JOURNAL OF BACTERIOLOGY, Apr. 1992, p. 2338–2343
0021-9193/92/072338-06$02.00/0

Vol. 174, No. 7

Spectroscopic and Genetic Evidence for Two Heme-Cu-Containing Oxidases in *Rhodobacter sphaeroides*

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Received 4 November 1991/Accepted 22 January 1992

It has recently become evident that many bacterial respiratory oxidases are members of a superfamily that is related to the eukaryotic cytochrome *c* oxidase. These oxidases catalyze the reduction of oxygen to water at a heme-copper binuclear center. Fourier transform infrared (FTIR) spectroscopy has been used to examine the heme-copper-containing respiratory oxidases of *Rhodobacter sphaeroides* Ga. This technique monitors the stretching frequency of CO bound at the oxygen binding site and can be used to characterize the oxidases in situ with membrane preparations. Oxidases that have a heme-copper binuclear center are recognizable by FTIR spectroscopy because the bound CO moves from the heme iron to the nearby copper upon photolysis at low temperature, where it exhibits a diagnostic spectrum. The FTIR spectra indicate that the binuclear center of the *R. sphaeroides* aa_3-type cytochrome *c* oxidase is remarkably similar to that of the bovine mitochondrial oxidase. Upon deletion of the *ctaD* gene, encoding subunit I of the aa_3-type oxidase, substantial cytochrome *c* oxidase remains in the membranes of aerobically grown *R. sphaeroides*. This correlates with a second heme-copper oxidase in these membranes revealed by FTIR spectroscopy. When wild-type *R. sphaeroides* is grown photosynthetically, the chromatophore membranes lack the aa_3-type oxidase but have this second heme-copper oxidase. Subunit I of the heme-copper oxidase superfamily contains the binuclear center. Amino acid sequence alignments show that this subunit is structurally very highly conserved among both eukaryotic and prokaryotic species. The polymerase chain reaction was used to show that the chromosome of *R. sphaeroides* contains at least one other gene that is a homolog of *ctaD*, the gene encoding subunit I of the aa_3-type cytochrome *c* oxidase. Presumably, this second gene codes for subunit I of the second heme-copper oxidase.

Rhodobacter sphaeroides is a gram-negative facultative photosynthetic bacterium (8). This bacterium, like many bacteria, has a branched respiratory chain. When *R. sphaeroides* is grown aerobically, the respiratory chain contains a cytochrome *c*-dependent branch and a cytochrome *c*-independent branch (27). At least three terminal oxidases have been detected in *R. sphaeroides*. The aa_3-type cytochrome *c* oxidase has been purified (15), and the structural genes for this protein have been isolated and sequenced (2, 21). A second cytochrome *c* oxidase was recently found by examining strains lacking the aa_3-type oxidase (21). Furthermore, aerobic growth of cytochrome bc_1-deficient strains demonstrated the presence of a third terminal oxidase, which is a ubiquinol oxidase (27). Neither the ubiquinol oxidase nor the alternate cytochrome *c* oxidase contains *a*-type heme (21).

Cloning and sequencing of the genes encoding respiratory oxidases from a variety of bacteria have revealed that many of these oxidases are members of a large oxidase superfamily (14, 19). This superfamily includes cytochrome *c* oxidases and quinol oxidases. Furthermore, a single bacterial species may contain more than one member of this superfamily (13, 17, 22, 23). Oxidases in this family (14, 19) all show homology with the eukaryotic aa_3-type cytochrome *c* oxidase (3–5, 19). The eukaryotic oxidase contains two hemes (one low spin and one high spin) and two coppers (5). One of these coppers is within 0.5 nm of the high-spin heme, and this heme-copper binuclear center is the site of oxygen reduction. Bacterial oxidases that belong to this superfamily also have a low-spin heme and a binuclear center (19). However, they do not all contain *a*-type heme; some contain *b*- and/or *o*-type hemes (6, 16). It is currently unknown whether the alternate cytochrome *c* oxidase or the ubiquinol oxidase of *R. sphaeroides* is a member of this heme-copper oxidase superfamily.

In this study, low-temperature Fourier transform infrared (FTIR) spectroscopy was used to detect and characterize the heme-copper oxidases in the membrane of *R. sphaeroides*. FTIR of carbon monoxide adducts of heme-copper oxidases has provided the strongest evidence of a binuclear center in aa_3-type oxidases (11). CO is a useful probe because of its strong infrared absorption and its high affinity for oxidases. The center frequency and the band shape of the CO stretching mode are very sensitive to the environment (1). In the aa_3-type oxidases, the iron of the high-spin heme a_3 has been identified as the binding site of CO. When the Fe-CO bond is photolyzed at low temperatures, the CO is trapped in the oxygen binding pocket and binds to the copper (Cu_B) (11). Both Fe-CO and Cu-CO have distinct ranges of center frequencies for the CO stretching mode (1). Detection of a Cu-CO signal after photolysis at low temperatures is diagnostic of the heme-copper binuclear center. If no copper were present in the binding pocket, the CO would associate weakly with the protein, as occurs in myoglobin (10). The only other bacterial heme-copper oxidases that have been studied with FTIR are the ba_3 and c_1aa_3 oxidases of *Thermus thermophilus* (9) and the *bo*-type oxidase of *Escherichia coli* (7). These oxidases all give spectra diagnostic of a heme-copper binuclear center. However, the peak center frequencies and band widths are different than those of either the aa_3-type oxidase of *R. sphaeroides* or the beef heart oxidase.

FTIR spectra of CO adducts indicate the presence of two

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heme-copper oxidases in the membranes of *R. sphaeroides* Ga. Genetically modified strains and various growth conditions were used to determine the spectroscopic characteristics of each oxidase. Based on this evidence, the polymerase chain reaction (PCR) was used to isolate a DNA fragment coding for a portion of a subunit homologous to subunit I of the aa_3-type oxidase. Evidence that this second heme-copper oxidase is the alternate cytochrome *c* oxidase is discussed.

MATERIALS AND METHODS

Materials. All reagents used were scientific grade. CO was purchased from Matheson and was 99.5% pure. Glycerol was purchased from Aldrich and was anhydrous grade. Sequenase enzymes and Sequenase kits were purchased from U.S. Biochemical. Taq polymerase was purchased from Perkin-Elmer Cetus.

R. sphaeroides Ga was grown both aerobically and photosynthetically at 30°C in Sistrom medium (8). *R. sphaeroides* JS100 (21) was cultured aerobically in the presence of 50 μg each of streptomycin and spectinomycin per ml. Cells cultured aerobically were grown in the dark with vigorous shaking. Photosynthetic growth was in sealed bottles in a temperature-controlled water bath illuminated by a bank of incandescent lights.

Cells were harvested and lysed by two passages through a French pressure cell at 20,000 lb/in^2. Cell debris was removed by two-low speed spins at 17,000 × *g*. Membranes were pelleted by ultracentrifugation for 2 h at 160,000 × *g*. Membranes were homogenized in 20 ml of 50 mM Tris (pH 7.5) (T buffer). Inner membranes were prepared by using a sucrose step gradient. The gradient was prepared in screw-cap Ti45 tubes (Beckman) by overlaying 30 ml of 55% sucrose with 22 ml of 25% sucrose. Then 10 to 12 ml of the homogenized membranes made up to 5% sucrose was layered on top. The gradient was spun for 10 to 12 h at 40,000 rpm in a Ti45 ultracentrifuge rotor (Beckman). The interface layer containing the inner membranes was removed, diluted fourfold with T buffer, and spun at 160,000 × *g*. Pelleted membranes were resuspended in 2 to 3 ml of T buffer and frozen at −70°C.

The CO adducts were prepared by adding 1 ml of the inner membranes to 14 ml of T buffer in a screw-cap Ti60 tube (Beckman). The tube was sealed with a rubber septum and made anaerobic by repeated cycles of vacuum and argon flushing. Anaerobically prepared sodium dithionite was added to a final concentration of 60 mM by syringe through the septum, and the sample was subjected to cycles of vacuum and argon flushing again. CO was added to the reduced anaerobic membranes by passing 1 atm of CO over the surface of the solution for 10 min. While the sample was under a flow of CO gas, the serum stopper was replaced with the standard Ti60 caps. The membranes were pelleted at 160,000 × *g* for 2 h. The supernatant was decanted under a flow of CO, and the pellet was overlaid with CO-saturated glycerol to dehydrate the sample. The sample was extracted for a minimum of 24 h at 4°C.

A portion of the dehydrated sample was placed between two $CaFl_2$ windows and pressed to the desired thickness. Infrared spectra were obtained with a Mattson Sirius 100 FT-IR interferometer at a resolution of 0.5 cm^{-1}. Temperatures were maintained with a Lake Shore Cryotronics closed-cycle helium refrigerator. Spectra were obtained in the range of 1750 to 3000 cm^{-1} with a liquid N_2 cooled indium atinimide detector to detect both bound and photolyzed CO. Interferograms were detected in a single-beam mode. Spectra are presented as difference spectra, for which the spectrum before photolysis (dark) is subtracted from the spectrum obtained during photolysis (light). Photodissociation was achieved by using continuous radiation from a focused 500-W tungsten bulb. Heat and UV radiation from the lamp were attenuated by passage through water and glass. Light spectra were begun after 10 min of sample illumination. Light and dark spectra are the averages of 512 scans. Peak areas were determined by cutting out and weighing each peak.

For Western immunoblotting, proteins were separated with sodium dodecyl sulfate–10% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Nonspecific binding to the membranes was blocked with 3% gelatin. Antibodies used to detect subunit I were raised against the aa_3 complex of *Paracoccus denitrificans* and were previously shown to react to subunit I of the aa_3-type oxidase of *R. sphaeroides* (15). The antibodies were kindly provided by Bernd Ludwig (Lubeck, Germany). The anti-subunit I antibodies were detected by using anti-rabbit immunoglobulin G and then rabbit peroxidase anti-peroxidase (Bio-Rad).

PCR amplification was carried out with two primers: bc2e, 5′ GCGCGAATTC(GC)AC(GA)TA(AG) TGGAA(AG)TG (CG)GC(GC)AC 3′; and bc1h, 5′ GCGCAAGCTTTTCGGC CA(TC)CCGGA(AG)GT(GC)TAC 3′. Both primers have a GC cap and restriction enzyme recognition sequences 5′ of the binding region. Bc2e has an *Eco*RI site, and bc1h has a *Hin*dIII site. These primers are from regions containing the conserved histidines in putative membrane-spanning helices VI and X in subunit I. These are residues 282 through 288 and 417 through 423, respectively, of the *R. sphaeroides* subunit I sequence (21). Temperature cycles for amplification were 1 min of denaturation at 92°C, annealing at 38°C for 3 min, and 2 min of amplification at 75°C for a total of 30 cycles. The resulting fragment was purified by using low-melting-point agarose (SeaPlaque) and cloned into pT7T3-18U (Pharmacia). The sequence was obtained from single-stranded DNA by the dideoxy-chain termination method (18).

RESULTS

The FTIR difference spectrum of inner membranes of aerobically grown *R. sphaeroides* Ga is shown in Fig. 1A. Inner membranes were used because crude membrane preparations bound little CO under these conditions and absorbance peaks were often indistinguishable from background noise. The two absorption bands that point downward (Fig. 1) are caused by CO bound to a ferrous heme. These spectra are light-minus-dark difference spectra, meaning that the spectrum taken during photolysis was subtracted from the spectrum taken after photolysis. The difference is caused by the movement of CO from the heme to the copper upon photolysis. Hence, the spectroscopic features caused by Fe-CO point downward in this representation. The absorption bands that point upward are due to photolyzed CO and represent CO bound to copper. The two Fe-CO absorbance bands have maxima at 1950 and 1964 cm^{-1} (Table 1). The band at 1964 cm^{-1} is very narrow with a band width of 2.4 cm^{-1} at half-peak height. The 1950-cm^{-1} band has a width of 7.0 cm^{-1} at half-peak height. The Cu-CO absorbance peaks are at 2039 and 2060 cm^{-1}. The 2060-cm^{-1} band is asymmetric, with a shoulder at about 2064 cm^{-1}.

Figure 1B shows the FTIR difference spectrum of inner

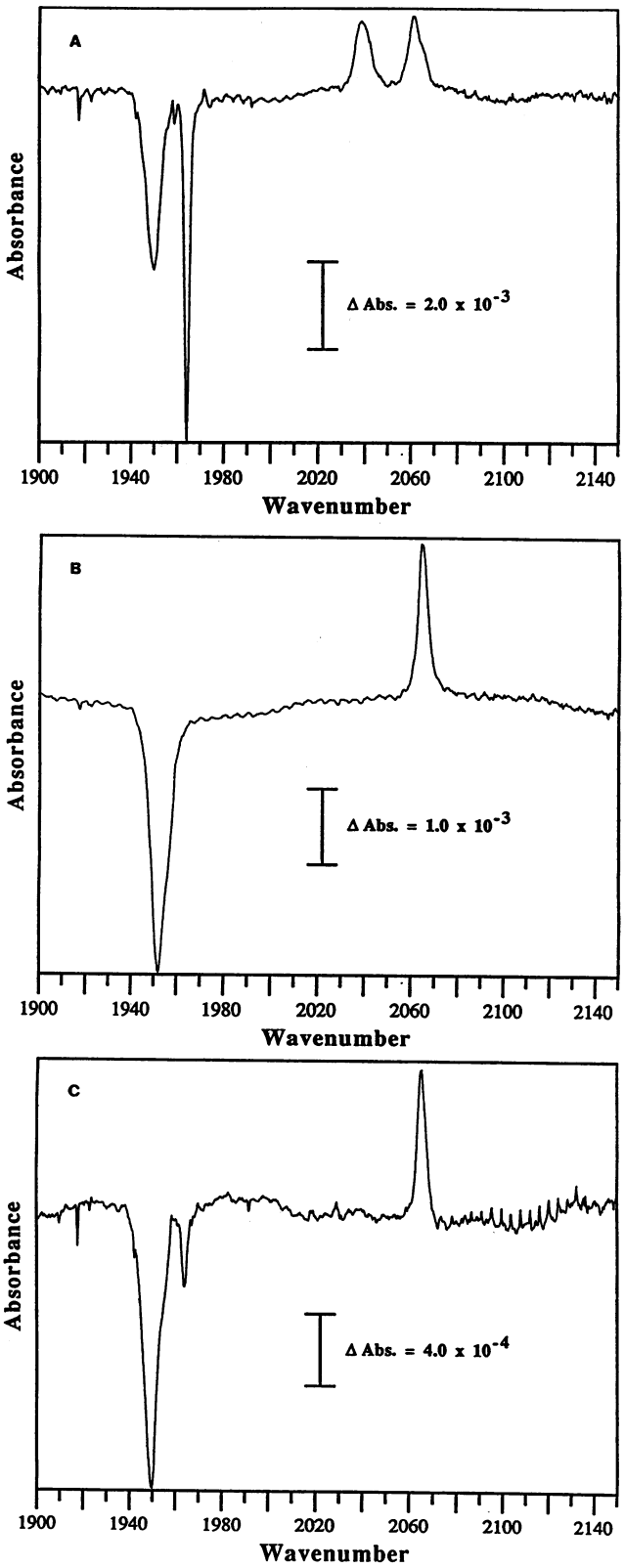

FIG. 1. (A) Infrared absorbance spectra (light minus dark) of CO complexes of glycerol-extracted inner membranes of aerobically grown *R. sphaeroides* Ga. Fe-CO bands are centered at 1950 and 1964 cm^{-1}, and CuCO bands are centered at 2039 and at 2060 cm^{-1}. (B) Infrared absorbance spectra (light minus dark) of CO complexes of glycerol-extracted inner membranes of *R. sphaeroides* JS100. JS100 does not express the aa_3-type oxidase because of a deletion of the gene encoding subunit I of the complex (21). The major Fe-CO peak is at 1952 cm^{-1}, and the major Cu-CO peak is at 2064 cm^{-1}. (C) Infrared absorbance spectra (light-minus-dark) of CO complexes of inner membranes of photosynthetically grown *R. sphaeroides* Ga. The major Fe-CO band is centered at 1950 cm^{-1}, and the minor Fe-CO band is centered at 1965 cm^{-1}. The Cu-CO band is centered at 2065 cm^{-1}. All spectra were recorded at 10 K.

TABLE 1. Infrared absorption band center frequencies of cytochrome oxidases in glycerol at 10 K

Source of cytochrome oxidase	ν (cm^{-1})	
	Fe-CO	Cu-CO
Ga (aerobically grown)[a]	1950, 1964	2039, 2060
Ga (photosynthetically grown)[a]	1950	2065
JS100 (aa_3 deletion mutant)[a]	1952	2064
Mitochondria[b]		
α form	1964	2055, 2065
β forms	1945, 1950	2039

[a] Data from this work.
[b] Data from reference 11.

membranes prepared from JS100, which is an aa_3-type cytochrome *c* oxidase-deficient strain because of a deletion of the *ctaD* gene (21). There is a major Fe-CO absorbance peak at 1952 cm^{-1} and one Cu-CO absorbance peak at 2064 cm^{-1} (Table 1). The lack of the 1964-cm^{-1} band in this spectrum indicates that this absorbance in the parent strain (Ga) is due to the aa_3-type oxidase. This frequency is identical to the 1964-cm^{-1} FeCO absorbance peak of the α form of the bovine mitochondrial oxidase (11).

The Cu-CO regions of strains Ga (wild type) and JS100 (aa_3 deletion mutant) are shown in Fig. 2. The 2060-cm^{-1} region of Ga is a composite of peaks from the aa_3-type oxidase and another heme-copper-containing oxidase. The aa_3-type oxidase has two CO absorption peaks at 2039 and 2060 cm^{-1}. The other heme-copper oxidase has only one Cu-CO absorbance peak at 2065 cm^{-1}. This appears as a shoulder in wild-type CO difference spectra.

The FTIR difference spectrum of photosynthetically grown Ga is shown in Fig. 1C. The major absorbance bands are almost identical to those in JS100 (aa_3 deletion mutant) (Table 1). There is a small Fe-CO absorbance peak at 1,965 cm^{-1} that may be from the aa_3-type oxidase, although there is no evidence for Cu-CO absorbance bands from this oxidase. A Western blot of membranes from aerobically and photosynthetically grown Ga and aerobically grown JS100 is shown in Fig. 3. Subunit I of the aa_3-type oxidase is lacking in photosynthetically grown Ga and JS100. The FTIR and Western blot assays show that the aa_3-type oxidase either is not present or is expressed at very low levels under photosynthetic conditions.

FTIR of CO complexes of beef heart cytochrome *c* oxidase reveals two molecular forms that have been designated α and β (11). The Fe-CO absorbance band of the α form has the same center frequency and band width as the 1964-cm^{-1} band in *R. sphaeroides* Ga (11). The β form has two major Fe-CO absorbance peaks at 1945 and 1950 cm^{-1} (11) (Table 1). The presence of a second oxidase that absorbs in the 1950-cm^{-1} region makes it difficult to determine whether

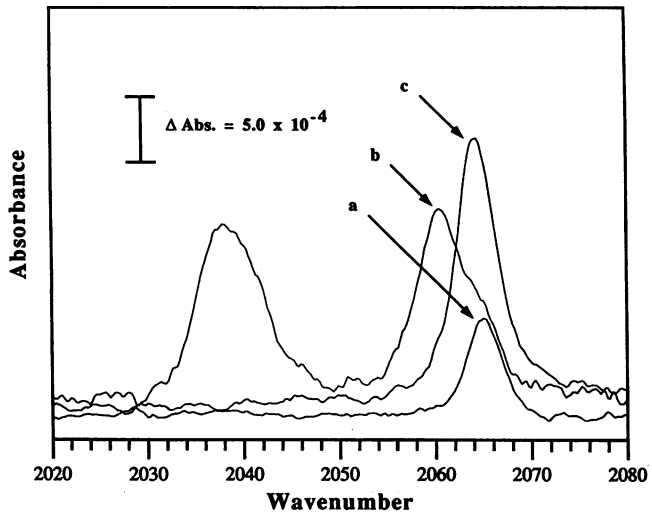

FIG. 2. Scale expansion of the Cu-CO absorbance bands of photosynthetically grown Ga (a), aerobically grown Ga (b), and aerobically grown JS100 (c). The spectra were recorded at 10 K and adjusted to the same absorbance scale.

Fe-CO bands analogous to the β forms are present in the Ga spectra.

To determine whether there are additional bands in this region of the spectrum, the band areas were determined. Band area is directly related to the amount of bound CO (2). Therefore, the areas of the Fe-CO bands and the Cu-CO bands of heme-copper oxidases should reflect the 1:1 stoichiometry of these species. This has been demonstrated in beef heart oxidase, in which the relative ratios of the band areas of the α and β forms of the oxidase change between samples but in which the ratio of the total Cu-CO band area to the total Fe-CO band area is always approximately the same (11). In spectra of both aerobically and photosynthetically grown *R. sphaeroides* Ga, the ratio of the total area of the Cu-CO bands to the total area of the Fe-CO bands is the same as that in the beef heart enzyme, indicating 1:1

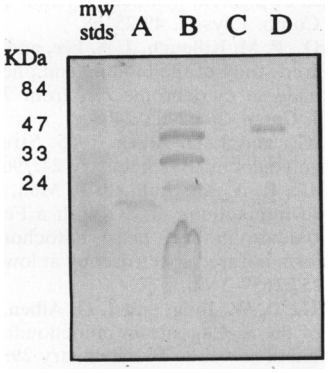

FIG. 3. Western blot of membranes from photosynthetically grown *R. sphaeroides* Ga (A), purified *P. denitrificans* aa_3-type oxidase (B), membranes from the JS100 mutant (C), and membranes from aerobically grown Ga (D). The polyclonal antibodies were raised against the aa_3-type oxidase of *P. denitrificans* and cross-reacted with subunit I of the aa_3-type oxidase in *R. sphaeroides*. About 40 μg of protein was added to each of lanes A, C, and D, and 5 μg of protein was added to lane B.

TABLE 2. Ratios of areas under CO vibrational absorption bands in cytochrome oxidases

Source of cytochrome oxidase	ΣCu-CO/ΣFe-CO
Ga (aerobically grown)[a]	0.42
Ga (photosynthetically grown)[a]	0.32
JS100 (aa_3 deletion mutant)[a]	0.37
Mitochondria[b]	0.37–0.43

[a] Values were calculated from data shown in Fig. 1.
[b] Values were calculated from data in reference 11.

stoichiometry (Table 2). If it is assumed that the 1964-cm^{-1} band is the only Fe-CO absorption peak from the aa_3-type oxidase, then the ratio of the total Cu-CO area to that of the 1964-cm^{-1} band should be similar to the ratio calculated for the beef heart enzyme. However, it is larger by a factor of 2. This discrepancy is not due to the Cu-CO band of the second heme-copper oxidase. The Cu-CO band at 2065 cm^{-1} from this component is estimated to account for only about 13% of the total intensity of the band centered at 2060 cm^{-1} (Fig. 2). There is not enough of the 1964-cm^{-1} Fe-CO species to give 1:1 stoichiometry with the CO bound to the two Cu-CO forms of the aa_3-type oxidase. This suggests that there must be one or more additional Fe-CO bands associated with the aa_3-type oxidase. These additional Fe-CO bands probably account for the majority of the absorbance at 1950 cm^{-1} (Fig. 1A), since the second heme-copper oxidase is present only as a minor component. This 1950-cm^{-1} band is similar to the β form of the mammalian aa_3-type oxidase (11).

There is significant homology among the amino acid sequences of subunits of the heme-copper oxidases (22). The highest levels of homology are found in subunit I of the complex, which contains the residues that are the ligands of both hemes and the copper in the binuclear center. Since the FTIR spectra indicate two heme-copper oxidases in *R. sphaeroides*, PCR probes with sequences based on the most highly conserved regions of subunit I were synthesized. These were used to probe chromosomal DNA from JS100, which lacks these regions of *ctaD*, the gene encoding subunit I of the aa_3-type oxidase (24). Amplification with these probes yielded a single fragment of approximately 350 bp. This was cloned and partially sequenced, and the deduced amino acid sequence is shown in Fig. 4. This sequence shares 70% identical residues with subunit I of the aa_3-type oxidase of *R. sphaeroides*. The G+C content of the DNA sequence is 60%, consistent with the high G+C content of the *R. sphaeroides* sequence (18). The G+C content of *ctaD* over the same stretch of residues is 62%.

DISCUSSION

Two oxidases with heme-copper binuclear centers were detected in *R. sphaeroides* by using FTIR spectroscopy of

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1 EVYILILPGFGMISQIVSTFSRKPVFGYLGMAYAMVALGFLGFIVWAH
  |||| || || |   || || |||| | ||||| | ||| ||||
2 EVYIIVLPAFGIVSHVIATFAKKPIFGYLPMVYAMVAIGVLGFVVWAH

1 HMYTVGMSSASTAYFVAATMVIAVPTGVKIFSWIATMWAARLNSYSES
  |||| | |    ||  |||||||||| ||||||||||
2 HMYTAGLSLTQQSYFMMATMVIAVPTGIKIFSWIATMWGGSIELKTPM
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FIG. 4. Alignment of the deduced amino acid sequences of the fragment derived by PCR from the strain in which the gene encoding subunit I of the aa_3-type oxidase has been deleted (rows 1) and the same region of the gene encoding subunit I of the aa_3-type oxidase (rows 2). Vertical lines indicate identical residues.

CO-oxidase adducts. With a strain that was deficient in the aa_3-type oxidase, the absorbances of CO bound to each oxidase were distinguished. The aa_3-type oxidase has a major Fe-CO absorbance peak at 1964 cm^{-1}. This band is very narrow, with a width of 2.4 cm^{-1} at half-peak height. This narrow band width indicates a highly ordered or nonpolar environment in the local environment of the high-spin heme (1).

The deletion of the *ctaD* gene eliminates the 1964-cm^{-1} Fe-CO band and both the 2038- and 2060-cm^{-1} Cu-CO bands in *R. sphaeroides*. Apparently, the molecular forms of the enzyme responsible for these bands are encoded by one gene. This conclusion is supported by the lack of these bands in the spectra of photosynthetically grown Ga. Cells grown under these conditions do not express detectable levels of the aa_3-type oxidase. In mammalian mitochondria, it has been suggested that the α and β forms are isozymes (12). However, given the similarity of the *R. sphaeroides* and mammalian aa_3-type oxidases, it is likely that the mitochondrial α and β forms are both encoded by a single gene. It is unclear what causes multiple molecular forms of these oxidases. One possibility is that the α and β forms represent different quaternary states of the enzyme (e.g., monomer or dimer). Another possibility is that there are differences in ligands of the metal centers or residues in the local environment of the binuclear center as a result of different conformational states of the enzyme.

The spectroscopic similarities between the aa_3-type oxidases of the *R. sphaeroides* and mammalian mitochondria are striking. The center frequency and band width of the CO stretching mode are very sensitive indicators of the environment of the CO binding pocket (1). The spectra indicate a very similar binding pocket for both of these oxidases. This suggests not only that the metal ligands of the binuclear center are the same in both systems but also that the local molecular environments are similar. The subunit composition of the *R. sphaeroides* enzyme is much simpler than that of the beef heart enzyme; 3 and 13 subunits, respectively, are found in each complex (3). The subunits in the mitochondrial oxidase which are encoded in the nucleus appear to have no role in the structure of the binuclear center pocket, since the three-subunit bacterial complex gives an almost identical FTIR spectrum. These data are also consistent with the hypothesis that the progenitor of the mitochondria came from the α subdivision of the purple sulfur bacteria (25), which includes *R. sphaeroides* (24).

Cells in which the gene encoding subunit I of the aa_3-type cytochrome *c* oxidase has been deleted still possess high levels of cytochrome *c* oxidase activity (21). There is no detectable *A*-type heme in these cells, indicating that this alternate cytochrome *c* oxidase is probably an *o*-type oxidase. Photosynthetically grown cells also possess significant cytochrome *c* oxidase activity but no detectable heme *A* (20). The cytochrome *c* oxidase activities of the deletion strain and of cells grown photosynthetically have very similar sensitivities to cyanide, about 10 μM (23; unpublished data), which is similar to the cyanide sensitivity of the aa_3-type oxidase. Heme-copper oxidases are, in general, very sensitive to cyanide. This may be due to cyanide binding with high affinity to the copper (26) of the binuclear center as well as the heme. It is likely that the alternate cytochrome *c* oxidase is the heme-copper oxidase detected by FTIR in JS100 and in photosynthetically grown strain Ga.

The DNA fragment generated by PCR from the *ctaD* deletion strain (JS100) shows that more than one gene encoding a subunit I-type protein is present in *R. sphaeroides*. It is currently not known whether this fragment is from a gene encoding an expressed protein. In the closely related bacterium *P. denitrificans*, two genes encoding subunit I-type proteins were sequenced (17). However, the functions of these two polypeptides are not clear. It is tempting to speculate that one of these genes (*ctaDII*) codes for the aa_3-type oxidase subunit I and that the other (*ctaDI*) codes for a second heme-copper oxidase, as may be the case in *R. sphaeroides*.

ACKNOWLEDGMENTS

We thank Bernd Ludwig (University of Lübeck, Lübeck, Germany) for providing the antibodies and the sample of *P. denitrificans* oxidase used in this work and C. Russ Hille (Ohio State University) for the use of his laboratory and facilities.

This work was supported by Public Health Service grant HL16101 (to R.B.G.) from the National Institutes of Health, by a grant from the Human Frontier Science Program (to R.B.G.), and by grant DMB89-04614 (to J.O.A.) from the National Science Foundation.

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