Modified, Large-Scale Purification of the Cytochrome *o* Complex (*bo*-Type Oxidase) of *Escherichia coli* Yields a Two Heme/One Copper Terminal Oxidase with High Specific Activity†

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ABSTRACT: The cytochrome *o* complex is a *bo*-type ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli*. This complex has a close structural and functional relationship with the eukaryotic and prokaryotic aa_3-type cytochrome *c* oxidases. The specific activity, subunit composition, and metal content of the purified cytochrome *o* complex are not consistent for different preparative protocols reported in the literature. This paper presents a relatively simple preparation of the enzyme starting with a strain of *Escherichia coli* which overproduces the oxidase. The pure enzyme contains four subunits by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Partial amino acid sequence data confirm the identities of subunit I, II, and III from the SDS–PAGE analysis as the *cyo*B, *cyo*A, and *cyo*C gene products, respectively. A slight modification of the purification protocol yields an oxidase preparation that contains a possible fifth subunit which may be the *cyo*E gene product. The pure four-subunit enzyme contains 2 equivs of iron but only 1 equiv of copper. There is no electron paramagnetic resonance detectable copper in the purified enzyme. Hence, the equivalent of Cu_A of the aa_3-type cytochrome *c* oxidases is absent in this quinol oxidase. There is also no zinc in the purified quinol oxidase. Finally, monoclonal antibodies are reported that interact with subunit II. One of these monoclonals inhibits the quinol oxidase activity of the detergent-solubilized, purified oxidase. Hence, although subunit II does not contain Cu_A and does not interact with cytochrome *c*, it still must have an important function in the *bo*-type ubiquinol oxidase.

The aerobic respiratory chain of *Escherichia coli* contains two terminal oxidases, the cytochrome *o* complex (*bo*-type oxidase) and the cytochrome *d* complex (*bd*-type oxidase)-(Anraku, 1988; Anraku & Gennis, 1987). Each of these enzymes functions as a quinol oxidase and reduces molecular oxygen to water (Minghetti & Gennis, 1988). Under conditions where the oxygen tension is high in the growth medium, the cytochrome *o* complex predominates, whereas the cytochrome *d* complex is present at high levels only when oxygen becomes limited or under anaerobic growth conditions (Kranz & Gennis, 1984; Rice & Hempfling, 1978). These enzymes are of interest not only because they are quinol oxidases but also because electron flow through either enzyme results in the generation of a proton motive force across the membrane. This has been demonstrated with preparations of each of the purified enzymes reconstituted in phospholipid vesicles (Carter & Gennis, 1985; Kita et al., 1982; Matsushita et al., 1984; Miller & Gennis, 1985).

The *bo*-type ubiquinol oxidase is of particular interest because of its structural and functional relationship to the aa_3-type cytochrome *c* oxidases (Chepuri et al., 1990; Gennis, 1991; Saraste, 1990). Despite the fact that the substrates are different (ubiquinol vs cytochrome *c*) and the hemes are different (heme *o* vs heme *a*) (Puustinen & Wikström, 1991), these enzymes appear to be members of a single superfamily of heme/copper oxidases (Gennis, 1991; Saraste, 1990). This is evident from the comparison of the sequences of the subunits of the *bo*-type oxidase with those of the eukaryotic or prokaryotic aa_3-type cytochrome *c* oxidases (Chepuri et al., 1990; Saraste, 1990). The mitochondrial (Chan & Li, 1990; Wikström, 1989) and several prokaryotic cytochrome *c* oxidases (Ludwig, 1987; Solio et al., 1982; Sone & Yanagita, 1984) have been demonstrated to be redox-gated proton pumps, and understanding the mechanisms for both the gating and vectorial proton translocation provides much of the motivation for studying these enzymes. Initial data using purified cytochrome *o* complex reconstituted into proteoliposomes indicated that this ubiquinol oxidase does not catalyze vectorial proton translocation, although the chemistry of oxidation of ubiquinol results in the release of protons into the periplasm (Matsushita et al., 1984). However, more recent work with spheroplasts of *E. coli* has demonstrated vectorial proton pumping in situ by the *bo*-type oxidase, similar to that observed with the cytochrome *c* oxidases (Puustinen et al., 1991, 1989).

EPR[1] studies have indicated that the *bo*-type oxidase contains a heme/copper binuclear center where oxygen is reduced to water, very similar to that of the cytochrome *c*

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxyethyl)aminomethane; TBS, 10 mM Tris-HCl and 160 mM NaCl, pH 8.0; PBS, 10 mM sodium phosphate and 160 mM NaCl, pH 8.0; PMSF, phenylmethanesulfonyl fluoride.

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oxidases (Salerno et al., 1989, 1990). In addition to the copper within the binuclear center (Cu_B), the cytochrome *c* oxidases contain at least one other copper (Cu_A), which is EPR-detectable and is involved in the oxidation of cytochrome *c* (Chan & Li, 1990; Li et al., 1987; Morgan et al., 1989; Stevens et al., 1982). Sequence comparisons and biochemical studies suggest that this copper (Cu_A) is located within subunit II of the cytochrome *c* oxidases (Capaldi et al., 1987; Covello & Gray, 1990; Hall et al., 1988; Holm et al., 1987; Li et al., 1987; Saraste, 1990). It has been proposed that this EPR signal is due to a two-copper center (Kroneck et al., 1990), although this has been disputed (Pan et al., 1991). It has also been suggested that the redox chemistry of Cu_A may gate the proton pumping activity of cytochrome *c* oxidase (Chan & Li, 1990; Gelles et al., 1987; Li et al., 1988; Nilsson et al., 1988). Of interest in this regard is the fact that none of the amino acid residues proposed to be ligands for Cu_A are conserved in subunit II of the *E. coli bo*-type oxidase, which is the homologue of subunit II of the cytochrome *c* oxidases (Chepuri et al., 1990; Saraste, 1990). Since the *bo*-type oxidase has been shown to pump protons (Puustinen et al., 1991), the copper content of the purified oxidase is of particular interest.

Several purification protocols of the cytochrome *o* complex have been reported (Georgiou et al., 1988; Kita et al., 1984; Matsushita et al., 1984; Puustinen et al., 1991). The preparation of Kita et al. (1984) is reported to have two subunits, 2 equivs of tightly-bound copper, a high specific activity (315 μmol of quinol oxidized min^{-1} mg^{-1}), and EPR-detectable copper (Hata et al., 1985). Matsushita et al. (1984) and Georgiou et al. (1988) each describe a four-subunit enzyme, but with relatively low specific activity (both about 88 μmol quinol oxidized min^{-1} mg^{-1}). Puustinen et al. (1991) also purified a four-subunit oxidase, which has only 1 equiv of copper, presumably Cu_B, and no EPR-detectable Cu_A. The specific activity of this preparation is similar to that of Kita et al. (1984). Since tnis preparation is reported to have a substantial cytochrome *b* contaminant (Puustinen & Wikström, 1991), the meaning of the reported Cu/Fe ratio of 0.5 in this preparation is uncertain.

This paper reports a simple protocol for purifying large amounts of the cytochrome *o* complex, starting with a strain of *E. coli* which overproduces the enzyme (Au & Gennis, 1987). The protocol is a substantial improvement over those previously reported and yields very high quality enzyme in amounts suitable for biophysical characterization. The purified enzyme has a high specific activity, four subunits, and only 1 equiv of tightly bound copper. EPR data show no evidence of Cu_A. Subunits I, II, and III from this preparation are shown by partial amino acid sequence data to correspond to the *cyo*B, *cyo*A, and *cyo*C gene products, respectively. Using a modified purification protocol, a possible fifth subunit is also present. Finally, a monoclonal antibody is reported that binds to subunit II and inhibits the quinol oxidase activity of the detergent-solubilized oxidase. Hence, although this subunit does not bind Cu_A, as in the cytochrome *c* oxidases, it probably has an important function in the ubiquinol oxidase.

MATERIALS AND METHODS

Materials. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): alumina WN-3 (neutral), sodium DL-lactate, sodium cholate, phenylmethanesulfonyl fluoride (PMSF), bovine serum albumin (Cohn fraction V), Tween-20, *N*-lauroyl sarcosine (Sarkosyl), egg lysozyme, pancreatic DNase (type 1), 2,3-dimethoxy-5-methyl-1,4-benzoquinone, aminopterin, hypoxanthine, chloramine T, thymidine, sodium dextran, glutamine, barbital, and protein-A Sepharose CL-4B. From Difco (Detroit, MI) the following were purchased: complete and incomplete Freund's adjuvant, beef extract, peptone, and yeast extract. L-Norleucine and the BCA assay system for protein determination were purchased from Pierce Chemical Co. (Rockford, IL). From Calbiochem (La Jolla, CA) were obtained Triton X-100 and pansorbin. DEAE-Sepharose CL-6B and Sephadex G-25 were purchased from Pharmacia (Piscataway, NJ). Bio-Rad (Richmond, CA) was the source for Affi-Gel-10, sodium dodecyl sulfate, Coomassie brilliant blue R-250, DNA-grade hydroxyapatite, and all electrophoresis reagents. Antifoam A was obtained from Dow Corning (Midland, MI). Glycine 2,6,10,14-tetramethylpentadecane, 3-cyclohexylamino-1-propanesulfonic acid (CAPS), dioxane, potassium bisulfate, silver(II) oxide, and 3-methyl-2-buten-1-ol were obtained from Aldrich Chemical Co. (Milwaukee, WI). Kodak Laboratories (Rochester, NY) supplied sodium dithionite and dithioerythritol. Polyethyleneglycol 1450, Dowex 1-X8, Ultrex hydrochloric acid, and Ultrex hydrogen peroxide were purchased from Baker Chemical Co. (Phillipsburg, NJ). Sodium hydroborate was purchased from Ventron Alfa Division (Danvers, MA). Potassium ferricyanide, pyridine, and Florisil were obtained from Fisher Scientific Co. (Pittsburgh, PA). High molecular weight standards, prestained high molecular weight standards, ultrapure agarose, and ultrapure urea were obtained from BRL (Bethesda, MD). Silica gel (0.05–0.2 mm) was obtained from Brinkman Instrument Co. (Westbury, NY). 1-*O*-Octyl-β-D-glucopyranonoside and leupeptin were purchased from Boehringer Mannheim (Indianapolis, IN). Proteases were purchased from either Sigma Chemical Co. or Boehringer-Mannheim. Immobilon (PVDF membrane) was obtained from Millipore. The following were obtained from Hyclone Laboratories (Logan, UT): goat anti-rabbit IgG–horseradish peroxidase conjugate; goat anti-mouse IgG–horseradish peroxidase conjugate; and *o*-phenylenediamine tablets. Protein A from *Staphylococcus aureus* Cowan strain was purchased from Repligen (Cambridge, MA). GIBCO (Grand Island, NY) supplied Earle's balanced salt solution and fetal calf serum (heat inactivated and IgG-free). Iodine-125 (iodide, carrier free) was obtained from Amersham (Arlington Heights, IL).

Strains and Growth Conditions. E. coli RG145 contains a cosmid into which the *cyo* operon has been closed, resulting in a 5-fold increase in oxidase production (Au & Gennis, 1987). The cells were grown aerobically to early logarithmic phase in 200 L at 37 °C with a rich medium (Georgiou et al., 1988). Approximately 600 g of cells was harvested from 200 L of media.

Preparation of Membranes. Cells were harvested by centrifugation and washed once with 10 mM Tris-HCl, pH 8.0. Washed cells (300 g) were resuspended in 750 mL of 10 mM Tris-HCl, pH 8.0, containing 5 mM magnesium sulfate, 20 mg pancreatic DNase (type 1), 1 mM phenylmethanesulfonyl fluoride freshly prepared in ethanol, and 0.5 mg/mL leupeptin. This suspension was passed twice through a French pressure cell (SLM-Aminco, Urbana, IL) at 20 000 psi, and unbroken cells were removed by centrifugation at 10000*g* for 10 mi, 4 °C. The supernatant was then centrifuged at 150000*g* for 1 h to sediment the membrane fraction.

Large-Scale Purification Protocol for the Cytochrome o Complex. All steps were performed at 4–8 °C unless noted otherwise. Membranes prepared as described above were washed with urea and cholate (Matsushita et al., 1983, 1984).

The membranes from 300 g of cells were suspended using a tissue homogenizer in 200 mL of 50 mM potassium phosphate, pH 7.5, and mixed with an equal volume of 10 M urea (60 g in 100 mL of water), which was freshly prepared at room temperature. The urea–membrane solution was mixed for 1 h and then centrifuged at 150000*g* for 1 h. The pellet was suspended with the use of a homogenizer in 200 mL of 50 mM potassium phosphate, pH 7.5. To the suspended membranes was added 200 mL of 12% sodium cholate, pH 7.8. After being thoroughly mixed with a homogenizer, the suspension was centrifuged at 150000*g* for 1 hour, and the pellet was retained. The washed membranes were resuspended in 400 mL of 50 mM potassium phosphate, pH 7.5, and pelleted by centrifugation at 150000*g* for 1 h. The pellet became brown in color after the phosphate wash.

This brown, opaque pellet was resuspended in 400 mL of 50 mM potassium phosphate, pH 7.5, 1% Triton X-100, and 1% octylglucoside. The solution was thoroughly mixed with a homogenizer and centrifuged at 150000*g* for 1 h. The red supernatant was retained and applied to a DEAE-Sepharose CL-6B column. At this stage, the solubilized fraction contained approximately 1.25 mg/mL of protein and was applied to a 300-mL column (4.5 cm × 20 cm) equilibrated with 10 mM Tris-HCl and 0.1% Triton X-100, pH 8.0. After application of the sample, the column was washed with one bed volume of equilibrating buffer. The cytochrome *o* complex was then eluted with 2 L of 10 mM Tris-HCl and 0.1% Triton X-100, pH 8.0, using a linear gradient of 0–300 mM NaCl at a flow rate of 100 mL/h. The cytochrome *o* complex eluted as a single peak at 200–220 mM sodium chloride. Fractions containing ubiquinol-1 oxidase activity were pooled and dialyzed overnight at 4 °C against 10 mM Tris-HCl, 0.1% Triton X-100, and 10 mM EDTA, pH 7.5. The fraction at the center of the peak, comprising about 25% of the total activity units, was characterized in detail. The cytochrome *o* complex was frozen in liquid nitrogen and stored at −80 °C.

Modified Purification Protocol for Obtaining Amino Acid Sequence Data. To avoid potential problems of N-terminal blocking, the preparation was initiated with cytoplasmic membranes instead of whole envelopes and the urea wash step was omitted. Briefly, after cell disruption using the French pressure cell, the membranes from 50 g of cells were suspended in 60 mL of 50 mM potassium phosphate, pH 7.5, followed by a sucrose gradient step. Eight milliliters of membrane suspension was layered on top of 17.5 mL of a 44% sucrose solution (w/w) in screw cap ultracentrifuge tubes. These tubes were centrifuged using a 60 Ti rotor at 100000*g* for 12 h at 4 °C. The cytoplasmic membranes were collected, pelleted, and resuspended in 70 mL of 50 mM potassium phosphate, pH 7.5. The membranes were washed with cholate, omitting the urea wash, and the remainder of the purification was performed as described above. To concentrate the protein following the DEAE column, an aliquot of protein was dialyzed against 50 mM Tris-HCl, pH 7.5, and 1% Triton X-100 to reduce the salt concentration. The dialyzed, pure protein was loaded onto a 2-mL DEAE-Sepharose CL-6B column which had been washed with 20 mL of 50 mM potassium phosphate, pH 7.5, and 1% Triton X-100. After loading the protein, the column was washed with 20 mL of 10 mM Tris-HCl, pH 7.5, and 1% Triton X-100 and eluted with 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, and 300 mM NaCl. All of the protein eluted in about 1.5 mL. This preparation was used for proteolysis experiments.

Electrophoresis, Elution, and Transfer of Protein. Different gel systems were used for the various purposes. For isolation of the subunits by electroelution (Hunkapiller et al., 1983), the system of Schägger and Von Jagow was used (Schägger et al., 1988; Schägger & Von Jagow, 1987) as previously described (Runswick et al., 1989).

When subunits of the cytochrome *o* complex were to be electrophoretically transferred to immobilon, a 15% SDS–PAGE system (Laemmli, 1970) was used as previously described (Matsudaira, 1987). Stained bands were excised with a razor blade and subjected to deblocking procedures prior to sequencing.

A modified gel system containing glycerol and urea (Dispirita et al., 1986; Kadenbach et al., 1983) was also used for analyzing purified cytochrome *o* complex. This system allows clear visualization of the smallest subunit in the purified complex.

Western immunoblotting was performed as described (Burnette, 1981) with the following modifications: 10% γ globulin-free fetal calf serum replaced BSA; Tween-20 replaced Nonidet P-40; and the transfer buffer contained 10 mM Tris, 150 mM glycine, and 0.2 M urea in 10% 1-propanol.

Protein Estimation. Protein concentration was estimated using the BCA Protein Assay Procedure (Pierce Chemical Co.). Bovine serum albumin (BSA, Cohn fraction V) was used as a standard. The concentration of BSA was determined spectrophotometrically using $\epsilon_{280,1\%} = 6.7$ (Foster & Sterman, 1956).

Metal Analysis. The dialyzed, pure protein was analyzed by the University of Illinois School of Chemical Sciences Microanalytical Laboratory using a Perkin-Elmer plasma II with an argon plasma, sequential unit, and dual monochrometers. The metal content of the dialysis buffer was determined as a control.

Protoheme Analysis. The concentration of protoheme in the cytochrome *o* complex was determined by the pyridine hemochromogen method (Fuhrop & Smith, 1985). This technique yields the sum of heme *b* (protoheme IX) plus heme *o* (Puustinen & Wikström, 1991).

Optical Spectroscopy. Absorption spectra (900–300 nm) were recorded at room temperature with a Beckman DU-7 HS scanning spectrophotometer, an Aminco DW2, or a Varian 219 spectrophotometer. Low-temperature spectroscopy was performed at 77 K with the Aminco DW2 low-temperature accessory. Samples were reduced with sodium dithionite and oxidized with either 1 mM potassium ferricyanide or air.

EPR Spectroscopy. EPR spectra were recorded on a Varian E-line Century Series X-band spectrophotometer with a modulation frequency of 100 kHz. Sample temperature was maintained at 77 K by immersion of the sample in liquid nitrogen or at 10 K by a liquid helium cryostat (Air Products or Oxford Instruments). Oxygen was removed from EPR samples by equilibration with argon gas immediately prior to freezing the sample.

EPR Sample Preparation. The Triton X-100 present in the sample following DEAE-Sepharose CL-6B chromatography was partially exchanged for Sarkosyl by dialyzing overnight against 50 mM potassium phosphate and 0.025% Sarkosyl, pH 7.5. The Triton X-100 was more effectively removed by dialysis using two changes of 20 vol of dialysis buffer for 12 h each. Following dialysis, this sample was concentrated at 4 °C to 50–100 μM cytochrome *o* by ultrafiltration using XM-50 or XM-100 membranes under 10 psi argon followed by use of an Amicon Centricon 30 unit.

Measurement of the Rate of Oxygen Consumption. The rate at which the oxidase consumes oxygen was measured with a YSI Model 53 oxygen electrode (Yellow Springs

Table I: Purification of the Cytochrome *o* Complex[a]

step	total *b*-type heme,[b] nmol	total protein, mg	specific heme content,[b] nmol/mg	ubiquinol-1 oxidase sp act.,[c] μmol min^{-1} mg^{-1}	yield (based on heme), %
membranes	1040	800	1.3	3.7	100
washed membranes	444	240	1.85	15	43
solubilization in octylglucoside/Triton X-100	291	55	5.3	109	28
DEAE-Sepharose	270	15	18.1	500	26

[a] Starting with 50 g wet weight of cells. The protocol described in the text is scaled up to 300-g cells. [b] Sum of heme *b* plus heme *o* (Puustinen & Wikström, 1991) calculated from pyridine hemochromogen. [c] These values were obtained at 25 °C. At 37 °C the purified oxidase has a specific activity which is approximately double the value shown. The total units of activity increase during the preparation due to the sensitivity of the assay to the lipids and detergents present.

Instrument Co., Yellow Springs, OH). Measurements were made at 25 or 37 °C in 50 mM potassium phosphate, pH 7.5. When purified oxidase was assayed, either 0.025% Tween-20 or 0.05% Sarkosyl was added to the buffer. Ubiquinone-1 was used at a concentration of 50–100 μM (ϵ_{275} = 12.25 mM^{-1} cm^{-1} in ethanol) (Redfearn, 1967) unless specified. Ubiquinone-1 was kept reduced by dithioerythritol which was present in the assay solution at a concentration of 2 mM. For spectroscopic assays, ubiquinone-1 was reduced by the method of Hatefi and Galante (1980). Ubiquinone-1 was synthesized following a procedure provided by Dr. Chang-An Yu (University of Oklahoma, personal communication).

Protease Digestion Experiments. Purified subunit II, obtained by electroelution and precipitation, was incubated with *Staphyloccoccus aureus* V8 protease at a 1:120 (w/w) ratio for 20 min at room temperature. The reaction was stopped by addition of 1 mM PMSF and transferred to ice. Protease digestion experiments using trypsin, chymotrypsin, and subtilisin were tested at ratios from 1:10 to 1:1000 as described by Gonzalez-Halphen et al. (1988).

Removal of Amino-Terminal Blocking Group. The amino-terminal blocking group was removed from two of the subunits by two different procedures. In the first procedure, a strip of immobilon containing the protein of interest was incubated in 280 μL of methanol and 20 μL of concentrated HCl for 24 h at room temperature. In the second protocol, the immobilon strip was soaked in 50% formic acid at 42 °C for 24 h. The samples were then submitted for protein sequencing by the Biotechnology Center, University of Illinois–Urbana using an Applied Biosystems gas-phase sequencer.

Monoclonal Antibody Production. Balb/c mice were immunized intraperitoneally with 200 μg of purified cytochrome *o* complex emulsified in complete Freund's adjuvant. Mice were boosted with 50 μg of cytochrome *o* in incomplete Freund's adjuvant one month later. Two weeks later a second booster was administered with 50 μg of cytochrome *o* in incomplete Freund's adjuvant. Four days following this booster, lymphocytes from the spleen were harvested for cell fusion. Procedures detailing cell fusion and cloning have been described previously (Galfre & Milstein, 1981).

Purification and Characterization of Monoclonal Antibodies. Ascites fluid was collected from each mouse. Red blood cells and fibroblasts were removed by centrifugation, and the serum was purified with sodium dextran and ammonium sulfate as described (Watt et al., 1980). Antibodies were then retained on a Protein A–Sepharose CL-4B column in 10 mM Tris-HCl and 160 mM NaCl, pH 8.0 (TBS), and eluted with 0.23 M glycine, pH 2.6. Purified antibodies were dialyzed against 10 mM sodium phosphate and 160 mM NaCl, pH 8.0 (PBS), overnight at 5 °C. The antibodies were stored at −80 °C in 0.002% azide.

Monoclonal anti-cytochrome *o* antibodies were characterized by a solid-phase radioimmunoassay using Dynatech immulon wells containing 1 μg of purified cytochrome *o* which had been dried. Monoclonal antibody classes and subclasses were determined by using a Mouse Monoclonal Sub-Isotyping Kit (HyClone, Logan, UT).

Inhibition Studies. Cytochrome *o* complex (about 5 μg) was added to various amounts of antibody in a total volume of 200 μL of PBS, pH 8.0. The antibody–antigen solutions were incubated overnight at 5 °C in the presence of 0.025% Tween-20. Enzyme activity was measured with an oxygen electrode as described above.

Precipitation of the Antibody–Antigen Complex. To precipitate the antibody–antigen complex, 50 μL of pansorbin in TBS, pH 8.0, was added and incubated for 1 h at room temperature. The antibody–antigen complex was pelleted in an Eppendorf centrifuge for 30 s. The supernatant was carefully removed and assayed for ubiquinol-1 oxidase activity.

RESULTS

The preparative protocol described in this work is designed to facilitate the purification of large amounts of the cytochrome *o* complex and takes advantage of a strain which overproduces the enzyme. The protocol combines aspects of two published protocols (Kita et al., 1984; Matsushita et al., 1984). The results of the purification are summarized in Table I. From 50 g wet weight of cells, one can obtain 15 mg of highly purified oxidase from the DEAE-Sepharose column. The remaining oxidase from the wings of the peak is about 80% pure and can be further purified by repeating the DEAE chromatography. The preparation has been routinely performed starting with 300 g of cells to yield as much as 200 mg of the purified cytochrome *o* complex. Some characteristics of the preparation are described below.

(*1*) *Subunit Composition.* As with all previous preparations of the cytochrome *o* complex (Georgiou et al., 1988; Matsushita et al., 1984; Puustinen et al., 1991), with the exception of the protocol described by Kita et al. (1984), this preparation contains four subunits by SDS–PAGE analysis. Figure 1 shows that the SDS–PAGE pattern of the purified oxidase is virtually identical for the enzyme prepared using this protocol or using the protocol previously described by Georgiou et al. (1988). Interestingly, in some preparations, a minor band is present in the SDS–PAGE gels just below subunit II. This band has an apparent molecular weight of about 29K. In modified preparations (see Methods section) in which the cytoplasmic membranes are isolated and the urea wash is omitted, this "impurity" is present to the same extent as the authentic subunits of the oxidase. This band is shown in Figure 2 and is referred to as band IIa. Attempts to directly sequence the protein present in band IIa (Figure 2), after elution of the protein from the gel or transfer to immobilon, were unsuccessful.

The presence of a fifth subunit in a preparation of purified cytochrome *o* complex is of interest because the *cyo* operon

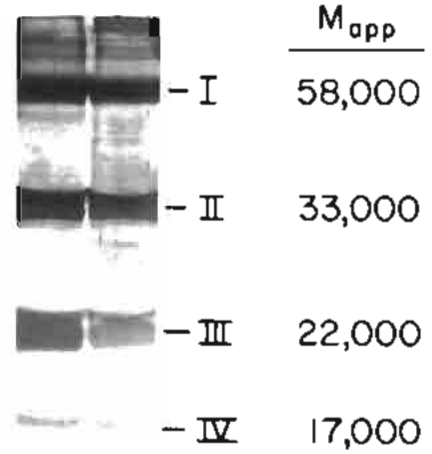

FIGURE 1: SDS–PAGE analysis of preparations of the cytochrome *o* complex (*bo*-type oxidase). Lane 1 contains 40 μg of protein purified using the protocol described in this work. Lane 2 contains an equal amount of protein isolated using the protocol of Georgiou et al. (1988). Often subunit II is observed as a doublet, but this is not resolved in the gel which is shown.

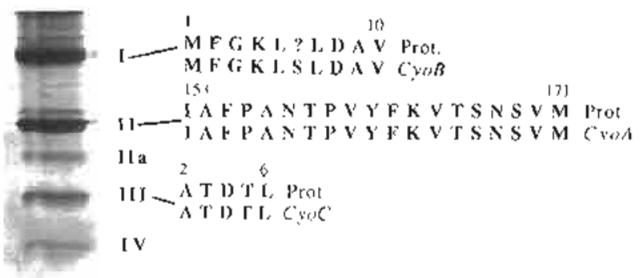

FIGURE 2: Correspondence of protein sequence and sequences deduced from genes in the *cyo* operon (Chepuri et al., 1990). SDS–PAGE of the cytochrome *o* complex prepared by a modified protocol in which purified cytoplasmic membranes rather than crude membranes were solubilized and the urea wash was omitted. There are more contaminants in the preparation, but the presence of band IIa is clearly shown. The lane contained 20 μg of protein and is Coomassie-stained. Protein sequences obtained from subunits I–III are shown in relation to the sequence deduced from the *cyo*B, *cyo*A, and *cyo*C genes, respectively (Chepuri et al., 1990).

has been shown to encode five open reading frames (Chepuri et al., 1990). The gene products of the first three of these open reading frames have been correlated with subunits of the purified protein by the work presented in this paper in the work from the Anraku laboratory (Mogi & Anraku, 1990). The calculated molecular weights of the remaining two open reading frames, *cyo*D and *cyo*E, correspond to the apparent molecular weights of the remaining two subunits. The deduced molecular weight of the open reading frame encoding *cyo*E is 32K, which correlates well with the molecular weight of band IIa. Although the role of the fifth open reading frame (*cyo*E) is uncertain, deletion of *cyo*E from the operon has been shown to prevent the formation of a functional oxidase (unpublished data). Even though no direct evidence is present to demonstrate that band IIa is part of the cytochrome *o* complex, it seems likely that it is the gene product of the *cyo*E open reading frame. Independent studies by Mogi and Anraku (1990) have led to the same conclusion.

(*2*) *Correspondence between Subunits and Genes.* Previous subcloning and immunological work demonstrated that *cyo*A and *cyo*B encode subunits II and I, respectively (Nakamura et al., 1990). Attempts were made to obtain partial protein sequence from each of the subunits following resolution by SDS–PAGE. Figure 2 shows the data obtained from subunits I–III. The data confirm the previous assignments of *cyo*A and *cyo*B and, in addition, show that subunit III is the *cyo*C gene product.

(*a*) *Subunit I.* Data were obtained after protein transfer to immobilon. Sequence was obtained only after the strip containing the subunit was soaked in methanolic HCl for 24 h. The N-terminus includes the initial methionine and corresponds to *cyo*B. Efforts to obtain internal sequence data using various proteases or CNBr were unsuccessful.

(*b*) *Subunit II.* All efforts to obtain N-terminal sequence were unsuccessful, so it is likely that the N-terminus is blocked, as in the case of subunit II of the aa_3-type cytochrome *c* oxidase from *Paracoccus denitrificans* (Steinrucke et al., 1987). The possibility that this protein has a cleaved N-terminal signal sequence (Chepuri et al., 1990) could not be evaluated. Internal sequence data were obtained by electroeluting the subunit following SDS–PAGE and digesting with *S. aureus* protease V8 (20 min at 1:120 ratio). Two major fragments resulted, but only one sequence was found upon sequencing the mixture. This sequence corresponds to residues 153–172 (Figure 2).

(*c*) *Subunit III.* Many attempts to obtain sequence data from subunit III were unsuccessful, but partial N-terminal sequence data were obtained by soaking the immobilon strip in 50% formic acid for 24 h at 42 °C. The five residues correspond to positions 2–6 in *cyo*C (Chepuri et al., 1990).

(*d*) *Subunit IV and Band IIa.* Efforts to obtain sequence data were unsuccessful.

(*3*) *Copper Content.* Table II shows the heme and metal content of the purified oxidase. The pyridine hemochromogen determination of protoheme IX indicates 18.1 nmol/mg. This correlates well with the iron content, 19.5 nmol/mg, and is similar to the values obtained from previous preparations (Kita et al., 1984; Matsushita et al., 1984; Puustinen et al., 1991). The copper content is 7.5 nmol/mg, approximately half of the value for iron. These data indicate that the purified oxidase contains 2 equivs of heme iron but only one of copper per mole of enzyme. This is in contrast to Kita et al. (1984) who measured 2 mol of copper/mol of enzyme, but is consistent with the data obtained using the preparation of Puustinen et al. (1991). However, in the latter preparation the subsequent finding of a substantial contaminating cytochrome *b* (Puustinen & Wikström, 1991) makes the interpretation of the data problematic. Since all of the specific activity, heme, and metal content determinations in the current work were carried out on the same preparations following extensive dialysis in the presence of EDTA, it can be definitely concluded that highly

Table II: Properties of the Purified Cytochrome *o* Complex[a]

specific heme content,[b] nmol/mg	18.1
Fe content, nmol/mg	19.5
Cu content, nmol/mg	7.5
Cu/Fe ratio	0.38
$\epsilon_{560-580}$ (red–ox)/mol of complex,[c] mM^{-1} cm^{-1}	20.5
ubiquinol-1 sp act. (37 °C),[d] μmol min^{-1} mg^{-1}	960

[a] The data are averaged for four preparations, with a variance of less than 10%. [b] Determined by pyridine hemochromagen technique (Fuhrop & Smith, 1985). The pyridine hemochrome absorbance peak is at 553 nm rather than the expected 556 nm, due to the presence of heme *o*, a modified protoheme IX (Puustinen & Wikström, 1991). [c] The amount of cytochrome *o* complex was determined by assuming that each mole of complex contains 2 mol of heme. [d] Determined by using an oxygen electrode as described in the text.

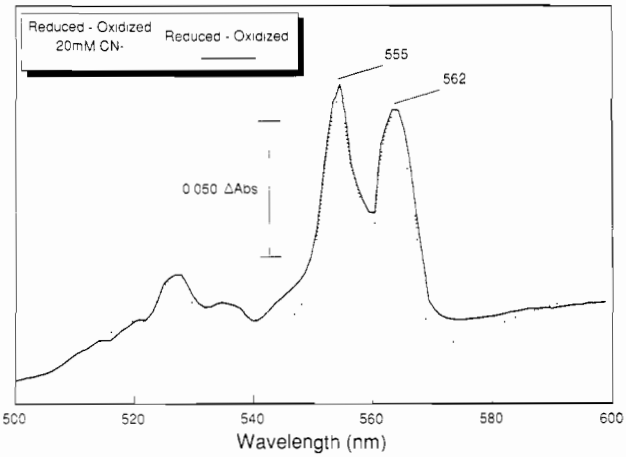

FIGURE 3: Low-temperature (77 K) reduced-*minus*-oxidized spectrum of the purified *bo*-type oxidase in the absence or presence of 20 mM cyanide. The enzyme was reduced by 6 mM ascorbate plus 0.3 mM TMPD and was incubated for 90 min at room temperature in a sealed tube prior to recording the spectrum. The changes in the α-region are nearly all due to the reduction of the heme component that does not bind to cyanide. It is presumed that cyanide will prevent the reduction of the high spin heme component of the oxidase (Salerno et al., 1990).

active oxidase contains only one copper atom. Presumably, this corresponds to the copper within the binuclear center (Salerno et al., 1989, 1990).

(*4*) *Optical and EPR Spectroscopy*. EPR spectra of membranes containing a high content of cytochrome *o* (Salerno et al., 1990) and of purified preparations (Hata et al., 1985; Puustinen et al., 1991) have been published previously. The low-temperature (77 K) EPR spectra obtained using this new preparative protocol are similar (not shown) and show no evidence for the EPR-detectable Cu_A center which is characteristic of the aa_3-type cytochrome *c* oxidases (Blair et al., 1983; Chan & Li, 1990; Kroneck et al., 1990; Stevens et al., 1982). This is not surprising, since there is only one copper, presumably the equivalent of Cu_B in the binuclear center (Salerno et al., 1989, 1990) and since the putative ligands for Cu_A within subunit II are not conserved in the *E. coli* sequence (Chepuri et al., 1990; Saraste, 1990). The optical absorbance band at 820–840 nm, another characteristic of Cu_A (Beinert et al., 1980; Boelens & Wever, 1980; Eglinton et al., 1980), is also absent in the purified *E. coli* oxidase (not shown).

Figure 3 shows the low-temperature (77 K) reduced-*minus*-oxidized difference spectrum of the cytochrome *o* complex taken in the presence of cyanide. These data show that when the high-spin cytochrome component (Salerno et al., 1989, 1990) is maintained in the oxidized state by cyanide ligation, the reduction of the low-spin center accounts for virtually all of the absorption changes observed in the α-region of the spectrum (around 560 nm). Hence, the low-spin cytochrome *o* component of the oxidase, referred to as cytochrome b_{562}, must have a split α-band with peaks at around 555 and 562 nm. The high-spin cytochrome component, previously called cytochrome b_{555} or *o*, contributes little to the reduced-*minus*-oxidized spectrum in the visible region. In the presence of cyanide, the difference spectrum in the Soret is reduced in intensity and is narrower. This situation is similar to the relative spectroscopic contributions of the analogous low-spin cytochrome *a* and high-spin cytochrome a_3 components of the aa_3-type cytochrome *c* oxidases (Wikström et al., 1981, 1976). The calculated extinction coefficient (Table II) of 20.5 mM^{-1} cm^{-1} is typical for protoheme-containing cytochromes, consistent with the interpretation that only one of the two hemes in the complex is responsible for the absorption changes in the α-region which are observed upon reduction. Similar data have been presented using the cytochrome *o* complex purified by Puustinen et al. (1991).

Table III: Summary of Properties of Monoclonal Antibodies against the Cytochrome *o* Complex

antibody	isotype	subunit recognized by immunoblotting	inhbn of ubiquinol oxidase activity	precptn of native oxidase with pansorbin
A	IgG_3	none[a]	yes	yes
B	IgG_3	none[a]	no	yes
D	IgG_3	none[a]	no	no
E	IgG_3	II	no	no
F	IgG_3	II	no	yes
G	IgA	II	yes	yes
I	IgG_3	II	no	no
J	IgG_{2b}	II	no	no

[a] The lack of Western blotting suggests that the epitope recognized by these antibodies are destroyed by SDS denaturation.

The preparation does not contain the low potential cytochrome component which was reported by Puustinin and Wikström (1991). The addition of excess ascorbate (2 mM) and *N,N,N′,N′*-tetramethylphenylenediamine (TMPD) (0.4 mM) to a sample of the oxidase which had been deoxygenated resulted in full reduction of the hemes within 10 min. The spectrum is identical to that obtained by using dithionite as reductant. However, if ascorbate plus TMPD are added to the enzyme in the presence of oxygen, the preparation exhibits kinetic heterogeneity. Upon depletion of oxygen in the solution, only a portion of the cytochromes are rapidly reduced whereas the remaining portion, in some samples, took several hours to fully reduce. The cause of this kinetic heterogeneity is not known, but it is certain that it is not due to a low potential cytochrome contaminant in the preparation.

(*5*) *Monoclonal Antibodies*. Eight monoclonal antibodies were obtained, and their properties are summarized in Table III. Five of the eight monoclonals immunoblot to subunit II (Figure 4) and of these, only two (F and G) recognize epitopes in the native detergent-solubilized oxidase. Of these two, only monoclonal G inhibits the ubiquinol-1 oxidase activity of cytochrome *o* complex. These data are shown in Figure 5. No inhibition is observed if the cytochrome *o* complex is incorporated into proteoliposomes. The inhibition data, however, demonstrate that although the role of subunit II in the quinol oxidase must be different from its presumed role in the cytochrome *c* oxidases, i.e., interacting with cytochrome *c* and binding to Cu_A (Bisson et al., 1982; Capaldi et al., 1987; Covello & Gray, 1990; Hall et al., 1988; Holm et al., 1987), it still must play some critical function in the cytochrome *o* complex.

DISCUSSION

This paper describes a practical, reliable protocol for preparing large quantities of the four-subunit cytochrome *o* complex with a high specific activity. This will facilitate biophysical studies which often require large quantities of pure protein. The characterization of the enzyme helps to clarify several points that have been either unclear or in dispute in previous reports (Georgiou et al., 1988; Kita et al., 1984; Matsushita et al., 1984; Puustinen et al., 1991).

(1) The enzyme contains four subunits, as do cytochrome *o* preparations from other organisms (Dokter et al., 1990; Georgiou & Webster, 1987; Matsushita et al., 1987; Schrattenholz et al., 1989; Sone et al., 1990a) as well as the similar aa_3-type cytochrome *c* oxidase from *Bacillus* PS3 (Sone et

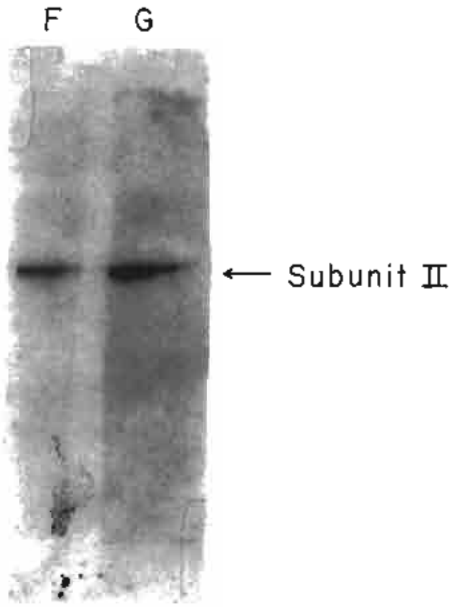

FIGURE 4: Western immunoblotting of purified cytochrome *o* complex with monoclonal antibodies F and G. Each lane contained 40 μg of the purified oxidase. The monoclonal antibodies were affinity purified with Protein A–Sepharose, and the antigen–antibody complexes were visualized by [^{125}I]-labeled protein A. Results show that both monoclonals F and G bind to subunit II.

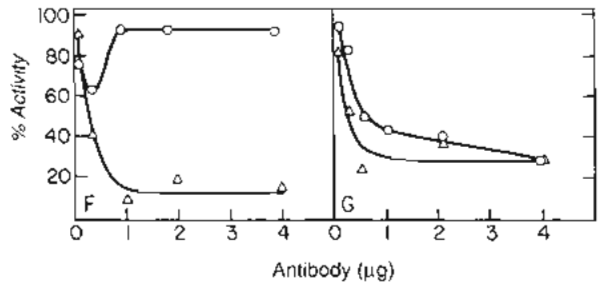

FIGURE 5: Binding and inhibition studies of monoclonal antibodies F and G to the purified cytochrome *o* complex in detergent. Cytochrome *o* complex (5 μg) was incubated with the antibody in 200 μL of PBS overnight at 5 °C. The sample was divided in half, and one portion was used to assay for ubiquinol-1 oxidase activity. To the second portion, 50 μL of pansorbin was added, the mixture was incubated for 1 h, and then the supernatant was assayed for ubiquinol-1 oxidase activity after centrifugation. Activity is expressed as a percent relative to a control which contained no antiserum or which contained an antiserum against the cytochrome *d* complex (Kranz & Gennis, 1984). The letters in each panel refer to the monoclonals used in the experiments (see Table III). Activity remaining after precipitation is shown by the triangles, and activity prior to precipitation is shown by circles.

al., 1990b). However, the strong possibility remains that the *cyo*E gene encodes a fifth subunit that may correspond to band IIa in Figure 2. A small amount of this band (IIa) is often observed using the large-scale preparative protocol described, but it is present to a much greater extent if cytoplasmic membranes are purified rather than washing crude membranes with urea. A five-subunit preparation of the cytochrome *o* complex has also been reported by Mogi and Anraku (1990).

The *cyo*E gene product is homologous to a yeast nuclear gene, COX10, which is necessary for the assembly of mitochondrial cytochrome *c* oxidase (Nobrega et al., 1990). Both COX10 and *cyo*E are homologous to the ORF1 gene in the operon encoding subunits of the aa_3-type cytochrome *c* oxidase from *P. denitrificans* (Raitio et al., 1987) and in other bacteria (Saraste, 1990). Whatever the function of the *cyo*E gene product, it appears to be a loosely associated subunit of the ubiquinol oxidase and is not essential for catalytic function in vitro.

(2) The purified *bo*-type ubiquinol oxidase contains only one copper, and there is no spectroscopic evidence for Cu_A. Hence, the redox gating mechanism for the proton pumping activity of the *bo*-type oxidase (Puustinen et al., 1991, 1989) cannot involve Cu_A, as hypothesized for cytochrome *c* oxidases (Chan & Li, 1990; Gelles et al., 1987). The *bo*-type oxidase also does not contain any equivalent of Cu_x or any Zn, which are both associated with cytochrome *c* oxidase preparations (Bombelka et al., 1986; Pan et al., 1991; Steffens et al., 1987; Yewey & Caughey, 1987).

(3) The two cytochrome components of the *bo*-type oxidase do not contribute equally to the absorption peak in the α-region of the reduced-*minus*-oxidized spectrum. The low-spin cytochrome center, which does not bind to cyanide, is responsible for nearly all of the absorbance, and more importantly, for both of the observed peaks at 555 and 562 nm. This is clearly shown for the first time in the low-temperature difference spectrum in Figure 3. Hence, claims that the component that absorbs at 562 nm (or 564 nm) is the CO or oxygen-binding high-spin component are not correct (Bolgiano et al., 1991; Withers & Bragg, 1990). The lack of absorption change in this region of the spectrum upon reduction of a high-spin cytochrome is to be expected and is similar to the situation with the analogous cytochrome a_3 (Wikström et al., 1981, 1976). More importantly, this means that the presence of the spectroscopic feature near 562 nm in the reduced-*minus*-oxidized spectrum is diagnostic of the low spin cytochrome center, which is presumably directly involved in the oxidation of ubiquinol. This has been confirmed by site-directed mutagenesis studies (Lemieux et al., 1992). It is interesting to note that the cytochrome component of the bc_1 complex, which is at the ubiquinol oxidase site (cytochrome b_L), also has a split α-band (Meinhardt & Crofts, 1983). The confusing potentiometric behavior of the purified cytochrome *o* complex (Bolgiano et al., 1991) is apparently complicated by heme–heme interactions (Salerno et al., 1989, 1990) and requires further analysis.

(4) Since the *bo*-type oxidase does not interact with cytochrome *c* and does not contain Cu_A, the role of subunit II in the ubiquinol oxidase is not as clear as in the cytochrome *c* oxidases. The monoclonal antibody data, however, suggest that this subunit must still be important in the function of the cytochrome *o* complex, though the nature of its role will require further experimentation.

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