Articles

Large-Scale Purification and Characterization of a Highly Active Four-Subunit Cytochrome bc_1 Complex from *Rhodobacter sphaeroides*†

Katherine M. Andrews,‡,∥ Antony R. Crofts,§ and Robert B. Gennis*,∥

Departments of Biochemistry and of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801

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ABSTRACT: A highly active, large-scale preparation of ubiquinol:cytochrome c_2 oxidoreductase (EC 1.10.2.2; cytochrome bc_1 complex) has been obtained from *Rhodobacter sphaeroides*. The enyzme was solubilized from chromatophores by using dodecyl maltoside in the presence of glycerol and was purified by anion-exchange and gel filtration chromatography. The procedure yields 35 mg of pure bc_1 complex from 4.5 g of membrane protein, and it consistently results in an enzyme preparation that catalyzes the reduction of horse heart cytochrome *c* with a turnover of 250–350 (μmol of cyt *c* reduced)·(μmol of cyt c_1)$^{-1}$·s^{-1}. The turnover number is at least double that of the best preparation reported in the literature [Ljungdahl, P. O., Pennoyer, J. D., Robertson, D. C., & Trumpower, B. L. (1987) *Biochim. Biophys. Acta 891*, 227–241]. The scale is increased 25-fold, and the yield is markedly improved by using this protocol. Four polypeptide subunits were observed by SDS–PAGE, with M_r values of 40K, 34K, 24K, and 14K. N-Terminal amino acid sequences were obtained for cytochrome c_1, the iron–sulfur protein subunit, and for cytochrome *b* and were identical with the expected protein sequences deduced from the DNA sequence of the *fbc* operon, with the exceptions that a 22-residue fragment is processed off of the N-terminus of cytochrome c_1 and the N-terminal methionine residue is cleaved off both the *b* cytochrome and iron–sulfur protein subunits. Western blotting experiments indicate that subunit IV is not a contaminating light-harvesting complex polypeptide. The pure enzyme was characterized by a variety of biochemical and biophysical methods, and values for ubiquinol, phospholipid, and iron content were determined. The quinol:cytochrome *c* reductase activity was stimulated by dodecyl maltoside and soybean lecithin and was inhibited in the presence of Triton X-100. Full visible spectrum redox titrations showed the presence of two thermodynamically distinct *b* hemes and cytochrome c_1 with spectral properties and E_m values similar to those found in chromatophores. Midpoint potentials of +280 and +325 mV were observed for the Rieske iron–sulfur cluster in the absence and presence of 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole, respectively, by use of EPR spectroscopy. The E_{m7} for the Rieske cluster was shifted to a value greater than +450 mV when stigmatellin was added. The redox properties of the antimycin-sensitive ubisemiquinone species were also studied by EPR spectroscopy, and a pH dependence of −53 mV/pH unit was determined for the E_m of the bound ubiquinol/ubiquinone couple.

Ubiquinol:cytochrome *c* oxidoreductase complexes (cytochromes bc_1) function as essential components of both respiratory and photosynthetic electron transport chains. Cytochrome bc_1 complexes have been isolated from a variety of sources, including yeast (Katan et al., 1976; Siedow et al., 1978) and bovine heart mitochondria (Hatefi et al., 1962; Rieske et al., 1964; Engel et al., 1980), *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* (Gabellini et al., 1982;[1] Yu, 1982; Takamiya et al., 1982; Ljungdahl et al., 1987), *Paracoccus denitrificans* (Yang & Trumpower, 1986), and thermophilic *Bacillus* PS3 (Kutoh & Sone, 1988). Although their polypeptide compositions vary from three to twelve subunits, all of the cytochrome bc_1 complexes contain one *c*-type cytochrome, a two-heme *b*-type cytochrome, and one iron–sulfur cluster. They share varying amounts of sequence homology in their prosthetic group containing subunits (Hauska et al., 1988), and they carry out electron transfer from dihydroquinol to cytochrome *c* by a common mechanism, termed the proton-motive Q-cycle (Mitchell, 1976; Hauska et al., 1983; Rich & Heathcote, 1983; Crofts, 1986b).

The photosynthetic bacterium *Rb.*[2] *sphaeroides* serves as an excellent model system for studying the relationships of structure and function with electron-transfer reactions in the cytochrome bc_1 complex. The kinetics of electron transfer can be rapidly initiated by illumination and have been extensively

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* Correspondence should be addressed to this author.

‡ Present address: Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110.

∥ Department of Biochemistry.

§ Department of Physiology and Biophysics.

[1] The *Rhodobacter* species used by Gabellini et al. (1982) for the purification of the bc_1 couple was originally reported as *Rb. sphaeroides*. The *fbc* operon was cloned and sequenced (Gabellini & Sebald, 1986). However, subsequent work by Davidson and Daldal (1987), comparing DNA sequences, showed that Gabellini et al. had actually employed a green derivative of *Rb. capsulatus* in her work.

[2] Abbreviations: E_m, oxidation–reduction midpoint potential; E_h, ambient redox potential; EPR, electron paramagnetic resonance; $Q^{\cdot -}$, ubisemiquinone anion; Q, ubiquinone; QH_2, ubiquinol; Q_i, Q at quinone reductase site; Q_o, Q at quinol oxidase site; QCR, quinol:cytochrome *c* oxidoreducase; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzohydroquinone; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PC, phosphatidylcholine; PMSF, phenylmethanesulfonyl fluoride; *Rb.*, *Rhodobacter*; cyt, cytochrome.

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characterized (Crofts, 1986a). The *Rb. sphaeroides* enzyme has a relatively simple polypeptide composition compared to its mitochondrial counterpart, which greatly simplifies studies of assembly, topology, and molecular architecture of the catalytic sites, as well as simplifying computer modeling. Finally, the genetics of *Rb. sphaeroides* have been well characterized (Kiley & Kaplan, 1988), and the genes encoding the *fbc* operon have been cloned, sequenced, and manipulated on plasmids (C.-H. Yun, R. Beci, A. Crofts, S. Kaplan, and R. B. Gennis, unpublished results), facilitating a genetic approach to the problem of structure and function.

Several procedures have been published for purification of the cytochrome bc_1 complex from *Rb. sphaeroides* or *Rb. capsulatus* employing various detergents for solubilization, followed by sucrose gradients and/or salt precipitation steps (Gabellini et al., 1982; Yu & Yu, 1982). Some procedures are not suitable for scaling up, and the resulting preparations are often characterized by low catalytic activities. Recently, Ljungdahl et al. (1987), published a purification procedure utilizing the nonionic detergent dodecyl maltoside for solubilization and subsequent purification of the bc_1 complex from a variety of sources, including *Rb. sphaeroides*. The procedure yields a pure enzyme which possesses a turnover number greater than 100 (μmol of cyt *c* reduced)·(μmol of cyt c_1)$^{-1}$·s^{-1}. While this procedure represents a vast improvement over those previously published, it was reported only on a small scale, yielding about 1 mg of purified protein. Furthermore, greater than 90% of the total activity was lost during the purification, predominantly in the first two steps. In order to approach many biochemical and biophysical questions, it is desirable to develop a reliable purification procedure, performed on a preparative scale, which results in pure enzyme with high activity, and with biochemical and biophysical properties identical with those of the intact cytochrome bc_1 complex in chromatophores. The purification protocol described here is scaled up approximately 25-fold over that reported by Ljungdahl et al. (1987) and has a greatly improved yield and high specific activity, possessing a turnover number of 250–350 (μmol of cyt *c* reduced)·(μmol of cyt c_1)$^{-1}$·s^{-1}. The pure enzyme has been characterized by a variety of biochemical and biophysical methods and behaves similarly to the bc_1 complex in chromatophores.

Experimental Procedures

Dodecyl maltoside obtained from either Calbiochem or Boehringer-Mannheim was used with equal success. Bio-Rad was the supplier for DEAE-Bio-Gel A and all SDS–PAGE reagents. DEAE-Sepharose CL-6B and Sepharose CL-4B were obtained from Pharmacia. Bovine serum albumin, horse heart cytochrome *c*, soybean lecithin, and antimycin A were obtained from Sigma. UHDBT was obtained from Dr. Bernard L. Trumpower, Dartmouth Medical School, Hanover, NH. Stigmatellin was a generous gift of Dr. G. Höfle, Gesellschaft für Biotechnologische Forschung mbH, Abteilung Naturstoffchemie. The substrate 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DBQ) was prepared as described (Wan et al., 1975), from undecanoyl chloride and 2,3-dimethoxy-5-methyl-1,4-benzoquinone obtained from Fluka. The reduced form of substrate 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzohydroquinone (DBH) was prepared and stored as described (Trumpower & Edwards, 1979).

Determination of protein content was done by the method of Lowry et al. (1951), modified such that sample solutions contained 1% (w/v) SDS, and using bovine serum albumin as standard. Quinol:cytochrome *c* reductase and cytochrome *c* oxidase activities were assayed as described by Berry and Trumpower (1985). The reductase assay mixture was modified to contain 40 μg/mL dodecyl maltoside and in some cases phospholipid. Analytical electrophoresis was done according to Laemmli (1970). The best results were obtained with gels containing 7% (v/v) glycerol and 15% acrylamide. Samples contained 0.5% 2-mercaptoethanol and were incubated at temperatures no higher than 30 °C. Preparative SDS–PAGE was as described (Schagger et al., 1986). Protein bands were electroeluted, lyophilized, and resuspended in 10% formic acid prior to sequencing. N-Terminal peptide sequencing was done in the University of Illinois Biotechnology Center, and by Dr. Chang-An Yu, Oklahoma State University, Stillwater, OK, using the automated Edman degradation method (Edman & Begg, 1967). Lipids were extracted for phospholipid analysis with 2:2:1 (v/v) chloroform/methanol/water. Phosphate content was determined after wet-ashing by the procedure of Joworowski et al. (1981). Extraction and quantitation of quinone from the purified enzyme were done by the method of Kroger (1978). Metal analysis was performed by the Illinois State Water Survey using atomic absorption spectroscopy.

Optical absorption spectra were obtained by using a home-built scanning single-beam spectrophotometer (Meinhardt, 1984) interfaced to a Zenith Z-100 microcomputer. Data acquisition and manipulation software was written by Dr. Edward Berry. Cytochrome *b* concentrations in solubilized membranes were determined by using an extinction coefficient of 28 mM^{-1} cm^{-1} at 560–574 nm for the dithionite-reduced *minus* ascorbate-reduced spectrum (Berden & Slater, 1970). Cytochrome c_1 was quantitated by using an extinction coefficient of 20 mM^{-1} cm^{-1} at 551–542 nm for the ascorbate-reduced *minus* ferricyanide-oxidized spectrum (Wood, 1980). Total heme *b* and heme *c* content in the pure enzyme was also determined by the pyridine hemochromagen method (Fuhrop & Smith, 1975), and the spectra were deconvoluted as described by Berry and Trumpower (1987). Optical redox titrations were done as described by Dutton (1978). Redox mediators were added from frozen DMSO stocks.

EPR spectra were recorded on a Bruker 220D X-band spectrometer interfaced to an IBM-PC computer, using data acquisition and manipulation software developed by Dr. Philip D. Morse, II, at the Illinois ESR Research Center. Experiments at 70–200 K were done by using a Bruker liquid nitrogen evaporator unit. Temperatures of 9–70 K were reached by using an Air Products liquid helium cryostat and variable-temperature unit. Microwave frequency was monitored with an EIP Microwave, Inc., frequency counter; *g* values were determined by measuring magnetic field with a Varian NMR gauss meter. EPR redox titrations were done as described by Dutton (1978) with mediators added from DMSO stock solutions. EPR samples were prepared and rapidly frozen as described by Salerno et al. (1986).

Rb. sphaeroides Ga was grown to mid-logarithmic phase phototrophically at 29 °C either in sealed 1-L Rous bottles or in a 20-L light-equipped fermentation tank. Chromatophores were prepared from French-pressure cell extracts by centrifugation at 10000*g* for 20 min, followed by centrifugation of the supernatant at 120000*g* for 90 min. Membranes were washed in 50 mM Tris-HCl and 50 mM NaCl, pH 7.5, and pelleted a second time. Chromatophores were resuspended in a small volume of Tris buffer and used immediately or diluted 1:1 with glycerol and stored at −70 °C.

Purification of cytochrome bc_1 was done by a procedure similar to that of Ljungdahl et al. (1987), with several modifications according to the flow diagram in Figure 1. Major modifications were the elimination of sucrose gradients during

Table I: Purification of the Cytochrome bc_1 Complex (Ubiquinol:Cytochrome c_2 Oxidoreductase) from *Rb. sphaeroides*[a]

step	total units of QCR activity [(μmol of cyt *c* reduced)·min^{-1}]	total yield (activity) (%)	total heme *b* (nmol)	total heme *c* (nmol)	protein (mg)	specific heme *c* content [(nmol of cyt c_1)·(mg of protein)$^{-1}$]
chromatophores (+dodecyl maltoside)	15750	100	5760	3765	4500	0.8
supernatant (dodecyl maltoside extract)	12200	77	4395	2910	2250	1.3
DEAE-Bio-Gel A pool	5900	37	1398	1184	188	6.3
DEAE-Sepharose CL-6B pool	3205	20	760	479	58	8.3
Sepharose CL-4B	3180	20	574	310	35	8.9

[a] Quinol:cytochrome c reductase activity was assayed at room temperature by adding 2–30 μL of enzyme to a 1-mL cuvette containing 40 mM sodium phosphate, pH 7.5, 0.5 mM EDTA, 50 μM horse heart cytochrome *c*, 40 μg/mL dodecyl maltoside, and 20 mM DBH substrate.

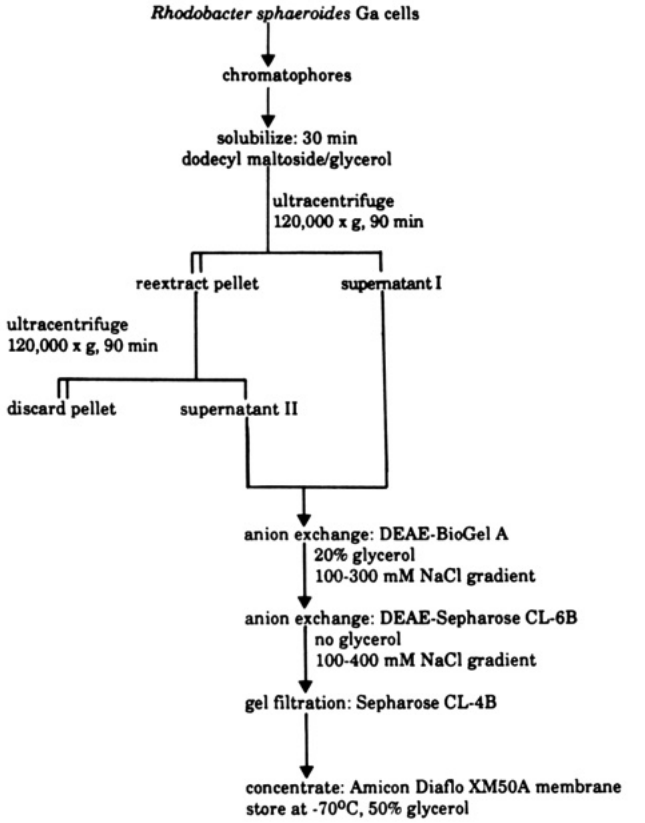

FIGURE 1: Flow scheme for the purification of the cytochrome bc_1 complex from chromatophores of *Rhodobacter sphaeroides*.

the chromatophore preparation, the addition of glycerol in the first two steps, a 40-fold scale-up of total protein in the starting material, and use of gel filtration as the final chromatography step. All steps were performed at 4 °C.

Chromatophores were diluted to 10 mg/mL protein in a solubilization mixture containing the following: 50 mM Tris-HCl, pH 8.0, 20% (v/v) glycerol, 100 mM NaCl, 1 mM PMSF, and 6.6 mg/mL dodecyl maltoside. The mixture ws stirred 30 min and then centrifuged at 120000*g* for 90 min. The brownish green supernatant was decanted and reserved, and the pellet was reextracted under the same conditions. The combined supernatants were loaded onto a 5 × 40 cm DEAE-Bio-Gel A column equilibrated with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20% glycerol, and 0.1 mg/mL dodecyl maltoside (buffer A). After washing with 1 column volume of buffer A, elution was carried out by using a 200–300 mM NaCl linear gradient. Fractions containing maximal quinol:cytochrome *c* reductase activity and minimal cytochrome *c* oxidase activity were pooled and concentrated by ultrafiltration to approximately 70 mL by using an Amicon XM50A membrane. The solution was loaded onto a 2.5 × 30 cm DEAE-Sepharose CL-6B column equilibrated with 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 0.1 mg/mL dodecyl

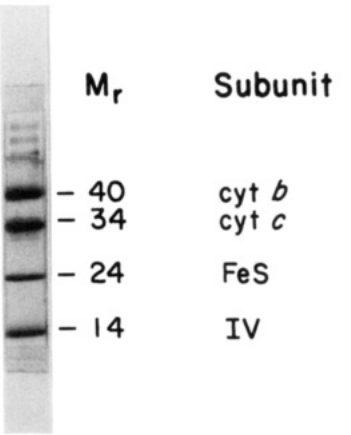

FIGURE 2: SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of the pure cytochrome bc_1 complex. Forty micrograms of protein was loaded onto a 15% polyacrylamide gel. Apparent molecular weight values, in K, are shown for each of the four subunits.

maltoside (buffer B). The column was washed with 1 column volume of buffer B and eluted with a 100–400 mM NaCl linear gradient. Pooled cytochrome fractions were concentrated to less than 1% of the gel filtration column volume by using an Amicon Diaflo XM50A ultrafiltration membrane. The enzyme was applied to a 2.5 × 90 cm Sepharose CL-4B column equilibrated in buffer B at a flow rate of 0.5 mL/min. Cytochrome fractions in the eluate were combined, concentrated by ultrafiltration over an XM50A membrane, and stored as 50% glycerol stocks at −70 °C.

Results and Discussion

Purification. The representative purification of *Rb. sphaeroides* cytochrome bc_1 complex is shown in Table I. The initial conditions are similar to those previously described (Ljungdahl et al., 1987); however, the presence of glycerol substantially improves the yield of activity in both the solubilization and DEAE-Bio-Gel A steps. The addition of a gel filtration step resulted in rates of turnover [(μmol of cyt *c* reduced)·(μmol of cyt c_1)$^{-1}$·s^{-1}] double those previously reported (Ljungdahl et al., 1987) and typically increased the heme *b*:heme *c* ratio from values of 1.4–1.5 to 1.75. Scaling up the entire purification resulted in total protein yields at least 25-fold higher than reported by Ljungdahl et al. (1987). The data in Table I indicate that while a negligible amount of activity is lost in the final gel filtration step, approximately 20% and 35% of hemes *b* and *c*, respectively are lost, along with 60% of the total protein applied to the column. This may represent fragments of bc_1 complex that have partially lost their structural integrity and catalytic competence during membrane solubilization and subsequent purification.

Polypeptide Composition. Figure 2 shows a 15% SDS–polyacrylamide gel of pure cytochrome bc_1 complex. The enzyme is composed of four polypeptides, identified as cyto-

Table II: Comparison of N-Terminal Amino Acid Sequences Obtained for the Cytochrome *b*, Cytochrome c_1, and Iron–Sulfur Protein Subunits with the Amino Acid Sequences Deduced from the DNA Sequence of the *fbc* Operon of *Rb. sphaeroides*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
b (deduced)	M	S	G	I	P	H	D	H	Y	E	P	R	T	G	I	E	K	W	L	H	S	R	L	P	I	V	A	A	L	A	Y	D	T	I	M	I	P				
b (N-term)		S	G	I	P	H	X	H	Y	Z	P	R	T	G	I	X	X	W	L																								
c (deduced)	M	I	R	K	L	T	L	T	A	A	T	A	L	A	L	S	G	G	A	A	M	A	A	G	G	G	H	V	E	D	V	P	F	S	F	E	G	P	F	G	T	F
c (N-term)																							A	G	G	G	H	V	E	D	V	P	F	S	F	E	G	P	F	G	T	F
f (deduced)	M	S	H	A	E	D	H	A	G	T	R	R	D	F	L	Y	Y	A	T	A	G	A	G	A	V	A	T	G															
f (N-term)		S	H	A	E	D	X	A	G	T	X	X	D	F	X	Y	Y	A	T																								

chrome *b*, cytochrome c_1, iron–sulfur subunit, and subunit IV, in order of decreasing apparent molecular weight values. The M_r values determined by SDS–PAGE are 40K, 34K, and 24K for the *b*, c_1, and iron–sulfur subunits, respectively, as compared to values of 50K, 31K, and 20K calculated from the amino acid sequences deduced by DNA sequencing (C.-H. Yun, R. Beci, A. Crofts, S. Kaplan, and R. B. Gennis, unpublished results). N-Terminal amino acid sequences obtained for cytochrome c_1, the iron–sulfur protein, and preliminarily for cytochrome *b* are listed in Table II and are compared with the sequences deduced from the DNA sequences of the respective genes. The two sets of sequences are identical with the exceptions that cytochrome c_1 appears to have a 22 amino acid cleaved signal sequence and N-terminal methionine residues have been cleaved off of the cytochrome *b* and the iron–sulfur subunits.

The N-terminus of subunit IV appears to be blocked, so it is unlikely that subunit IV is a proteolytic fragment of any of the three higher M_r subunits. Furthermore, preliminary N-terminal amino acid sequences obtained from proteolytic fragments of subunit IV do not match those of the cytochrome *b*, cytochrome c_1, or iron–sulfur protein subunits. It has been suggested that a fourth subunit in some preparations of the bc_1 complex from *Rb. capsulatus* (Gabellini et al., 1982)[1] is a contaminating light-harvesting complex polypeptide (Gabellini, 1988). The preparation of Ljungdahl et al. (1987) from *Rb. capsulatus* appears to contain four subunits when analyzed by SDS–PAGE. However, recent immunoblotting experiments using monocloral antibodies indicate that the two low-M_r bands (21K and 19.8K) both represent the Fe/S protein and are not discrete subunits (D. Robertson and F. Daldal, personal communication). Antibodies raised against the B800 and B850–875 α and β polypeptides of the light-harvesting complex did not cross-react with pure cytochrome bc_1 from *Rb. sphaeroides* in Western blotting experiments (not shown). Furthermore, bc_1 complex subunit IV migrates as a larger polypeptide than any of the subunits of the light-harvesting complex under the gel conditions used. The 14K subunit IV has been observed in two other preparations of the bc_1 complex from *Rb. sphaeroides* (Yu & Yu, 1982; Ljungdahl et al., 1987), and recently a four-subunit quinol:cytochrome *c* oxidoreductase has been isolated from the thermophilic *Bacillus* PS3 (Kutoh & Sone, 1988). However, the *Bacillus* complex contains a small-M_r cytochrome *b* subunit, similar to that of spinach chloroplast b_6f. In spinach, the two smallest M_r subunits have each been shown to be homologous to portions of the 48K cytochrome *b* subunit of the mitochondrial bc_1 complex (Widger et al., 1984). The gene encoding subunit IV from the *B. sphaeroides* bc_1 complex has not yet been located, and its function is currently unknown, although a small-M_r subunit has been implicated in the binding of ubiquinone and antimycin (Yu & Yu, 1987; Wilson et al., 1985).

Table III: Prosthetic Group Composition of the Pure Cytochrome bc_1 Complex from *Rb. sphaeroides*

prosthetic group	specific content (nmol/mg of protein)	stoichiometry (nmol/nmol of c_1)
heme *b*	15.3	2
heme *c*	8.9	1
iron–sulfur center	6.3	1
total iron	38.3	4–5
total copper	<2	0
ubiquinone	34	4
phospholipid	9	1
antimycin-sensitive semiquinone	3	0.3

Prosthetic Group Composition. The specific content and stoichiometry values for the redox centers, ubiquinone, and phospholipid present in the pure enzyme are listed in Table III. The expected stoichiometry of 2:1:1 heme *b*:heme *c*: Rieske iron–sulfur cluster was obtained. Pure cytochrome bc_1 typically contains 4–6 ubiquinone molecules and approximately 1 phospholipid per complex. The small amount of protein-bound phospholipid may reflect highly efficient detergent solubilization in the presence of glycerol.

Catalytic Activity. Figure 3 shows typical quinol:cytochrome *c* reductase assay traces for the pure enzyme in the presence of added detergents or phospholipid. The activity was enhanced in the presence of dodecyl maltoside and inhibited by Triton X-100. The inhibition by Triton X-100 is complex, as seen in Figure 3B, where the effect appears to be not so much on the initial velocity as on the length of time during which the enzyme is active in the assay cuvette. The adverse effects of Triton X-100 have previously been reported (Von Jagow et al., 1977; Gabellini et al., 1982;[1] Engel et al., 1983), although the detergent has been used in two different cytochrome bc_1 preparations from *Rb. capsulatus* (Gabellini et al., 1982)[1] and *Rb. sphaeroides* (Yu & Yu, 1982). Dissociation of the iron–sulfur protein in the presence of Triton X-100 may explain the low activities of these preparations. Octyl glucoside added to the assay medium at 0.5 mg/mL had no effect on the enzyme activity (data not shown). Soybean lecithin further enhanced the quinol:cytochrome *c* reductase activity when added in the presence of dodecyl maltoside. The largest enhancement of activity was observed when the complex was preincubated with phospholipid as well. Turnover

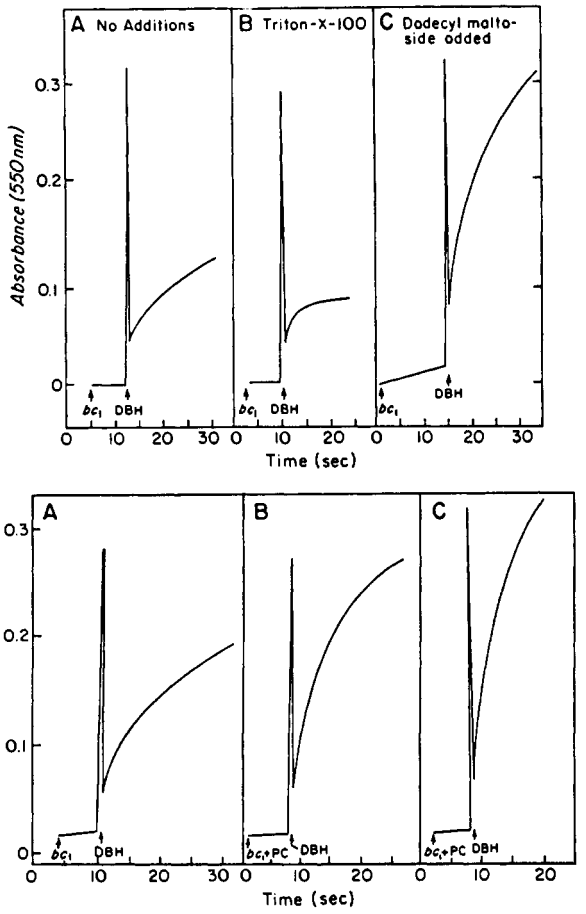

FIGURE 3: Effects of detergents and phospholipid on quinol:cytochrome *c* reductase activity of the pure cytochrome bc_1 complex. Each assay mixture contained 40 mM sodium phosphate, 0.5 mM EDTA, 50 μM horse heart cytochrome *c*, and 0.3 mM DBH, at pH 7.5. (Top panel) Approximately 24 pmol of enzyme was added for each assay: (A) no detergent added; (B) 50 μg/mL Triton X-100 added: (C) 40 μg/mL dodecyl maltoside added. (Bottom panel) Approximately 12 pmol of enzyme was added for each assay in the presence of 40 μg/mL dodecyl maltoside: (A) no phospholipid added; (B) 40 μg/mL soybean lecithin (PC) added; (C) 40 μg/mL soybean lecithin added to the assay mixture following preincubation of the enzyme with 40 μg/mL soybean lecithin. Specific activities calculated from the initial slopes of these traces are (top panel) (A) 23, (B) 24, (C) 57; (bottom panel) (A) 60, (B) 146, (C) 206 μmol of cytochrome *c* reduced)·min^{-1}·mg^{-1}.

numbers of 250–350 (μmol of cyt *c* reduced)·(μmol of cyt c_1)$^{-1}$·s^{-1} were observed following gel filtration chromatography.

Absorption Spectra and Heme Midpoint Potentials. Redox-resolved spectra for the three hemes associated with the bc_1 complex were obtained by successively reducing the sample with ascorbate, menadiol, and dithionite and taking difference spectra, shown in Figure 4. Cytochromes b_h, (*b*-high) b_1 (*b*-low), and c_1 have α-band wavelength maxima of 559.5, 565, and 552 nm, respectively. The peaks are slightly blue-shifted compared to those observed in chromatophores (Bowyer & Crofts, 1981), possibly due to detergent binding. The relative sizes and positions of the peaks, as well as retention of the characteristic split-α band, indicate that the enzyme has not lost significant amounts of heme during the purification and that its structural integrity has not been disrupted in such a manner as to perturb the heme absorption spectra. Addition of the inhibitor antimycin results in a 2-nm red shift of the cytochrome *b* spectrum, an effect that has been observed previously in chromatophores (Van den Berg et al., 1979). The presence of the inhibitors myxothiazol and stigmatellin also causes perturbations in the heme *b* α and Soret absorption bands. Full-spectrum redox titrations yielded E_m values of

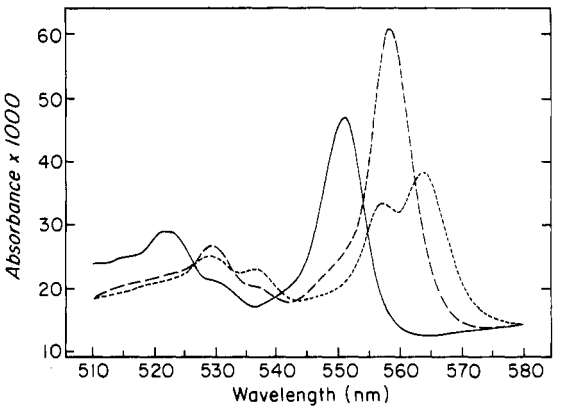

FIGURE 4: Resolved absorption difference spectra for cytochrome c_1, heme b_1, and heme b_h obtained from "redox cuts" using the pure bc_1 complex. (—) Ascorbate-reduced minus ferricyanide-oxidized difference spectrum, representing cytochrome c_1. (– –) Menadiol-reduced minus ascorbate-reduced spectrum, representing the high-potential heme *b* (b_h). (- - -) Dithionite-reduced minus menadiol-reduced difference spectrum, showing the low-potential heme *b* (b_1). The pure enzyme was present at 0.17 mg/mL in 50 mM Tris-HCl, 100 mM NaCl, and 0.1 mg/mL dodecyl maltoside at pH 7.5.

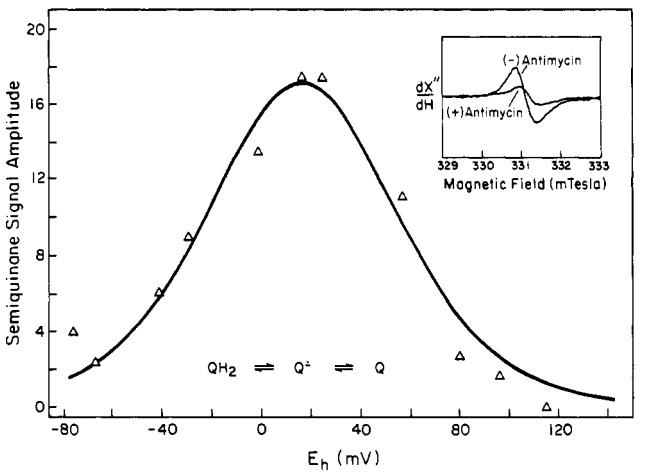

FIGURE 5: EPR redox titration of the antimycin-sensitive semiquinone in the pure bc_1 complex. Samples containing 5 μM cytochrome bc_1 were anaerobically redox-poised at 18 °C in 50 mM Tris-HCl, 100 mM NaCl, and 0.1 mg/mL dodecyl maltoside, at pH 8.7, transferred to EPR tubes, and rapidly frozen. The following mediators were present at final concentrations of 40 μM each: 1,2-naphthoquinone, 1,4-naphthoquinone, 1,4-naphthoquinone-2-sulfonate, *p*-benzoquinone, pyocyanine, and 2,3,5,6-tetramethyl-*p*-phenylenediamine. Duroquinone was present at a final concentration of 60 μM. Each point on the titration curve was obtained by subtracting the antimycin-insensitive signal from the signal obtained in the absence of antimycin (shown inset). The peak-to-trough amplitude of each resultant spectrum was then plotted vs E_h. EPR conditions were as follows: microwave frequency 9.3 GHz, modulation amplitude 0.25 mT, microwave power 10 μW, temperature 150 K.

−100, +48, and +240 mV for cytochromes b_1, b_h, and c_1, respectively.

Antimycin-Sensitive Semiquinone. When the purified cytochrome bc_1 complex from *Rb. sphaeroides* is appropriately redox-poised, a free-radical signal is observed by EPR spectroscopy. The isotropic signal is centered at $g = 2.004$ with a peak-to-trough line width of 0.7 mT. The radical species has also been observed in purified bc_1 complex from *P. denitrificans* (Meinhardt et al., 1987) as well as in many preparations of the enzyme from other sources (de Vries et al., 1979; Ohnishi & Trumpower, 1980; Nagaoka et al., 1981; de Vries et al., 1982; Matsuura et al., 1983; Robertson et al., 1984), and it is attributed to a bound ubisemiquinone radical. This signal is abolished in the presence of antimycin (Siedow et al.,

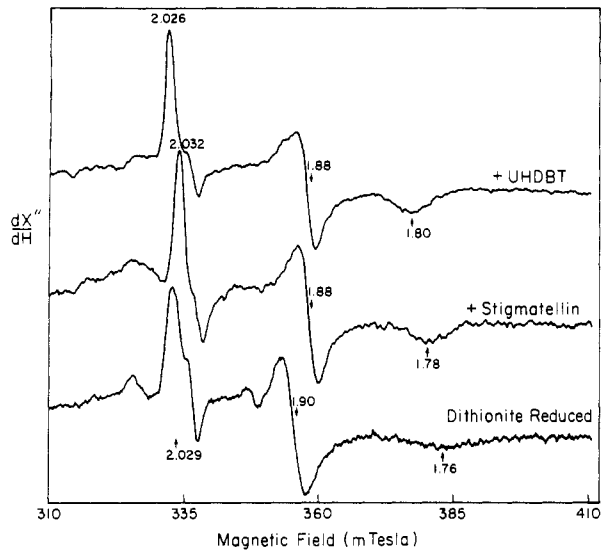

FIGURE 6: EPR spectra of the Rieske iron–sulfur cluster in pure cytochrome bc_1 complex from *Rb. sphaeroides* showing inhibitor-induced shifts in the resonance frequencies. For each spectrum the enzyme was suspended at a concentration of 5 μM in 50 mM Tris-HCl, 100 mM NaCl, and 0.1 mg/mL dodecyl maltoside, at pH 8.0. For the bottom spectrum, the sample was dithionite-reduced as indicated. For the upper two spectra, ascorbate was added to each sample prior to the addition of 30 μM UHDBT or 10 μM stigmatellin. In the absence of inhibitor, the ascorbate-reduced spectrum exhibited g_x, g_y, and g_z values of 1.81, 1.90, and 2.03, respectively (not shown). EPR spectrometer conditions were as follows: temperature 15 K, microwave frequency 9.489 GHz, modulation amplitude, 1.4 mT, microwave power 2 mW.

1978; Nagaoka et al., 1981). A typical redox titration of the antimycin-sensitive semiquinone is shown in Figure 5, with representative EPR spectra inset. The midpoint of the QH_2/Q couple shifts approximately −53 mV/pH unit at 18 °C, close to the predicted pH dependence at that temperature for a process involving one proton and one electron. In the pH range 7.8–9.5 no changes in the absolute concentration of semiquinone were observed. A typical value of 0.3 equiv of semiquinone per bc_1 complex was found. Extrapolation to pH 7.0 gives an E_{m7} value near +140 mV, much higher than the midpoint of +90 mV measured for the QH_2/Q couple in the free quinone pool of chromatophores (Takamiya & Dutton, 1979). The results are similar to those observed in *Rb. sphaeroides* chromatophores (Matsuura et al., 1983) and indicate that the Q_i site is intact in the pure cytochrome bc_1 complex.

Rieske Iron–Sulfur Cluster. The EPR spectra and redox titrations of the Rieske center are shown in Figures 6 and 7. The spectrum is characteristic of this type of redox center (Siedow et al., 1978; de Vries et al., 1979; Ohnishi & Trumpower, 1980; Bowyer et al., 1982) with g_x, g_y, and g_z values of 1.76, 1.90, and 2.03 in the dithionite-reduced sample, in the absence of inhibitors. When the enzyme is reduced with ascorbate, g_x shifts to 1.81 and becomes significantly more narrow. When added to the ascorbate-reduced sample in saturating amounts, UHDBT causes all three g values to shift. Stigmatellin similarly causes shifts in the EPR spectrum of the iron–sulfur cluster. In the absence of inhibitor, an E_{m7} value of +280 mv was measured for the Rieske center. In the presence of UHDBT, the E_{m7} shifted to +325 mV, and saturating concentrations of stigmatellin shifted the midpoint to greater than +450 mV. These effects have been observed in the bc_1 complexes from a number of organisms (Leigh & Erecinska, 1975; Prince et al., 1975; de Vries et al., 1979; Bowyer et al., 1980; Von Jagow & Ohnishi, 1985).

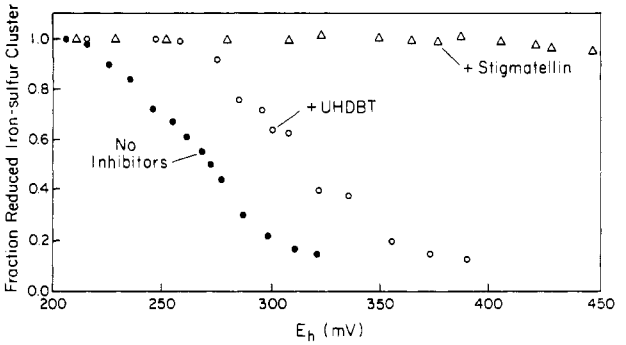

FIGURE 7: EPR redox titrations of the Rieske iron–sulfur cluster of the pure bc_1 complex showing inhibitor-induced shifts in the midpoint potential. Pure cytochrome bc_1 complex was suspended at a concentration of 5 μM in 50 mM Tris-HCl, 100 mM NaCl, and 0.1 mg/mL dodecyl maltoside, at pH 7.5. Peak-to-trough amplitudes of the g_x signal were measured at each E_h value and plotted. The following mediators were present at final concentrations of 40 μM: *p*-benzoquinone, 1,2-naphthoquinone, 1,4-naphthoquinone, 1,4-naphthoquinone-2-sulfonate, and 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD). UHDBT and stigmatellin were added at concentrations of 50 and 30 μM, respectively. EPR spectrometer conditions were similar to those described for Figure 6.

CONCLUSIONS

The purification protocol described is simple and reproducible, and it is carried out on a scale large enough to provide the quantities of enzyme required for magnetic resonance spectroscopy, crystallization, and many other biochemical and biophysical techniques. Biochemical and biophysical analyses indicate that the enzyme has very high specific activity, that each of the prosthetic groups is present at the expected ratios, and that each of the redox centers and prosthetic groups functions as in chromatophores.

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Registry No. EC 1.10.2.2, 9027-03-6; cytochrome *b*, 9035-37-4; cytochrome c_1, 9035-42-1.

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