited by K. M. Smith. Amsterdam: Elsevier Scientific Publishing Co.
- JURTEHUK, P., MUELLER, T. J. & ACORD, W. C. (1975). Bacterial terminal oxidases. *CRC Critical Reviews in Microbiology* **3**, 399–468.
- KEEVIL, C. W., HOUGH, J. S. & COLE, J. A. (1979). Regulation of 2-oxoglutarate dehydrogenase synthesis in *Citrobacter freundii* by traces of oxygen in commercial nitrogen gas and by glutamate. *Journal of General Microbiology* **114**, 355–359.
- KOLAND, J. G., MILLER, M. J. & GENNIS, R. B. (1984). Potentiometric analysis of the purified cytochrome d terminal oxidase complex from *Escherichia coli*. *Biochemistry* **23**, 1051–1056.
- MILLER, M. J. & GENNIS, R. B. (1983). The purification and characterisation of the cytochrome d terminal oxidase complex of the *Escherichia coli* aerobic respiratory chain. *Journal of Biological Chemistry* **258**, 9159–9165.
- MURPHY, M. J., SIEGEL, L. M., KAMIN, H. & ROSENTHAL, D. (1973). Reduced nicotinamide adenine dinucleotide phosphate-sulphate reductase of Enterobacteria. II. Identification of a new class of heme prosthetic group: an iron-tetrahydroporphyrin (isobacteriochlorin type) with eight carboxylic acid groups. *Journal of Biological Chemistry* **248**, 2801–2814.
- POOLE, R. K. (1983). Bacterial cytochrome oxidases. A structurally and functionally diverse group of electron-transfer proteins. *Biochimica et biophysica acta* **726**, 205–243.
- POOLE, R. K., SCOTT, R. I. & CHANCE, B. (1981). The light-reversible binding of carbon monoxide to cytochrome a_1 in *Escherichia coli* K12. *Journal of General Microbiology* **125**, 431–438.
- SMITH, M. L. & CAUGHEY, W. S. (1978). New methods for isolation and characterization of hemes. *Methods in Enzymology* **52**, 421–436.
- TANAKA, Y., FUKUMORI, Y. & YAMANAKA, T. (1983). Purification of cytochrome a_1c_1 from *Nitrobacter agilis* and characterization of nitrite oxidation system of the bacterium. *Archives of Microbiology* **135**, 265–271.
- WOOD, P. M. (1984). Bacterial proteins with CO-binding b or c type haem: functions and absorption spectroscopy. *Biochimica et biophysica acta* (in the Press).