A Novel Cytochrome *c* Oxidase from *Rhodobacter sphaeroides* That Lacks Cu_A[†]

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ABSTRACT: *Rhodobacter sphaeroides* contains at least two different cytochrome *c* oxidases. When these bacteria are grown with high aeration, the traditional aa_3-type cytochrome *c* oxidase is present at relatively high levels. However, under microaerophilic growth conditions or when the bacteria are grown photosynthetically, the amount of the aa_3-type oxidase is greatly diminished and an alternate cytochrome *c* oxidase is evident. This alternate oxidase has been purified and characterized. The enzyme consists of three subunits by SDS–PAGE analysis (M_{app} 45, 35, and 29 kDa). Two of the three subunits (M_{app} 35 and 29 kDa) contain covalently bound heme C. Metal and heme analyses indicate that the oxidase contains heme C, heme B (protoheme IX), and Cu in a ratio of 3:2:1. Cryogenic Fourier transform infrared (FTIR) difference spectroscopy of the CO adduct of the reduced enzyme shows that the oxidase contains a heme–copper binuclear center and, thus, is a member of the heme–copper oxidase superfamily. In contrast to other members of this superfamily, however, this oxidase does not contain either heme O or heme A as a component of the binuclear center, but has heme B at this site. The single equivalent of Cu found in the oxidase is accounted for by the Cu_B component at the binuclear center. This suggests that this oxidase does not contain Cu_A, which is found in all other well-characterized cytochrome *c* oxidases. Both EPR and optical spectroscopic studies are consistent with this conclusion, also indicating that this oxidase does not contain Cu_A. Since the purified enzyme has a turnover number of greater than 900 s^{-1} using horse heart cytochrome *c* as a substrate, it is not likely that the lack of Cu_A is the result of damage incurred during the purification procedure. It is concluded that the alternate cytochrome *c* oxidase is a novel cbb_3-type of the heme–copper oxidase superfamily that contains heme B at the binuclear center (not heme O), and which lacks Cu_A.

During the last few years, it has become evident that many prokaryotic respiratory oxidases belong to a large superfamily which also includes the mitochondrial aa_3-type cytochrome *c* oxidase (Hosler et al., 1993; Saraste, 1990; Brown et al., 1993). These enzymes have in common a binuclear center consisting of a high-spin, five-coordinate heme and a copper (Cu_B), and this binuclear center is the site where oxygen is reduced to water. All of the members of this superfamily also appear to contain at least one other heme component which is low spin and six-coordinate and which serves to pass electrons to the binuclear center. Both hemes as well as Cu_B are ligated to amino acid residues within a single subunit whose sequence is very highly conserved within the superfamily. This subunit corresponds to the largest subunit of the mitochondrial oxidase and is referred to as subunit I (Hosler et al., 1993; Brown et al., 1993; Saraste, 1990). Unambiguous membership in the heme-copper oxidase superfamily can be determined for a respiratory oxidase either by the demonstration of a subunit whose sequence is homologous to subunit I or by the spectroscopic demonstration of the heme–copper binuclear center. In many other respects, members of this superfamily exhibit extraordinary diversity, in particular, with respect to the heme and Cu composition and with respect to the substrate oxidized.

The heme–copper oxidase superfamily has two branches: cytochrome *c* oxidases and quinol oxidases (Hosler et al., 1993). The primary differences between these two branches do not appear to reside within subunit I but, rather, subunit II. In all the cytochrome *c* oxidases previously analyzed, including the mitochondrial enzyme, there are sets of highly conserved residues in subunit II which either have been implicated in the binding to cytochrome *c* or have been found to constitute the Cu_A binding site (Saraste, 1990). The Cu_A redox center is the primary site accepting electrons from reduced cytochrome *c* (Hill, 1993). A substantial body of evidence now indicates that the Cu_A center actually contains two copper atoms (Malmström & Aasa, 1993; Kelly et al., 1993), which provides an explanation for the fact that many preparations of either the mitochondrial or prokaryotic cytochrome *c* oxidases contain 3 equivalents of copper/mol of oxidase (Steffens et al., 1987).

Numerous heme–copper oxidases do not utilize cytochrome *c* as a substrate but, rather, oxidize quinols such as ubiquinol (Hosler et al., 1993; Brown et al., 1993). Although the quinol oxidases which are within this superfamily contain a homologue of subunit II, they do not contain Cu_A, and the residues implicated in Cu_A ligation or in the binding of cytochrome *c* are not conserved in subunit II of the quinol oxidases. The best characterized examples of quinol oxidases are cytochrome *bo* from *Escherichia coli* (Minghetti et al., 1992; Chepuri et al., 1990) and cytochrome aa_3 from *Bacillus subtilis* (Santana et al., 1992; Lauraeus et al., 1991). Only a single equivalent of copper is present in these quinol oxidases, i.e., Cu_B. These two enzymes also illustrate the variety of hemes which are found within the oxidase superfamily. The aa_3-type oxidases contain 2 equivalents of heme A, providing both the low-spin (heme *a*) and high-spin (heme a_3) components of the oxidase. The *bo*-type oxidase contains 1 equivalent each of heme B, at

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the low-spin site (heme b_{562}), and heme O, at the high-spin site (heme *o*) (Wu et al., 1992; Puustinen et al., 1992). There are also *o*-type oxidases which are cytochrome *c* oxidases (Sone & Fujiwara, 1991). There is no correlation between the substrate utilized by a particular oxidase and the heme types present, with one exception. Several prokaryotic cytochrome *c* oxidases contain heme C located within a domain at the carboxy-terminus of subunit II, near the Cu_A binding domain (Saraste, 1990; Fee et al., 1993; Ishizuka et al., 1990). No quinol oxidase has been identified to date within this superfamily which contains a heme C component.

One common feature of all the oxidases in this superfamily which have been characterized to date is that the high-spin heme component at the binuclear center is either heme O or heme A. Each of these hemes contains a farnesyl side chain (Wu et al., 1992).

In the current work, a novel member of the heme–copper oxidase superfamily is described. This oxidase is most apparent in the membranes of *Rhodobacter sphaeroides* grown under microaerophilic or photosynthetic conditions and is distinct from the aa_3-type oxidase which is present when the bacteria are grown with high aeration (Shapleigh et al., 1992). The alternate cytochrome *c* oxidase is also evident in membranes from mutants of *Rb. sphaeroides* which have a nonfunctional or defective aa_3-type oxidase (Shapleigh et al., 1992). This novel, three-subunit alternate oxidase is demonstrated to be in the heme-copper superfamily by low-temperature Fourier transform infrared (FTIR) absorption difference spectroscopy of the CO adduct, which reveals the presence of Cu_B (Shapleigh et al., 1992). Analysis of the metal content reveals less than 1 equiv of copper/mol of oxidase (i.e., Cu_B), with no indication of Cu_A. Iron is also present and is accounted for by three heme C and two heme B prosthetic groups. Two of the three subunits are shown to contain covalently bound heme C. This cbb_3-type cytochrome *c* oxidase contains no heme O or heme A and yet exhibits a high turnover of greater than 900 s^{-1} using horse heart cytochrome *c* as a substrate.

Hence, this unusual cytochrome *c* oxidase is novel in several respects. It contains neither heme O or heme A, but uses heme B at the binuclear center, establishing that the farnesyl side chain is not an essential feature of the heme component at this site. Also, there is no Cu_A, establishing that this redox center is also not an essential feature of cytochrome *c* oxidases.

Because of the presence of low-spin heme *c* and heme *b* components, and a high-spin heme *b* at the oxygen binding site, this oxidase is referred to as a cbb_3-type oxidase, where the "b_3" notation designates a heme B as the oxygen binding species.

MATERIALS AND METHODS

Materials. Sepharose CL-6B and Sepharose FF-6B were obtained from Pharmacia, Inc. The β-D lauryl maltoside was purchased from Anatrace (Maumee, OH). Tris buffer, tetramethylphenylenediamine (TMPD), ascorbate, and horse heart cytochrome *c* type S-II were from Sigma Chemical Co. (St. Louis, MO). Tricine, sodium dodecyl sulfate (SDS), acrylamide, and bis(acrylamide) were obtained from Bio-Rad. All other reagents were reagent grade.

Bacterial Strains and Growth Conditions. Previous work has shown that the cbb_3-type cytochrome *c* oxidase is present under aerobic growth conditions in the membranes of a mutant of *Rh. sphaeroides* from which the gene encoding subunit I of the aa_3-type oxidase has been deleted (Shapleigh & Gennis, 1992). This deletion mutant (JS100) was cultured as described previously (Shapleigh & Gennis, 1992), using Sistrom's medium (Sistrom, 1962) and constant sparging with air. Cells were harvested by centrifugation and were frozen at −80 °C until used.

Purification of the cbb_3-Type Cytochrome c Oxidase. The thawed cell paste was suspended in buffer and disrupted using a French press as previously described (Shapleigh & Gennis, 1992). After collecting the crude membranes by centrifugation, the inner membranes were purified by sucrose gradient centrifugation as reported (Hosler et al., 1992). The membranes were then washed with buffer containing 100 mM Tris-HCl and 10 mM EDTA, pH 8.8. The washed membranes were resuspended in buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8) and stored at −70 °C. Approximately 2 g of cytoplasmic membrane protein was resuspended in buffer containing 0.7% β-D-lauryl maltoside, 100 mM NaCl, and 20 mM Tris-HCl, pH 8, to approximately 7.5 mg of protein/mL. This was stirred for 25 min and then centrifuged at 140000*g* for 30 min. The supernatant was loaded onto a Sepharose CL-6B column (150 × 20 cm) equilibrated with 0.01% lauryl maltoside, 100 mM NaCl, and 20 mM Tris-HCl, pH 8. The protein eluted in two peaks. The first sharp peak contained high molecular weight particles with 8–14% of the total protein, but less than the 5% of the total cytochrome *c* oxidase activity. The second peak of protein was very broad and contained most of the oxidase activity. Material in this peak was pooled, NaCl was added to a final concentration of 200 mM, and the sample was loaded onto a DEAE-Sepharose FF-6B column (7.5 × 30 cm) equilibrated with 0.01% lauryl maltoside, 200 mM NaCl, and 50 mM Tris-HCl, pH 8 (buffer A). After washing with 2 column volumes of buffer A, the protein was eluted with a 2-L linear gradient of NaCl from 200 to 300 mM. This was followed by a final wash with 300 mM NaCl. The cytochrome *c* oxidase was eluted after about 1.5 column volumes of the last wash. Fractions with oxidase activity were pooled, diluted to 200 mM NaCl with cold, distilled water, and then loaded onto a second DEAE-Sepharose FF-6B column (5 × 20 cm) equilibrated with buffer A. Elution was performed the same way as in the first DEAE column. Fractions with activity were pooled, diluted to 200 mM, and loaded onto an FPLC DEAE-TSK 5 PW glass column (Waters) equilibrated with buffer A. The oxidase eluted in a sharp peak around 350 mM NaCl, using a linear gradient from 200 to 600 mM NaCl. The enzyme was concentrated to 5 mg/mL by ultrafiltration using an Amicon YM30 membrane, glycerol was added to a final concentration of 10%, and the sample was stored frozen at −70 °C. No loss of oxidase activity was observed due to freezing under these conditions for up to 4 months.

Spectroscopic Techniques. Absorption spectroscopy was performed using an SLM-Aminco DW-2000 UV/visible spectrophotometer.

FTIR spectroscopy of the purified protein was performed as described previously (Hill et al., 1992). The sample was prepared starting with 1 mL of a solution containing 50 μM of the cbb_3-type oxidase in 0.01% lauryl maltoside, 200 mM NaCl, and 50 mM Tris-HCl, pH 8. The CO adduct was prepared in a 75 Ti centrifuge tube by diluting the sample into 15 mL of CO-saturated buffer which had been made anaerobic by sparging with argon and to which dithionite had been added to a final concentration of 67 mM. The protein was then pelleted by centrifugation for 24 h at 75 000 rpm in a Beckman 75 Ti rotor (4 °C). Under a flow of CO, the supernatant was decanted and CO-saturated glycerol was added to dehydrate the sample. The tube was purged with CO, and the cap was replaced.

The oxidases present in membranes from photosynthetically grown *Rb. sphaeroides* Ga were also analyzed by FTIR

spectroscopy. These membranes were isolated as described previously from photosynthetically grown cells, and the sample was prepared for FTIR spectroscopy as detailed earlier (Shapleigh et al., 1992).

A portion of the sample was placed between two CaF_2 windows (Janus Technology, Inc.) and pressed to an appropriate thickness (Shapleigh et al., 1992). A Mattson Sirius FTIR interferometer equipped with a Lake Shore Cryotronics closed-cycle helium refrigerator and a liquid nitrogen-cooled indium antimonide detector was used to record the FTIR spectra. Interferograms were detected in the single-beam mode and are presented as the "light" *minus* "dark" absorbance difference spectrum with a resolution of 0.5 cm^{-1}. The dark spectrum was recorded before photolysis. The light spectrum was recorded during continuous irradiation of the sample using a 500-W tungsten bulb filtered through glass and water. The light and dark spectra are each the average of 512 scans collected at 15 K.

Electron spin resonance (EPR) spectroscopy was performed at 15 K with a Bruker ESP 300 spectrometer equipped with a liquid helium system. The sample contained approximately 20 μM oxidase in buffer containing 0.05% lauryl maltoside, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.8.

Analytical Techniques. Cytochrome *c* oxidase activity was measured at 30 °C using a Clark-type electrode (YSI Model 53, Yellow Springs, OH) by diluting aliquots into the following medium: 0.01% lauryl maltoside, 25 μM TMPD, 150 μM horse heart cytochrome *c*, 2.5 mM ascorbate, 1 mM EDTA, and 50 mM potassium phosphate, pH 7.0.

Protein was measured by using the BCA method with reagents obtained from the Pierce Chemical Co. (Rockford, IL). The accuracy of the protein estimation was checked by comparing the values obtained by quantitative amino acid analysis of the purified oxidase performed at the University of Illinois Genetic Engineering Center. The concentration of protein obtained by the BCA spectrophotometric assay agreed to within 10% of the value obtained by amino acid analysis.

Qualitative analysis of the hemes present was performed by using the extraction protocol and HPLC technique described previously (Puustinen & Wikström, 1991) and modified as reported (Hill et al., 1992). Quantitation of the heme B and heme C content was obtained by two methods. The reduced-*minus*-oxidized spectrum was used, assuming the following extinction coefficients: For cytochrome *c*, 20 mM^{-1} cm^{-1} for the wavelength pair 550 nm *minus* 540 nm, and for cytochrome *b*, 25 mM^{-1} cm^{-1} for the wavelength pair 560 nm *minus* 580 mn. Alternatively, the hemes were quantified by forming the pyridine hemochromes, using the previously described procedure (Berry & Trumpower, 1987).

Metal analysis was performed by the Microanalytical Laboratory at the University of Illinois School of Chemical Sciences using a Perkin-Elmer plasma II with an argon plasma sequential unit and dual monochromators. The enzyme sample was extensively dialyzed against several changes of buffer containing 50 mM Tris-HCl, and 5 mM EDTA, pH 8. The oxidase retained full enzymatic activity after this treatment. The metal content of a sample of the buffer was used as a control for each determination.

SDS–PAGE analysis was performed using the Tris–tricine system described (Schägger & Von Jagow, 1987). Heme-containing polypeptides were visualized on unstained gels by the intrinsic fluorescence of the heme-containing proteins following SDS–PAGE as reported (Katan, 1976).

Protein sequence was obtained following resolution of the subunits by SDS–PAGE and transfer of the polypeptides to PVDF membranes (Applied Biosystems) using conditions specified by the manufacturer. After transfer, the polypeptides were visualized by staining with Coomassie Blue, dried, excised, and submitted for amino-terminal sequencing by the Genetic Engineering Facility at the University of Illinois.

Table 1: Purification of ccb_3-Type Cytochrome *c* Oxidase from *Rb. sphaeroides*[a]

step	protein (mg)	sp act. [nmol/(min·mg)]	heme B (nmol/mg)	heme C (nmol/mg)
crude membranes	2618	3 600	0.12	0.21
sucrose gradient	1875	15 500	0.87	0.55
detergent extraction	1430	17 300	0.76	1.3
gel filtration	1005	30 100	1.1	1.56
1st DEAE-Sepharose	188	110 100	4.4	6.9
2nd DEAE-Sepharose	46	223 200	6.2	9.9
DEAE TSK-5pw	16	530 000	9.9	14.4

[a] Protein determined by the BCA method. Oxidase activity is given as nmol of cytochrome *c* oxidized/(min·mg of protein), determined at 30 °C with an oxygen electrode. The heme B and heme C contents were estimated by the reduced-*minus*-oxidized spectrum as detailed in the text. Only low-spin heme B will be included in this measurement, and this measurement should be considered less reliable than the heme analysis following extraction (Table 2).

RESULTS

Purification. Results obtained previously from this laboratory demonstrated that the deletion of the gene encoding subunit I of the aa_3-type cytochrome *c* oxidase did not eliminate the cytochrome *c* oxidase activity from membrane preparations of aerobically grown *Rb. sphaeroides* (Shapleigh et al., 1992). This strain of *Rb. sphaeroides* (JS100) which does not contain the aa_3-type oxidase was used as the source of membranes for the isolation of the "alternate" cytochrome *c* oxidase. Cells were grown aerobically with continuous sparging with air to maintain the dissolved oxygen concentration in the growth medium. This was done to avoid the induction of the photosynthetic apparatus and the accompanying pigments. The presence of these pigments interferes with the purification protocol.

Cytoplasmic membranes from the aerobically grown cells were prepared and then solubilized in 0.7% β-D-lauryl maltoside, and the solubilized material was passed through a gel filtration column. Even though there is no significant improvement of the oxidase-specific activity resulting from this step (see Table 1), this column eliminates large particles, probably rich in membrane phospholipids, that affect further ion-exchange steps. Omitting this step, the resolution of the oxidase from other membrane proteins by the following DEAE chromatography steps is unsatisfactory.

After gel filtration, the enzyme was purified by anion-exchange chromatography. The first DEAE column removes much of the non-heme protein, and a major contaminant remaining is the bc_1 complex. This was revealed by Western immunoblotting using bc_1-specific antibodies. The second DEAE column eliminates most, but not all, of the bc_1 complex from the fractions containing the oxidase, but analysis by SDS–PAGE indicates many polypeptide impurities. The final column chromatography step utilizes an FPLC system to improve resolution. The oxidase elutes in a symmetrical peak, and attempts at further improving the specific oxidase activity beyond that obtained after this step were unsuccessful.

SDS–PAGE analysis of the enzyme prepared using the protocol described is presented in Figure 1. The gel shows three polypeptides visualized by staining with Coomassie Blue with apparent molecular masses of 45, 35, and 29 kDa (Figure 1). A band of material at about 80 kDa is also present to varying degrees in different preparations. This is probably

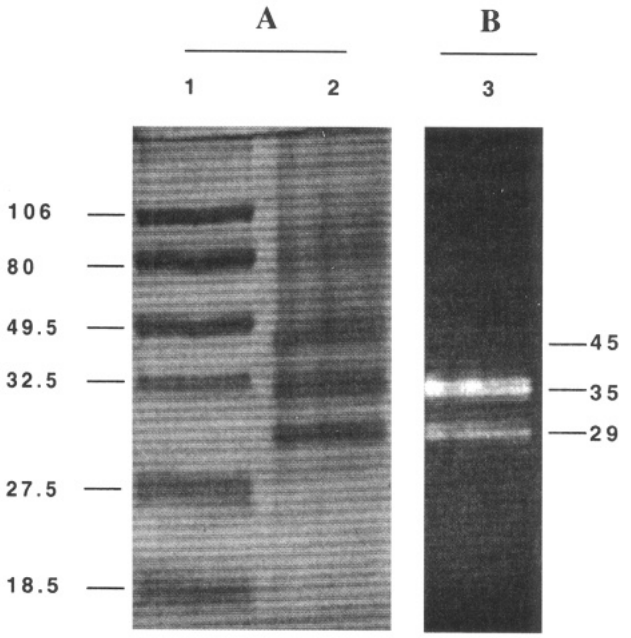

FIGURE 1: SDS–PAGE of the purified cbb_3-type oxidase from *Rb. sphaeroides*. Approximately 5 μg of the purified enzyme was precipitated with 5% TCA to remove salts and detergent. The pellet was washed with distilled water, and then the protein was resuspended in standard sample buffer and incubated at room temperature for 5–6 h before loading onto the gel (lanes 2 or 3). Electrophoresis was run as described in the text using a 16% acrylamide–4 M urea gel. The gel was photographed using a near-UV transilluminator (panel B) and then stained with Coomassie Blue (panel A). Prestained SDS–PAGE molecular weight markers (Bio-Rad, Madison, WI) were run in a lane beside (lane 1). Apparent molecular masses in kDa are indicated.

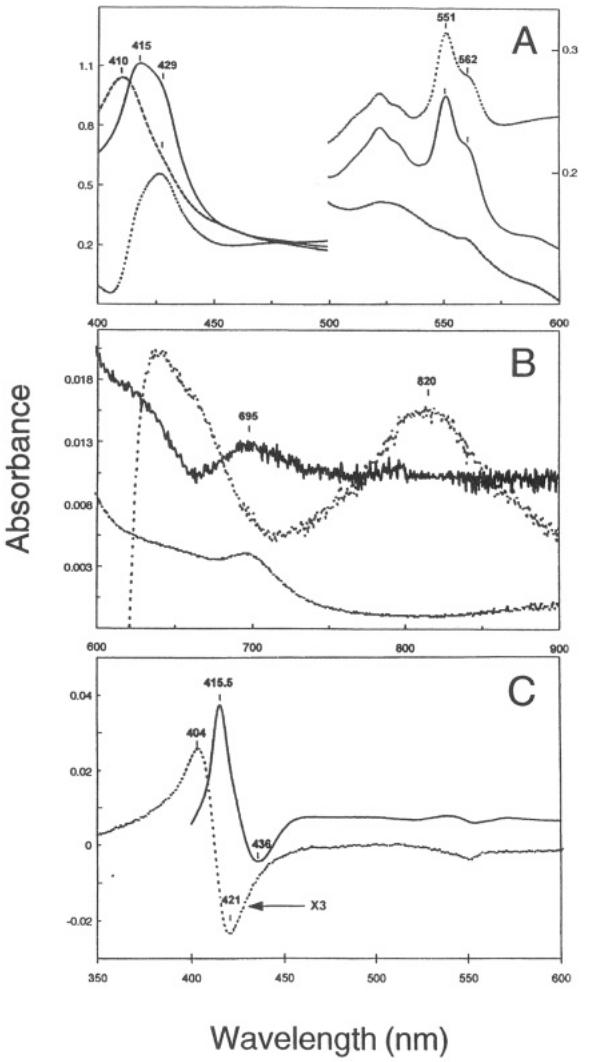

FIGURE 2: UV/visible spectra of the purified cbb_3-type oxidase from *Rb. sphaeroides*. Approximately 50 μM of pure enzyme was used. All the spectra were recorded at 25 °C. Panel A: Dithionite-reduced (—), persulfate-oxidized (- - -), and the reduced-*minus*-oxidized difference spectrum (···). Panel B: Near-infrared region, air-oxidized-*minus*-dithionite-reduced cbb_3-type oxidase (—). The air-oxidized-*minus*-dithionite-reduced *Rb. sphaeroides* cytochrome aa_3 spectrum (···), and the air-oxidized-*minus*-dithionite-reduced horse heart cytochrome *c* spectrum (- · · · -) are shown for reference. Panel C: Reduced plus CO-*minus*-reduced difference spectrum (—) and the air-oxidized-*minus*-air-oxidized plus CN^- difference spectrum (amplified 3×) (···).

an impurity or an aggregate of denatured subunits. The two smaller subunits (35 and 29 kDa) have covalently bound heme, presumably heme C, as revealed either by a peroxidase test (Goodhew et al., 1986) (not shown) and by the porphyrin intrinsic fluorescence (Figure 1, lane 3). N-Terminal analysis showed that the the amino termini of subunits I and II are chemically blocked. The following sequence was obtained from subunit III: GILAKHKILETNATLLIFSFTVVTIG-GLV. Computer searches did not reveal significant homology with other polypeptides. Although the oxidase activity of the isolated enzyme was stable at 4 °C for several days, SDS–PAGE analysis demonstrated limited proteolysis of subunit III under these conditions (not shown). There is no evidence to suggest that subunit III is proteolytically derived from subunit II. The ratio of these subunits appears constant in different preparations of the oxidase.

If the oxidase is considered to consist of one copy each of subunits I–III, the turnover of the purified oxidase can be estimated to be 963 s^{-1} (cytochrome *c* oxidized per second). Protein was determined for this estimate by both the BCA technique and quantitative amino acid analysis. The oxidase does not utilize ubiquinol-1 as a substrate and is not a quinol oxidase.

Heme and Metal Content. The UV/visible absorption spectra of the purified enzyme are shown in Figure 2. The reduced-*minus*-oxidized difference spectrum has a peak in the visible at 551 nm with a distinct shoulder at 562 nm. This is suggestive of the presence of both low-spin heme C and heme B in the complex. The magnitudes of the peaks at 551 and 562 nm can be used to crudely estimate the content of heme C and heme B, respectively. This was done for three independent preparations of the oxidase. Using this technique, the oxidase contains 14.4 nmol of heme C and 9.9 nmol of heme B/mg of protein (Tables 1 and 2). Since several other oxidases have been shown to contain heme O, which has very similar absorption properties as does heme B, the hemes were extracted and identified by HPLC analysis. Figure 3 shows the elution profile of the heme extracted from the purified oxidase in comparison to the elution positons of heme B and heme O standards from cytochrome *bo* from *E. coli*. It is evident that the purified oxidase contains heme B and not heme O, and this is responsible for the absorbance at 562 nm (Figure 2).

Both heme C and heme B contents of the oxidase were also determined by forming the pyridine hemochromes using standard techniques (Berry & Trumpower, 1987). The heme B was extracted from the protein prior to analysis. The heme contents in both cases were consistently lower by about 15% than the estimates made by quantifying the magnitude of the α-band (Table 2). The lower values could be due to losses incurred during the procedure. Both techniques indicate the presence of more heme C than heme B with a ratio of about 3:2. However, one would expect the oxidase to have a high-spin heme component, which would not have a substantial

Table 2: Metal and Heme Content of the cbb_3-Type Cytochrome *c* Oxidase from *Rb. sphaeroides*

group	content (av of 3 preparations) specific content (nmol/mg)	mol/ (109 000 g of protein)	ratio
Cu[a]	4.8 ● 0.3	0.5	1
Fe[a]	34 ± 7	3.7	7.0
heme B[b]	8.7 ● 2 (9.9)[c]	0.9 (1.08)[c]	1.8 (2.1)[c]
heme C[b]	11.5 ± 3 (14.4)[c]	1.25 (1.57)[c]	2.4 (3.0)[c]

[a] Determined by atomic absorption. See text for details. [b] Determined by the pyridine hemochromagen method (Berry & Trumpower, 1987). [c] The values in parentheses are based on the heme content based on spectrophotometric analysis of the reduced-*minus*-oxidized spectrum of the purified oxidase. The heme B content as determined by the simple analysis of the absorption intensity of the α-band (9.9 nmol/mg) should be less than the value obtained by the pyridine hemochromagen technique (8.7 nmol/mg) since the simple spectrophotometric technique will underestimate contributions from high-spin heme B. The fact that this is not the case may be the result of losses during heme extraction, or due to overlapping spectroscopic contributions from the various heme components, rendering the quantitative analysis based on the absorption in the α-band at two wavelengths inappropriate.

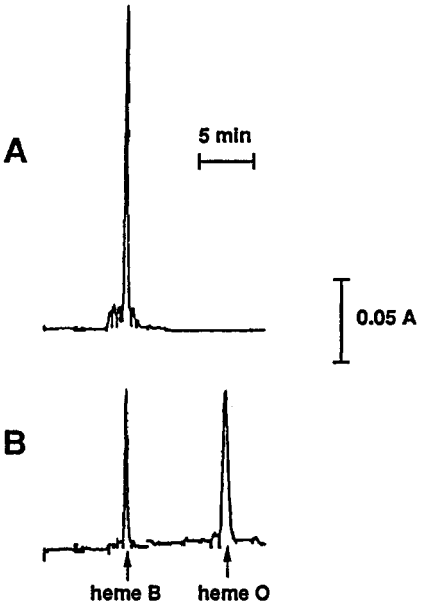

FIGURE 3: Reversed-phase HPLC analysis of the hemes extracted from purified cbb_3-type cytochrome c oxidase from *Rb. sphaeroides*. Elution profile for the hemes from the cbb_3 oxidase (A) and *E. coli* cytochrome *bo* (B) are shown. The elution times for protoheme IX (heme B) and heme O are 5.58 and 13.45 min, respectively. Procedures are given in the text.

absorption in the reduced-*minus*-oxidized spectrum at 562 nm. The presence of a high-spin heme B is suggested by the spectroscopic changes concomitant with CO binding to the reduced oxidase and CN^- binding to the oxidized enzyme (Figure 2C). The spectroscopic changes are consistent in both cases with a single high-spin heme B binding the exogenous ligands. If the oxidase contains 1 equiv each of the three subunits, then the molar extinction coefficients can be estimated for the following wavelength pairs: 415.5 and 436 nm (CO binding), 190 mM^{-1} cm^{-1}; 404 and 420 nm (CN^- binding), 52 mM^{-1} cm^{-1}.

Potentiometric titrations were performed in an effort to resolve the spectroscopic features of the different heme components. The results (not shown) did not clearly resolve the cytochrome *b* and cytochrome *c* components of the oxidase. However, it was demonstrated that all the hemes are fully reduced at a solution potential of 100 mV.

The reduced-*minus*-oxidized spectrum also has a small band centered at 695 nm (Figure 2). This is typical for six-coordinate heme C with methionine as an axial ligand (Pettigrew & Moore, 1987). The intensity of this band is about 20% of the intensity expected per mole of heme C in comparison to horse heart cytochrome *c* (Pettigrew & Moore, 1987). This suggests the possibility that not all of the heme C prosthetic groups in the oxidase have methionine as an axial ligand. Note also that the cbb_3-type oxidase lacks an absorbance band at 820 nm, which is present in the spectrum of the aa_3-type oxidase and is diagnostic of Cu_A (Beinert et al., 1980) (Figure 2).

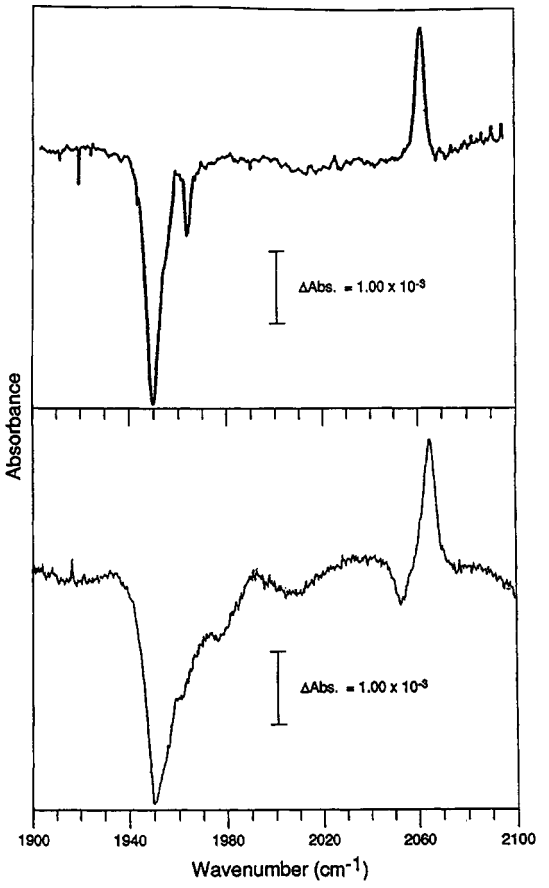

FIGURE 4: Cryogenic FTIR absorbance spectra (light-*minus*-dark) of CO complexes of chromatophores from wild-type *Rb. sphaeroides* (top) and the cbb_3-type cytochrome *c* oxidase purified from *Rb. Sphaeroides* stain JS100 (bottom). Conditions are given in the text.

The iron and copper content of the oxidase is shown in Table 2. These values are the average of three independent preparations. The enzyme was extensively dialyzed against EDTA to minimize contaminating metals, and the oxidase activity was determined to be unchanged as a result of this procedure. The oxidase contains 4.8 nmol of copper and 34 nmol of iron/mg of protein. The iron values varied considerably (±20%) in different preparations and probably include some adventitious metal, despite the dialysis. The iron content is slightly higher than that anticipated from the heme content (34 *vs* 20–25 nmol/mg, respectively). The copper value value is reproducible and represents about 0.5 equiv/mol of oxidase (i.e., 109 000 g of protein).

Low-Temperature FTIR Absorbance Difference Spectroscopy. The question of whether the cbb_3-type oxidase is a member of the heme–copper oxidase superfamily was addressed by examining the CO adduct of the dithionite-reduced enzyme by cryogenic FTIR absorption difference spectroscopy. The light-*minus*-dark difference spectrum is shown in Figure 4. The FTIR spectrum of the CO adduct is taken at cryogenic temperatures (e.g., 15 K) both in the dark and during continuous illumination with white light. In the

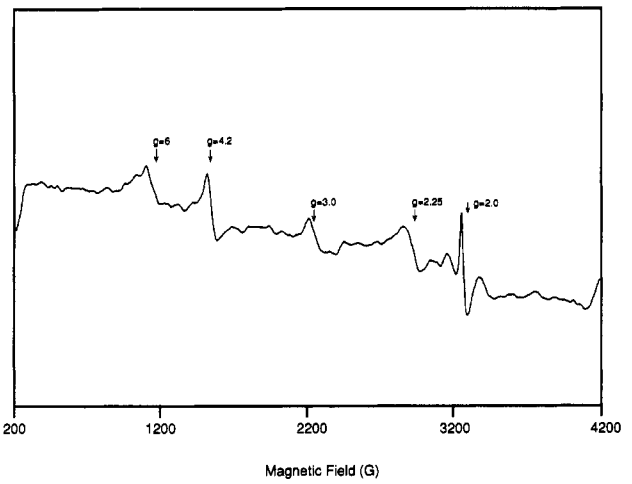

FIGURE 5: Electron paramagnetic resonance spectrum of the cbb_3-type cytochrome *c* oxidase from *Rb. sphaeroides*. The spectrum of the purified *Rb. sphaeroides* EPR spectrum was taken with an air-oxidized sample of the cbb_3-type oxidase. The EPR spectrometer conditions were as follows: Modulation amplitude, 8 G; temperature, 15 K; microwave power, 10 mW; frequency, 9.06 GHz. The cbb_3-type oxidase concentration was approximately 20 μM in 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, and 0.05% dodecyl maltoside.

"dark" spectrum, the CO is bound to the ferrous heme, and the CO stretching frequency is typical of heme Fe—C≡O adducts. Illumination results in the photolytic dissociation of CO from the heme, and in the heme–copper oxidases at the low temperatures utilized, the photolyzed CO binds to Cu_B. The stretching frequency of CO bound to Cu is higher than when bound to the heme Fe, and this shift in the CO infrared band is the most salient feature when the dark spectrum is subtracted from the light spectrum. The trough centered at 1950 cm^{-1} is due to the Fe—C≡O, whereas the peak at 2065 cm^{-1} is typical of Cu—C≡O species. These data conclusively demonstrate that the cbb_3-type oxidase has a heme–copper binuclear center and must be a member of the heme–copper oxidase superfamily.

Also shown in Figure 4 is the FTIR difference spectrum of the CO adduct to chromatophore membranes from a wild-type photosynthetically grown strain (Ga) of *Rb. sphaeroides*. The spectroscopic features are virtually identical to those of the purified cbb_3-type oxidase. The absorption bands from the purified oxidase are more broad than those from the membrane-bound species, probably resulting from some loss of structural integrity upon detergent solubilization and isolation. The well-characterized spectroscopic features of the aa_3-type oxidase are not present in the membranes. These data demonstrate that the alternate cytochrome *c* oxidase is predominant in the membranes of photosynthetically grown *Rb. sphaeroides*.

Electron Spin Resonance Spectroscopy. The FTIR analysis shows that the oxidase must have at least 1 equiv of copper/mol to account for the Cu_B component of the binuclear center. If Cu_A were also present, this would require at least one, and more likely two, additional equivalents of copper. The low amount of copper which is present as measured by atomic absorption and the lack of an 820-nm absorbance band suggest that Cu_A is not present. This was further examined by EPR spectroscopy of the oxidized enzyme. An EPR spectrum of the enzyme is shown in Figure 5. The distinct spectroscopic features near $g = 2$ due to Cu_A which are very apparent in the spectrum of the aa_3-type oxidase from *Rb. sphaeroides* (Hosler et al., 1992) are not observed with the cbb_3-type oxidase. A small free radical signal is present in the spectrum of the cbb_3-type oxidase near $g = 2.0$ whose origin is unknown. The EPR signals from the multiple low-spin hemes in the oxidase are apparent near $g = 3.0$. The spectrum also shows that the cbb_3-type oxidase does not have a large high-spin heme signal near $g = 6$, which is expected since the high-spin heme component of the binuclear center is coupled to the adjacent Cu_B and is EPR silent in the heme–copper oxidases. The EPR data are consistent with the lack of Cu_A, and with the presence of a heme–copper binuclear center. The lack of Cu_A is also consistent with the absence of an absorbance band around 820 nm, which is due to a charge-transfer electronic transition associated with Cu_A (Figure 2B).

DISCUSSION

Previously published work has established that *Rb. sphaeroides* contains two different cytochrome *c* oxidases (Shapleigh et al., 1992). This is most evident from the fact that the deletion of the gene-encoding subunit I of the well-characterized aa_3-type oxidase does not completely eliminate the cytochrome *c* oxidase activity in membrane preparations of aerobically grown cells. The FTIR analysis of the CO adduct formed by the oxidase(s) in these membranes clearly shows that a second heme–copper oxidase, distinct from the aa_3-type oxidase, is present in these membranes. The work presented in this paper describes the purification and characterization of this alternate cytochrome *c* oxidase. The oxidase is shown to have a heme–copper binuclear center, but is different from other well-characterized members of this oxidase superfamily in two respects. (1) The oxidase lacks both heme O and heme A, and hence, the heme component of the binuclear center (heme B) does not have the farnesyl side chain found in other heme–copper oxidases. (2) The oxidase lacks Cu_A, which is found in all other cytochrome *c* oxidases which have been demonstrated to be in this superfamily.

SDS–PAGE analysis shows that the oxidase contains three different subunits: subunit I (45 kDa), subunit II (35 kDa), and subunit III (29 kDa). Both subunits II and III contain covalently bound heme C. Using the subunit molecular weight estimates obtained from the SDS–PAGE analysis, and assuming that the native oxidase contains a single copy of each subunit, the minimal molecular weight of the oxidase is 109 000. The turnover number of the oxidase with horse heart cytochrome *c* is greater than 900 s^{-1}, indicating that the oxidase is a highly active species and is not denatured during purification.

Chemical and spectroscopic analyses indicate the following:

(i) The oxidase contains heme B, which is noncovalently bound, and heme C, which is covalently bound to subunits II and III. Heme O and heme A are not present. The α-band absorption at 562 nm suggests the presence of a low-spin, six-coordinate heme *b*. The adducts formed with CO and CN^- to the reduced and oxidized forms of the enzyme, respectively, indicate the presence of a high-spin heme component, probably heme *b*. Hence, 2 equiv of heme B/mol of oxidase are likely.

(ii) The enzyme must contain at least 2 equiv of heme C/mol of enzyme since heme C is present on both subunits II and III. Heme analysis based on pyridine hemochromogen (Berry & Trumpower, 1987) indicates heme C and heme B are present in a 3:2 ratio. The iron content is accounted for by the hemes, with no evidence of any other iron-containing prosthetic group.

(iii) FTIR analysis indicates unequivocally the presence of a heme–copper binuclear center. This is consistent with the presence of a high-spin heme *b* which is EPR silent, as found. This also indicates a minimum requirement of 1 equiv of copper/mol of oxidase.

(iv) The low copper content, measured by atomic absorption analysis (0.5 equiv/mol of oxidase), suggests that Cu_B is the only copper present in the enzyme and that Cu_A is absent. This is verified by the EPR spectrum and by the absence of an absorption band near 820 nm.

The spectroscopic and analytical data indicate a minimal composition of 1 copper, 2 heme B, and 3 heme C per oxidase molecule. The measured values of both copper and heme are all too low by about a factor of 2: 0.5 copper, 1 heme B, and 1.5 heme C (Table 2). One possible explanation of this discrepancy is that there is a protein impurity remaining in the preparation (e.g., the variable band at about 80 kDa). Some of this impurity, perhaps, aggregates and does not enter the SDS–PAGE gel. In any event, if the heme content is normalized to the minimal requirement of 1 copper per oxidase molecule, the data indicate a ratio of copper:heme B:heme C of 1:2:3. This would be consistent with Cu_B as the only copper present, one high-spin and one low-spin heme B, and three heme C's. This would predict that either subunit II or subunit III would be a diheme cytochrome *c*.

The properties of this oxidase are remarkably close to those expected from the cloned genes of a microaerobically induced oxidase from *Bradyrhizobium japonicum* which have been recently reported (Preisig et al., 1993). The gene cluster encodes a subunit I homologue of the heme–copper oxidases, plus two cytochrome *c*'s which have approximately the same molecular weights of subunits II and III of the microaerobically induced *Rb. sphaeroides* oxidase reported here. Interestingly, one of the cytochrome *c*'s has a sequence strongly suggesting two heme C binding sites. These sequences are consistent with an oxidase containing heme C, heme B, and copper in a ratio of 3:2:1, as found for the *Rb. sphaeroides* oxidase. Since the three subunits of the cbb_3-type oxidase from *Rb. sphaeroides* are likely to correspond the the genes isolated from *B. japonicum* (Preisig et al., 1993), this would indicate that not only is Cu_A absent in this cytochrome *c* oxidase, but that the traditional subunits II and III of most other heme–copper oxidases (i.e., homologues of the mitochondrial oxidase subunits) are also absent. Since these subunits appear to be essential components of most other members of the heme–copper oxidase superfamily (both cytochrome *c* oxidases as well as quinol oxidases), this would be another unique feature of the cbb_3-type oxidase. Further protein sequencing as well as genetic analysis is required to verify this.

This conclusion is given further credibility by the recently reported isolation of a similar cbb_3-type oxidase from another purple photosynthetic bacterium, *Rhodobacter capsulatus*, by Daldal and colleagues (Gray et al., 1994). The biochemical properties of this oxidase are very similar to those reported here for the *Rb. sphaeroides* oxidase, i.e., two heme C-containing subunits, no Cu_A, and no homologues of the mitochondrial oxidase subunits II and III. It seems that *B. japonicum*, *Rb. capsulatus*, and *Rb. sphaeroides* all contain a similar cbb_3-type cytochrome *c* oxidase which has structural features which are distinct from those of other cytochrome *c* oxidases. Of particular interest is the question of whether these oxidases can pump protons as do many of the more traditional cytochrome *c* oxidases. Such experiments are in progress.

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