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A CYTOCHROME aa_3-TYPE TERMINAL OXIDASE OF A THERMOPHILIC BACTERIUM

PURIFICATION, PROPERTIES AND PROTON PUMPING

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Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase EC 1.9.3.1) was purified from thermophilic bacterium PS3 by ion-exchange and hydroxyapatite chromatography in the presence of Triton X-100. The enzyme possessed 15.9 nmol heme *a*, 8.9 nmol heme *c* and 16–18 ng atom Cu per mg protein and was composed of three subunits of relative molecular mass 56000, 38000 (heme *c*-binding) and 22000. Since the ratio of the subunits was 1:1:1, one enzyme unit may be composed of one molecule each of the three subunits containing two hemes *a*, one heme *c* and two copper atoms. The enzyme shows very similar absorption characteristics, including the CO difference spectrum, to the mitochondrial enzyme, indicating that the PS3 enzyme is of the cytochrome aa_3 type with a firmly bound cytochrome *c*. The enzyme rapidly oxidizes cytochrome *c*-551 from PS3 and cytochrome *c*-552 from *Thermus thermophilus*, and has a lower affinity for yeast cytochrome *c* from *Candida krusei*. The reduction product of oxygen is concluded to be water on the basis of stoichiometric measurements. The enzyme, when reconstituted into proteoliposomes, can translocate H^+ in an uncoupler-sensitive fashion, indicating that the enzyme is a proton pump.

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1), the terminal enzyme of the respiratory chain, is known to catalyze oxidation of ferrocytochrome *c* by molecular oxygen along with concomitant transformation of energy to an electrochemical proton gradient [1,2]. The enzyme of mitochondria from beef heart and yeast is believed to have two hemes *a* and two copper prosthetic groups, and to be composed of seven kinds of subunits [3–5]. Out of the seven kinds of subunits, the largest three peptides are known to be coded for by mitochondrial DNA [6,7]. The complete sequence of human mitochondrial DNA has been determined very recently [8]. Recent observations [9–12] suggest that the enzyme pumps H^+ in the reverse direction in addition to simply carrying electrons across the membrane [13,14].

The participation of corresponding enzymes has been established in aerobic bacteria [15], but studies at the molecular level have only a short history. We have purified cytochrome oxidase from a thermophilic bacterium PS3 [16], aided by the stable nature of the enzyme and ease of large-scale culture. The purified enzyme shows a simpler subunit composition but very similar enzymatic properties [16] to those of mitochondrial cytochrome oxidase. Very recently, similar enzymes have been purified

Abbreviations: Mes, 4-morpholineethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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also from *Thiobacillus novellus* [17], *Thermus thermophilus* [18,19], *Paracoccus denitrificans* [20] and *Nitrobacter agilis* [21]. All these bacterial enzymes were reported to be composed of two kinds of subunit while still demonstrating very similar enzymatic and spectral properties to the mitochondrial enzyme (Refs. 16–21; see also Ref. 22 for a review). However, in the cases of the thermophilic bacteria, PS3 and *T. thermophilus,* a *c*-type cytochrome is found to bind firmly [16,18,19]. Bacterial cytochrome oxidase can also convert redox energy to chemiosmotic energy; the enzyme from PS3 generates a membrane potential when reconstituted into proteoliposomes [16], and reconstituted *Paracoccus* enzyme is reported to show 'respiratory control' [20].

Here we describe the purification and properties of heat-stable PS3 cytochrome oxidase which is composed of three kinds of subunit and is capable of H^+ pumping. The correspondence of these three subunits to the largest three subunits of the eukaryote enzyme, which are coded for by mitochondrial DNA, is also discussed.

Experimental Procedure

Materials. Bacterial cytochrome *c*-552 from *T. thermophilus* HB8 [23] was kindly donated by Dr. Hon-nami of Mitsubishi-kasei Institute of Life Science, Machida. Methods for purification of PS3 cytochrome *c*-551 will be described elsewhere. Yeast cytochrome *c* from *Candida krusei* was purchased from Sankyo Co., Tokyo. Bovine serum albumin, sperm whale myoglobin, ovalbumin, equine cytochrome *c*, dithiothreitol, Mes, and Tricine were products of Sigma, St Louis. Glucose oxidase, valinomycin and FCCP were products of Boehringer Mannheim. Bathocuproinesulfonic acid (disodium salt) and sodium *N*-lauroylsarcosinate were purchased from Wako Pure Chemicals, Osaka. DEAE-Toyopearl 650M (DEAE derivative of hydrophilic vinyl polymer gel) was a product of Toyo Soda Co. Tokyo.

Assay of oxidase activity. Ascorbate oxidation via a substrate of cytochrome oxidase was measured at 40°C with an oxygen electrode (YSI 4001). Oxidation of cytochrome *c* was also measured spectrophotometrically in 1 ml of 20 mM sodium phosphate buffer (pH 6.1) containing 15 μM ferrocytochrome *c*. Ferrocytochrome *c* was prepared by reduction with $Na_2S_2O_4$ and successive removal of $Na_2S_2O_4$ by the centrifuge-column method [24], using Sephadex G-15 or Biogel P-6 (Bio-Rad) as a packing material.

Assay of prosthetic groups. Absorption spectra were recorded with a Union-giken spectrophotometer (model SM-401), or a Shimadzu UV-200 spectrophotometer using 1 cm light-path cuvettes. Heme *a* and heme *c* contents were determined after converting them to pyridine ferrohemochromes using millimolar extinction coefficients of 21.7 $mM^{-1} \cdot cm^{-1}$ (587–620 nm, reduced minus oxidised) for heme *a*, and 24.3 $mM^{-1} \cdot cm^{-1}$ (550–535 nm, reduced minus oxidised) for heme *c* [25]. Final concentrations of NaOH and pyridine were 0.05 M and 10% (v/v), respectively. Then sodium ascorbate (about 2 mg) and a small amount of $Na_2S_2O_4$ were added to the sample cuvette. After 5 min the spectrum was recorded. Copper content was determined by atomic absorption with a Varian Techtoron atomic absorption spectrophotometer (model 1100), or by colorimetry with bathocuproinesulfonic acid according to the method of Tsudzuki et al. [26].

Other analytical methods. Amino acid composition was determined in a Nihon-Denshi amino acid analyzer (model JLC-6AH). The sample was carboxymethylated with monoiodoacetic acid in the presence of 0.1% SDS and hydrolyzed in 4 M methanesulfonic acid for 24 h at 115°C in vacuo [27]. Polyacrylamide gel electrophoresis in the presence of SDS was carried out by the method of Weber and Osborn [28] with 8 M urea when described. Malachite green was used as a front marker. Protein in the gel was stained with Coomassie brilliant blue R-250, Amido black 10B or Fast green, and recorded with a Fuji-riken FD-A4 densitometer. Heme staining in the gel was carried out with *o*-tolidine as described by Reid and Ingledew [29]. Protein content was determined by the method of Lowry et al. [30], except for adding SDS (10 mg) to avoid interference by Triton X-100.

Reconstitution of proteoliposomes capable of H^+ translocation. Proteoliposomes containing PS3 cytochrome oxidase were prepared by freeze-thawing [31] as follows: 40 mg of acetone-washed soybean phospholipids [32] in 1 ml of 5 mM Mes-KOH

buffer (pH 6.4) containing 0.2 mM EDTA and 2 mM K_2SO_4 were sonicated for 1 min at 5–15°C under an argon atmosphere with a probe-type sonifier (Branson model 200 at an amplitude of 2). PS3 cytochrome oxidase (0.18 mg) was then added and the mixture was sonicated briefly (5 s). The mixture in the test tube was then quickly frozen, thawed at room temperature, and sonicated for 5 s. The freeze-thawing-sonication cycle was repeated once more. The H^+-pumping activity due to addition of ferrocytochrome *c* was measured with a Beckman combination pH electrode No. 39030.

Purification of PS3 cytochrome oxidase

Steps 1, 2 and 3: The starting material was washed membranes prepared from 500 g of wet cells of the thermophilic bacterium PS3 cultured with vigorous aeration [33]. Pretreatment of membranes, i.e., washing with cholate plus deoxycholate (step 1) and washing with LiCl (step 2), was carried out as described previously [2], except that 2.5 M LiCl was used instead of 4 M LiCl. The resulting residues were extracted with 200–250 ml of a mixture of 6% Triton X-100, 0.05 M NaCl and 50 mM Tris-HCl buffer (pH 8.0) instead of 4% Triton X-100 containing 0.1 M NaCl.

Step 4: DEAE-cellulose column chromatography. The amber-colored Triton X-100 extract from step 3 was diluted with water (about 3 vol.) until its conductance became 0.7 mmho (cm^{-1}), it was then applied to a DEAE-cellulose (Whatman DE-52) column (8 × 6 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0), containing 0.5% Triton X-100. Most of the cytochrome oxidase (cytochrome *c-aa*$_3$ complex) was not absorbed.

Step 5: DEAE-Toyopearl column chromatography. At this step DEAE-Toyopearl (DEAE-derivative of vinyl polymer gel) was used instead of Whatman DE-52 because of its superior separation. The unabsorbed fraction of step 4 was diluted with water until its conductance became 0.4 mmho (cm^{-1}) and then applied to a DEAE-Toyopearl column (3 × 8 cm). The column had been equilibrated with 25 mM Tris-HCl buffer (pH 8.0) containing 0.25% Triton X-100. Cytochrome oxidase was adsorbed, while most *b*-type cytochromes were not. The column was then washed with 300 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 1.5% Triton X-100, followed by 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM NaCl and Triton X-100. Then the concentration of NaCl was raised to 6 mM which caused slow movement of a greenish-brown band, leaving a red band containing *b*- and *c*-type cytochromes on the top of the column.

Steps 6 and 7: Hydroxyapatite column chromatography (step 6) was carried out as described previously [16] except that the column dimensions were 2.5 × 3 cm. The subsequent ammonium sulfate precipitation (step 7) of the column eluate mixed with sodium cholate (1.5% in a final concentration) was also similar to the previous method [16] except that the rate of ammonium sulfate addition was 230 mg/ml instead of 220 mg/ml.

Preparation of subunits

Subunits I and II were prepared from PS3 cytochrome oxidase (1.3 mg protein) by fractionation on a Sephacryl S-200 (Pharmacia) column (1.6 × 95 cm) equilibrated with 0.1% SDS containing 0.1 M NaCl and 0.01 M Tris-HCl buffer (pH 8.0). The elution pattern, similar to the electrophoretic pattern shown in Fig. 5, was monitored with an ultraviolet monitor (Isco, UA-5) at 280 nm and a recycling technique was applied to ensure good separation. The fractions containing subunits I or II were collected and then proteins were recovered by the addition of ethanol to give 50% (v/v) and successive centrifugation.

Results

Purification

Results of a typical purification of PS3 cytochrome oxidase are summarized in Table I. The purity of the oxidase was monitored by following the absorption bands of reduced cytochrome *aa*$_3$ and expressed as the specific heme *a* content. The heme *a* content of the final preparation was about 16 nmol/mg protein. Before extracting cytochrome oxidase with Triton X-100, the membranes were preextracted with LiCl as well as cholate plus deoxycholate. LiCl treatment did not raise the specific heme *a* content much, but this treatment removed a part of the *b*-type cytochrome(s) wich otherwise sometimes contaminated the final preparation. The enzymatic activity for oxidizing yeast cytochrome *c* was also purified in a similar manner

TABLE I

PURIFICATION OF PS3 CYTOCHROME OXIDASE

Step	Protein (mg)	Heme *a* (nmol)	Heme *a*/protein (nmol/mg)	Yield (%)
Membranes	11500	4140	0.36	100
1. Bile acid treatment	4020	3294	0.82	80.0
2. LiCl treatment	2700	2971	1.10	71.8
3. Triton X-100 extract	2185	2647	1.21	63.9
4. DEAE-cellulose eluate	707	2142	3.03	51.7
5. DEAE-toyopearl eluate	81.6	750	9.19	18.1
6. Hydroxyapatite eluate	27.5	410	14.9	9.9
7. $(NH_4)_2SO_4$ precipitate	23.8	349	16.3	8.4

to cytochrome aa_3, but the final purification was 33-fold, while the enrichment of heme *a* content was 45-fold. In some cases the yield of step 4 was not good because of the partial absorption of cytochrome oxidase on DEAE-cellulose. This absorption did not occur when a sufficient amount of Triton X-100 was present. The addition of an excess amount of Triton X-100 as described in Materials and Methods seems important.

Spectral properties and prosthetic groups

Fig. 1 shows spectra of oxidized (as purified) and dithionite-reduced forms of PS3 cytochrome oxidase. Absorption peaks of the reduced form were found at 550 and 604 nm, suggesting that a *c*-type cytochrome was copurified with cytochrome

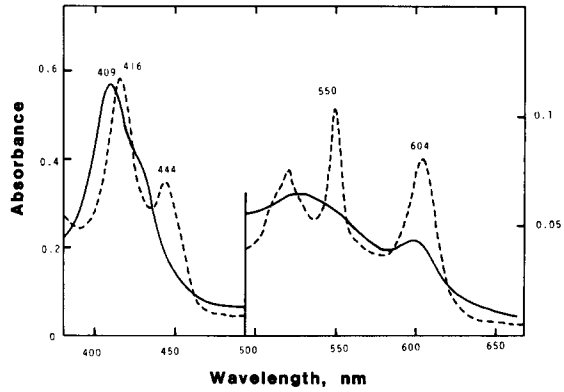

Fig. 1. Absorption spectra of PS3 cytochrome oxidase. The enzyme (4.2 μM in heme *a*) was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5% Triton X-100. (———) Oxidized (as purified), (— — —) reduced with sodium dithionite.

TABLE II

OPTICAL EXTINCTION COEFFICIENTS OF PS3 CYTOCHROME OXIDASE

	Cytochrome	Wavelength (pair) (nm)	$\Delta\epsilon$ ($mM^{-1} \cdot cm^{-1}$)
Reduced			
	aa_3	(604−630)	16.6 [a]
	c	(550−538)	21.2
Reduced minus oxidized			
	aa_3	604	10.5 [a]
	aa_3	(604−630)	11.6 [a]
	c	550	21.2
	c	(550−541)	21.2

[a] Values for heme *a*.

aa_3 as reported previously [16]. The pyridine ferrohemochromes showed the peaks at 548 and 587 nm, indicating that these peaks were due to heme *c* and heme *a*, respectively. Tentative extinction coefficients of PS3 cytochrome oxidase are given in Table II. The molar extinction coefficients used previously [16] are slightly underestimated. The ratio of both types of hemes seems to be 2:1; heme *c* content was 8.3 nmol/mg protein, while that of heme *a* was 15.9 nmol/mg protein in a typical preparation. Determination of the amount of copper in the same sample but after overnight dialysis against 1 mM EDTA was carried out both by atomic absorption spectrophotometry and by colorimetry with bathocuproinesulfonic acid. The amount of total copper was estimated to be 17.6 (atomic absorption) − 18.2(bathocuproine) ngatom/mg protein. Intrinsic copper, which is reactive with bathocuproinesulfonic acid in the presence of SDS but not reactive without SDS [25], was 16.2 ngatom/mg protein. Without dialysis against 1 mM EDTA slightly higher values (about 22 ngatom/mg protein) were found for total copper [16]. These results suggest that the copper/heme *a* ratio is 1:1 as in the mitochondrial enzyme.

The effects of CO on the reduced form are shown in Fig. 2. Peaks appeared at 417, 540 and

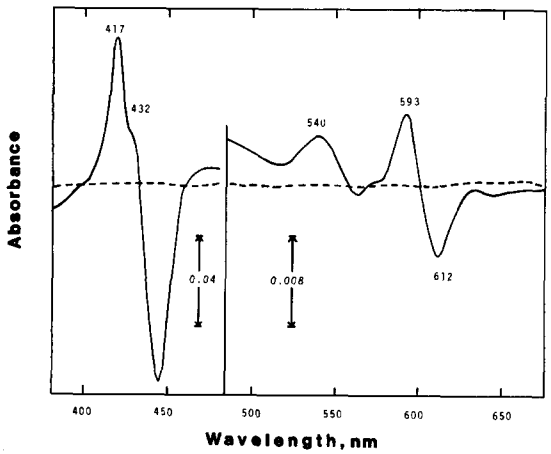

Fig. 2. Effect of CO on the reduced PS3 cytochrome oxidase. The sample (3.5 μM in heme *a*) was reduced with sodium dithionite and ascorbate plus TMPD (10μM), and CO was bubbled through the sample cuvette for 2 min. (———) CO reduced minus reduced, (— — —) baseline.

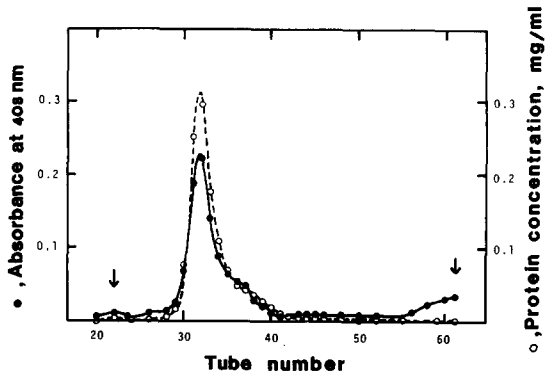

Fig. 3. Gel filtration of PS3 cytochrome oxidase. The enzyme (0.72 mg protein) was applied to a column (0.9×120 cm) of Sephacryl S-300 equilibrated with 0.2% *N*-lauroylsarcosinate, 0.2 M NaCl, 1 mM EDTA and 25 mM sodium phosphate buffer (pH 6.5), and eluted at a flow rate of 6.8 ml/h. The fraction volume was 1.3 ml each. Ferricyanide and blue dextran were used to determine the total volume and the void volume (downward arrows).

593 nm and troughs at 444, 560 and 612 nm in the CO difference spectrum. This spectrum was very similar to that reported from mitochondrial cytochrome oxidase from beef heart [34] and thus indicated that PS3 cytochrome oxidase is also a cytochrome aa_3-type. However, the peak at 417 nm and a shoulder at 432 nm suggested that a small amount of cytochrome *o*-like pigment was present. The cytochrome oxidase prepared from not sufficiently aerated cells contained much cytochrome *o*-like pigment. In such preparations the main trough of the CO difference spectrum was at 434 nm, and 536 and 568 nm peaks were prominent [35]. The effects of aeration during the culture on the CO-binding pigments in PS3 membranes will be described elsewhere.

Gel filtration and molecular weight

The purification procedure did not include any gel filtration step. Fig. 3 shows an elution pattern of a PS3 cytochrome oxidase sample from a column of Sephacryl S-300 in the presence of *N*-lauroylsarcosinate. The absorbance due to a Soret peak at 408 nm was almost coincident with that of protein peak. The apparent molecular mass of PS3 cytochrome oxidase in the presence of *N*-lauroylsarcosinate was estimated to be 250000 using bovine serum albumin, glucose oxidase and F_1-

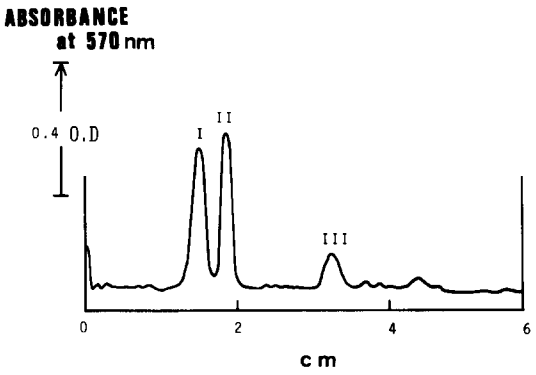

Fig. 4. Densitometric tracing of PS3 cytochrome oxidase electrophoresed in the presence of SDS and urea. PS3 cytochrome oxidase (4 μg), denatured in 6% SDS and 6 M urea at 25°C for 30 min, was applied on a 7.5% polyacrylamide gel containing 0.1% SDS and 8 M urea. Staining was carried out with Coomassie brilliant blue. The absorbance was monitored at 570 nm.

ATPase from the thermophilic bacterium PS3 (TF_1) as the standards. Considering the minimum molecular mass for two molecules of heme *a* (about 125000) and supposing that no severe binding of surfactant occurs, dimers seem to be the prevalent form.

Subunit structure

Fig. 4 shows a typical electrophoretic pattern of PS3 cytochrome oxidase on a SDS-polyacrylamide gel containing urea. PS3 cytochrome oxidase seemed to be composed of three different subunits (bands I, II and III). The other weak bands were probably contaminants, since their absorbances were low and varied from preparation to preparation. The molecular masses were calculated to be 49000 for band I, 38000 for band II and 21000 for band III by comparison with standard proteins such as bovine serum albumin, ovalbumin, trypsin, myoglobin and equine cytochrome *c*. However, the mobility of band I protein was highly dependent on the polyacrylamide concentration of the gel; the R_{fo} value of band I was 0.84, while those of bands II, III and the standard proteins were 0.68 $\pm$ 0.05. The molecular masses of the subunits of PS3 cytochrome oxidase were determined by plotting the retardation coefficient (the slope of the Ferguson plot) versus molecular mass for the marker proteins; bands I, II and III had molecular masses of 56000, 38000 and 22000, respectively. When *o*-tolidine was used for detection of hemes [29], only band II was stained, indicating that heme *c* is attached on this subunit (not shown).

Quantitation of the protein staining of the gel gives an estimate of the ratio among the subunits. When the gel was stained with Coomassie blue the ratio of the absorbances at 570 nm was 1:0.88:0.32 for I:II:III (Fig. 4). The ratio of intensities obtained with Amido black 10B was slightly different (1:0.67:0.33). Since different hydrophobic proteins do not necessarily stain with the same intensity, the stoichiometry was further substantiated by measuring the absorbance at 220 nm of the subunits separated by gel filtration in the presence of SDS. As shown in Fig. 5 separation of subunit I from subunit II was not complete. However, the correct contribution of subunit II was ascertained by measuring the absorbance at 408 nm (shadowed part in the figure), since subunit II contains covalently-bound heme *c*. Since the ratio of subunit proteins based on the absorbances at 220 nm was 1:0.71:0.40, it is likely that this thermophilic cytochrome oxidase has three subunits in an equimolar ratio, since the ratio of molecular weights was

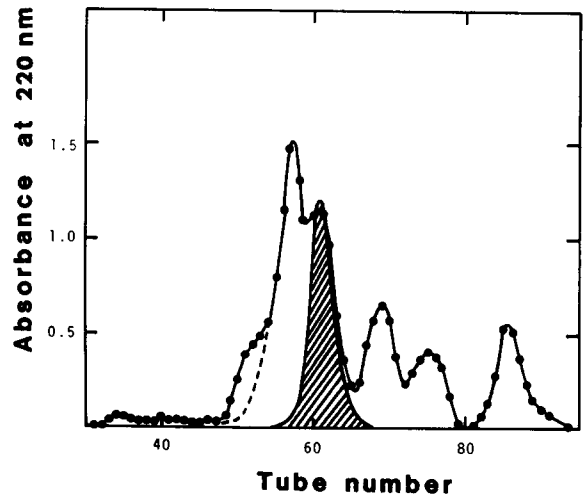

Fig. 5. Gel filtration of PS3 cytochrome oxidase in the presence of SDS. PS3 cytochrome oxidase (0.36 mg/ml) denatured in 0.3 ml of 6% SDS was applied on a column (0.9×120 cm) of Sephacryl S-300 equilibrated with 0.5% SDS containing 0.15 M sodium sulfate and 0.01 M sodium phosphate buffer, pH 7.2. The absorbance of each fraction (0.6 ml) was measured at 220 nm. The flow rate was 4.2 ml/h.

1:0.68:0.39. It is noteworthy that subunit I had a 'fuzzy' appearance after electrophoresis in a gel containing SDS, an effect which was even more prominent when urea was omitted (not shown).

Amino acid composition

Table III shows amino acid compositions of subunits I, II and total PS3 cytochrome oxidase. Subunit III was not analyzed because of the low yield. Subunit I was highly hydrophobic, while subunit II contained many aspartic acid (asparagine), glutamic acid (glutamine) and lysine residues and was not so hydrophobic. Since thioether bridges of cytochrome *c* may not be hydrolyzed by the treatment with 4 M methanesulfonic acid as with 6 M HCl and a very low content of cysteine residues is characteristic of the enzymes from thermophiles [37], the cysteine residues found in subunit II may be related to the binding site of copper as in the case of azurin [38].

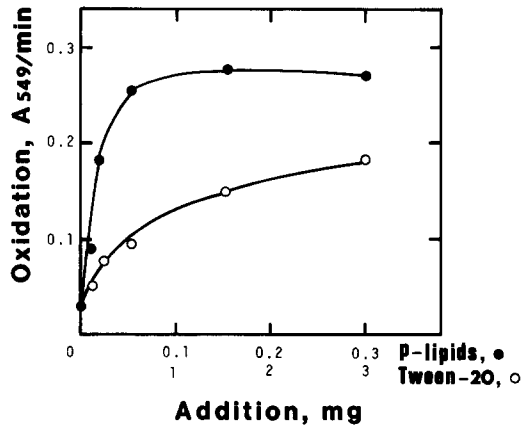

Fig. 6. Phospholipid requirement of PS3 cytochrome oxidase. PS3 cytochrome oxidase (4 μg) was incubated with soybean phospholipids (●) or Tween 20 (○) in the assay medium for 5 min at 25°C and then oxidation rate with yeast cytochrome *c* was determined spectrophotometrically as described in Experimental Procedures.

TABLE III

AMINO ACID COMPOSITION OF PS3 CYTOCHROME OXIDASE AND ITS SUBUNITS I AND II

PS3 cytochrome oxidase (100 μg), fractionated by gel filtration in a Sephacryl S-300 column as used in Fig. 3, and subunit I (40 μg) and subunit II (60 μg), prepared as described in Experimental Procedure, were used for amino acid analysis. The polarity index represents the sum of the mol% of Asp, Thr, Ser, Glu, His, Lys and Arg [36].

Amino acid	mol%		
	Whole enzyme	Subunit I	Subunit II
Asp	7.1	6.2	10.4
Thr	5.7	5.4	5.7
Ser	4.0	4.2	3.5
Glu	6.9	5.5	10.5
Pro	5.0	4.8	6.9
Gly	9.2	11.4	7.3
Ala	8.3	8.1	7.8
Cys	0.2	0.0	0.5
Val	6.6	5.8	7.0
Met	4.1	4.2	2.7
Ile	6.3	7.0	4.9
Leu	10.9	10.8	9.0
Tyr	3.8	2.9	2.1
Phe	8.7	13.0	6.2
His	2.7	2.8	2.0
Lys	5.2	3.7	8.5
Arg	2.9	2.3	3.8
Trp	2.4	1.9	1.2
Polarity index (%)	34.5	30.1	44.4

Catalytic activities

Purified PS3 cytochrome oxidase rapidly oxidized yeast cytochrome *c* when phospholipids were supplied. Fig. 6 shows the effects of addition of soybean phospholipids or Tween 20. As much as a 10-fold activation was observed with the addition of soybean phospholipids.

The stoichiometry of ferrocytochrome *c* oxidized to oxygen reduced is about 4:1; the addition of 250 nmol of yeast ferrocytochrome *c* was followed by 65 nmol of oxygen consumption. The addition of catalase did not change the trace of oxygen consumption. Thus, the following reaction is likely to occur:

$$4 \text{ cytochrome } c(\text{II}) + O_2 + 4H^+ = 4 \text{ cytochrome } c(\text{III}) + 2H_2O$$

Table IV shows the substrate specificity of PS3 cytochrome oxidase as measured polarographically. Both cytochrome *c*-551 from this bacterium and cytochrome *c*-552 from *T. thermophilus* were oxidized much faster than yeast and equine cytochromes *c* at 25 μM. These apparent differences of the rate constants are partly due to a different K_m for each cytochrome *c*. V_{max} values for yeast cytochrome *c* and PS3 cytochrome *c*-551 were 16.2 and 16.0 μgatom oxygen/min per mg protein, while K_m values were 67 and 11 μM, respectively. In the case of equine cytochrome *c*, a smaller V_{max} (5.6 μgatom/min per mg protein) and a larger K_m value (130 μM) were obtained. Besides cytochromes *c*, phenazine methosulfate served as a good substrate. TMPD and 2,6-dichlorophenolindophenol were also oxidized. These experiments were carried out at the optimal pH of 6.1. At pH 5.0 or 7.1 the enzyme oxidized yeast cytochrome *c* with two-thirds of the optimal activity (not shown). The activity was also highly dependent on the ionic strength of the medium as in the case of mitochondrial cytochrome oxidase; the activity in 100 mM phosphate buffer was only one-half of that measured in 20 mM (the standard assay condition).

Proton translocating activity

As reported previously [16], PS3 cytochrome oxidase was able to generate a membrane potential upon oxidation when reconstituted into proteoliposomes. To answer the question as to whether cytochrome oxidase is an H^+ pump [1] or an electron half-loop [13], the measurement of H^+ movement across membranes during oxidation has key importance. Fig. 7 shows that H^+ ejection occurred when a small amount of yeast cytochrome *c* was added as a reductant pulse [10]. In the presence of the uncoupler FCCP, this H^+ ejection was not observed and a corresponding net H^+ consumption due to production of H_2O occurred. The H^+/e ratio, calculated as the maximal amounts of H^+ translocated per ferrocytochrome *c* added, was about 0.45 in this experiment. How-

TABLE IV

SUBSTRATE SPECIFICITY OF PS3 CYTOCHROME OXIDASE

The reaction mixture contained 2 mM sodium ascorbate and one of the substrates in 2.8 ml of 20 mM sodium phosphate buffer (pH 6.1). The reaction was started by the addition of proteoliposomes containing 13 μg protein of PS3 cytochrome oxidase and 2 mg of phospholipids in 0.1 ml and measured at 40°C. The autooxidation rate was subtracted from each result.

Substrate	Concentration (μM)	Oxidation rate (μg atom O/min per mg)
None (ascorbate)		0
PS3 cytochrome *c*-551	25	11.3
T. thermophilus cytochrome *c*-552	25	10.6
C. krusei cytochrome *c*	25	8.0
Equine cytochrome *c*	25	2.0
Phenazine methosulfate	5	36.0
TMPD	100	13.0
2,6-Dichlorophenolindophenol	100	5.2

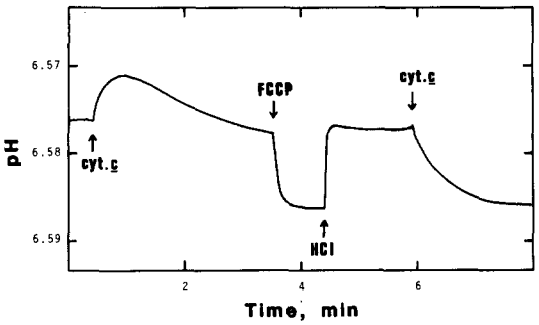

Fig. 7. pH change due to H^+ pumping during cytochrome *c* oxidation by proteoliposomes reconstituted from PS3 cytochrome oxidase. Proteoliposomes containing 18 μg PS3 cytochrome oxidase and 4 mg phospholipids in 0.1 ml were suspended in 2 ml of the reaction medium containing 25 mM K_2SO_4, 2.5 mM $MgSO_4$, 0.1 mM Mes-KOH buffer and 0.1 μg/ml valinomycin at 32°C. Additions were yeast ferrocytochrome *c* (4.1 nmol), FCCP (0.1 μg) and HCl (5 nmol).

ever, this relatively low value of $\vec{H}^+/e$ seemed to be due to the relatively slow oxidation rate, since only 60% of ferrocytochrome *c* was oxidized when H^+ ejection reached its maximum at 30 s after cytochrome *c* addition. Thus, it is likely that PS3 cytochrome oxidase is an H^+ pump and translocates at least one H^+ per one electron transfer.

Discussion

The present investigation shows that cytochrome oxidase from the thermophilic bacterium PS3 is probably composed of three kinds of subunits. The minimum molecular mass of about 125000 for two hemes *a* and two coppers was close to 116000, i.e., the sum of the molecular weights of the three subunits. Thus, it is likely that a complex containing equimolar amounts of subunits I, II and III constitutes one oxidase molecule containing two hemes *a*, one heme *c* and two copper atoms. In the presence of surfactants such as *N*-lauroylsarcosinate (Fig. 3) the enzyme may exist as dimers.

We previously reported that PS3 cytochrome oxidase had two kinds of subunits although a single band was observed on the gel electrophoresed with SDS [16]. This observation was reproduced in another laboratory [22]. One reason why we could not observe two or three bands previously was an inappropriate pretreatment (95°C) for the denaturation of the oxidase. Heating of the oxidase in the presence of SDS above 70°C causes subunits I and III to aggregate to soluble forms which would still not enter, and so a single band was observed as reported [16]. Another reason seemed to be an abnormal extrapolated R_f value at 0% acrylamide concentration. The R_f values of subunits I and II were rather similar at a lower acrylamide concentration.

PS3 cytochrome oxidase, which consists of three kinds of subunit, could translocate H^+ upon oxidation (Fig. 7) just as beef heart enzyme containing as many as seven subunits could [9–12]. Other cytochrome oxidases hitherto separated from bacteria are known to contain at least two kinds of subunits [17,18,20,21]. Ludwig and Schatz [20] reported that vesicles reconstituted from *Paracoccus* cytochrome oxidase showed 'respiratory control', but their $\vec{H}^+/e$ ratio was low and Ludwig [22] suggested that this may be due to the loss of subunit III in their preparation. Although this suggestion coincides with our present results that PS3 enzyme containing subunit III shows a good $\vec{H}^+/e$ ratio, it was reported very recently that the *Paracoccus* enzyme showed an appreciable H^+-pump activity [39]. Some bacterial cytochrome oxidases composed of just two kinds of subunits may translocate H^+.

It has been shown that the three largest polypeptides of mitochondrial cytochrome oxidase are coded for by mitochondrial DNA [6–8]. Since the properties of PS3 cytochrome oxidase are very similar to those of mitochondrial enzyme, it is tempting to speculate that the three large subunits are the minimal assembly for a complete enzyme. Winter et al. [5] reported very recently that subunit I bears one heme *a*, subunit II, one heme *a* and two coppers in beef heart cytochrome oxidase. Evidence suggesting that subunit III from beef heart mitochondria plays an important role in H^+ translocation has been reported; dicyclohexylcarbodiimide covalently binds to subunit III with a concomitant loss of H^+-translocating activity [40], and a six-subunit preparation without subunit III fails to show H^+ translocation [41].

The role of *c*-type cytochrome in PS3 cytochrome oxidase is not known at present. However, this cytochrome *c* at least seems to activate the

cytochrome aa_3 part of the enzyme, since many dyes and $Na_2S_2O_4$ could be oxidized rapidly without adding an extrinsic cytochrome *c*. The mitochondrial enzyme is known to oxidize these compounds only slowly without addition of cytochrome *c* [42,43]. The fact that oxidation occurs at $-80°C$ from this cytochrome *c* to the cytochrome aa_3 part after flash photolysis of the CO complex in the presence of molecular oxygen (Sone, N., Yang, E. and Chance, B., unpublished observations) also suggests that this cytochrome *c* is not a contaminant. On the contrary, the true extrinsic substrate for PS3 cytochrome oxidase (caa_3 complex) seems to be cytochrome *c*-551, a slightly hydrophobic *c*-type cytochrome (0.45 in the polarity index) which can be extracted by cholate from PS3 membranes (Yanagita, Y. and Sone, N., unpublished observations). Soluble cytochrome *c*-550, reported previously by Hon-nami et al. [44], was not found in the present investigation. Cytochrome *c*-551 which has a high affinity for PS3 cytochrome oxidase will be useful for H^+-pulse experiments, since a slow rate of oxidation with yeast cytochrome *c* was the reason that the $\vec{H}^+/e$ ratio (with the usual meaning) was not so high (Fig. 7).

Each subunit of PS3 cytochrome oxidase seems to correspond, respectively, to the largest three subunits of the mitochondrial enzyme. The anomalous properties of PS3 subunit I on gel electrophoresis in the presence of SDS have also been reported for the mitochondrial [45], *Paracoccus* [20] and *Nitrobacter* [21] enzymes. The subunit II of PS3 cytochrome oxidase bearing heme *c* seems somewhat different from the mitochondrial subunit II or the minor subunits of *Paracoccus* or *Nitrobacter* oxidases. Ludwig [22], however, recently reported that the PS3 subunit II reacts immunologically with antiyeast subunit II as in the case of the *Paracoccus* subunit II. Cytochrome oxidase from *T. thermophilus* is also known to contain a *c*-type cytochrome [18,19]. Our preliminary experiment shows that the subunit III of PS3 cytochrome oxidase is also labeled by [^{14}C]dicyclohexylcarbodiimide, an inhibitor of H^+-translocating F_0F_1-ATPase, with a loss of H^+-translocating activity as in the case of cytochrome oxidase from beef heart [10,40,46].

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