LIGAND-BINDING CYTOCHROMES a_3, c and o IN MEMBRANES FROM THE THERMOPHILIC BACTERIUM PS3

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1. Introduction

The isolation and characterization of a highly stable adenosine triphosphatase from the thermophilic bacterium PS3 [1] has given new impetus to studies of the electron-transferring and energy-transducing proteins from such bacteria. Interest has been heightened by the finding that the cytochrome oxidases of thermophiles and of certain other bacteria bear a remarkable resemblance to the cytochrome *c* oxidase (EC 1.9.3.1) of eukaryotic mitochondria, but appear to have much simpler subunit compositions [2]. The extreme case appears to be PS3 in which the purified cytochrome oxidase has been reported to have only one subunit, and yet possesses haems *a* and a_3, cytochrome *c* and intrinsic copper [3]. However, Fee et al. [4] were unable to demonstrate any perturbation of the optical spectra of PS3 membranes by CO, the classical a_3-binding ligand. Later experiments (Yang, E. and Sone, N., unpublished; cited in [2]) using low temperature spectroscopy revealed that an a_3 component was missing and that the complex contained haems *a*, *o* and *c* in molar ratios 5:1:5.

The purpose of the present paper is to clarify this situation and report the binding of CO, cyanide and NO by cytochromes in membranes isolated from PS3. A ligand-binding *a*-type cytochrome (thus classifiable as a_3 [5]), together with cytochrome *o* and a ligand-binding cytochrome *c* are found.

2. Experimental

2.1. *Growth and harvesting of PS3*

The thermophilic bacterium PS3 (believed to be *Bacillus stearothermophilus*) was the generous gift of Dr Tairo Oshima. It was grown in a medium containing (%, w/v); proteose peptone (Oxoid), 0.5; yeast extract (Difco), 0.3; NaCl, 0.2. The pH was 7.0. Stocks were maintained by adding sterile glycerol to a small portion of culture to give a final concentration of 12.5% (w/v) and storing at −20°C. Starter cultures (100 ml medium in 250 ml screw-top Erlenmeyer flasks) were inoculated with a few drops of thawed stock culture and incubated with vigorous magnetic stirring at 65°C for ~3 h. The entire culture was then inoculated into 4.5 l of the same medium in a Gallenkamp CeCa fermenter vessel maintained at 65°C and stirred at 900 rev./min. Air was passed into the head space above the culture at 4.5 l/min. The mean generation time was ~20 min. Cells were harvested by continuous-flow centrifugation when the E_{600} (1 cm light/path) had reached ~1.0, and then washed once in 50 mM Tris–sulphate (pH 8.0).

2.2. *Preparation of washed membranes*

Membranes were prepared and washed as in [1] except that lysozyme (egg white; Schwarz/Mann) was increased to 2 mg/g wet wt cells.

2.3. *Analytical methods and materials*

Membranes were resuspended in the same buffer and difference recorded at room and low temperatures using the split beam apparatus in [6]. During gassing of cuvettes with CO (CP grade) or NO (both from BOC Special Gases, Morden, London SW19), foaming was controlled by a smear of silicone MS Antifoam A (Hopkin and Williams Ltd) around the cuvette top. Protein was determined using the Lowry technique [7].

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3. Results

3.1. *Effects of CO on difference spectra*

According to [8], cytochrome a_3 is that portion of the mitochondrial cytochrome aa_3 complex that binds CO. It contributes relatively little to the α absorption at ~605 nm, but both components contribute almost equally to the Soret band at ~445 nm. At room temperature, CO reduced minus reduced difference spectra of PS3 membranes clearly revealed the presence of cytochrome a_3 at 444 and 608 nm (fig.1). The peak at 590 nm is the absorption of the a_3–CO complex; the corresponding Soret absorption expected at ~430 nm is presumably obscured by the intense band at ~416 nm (peak) and 431 nm (trough). This component, which also contributes to the minimum at 562 nm, is presumably a CO-binding *b*-type cytochrome, i.e., cytochrome *o* [9]. The a_3 and *o* components have apparently different affinities for CO (fig.1; inset); the reaction of a_3 with CO is complete within 30 s of treatment with CO, whilst the intensities of bands attributable to cytochrome *o* and its CO complex continue to increase for up to 2 min. A similar kinetic distinction is possible between cytochrome a_3 and haemoglobin [10].

A comparison of the troughs due to cytochrome a_3 in fig.1 and the aa_3 maxima in a reduced minus oxidised difference spectrum (not shown) revealed that cytochrome a_3 contributed ~40% to the Soret absorbance of aa_3 but only ~10% of the α band of aa_3.

At 77 K (fig.2) the absorption bands were intensified and showed the trough attributed to cytochrome *o* to be split, with components at 547 and 554 nm. The former is presumably a CO-binding cytochrome *c* [11,12] whilst the latter is broad and may contain components other than cytochrome *o*. Cytochrome a_3 and its CO complex were seen at 604–605 nm (A,B) and 588 nm (fig.2B), respectively.

3.2. *Difference spectra in the presence of NO*

The spectrum of a reduced membrane preparation bubbled with NO for 30 s minus that of a reduced sample was qualititatively similar to an analogous CO spectrum at both room temperature (fig.3B) and 77 K (fig.3C), consistent with the known formation of NO–ferrocytochrome a_3 [13]. However, the relative intensities of troughs due to ligand-binding cytochromes was altered in both the α and β regions (compare fig.3C,2B), lending support to the proposal that both *b*- (i.e., *o*-) and *c*-type [5] cytochromes bind these ligands. No maxima analogous to the absorbances of the CO complexes of cytochromes a_3 and *o* were visible.

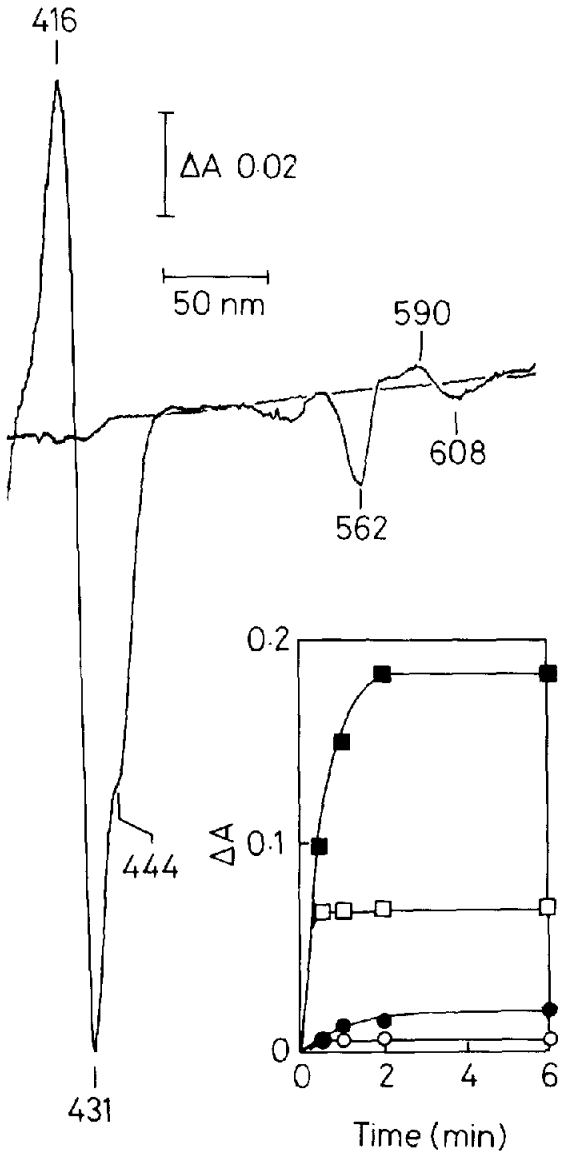

Fig.1. CO-reduced minus reduced difference spectrum of PS3 membranes and the effect of CO at room temperature. Reduction was with dithionite and CO was bubbled into the reduced suspension for 2 min (for the spectrum shown) or other times as indicated on the abscissa to the inset. The latter shows absorbance changes at 416–431 nm (–▪–), 444–463 nm (–□–), 562–550 nm (–•–) and 608–628 nm (–○–). Spectral conditions were: spectral bandwidth, 0.8 nm; scan speed, 1 nm/s; pathlength, 10 mm. Protein was 2.89 mg/ml.

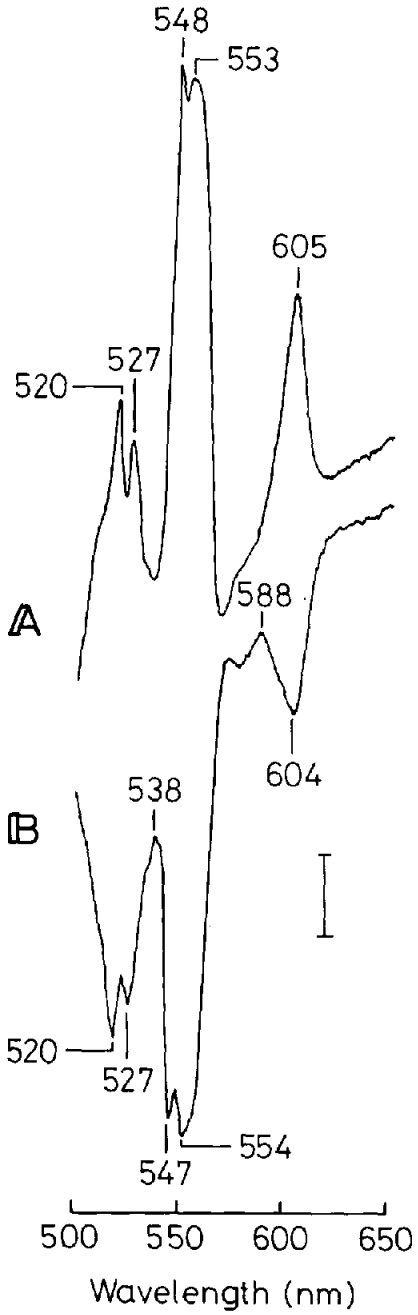

Fig.2. Reduced minus oxidised (A) and CO-reduced minus reduced (B) difference spectra of PS3 membranes at 77 K. Reduction was with dithionite; oxidation was accomplished by addition of H_2O_2. (B) CO was bubbled into the reduced cell suspension for 3 min. Spectral conditions were: spectral bandwidth, 0.95 nm; scan speed, 1 nm/s; pathlength, 2 mm. The vertical bar denotes ΔA 0.03 (A) or ΔA 0.016 (B); protein was 6.97 mg/ml.

3.3. *Identification of cytochromes* a *and* a_3 *with cyanide*

Reaction of KCN with reduced cytochrome a_3 resulted in an absorption minimum at 608 nm in the room temperature difference spectrum of fig.4a (compare with fig.1) and a maximum at 590 nm, corresponding to the ferrous cytochrome a_3—CN^- complex [14]. At 77 K (fig.4B), similar features were seen and ligand-binding *b*- and *c*-type cytochromes were again revealed. For the difference spectra in fig.4C,D, the contents of both cuvettes were oxidized by shak-

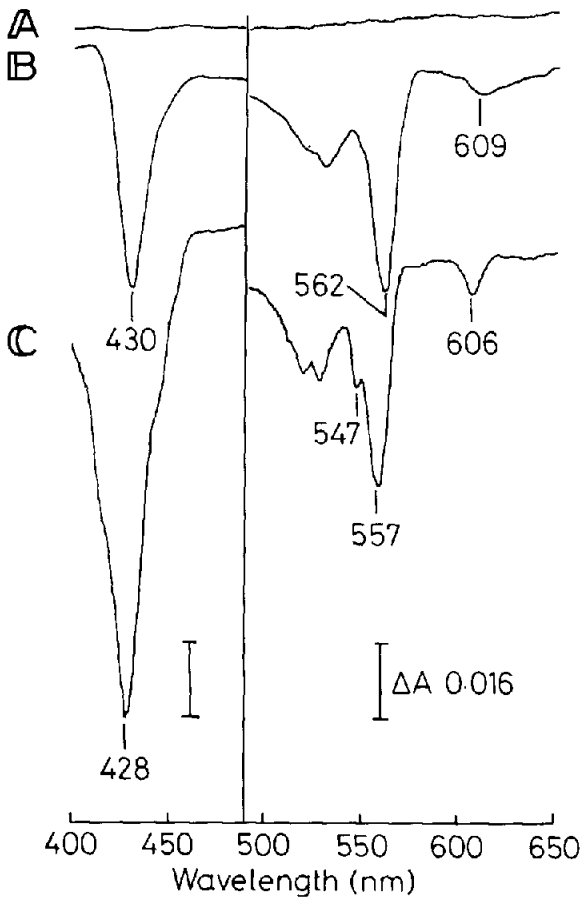

Fig.3. NO-reduced minus reduced difference spectra of PS3 membranes at room temperature (B) and 77 K (C). A, shows the reduced minus reduced baseline at room temperature. (A,B) Spectral conditions were: spectral bandwidth, 1.5 nm; scan speed, 2 nm/s; 10 mm pathlength and the vertical bar to the left denotes ΔA 0.11. (C) Spectral conditions were: spectral bandwidth, 0.9 nm; scan speed, 2 nm/s; 2 mm pathlength and the vertical bar to the left denotes ΔA 0.03. Protein was 2.2 mg/ml.

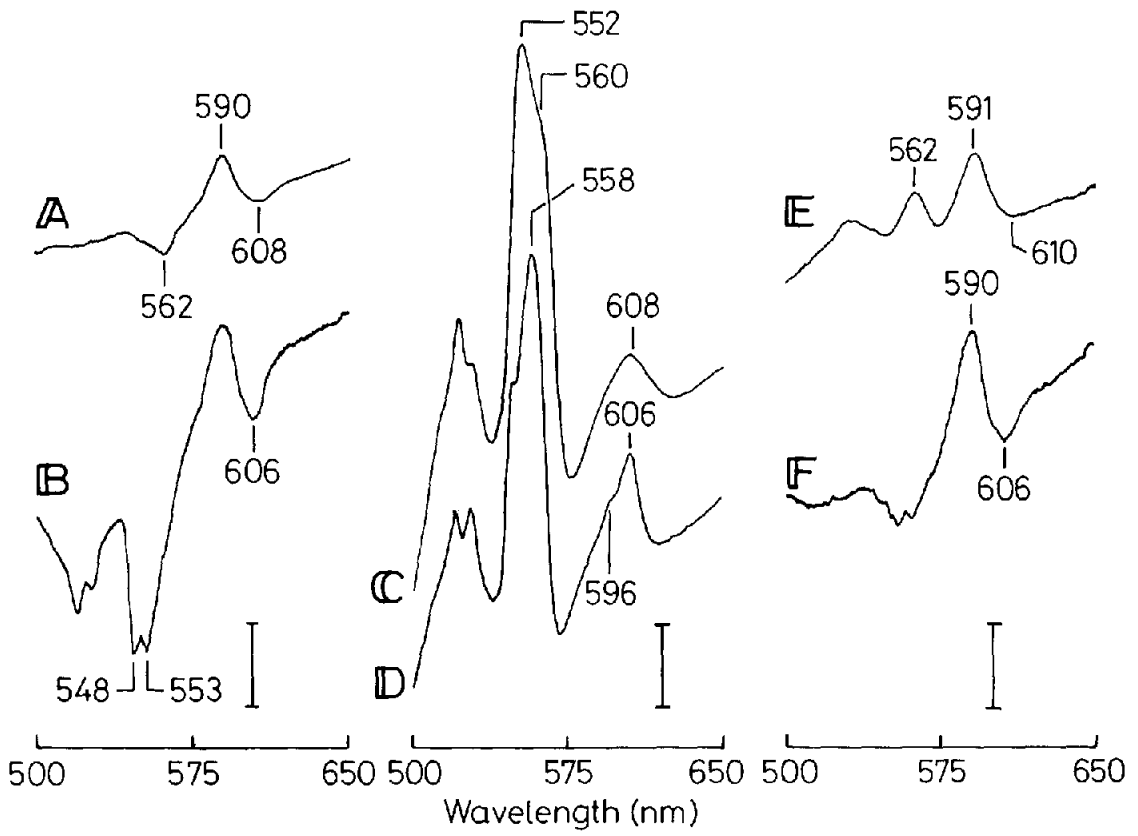

Fig.4. Effects of cyanide on difference spectra of PS3 membranes recorded at room temperature (A,C,E) and 77 K (B,D,F). (A,B) Contents of both cuvettes were reduced with $Na_2S_2O_4$ and then 5 mM KCN was added to the sample cuvette. (C,D) Contents of both cuvettes were oxidized by aeration and 5 mM KCN was added to both. The sample cuvette was then reduced with dithionite. (E,F) Contents of both cuvettes were aerated and 5 mM KCN added to the sample cuvette only; $Na_2S_2O_4$ was then added to each. Spectral bandwidth for spectra A, C and E was 1.5 nm, 1.0 nm for B and 0.9 nm for D and F. Scan speed was 2 nm/s for A, C and E, and 1 nm/s for B, D and F. The vertical bar denotes ΔA 0.016 for A, B, E and F, and 0.032 for C and D. Protein throughout was 3.55 mg/ml.

ing and then 5 mM KCN added. Sodium dithionite was added to the contents of the sample cuvette only and the difference spectra recorded. Since cytochrome a_3 is not readily reduced when complexed with CN^- [5], it is effectively excluded from the spectrum. Cytochrome *a*, however, does not react with CN^- and its reduced minus oxidized spectrum, together with that of other non-CN^- reacting components was obtained. A peak at 608 nm (room temperature) or 606 nm (77 K) was thus attributed to cytochrome *a*. The identity of a small shoulder appearing at 596 nm has not been determined. Comparison of fig.4C with a reduced minus oxidized difference spectrum and with a cyanide reduced minus reduced spectrum (fig.4A) at room temperature indicated that cytochrome *a* contributed ~90% to the aa_3 band in the α region.

In fig.4E,4F, both cuvettes initially contained aerated mitochondria. KCN was added to the sample cuvette only and then $Na_2S_2O_4$ was added to both cuvettes. The CN^--reacting a_3 was seen as a minimum at ~610 nm (room temperature; fig.4E) or 606 nm (77 K; fig.4F).

4. Discussion

These results confirm the original description of the PS3 oxidase as an *a*- and a_3-containing enzyme [3] (although no evidence for ligand binding was

reported in [3]). Support for the presence of cytochrome *o* in these cells (Yang, E. and Sone, N., unpublished; cited in [2]) is also provided. Fee et al. [4] were unable to demonstrate binding of CO to cytochromes in membranes from either PS3 or *Bacillus caldolyticus* and suggested that this may represent an adaptation that avoids poisoning from the CO that may be present in the natural environment of these organisms. Although aerobic oxidation of CO is demonstrated by diverse bacteria, it does not appear to be accompanied by any obvious modification of the electron-transport chain to avoid CO poisoning [15]. However, in [4] CN^- was reported to produce 'minor modifications in the Soret region of the reduced minus oxidized and cyanide spectrum as would be expected for an aa_3-type system'.

In conclusion, washed membranes prepared from lysozyme-treated cells of PS3 contain an aa_3-type cytochrome that bears several spectral similarities to the much-studied mitochondrial cytochrome *c* oxidase. These similarities are:

(1) The position of the α and Soret peaks of the reduced form;
(2) The binding by a component (a_3) of the cytochrome to cyanide, CO and NO;
(3) The small contribution (~10%) made by the CO- and CN^--binding a_3 compared to the total absorption in the α region;
(4) The much larger contribution (~40%) made by a_3 to the Soret absorption at 444 nm.

In addition, these membranes contain cytochrome *o*, which may constitute a second oxidase, and a *c*-type cytochrome of unknown function that binds CO, NO and CN^-. This preliminary identification of cytochrome a_3 has important consequences for future studies of oxidase function in this organism, and suggests that PS3 should not be regarded as a special case in discussion of bacterial *a*-type oxidases [2].

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