Respiratory Proteins from the Extremely Thermophilic Aerobic Bacterium, Thermus thermophilus

PURIFICATION PROCEDURES FOR CYTOCHROMES c₅₅₂, C₆₅₅₋₅₄₉, AND C₁₆₀₃, AND CHEMICAL EVIDENCE FOR A SINGLE SUBUNIT CYTOCHROME aa₃

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We have developed a chemically defined, minimal growth medium for Thermus thermophilus which is suitable for nutritional studies, isotopic enrichment, and genetic manipulation of the organism. Reliable procedures are described for the large scale purification of cytochrome c₅₅₂ from the periplasm and for cytochrome c₆₅₅₋₅₄₉ and cytochrome c₁₆₀₃ from the plasma membrane. In contrast to a previous report (Fee, J. A., Choc, M. G., Findling, K. L., Lorence, R., and Yoshida, T. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 147-151) which suggested a molecular weight near 200,000, the cytochrome c₁₆₀₃ complex was shown by protein and amino acid analyses to have M, ~ 98,000. Sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis and reversed phase high performance liquid chromatography, combined with amino acid analyses, revealed the presence of only two proteins in a 1:1 ratio: C-protein has M, ~ 33,000, binds heme C, and is thought to correspond to cytochrome c₁. A-protein has M, ~ 58,000 and is thought to bind the four redox components (2 heme A and 2 Cu) of cytochrome c₁₆₀₃.

In recent years, there has been a renewed interest in the respiratory proteins of bacterial membranes (1, 2). Several respiratory enzymes have been purified from a variety of bacterial sources, and present information supports the concept that similar function throughout the phyla derives from a common ancestor (3, 10). Phylogenetic relationships of the respiratory enzymes have been proposed which place the eucaryotic enzyme between the prokaryotic enzymes and the mitochondria (4, 5). Protein and amino acid analyses of the cytochrome c₁₆₀₃ subunits (6) suggest that the environments of the heme A and Cu cofactors are nearly identical with those of the extensively studied beef heart mitochondrial enzyme (15), and all have been shown to pump protons when reconstituted into phospholipid vesicles (16, 17).

A pervasive problem in the biochemical study of respiration is the difficulty of obtaining highly purified, stable enzymes which are amenable to detailed study. Nevertheless, procedures have evolved over the past decades which allow many respiratory enzymes to be removed from their natural environment in apparently intact form (18). In many cases, it has been possible to reconstitute biological activities with purified enzymes (19), and such studies have contributed greatly to our present understanding of respiration. However, the difficulty of stabilizing these enzymes outside their natural environment remains an impediment.

Several years ago, we undertook a program aimed at providing additional biophysical characterizations of certain bacterial respiratory proteins with the hope that these would facilitate understanding their functions at the molecular level. After a brief survey of thermophilic organisms (20), we chose the strictly aerobic, Gram-negative, extremely thermophilic bacterium, Thermus thermophilus, for these studies. Our rationale for selecting a thermophilic aerobe was predicated on two contentions. First, the membrane-associated enzymes would show a higher resistance to denaturation than analogous enzymes isolated from mesophilic organisms, and second, enzymes from extreme thermophiles utilize the same redox cofactors as do their mesophilic counterparts. The first of these contentions finds ample support in a long history of comparing the stabilities of enzymes obtained from organisms having widely differing preferences in growth temperature (21), and the elegant work of Kagawa et al. (22) with the F₁F₀ complex serves as an example of the usefulness of studying thermostable enzymes. The second is simply an assertion that the principle of biochemical unity (23) holds at high temperature. Our preliminary reports (11, 14), concerned with the spectroscopic properties of cytochrome c₁₆₀₃ isolated from T. thermophilus, fully justify the rationale of this undertaking. These studies showed that the cytochrome c₁₆₀₃ was, in a spectroscopic sense, fundamentally similar to eucaryotic enzymes and that the cytochrome c had properties identifiable...
with eucaryotic cytochrome c1 (11). We will continue to use the notation cytochrome c\textsubscript{ oxid} for this protein.

The present study details our procedures for the growth of \textit{T. thermophilus}, the initial fractionation of the plasma membrane into various respiratory activities, the purification of cytochromes \textit{C} subunits, and chemical characterization of cytochrome \textit{c} as a two-subunit enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

\textit{T. thermophilus} HB8 cultures were obtained from the American Type Culture Collection (No. 27634). Tris base,\(^1\) Triton X-100, ascorbic acid, \(d\)-biotin, TMPD, EDTA, EDTA, lithium dodecyl sulfate, avidin, hexafluoracetone, and deoxycholic acid were obtained from Sigma Chemical Co. (St. Louis, MO); Bio-Gel P-30 and P-100, SDS, urea, acrylamide, Bis, and a molecular weight standards kit for SDS-PAGE were obtained from Bio-Rad Laboratories (Richmond, CA); thin layer isoelectric focusing gels (Servalyt Precotes 3-10), running buffers, and isoelectric point marker proteins were obtained from Serva Fine Chemicals (Garden City Park, NY); octylglucoside was obtained either from Sigma Chemical Co. or Calbiochem-Behring (La Jolla, CA); laurylmaltoside was obtained from Calbiochem-Behring; and sodium deoxycholate, lysozyme (2X crystallized), and monosodium glutamate were obtained from United States Biochemical Corp., Cleveland, OH). DEAE- and CM-celluloses (DE52, CM52) were obtained from Whatman Inc. (Clifton, NJ). DEAE-Sephadex, Sephadex G-75-200, and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Yeast extract, peptone, and agar was purchased from Difco (Detroit, MI) and BBL (Cockeysville, MD). The water used was of reagent grade, except for 200-liter cultures, where distilled water was used. The inorganic salts used were of reagent grade.

**General Methods**

**Assays and Analyses**—Optical spectra were recorded with a Perkin-Elmer model 599 dual beam spectrophotometer equipped with digital background subtraction. Reduced minus oxidized (redox) spectra were obtained by recording the spectrum of the oxidized protein into memory, reducing the sample with a small excess of dithionite while keeping the sample anaerobic, then recording the spectrum of the reduced protein, and subtracting the spectrum stored in memory. Reduced minus oxidized difference spectra were obtained by recording the spectrum of the fully reduced protein into memory, flushing the cell several times with CO, then recording the spectrum of the CO-reduced protein complex, and subtracting the spectrum stored in memory. Pyridine hemochrome assay was done according to Paul et al. (24) using \(e_{412} = 24 \text{ M}^{-1}\) for heme A and \(e_{578} = 29.1 \text{ M}^{-1}\) for heme C. Concentrations of 0.5 mM NaOH and 3 mM pyridine were required to fully develop the hemochromogen, and protein was added to the basic pyridine mixture to avoid an irreversible precipitation. Iron and copper analyses were carried out by atomic absorption using a Perkin-Elmer model 500 spectrophotometer with a graphite furnace, in the laboratory of B. Rosman, Great Lakes Research Division. Phosphorus was quantitated by the method of Ames (25). Protein was measured by a modified method of Lowry et al. (26), where 0.5% SDS was included in the alkaline copper solution (27), and bovine serum albumin was used as the standard. Amino acid analyses were carried out by two methods: first as described by Jones and Williams (28) using conventional methodology, and second using a reversed phase liquid chromatographic separation of the phenylboranly amino acids.\(^2\) Tests for \(N\)-terminal residues were done according to the method of Tarr (30).

SDS-PAGE in the presence of 8 M urea was carried out, with minor modifications, according to Dwoer et al. (31), using a Hoeffer model SE 500 slab gel system with 1.5-mm spacers. Total acrylamide concentration was 7%, with Bis-acrylamide of 1:1. About 10% of 3.5% gel (1:1.5 Bis-acrylamide) was used as a nondiscontinuous stacking gel on top of the separating gel. Preparative scale purification of subunits was carried out using the same gel composition described above, cast with 6-mm spacers. Small strips of the gel were cut out, stained, and used as guides to obtain portions of the gel containing individual proteins. The gel was removed from the well by homogenizing, dialyzing against 0.1% SDS, and 1 mM Tris-Cl, pH 7.8, centrifuging to remove the gel particles, and finally concentrating by lyophilization. The molecular weights of the subunits were estimated from an extrapolated relationship between the free retardation coefficients and the molecular weight standards. The standard protein was extended lysozyme (12,000 Da). SDS-PAGE in the presence of 8 M urea was carried out using a Beckman model 320 gradient liquid chromatograph equipped with a Beckman model 160 detectors (229 and 254 nm) in tandem with a Kratos model FS970 spectrofluorometer. Both fluorescence and absorbance were used to detect protein in the eluant. Samples for HPLC were prepared either by direct precipitation of the protein with added ethanol or dialysis against ethanol, followed by dissolution of the precipitate in a small amount of hexafluoroacetone and dilution into 0.1% trifluoroacetic acid. Such samples were free of heme A but contained covalently bound heme C. The succinic dehydrogenase assay was done according to King (33), and the NADH dehydrogenase assay was done according to Dancen and Shapiro (34). Cytochrome oxidase activity was measured as \(O_2\) uptake in the presence of TMPD and ascorbate in a temperature-controlled cell (Gibson Medical Electronics, Inc., Middleton, WI), according to Kimmelberg and Nicholls (35). A YSI 5331 Clark electrode equipped with a high sensitivity membrane (Yellow Spring Instruments, Yellow Spring, OH), in conjunction with a Chemtrix Type 30 \(O_2\) meter (Chemtrix Inc., Hillsboro, OR), was used to measure oxygen concentration. The conditions of the standard assay were: 50 mM Tris-Cl, pH 7.8, 0.1 mM Triton X-100, 0.5 mM EDTA, 5 mM ascorbate, 0.15 mM (M)TMPD at 25 °C.

**Centrifugations**—Cells were harvested using a Sharples model A512 continuous flow centrifuge operating at 10,000 rpm. Membranes were collected using a Beckman L-5-65 ultracentrifuge equipped with a type Ti-65 rotor, 564-mI capacity, operating at 45,000 rpm (245,000 \(x g)\). An IEC model 66-6 centrifuge with four 1-liter bottles running at 3,000 rpm (2,500 \(x g)\) was used for the cell wash and cytochrome \(c_552\) extraction steps. For all other centrifugation steps, a DuPont Sorval RC2B centrifuge was used with SS34, GSA, and GS3 rotors operating at their maximum rated speeds.

**Preparative Concentration and Filtration Techniques**—The large scale concentration of membrane suspensions, typically from 40 to 50 liters, was carried out with a Pellicon high volume concentrator cell (Millipore Corp., Bedford, MA), equipped with 3.5 square feet of 100,000-Da cut-off membranes. Small scale concentrations, in the presence of low concentration of detergents, were performed in 400- or 500-ml Amicon stirred cell concentrators equipped with an XM 50 Diaflo membrane operating under 50 p.s.i. of nitrogen pressure (Amicon Corp., Danvers, MA).

**Growth of Bacteria**—The description of a chemically defined growth medium and the details of our microbiological procedures are presented in the Miniprint.\(^3\)

\(^1\)Extreme cytochrome c is not required for this type of activity. Kinetic studies have shown that the \(K_m\) for TMPD is \(\approx 0.25 \text{ mM}\), thus, the enzyme is operating at \(\approx 38\%\) efficiency under these conditions. We have also demonstrated that water, rather than peroxide, is the product of oxygen reduction (T. Yoshida, B. Zimmermann, and J. A. Fee, unpublished results).

\(^2\) Portions of this paper (including a supplementary description of procedures, Figs. 1 and 2, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-1401, cite the authors, and include a check or money order for \$3.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
RESULTS

Purification of Proteins

The following purification procedures were specifically developed for cells grown on the minimal, defined culture medium. They will work equally well, however, for bacteria grown on any of the three culture media described in the Miniprint.

A typical preparation of respiratory proteins is begun with 2 kg of frozen cell paste. The procedure involves handling volumes as large as 40 liters and the use of a 10 cm (inner diameter) × 60 cm fractionating column. An overview of the entire procedure is shown in the flow chart of Fig. 1.

Initial Wash of the Cells and Cytochrome c552 Removal—The harvested cells were suspended in TE-buffer to a volume of 1.3 liters/kg of wet cells by using a Waring blender (low speed for 1 min). The suspension was centrifuged at 14,700 × g for 45 min and the above procedure was repeated once. Cytochrome c552 was then extracted by resuspending the washed cells in 200 mM Tris-Cl, pH 7.8, 0.1 mM EDTA (1.3 liters/kg of wet cells) in the blander at low speed for 1 min. The suspension was centrifuged at 14,700 × g for 60 min, and the supernatant was set aside for further purification of cytochrome c552 (see below). This treatment was repeated once to achieve the maximal extraction of cytochrome c552.

Spheroplast Formation—The cell paste was suspended in water (8 liters/kg of wet cells) containing lysozyme (4.0 μg/kg of wet cells) by blending at low speed for 2 min. The suspension was placed on a magnetic stirrer/hot plate and heated with stirring until its temperature reached 70 °C. It was then removed from the hot plate and allowed to cool to room temperature. The extent of spheroplast formation was estimated by observing the suspension under a phase contrast microscope: spheroplasts appear as small spheres and intact cells as thin rods. Occasionally, the transformation from intact cells to spheroplasts was incomplete after this treatment, and the cells looked like swollen grains of rice. Because it is very important to strip away as much of the outer membranes of the cells as possible, these cells were further treated by adding more lysozyme (4 g/kg of wet cells), introducing 0.01% SDS, and reheating the suspension to 70 °C as before. Deoxyribonuclease I (5 mg/kg of wet cells) was added to the cooled suspension, the suspension was centrifuged at 14,700 × g for 60 min, and the spheroplasts were collected as a pellet. Up to this stage, all the operations were carried out at room temperature, while all subsequent operations were carried out at 4 °C unless otherwise noted.

Membrane Separation—The spheroplasts were resuspended in TE-buffer (20 liters/kg of wet cells) containing deoxyribonuclease I (20 mg/kg of wet cells) by blending at low speed for 2 min. The suspension was then cooled to 4 °C, and the cells were broken in a Manton-Gaulin laboratory homogenizer (Gaulin Co., Everett, MA) operating at 9000 p.s.i. The homogenate was concentrated to 1/6 of volume using a Pellicon concentrator; this process takes about 6 h. The cytosol may be discarded at this point. The membranes were washed once by diluting the final solution with 3× volume of TE-buffer and then recentrifuging to the original volume. The suspension was then centrifuged at 235,000 × g for 6 h to pellet the membranes. The membranes were separated from tightly packed brown material, presumably incompletely broken cells, by gently shaking with TE-buffer. The membranes were washed once by homogenizing in a Potter-Elvehjem homogenizer with TE-buffer, approximately 1 liter/kg of wet cells, then pelleted by centrifugation at 235,000 × g for 3 h. Membranes could be stored at −17 °C for long periods of time without apparent damage.

Detergent Extraction—Frozen membranes were thawed and suspended in TE-buffer (2 liters/kg starting wet cell weight) by blending at low speed for 2 min. A 20% solution of Triton X-100 was added to make the final concentration of detergent 2%. The detergent-membrane mixture was stirred for 30 min or more at 4 °C and then centrifuged at 48,200 × g for 30 min. If the pellet still retained the original yellow-brown color, it was re-extracted by homogenizing in a Potter-Elvehjem homogenizer, with 2% Triton X-100, TE-buffer (1 liter/kg of wet cells), and centrifuged as before. Occasionally, we had to repeat this step several times, until the pellet became gray colored and the volume decreased to less than 5% of starting membrane. The membrane’s inability to dissolve in the detergent at once seems to be due to contamination by unbroken spheroplasts. This problem can be avoided if one is careful to take only the translucent portion of the membranes during the initial centrifugation step.

Initial Fractionation—The extracts were pooled, diluted with 2% Triton X-100 to an appropriate conductivity (~330 μmho/cm), and loaded onto DEAE-cellulose (Whatman DE52) column (2 liters of cellulose/kg of starting wet cells, for 2 kg, a 10 × 60 cm column) pre-equilibrated with 2% Triton X-100 in TE-buffer at room temperature. The column was washed exhaustively at room temperature with 2% Triton X-100, TE-buffer until the effluent became colorless (typically 6 column volumes). The column was then moved into a 4 °C room, washed with 3 column volumes of 0.1% Triton X-100, TE-buffer, and eluted with a linear NaCl gradient consisting of 2 column volumes of 0.0-0.25 M NaCl in 0.1% Triton X-100. TE-buffer followed by 1.5 column volumes of 0.25-1.0 M NaCl in the same buffer at a flow rate of 3.7 ml cm−2 h−1. Absorption of the eluant at 420 nm was recorded (Fig. 28), and the eluant was assayed for NADH dehydrogenase and

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**FIG. 1.** Flow diagram representing purification procedures. (1), cytochrome c552; (2), cytochrome c650-c645; (3), cytochrome c550 a crude fraction (4) containing the Rieske protein, NADH and succinic dehydrogenases from T. thermophilus; and (5) the ferredoxin. The circular arrows denote centrifugations with supernatants on the left and precipitates on the right.
succinic dehydrogenase activities (Fig. 2A). Reduced minus oxidized spectra of the corresponding fractions, including the membrane extract, are shown in Fig. 2, C-H.

The bacteria contain large amounts of carotenoid having strong absorbance near 450 nm (36); this is completely removed by the above DE52 column. Also, a large amount of cytochrome \( b \) is removed from the column at this stage. The redox spectrum of the combined wash is shown in Fig. 2D. Fig. 2B shows a typical gradient elution profile of the DE52 column. The first major peak contains cytochrome \( c_5, a_3 \) complex, as shown in Fig. 2E. A minor peak containing a cytochrome \( a/b \) complex occasionally precedes the cytochrome \( c_5, a_3 \) peak, depending on the extent of removal during the washing steps.\(^5\) The latter third of the \( c_5, a_3 \) peak contains varying amounts of cytochrome \( c_{552} \) which was not extracted from the whole cells during the high salt treatment. Succinic and NADH dehydrogenase activities (Fig. 2A) eluted after \( c_{552} \) and are often superimposed on each other. At the present time, we are investigating procedures to purify these dehydrogenases.

In addition, NADH dehydrogenase fractions contain the Rieske protein which has been purified and characterized in detail (37). The second major cluster of peaks on the profile consists of mixture of cytochromes \( a, b, \) and \( c_{552} \), and the third peak contains the “split-alpha” cytochrome \( c_{555,549} \) (Fig. 2G). Some seven-iron ferredoxin (38) occasionally elutes as a major peak toward the end of the first part or early in the second part of the gradient (Fig. 2H). The presence of appreciable amounts of ferredoxin seemed to be correlated with contamination by unbroken spheroplasts in the extracted membranes. Individual protein fractions were pooled, as indicated in Fig. 2B, and frozen at \(-10^\circ\) C, for subsequent purification.

**Purification of Cytochrome \( c_{552} \)**—The combined high salt extracts of whole cells were diluted 8-fold with water, stirred overnight with CM52 cation exchanger (100 g of resin/kg of wet cells), and equilibrated with 20 mM Tris-Cl, pH 7.8, 0.1 mM EDTA buffer. The pink-colored CM52 resin having bound cytochrome \( c_{552} \) was poured onto a CM52 column (0.35 liter/kg of wet cells) equilibrated with TE-buffer, and the resulting column was washed with 25 mM Tris-Cl, pH 7.8, 0.1 mM EDTA buffer (2.5 liters/kg of wet cells). The cytochrome \( c_{552} \) was then eluted with a linear gradient (4 liters/kg of wet cells) of 25 to 150 mM Tris-Cl, pH 7.8, 0.1 mM EDTA.

The cytochrome-containing fractions were pooled, dialyzed against TE-buffer, and adsorbed onto another CM52 column (0.5 liter/kg of wet cells), equilibrated with TE-buffer. The cytochrome was then eluted with a linear gradient of 0-0.2 M NaCl in TE-buffer (4 liters/kg of wet cells).\(^6\) The purer fractions of cytochrome \( c_{555} \) were combined and dialyzed for approximately 4 h against TE-buffer and then concentrated onto a small CM52 column (10 ml/kg of wet cells). The protein was eluted with 0.5 M NaCl, TE-buffer, and dialyzed against 20 mM Tris-Cl, pH 7.8, 0.1 mM EDTA. Passage through a Bio-Gel P-100 column (40 ml/kg of wet cells, 85 cm long) at room temperature using TE-buffer as eluant, yielded purified cytochrome \( c_{552} \). Table I summarizes the efficiency of each purification step.

**Purification of Cytochrome \( c_{555,549} \)**—The cytochrome fractions containing cytochrome \( c_{555,549} \) from the initial DE52 column (cf. Fig. 2) were combined, dialyzed against TE-buffer, and concentrated onto a small DE52 column (8 ml/kg of wet cells). After elution with 0.7 M NaCl, TE-buffer, and dialysis against 15 mM Tris-Cl, pH 7.8 buffer, 1 ml of this cytochrome solution was mixed with 0.5 ml of 100% Triton X-100 and loaded onto a Sephadex G-75-120 column (0.45 liter/kg of wet cells, 82 cm) equilibrated with 2% Triton X-100, TE-buffer. Developing the column with TE-buffer resulted in the elution of most of the contaminants in a Triton X-100-rich fraction at the void volume of the column. The combined cytochrome \( c_{555,549} \) fractions, which eluted later, were concentrated onto a small DE52 column (8 ml/kg of wet cells) and most of the detergent was removed from the protein by washing with \(-30 \) column volumes of TE-buffer. After elution with 0.7 M NaCl, TE-buffer, the cytochrome \( c_{555,549} \) was further purified by passage through a Bio-Gel P-30 column (0.42 liter/kg of wet cells, 82 cm length) equilibrated with 0.7 M NaCl, TE-buffer. Table I presents a summary of the purification steps and shows the

\(^5\) We estimate that as much as half the heme A applied to the column appears in the wash. On one occasion, the material was purified by procedures similar to those developed for the cytochrome \( c_{552} \), and it was characterized by gel electrophoresis, and optical and EPR spectrosopies. This material appears to be a cytochrome \( ba_{3} \) complex: gels indicated the presence of \( 54-, 39-, \) and 18-kDa bands, the latter being relatively weakly stained. The optical spectra of the corresponding fractions, including the membrane extract, are shown in Fig. 2, C-H.

\(^6\) Occasionally, cytochrome \( c_{552} \) may already be quite pure at this point. In such cases, the protein forms a very tight band at the top of the column and a higher concentration of salt is required for its removal.
Purification of low molecular weight c-type cytochromes from *T. thermophilus*

| Purification step               | Total protein | Cytochrome c | Purity index | Heme/protein |
|--------------------------------|--------------|--------------|--------------|--------------|
| Salt wash of whole cells       | 1800         | 400*         | 0.26         |              |
| First CM52 column              | 170          | 398          | 2.3          |              |
| Second CM52 column             | 8            | 280          | 2.9*         | 36           |
| Gel filtration (P-100)         | 1.8          | 135          | 5.1          | 75           |
| Cytochrome c555,549            |              |              |              |              |
| DE52 column                    | 100          | 140          | 1.3          |              |
| First phase separation/gel filtration | 1.2          | 68           | 57           |              |
| Second phase separation/gel filtration | 0.57       | 45           | 5.5*         | 79           |
| Gel filtration (P-30)          | 0.23         | 20           | 7.9          | 87           |

* Determined from the reduced minus oxidized difference spectrum using $\Delta A_{552} = 14.3 \text{mM}^{-1} \text{cm}^{-1}$ (39).

* $A_{408}/A_{552}$ of oxidized protein.

* Determined from the reduced minus oxidized difference spectrum using $\Delta A_{552} = 15.2 \text{mM}^{-1} \text{cm}^{-1}$ (40).

* $A_{408}/A_{552}$ of oxidized protein.

effectiveness of the detergent phase-separation of the Sephadex column step.

**Purity of Cytochromes c552 and c555,549**—As indicated by optical spectra of their solutions and SDS-PAGE, both of the small c-cytochromes were obtained in high purity. The ratios of Soret absorbance ($c_{552}$: 408 nm; $c_{555,549}$: 409 nm) to protein absorbance at 280 nm in the oxidized state was 0.1 for cytochrome c552 and 7.9 for cytochrome c555,549; these values compare favorably to previously reported values of 4.7 (41) and 7.7 (39). Table I shows purity values in terms of nanomoles of cytochrome/mg of protein. Our values of 75 and 87 for cytochrome c552 and c555,549 compare favorably to the theoretical values of 67 and 100 calculated from previously estimated $M_r = 15,000$ (41) and 10,000 (39).

SDS-PAGE and thin layer isoelectric focusing revealed a single band for purified cytochrome c552 with $M_r = 14,000$ and pI ~ 10.8 when the gels were stained with Coomassie blue R. However, we were unable to find conditions under which cytochrome c555,549 could be properly stained in these gel systems. Therefore, we resorted to electrophoresis in a glass tube followed by optical and spectral scanning of the gel at 280 and 408 nm. Purified cytochrome c555,549 revealed two bands in such gels, one corresponding to a molecular weight near 11,000 and one near 23,000. Since the $A_{408}/A_{552}$ ratio was approximately the same in both bands, we speculate that the higher molecular weight material arises from noncovalently associated, dimeric material.

**Purification of Cytochrome c553 Complex**—The pooled fractions containing cytochrome c553 (Fraction e, Fig. 2B) were concentrated to approximately $V_{40}$ to $V_{50}$ of the original volume. Octylglucoside was then added to this solution, to a final concentration of 70 mM, and stirred for at least 2 h at 4 °C. This solution was then dialyzed for at least 12 h, with three changes, against 40 volumes of 0.1% Triton X-100, TE-buffer. Octylglucoside was added to this solution again, to make 70 mM in final concentration, stirred for at least 2 h at 4 °C, and dialyzed as before.

The protein solution was brought to 2% Triton X-100, and loaded onto a DEAE-Sephacel column (60 ml/μmol of heme A₃ for 2 kg of preparation, 2.6 × 80 cm), pre-equilibrated with 2% Triton X-100, TE-buffer. The column was washed with approximately 5 column volumes of 2% Triton X-100, TE-buffer, until the eluant became free of any color. At this wash, complete removal of cytochrome b and a cytochrome ab complex was accomplished (cf. Footnote 5). The Triton X-100 concentration was lowered by washing with 3 column volumes of 0.1% Triton X-100, TE-buffer, and the protein was eluted in a small volume with 0.5 M NaCl, 0.1% Triton X-100, TE buffer. The solution was diluted 4-fold with TE-buffer and concentrated to the original volume. This procedure was repeated once with the solution finally being concentrated to approximately 5% of the column volume required for the following gel filtration step.

The protein solution was loaded onto a Sephacryl S-300 column (220 ml, 5 × 40 cm) equilibrated with 1% sodium deoxycholate, TE-buffer, maintained at room temperature, and eluted at a flow rate of approximately 1.8 ml h⁻¹ cm⁻². A typical elution profile (420 and 280 nm absorption) is shown in Fig. 3A. The first major peak contains the cytochrome c553 complex, and the large 280 nm peak contains Triton X-100. Occasionally, when the cytochrome c553 Contamination is high, it appears as a shoulder after the cytochrome c553 peak. The cytochrome c553-containing fractions, including containing cytochrome c553, were pooled, diluted 10-fold with TE-buffer, and concentrated in the Amicon-stirred concentrator at 4 °C. Several dilution-reconcentration steps may be necessary to obtain a low viscosity concentrate. The volume was finally reduced to the starting volume of the previous column, and chromatographed on the same Sephacryl S-300 which had been exhaustively washed with 1% sodium deoxycholate, TE-buffer. An elution profile similar to Fig. 3A is observed in which the Triton X-100 peak is replaced by a minor colorless peak.

The pooled fractions containing cytochrome c553 were brought to 0.5% deoxycholate, 2% Triton X-100, TE-buffer, and the conductivity was adjusted to ~330 μMho/cm with
2% Triton X-100 in water. This solution was then loaded onto a DEAE-Sephacel column (100 ml/mmol of heme A; 2.6 x 70 cm) equilibrated with 2% Triton X-100, TE-buffer. The column was washed with 3 column volumes each of 2% Triton X-100, TE-buffer and 0.1% Triton X-100, TE-buffer, respectively, and developed with 5 column volumes of 0-0.4 M linear NaCl gradient in 0.1% Triton X-100, TE-buffer. A typical elution profile is shown in Fig. 3B. The 420 nm absorption profile usually consists of one major peak with a very sharp leading edge and a slowly decreasing tail, although spectra of fractions throughout this peak are identical. However, SDS-urea-PAGE gels of different fractions clearly show that the purest materials were located in the middle section of the peak. The initial part of the peak contains lower molecular weight contaminants whereas the latter part of the peak contains increasing amounts of high molecular weight contaminants. The fractions indicated in Fig. 3B were pooled and subsequently used for characterization of this protein. The yields of cytochrome c$_{1aa3}$ complex are shown in Table II.

**General Characterization of Cytochrome c$_{1aa3}$**

Indications of purity were obtained primarily from SDS-urea-PAGE experiments (see below). However, when the protein was electrophoresed under nondenaturing conditions, in the absence of SDS, only a single band was revealed by both Coomassie and TMPD staining (42); gel filtration HPLC using a Synchropak GPC500 column also revealed a single band of material (data not shown).

Several compositional features of the protein have been examined. The combined results of atomic absorption, pyridine hemochromogen, and protein analyses are presented in Table III. Within the indicated error limits, cytochrome c$_{1aa3}$ contains 2 heme A, 2Cu, and 1 heme C per ~94,000 Da of protein as determined by the method of Lowry et al. (26). The ratio of heme iron to total iron is approximately 1 and the ratio of total Fe to Cu is approximately 3:2, indicating the absence of extraneously bound metal. With the exception of the molecular weight, these results are similar to those reported earlier (11). Some phospholipid was found to be associated with the purified protein. The purest preparations contained ~1P/enzyme molecule and less pure material contained greater amounts.

It is important to be certain that the preparation is free of cytochrome o (a CO-reactive b-type cytochrome). That this is true was demonstrated in two ways. Shown in Fig. 4 are spectra of pyridine hemochromogen samples prepared from various cytochromes. Spectrum a was derived from a membrane extract and thus contains a mixture of hemes A, B, and C. Spectrum b was obtained from a cytochrome ab complex (see Footnote 5) and shows the prominent band at 555 nm due to heme B. Spectrum c was obtained from purified cytochrome c$_{1aa3}$, which can be compared with that obtained from a 1:1 mixture of horse heart cytochrome c and beef heart cytochrome az$_{5}$ (Spectra d-f). Note the narrow, nearly symmetric peak of the heme C at 548 nm in spectrum c, indicating the absence of b-type cytochromes in the cytochrome c$_{1aa3}$ complex. Fig. 4 further demonstrates the interference of heme A in the determination of heme C. We have determined the base-line for the C-heme to be 0.25 the absorbance of the peak at 586 nm due to heme A. An additional test for the presence of cytochrome c involves recording the difference spectrum of reduced + CO minus reduced protein, since c-type cytochromes will have a characteristic peak in the region of 416 nm (43) (see also Miniprint). We find, however, a peak

| Sample        | Protein | Heme A | Heme A/Protein | O$_2$ uptake/ | O$_2$ uptake/ |
|---------------|---------|--------|----------------|--------------|--------------|
|               |         | mg     | µmol/mg        | protein-min  | protein-min  |
| Washed        |         | 54,000 | 1.85           | 3.37         | 1.1          |
| Membrane      |         | 44,000 | 1.85           | 3.37         | 1.1          |
| After first DE52 | 1,000  | 5.72   | 5.67           | 2.73         | 24.0         |
| After second DE52 | 687    | 4.58   | 7.19           | 5.09         | 354         |
| After first S-300 | 611    | 4.46   | 7.30           | 5.29         | 369         |
| After second S-300 | 497    | 3.92   | 7.90           | 5.64         | 357         |
| After DEAE-Sephalcel | 678    | 1.42   | 21.3           | 15.9         | 350         |

* Moles of O$_2$/mol of c$_{1aa3}$.min.
* There are also 1.42 µmol of heme A with 18.8 nmol heme A/mg protein, which show slight contamination in SDS-urea gel. The optical and EPR spectra are identical with the purified enzyme.

**Table III**

| Composition of purified cytochrome c$_{1aa3}$ |
|---------------------------------------------|
| Heme Az/protein  | Fe/c$_{1aa3}$ | Cu/c$_{1aa3}$ | Phosphorus/c$_{1aa3}$ | heme C'/heme A |
|-----------------|--------------|--------------|----------------------|----------------|
| 21.3$^a$       | 3.37±0.10    | 1.85±0.04    | 1.1                  | 0.37-0.5$^b$  |

$^a$ Determined from the pyridine hemochromogen.
$^b$ Determined by the modified Lowry method.

**Footnote 5**

Elemental analyses referred to the holoenzyme assumed to be composed of one heme C and two heme A and generally based on the optical measurements of reduced enzyme + CO versus reduced enzyme.

**Fig. 4. Pyridine hemochrome spectra of beef heart cytochrome c oxidase, horse heart cytochrome c, and T. thermophilus cytochrome complexes from various stages of the preparation.** The spectra were taken under the conditions described under "General Methods." A, 2% Triton X-100 extract of purified T. thermophilus membrane. B, cytochrome ba$_{5}$ complex from T. thermophilus (cf. Footnote 5). C, purified c$_{1aa3}$ complex from T. thermophilus. D, summation of spectra E and F, corresponding to 1 mol of heme C and 2 mol of heme A. E, horse heart cytochrome c. F, beef heart cytochrome c oxidase.
Absorption spectra were recorded from oxidized, reduced, reduced minus oxidized, and reduced + CO minus reduced proteins, all of which had been carefully characterized by their pyridine hemochromogen spectra. Extinction coefficients at pertinent wavelengths are given in Table IV. These values are slightly different from those reported earlier (11) and are considered more reliable.

The specific activity of the cytochrome c_3a_3, defined as moles of O_2 consumed/mol of enzyme-min, is relatively constant at ~350 throughout the purification procedure (see Footnote 2). Addition of phospholipids or other detergents such as Tween 20, Tween 80, cholate, deoxycholate, octylglucoside, and laurylmaltoside had only minor effects on the activity in this system.

Protein subjected to SDS-urea-PAGE under several conditions and after a variety of denaturing procedures revealed only two bands as shown in Fig. 5, a–c. The lower molecular weight band is generally quite sharp while the higher molecular weight band is diffuse. The relative ratios of these bands to each other and to higher molecular weight material, including that which does not enter the gel, is strongly dependent on the particular sample and the method of denaturation. We have suggested the denaturing conditions which generally avoid aggregation while optimizing resolution of the two bands.

We expected that C-heme would be associated with one of the bands appearing on the SDS-PAGE gels, and this was examined by staining gels for heme peroxidase activity (44). Heme A was extracted into acetone-HCl according to the method of Takemori and King (45), and the protein was electrophoresed under the same conditions except that 2-mercaptoethanol, which interferes with the assay, was omitted. Exclusion of 2-mercaptoethanol from the denaturing medium did not affect the gel pattern as the Coomassie-stained gel indicates (Fig. 5f), although precipitation of the protein by cold acetone caused a portion of the protein to irreversibly aggregate. As shown in Fig. 5e, the lower molecular weight band and aggregated material showed the peroxidase activity but none was detected in the high molecular weight band. Further, pyridine hemochromogen analysis showed that the acetone-HCl extract contained only heme A. Horse heart cytochrome c was used as a positive control, and beef heart cytochrome oxidase, from which all heme is extractable, was used as a negative control. These observations strongly suggest that the C-heme is associated with the 33,000-Da band and that this peptide corresponds to cytochrome c_1. Further support for this conclusion was obtained by separating small quantities of the c cytochrome from c_3a_3 by preparative gel electrophoresis and carrying out SDS-PAGE/peroxide staining (data not shown).

An electrophoretic determination of the apparent molecular weights of the two polypeptides was carried out by the method of Ferguson (32) in which the percentage of acrylamide in the gels was varied over the range 4 to 11% (Fig. 6). The values

| Table IV | Extinction coefficients of purified cytochrome c_3a_3 complex |
|----------------|-----------------------------------------------|
| **State** | **Wavelength (nm)** | **ε or Δε of c_3a_3 complex** |
| Oxidized | 604 | 18.6 |
| Reduced | 604 | 36.4 |
| Reduced-oxidized | 604–640 | 21.4 |
| (Reduced/CO)-reduced | 591–612 | 8.1 |

**Fig. 5.** SDS-urea-PAGE of purified cytochrome c_3a_3 from *T. thermophilus*. Gel composition and denaturing conditions are given under "General Methods" except that 2-mercaptoethanol was omitted from c and f. The molecular weights of the standards (gels d and g) are given in Fig. 6. a through c: Coomassie blue R-stained gel of highly purified cytochrome c_3a_3. Approximately 1, 2, and 5 μg were loaded, respectively. c: heme activity stain of slightly impure protein samples. f: identical gel after removing activity stain and restaining it with Coomassie blue R.

**Fig. 6.** Apparent molecular weights of the subunits of *T. thermophilus* cytochrome c_3a_3. The free mobilities of the standards measured by the method of Ferguson (32), with horse heart cytochrome c as an internal reference, are plotted against their molecular weights: lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase b (92,500). See text for experimental details of the gel system and denaturing condition.
of 33,000 and 52,000 ± 3,000 Da were determined for the cytochrome c and the largest subunit, respectively. A measure of the ratio of these values to the 52,000-Da proteins can be estimated from the peak areas of densitometer traces of gels stained with Coomassie blue R. Assuming the staining efficiency of each band with Coomassie blue R to be identical on a weight basis, and using molecular masses of 33 and 52 kDa, one obtains a ratio of 0.95 (± 8%). Denatured protein was also subjected to electrophoresis in a 10% gel (1:34, Bis-acrylamide) at pH 4.5 in the presence of lithium dodecyl sulfate as denaturant (46). Only two bands were observed having apparent $M_r = 36,000$ and 61,000. Since the sums of the $M_r$ values obtained for the two protein bands by two different gel electrophoresis systems are consistent with the minimum molecular weight indicated by analyses of protein content (∼94,000), and the staining ratio is near unity, we tentatively conclude that the enzyme is composed of only two polypeptide chains. The smaller one is designated as C-protein and the larger one as A-protein.

The cytochrome $c_{o_{2}}$ was further characterized by amino acid analyses. The amino acid composition of the purified protein indicates a minimum $M_r = 93,000$ (Table V). These data are consistent with estimations of protein content by the Lowry method and further suggest that the $M_r$ values obtained for the individual polypeptides are approximately correct. In addition, the two subunits were separated from each other by preparative gel electrophoresis under strongly denaturing conditions. Both proteins were analyzed for their amino acid compositions, and the results are given in Table V. The data were subjected to the minimization procedure of Hoy et al. (47) and the analysis pointed to a molecular weight near 53,500 for the A-protein and 37,500 for the C-protein. These values are consistent with the $M_r$ values from SDS-urea-PAGE. The sum of the compositions of the A- and C-proteins satisfactorily matches that of the intact complex (Table V). Both A- and C-proteins are extremely hydrophobic, with polarity indices equal to ∼0.35, which is similar to the subunit I of yeast cytochrome oxidase (48). Beyond this there appears to be nothing extraordinary about the amino acid compositions of the two proteins.

While the above correlations strongly support the idea that cytochrome $c_{o_{2}}$, is comprised of only two subunits, there remains the possibility that the denaturation conditions are inadequate for these extremely robust proteins, and that electrophoresis is revealing only an intermediate level of disaggregation. Unfortunately, we were unable to detect free NH$_2$-terminal residues in the complex beyond a trace of methionine. Therefore, the composition of the protein was further examined by combined HPLC/AMINO acid analyses. Protein freed of heme A, detergents, and other lipophilic substances by ethanol precipitation (see "General Methods"), was applied to an Altex C$_3$ column. Protein was eluted with 0.1% trifluoroacetic acid and a 40–100% gradient of acetonitrile.

### Table V

| Amino acid | Complex $^a$ | A-Protein $^b$ | C-Protein $^b$ | Sum of A + C |
|------------|-------------|----------------|----------------|--------------|
| mol/mol $c_{o_{2}}$ | Residues/53,500 | Residues/37,500 | |
| Asx | 47.5 | 31 | 23 | 54 |
| Glx | 71.1 | 41 | 1 | 82 |
| Arg | 40.6 | 16 | 16 | 34 |
| His | 20.2 | 7 | 24 |
| Ser | 48.0 | 35 | 14 | 49 |
| Gly | 78.8 | 62 | 37 | 99 |
| Thr | 56.5 | 25 | 12 | 37 |
| Pro | 47.8 | 32 | 27 | 59 |
| Ala | 88.6 | 52 | 34 | 86 |
| Tyr | 35.1 | 26 | 8 | 34 |
| Val | 65.8 | 40 | 30 | 70 |
| Met | 18.2 | 12 | 6 | 18 |
| Ile | 32.7 | 24 | 15 | 39 |
| Leu | 117.0 | 72 | 38 | 110 |
| Phe | 68.1 | 42 | 26 |
| Lys | 25.8 | 14 | 14 | 28 |
| Cys $^c$ | 5 | 4 | 9 |
| Trp | |

$^a$ Heme content measured by pyridine hemochromogen. Amino acids determined by the phenylisocyanamyl method (cf. Footnote 2). Estimated error of these data is ±5%.

$^b$ Molecular weight and residue number were estimated using the minimization procedure of Hoy et al. (47). Hydrolyses were carried out for 24, 48, and 72 h and the results were obtained by extrapolation to time zero.

$^c$ Determined as cysteic acid after hydrolysis for 48 h in the presence of dimethyl sulfoxide.

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**Figure 7.** Reversed phase liquid chromatograms of the subunits of *T. thermophilus* cytochrome *c*$_{o_{2}}$ complex. The experiments were performed with an Altex Ultrapore C$_3$ (methyl) 4.6 × 75 mm column with 10-μm spherical particles having 300-A pores. The solvent system consisted of 0.1% aqueous trifluoroacetic acid versus 0.1% trifluoroacetic acid in a 5:1 mixture of acetonitrile and isopropanol (cf. Ref. 49). The flow rate was 1 ml/min at a gradient rate of 2%/min. Protein samples were prepared as described under "General Methods" and all solutions and buffers were filtered and degassed shortly before use. The solvent gradient is indicated by the dashed line and is offset to reflect conditions at the detector. Absorbance was recorded at 229 nm (upward sloping traces). Excitation of fluorescence was at 273 nm and the emission filter cut off below 370 nm (traces with flat base-lines). AC (lower traces): purified, denatured cytochrome *c*$_{o_{2}}$. The peak near 21 min (~70% organic) is C-protein and the peak near 29 min (~85% organic) is A-protein. C (middle traces): C-protein obtained by preparative gel electrophoresis. A (upper traces): A-protein obtained by preparative gel electrophoresis. The vertical dashed lines indicate the reproducible features of the chromatograms.
trile:isopropanol (3:1) (see Fig. 7, trace AC). Only two peaks of protein were eluted by this procedure: the first appeared between 19 and 20 min at ~70% organic solvent and the other between 28 and 29 min at ~85% organic solvent. Fractions were collected across each peak and analyzed for amino acid compositions. The second peak had the same amino acid composition as purified A-protein and was constant throughout. Also shown in Fig. 7 are elution profiles of isolated A- and C-proteins obtained from preparative SDS-urea-PAGE. While there is evidently some cross-contamination, the major peaks correspond to the assignments made above. (The small peak appearing at ~26 min in the A-protein sample is not a constant feature of the system.) It is possible to recover the C-protein from the organic solvent and show by gel electrophoresis that it migrates with the same RF as original material. By contrast, we were not able to identify the A-protein in this manner, as it seems to have been irreversibly polymerized and would not enter the gel under various denaturing conditions. (Similar behavior was observed after acetone-HCl extraction, cf. Fig. 5f.) The protein is not fully recovered from HPLC columns. For example, no A-protein eluted from C18 at any organic solvent composition and the yields from C8 were rather low. The following recoveries, based on valine analyses, were observed (cf. Fig. 7a): from a 1:1 mixture of A- and C-proteins (Fig. 7Ac), 10.3 and 22.6%, respectively; from purified C-protein (Fig. 7c), 11%; and from purified A-protein (Fig. 7a), 2%. While these recoveries are low, the unique amino acid composition found in each peak proves the identity of the individual component. Therefore, these results provide good support for the idea that the cytochrome c555.549 complex of Thermus is composed of only two subunits.

DISCUSSION

Several growth media for T. thermophilus have previously been described (cf. Ref. 50 and references therein). Most recently, Sonnleitner et al. (50) have carried out an extensive study of T. thermophilus growth kinetics using steady state culture techniques. With one important exception, our results are consistent with theirs: we find that the strain of T. thermophilus obtained from the American Type Culture Collection (No. 27834) definitely requires biotin for growth. In order to demonstrate this requirement, the culture medium was pretreated with avidin, and at least two and occasionally several passages of a culture in liquid minimal medium were required. This suggests that the intracellular concentration of biotin is maintained at a higher level than is necessary for growth, thereby requiring an extended period in a biotin-free medium before the need becomes apparent. The pulse procedure of Sonnleitner et al. (50) may thus not have detected this requirement. The medium described herein is suitable for isotope substitution (cf. Ref. 14) and isolation of auxotrophic mutants of T. thermophilus.  

Growth of T. thermophilus provided by the American Type Culture Collection is supported by a rather limited number of C-sources. For our purposes, the most important observation was the quantitative variation in the type of terminal oxidase expressed by the cells. As evidenced by the relative amplitudes of the 600 nm bands in the reduced minus oxidized spectra of cell extracts (cf. Fig. 2, Miniprint), cells grown aerobically on glutamate produce significant amounts of a-type cytochrome while cells grown aerobically on glucose synthesize very little of this pigment. However, the cells grown on glucose synthesize primarily a CO-sensitive terminal oxidase having optical properties similar to cytochrome o (43). Previous observations (51) showed that quantitative variations also occurred in the C-type cytochromes. While the cause of these alterations is not presently understood, their occurrence is important since the presence of the terminal oxidase may determine the outcome of experiments directed at the kinetic and metabolic growth yields and other bioenergetic considerations (50, 52). As well as the yields of respiratory proteins isolated from the membranes.

Turning now to purification of the respiratory components, we report reasonably efficient procedures for purification of the three known C-type cytochromes present in T. thermophilus (one as a complex with cytochrome aa3). The procedures developed allow cytochromes c552 and c555.549 to be isolated in milligram quantities from kilogram amounts of cell paste. The latter have previously been purified from whole cells by Honnami and Oshima (40, 41) and characterized to varying degrees by Oshima and his collaborators (53, 54).

Cytochrome c552 has a molecular weight near 15,000 with pl = 10.8 (40). We found earlier (51) that the protein resides in the periplasmic space and can readily be removed from whole cells by washing them with 0.2 m Tris-HCl and have taken advantage of this behavior in developing a relatively straightforward method for purifying the protein. Scholes et al. (55) demonstrated that a cytochrome c550 having similar properties could be extracted from Paracoccus denitrificans cells with 0.5 m KCl. The effectiveness of this high salt treatment may be due to electrostatic attachment of the cytochrome to the membrane, as appears to be the case in mitochondria (56). Kinetic studies of cytochrome c oxidation reveal that cytochrome c552 is an effective electron donor to cytochrome c555.549 (39) and may thus serve as a donor to the terminal oxidase under physiological conditions.

Cytochrome c555.549 is a small, acidic protein (M, = 10,000, pl = 4.0) which is clearly associated with the plasma membrane but which has been isolated as a soluble protein (41). We have developed a purification procedure novel in approach compared to that used by Hon-nami and Oshima (41). Application of the cytochrome sample along with other, contaminating membrane proteins in a detergent-rich (Triton X-100) layer to a gel filtration column led to separation of the hydrophilic cytochrome from most of the contaminants. This technique of phase separation of integral membrane proteins using Triton detergents is similar to that developed by Bordier (57), except that it uses a gel filtration column to separate the detergent-rich layer containing the more hydrophobic proteins from the aqueous layer; this may be generally useful in resolving membrane proteins. The function of this cytochrome c in the respiration of Thermus is not known.

Cytochrome c552 or c555.549 proved to be much more difficult to purify than either cytochromes c552 or c555.549. The procedure developed, however, was found to be quite reproducible if one did not deviate far from the recommended conditions. Several of the manipulations presently have no apparent molecular explanation but are important to the overall success of the procedure. (a) The Triton X-100 extract of the membrane must be passed over the DE52 resin at room temperature to obtain efficient binding of cytochrome c552. However, subsequent washing of the resin at 4 °C does not remove the cytochrome. By contrast, if the extract is applied at 4 °C the cytochrome c552 may not bind. (b) Treatment of the combined fractions of cytochrome c552 obtained from the large

7 We have recently succeeded in isolating stable auxotrophs for methionine and histidine (K. L. Findling, and J. A. Fee, unpublished results).

8 T. Yoshida, and J. A. Fee, submitted for publication.
plays in biological function.

The demonstration that the four redox components of the enzyme, and enzymes having very similar properties (17, 59) are able to extrude protons when reconstituted into phospholipid vesicles suggests that many functions of cytochrome oxidases may be localized to a single protein subunit.

During the preparation of this paper, Sone and Yanagita (59) published a purification procedure for a cytochrome $c_{uu3}$ complex from the thermophilic bacillus PS3. The protein had three subunits of 56,000, 38,000 (heme C binding), and 22,000 Da. It was proposed that these subunits correspond, respectively, to subunits I, II, and III of mitochondrial cytochrome oxidase. However, because the PS3 cytochrome $c_{uu3}$ complex was contaminated with cytochrome o (see Fig. 2, Ref. 59), additional work will be needed to determine the differences between the cytochrome oxidase complexes from these bacteria.

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Additional references are found on p. 123.
Supplemental material to: Respiratory Proteins from Thermus thermophilus

A CHEMICALLY DEFINED MEDIUM FOR THE LARGE-SCALE CULTURE OF THERMUS THERMOPHILUS

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The starting cultures of T. thermophilus were grown in American Type Culture Collection medium No. 609 without agar, and aliquots were stored at -70°C in 20% glycerol. Inoculums were made by transferring an aliquot and culturing on the surface at 70°C before transferring to the minimal medium. The growth temperature was 70°C.

The ATCC medium consists of the following (in g liter-1): yeast extract (4 g), polygalacturon (4 g), NaCl (1 g), MnSO4.4H2O (0.5 g) and HEPES (1.02 mM). Glucose (20 g), KH2PO4 (2 g), K2HPO4 (1 g), MgSO4.7H2O (1 g), CaCl2 (0.05 g), and MgCl2.6H2O (0.5 g) were added to 800 ml of HEPES buffer, pH of the medium at 7.0°C. The pH was adjusted to 7.0 at room temperature. After autoclaving, the medium was supplemented with 0.4% yeast extract (final) and stored at 4°C.

Stabilization by filtration was accomplished by feeding the solutions successively through 0.45 and 0.2 micron nitrocellulose filters followed by passage through a 0.2 micron Millipore polysulfone membrane. The last step was necessary to remove conamination, stabilizing which were found in large numbers and certain members of the medium. All pH measurements were carried out at room temperature. Taking into account the temperature coefficient of this buffer, pH of the medium at 70°C is estimated to be 6.2.

Aerobic cultures were grown in 300 ml Erlenmeyer flasks in a New Brunswick shaker bath (Model 205) with vigorous shaking. Medium scale cultures (1 l) were grown in 2 l glass fermenter with gas dispersing tubes for aeration, and sampling tubes for anaerobic sampling, which were adapted for this purpose. Air was introduced by bubbling through 10-20 ml of sterile distilled water at room temperature, it was introduced at a flow rate of 3 cubic feet per minute. The inoculum for the 1 l cultures was 100 ml of a growth medium. 1 l cultures were grown in 250 ml flasks. When growth reached a maximum it was killed by cooling in an ice bath and stored at -20°C in 10% glycerol. Experiments were begun by thawing an aliquot and diluting to 1 l with 0.5 M NaCl to 10 ml. Solutions were concentrated by filtration (see below) and stored at 4°C.

Carbon dioxide was removed by bubbling through a 0.2 micron polycarbonate membrane. The latter was distilled water (with a final volume of 30 ml of distilled water) and the latter was diluted to 1 l. The solution required for the growth of T. thermophilus in the ATCC medium was sterilized by filtration (see below) and stored at 4°C.

Colonies were grown on emulsion: use of antifoam B emulsion resulted in prolonged growth. Other growth media are discussed below. Inclusion of EGTA prevented the inhibition of the growth of T. thermophilus by any of the following substances: acetate, L-glutamine, L-asparagine and L-serine (0.04% yeast extract or lactate required): butyric acid (1.6 mM), glycine (1.6 mM), L-glutamate required): glycerol (1.6 mM), triacetate (0.75 g ml-1), and 0.2% for the artificial medium. Inclusion of EGTA prevented the inhibition of the growth of T. thermophilus on either glutamate or lactate. (see below) and stored at 4°C.

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In Fig. 1, growth curves of T. thermophilus in ATCC medium (left), the chemically defined medium described in the text (right), and the defined medium without 0.4 yeast extract (center). Cultures grown in 1 l flasks.

Fig. 1. Growth curves of T. thermophilus in ATCC medium (left), the chemically defined medium described in the text (right), and the defined medium without 0.4 yeast extract (center). Cultures grown in 1 l flasks.

Fig. 2. Influence of carbon source on the cytochrome composition of T. thermophilus. Reduced and oxidized difference spectra of cytochromes a and b were obtained from cultures grown at glucose (lower panel) and glucose (upper panel). From Ref. 1.

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In our experience, there were few operational problems with large-scale culture of T. thermophilus in the ATCC or chemically supplemented minimal medium. Large-scale growth of the organism on the minimal medium, however, proved more demanding. Occasionally, lower growth rates and difficulties with contaminations were encountered.

The relationship between optical density at 600 nm, viable cell count and dry weight was found to be 1.0: 2 x10^11: 5.1 x10^13 g/cm^3.

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Respiratory proteins from the extremely thermophilic aerobic bacterium, Thermus thermophilus. Purification procedures for cytochromes c552, c555,549, and c1aa3 and chemical evidence for a single subunit cytochrome aa3.

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