Identification of the Structural Subunits Required for Formation of the Metal Centers in Subunit I of Cytochrome *c* Oxidase of *Rhodobacter sphaeroides*†

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ABSTRACT: Genetic manipulation of the aa_3-type cytochrome *c* oxidase of *Rhodobacter sphaeroides* was used to determine the minimal structural subunit associations required for the assembly of the heme A and copper centers of subunit I. In the absence of the genes for subunits II and III, expression of the gene for subunit I in *Rb. sphaeroides* allowed purification of a form of free subunit I (subunit I_a) that contained a single heme A. No copper was present in this protein, indicating that the heme a_3-Cu_B active site was not assembled. In cells expressing the genes for subunits I and II, but not subunit III, two oxidase forms were synthesized that were copurified by histidine affinity chromatography and separated by anion-exchange chromatography. One form was a highly active subunit I−II oxidase containing a full complement of structurally normal metal centers. This shows that association of subunit II with subunit I is required for stable formation of the active site in subunit I. In contrast, subunit III is not required for the formation of any of the metal centers or for the production of an oxidase with wild-type activity. The second product of the cells lacking subunit III was a large amount of a free form of subunit I that appeared identical to subunit I_a. Since significant amounts of subunit I_a were also isolated from wild-type cells, it is likely that subunit I_a will be present in any preparation of the aa_3-type oxidase isolated via an affinity tag on subunit I.

Cytochrome *c* oxidase is a multisubunit protein complex that uses energy derived from electron transfer from cytochrome *c* to O_2 to effect transmembrane charge separation in mitochondria and many aerobic bacteria (*1*). The three largest subunits of the mitochondrial oxidase are integral membrane proteins encoded by mitochondrial DNA (*2*) that form the "catalytic core" of the enzyme (*3*, *4*). Mitochondrial cytochrome oxidase contains up to 10 additional, nuclear-encoded subunits (*3*); the function of these is largely undetermined. The proteobacteria *Rhodobacter sphaeroides* and *Paracoccus denitrificans* each synthesize an aa_3-type cytochrome oxidase (*5*, *6*) with a structure that is nearly identical to the three-subunit catalytic core of the mitochondrial oxidase (*7*−*11*). In both the mitochondrial oxidase and these bacterial oxidases, reduced cytochrome *c* binds to subunit II to transfer its electron to the dicopper Cu_A site in subunit II (*12*, *13*). The electron is then transferred to the six-coordinate heme *a* center in subunit I and then onto the five-coordinate heme a_3-Cu_B active site in subunit I (*14*). Molecular oxygen binds to reduced heme a_3 and accepts four electrons and four protons to form two water molecules. Subunit III binds no metals and thus does not participate directly in electron transfer.

The assembly of cytochrome oxidase is beginning to be understood in terms of the order of subunit association. Little is known, however, about the formation of the metal centers. Recent experiments in human cells have established an assembly pathway in which the first intermediate is a free form of subunit I (*15*). Accumulation of free subunit I has also been demonstrated in *Neurospora*, pea, and rat mitochondrial membranes (*16*−*18*). The metal content of this free form of subunit I has not been established. The fact that the addition of exogenous heme A speeds the association of subunit I with subunits II and III has led to the proposal that both hemes of subunit I are inserted before it binds to the other two core subunits (*15*, *19*). However, the apparent absence of heme a_3 from a mutant oxidase in which the interaction between subunits I and II is disrupted suggests that subunit I does not contain both of its heme A centers before it binds with subunit II (*20*).

It remains unclear whether subunit III, the only metal-free subunit of the catalytic core, plays any role in the assembly of the metal centers of subunits I and II. A recent study determined that one role of subunit III is prevention of suicide inactivation of the oxidase, apparently by protect-

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ing the structure of the active site during the oxygen reduction cycle (*21*). This observation indicates that subunit III maintains long-distance interactions that affect the structure of the active site. A reasonable extension of this is that subunit III plays a role in the assembly of the metal centers of subunit I. Indeed, subunit III has been proposed to be necessary for the proper alignment of the heme *a* center (*22*), although at least one preparation of a two-subunit oxidase synthesized in the absence of subunit III showed an apparently normal heme *a* (*23*). It has also been proposed that the addition of subunit III, as a final step in oxidase assembly, activates electron transfer between heme *a* and the heme a_3−Cu_B center (*24*).

To directly test the requirement for subunit interactions in the formation of the oxidase metal centers, we have expressed the gene for subunit I independently or in combination with the gene for subunit II. The oxidase forms resulting from these manipulations have been purified and characterized. With expression of the gene for subunit I only, *Rb. sphaeroides* cells produced a free form of subunit I that contained a single heme A and lacked an intact heme a_3−Cu_B active site. Expression of the genes for both subunits I and II revealed that the formation of a complete heme a_3−Cu_B active site in subunit I required the association of these two structural proteins. Finally, the results confirm that subunit III is not required for the normal assembly of any of the metal centers of cytochrome oxidase or for the production of an oxidase with wild-type activity.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors. The expression of ΔCoxIII (described below) was driven by pMA120H, a plasmid that contains the genes for subunits I and II (*coxI* and *coxII*) and the genes for assembly factors Cox10p and Cox11p (*cox10* and *cox11)*, but not the gene for subunit III (*coxIII*) (*25*, *26*). *CoxIII* was deleted from the *coxII/III* operon (*26*) by removing a 956 bp *Sma*I fragment that contained all of *coxIII* from pYJ100 (*27*) to create pCF200. The truncated *coxII/III* operon was removed from pCF200 on a 3.3 kb *Pst*I−*Sma*I fragment and cloned into pJS3-X6H, a plasmid containing a version of *coxI* encoding a six histidine tag at the C-terminus (*28*), to create pMB307. *CoxI* and the truncated *coxII/III* operon are located on opposite DNA strands in pMB307. A 5.3 kb *Eco*RI−*Hin*dIII fragment containing *coxI*, *coxII*, *cox10*, and *cox11* of pMB307 was transferred to the broad-host range vector pRK415-1 (*29*) to yield the expression vector pMA120H. Conjugation of pMA120H into YZ200, a strain of *Rb. sphaeroides* in which the *coxII/III* operon has been deleted (*27*), yielded strain MA120H.

Expression of subunit I_a (described below) was driven by pLH361, a derivative of pMA120H in which a 210 bp in-frame deletion was created within *coxII* (*30*) by removing an *Xho*I fragment. This deletion removed amino acid residues 30−99 of subunit II, leaving full-length copies of *coxI*, *cox10*, and *cox11*. Conjugation of pLH361 into *Rb. sphaeroides* YZ200 created strain LH361. Expression of subunit I_a was also driven by pLH364, a vector that contains full-length copies of *coxI* and *cox10*, but not *cox11*. The creation of pLH364 began with the removal of the 956 bp *Sma*I fragment containing *coxIII* from pLAJ200, a pUC-based vector containing both oxidase operons and an in-frame deletion in *cox11* (*31*). A 5 kb *Eco*RI−*Hin*dIII fragment containing *coxI*, *coxII*, *cox10*, and the inactive version of *cox11* was cloned into pRK415-1 to make pLH334. The removal of the 210 bp *Xho*I fragment from *coxII*, as described for pLH361, created pLH364. Conjugation of pLH364 into *Rb. sphaeroides* YZ200 created strain LH364.

Bacterial Growth, Cytoplasmic Membrane Isolation, and Oxidase Purification. *Rb. sphaeroides* strains MA120H, LH361, LH364, as well as YZ300, which expresses the normal three-subunit oxidase (*27*), were grown as in Bratton et al. (*21*). Cytoplasmic membranes were prepared as previously described (*6*). The oxidase forms were initially purified from the membranes as in Zhen et al. (*27*) by affinity chromatography on Ni^{2+}-NTA[1] agarose.

High-Resolution Anion-Exchange Chromatography. Affinity-purified cytochrome oxidase was briefly incubated in dodecyl maltoside at a milligram of detergent to milligram of protein ratio of 40 in a buffer composed of 10 mM Tris-HCl, pH 8.0, and 20 mM KCl. The oxidase samples were then loaded onto a DEAE−5PW column (7.5 cm × 8 mm, Toso-Haas) previously equilibrated in 10 mM KH_2PO_4, pH 7.8, 1 mM EDTA, 0.1% dodecyl maltoside, using a Biologic HR chromatography system (Bio-Rad). Following a 20 mL wash with this buffer, the column was developed with a 35 mL gradient of 0 to 0.4 M KCl in the same buffer. Fractions containing protein were collected and stored at −80 °C.

Oxygen Reduction Activity and Ligand Binding. Measurements of the initial rates and V_{max} values of O_2 reduction were performed as in Bratton et al. (*21*). Carbon monoxide (CO) and cyanide (CN^-) binding were performed as previously described (*21*).

Copper, Iron, and Heme A Content. Oxidase samples for metal content analysis were treated with 25 mM EDTA in a buffer of 10 mM Tris-HCl, pH 8.0, 40 mM KCl, and then washed in this same buffer without EDTA, using a 50 kDa molecular mass cutoff Ultrafree-4 filtration device (Millipore), until the EDTA concentration was below 1 mM. Samples were concentrated to 10−25 μM and shipped to the University of Georgia Chemical Analysis Laboratory for inductively coupled plasma atomic emission spectroscopy (ICP-AES).

The heme A to protein stoichiometry of each of the purified oxidase forms was determined using the pyridine hemochrome method to measure the amount of heme A (*6*, *32*) and the absorbance at 280 nm to determine the amount of protein. Extinction coefficients for the 280 nm absorbance (as $A_{280nm} - A_{312nm}$) of each oxidase form were calculated by summing the extinction values for tryptophan and tyrosine (*33*); the number of these residues was taken from the DNA-derived amino acid sequences of subunits I, II, and III (*25*, *26*, *30*). An extinction coefficient of 153 mM^{-1} cm^{-1} was used for subunits I_a and I'_a; 215 mM^{-1} cm^{-1} for the I−II oxidase, 354 mM^{-1} cm^{-1} for the normal three-subunit oxidase, and 368 mM^{-1} cm^{-1} for ΔCoxIII (assuming equal amounts of subunit I'_a and the I−II oxidase in ΔCoxIII). Protein concentrations determined by UV absorbance were found to be in reasonable agreement (±8%) to concentrations

[1] Abbreviations: NTA, nitrilotriacetic acid; ICP-AES, inductively coupled plasma atomic absorption spectroscopy; EPR, electron paramagnetic resonance; BCA, bicinchoninic acid.

Table 1: Activity, Optical Analyses, Heme A Content, and the Cu/Fe Value of Five Purified Oxidase Forms

oxidase form	V_{max} of O_2 reduction (s^{-1})	α peak maxima (nm)	Soret peak maxima (nm)	Soret/α (amplitude)	heme A/ protein[a]	Cu/Fe[a]
normal	1983	605	444	5.3	2.2 ± 0.2 (3)	1.42 ± 0.16 (7)
ΔCoxIII	1891	603	442	4.9	2.9 ± 0.2 (3)	0.88 ± 0.13 (6)
I−II oxidase	1955	605	444	5.2	1.9 ± 0.1 (3)	ND[b]
subunit I'_a	0	599	440	4.6	1.2 ± 0.2 (5)	0 (1)
subunit I_a	0	599	440	4.6	0.9 ± 0.2 (4)	0 (1)

[a] The number in parentheses is the number of independent determinations. [b] Not determined.

determined by the more laborious BCA assay (Pierce). Previous studies of the *Rb. sphaeroides* oxidase (*6*) show that the BCA assay and a modified Lowry assay are no more accurate than the UV absorbance method. The UV absorbance method was also used in the calculation of the extinction coefficients of the Soret and α-band transitions of subunits I_a and I'_a.

RESULTS

Expression, Purification, and Characterization of Free Subunit I. To test the minimal subunit composition necessary for metal center assembly in subunit I of the aa_3-type oxidase of *Rb. sphaeroides*, the gene for subunit I (*coxI*) was expressed in the absence of the genes for subunits II and III. All of the genes coding for cytochrome oxidase assembly factors were present in either the expression vector or in the bacterial genome. This was accomplished by creating *Rb. sphaeroides* LH364 or LH361, strains that contained episomal copies of the genes for subunit I and the assembly factors Cox10p or both Cox10p and Cox11p, respectively, under control of their natural promoters. The genes for subunits II and III were deleted (see Experimental Procedures). [It should be noted that the expression of a mutant aa_3-type cytochrome oxidase with no activity does not affect aerobic growth of *Rb. sphaeroides*, since the bacterium constitutively expresses a cbb_3-type cytochrome *c* oxidase with redundant function (*34*)]. The oxidase forms expressed from LH361 and LH364 were spectroscopically indistinguishable and were termed "subunit I_a". Spectra of purified cytoplasmic membranes of cells expressing subunit I_a showed a significant α-band at 602 nm (Figure 1), indicating the accumulation of a cytochrome containing heme A. Subunit I_a was isolated from purified cytoplasmic membranes by virtue of a six histidine tag at its carboxy-terminus and was shown to have full-length subunit I (Figure 2). The purified protein exhibited no oxygen reduction activity.

The metal center content of subunit I_a was determined. In contrast to subunit I of the normal oxidase, which contains two heme A centers (*35*), subunit I_a contained only one heme A molecule (Table 1). Metal analysis showed that subunit I_a contained a single equivalent of iron, consistent with the presence of a single heme A, and no copper (Table 1). Thus, the heme a_3-Cu_B active site was clearly not intact in subunit I_a.

The environment of the heme in subunit I_a was examined by optical and EPR spectroscopy and by ligand binding assays. The optical spectrum of reduced cytochrome oxidase is dominated by the absorption of low-spin heme *a* and high-spin heme a_3 (*36*, *37*); the α band at 605 nm is primarily due to heme *a*, while the larger Soret peak at 444 nm results from roughly equal absorbance by both heme centers (*6*, *36*−*38*). The absolute reduced spectrum of subunit I_a does show a significant α band (Figure 3A). The Soret to α amplitude ratio of subunit I_a (4.6; Table 1) is greater than that of low-spin heme *a* in the normal oxidase (Soret/α ≈ 3), but distinctly different from the Soret to α value of high-spin heme a_3 (Soret/α ≈ 17) (*36*). Extinction coefficients of 86 and 20 mM^{-1} cm^{-1} were calculated for the Soret and α transitions, respectively, for subunit I_a (see Experimental Procedures). These values are significantly lower than the corresponding values for either heme *a* or heme a_3 of the normal oxidase (*36*, *38*). Similar to the extinction coefficients of reduced subunit I_a, the extinction coefficient of the Soret transition of oxidized subunit I_a was lower than either normal heme *a* or heme a_3 (*36*, *38*). This argued against the possibility that the low extinction values were due to incomplete reduction of the heme of subunit I_a. Another indication that the environment of the heme of subunit I_a was abnormal was that its α and Soret peaks were blue-shifted by 4−6 nm (Figure 3A and Table 1). In the complete oxidase, the position of these peaks is strongly influenced by the strength of a hydrogen bond between an arginine of subunit I and the formyl group of heme *a* (*39*−*41*). The EPR spectrum of subunit I_a was similar to one previously published for a preparation of free subunit I isolated from *P. denitrificans* TN-57, a strain that lacks the gene for subunit III (*42*). A broad low-spin heme

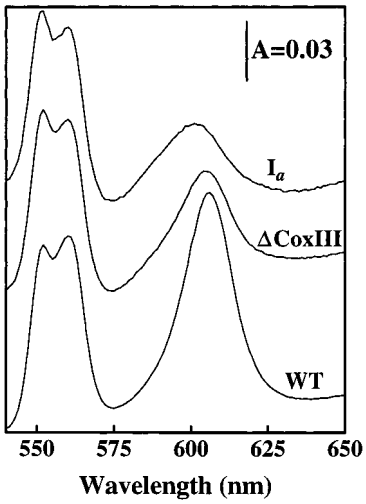

FIGURE 1: Cytochrome content of purified *Rb. sphaeroides* cytoplasmic membranes containing the normal oxidase (WT), ΔCoxIII, and subunit I_a. Dithionite-reduced minus ferricyanide-oxidized spectra of membranes from YZ300 (WT), MA120H (ΔCoxIII), and LH361 (I_a) were recorded using a Hitachi U-3010 dual beam spectrophotometer. The α peaks ~600−606 nm arise from the aa_3-type oxidase forms in the membranes, while the α peaks at 560 and 550 nm are due to membrane-bound *b* and *c* cytochromes, respectively. The three spectra have been normalized so that the amplitudes of the *b*-cytochrome peaks at 560 nm are the same.

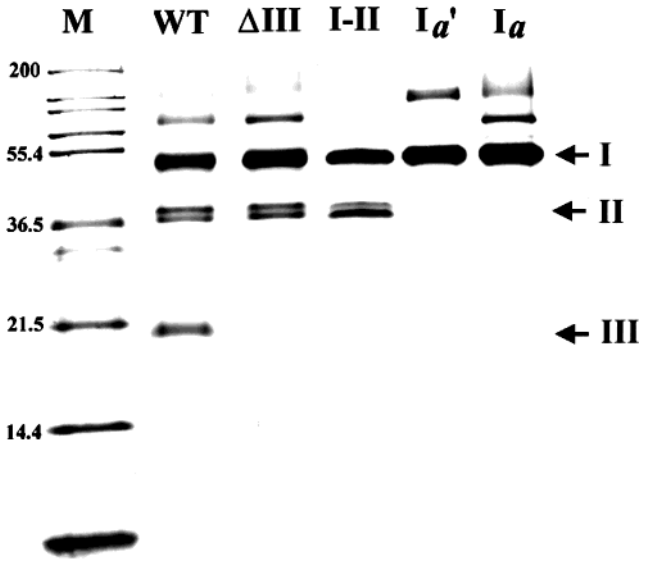

FIGURE 2: Subunit composition of purified cytochrome oxidase forms. Oxidase samples were separated on SDS−polyacrylamide gels containing urea as described in Hosler et al. (*6*) and stained with Coomassie Blue. The location of subunits I, II, and III are indicated by the arrows to the right. The molecular masses of protein standards (M) are given in kDa.

signal at $g = 3$ was seen consistently (Figure 4B), along with variable amounts of a broad signal $\approx g = 6$ (not shown) indicative of high-spin heme (*43*). The broad nature of the EPR signals of subunit I_a, the shift away from the normal low-spin g_z value of 2.83 (*6*), and the variable amount of high-spin heme indicated substantial structural heterogeneity of the heme binding site in this protein. Consistent with the presence of some high-spin (five-coordinate) heme, subunit I_a bound small amounts of CO and CN^- in its reduced and oxidized states, respectively (spectra not shown). The absolute amount of ligand binding could not be determined since the extinction values used to measure the extent of CO and CN^- binding to heme a_3 of the normal oxidase are unlikely to apply to the altered heme of subunit I_a.

Isolation of Two Oxidase Forms That Accumulate in the Absence of Subunit III. To examine the role of subunit II in metal center assembly, as well as to gain insight into the role of subunit III by inference, *Rb. sphaeroides* strain MA120H was created (see Experimental Procedures). This strain contained the genes for both subunits I and II (as well as the genes for both Cox10p and Cox11p), but not the gene for subunit III. MA120H also synthesized a cytochrome containing heme A (Figure 1); this protein was termed "ΔCoxIII" since it assembled in the absence of subunit III. Following affinity purification on Ni^{2+}-NTA agarose, ΔCoxIII was found to contain subunits I and II (Figure 2).

Visual inspection of the staining pattern of ΔCoxIII on denaturing protein gels (such as Figure 2) suggested that affinity-purified ΔCoxIII contained more subunit I per subunit II than the normal, three-subunit oxidase. This was confirmed by gel densitometry measurements comparing the subunit I/subunit II ratio for ΔCoxIII to that of the wild-type oxidase. The mean of five measurements of the subunit I/subunit II ratio for ΔCoxIII divided by the mean of seven measurements of the same ratio for the wild-type oxidase gave a value of 1.8 ± 0.3. ΔCoxIII was further purified by high-resolution anion exchange chromatography. Cytochrome

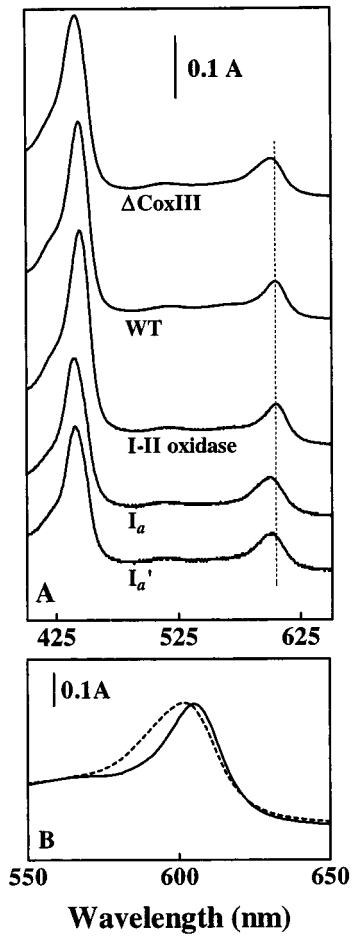

FIGURE 3: Absolute spectra of the reduced oxidase forms. Each purified oxidase form was reduced by the addition of solid sodium dithionite to a final concentration of 5 mM in a buffer of 50 mM KH_2PO_4, pH 7.2, 1 mM EDTA, and 0.1% dodecyl maltoside. Spectra were recorded using a Hitachi U-3010 dual beam spectrophotometer. The dotted line in panel A indicates the peak position of the α bands of the normal oxidase and the I−II oxidase at 605 nm. Panel B is an enlargement that shows how the presence of subunit I_a' alters the shape of the α-peak of ΔCoxIII (dotted line), as compared to the wild-type oxidase (solid line).

oxidase appears to bind to DEAE via a cluster of exposed carboxylate residues on subunit II as opposed to the histidine tag on subunit I used for the initial purification step. The chromatogram of the anion-exchange separation of ΔCoxIII was essentially the same as that shown previously for the wild-type oxidase (*27*), except that large amounts of weakly bound protein appeared in the flow-through fractions. These fractions contained free subunit I, which was termed "subunit I_a". Like subunit I_a, subunit I_a' contained full-length subunit I (Figure 2) and showed no O_2 reduction activity. Two other fractions, which differed only in the degree to which subunit II was processed to its shorter form (*27*), eluted between 200 and 300 mM KCl and contained a highly active oxidase composed of subunits I and II. These complexes, termed the "I−II oxidase", contained only as much subunit I per subunit II as the normal three-subunit oxidase purified by anion-exchange chromatography (data not shown). Thus, the cytochrome termed "ΔCoxIII" is actually a mixture of subunit I_a' and the I−II oxidase. These distinct oxidase forms copurify on Ni^{2+}-NTA agarose due to the six histidine tag present in each. In agreement with the densitometry results reported above, a stoichiometry of 0.9 ± 0.3 copies of subunit

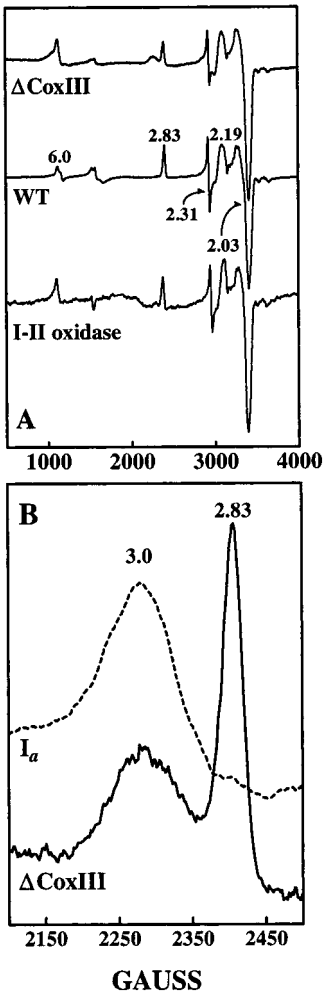

FIGURE 4: EPR spectra of ΔCoxIII, the normal oxidase (wild type), the I−II oxidase, and subunit I_a. Panel A compares the wide-field spectra of ΔCoxIII, the wild-type oxidase (WT) and the I−II oxidase. Panel B compares the $g = 3$ region of ΔCoxIII and subunit I_a, whose spectrum is identical in this region to I'_a. The spectra of ΔCoxIII (panels A and B) and the wild-type oxidase (WT; panel A) were recorded at X-band using a Bruker ESP300E spectrometer at Michigan State University, Department of Chemistry. Each spectrum is an average of four scans taken at 10 K using 2 mW microwave power at 9.483 GHz. The modulation amplitude was 12.5 G (peak-to-peak), the sweep time was 335 s, and the time constant was 163 ms. The spectrum of the I−II oxidase (panel A) was recorded at X-band using a Varian Century Series spectrometer at the National Biomedical ESR Center, Milwaukee, WI. This spectrum is an average of four scans, taken at 8.5 K using 2 mW power at 9.2348 GHz. The modulation amplitude was 5 G, the sweep time was 240 s, and the time constant was 128 ms. The spectrum of the $g = 3$ region of subunit I_a (panel B; dotted line), obtained with the same instrument, is an average of 50 scans, taken at 12 K using 2 mW power at 9.239 GHz. The modulation amplitude was 10 G, the sweep time was 60 s and the time constant was 250 ms. The actual concentrations of the oxidase forms during data collection were 22 μM for the I−II oxidase, 51 μM for subunit I_a, 33 μM for ΔCoxIII, and 50 μM for the normal oxidase. The amplitudes have been adjusted for comparison. Relevant *g* values are indicated.

I'_a per copy of the I−II oxidase was obtained for three different preparations of ΔCoxIII by integrating the peaks of the anion-exchange chromatograms.

Characterization of the I−II Oxidase and Subunit I'_a. The I−II oxidase contained two hemes A (Table 1). Consistent with the presence of both hemes *a* and a_3, the absolute reduced spectrum of the I−II oxidase isolated from ΔCoxIII was identical to that of the normal three-subunit oxidase (Figure 3A). The I−II oxidase bound normal amounts of CO, and its V_{max} of O_2 reduction was essentially the same as the normal three-subunit oxidase (Table 1). The EPR spectrum of the I−II oxidase was the same as that of the normal oxidase (Figure 4A). Signals arising from low-spin heme *a* are seen at $g = 2.83$ and 2.31. A small amount of high-spin heme gave a signal $\approx g = 6$, while the signals at $g = 2.19$ and 2.03 are assigned to Cu_A (*6*). Thus, the redox-active metal centers of the I−II oxidase, which assembled in the absence of subunit III, appear completely normal. Despite the high initial activity of the I−II oxidase, this enzyme did suicide inactivate in the same manner as the two-subunit oxidase prepared by removing subunit III with Triton X-100 (*21*).

Subunit I'_a appeared identical to subunit I_a in terms of its metal center content and structure. It contained only one heme A per subunit I (Table 1), and its optical spectrum and extinction values were the same as subunit I_a (Figure 3A). The metal content of subunit I'_a was identical to that of subunit I_a: 1 equiv of iron and no detectable copper. The EPR spectrum of subunit I'_a (not shown) was the same as that of subunit I_a, and subunit I'_a also bound undetermined, but seemingly low, levels of CO and CN^-.

Spectroscopic Characteristics of ΔCoxIII. The presence of subunit I'_a significantly alters the spectroscopic characteristics of the I−II oxidase. Near stoichiometric amounts of subunit I'_a and the I−II oxidase in affinity-purified ΔCoxIII were observed by densitometry of stained gels and by integration of chromatographic peaks (see above). The presence of significant amounts of subunit I'_a in ΔCoxIII was confirmed by analyzing the metal content using ICP-AES. If ΔCoxIII, as isolated from the Ni^{2+}-NTA column, is composed of roughly equal amounts of subunit I'_a and the I−II oxidase, the mixture should contain one more heme A than wild type for a total of three hemes A and three copper atoms. Consistent with this model, a Cu/Fe value of ~1.0 was measured for ΔCoxIII, and pyridine hemochrome analysis indicated the presence of three hemes A (Table 1). In contrast, the normal oxidase exhibited a heme A content and a Cu/Fe value consistent with its known structure (Table 1). The presence of subunit I'_a in ΔCoxIII led to a lower Soret/α ratio, blue-shifted peak maxima, and a broader α band in its optical spectrum, as compared to either the I−II oxidase or the wild-type oxidase (Figure 3). The contribution of subunit I'_a to the EPR spectrum of ΔCoxIII is most clearly seen in the broad low-spin heme signal $\approx g = 3$ (Figure 4).

DISCUSSION

We have produced, purified, and characterized three partially assembled forms of the three-subunit aa_3-type cytochrome *c* oxidase of *Rb. sphaeroides*. Upon expression of the gene for subunit I, in the absence of the genes for subunits II and III, the cells accumulate a free form of subunit I (subunit I_a) that contains a single heme A and lacks an intact heme a_3−Cu_B active site. Coexpression of the genes for subunits I and II, but not subunit III, yields a more complicated oxidase form, termed ΔCoxIII, that was isolated via histidine-affinity chromatography. Anion-exchange chromatography resolved ΔCoxIII into two components: a free form of subunit I (subunit I'_a) and a highly active two-subunit

oxidase (I−II oxidase). While the anion-exchange separation clearly shows that subunit I_a' and the I−II oxidase exist as separate entities in detergent solution, their near 1:1 stoichiometry in the ΔCoxIII mixture initially purified by histidine-affinity chromatography suggests that these two forms are loosely associated in the intact cell membrane.

Association of Subunit II with Subunit I Allows Stable Formation of the Heme a_3−Cu_B Active Site. In contrast to subunit I_a and I_a', the I−II oxidase purified from ΔCoxIII contained normal structures and normal amounts of all of the oxidase metal centers, and it was highly active. Since the only difference in the bacterial strain producing subunit I_a and the strain producing the I−II oxidase is the presence of the gene for subunit II, it is concluded that the association of subunit II with subunit I, presumably subunit I_a, is required for stable assembly of the heme a_3−Cu_B active site. The mechanism of this stabilizing function is undetermined; however, residues in the extramembrane domain of subunit II interact closely with subunit I (*7*, *8*, *10*, *11*, *44*). One principal interaction is mediated by the simultaneous binding of a magnesium atom by residues in subunit II and subunit I (*7*, *44*, *45*). In the normal oxidase, the magnesium ligand histidine 411 (subunit I) forms at least one hydrogen bond with the A-ring propionate of heme a_3 (*46*). However, the production of site-directed mutants of cytochrome oxidase that fail to bind magnesium, but retain activity (*47*, *48*), indicates that formation of the magnesium center is not required for formation of a functional heme a_3−Cu_B active site.

Free Subunit I Can Accumulate in the Membrane in the Absence of Subunits II and III. Subunit I_a contains a single heme A and no copper; the heme a_3−Cu_B active site does not assemble. The accumulation of subunit I_a was achieved by the overexpression of the genes for subunit I and the assembly factor Cox10p, a farnesyl transferase required for heme A synthesis (*49*, *50*). Significant accumulation of a pool of free subunit I in the cell membrane may require the insertion of heme A, perhaps for the formation of the low-spin heme *a* center that connects transmembrane helix 2 to helix 10 (*7*, *8*, *10*, *35*, *44*). The optical and EPR signals exhibited by subunit I_a (and I_a'), however, are considerably altered from those of normal heme *a*. The optical transitions are blue-shifted and the extinction coefficients are lower than the published values for either heme *a* or heme a_3 (*36*, *38*). The single heme appears primarily low-spin, with EPR signals consistent with bis-histidine ligation (*51*), but structural heterogeneity in the heme-binding site is evidenced by the broad nature of the EPR signals and the presence of variable amounts of high-spin heme. Despite the ambiguity in the identification of the heme-binding site in subunit I_a, it is clear that stabilization of subunit I in the membrane does not require complete formation of the heme a_3−Cu_B center. In addition, our results show that Cox11p, which is required for the insertion of Cu_B in the normal oxidase (*31*), does not insert Cu_B in the absence of subunit II.

Subunit I_a may be an early intermediate in the normal assembly of cytochrome oxidase in both bacteria and mitochondria. Subunit I_a is present in appreciable amounts in *Rb. sphaeroides* cells (YZ300) synthesizing the wild-type oxidase (one copy of subunit I_a for every four to five copies of the normal oxidase; data not shown). In addition, recent work in human cells identified free subunit I as the earliest intermediate in the assembly of mitochondrial cytochrome oxidase (*15*). If subunit I_a is an intermediate, it should be driven to accumulate by blocking later assembly steps (e.g., by preventing its association with subunit II) or by shifting the equilibrium of early assembly steps to favor its accumulation (e.g., by weakening the association of subunits I and II). Several observations are consistent with this. First, subunit I_a accumulates in the bacterial cell to high levels in the absence of subunits II and III. Second, two mutants of the *Rb. sphaeroides* oxidase that eliminate magnesium binding at the interface of subunits I and II and likely weaken the binding interaction between these subunits, appear to accumulate subunit I_a' along with a I−II oxidase as evidenced by their EPR spectra and Cu/Fe values (*48*). Last, an examination of a disease-causing mutant in humans that disrupts the binding of subunit II to subunit I showed that heme a_3 was not present in the subunit I that accumulated to near normal levels in the affected mitochondria (*20*). Thus, it seems likely that the subunit I identified as the earliest intermediate in human mitochondria lacks the heme a_3−Cu_B active site, as does *Rb. sphaeroides* subunit I_a.

The isolation of bacterial terminal oxidases using affinity tags is now common, since it is much faster than multistep chromatography procedures (*28*, *52*, *53*). It should be recognized, however, that significant amounts of free subunit I are likely to co-purify with terminal oxidases that are purified by an affinity tag attached to subunit I. Subunit I_a (or I_a') is present, in variable amounts, in cells expressing the wild-type oxidase of *Rb. sphaeroides* (see above) as well as in cells expressing every mutant oxidase that we have examined. The presence of subunit I_a can affect spectroscopic analyses as well as the determination of oxidase concentration by α-band absorbance. For example, the "heme-containing impurity" that contributes to the resonance Raman spectrum of an affinity-purified mutant of arginine 52 of subunit I (*41*) is likely to be subunit I_a.

Role of Subunit III. At least two of the possible roles for subunit III in cytochrome oxidase assembly can be eliminated. Spectroscopic examination of the I−II oxidase purified from ΔCoxIII shows that subunit III is not necessary for the formation or alignment of any of the metal centers of cytochrome oxidase. In addition, subunit III is not necessary for the initiation of normal electron transfer within subunit I, as previously proposed (*24*), since the V_{max} values of the I−II oxidase and the wild-type oxidase are nearly identical (Table 1). The lack of subunit III *does* cause the I−II oxidase to suicide inactivate during O_2 reduction (*21*), regardless of its high initial activity.

The presence of normally assembled metal centers in the I−II oxidase does not exclude other roles for subunit III in cytochrome oxidase assembly. The aa_3-type oxidase accumulates to a lesser extent in cells that lack subunit III (Figure 1). It is possible that subunit III enhances the rate of oxidase assembly by stabilizing the structure of a labile assembly intermediate.

In conclusion, we find that the association of subunits I and II is required for the formation of the active site of cytochrome *c* oxidase, even though the active site is entirely within subunit I. The absence of subunit II reveals a free form of subunit I that has incorporated one molecule of heme A in a distorted binding site; this cytochrome is likely to be an early assembly intermediate. The stable addition of a

second heme A and the Cu_B center forms an oxidase with maximal activity; this process requires subunit II, with Cu_A, but not subunit III.

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