Cox11p Is Required for Stable Formation of the CuB and Magnesium Centers of Cytochrome c Oxidase*

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Assembly of the core subunits of the aa3-type cytochrome c oxidase in mitochondria and aerobic bacteria such as Rhodobacter sphaeroides requires the association of three subunits and the formation of five to seven metal centers. Several assembly proteins are required for the late stages of oxidase assembly in eukaryotes; some of these are also present in Rb. sphaeroides. To investigate the role of one of these proteins, Cox11p, the mitochondrial-like oxidase of Rb. sphaeroides was overexpressed and purified from cells that lacked cox11, the gene for Cox11p. The oxidase that assembled in the absence of Cox11p lacked CuB at the active site and contained greatly reduced amounts of metal at the magnesium/manganese-binding site between subunits I and II. This inactive oxidase, however, did contain hemes α and α3, CuA, and all three subunits. These results indicate that Cox11p is required at a late, perhaps final, step in the assembly of cytochrome oxidase, most likely the insertion of CuB Oxidase which assembled in a strain with the assembly of cytochrome oxidase, most likely the in-

able for binding O2. CuB is ligated by three histidines and is located 4.5–5.2 Å from the iron of heme α3 (3, 4, 6, 8, 12). A non-redox active magnesium center is present at the interface of subunits I and II (4, 8, 13). At least two residues from subunit I and two from subunit II are necessary for coordination of this metal (8, 12–14). Manganese can replace magnesium at this site without altering oxygen reduction activity (13).

The assembly of the multisubunit oxidase with its metal centers apparently occurs in an ordered sequence; most of the details, however, remain unresolved (15–17). In Saccharomyces cerevisiae, genetic analysis indicates that more than a dozen genes are involved in holoenzyme assembly (18, 19). Orthologs of some of these genes are found in bacteria, suggesting that they are required for assembly of the core subunits and the metal centers (20, 21). Two such genes, cox10 and cox11, are present in the Rb. sphaeroides operon that encodes subunits II and III of the oxidase (22). The importance of cox10 and cox11 for cytochrome oxidase assembly is underscored by the finding that orthologs of these genes are present in the human genome (23, 24).

In S. cerevisiae, cox10 and cox11 mutants lack an optically detectable cytochrome c oxidase even though all of the structural subunits are synthesized (25, 26). The product of cox10 (Cox10p) has farnesytransferase activity and catalyzes the conversion of protoheme to heme O, a precursor of heme A (27, 28). Because heme A is required for function and optical detection of the aa3-type oxidase, the phenotype of the yeast cox10 mutant is readily understood. Based on the similar phenotypes of the cox10 and cox11 mutants in yeast, it has been suggested that Cox11p also functions in heme A biosynthesis (25, 26), specifically in the formylation of the heme (29).

Cox11p is not found in isolated cytochrome oxidase, and its sequence (22) does not reveal any homology to proteins of known function. Sequence analysis predicts that Cox11p of Rh. sphaeroides is a 20.7-kDa protein with a single transmembrane helix and a large globular domain (22). Immunological studies in yeast have shown that Cox11p is localized to the mitochondrial inner membrane (26). Our investigation into the function of Cox11p has revealed an essential role for this protein in the formation of the CuB center. In addition, Cox11p was necessary for proper alignment of hemes α and α3 and stability of the magnesium/manganese center. The presence of Cox11p was not required for heme A biosynthesis, heme A insertion, CuA assembly, and association of the subunits of the oxidase. Experiments not altering the copy number of cox11 suggested that Cox11p was required in only substoichiometric amounts relative to the structural subunits.

EXPERIMENTAL PROCEDURES

Materials—Horse heart cytochrome c (Sigma C-7752) was resuspended in distilled, deionized water and stored at −80 °C. t-α-Phosphatidylcholine (Sigma) was solubilized to 40 mg/ml in 2% sodium
Role of Cox11p in Oxidase Assembly

Expression and Purification of Oxidase Forms—In order to examine the role of Cox11p in oxidase assembly, the mitochondrial-like oxidase of Rb. sphaeroides was overexpressed in the presence of different copy numbers of cox11. A derivative of pRKpAH1H32, a plasmid containing both coxI operons with their natural promoters, was prepared that contained a 300-base pair in-frame deletion in coxl. This Δcox1 plasmid, termed pRKpLAJ200, was conjugated into wild-type Rb. sphaeroides 2.4.1 (37) and YZ200, a strain in which the genomic copy of the coxII/III operon has been deleted (31), for overproductions and purification of cytochrome c oxidase. The enzyme isolated from 2.4.1 (wild type) cells harboring the Δcox11 plasmid was termed Δcox11 (for “low cox11”) because it was assembly in a strain with a single copy of coxl (in the 2.4.1 genome) and about five plasmid-borne copies of coxI, coxII, cox10, and coxIII. Correspondingly, Δcox1 oxidase was isolated from cells with no copies of coxl by introducing the same Δcox11 plasmid into YZ220 (ΔcoxII/III). The purified wild-type oxidase used in this study was prepared from strain YZ300, which is a deletion strain YZ200 containing pRK-pYJ123H (31).

The level of expression of Δcox11 and Δcox11 was compared with that of the wild-type oxidase (Fig. 1). Because Δcox11 and Δcox11 result from expression of the same plasmid (pRKpLAJ200) in two different host strains (YZ200 and 2.4.1), expression of the wild-type oxidase from the corresponding plasmid, pRKpAH1H32, was measured in both strains. Compared with the expression of the wild-type oxidase in the same host strain, Δcox11 accumulated to 100% (Fig. 1, 3rd and 4th columns), whereas Δcox11 accumulated to approximately 50% (Fig. 1, 1st and 2nd columns). When coxl is deleted in yeast, no cytochrome oxidase can be detected by optical spectroscopy (26). Thus, a mutation that causes a catastrophic phenotype in yeast had a less severe defect in Rb. sphaeroides. The reduction in oxidase expression observed in the absence of coxl was completely reversed by a single copy of coxl (Fig. 1), suggesting that Cox11p was not required in stoichiometric amounts with the structural subunits for oxidase assembly.

Spectral and Heme Analysis—The absolute reduced spectrum of purified Δcox11, but not of Δcox11, revealed a 3–4 nm blue shift in both the α (602 versus 605 nm for wild type) and Soret (441 versus 444 nm) peaks (Fig. 2). Because the α band is primarily due to absorption by heme a, whereas the Soret band includes roughly equal contributions from both hemes a and a3 (33, 38), the observed shifts indicated altered environments around both heme centers in Δcox11. Indeed, resonance Raman analysis (not shown) revealed a disturbed heme a environment in Δcox11, similar to mutant oxidases containing alterations around heme a3 (39). In contrast, there was no evidence for disturbance of the heme centers in Δcox11 by optical or resonance Raman spectroscopy.

The pyridine hemochrome spectrum of Δcox11 was indistinguishable from that of heme extracted from the purified wild-type or Δcox11 oxidases (Fig. 3), indicating that Δcox11 contained bona fide heme A. This argued against a role for Cox11p in heme A synthesis. Furthermore, the heme A content of Δcox11 was nearly the same as that of the wild-type oxidase (Table I), indicating that Cox11p was not required for insertion of heme A during oxidase assembly.

Subunit Association—SDS-polyacrylamide gel electrophoresis analysis of purified Δcox11 and Δcox11 revealed that both protein complexes contained all three of the structural subunits (Fig. 4). This showed that Cox11p was not essential for association of the oxidase subunits.

O2 Reduction Activity—The O2 reduction activity of cytochrome c oxidase purified from strains with varying amounts of...
The absence of CuB (40). The heme oxidized cytochrome oxidase, and it is also used to detect the pLAJ200. The host strains were YZ200 (mined by inductively coupled plasma atomic absorption spec-

amplitude of the cytochrome b ratio of the amplitude of the cytochrome coppers (CuB and the di-copper CuA center) and two hemes (7).

Based on previous analysis of the heme a signals of the mitochondrial-like oxidase of Rb. sphaeroides, this change indicated disruption of the hydrogen bonds to the histidine ligands of heme a in ΔCox11 (1). The blue-shifted optical spec-

cox11 is listed in Table I. The ΔCox11 oxidase was unable to reduce oxygen efficiently. Measurable activity (80 s⁻¹) using nearly saturating amounts of cytochrome c (90 μM) was ob-

served in only one of three ΔCox11 preparations. The high activity of ΔCox11 (Table I) indicated that substoichiometric amounts of Cox11p were sufficient for assembly of an active oxidase. This was consistent with the high expression of λCox11 (Fig. 1).

EPR Spectroscopy and Metal Content—Because both of the heme centers were present in ΔCox11, the loss of activity suggested that one of the other metal centers was disrupted. Electron paramagnetic resonance spectroscopy (EPR) is a sensitive method for examining the environments of CuA and heme a of oxidized cytochrome oxidase, and it is also used to detect the absence of CuB (40). The heme a₃ and CuB centers are normally EPR-silent due to spin coupling of the metals. However, in the absence of CuB, a strong signal for five-coordinate, high spin heme a₃ is seen at approximately g = 6 (41). The ΔCox11 spectrum (Fig. 5) showed a signal for high spin heme a₃ at g = 6 with an amplitude similar to oxidases known to lack CuB (41). This strongly suggested that ΔCox11 lacked CuB. In contrast, signals in the g = 2 region of the EPR spectrum showed that ΔCox11 oxidases contained wild-type amounts of a normal CuA center.

The extent to which CuB was lost from ΔCox11 was determined by inductively coupled plasma atomic absorption spectroscopy. The wild-type and ΔCox11 oxidases gave Cu:Fe values of about 1.5 (Table I) as expected for an enzyme with three coppers (CuA and the di-copper CuA center) and two hemes (7). The Cu:Fe value of ΔCox11 was 0.99 (Table I), suggesting complete loss of CuB. As predicted from the heme A content, iron levels were normal in all three of the oxidases (data not shown).

In addition to loss of CuB, the EPR spectra showed significant disturbance of the heme a environment of ΔCox11 and, to a lesser extent, of λCox11. The wild-type heme a signal at g = 2.83 (g₂) was replaced by a broad signal around g = 3 in ΔCox11 (Fig. 5). Based on previous analysis of the heme a signals of the mitochondrial-like oxidase of Rb. sphaeroides, this change indicated disruption of the hydrogen bonds to the histidine ligands of heme a in ΔCox11 (1). The blue-shifted optical spec-

trum of ΔCox11 (Fig. 2) was consistent with this interpretation. The loss of strong hydrogen bonds to the heme a ligands will cause the electron density on the heme iron to decrease, thus forcing the electronic transitions of heme a in ΔCox11 to occur at higher energy (see Ref. 1). The heme a signals of ΔCox11 (g₂ = 2.98 and g₇ = 2.30) were also diminished in intensity due to incomplete oxidation of heme a in resting ΔCox11 (data not shown).

In order to examine the magnesium/manganese center of cytochrome oxidase, ΔCox11 and λCox11 were prepared from bacteria grown in media supplemented with manganese salts. This allows substitution of manganese, which is EPR-visible, for magnesium at its binding site between subunits I and II (8, 12, 13, 42). Comparison of the EPR spectra of ΔCox11 and λCox11 (Fig. 6) showed that much less manganese was inserted into the oxidase in the absence of Cox11p. EPR spectra of acid-extracted Mn²⁺ (not shown) indicated that ΔCox11 bound approximately 15% of the manganese of λCox11. Similarly, metal analysis showed that reduced levels of magnesium (29 ± 11% of the normal oxidase) were incorporated into ΔCox11 purified from cells grown in normal media.
DISCUSSION

Previous work in yeast identified Cox11p as one of several proteins required for the post-translational stages of the assembly of cytochrome c oxidase (26, 29). Here, we have further defined the role of Cox11p by characterizing the oxidase that was synthesized in its absence. Due to the presence of an alternative cytochrome c oxidase in *Rb. sphaeroides* (43, 44), it is possible to grow strains without the mitochondrial-like oxidase; this permits the study of inactive oxidases. Cells in which most of the cox11 gene was deleted produced a partially assembled oxidase, termed ΔCox11, that contained all three structural subunits, both hemes a and a3, and the Cu A center. Contrary to a previous proposal based on experiments in yeast (26, 29), the presence of the heme A centers in ΔCox11 argued against a role for bacterial Cox11p in heme A synthesis. Roles for Cox11p in the association of the oxidase subunits, insertion of heme A, and the formation of the CuA center were also eliminated. The principal defects identified in ΔCox11 were diminished accumulation in the membrane, the absence of CuB, a 70–85% decrease in magnesium/manganese content, and altered heme environments.

The complete absence of CuB indicated that Cox11p normally functions in the insertion of this metal. In ΔCox11, the magnesium/manganese center was incompletely formed, and perturbation of the heme centers was observed. Therefore, the insertion of CuB is likely to facilitate the stable formation of other structures in subunit I. This seems reasonable because the metal centers of subunit I are close together. For example, CuB and the magnesium atom are only 13.5 Å apart, and imidazole

**TABLE I**

| Oxidase     | Heme A Oxidase | Cu:Fe | V<sub>max</sub> | g<sup>-1</sup> |
|-------------|----------------|-------|----------------|----------------|
| Wild type   | 2.20 ± 0.20 (<i>n</i> = 4, 2 preparations) | 1.62 ± 0.11 (<i>n</i> = 3) | 1700 |
| ΔCox11      | 1.91 ± 0.08 (<i>n</i> = 4, 3 preparations) | 0.99 ± 0.08 (<i>n</i> = 3) | <100 |
| λCox11      | 2.21 ± 0.08 (<i>n</i> = 4, 2 preparations) | 1.48 (<i>n</i> = 1) | 1500 |

**FIG. 4.** SDS-polyacrylamide gel electrophoresis of purified cytochrome c oxidase. Sample preparation and electrophoresis were performed as described in Hosler et al. (1). The positions of subunits I, II (a doublet), and III are indicated on the left. The molecular mass of the standards (M) is shown on the right in M<sub>r</sub> × 10<sup>3</sup>. Approximately 20 pmol (WT) or 30 pmol (ΔCox11 and λCox11) oxidase were loaded.

**FIG. 5.** EPR spectra of purified ΔCox11, λCox11, and wild-type oxidases. Spectra of 55 μM oxidase samples were recorded at X-band at 10 K as in Hosler et al. (1) with the following exceptions. A Bruker ESP300E spectrometer was used. The sweep time was 335 s, and the time constant was 163 ms. The spectra presented are averages of 4 scans using 2 milliwatt microwave power at 9.48 GHz. Relevant g values are indicated by the arrows.

**FIG. 6.** EPR spectra of purified oxidase isolated from *Rb. sphaeroides* cells grown in 0.7 mM MnSO<sub>4</sub>. Spectra of ΔCox11 (---) and ΔCox11 (----) oxidase samples, each containing 50 μM oxidase, were recorded at 110 K with a sweep time of 163 s and a time constant of 82 ms. Other conditions were as in Fig. 5. The g value of the predominant manganese signal is marked. The six-line manganese signal largely or completely obscures the Cu<sub>A</sub> signal, g = 2.
side chains of ligands from each of these centers come within 7 Å (6, 8).

Cox11p may be a copper chaperone specific for the CuB center of cytochrome oxidase. Specific copper chaperones are required for the assembly of metalloprotein copper centers. For example, CopY, a repressor in Enterococcus hirae, accepts copper from CopZ but not from other copper chaperones (45). Sequence analysis of Cox11p orthologues from several species reveals a conserved CFCF motif that resembles the metal-binding regions of other metal chaperones (45–48). However, a role for Cox11p in formation of CuB does not require it to bind copper. It is also possible that by interacting with subunit I, Cox11p permits a conformational receptive for copper delivery by a different protein. The final position of the CuB center is not solvent-accessible and is within the transmembrane region of subunit I (3, 4, 6, 8). Thus, the involvement of an integral membrane protein such as Cox11p in the assembly of this site was not unexpected.

The model to which ΔCox11 accumulated in the bacterial membrane was one half of that of the normal oxidase that assembled in the presence of Cox11p (Fig. 1). An oxidase lacking CuB would be predicted to have reduced stability at the heme aoptically detectable cytochrome oxidase in the mitochondrial core of cytochrome oxidase.

In elucidating the mechanisms of the assembly of the catalytic core subunits of the eukaryotic oxidase, the latter enzyme is not structurally stable in the absence of the accessory subunits (17). Despite the near structural identity of the eukaryotic mitochondrial oxidase. In the mitochondria, association of several nuclear-encoded accessory subunits follows assembly of the catalytic core (17). From a different perspective, it is significant that the presence of Cox11p (Fig. 1). An oxidase lacking CuB would be membrane was one-half that of the normal oxidase that assembled in the membrane protein such as Cox11p in the assembly of this site was not unexpected.

In yeast, the deletion of cox11 eliminates the accumulation of optically detectable cytochrome oxidase in the mitochondrial membrane (26). The different results seen in yeast and Rh. sphaeroides are likely a consequence of the complexity of the mitochondrial oxidase. In the mitochondria, association of several nuclear-encoded accessory subunits follows assembly of the catalytic core (17). Despite the near structural identity of the simpler bacterial oxidases and the mitochondrially encoded core subunits of the eukaryotic oxidase, the latter enzyme is not structurally stable in the absence of the accessory subunits (49). In this respect, bacteria are likely to be of greater utility in elucidating the mechanisms of the assembly of the catalytic core of cytochrome oxidase.

In summary, the results presented here suggest that ΔCox11p has a primary defect in CuB insertion, with secondary effects being the loss of metal from the magnesium/manganese center and misalignment of the hemes. We propose a role for Cox11p in the insertion of CuB into the aa3-type cytochrome c oxidase at a late stage in assembly.

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REFERENCES

1. Hosler, J. P., Fetter, J., Tecklenburg, M. J., Espe, M., Lerma, C., and Ferguson-Miller, S. (1992) J. Biol. Chem. 267, 24264–24272
2. Haltia, T., Puustinen, A., and Finel, M. (1988) Eur. J. Biochem. 172, 543–546
3. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Nature 376, 660–660
4. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) Science 272, 1136–1144
5. Ostermeier, C., Iwata, S., and Michel, H. (1996) Curr. Opin. Struct. Biol. 6, 460–466
6. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Libes, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) Science 280, 1723–1729
7. Steffens, G. C. M., Biewald, R., and Buse, G. (1987) Eur. J. Biochem. 164, 295–300
8. Ostermeier, C., Harrenga, A., Ermier, U., and Michel, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10547–10553
9. Hill, B. C. (1991) J. Biol. Chem. 266, 2219–2226
10. Geren, L. M., Beasley, J. R., Fine, B. R., Saunders, A. J., Hidson, S., Pelak, G. J., Durham, B., and Millet, F. (1995) J. Biol. Chem. 270, 2466–2672
11. Ferguson-Miller, S., and Babcock, G. T. (1998) Chem. Rev. 96, 2889–2907
12. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) Science 269, 1069–1074
13. Hosler, J. P., Espe, M., Zhen, Y., Babcock, G. T., and Ferguson-Miller, S. (1995) Biochemistry 34, 7586–7592
14. Zhen, Y., Hoganan, C. W., Babcock, G. T., and Ferguson-Miller, S. (1999) J. Biol. Chem. 274, 38032–38041
15. Haltia, T., Puustinen, A., and Finel, M. (1988) Biochemistry 27, 24273–24284
16. Saiki, K., Mogi, T., Ogura, K., and Anraku, Y. (1993) Biochemistry 32, 3178–3187
17. Zhen, Y., Qian, J., Follmann, K., Hayward, T., Nilsson, T., Dahn, M., Hilmi, Y., Hamer, A. G., Hosler, P. J., and Ferguson-Miller, S. (1998) Protein Expression Purif. 13, 326–336
18. Thompson, D. A., and Ferguson-Miller, S. (1983) Biochemistry 22, 336, 343–349
19. Shapleigh, J. P., Hosler, J. P., Tecklenburg, M. M. J., Kim, Y., Babcock, G. T., Hamer, A. G., Hosler, P. J., and Ferguson-Miller, S. (1998) Protein Expression Purif. 13, 326–336
20. Saraste, M. (1990) J. Biol. Chem. 265, 2759–2764
21. Sistrom, W. R. (1960) J. Gen. Microbiol. 22, 778–785
22. Zhen, Y., Qian, J., Follmann, K., Hayward, T., Nilsson, T., Dahn, M., Hilmi, Y., Hamer, A. G., Hosler, P. J., and Ferguson-Miller, S. (1998) Protein Expression Purif. 13, 326–336
23. Thompson, D. A., and Ferguson-Miller, S. (1983) Biochemistry 22, 336, 343–349
24. Keen, N. T., Tamaki, S., Kobayashi, D., and Trollerling, D. (1988) Gene (Amst.) 70, 191–197
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Donohue, T. J., and Kaplan, S. (1991) Methods Enzymol. 204, 459–485
27. Liao, G.-L., and Palmer, G. (1996) Biochim. Biophys. Acta 1274, 109–111
28. Sistrom, W. R. (1960) J. Gen. Microbiol. 22, 778–785
29. Zhen, Y., Qian, J., Follmann, K., Hayward, T., Nilsson, T., Dahn, M., Hilmi, Y., Hamer, A. G., Hosler, P. J., and Ferguson-Miller, S. (1998) Protein Expression Purif. 13, 326–336
30. Thompson, D. A., and Ferguson-Miller, S. (1983) Biochemistry 22, 336, 343–349
31. Zhen, Y., Qian, J., Follmann, K., Hayward, T., Nilsson, T., Dahn, M., Hilmi, Y., Hamer, A. G., Hosler, P. J., and Ferguson-Miller, S. (1998) Protein Expression Purif. 13, 326–336
32. Thompson, D. A., and Ferguson-Miller, S. (1983) Biochemistry 22, 336, 343–349
33. Thompson, D. A., and Ferguson-Miller, S. (1983) Biochemistry 22, 336, 343–349
34. Keen, N. T., Tamaki, S., Kobayashi, D., and Trollerling, D. (1988) Gene (Amst.) 70, 191–197