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PURIFICATION AND PARTIAL CHARACTERIZATION OF TWO CYTOCHROME OXIDASES (caa_3 AND *o*) FROM THE THERMOPHILIC BACTERIUM PS3

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Two cytochrome oxidases, cytochrome aa_3 (EC 1.9.3.1) and cytochrome *o*, have been purified from the membranes of a thermophilic bacterium, PS3. The enzymes were solubilized with Triton X-100 and purified to apparent homogeneity on anion-exchange columns. The properties of the three-subunit cytochrome oxidase complex caa_3 obtained here are compared with the same enzyme isolated by Sone, N. and Yanagita, Y. (1982) (Biochim. Biophys. Acta 682, 216–226). On storage, the purified caa_3 enzyme undergoes denaturation; a shoulder at 432 nm seen in (CO-reduced)-minus-reduced difference spectra may be due in part to denaturation products of the enzyme. The purified cytochrome *o* is more stable. At room temperature, the reduced-minus-oxidized difference spectrum shows absorbance maxima at 427 and 559 nm; at 77 K, its α-band is split into 554 and 557 nm components. At room temperature, the CO-reduced-minus-reduced spectrum shows troughs at 430 nm and 560 nm. Dissociating polyacrylamide gel electrophoresis suggests that the purified cytochrome *o* is composed of one type of subunit with an apparent molecular mass of 47000–48000. Metal analysis of the purified enzyme demonstrated the lack of copper. Both oxidases, purified in the presence of Triton X-100, exist in highly polydisperse forms.

Introduction

The cytochrome oxidases of bacteria are structurally diverse, the main groups being cytochromes a_1, *o*, *d*, cd_1 and aa_3 [1,2]. The bacterial aa_3-type oxidases resemble most closely the mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) and several have now been purified. Their most striking feature is the relative simplicity of subunit composition. In contrast to the mitochondrial enzyme (comprising 7–13 subunits), the bacterial enzymes contain three or fewer subunits [2,3]. The aa_3-type enzymes from thermophilic bacteria co-purify with a cytochrome *c* [4,5].

In contrast, there are few detailed investigations on purified cytochrome *o*, a protohaem oxidase, and the most widely distributed bacterial cytochrome oxidase [2]. Purified cytochrome *o* from *Azotobacter vinelandii* [6–8] appears to consist of only one type of subunit ($M_r = 28000$) and two identical haem components. The oxidase from *Methylophilus methylotrophus* is a complex [9] composed of cytochrome *c* ($M_r = 21000$) and cytochrome *o* ($M_r = 29000$) in a 1 : 1 to 2 : 1 ratio. The well-characterized *Vitreoscilla* oxidase consists of two identical polypeptides ($M_r = 13000$) [10], but appears to be a special case, since it is soluble and its ligand-binding properties are more like those of an oxygen carrier [11]. More complex membranous cytochromes *o* have been purified from *Escherichia coli* [12] and *Pseudomonas aeruginosa* [13,14].

We have previously demonstrated that the thermophilic bacterium PS3 synthesizes CO-binding

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cytochromes of the *c*-, *o*- and *a*-types [15] and that photochemical action spectra identify a_3 and *o*, but not the CO-binding *c*-type cytochrome, as functional terminal oxidases [16]. We report here the purification of both oxidases from cytoplasmic membranes of PS3, and a preliminary characterization of cytochrome *o*. We also compare the caa_3-type oxidase purified in this work with the similar enzyme obtained by Sone and Yanagita [4] and comment on its supposed thermostability.

Materials and Methods

Organism, growth conditions and preparation of membranes

PS3 was donated by Dr. T. Oshima (Mitsubishi-Kosei Institute of Life Sciences, Machida-shi, Tokyo) and grown to early stationary phase at 65 °C in a 10 l Biostat-V fermenter (FT Scientific Instruments, Bredon, Glos., GL20 7HH, U.K.); sterile air was sparged into the medium at 5 l/min; the stirring speed was 500 rpm. The chemically defined growth medium contained 90 mM monosodium glutamate as major carbon and nitrogen source; full details will be described elsewhere. Membranes were prepared as described in Ref. 15, and rapidly frozen in liquid nitrogen prior to storing at −20 °C until required.

Reagents and Chemicals

Triton X-100, puriss grade, was obtained from Koch-Light Laboratories Ltd. DEAE Sephadex A 50 was from Pharmacia (U.K.) Ltd., Whatman DE 32 cellulose from Uniscience Ltd., and hydroxylapatite, HA-Ultragel from LKB Ltd. Horse cytochrome *c*, type VI, was purchased from Sigma.

Analytical methods

Difference spectra at 77 K were obtained as described by Salmon and Poole [17]. Cytochrome contents were determined using the wavelength pairs and extinction coefficients given by Smith [18]. Metal contents were determined on samples of purified oxidase, extensively dialysed against 1 mM EDTA. A measured volume of the dialysed sample was placed in a lyophilizing tube and dried in a stream of nitrogen. 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 manner. Analysis of the hydrolysate was carried out by electrothermal atomic absorption spectroscopy, with a Perkin-Elmer model 2380 atomic absorption spectrometer using deuterium arc background correction and a Perkin-Elmer HGA-400 graphite furnace. The programs used for copper [19] and iron [20] were based on published methods. Dissociating polyacrylamide gel electrophoresis in the presence of SDS and urea [21] and acid gels [22] were run using published methods. For SDS/urea electrophoresis, the dissociation of the protein (5 μg) was carried out in 6% SDS and 6 M urea containing 20 mM 2-mercaptoethanol, followed by dialysis against the buffer of the upper reservoir in the electrophoresis cell for 12 h at room temperature. The buffer system J 4179 [21] and 7.5% (w/v) polyacrylamide were used. Haem staining in the gels was carried out as described by Reid and Ingledew [23]. Protein was estimated by the Lowry method, except for adding SDS (10 $mg \cdot ml^{-1}$) and ensuring that the temperature was higher than 30 °C, to avoid interference by Triton X-100. Cytochrome *c* oxidase activity of the purified enzymes was measured at 40 °C using equine ferrocytochrome *c* [4] as electron donor. O_2 uptake measurements were made using a Clark-type O_2 electrode (Rank Bros., Bottisham, Cambs., U.K.). The steady-state concentration of ferrocytochrome *c* (total [cytochrome *c*], 25 μM) was maintained by using 5 mM hydroquinone in a total volume of 2 ml. All the solutions were prepared in 50 mM KH_2PO_4/NaOH buffer (pH 6.1). The reaction was initiated by addition of 20 μl enzyme solution at an appropriate protein concentration.

Purification of cytochrome oxidases

Steps 1, 2 and 3. Membranes prepared from 200 g wet weight cells (grown in defined medium) were used.

The initial steps [4] involved extraction with 1% sodium deoxycholate and 1% sodium cholate, followed by washing with 4 M-LiCl and extraction of the residues with 6% Triton.

All subsequent steps were modified in order to decrease the volumes loaded to ion-exchange col-

umns and overcome the problems associated with non-specific aggregation of the solubilized membrane proteins (see Results and Discussion).

Step 4. (a) First anion-exchange chromatography. The supernatant from step 3 (typically 220 ml) was concentrated in a stirred ultrafiltration cell to approx. one-tenth of the original volume, and the resultant concentrate diluted to the original volume with distilled water. This was applied to a column (20 × 2.5 cm) of DE 32 cellulose, preequilibrated with 50 mM Tris-HCl (pH 8.0)/0.5% (v/v) Triton X-100. After loading, the column was washed with the same buffer until $A_{412} \geq 0.01$. Cytochrome oxidase aa_3 failed to bind to this column and fractions rich in the enzyme were pooled to give fraction I. A linear gradient of up to 300 mM NaCl in the same buffer (total volume 1 l) was then applied and fractions (10 ml) were collected and analysed by measurement of A_{412}, reduced-minus-oxidized difference spectra and CO-reduced-minus-reduced spectra. Fractions rich in cytochrome *o*, eluting between about 75 and 115 mM NaCl, were pooled to give fraction II and treated as described below.

(b) Fraction I – a source of cytochrome oxidase caa_3. The pooled fractions were rapidly concentrated and diluted as before. This was applied to a column (20 × 2.5 cm) of DEAE Sephadex A50 pre-equilibrated with 10 mM Tris-HCl (pH 8.0)/0.5% (v/v) Triton X-100; all the material bound to this column. Elution was effected with three linear gradients (see later; Fig. 1). Fractions (8 ml) were collected and analysed as for the first ion-exchange column. Fractions rich in caa_3, and least contaminated with other cytochromes were pooled, concentrated and stored at 77 K or 4°C.

(c) Fraction II – a source of cytochrome oxidase o. The pooled fractions were concentrated, diluted and applied to a column (20 × 2.5 cm) of DEAE-Sephadex A50 pre-equilibrated with 50 mM Tris-HCl (pH 8.0), containing 100 mM NaCl and 0.5% (v/v) Triton X-100. All the material bound to the column, and elution was achieved by applying a linear gradient of 100–700 mM NaCl in the same buffer. Fractions (10 ml) were analysed as before and those rich in cytochrome *o*, eluting at about 400 mM NaCl were pooled, concentrated and stored at 77 K or 4°C.

Results and Discussion

In our earlier attempts to fractionate the cytochrome components of PS3 membranes, variable yields and activities of the purified enzyme were obtained. After the complete solubilisation of cytochromes by Triton X-100 (step 4), the protein components undergo non-specific aggregation in a time-dependent fashion making resolution of oxidases from the highly polydisperse aggregates difficult. In order to improve yields and reduce the contact period of solubilised proteins, a strategy of rapid concentration, followed by dilution to reduce the conductivity before loading on to the ion-exchange columns was used. This modification of the Sone and Yanagita [4] method reduced the

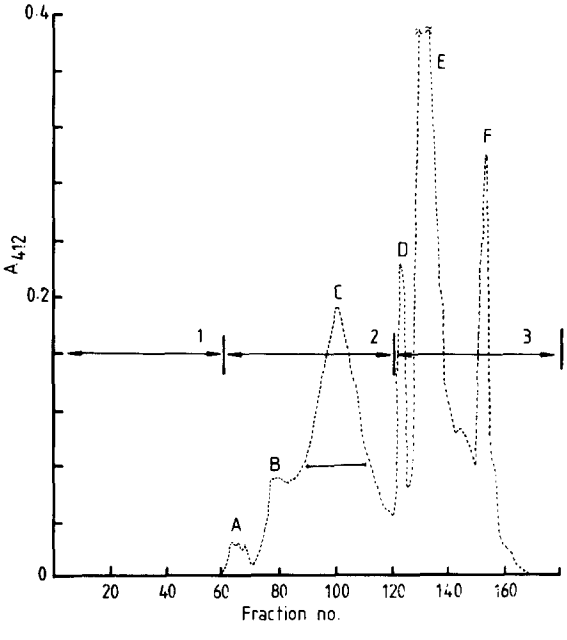

Fig. 1. Chromatography of pooled fractions I (see text) on DEAE, Sephadex A 50 column (20×2.5 cm). Gradient (1), 10 mM Tris-HCl (pH 8.0) containing 0.5% (v/v) Triton X-100 to 100 mM Tris-HCl (pH 8.0) containing 0.5% (v/v) Triton X-100 (total volume, 500 ml); gradient (2), 100 mM Tris-HCl (pH 8.0) containing 0.5% (v/v) Triton X-100, to 100 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 0.5% (v/v) Triton X-100 (total volume 500 ml); gradient (3), 100 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 0.5% (v/v) Triton X-100 to 100 mM Tris-HCl (pH 8.0) containing 1 M NaCl and 1% (v/v) Triton X-100 (total volume 500 ml). The fractions (8 ml) under peak C were pooled as indicated by the horizontal bar line, and concentrated to provide purified cytochrome oxidase caa_3.

extent of protein aggregation, although it did not alleviate it completely. Thus, fractions (Fig. 1) eluted at high salt concentrations (gradient 3) were composed entirely of various aggregated cytochrome components. A 77 K (dithionite-reduced)-minus-(persulphate-oxidized) spectrum of this aggregate showed α-bands at 545.5, 561, 564, 569 and 603 nm (results not shown). Since each purification yielded different amounts of the two oxidases, presumably dependent upon the extent of non-specific aggregation, a purification table is omitted. Further purification on a hydroxylapatite column did not increase the specific haem contents of the two oxidases.

Properties of the purified cytochrome caa_3

The 77 K [(dithionite-reduced)-minus-(persulphate-oxidised)] spectrum of the freshly prepared caa_3 complex was characterised by major α absorption peaks at 547, 604 and 561 nm (Fig. 2). The room temperature spectra of freshly prepared enzyme were identical to those obtained previously [4] and did not reveal any cytochrome *b* around 560–561 nm. By combined potentiometric and spectral analysis of PS3 membranes, we have suggested that the cytochrome *c* that co-purifies with cytochrome oxidase aa_3, and which has a λ_{max} at 550 nm at room temperature [24], is the 547 nm component observed at 77 K (E_{m7} + 229 mV) [25].

Further analysis of this purified oxidase complex was frustrated by its apparent instability at low temperatures. Thus, in samples stored at 4°C for periods of up to 30 days, a 562 nm component

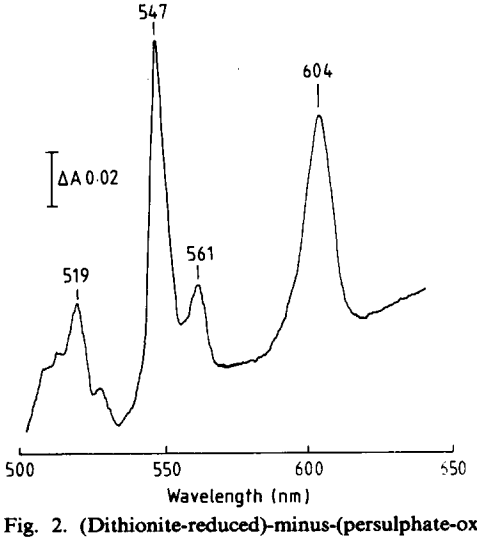

Fig. 2. (Dithionite-reduced)-minus-(persulphate-oxidised) difference spectrum at 77 K of the purified cytochrome oxidase caa_3 complex. The concentrated protein (0.5 ml) was diluted in 0.5 ml of 50 mM Tris-HCl (pH 7.4). The path length was 2 mm and the protein concentration was 1.8 mg·ml^{-1}.

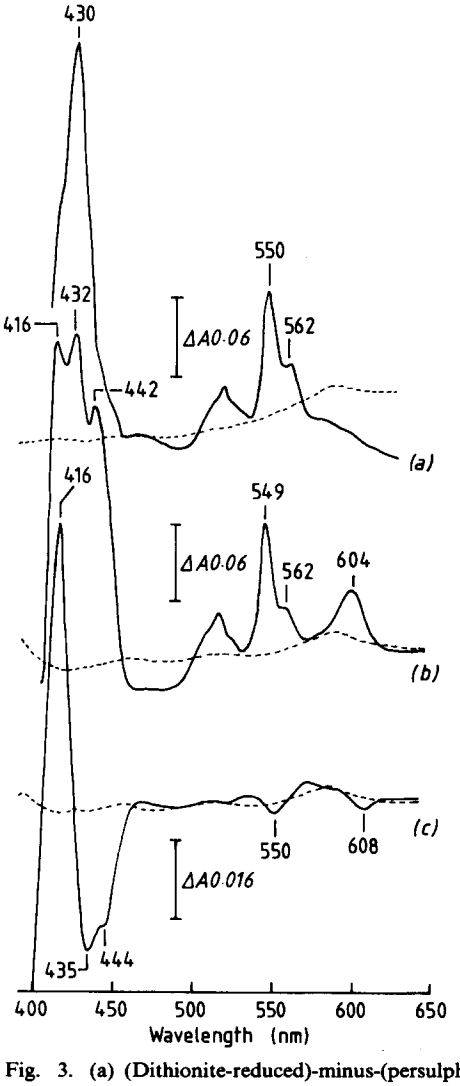

Fig. 3. (a) (Dithionite-reduced)-minus-(persulphate-oxidized) spectrum at room temperature of the purified cytochrome oxidase caa_3, stored at 4°C for 30 days. (b) (Dithionite-reduced)-minus-(persulphate-oxidised) spectrum at room temperature of the purified cytochrome oxidase caa_3 stored at 77 K for 30 days. (c) (CO + reduced)-minus-reduced difference spectrum of the purified cytochrome oxidase caa_3 stored at 77 K for 30 days. In each case, the protein was stored in the concentrated form and diluted (0.25 ml) into 0.75 ml of 50 mM Tris-HCl (pH 7.4). The path length was 10 mm and the protein concentration was 0.9 mg·ml^{-1}.

(in reduced-minus-oxidized, room temperature, spectrum) appeared paralleling the loss of oxidase activity and disappearance of the 604 nm absorbance band (i.e., aa_3). In the Soret region, a broad absorbance band centred at 430 nm appeared (Fig. 3a). The freshly prepared oxidase complex is characterized in the Soret region of the spectrum by absorbance bands at 416 and 444 nm (not shown, see Ref. 24). These spectral changes were slowed down, but not eliminated, by storage of the purified oxidase complex at 77 K (Fig. 3b). The CO-reduced-minus-reduced difference spectrum of the sample of oxidase stored at 77 K for 30 days is shown in Fig. 3c. An analogous spectrum of the freshly prepared enzyme was characterized by a shoulder at 432 nm on the 446 nm trough (results not shown; see Ref. 4).

Further indications that gross changes in the state of the purified oxidase had occurred were provided by metal analysis of the freshly prepared protein and of the protein stored at 77 K for 30 days. Freshly prepared oxidase complex contained almost equistoicheiometric amounts (0.92) of copper and haem *a*, whereas a stored sample gave a ratio of 0.66 (assuming the haem *a* content of freshly prepared oxidase complex; Table I). Analysis of the total iron content of the stored sample provided a value one-third of that expected, assuming that the protein comprises three haem groups (two haems *a* and one haem *c*). A possible explanation is that both haems *a* readily dissociate from the protein following prolonged storage at low temperatures, and that the iron and some copper are lost during dialysis against EDTA.

The results obtained here demonstrate that the new 562 nm component (in room temperature spectra) binds CO, and is open to misinterpretation as cytochrome oxidase *o*. Note that membranes derived from aerobically grown PS3 contain a cytochrome *b* (λ_{max} 561 nm at 77 K) characterised by an E_{m7} of −130 mV [25]. The negative midpoint potential of this cytochrome *b* suggests that it is unlikely to be cytochrome oxidase *o*.

Some properties of the purified caa_3 oxidase complex are summarised in Table I. The properties of the present preparation are similar to those reported previously [4], except for the somewhat lower relative molecular weight of subunit II. Detailed analyses of the catalytic and ligand-binding properties are in progress.

TABLE I

SOME PROPERTIES OF CYTOCHROME OXIDASES PURIFIED FROM PS3

(i) SDS/urea polyacrylamide gel electrophoresis. (ii) Acid polyacrylamide gel electrophoresis.

	caa_3 complex				Cytochrome *o*-containing complex	
	Freshly prepared protein (less than 7 days storage at 77 K)		Sample stored at 77 K for 30 days	Ref. 4		
Haem groups (nmol per mg protein)						
b	–		n.d.	?	20.2	
c	8.05		n.d.	8.3	–	
a	14.8		n.d.	15.9	–	
Subunits	(i)	(ii)				
I	54950	58200	n.d.	56000	(i)	(ii)
II	27850	28200	n.d.	38000	47300	48700
III	22400	26650	n.d.	21000		
Cu content (nmol per mg protein)	13.6		9.8	17.6–18.2	1.7	
Fe content (nmol per mg protein)	n.d.		8.6	n.d.	21.7	
Catalytic activities (per min per mg protein)	217 nmol cyt *c* oxidized		–	n.d.	62 nmol cyt *c* oxidized	
Ferrocytochrome $c \rightarrow O_2$						
Hydroquinone → cytc $c \rightarrow O_2$	162 nmol O_2 reduced		n.d.	n.d.	29 nmol O_2 reduced	

n.d. not determined.

–, none found.

Properties of the purified cytochrome oxidase o

Fig. 4 shows the room temperature difference [(dithionite-reduced)-minus-(persulphate-oxidized)] spectrum of the purified cytochrome *o* complex. The symmetrical α-band is at 559 nm and the Soret band at 427 nm. In the (CO-reduced)-minus-reduced spectrum, the α-band trough is at 560 nm and the corresponding Soret trough at 430 nm; the peak at 412 nm is due to the oxidase CO-adduct (Fig. 4B). At 77 K, the α-band of the reduced-minus-oxidised spectrum split into two components at 554 nm and 557 nm and the β maximum was at 526 nm (not shown). Three *b*-type cytochromes have been resolved in PS3 at 554 nm (E_{m_7}, −194 mV), 557 nm (E_{m_7}, +104 mV) and 561 nm (E_{m_7}, −130 mV) [25]. On the basis of its high redox potential, cytochrome *b*-557 was tentatively equated with the *o*-type oxidase of this organism. The results obtained in the present work support this assignment and provide evidence for the close association of cytochrome *b*-554 (of more negative potential) with the cytochrome *o* complex.

Fig. 5 shows typical electrophoretic patterns of PS3 cytochrome oxidases on SDS-polyacrylamide gels containing urea. Similar patterns were obtained in acid gels. PS3 cytochrome *o* is apparently composed of one type of polypeptide subunit of relative molecular mass 47000–48000 (Table I). This value is approx. twice the value obtained for cytochrome *o* purified from other sources (see

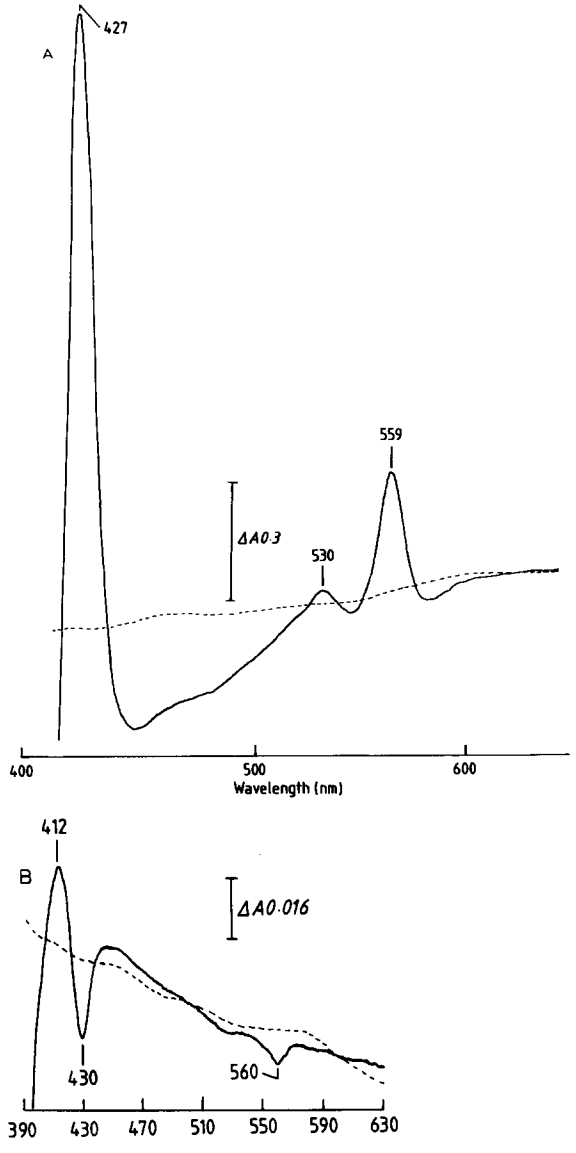

Fig. 4A. (Dithionite-reduced)-minus-(persulphate-oxidized) spectrum at room temperature of the purified cytochrome oxidase *o*. The concentrated protein (0.5 ml) was diluted in 0.5 ml of 50 mM Tris-HCl (pH 7.4). The path length was 10 mm and the protein concentration was 1.5 mg·ml^{-1}. Fig. 4B shows the (CO − reduced)-minus-reduced spectrum of the purified cytochrome oxidase *o* (path length 10 mm; protein concentration, 0.15 mg·ml^{-1}).

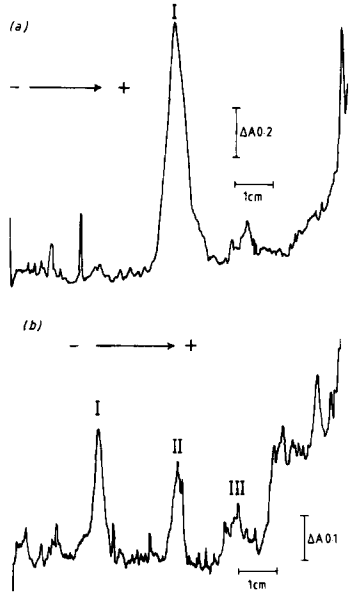

Fig. 5. Densitometric trace of PS3 cytochrome oxidase *o* (a) and caa_3 (b) on SDS/urea electrophoresis gels.

Introduction), but is similar to the largest subunit of aa_3-type oxidases. It is unlikely that the band is the result of incomplete dissociation, since two independent methods for dissociating the protein, i.e., the acid/phenol system [22] and SDS/urea at room temperature, provided identical results.

Attempts to obtain estimates of the apparent molecular weight of the purified cytochrome *o* complex were unsuccessful, due to the highly polydisperse nature of the purified protein, as evident by its exclusion on appropriate gel filtration columns.

The iron-to-heam *b* ratio in cytochrome *o* was 1 : 1 (Table I). In common with other cytochromes of the *o*-type previously isolated from *Ps. aeruginosa* [13,14] and Vitreoscilla [10], the PS3 cytochrome *o* lacks copper (Table I). Purified cytochrome *o* is stable when stored either at 4 °C or 77 K for 30 days.

The catalytic activities of purified cytochrome *o* were generally lower than those of the caa_3 oxidase. Direct comparison is not possible, since the most effective electron donors for the two systems are likely to be different. Detailed catalytic properties and the specificity of cytochrome *o* towards various electron donors have yet to be evaluated. We view the catalytic activities reported here with caution, since, in both cases, the purified oxidase complexes existed in a highly aggregated state, the extent of which is dependent upon the detergent used to solubilise membrane proteins. This, in turn, can have a strong influence on the function and structural properties of solubilised membrane-associated proteins [26,27]. Work directed towards minimising this limitation is in progress and will be reported elsewhere.

It is noteworthy that other proteins isolated from thermophilic bacteria have been described as thermostable, and are widely regarded as attractive experimental systems for that reason. Although PS3 cytochrome oxidase (caa_3) activity is stable at room temperature and against denaturing reagents [24], the present work has revealed a cold lability reminiscent of some other membrane-bound enzymes [28].

The lability of the *a*-type oxidases and the spectral characteristics of a degradation product, reminiscent of a *b*-type cytochrome, may explain the report [29] that certain preparations of the aa_3-type oxidase indicated an unusually high level of contamination with a cytochrome *o*-like pigment.

Acknowledgments

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