Identification of cytochromes *o* and a_3 as functional terminal oxidases in the thermophilic bacterium, PS3

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Low-temperature photodissociation spectra of membranes from the thermophile PS3 reveal cytochromes *o* and a_3. The latter reacts with O_2 at $-103°C$ to give a light-insensitive compound(s), but the initial stages of O_2 binding to cytochrome *o* could not be studied under these conditions. Photochemical action spectra identify cytochromes a_3 and *o*, but not a CO-binding *c*-type cytochrome, as functional terminal oxidases in this bacterium.

Cytochrome oxidases *Bacterial respiration* *Cytochrome* o *Cytochrome* aa_3

1. INTRODUCTION

Many heterotrophic bacteria (review [1]) and cyanobacteria [2] possess cytochrome oxidases of the aa_3 type that resemble the cytochrome *c* oxidase (EC 1.9.3.1) [3] found in the mitochondria of most eukaryotes. In some bacteria, these oxidases comprise only two or three types of subunits, making them attractive experimental systems. An evolutionary link between the bacterial and mitochondrial oxidases is suggested by:

(i) Immunological cross-reactivity between the subunit II from beef, yeast and *Paracoccus denitrificans* [1];
(ii) Cytochrome *c* specificities of the oxidases from diverse bacteria and cow [4];
(iii) Amino acid sequence homologies of subunits I [4];
(iv) The finding [5] that it is with the largest, mitochondrially synthesized subunits of the eukaryotic enzyme that the functionally essential chromophores (haems *a*, a_3 and two copper atoms) are associated.

In [6] a stable cytochrome oxidase, termed an aa_3-type, from the thermophilic bacterium PS3 had catalytic properties similar to the mitochondrial enzyme, contained *a* and *c*-type cytochromes together with copper, and yet was composed of only one subunit type. Later experiments [7] failed to demonstrate CO binding by the presumptive *a*-type cytochrome, but a CO-binding cytochrome *b* (i.e., cytochrome *o*) was detected (Yang, E. and Sone, N., unpublished; cited in [1]). It was suggested that the PS3 oxidase comprised an *o*-type ligand-binding cytochrome and multiple *a* and/or *c*-types in a novel configuration. However, membranes from PS3, grown aerobically under carefully controlled conditions [8] contained CO-binding cytochromes of *c*-, *o*- and *a*-types. The diminution of the α-peak of the *a*-type cytochrome by CO liganding was small (~10%) but clearly due to an a_3 component.

Here, we demonstrate that cytochromes *o* and a_3 form photodissociable CO complexes, are O_2-reactive, and are functional terminal oxidases in this bacterium.

2. MATERIALS AND METHODS

2.1. *Organism and growth conditions*

The thermophile PS3 was generously donated by Dr T. Oshima (Mitsubishi-Kosei Institute of Life Sciences, Machida-Shi, Tokyo). Large-scale growth

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at 65°C and preparation of washed membranes were as in [8]. For photochemical action spectra, 50 ml cultures (in 250 ml Erlenmeyer flasks) were vigorously shaken at 65°C until cells had reached the mid-exponential phase of growth. After harvesting by centrifugation, cells were resuspended in the supernatant giving ~8-fold concentration over the growing culture.

2.2. *Low-temperature photodissociation studies*

Membranes were suspended in a buffer that contained 30% (v/v) ethylene glycol and 50 mM Tris/H_2SO_4 (pH 8.0 at room temperature), and reduced with 1 mM NADH or 2 mM ascorbate plus 0.1 mM TMPD. The method was essentially that in [9,10]. Flash photolysis was achieved in the sample compartment of a Johnson Foundation microprocessor-controlled dual wavelength spectrophotometer, by means of a 200 J Xenon lamp.

2.3. *Photochemical action spectra*

Measurements of the respiration of suspensions (5 ml) of whole bacteria were made at 40°C in an open O_2 electrode vessel. The CO/O_2 gas mixture (19/1; v/v) flowed at 210 ml/min over the vortex of the stirred suspension. Spectra for the photolytic release of CO-inhibited respiration were obtained by irradiation of the suspension with a jet-stream dye laser using rhodamine 6G (567–634 nm) or rhadamine 110 (536–572 nm). The error inherent in the method was estimated by making 7 replicate measurements of the relief of respiration at 570 or 587 nm; the coefficient of variation (SD mean) was 2–4%. Full details of the method are presented in [11].

3. RESULTS AND DISCUSSION

At room temperature, CO binds to *a*, *c* and *b* (*o*)-type cytochromes in washed membrane particles [8]. Photodissociation of CO-liganded membranes at low temperatures (−85 to −103°C) gave maxima in the difference spectra at ~436 and 443 nm (fig. 1,2), assigned to cytochromes *o* and a_3, respectively, and 547, 553 and 605 nm (not shown). That the Soret band contains at least two components in such spectra was shown by the variation in the relative absorbances of the shoulders at 436 and 443 nm in different preparations (fig. 2) and the disappearance of the lower wavelength

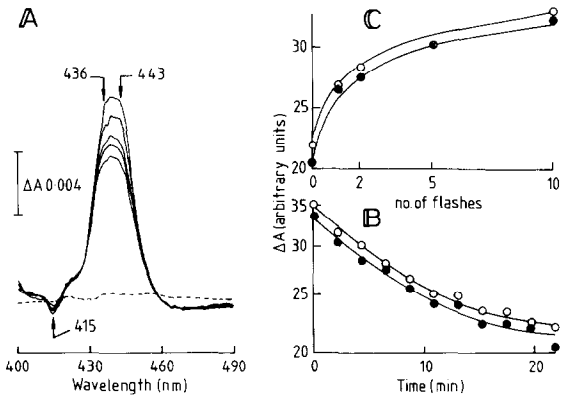


Fig. 1. Formation and photolysis of the CO compounds of cytochromes a_3 and *o* at −85°C. Membrane particles were reduced with NADH and bubbled with CO for 2.5 min in a 2 mm pathlength cuvette before freeze-trapping in the absence of O_2. The spectrum of the CO-liganded form was recorded, stored in the dual wavelength instrument at −85°C (reference wavelength 500 nm) and subtracted from all subsequent scans at this temperature. The next scan gave the baseline (– – –). The sample was then photolysed with 16 flashes from a Xenon lamp and spectra (top to bottom) were recorded 2.2, 6.5, 10.9, 15.3 and 21.8 min after photolysis (A). Absorbance changes in these and other scans are plotted against time (B). After ~22 min, the sample was exposed to 1, 2, 5 and 10 further flashes (C). In B and C, the ΔA at 436–456 nm (○) and 443–456 nm (●) are shown; protein was 3.2 mg/ml.

component when photolysis was performed in the presence of oxygen (fig. 3). Fig. 1(A,B) show that, at −85°C, the recombination of CO with both components can be readily observed with the wavelength scanning technique and that the rate of recombination was similar for cytochromes *o* and a_3. The rebinding was not a simple first-order process. After 22 min, the extent of photolysis at 436 and 443 nm when the sample was irradiated with the Xenon flash lamp (fig. 1C), demonstrated that the light sensitivity of CO-binding to both cytochromes was similar.

When oxygen (~400 μM) was introduced to a CO-liganded sample held at −25°C in the liquid state, and the freeze-trapped sample photolysed at −103°C, the difference spectrum (fig. 3) showed prominent bands at 443 and 602 nm; similar spectra are seen in cells or mitochondria where cytochrome aa_3 is the sole terminal oxidase [12,13].

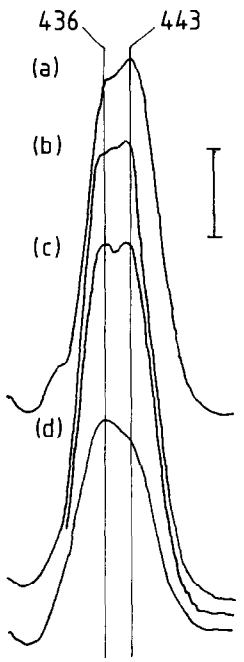
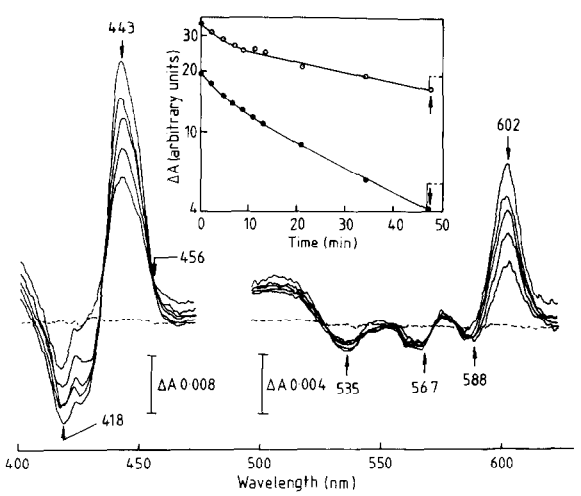


Fig. 2. Shapes of the Soret bands of photodissociable CO-binding cytochromes. Four experiments are shown, each performed essentially as in fig. 1 but with minor modifications. Each spectrum is the first after photolysis. The reductant, temperature (0°C), protein concentration (mg/ml) and ΔA corresponding to the bar were, respectively: (a) NADH, −100, 1.5, 0.008; (b) NADH, −103, 3.1, 0.008; (c) NADH, −100, 1.5, 0.008; (d) ascorbate plus TMPD, −100, 1.5, 0.02.



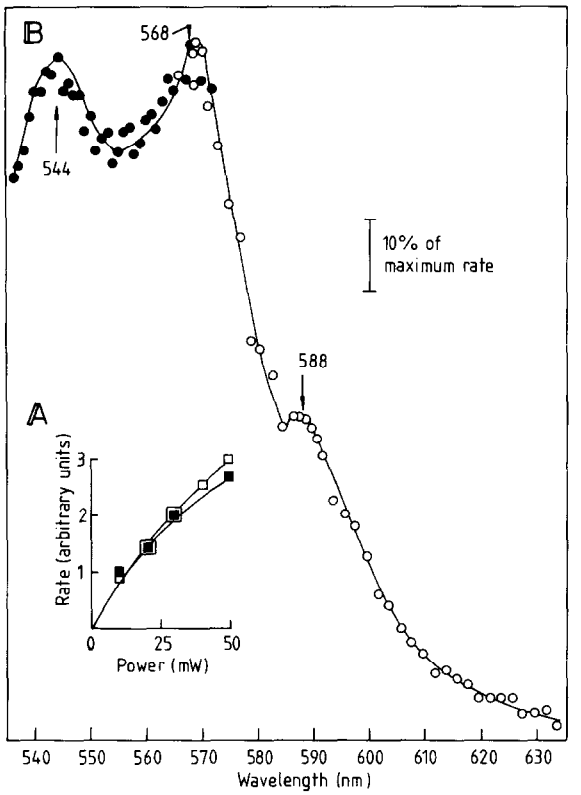


Fig. 4. Photochemical action spectrum of PS3. (A) Irradiation of a cell suspension at 570 nm (—■—; rhodamine 110) and at 590 nm (—□—; rhodamine 6G). Output of the dyes was varied by adjustment of power output of the argon-ion pump laser. (B) Irradiation from 536–572 nm used Rhodamine 110 (—●—; mean of two spectra), that from 567–634 nm rhodamine 6G, both at 30 mW. Spectra were normalized by comparison of data obtained at 571 nm. Respiration rate is plotted on the ordinate in arbitrary units; the O_2 concentration in the liquid phase was ~3.3 μM.

Fig. 3. Initial stages of O_2 binding to cytochrome a_3 in PS3 membranes. Methods were as shown in fig. 1 except that O_2 was present before freeze-trapping. Following photolysis (10 Xenon flashes) at −103°C, the sample was scanned repetitively; spectra initiated 2.2, 6.5, 10.9, 22.3 and 48.6 min later are shown. Absorbance stages are downward in the 443 and 602 nm regions and upward around 418 nm. The inset shows ΔA at 443–456 nm (—○—) and 603–582 nm (—●—). At the time shown by the arrows, 10 further flashes were given which resulted in the small ΔA shown by the dashed lines; protein was 3.1 mg/ml and pathlength 2 mm.

Surprisingly, maxima at 436 and between 540 and 560 nm, assigned to the photolysed CO complexes of *b*(*o*)- [9,10] and for *c*-type cytochromes were not observed. This phenomenon requires further study, but may result, for example, from:

(i) The displacement by O_2 of the CO from cytochrome *o* at −25°C during sample preparation and subsequent oxidation; or
(ii) The very fast combination of O_2 with the reduced cytochrome *o*.

The CO complex of cytochrome *d* in *Escherichia coli* is rather O_2-labile at this temperature (unpublished).

Successive scans of the photolysed sample held at −103°C revealed diminution of the peaks at 443 and 603 nm, the latter being somewhat faster (fig. 2 and inset). After 47 min, the sample was given 10 further flashes sufficient to produce almost complete photolysis of the CO–a_3 compound (fig. 1). The small absorbance changes resulting from this treatment (fig. 2 inset) suggest that the ligand binding observed is that of O_2, due to the lower quantum yield for photolysis of O_2-containing compounds [14].

Photochemical action spectra for the relief of CO inhibition of respiration were obtained to determine whether the CO-binding cytochromes act as functional terminal oxidases. Fig. 3A shows that illumination or bacteria under a gas phase of CO/O_2 (19/1) with monochromatic light at 570 or 590 nm gave stimulation of respiration that was proportional to the laser output up to 30 mW. The action spectrum revealed at least two oxidases. The prominent peak at ~568 nm corresponds to the CO complex of cytochrome *o* [8,15], whilst the shoulder at 588 nm (observed in 4 separate spectra) coincides with the absorption maximum of the CO-liganded form of cytochrome a_3 ([8]; fig. 2). The broad peak centred at 544 nm probably contains contributions from the β bands of cytochrome a_3 and *o*. The band at 588 nm could also be attributed (as it has been in *Azotobacter vinelandii* [16]) to cytochrome a_1; the distinction between a_1 and a_3 rests solely on the position of the α-band in the reduced form [17]. The action spectra do not provide any indication of a distinctive contribution from the CO-binding cytochrome *c* [8] as a functional oxidase.

We conclude that the thermophile PS3 contains at least two CO-binding cytochromes that act as functional oxidases; these are cytochrome a_3, as originally proposed [6], together with cytochrome *o*. This organism is probably *Bacillus stearothermophilus* (T. Oshima, personal communication) and thus related to *B. subtilis* which appears to possess the same oxidases [18].

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