Purification and Characterization of Cytochrome *c* Oxidase from *Thermus thermophilus* HB8†

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ABSTRACT: Cytochrome *c* oxidase (aa_3 type) containing heme *c* from *Thermus thermophilus* HB8 was purified to a hydrodynamically homogeneous state in Triton X-100. The oxidase contained two hemes *a*, one heme *c*, and two copper atoms per minimal unit with a heme *a* content of about 22 nmol/mg of protein and was composed of two subunits whose molecular weights were 71 000 and 34 000 (heme *c* binding), respectively. The molecular weights were estimated on the basis of a Ferguson plot. The enzyme was highly active with *T. thermophilus* cytochrome c_{552} and active with mitochondrial cytochrome *c*. The purified enzyme was reconstituted into phospholipid vesicles. Addition of reduced cytochrome c_{552} to these vesicles caused acidification of the extravesicular space with a stoichiometry of 0.8 H^+/e, which could not be observed in the presence of uncoupler. The data indicate that cytochrome *c* oxidase of *T. thermophilus* is a proton pump.

Bacterial cytochrome *c* oxidases of aa_3 type have attracted much attention in recent years since although their enzymatic and spectral properties are similar to those of mitochondrial cytochrome *c* oxidase, their subunit composition is much simpler than that of the mitochondrial counterpart [reviewed in Ludwig (1980)]. Very recently, it was found that *Paracoccus denitrificans* and thermophilic bacterium PS3 enzymes act as proton pumps (Solioz et al., 1982; Sone & Yanagita, 1982; Sone & Hinkle, 1982). Thus, it is expected that the study of bacterial enzymes might help to answer some of the structural and functional questions that have not been solved with mitochondrial enzymes (Ludwig & Schatz, 1980; Yamanaka & Fujii, 1980).

We previously showed that purified cytochrome *c* oxidase from an extreme thermophile, *Thermus thermophilus* HB8, has a simple subunit composition (Hon-nami & Oshima, 1980). This enzyme showed enzymatic and spectral properties similar to those of the mitochondrial enzyme but contained a heme *c* as an additional prosthetic group. This thermophilic enzyme was isolated by the Fee group independently of us (Fee et al., 1980). They studied the magnetic properties of chromophores in the enzyme by means of electron paramagnetic resonance and Mössbauer spectroscopies in detail (Fee et al., 1980; Kent et al., 1982). X-ray edge absorption and extended fine structure studies with this enzyme were also carried out by Powers et al. (1981). In this paper, we report on the purification and properties of thermostable *T. thermophilus* cytochrome *c* oxidase, which consists of only two kinds of subunits. Proton pumping by the purified enzyme, reconstituted into phospholipid vesicles, is also described.

Materials and Methods

Reagents. Calcium phosphate gel–cellulose was prepared according to the method of Cha (1969). Cytochrome *c* from both horse heart (type VI) and *Candida krusei* (type VII) were purchased from Sigma. Valinomycin and FCCP were products of Boehringer Mannheim. Chemical reagents used were of the highest purity commercially available.

Organism. *T. thermophilus* HB8 (ATCC 27634) was grown at 75 °C in a synthetic medium as described in previous papers (Oshima & Baba, 1981; Tanaka et al., 1981). Cultivation of the organism was performed in 20 L of the above medium in a stainless-steel jar fermenter of 30-L capacity with vigorous aeration and agitation. The cells were harvested at the late-log phase (about 300 Klett units) by centrifugation in a Sharples continuous-flow centrifuge and washed with 0.9% NaCl solution. The yield was 6–8 g wet wt of cells/L. The cells were stored at −20 °C before use.

Purification of Cytochrome c Oxidase. The frozen cells (2 kg wet wt) were thawed and suspended in 2 volumes of cold 50 mM Tris-HCl[1] buffer at pH 7.5, containing 1 mM EDTA, 10 mM $MgCl_2$, 50 mM 2-mercaptoethanol, and 135 mM KCl. The cells were then broken by sonication at 12 kHz for 5 min by 300-mL aliquots (Branson Sonic Power Co., sonifier Model 350), with cooling in an ice bath. The broken cell suspension was added to 10 mg of DNase I (Sigma, DN-25) and allowed to stand at room temperature for 1 h with gentle stirring. The cell debris was removed by centrifugation at 8500*g* for 30 min, and the supernatant was centrifuged at 22000*g* for 15 h. The sediments thus obtained were stored at −20 °C and used as membranes.

The membrane particles were resuspended in 50 mM Tris-HCl buffer at pH 7.5, containing 1 mM EDTA, and homogenized with a Potter-Elvehjem homogenizer. To the homogenate, were added Triton X-100 and KCl. The concentrations of Triton X-100, KCl, and protein were 1.5%, 1 M, and about 30 mg/mL, respectively. The mixture was stirred gently at 4 °C overnight and centrifuged at 22000*g* for 15 h. The supernatant was adjusted to 1.5% potassium cholate and fractionated with ammonium sulfate at room temperature. The precipitate between 25 and 33% saturation was collected by centrifugation at 8500*g* for 30 min and dissolved in buffer A (50 mM Tris-HCl at pH 7.5, 1 mM EDTA, 0.5% Triton X-100). The solution was adjusted to 2% cholate and refractionated with ammonium sulfate as above. The precipitate was dissolved in a minimal volume of buffer A and dialyzed against buffer A containing 0.1 M KCl. The dialyzed solution was divided into two equal amounts, both of which were separately passed through a column of Sephacryl S-300 (Pharmacia, 3 × 110 cm) equilibrated with the dialysis buffer. Fractions containing heme *a* were pooled, adjusted to 1% cholate, and precipitated with ammonium sulfate be-

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[1] Abbreviations: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid disodium salt; DEAE, diethylaminoethyl; CM, carboxymethyl; $NaDodSO_4$, sodium dodecyl sulfate; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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tween 25 and 35% saturation. The precipitate was suspended, dialyzed against buffer B (50 mM Tris-HCl buffer at pH 8.5, 1 mM EDTA, 0.5% Triton X-100), and applied to a DEAE-cellulose column (Whatman DE-32, 3 × 36 cm). The column was washed with buffer B, and the oxidase was eluted with buffer B containing 0.3 M KCl. The enzyme fractions were pooled, adjusted to 1% cholate, and precipitated between 25 and 33% ammonium sulfate as above. The precipitate was dialyzed against buffer A and poured onto a DEAE-Sephacel column (Pharmacia, 3 × 42 cm). The column was washed, and the enzyme was eluted with a linear salt gradient (400 mL each of buffer A and buffer A containing 0.3 M KCl). Cytochrome *b* was completely removed in this step. The greenish brown fractions were pooled and precipitated with the addition of solid ammonium sulfate of 33% saturation in the presence of 1% cholate.

The precipitate was dissolved in a minimal volume of buffer A, and the concentrated enzyme was passed through a Sephacryl S-200 column (Pharmacia, 2 × 200 cm) equilibrated with buffer A containing 0.1 M KCl. Fractions with the highest heme *a* to protein ratio were pooled, dialyzed against buffer A, and loaded onto the second DEAE-Sephacel column (2.5 × 40 cm). The column was washed, and the enzyme was eluted with a 500-mL linear gradient from 0 to 0.2 M KCl in buffer A. The fractions with the highest heme *a* to protein ratio were pooled and precipitated with ammonium sulfate of 35% saturation in the presence of 1% cholate. Although the preparation in this step was spectrally pure, some protein components, mainly 47 000 dalton, were contaminated. The enzyme precipitate was dialyzed against 10 mM potassium phosphate buffer at pH 7.0 containing 0.5% Triton X-100 and applied to a calcium phosphate cellulose–gel column (3 × 20 cm) equilibrated with the dialysis buffer. The column was washed and developed with a linear gradient of phosphate buffer concentration from 10 to 400 mM at pH 7.0 containing 0.5% Triton X-100 in a total volume of 500 mL. The oxidase was eluted out at a phosphate concentration of about 40 mM. The enzyme fractions not contaminated with the 47 000-dalton component were pooled, concentrated on an Amicon Diaflo assembly with a PM-10 membrane, and dialyzed against 50 mM Tris-HCl buffer at pH 7.5 containing 1 mM EDTA and 0.5% Triton X-100. The solution thus obtained was used as the purified enzyme preparation.

Preparation of Cytochrome c_{552}. The purification method was basically the saame as described previously (Hon-nami & Oshima, 1977) with slight modification. Cells were disrupted by the sonication described above. Ion-exchange chromatography was performed in a CM-cellulose column (Whatman CM-32, 3 × 30 cm) in place of the Amberlite CG-50 column. This step was repeated once more. Prior to gel filtration on Sephadex G-75, the protein solution was concentrated by ultrafiltration with a UM-2 membrane (Amicon). The purified protein was dialyzed against 50 mM NH_4HCO_3 solution and freeze-dried. The final yield was about 6 μmol from 4 kg of wet cells.

Isolation of Subunits. The purified enzyme was subjected to slab gel electrophoresis, which was performed on NaDod-SO_4–polyacrylamide gel in the Laemmli system (1970). The electrode buffer used in the system contained 0.5% Tris, 0.55% barbital, and 0.1% $NaDodSO_4$ at pH 8.3 (Maurer, 1971). The protein bands were stained and cut out. According to the method described by Sreekrishna et al. (1981), proteins were eluted out, dialyzed, and lyophilized.

Assay of Prosthetic Groups. Absorption spectra were recorded with a Cary 17 spectrophotometer. The heme *a* content was determined with a millimolar extinction coefficient of 26 mM^{-1} cm^{-1} (Morrison et al., 1960) at the α peak of the pyridine ferrohemochrome *a* prepared according to the method of Morrison & Horie (1965). The final concentrations of NaOH and pyridine were 0.1 M and 25% (v/v), respectively. The content of heme *c* associated with the oxidase preparation was determined from the reduced minus oxidized difference spectrum, by assuming a difference millimolar extinction coefficient at the α peak to be 18.5 mM^{-1} cm^{-1} (Orii et al., 1963). Copper was analyzed by flameless atomic absorption with a Varian AA-175 spectrometer or by colorimetry with bathocuproine according to the method of Van De Bogart & Beinert (1967).

Extraction and Estimation of Lipid. The purified preparation (12 mg of protein) dissolved in a 1% potassium cholate solution was precipitated by the addition of ammonium sulfate and dialyzed extensively against distilled water. The solution was freeze-dried and extracted 3 times with 5 mL of chloroform–methanol–25% aqueous ammonia (7:1:2 by volume). The extract was washed once with 3 mL of distilled water and evaporated to dryness under nitrogen. Chromatography of the extracted lipid was carried out on silica gel thin-layer plates (Merck; HP-TLC, No. 5641) in chloroform–methanol–water (65:25:4 by volume). Detection of lipid on the plates was performed by spraying with 0.02% rhodamin 6G in ethanol solution or anthron (Yamakawa et al., 1960) and acid molybdate (Dittmer & Lester, 1964) reagents. The hexose and phosphorus contents of the extracts were measured by the methods of Oshima & Yamakawa (1974) and Chen et al. (1956), respectively.

Assay of Enzymatic Activity. Activity of cytochrome *c* oxidase was measured spectrophotometrically at 24 °C. The reaction mixture contained 10 mM potassium phosphate buffer at pH 6.5 and 30 μM reduced cytochrome *c* in 1.0-mL volume unless otherwise stated. The reaction was initiated by the addition of 10 μL of enzyme solution dissolved in 10 mM potassium phosphate buffer at pH 6.5 containing 0.5% Triton X-100. The decrease in absorbance at the α peak of each cytochrome *c* was followed with time. The reaction rate was calculated from the initial slope and expressed as molecular activity (mole of reduced cytochrome *c* oxidized per mole of heme *a* per minute). Oxygen consumption was measured at 25 °C with a Clark-type oxygen electrode (Yellow Spring Instruments Co.). The reaction mixture containing 200–270 nmol of reduced cytochrome *c* in 2.4 mL of 10 mM potassium phosphate buffer at pH 6.5. Cytochrome *c* was reduced with $Na_2S_2O_4$, followed by chromatography on a Sephadex G-25 (Pharmacia, superfine) column with the same buffer used in the assay experiments.

Vesicle Reconstitution and Proton Pumping. Preparation of reconstituted vesicles and proton-pumping experiments were carried out in the laboratory of Dr. N. Sone, Jichi Midical School, according to the method of Sone & Yanagita (1982). Fourty milligrams of soybean phospholipids (Sigma, type IV-S) suspended in 1 mL of 10 mM Mops–KOH buffer at pH 6.4, containing 0.2 mM EDTA and 1 mM dithiothreitol, was dispersed to clarity by sonication with a Branson Model 200 sonifier equipped with a microtip with cooling in an ice bath. To this suspension was added 0.15 mg of protein of *T. thermophilus* enzyme (3.3 nmol of heme *a*), and the mixture was treated by pulse sonication for 10 s at an amplitude of 2. The mixture was frozen quickly at −80 °C, thawed at room temperature, and followed by sonication as above. This process was repeated until the mixture became clear after sonication. The reconstituted cytochrome *c* oxidase (0.165 nmol of heme

Table I: Purification of *T. thermophilus* Cytochrome *c* Oxidase

step	protein (mg)	heme *a* (nmol)	heme *a*/protein (nmol/mg)	yield (%)
membrane	87500	9210	0.10	100
Triton-KCl extract	39400	4710	0.12	51.1
ammonium sulfate precipitation	8290	3470	0.42	37.7
Sephacryl S-300	3270	2990	0.91	32.5
DEAE-cellulose	1090	2120	1.95	23.0
first DEAE-Sephacel	182	1620	8.91	17.6
Sephacryl S-200	93.2	1460	15.7	15.9
second DEAE-Sephacel	41.7	987	23.7	10.7
calcium phosphate gel-cellulose	15.7	350	22.2	3.8

a) was incubated with 0.2 μg of valinomycin in 1.5 mL of the reaction medium containing 25 mM K_2SO_4 and 0.1 mM Mops–KOH buffer at pH 6.4 in a thermostated vessel equipped with a magnetic stirrer. The pH changes in the medium resulting from the addition of reduced *T. thermophilus* cytochrome *c*-552 were monitored with a pH meter (Beckman, Model 4500) equipped with a glass electrode (Beckman, No. 39030) at 30 °C. Cytochrome *c* was reduced with $Na_2S_2O_4$, and excess reductant was removed according to the centrifuge–column method (Penefsky, 1979) with Bio-Gel P-8 (Bio-Rad) as a resin. One turnover of enzyme is defined as described in a literature (Solioz et al., 1982).

Miscellaneous. The concentrations of cytochromes *c* used in these experiments were determined spectrophotometrically on the basis of the following difference extinction coefficients: *T. thermophilus* cytochrome c_{552}, $\Delta\epsilon_{552nm}$ = 14.3 mM^{-1} cm^{-1}; horse cytochrome *c*, $\Delta\epsilon_{550nm}$ = 18.7 mM^{-1} cm^{-1} (Margoliash & Frohwirt, 1959); *C. krusei* cytochrome *c*, $\Delta\epsilon_{549nm}$ = 22.8 mM^{-1} cm^{-1} (Margalit & Schejter, 1970). Sedimentation equilibrium measurement was carried out with a Hitachi analytical ultracentrifuge, Model 282, with an RA-72 TC rotor and a double-sector cell. The protein concentration gradient was determined with a photoelectric scanner at a wavelength of 410 nm. Amino acid analysis was carried out with a Durrum 500 amino acid analyzer. Samples were hydrolyzed in vacuo for 24 h at 105 °C in constant-boiling HCl containing 0.2% phenol. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was carried out by the method of Weber & Osborn (1969) with 8 M urea. Malachite green was used as a front marker. The protein was stained with Coomassie brilliant blue R-250. Densitometric tracing of the gels was done at 600 nm on a Fujiriken densitometer, type FD-AIV. Heme staining was performed by the method of Reid & Ingledew (1980). Protein was assayed by the method of Lowry et al. (1951) or its modification as described by Wang & Smith (1975).

Results

Purification. Table I summarizes the typical purification of *T. thermophilus* cytochrome *c* oxidase. The heme *a* content of the final preparation was 22 nmol/mg of protein, and the overall purification was about 200-fold. The purified preparation was homogeneous, judging from the results of the sedimentation equilibrium analysis; a straight line was obtained by plotting ln *c* vs. r^2, where *c* and *r* refer to enzyme concentration as absorbance at 410 nm and the distance from the rotation center, respectively (data not shown).

Spectral Properties. Figure 1 shows the absorption spectra of the oxidized (as purified) and reduced forms of *T. thermophilus* cytochrome *c* oxidase. Absorption maxima in the reduced minus oxidized difference spectrum were at 443 and 603 nm and at 416, 519, and 548 nm. The pyridine ferrohemochrome spectrum of the purified preparation exhibited

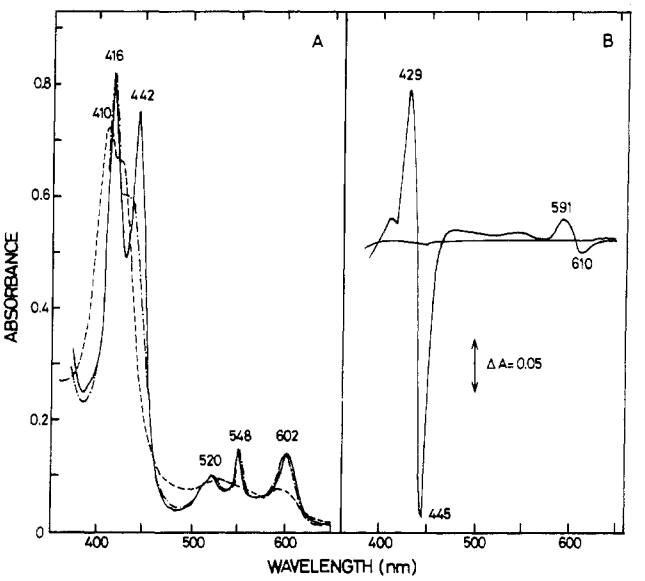

FIGURE 1: Absorption spectra of purified *T. thermophilus* cytochrome *c* oxidase. (A) The enzyme was dissolved in 50 m Tris-HCl buffer at pH 7.5 containing 1 mM EDTA and 0.5% Triton X-100. The concentration of heme *a* was 6.4 μM. (– –) Oxidized; (—) reduced with $Na_2S_2O_4$; (–·–) reduced plus CO (CO was bubbled through the solution for 1 min). (B) Reduced plus CO minus reduced difference spectrum.

peaks at 427 and 587 nm and at 414, 520, and 549 nm. The ferrohemochrome of the acid acetone extract showed absorption peaks at 430 and 587 nm. These spectral properties inducate that *T. thermophilus* cytochrome *c* oxidase is an *a*-type cytochrome containing heme *c* as reported previously (Hon-nami & Oshima, 1980).

The effect of CO on the spectrum of the reduced form is also shown in Figure 1. A remarkable change was observed in the Soret region of heme *a*. The reduced plus CO minus reduced difference spectrum exhibited maxima at 429 and 591 nm and troughs at 445 and 610 nm (Figure 1B). This spectrum resembles that reported for mitochondrial cytochrome *c* oxidase from beaf heart (Yonetani et al., 1960). Tentative extinction coefficients were estimated to be 19.1 ($\epsilon_{602-630}$) for the reduced form and 11.4 ($\epsilon_{603-630}$) for the reduced minus oxidized difference, on the basis of the extinction coefficient at the α peak of the pyridine ferrohemochrome *a* (Morrison et al., 1960).

The heme and copper contents of the purified preparation are given in Table II. The values given suggest that the ratio of heme *a* to heme *c* to copper is 2:1:2.

Subunit Structure. The purified preparation showed only two bands in $NaDodSO_4$–polyacrylamide gels when stained with Coomassie brilliant blue. Figure 2 illustrates a typical densitometric trace of a gel containing 8 M urea. Subunit I was stained as a broad band when urea was omitted from the gel. This band remained almost at the top of the gel, and its

Table II: Heme and Metal Components of *T. thermophilus* Cytochrome *c* Oxidase

component	content (nmol/mg of protein)	ratio
heme *a*	21.8	2
heme *c*	11.3	1
total copper	22.7,[a] 26.5[b]	2

[a] Estimated by the atomic absorption method. [b] Estimated by colorimetry with bathocuproine (Van De Bogart & Beinert, 1967).

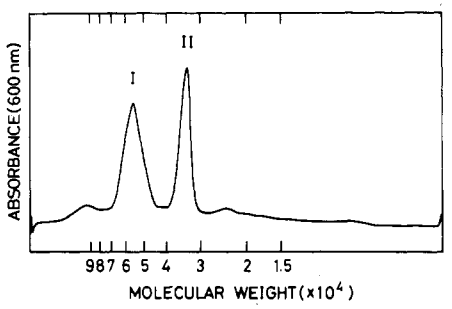

FIGURE 2: Densitometric tracing of $NaDodSO_4$–polyacrylamide gel of *T. thermophilus* cytochrome *c* oxidase. The purified enzyme (15 μg) was electrophoresed on a 7.5% polyacrlamide gel containing 0.1% $NaDodSO_4$ and 8 M urea and stained with Coomassie brilliant blue. The sample was dissociated in 1% $NaDodSO_4$, 1% 2-mercaptoethanol, and 8 M urea at 50 °C for 30 min before electrophoresis. Standard proteins used were obtained as a Pharmacia electrophoresis calibration kit: phosphorylase *b* (94000), bovine serum albumin (67000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100), and α-lactalbumin (14400).

intensity decreased when the sample was pretreated in boiling water. Subunit II, on the other hand, is a component with heme *c* covalently attached since only this band showed a peroxidase activity catalyzed by heme on the gels (data not shown). The apparent molecular weights were 55000 and 33000 for subunits I and II, respectively, in a 7.5% polyacrylamide gel system (Figure 2).

It was found that the largest subunit of cytochrome *c* oxidase derived from bacteria as well as mitochondria exhibits an anomalous behavior on $NaDodSO_4$–polyacrylamide gel electrophoresis; its value of free mobility (M_0), the relative mobility (R_f) extrapolated to 0% acrylamide in a Ferguson plot, is large relative to that (those) of the other subunit(s) and those of normal standard proteins (Yamanaka et al., 1981; Sone & Yanagita, 1982; Briggs et al., 1975; Poyton & Schatz, 1975). From a Ferguson plot of *T. thermophilus* cytochrome *c* oxidase, the M_0 value for subunit I was obtained at 0.79. This value is larger than 0.72 for subunit II and 0.68 ± 0.08 for standard proteins used. Tracy & Chan (1979) suggest that the effects of such anomalous behavior may possibly be eliminated by the linear relationship of molecular weight to retardation coefficient (K_r), the slope of a line in a Ferguson plot. A standard calibration curve was obtained by plotting K_r against molecular weight for standard proteins (data not shown). Thus, the molecular weights for subunits I and II were determined to be 71000 and 34000, respectively.

Amino Acid Composition. The amino acid composition of each separated subunit and that of the whole enzyme of *T. thermophilus* oxidase are given in Table III. The polarity index of subunit I was smaller than that of subunit II, suggesting that subunit I is more hydrophobic. Polarity indexes of the subunits are comparable to those reported for the corresponding subunits of other bacterial and mitochondrial oxidases (Ludwig & Schatz, 1980; Yamanaka & Fukumori, 1981; Sone & Yanagita, 1982; Downer et al., 1976; Poyton & Schatz, 1975). The amino acid composition of subunit I

Table III: Amino Acid Composition of *T. thermophilus* Cytochrome *c* Oxidase and Its Subunits[a]

amino acid	whole enzyme	mol % subunit I	mol % subunit II
Asp	6.5	6.4	6.9
Thr	5.5	6.1	3.8
Ser	4.6	5.3	4.2
Glu	7.7	6.8	12.8
Pro	6.8	6.6	9.5
Gly	10.1	10.1	10.2
Ala	11.4	10.4	10.2
Val	7.4	6.7	7.6
Met	2.7	3.0	1.8
Ile	3.3	3.1	3.5
Leu	11.7	13.1	9.5
Tyr	3.9	4.6	2.2
Phe	8.4	8.0	6.5
His	3.2	3.2	2.1
Lys	3.0	3.0	4.0
Arg	3.9	3.6	5.4
polarity index (%):	34.4	34.4	39.2

[a] Purified *T. thermophilus* cytochrome *c* oxidase (220 μg), subunit I (50 μg), and subunit II (120 μg) were used for the amino acid analysis. Subunits were prepared as described under Materials and Methods. Cys and Trp were not determined. The polarity index represents the sum of the mole percentages of Asp, Thr, Ser, Glu, His, Lys, and Arg (Capaldi & Vanderkooi, 1972).

of *T. thermophilus* enzyme is very similar to that of each subunit I of bacterial two-subunit enzymes from *P. dentrificans* (Ludwig & Schatz, 1980) and *Nitrobacter agilis* (Yamanaka & Fukumori, 1981) except that the Ile content of the thermophile subunit is lower than those of the mesophiles while the Leu content is higher by at least 2.2 mol %. The composition of thermophilic subunit II resembles those of mesophilic enzymes. It is evident, however, that the thermophilic subunit has higher contents of Pro (more than 2.4 mol %) and of Arg (2.3 mol %) and lower contents of Val (not less than 3.0 mol %) and of Ile (2.4 mol %).

Lipid Content. It was previously reported that a glycolipid constitutes a major portion of lipids present in his bacterium (Oshima & Yamakawa, 1974). Analysis of lipids including glycolipid and phospholipid in the purified oxidase preparation was performed by thin-layer chromatography. No spot could be detected except for those corresponding to detergents such as Triton X-100 and cholate; these could not be removed completely by dialysis.

The total hexose and phosphorus contents were determined to be 0.13 and 0.046 μg/mg of protein, respectively. These values corresponded to 0.36 μg of glycolipid and 1.0 μg of phospholipid/mg of protein, if one assumes average molecular weights of 1461 (that of the major glycolipid found in this thermophile) and 691 (that of dipalmitoylphosphatidylethanolamine) for glycolipid and phospholipid, respectively.

Enzymatic Properties. The purified oxidase was highly active with *T. thermophilus* cytochrome c_{552} and active with mitochondrial cytochromes *c* (Table IV). The activity of the purified enzyme was highly sensitive to phosphate concentration. Fifty percent inhibition was observed at 20 mM phosphate with horse cytochrome *c* as the substrate and at 75 mM with the thermophile cytochrome.

Figure 3 shows the effects of soybean phospholipid on the oxidation of reduced *T. thermophilus* cytochrome c_{552} catalyzed by the purified oxidase in 40 mM phosphate buffer. Addition of phospholipid increased the reaction rate; its stimulation was as much as 60% at a concentration of 0.2 mg of phospholipid/mL. The stimulation was highly intense when assayed with reduced horse cytochrome *c*; the reaction rate

Table IV: Substrate Specificity of *T. thermophilus* Cytochrome *c* Oxidase[a]

substrate	K_m (μM)	molecular activity[b]
T. thermophilus cytochrome c_{552}	13	3180
horse cytochrome *c*	41	233
C. krusei cytochrome *c*	74	500

[a] The reaction mixture of 1.0 mL of 10 mM potassium phosphate buffer at pH 6.5 contained various concentrations of cytochrome *c* and appropriate amounts of enzyme. The reactions were performed at 24 °C. [b] Molecular activity (mole of reduced cytochrome *c* oxidized per mole of heme *a* per minute) was calculated from V_{max}.

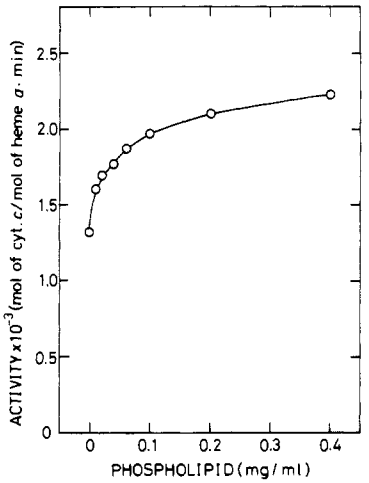

FIGURE 3: Effect of phospholipid on the activity of purified *T. thermophilus* cytochrome *c* oxidase. The reaction mixture of 1 mL contained 30 μM reduced *T. thermophilus* cytochrome c_{552}, 40 mM potassium phosphate buffer at pH 6.5, and various concentrations of soybean phospholipid. Phospholipid (Sigma, type IVS) was suspended in the same buffer used as the reaction medium and dispersed by sonication with cooling in an ice bath before being added to the reaction mixture.

reached a maximum value with about a 6-fold activation at a phospholipid concentration of 0.2 mg/mL.

The effects of nonionic detergents were also determined at 40 mM phosphate concentration with *T. thermophilus* cytochrome c_{552} as the substrate. The reaction was activated slightly by 0.5% Triton X-100 (10%) but inhibited by Tween series such as 0.5% Tween 20 (40%) and 0.5% Tween 80 (60%).

The stoichiometry of the reaction catalyzed by the enzyme was determined with horse cytochrome *c* as an electron donor. The molar ratio of reduced cytochrome *c* oxidized to oxygen consumed was 4.2 (average of four experiments). The reaction was not affected by the addition of catalase. Thus, this bacterial oxidase is likely to catalyze the transfer of four electrons from reduced cytochrome *c* to dioxygen, as mitochondrial oxidase does.

Proton Pumping. The purified *T. thermophilus* oxidase was incorporated into phospholipid vesicles, and the changes in pH of the vesicle suspensions were monitored. The addition of reduced *T. thermophilus* cytochrome c_{552} caused a rapid acidification of the suspending medium (Figure 4, trace A). A value of 0.82 was calculated for the molar ratio of the maximum number of protons extruded to the extravesicular space to the reduced cytochrome *c* added at 8.2 turnovers of the enzyme. In the presence of uncoupler, FCCP, on the other hand, which allows the free permeation of protons across the membrane, the initial small acidification was followed by rapid alkalinization (Figure 4, trace B). In this case, the molar ratio

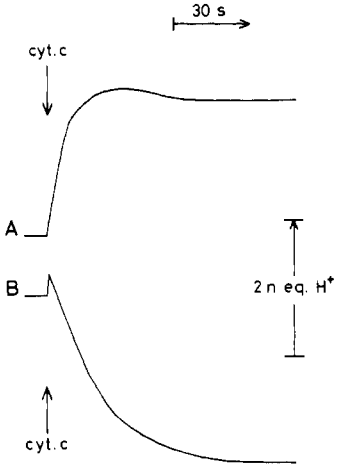

FIGURE 4: pH responses induced by cytochrome *c* oxidation in *T. thermophilus* cytochrome *c* oxidase reconstituted vesicles. Phospholipid vesicles containing *T. thermophilus* cytochrome *c* oxidase (0.165 nmol of heme *a*) were incubated in 1.5 mL of 0.1 mM Mops–KOH buffer at pH 6.4, 25 mM K_2SO_4, and 2 μg of valinomycin at 30 °C. To this suspension was added 2.7 nmol of reduced *T. thermophilus* cytochrome c_{552} in both the absence (A) and presence (B) of 0.1 μg of FCCP.

of proton consumed to reduced cytochrome *c* added was determined to be 0.91. This value is close to unity, as expected for the consumption of one proton per electron in the reaction catalyzed by this enzyme. These findings indicate that *T. thermophilus* oxidase acts as a proton pump.

Discussion

The present investigation shows that the purified cytochrome *c* oxidase from *T. thermophilus* with a heme content of about 22 nmol/mg of protein consists of two subunits (I and II) having apparent molecular weights of 55 000 and 33 000, respectively. Subunit I had an anomalous M_0 value in NaDodSO$_4$–polyacrylamide gels and was observed as a broad band in the absence of urea. In addition, this band remained at the top of the gel on treatment with boiling water before electrophoresis. These features are characteristic of subunit I of cytochrome *c* oxidases from bacteria (Ludwig & Schatz, 1980; Yamanaka et al., 1981; Sone & Yanagita, 1982) as well as mitochondria (Briggs et al., 1975; Poyton & Schatz, 1975).

In our previous paper (Hon-nami & Oshima, 1980), the purified enzyme preparation having a heme *a* content of 12.6 nmol/mg of protein, showed three major bands on a NaDodSO$_4$–polyacrylamide gel. Their apparent molecular weights on a 10% polyacrylamide gel were 52 000, 37 000, and 29 000 for bands I, II, and III, respectively. Band I would not correspond to subunit I since this component was observed as a sharp band on a NaDodSO$_4$–polyacrylamide gel in the absence of urea in spite of the dissociation of the sample in boiling water before electrophoresis. Subunit I probably remained at the top of the gel in the previous study: a strong band was seen at the top of the gel as illustrated in our previous paper (Hon-nami & Oshima, 1980). This band was not observed in the present study in which pretreatment with boiling water was omitted. Thus, band I is probably a contaminant corresponding to a polypeptide with an apparent molecular weight of 47 000 and was removed from the enzyme at the last purification step in the present investigation.

Band II, reported as a heme *c* containing subunit in the previous study, corresponds to subunit II, since only this subunit showed heme-catalyzed peroxiddase activity. Band III, observed as having a smaller molecular weight than that of heme *c* attached band, was removed completely by ion-

exchange chromatography prior to the last purification step, and no band was observed at a more forward site than subunit II in the purified sample in the present investigation (Figure 2).

The molecular weights of subunits I and II of *T. thermophilus* cytochrome *c* oxidase were determined to be 71 000 and 34 000, respectively, from the relationship of molecular weight to K_r. On the basis of the heme *a* content, the molecular mass of the enzyme was calculated to be 91 000 per two hemes *a*. This value is close to 105 000, which is the sum of the molecular weights of the two subunits. These results indicate that *T. thermophilus* oxidase contains two hemes *a*, one heme *c*, and two copper atoms, if one assumes that the enzyme complex consists of one copy of each subunit.

It is known that cytochrome *c* oxidase from bacteria such as *P. denitrificans* and thermophilic bacterium PS3 functions as proton pump (Solioz et al., 1982; Sone & Yanagita, 1982; Sone & Hinkle, 1982), while certain others, *Rhodopseudomonas sphaeroides* (Gennis et al., 1982) and *N. agilis* (Sone et al., 1983) enzymes, do not. Proton pumping by *T. thermophilus* cytochrome c oxidase was demonstrated in the reconstituted system with a stoichiometry of 0.82 H^+/e (Figure 4). This value is high compared to those reported for other bacterial enzymes (Solioz et al., 1982; Sone & Yanagita, 1982; Sone & Hinkle, 1982). This high efficiency may be due to the employment of a natural substrate (*T. thermophilus* cytochrome c_{552}) for this enzyme (Hon-nami & Oshima, 1980) as a reductant.

Subunits I and II of *T. thermophilus* enzyme seem to correspond to those of the PS3 enzyme, since subunit I of each thermophilic enzyme is highly hydrophobic (*Thermus*, 34.4%, PS3, 30.1% in polarity index) and shows anomalous features in $NaDodSO_4$–polyacrylamide gel electrophoresis and since subunit II of the *T. thermophilus* enzyme, like that of the PS3 enzyme, contains covalently attached heme *c* (Sone & Yanagita, 1982). In the *T. thermophilus* enzyme, however, a protein component corresponding to subunit III of the PS3 enzyme is absent. This subunit is considered to be analogous to subunit III of mammalian enzymes (Sone & Yanagita, 1982), and its role in proton pumping has attracted attention since dicyclohexylcarbodiimide inhibits proton-pumping activity through its covalent binding to subunit III (Casey et al., 1979; 1980) and the subunit III free enzyme loses this activity (Saraste et al., 1981).

In summary, *T. thermophilus* cytochrome *c* oxidase consisting of only two subunits does not require the third protein component corresponding to subunit III of mammalian enzymes to act as a proton pump. This is also the case for the *P. denitrificans* enzyme, which was demonstrated by the Ludwig group (Ludwig & Schatz, 1980; Solioz et al., 1982). They have speculated that the structures involved in subunit III functions are integrated into subunit II of this two-subunit enzyme and pointed out that whether dicyclohexylcarbodiimide binds covalently to the enzyme to inhibit proton-pumping activity is a matter of key importance. Our preliminary experiment showed that the *T. thermophilus* enzyme is resistant to even a relatively high concentration of this inhibitor.

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Registry No. Hydrogen ion, 12408-02-5; cytochrome *c* oxidase, 9001-16-5; cytochrome *c*, 9007-43-6; cytochrome c_{552}, 9048-78-6.

References

Briggs, M., Kamp, P.-F., Robinson, N. C., & Capaldi, R. A. (1975) *Biochemistry 14*, 5123–5128.
Capaldi, R. A., & Vanderkooi, G. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 930–932.
Casey, R. P., Thelen, M., & Azzi, A. (1979) *Biochem. Biophys. Res. Commun. 87*, 1044–1051.
Casey, R. P., Thelen, M., & Azzi, A. (1980) *J. Biol. Chem. 255*, 3994–4000.
Cha, S. (1969) *Methods Enzymol. 13*, 62–69.
Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.
Dittmer, J. C., & Lester, R. L. (1964) *J. Lipid Res. 5*, 126–127.
Downer, N. W., Robinson, N. C., & Capaldi, R. A. (1976) *Biochemistry 15*, 2930–2936.
Fee, J. A., Choc, M. G., Findling, K. L., Lorence, R., & Yoshida, T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 147–151.
Gennis, R. B., Casey, R. P., Azzi, A., & Ludwig, B. (1982) *Eur. J. Biochem. 125*, 189–195.
Hon-nami, K., & Oshima, T. (1977) *J. Biochem. (Tokyo) 82*, 769–776.
Hon-nami, K., & Oshima, T. (1980) *Biochem. Biophys. Res. Commun. 12*, 1023–1029.
Kent, T. A., Münk, E., Dunham, W. R., Filter, W. F., Findling, K. L., Yoshida, T., & Fee, J. A. (1982) *J. Biol. Chem. 257*, 12489–12492.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosengrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Ludwig, B. (1980) *Biochim. Biophys. Acta 594*, 177–189.
Ludwig, B., & Schatz, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 196–200.
Margalit, R., & Schejter, A. (1970) *FEBS Lett. 6*, 278–280.
Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570–572.
Maurer, H. R. (1971) *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis*, p 45, de Gruyter, Berlin.
Morrison, M., & Horie, S. (1965) *Anal. Biochem. 12*, 77–82.
Morrison, M., Connelly, J., Petix, J., & Stotz, E. (1960) *J. Biol. Chem. 235*, 1202–1205.
Orii, Y., Sekuzu, I., & Okunuki, K. (1963) *J. Biochem. (Tokyo) 51*, 204–215.
Oshima, M., & Yamakawa, T. (1974) *Biochemistry 13*, 1140–1146.
Oshima, T., & Baba, M. (1981) *Biochem. Biophys. Res. Commun. 103*, 156–160.
Penefsky, H. (1979) *Methods Enzymol. 56*, 527–530.
Powers, L., Chance, B., Ching, Y., & Nagiolillo, P. (1981) *Biophys. J. 34*, 465–498.
Poyton, R. O., & Schatz, G. (1975) *J. Biol. Chem. 250*, 752–761.
Reid, G. A., & Ingledew, W. J. (1980) *FEBS Lett. 109*, 1–4.
Saraste, M., Penttila, T., & Wikstrom, M. (1981) *Eur. J. Biochem. 115*, 261–268.
Solioz, M., Carafoli, E., & Ludwig, B. (1982) *J. Biol. Chem. 257*, 1579–1582.
Sone, N., & Hinkle, P. C. (1982) *J. Biol. Chem. 257*, 12600–12604.
Sone, N., & Yanagita, Y. (1982) *Biochim. Biophys. Acta 682*, 216–226.

Sone, N., Yanagita, Y., Hon-nami, K., Fukumori, Y., & Yamanaka, T. (1983) *FEBS Lett. 155*, 150–154.
Sreekrishna, K., Jones, C. E., Guetzow, K. A., Prasad, M. R., & Joshi, V. C. (1981) *Anal. Biochem. 103*, 55–57.
Tanaka, T., Kawano, N., & Oshima, T. (1981) *J. Biochem. (Tokyo) 89*, 677–682.
Tracy, R. P., & Chan, S. H. P. (1979) *Biochim. Biophys. Acta 576*, 109–117.
Van De Bogart, M., & Beinert, H. (1967) *Anal. Biochem. 20*, 325–334.
Wang, C. S., & Smith, R. L. (1975) *Anal. Biochem. 63*, 414–417.
Weber, K., & Osborn, M. J. (1969) *J. Biol. Chem. 244*, 4406–4412.
Yamanaka, T., & Fujii, K. (1980) *Biochim. Biophys. Acta 591*, 53–62.
Yamanaka, T., & Fukumori, Y. (1981) *Plant Cell Physiol. 22*, 1223–1230.
Yamakawa, T., Irie, R., & Iwanage, M. (1960) *J. Biochem. (Tokyo) 48*, 490–507.
Yamanaka, T., Kamita, Y., & Fukumori, Y. (1981) *J. Biochem. (Tokyo) 89*, 265–273.
Yonetani, T. (1960) *J. Biol. Chem. 235*, 845–852.

Anticoagulant Proteases from Western Diamondback Rattlesnake (*Crotalus atrox*) Venom†

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ABSTRACT: *Crotalus atrox* venom contains agents that render human fibrinogen and plasma incoagulable by thrombin. To elucidate the mechanism of alteration of fibrinogen clotting function by the venom, four immunochemically different proteases, I, II, III, and IV, were purified from the venom by anion-exchange chromatography and column gel filtration. All four proteases had anticoagulant activity rendering purified fibrinogen incoagulable. Proteases I and IV do not affect fibrinogen in plasma but in purified fibrinogen cleave the Aα chain first and then the Bβ and γ chains. Both enzymes are metalloproteases containing a single polypeptide chain with 1 mol of zinc, are inhibited by (ethylenedinitrilo)tetraacetate and human α_2-macroglobulin, and have an optimal temperature of 37 °C and an optimal pH of 7. Protease I has a molecular weight (M_r) of 20 000 and is the most cationic. Protease IV has an M_r of 46 000 and is the most anionic glycoprotein with one free sulfhydryl group. Proteases II and III degrade both purified fibrinogen and fibrinogen in plasma, cleaving only the Bβ chain and leaving the Aα and γ chains intact. Both enzymes are alkaline serine proteases, cleave chromogenic substrates at the COOH terminal of arginine or lysine, are inhibited by diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride, and have an optimal temperature of 50–65 °C. Protease II is a single polypeptide chain glycoprotein with an M_r of 31 000. Protease III is a two polypeptide chain protein with an M_r of 24 000, each of the two chains having an M_r of 13 000; its activity is not affected by major protease inhibitors of human plasma. Proteases II and III are enzymes with unique and limited substrate specificity by cleaving only the Bβ chain, releasing a peptide of M_r 5000 and generating a fibrinogen derivative of M_r 325 000, with intact Aα and γ chains and poor coagulability. Since the two enzymes are active in human plasma and serum, it is postulated that proteases II and III can mediate anticoagulant effects in vivo after envenomation.

Proteases, which in vitro convert fibrinogen to fibrin, have been found in venoms of snakes belonging to Crotalidae and Viperidae families (Iwanaga & Suzuki, 1979). Fibrinogen-clotting enzymes were purified from venoms of *Agkistrodon acutus* (Ouyang et al., 1971), *Agkistrodon rhodostoma* (Esnouf & Tunnah, 1967), *Agkistrodon contortrix contortrix* (Herzig et al., 1970), *Bitis gabonica* (Marsh & Whaler, 1974), *Bothrops atrox* (Stocker & Egberg, 1973), *Trimeresurus gramineus* (Ouyang & Yang, 1974), *Crotalus horridus horridus* (Bonilla, 1975), and *Crotalus adamanteus* (Markland & Damus, 1971). A fibrinogen-clotting activity was also found in the venom of juvenile specimens of *Crotalus atrox*, a western diamondback rattlesnake, but it disappeared after the snake reached the age of about 1 year (Reid & Theakston, 1978). Preliminary reports describing the absence of coagulant activity and the presence of fibrinolytic activity in *C. atrox* venom have been published (Deutsch & Diniz, 1955; Denson, 1969). Column gel filtration of the venom on Sephadex G-100 revealed two protein peaks of M_r 60 000 and 21 500 with fibrinolytic activity, as determined by the fibrin plate assay (Bajwa et al., 1980, 1981). However, the corresponding enzymes have not been purified and characterized.

A variety of proteases have been isolated from *C. atrox* venom. These include three proteases with caseinolytic activity, called α-, β-, and γ-proteases (Pfleiderer & Sumyk, 1961), five hemorrhagic toxins (*a*, *b*, *c*, *d*, and *e*) with proteolytic activity on dimethylcasein and dimethylhemoglobin (Bjarnason & Tu, 1978), and four complement-inactivating proteases (Man & Minta, 1977). Our studies with unfractionated *C. atrox* venom showed that human plasma or purified human fibrinogen was rendered incoagulable. The effect resulted from cleavage of the fibrinogen molecule. The degradation pattern of plasma fibrinogen, however, was different from that of purified fibrinogen, suggesting that the venom contained two different types of fibrinogenolytic enzymes: those that initially cleave the Aα chain of fibrinogen and other proteases that

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