Yeast Cox11, a Protein Essential for Cytochrome c Oxidase Assembly, Is a Cu(I)-binding Protein*

Cox11 is a protein essential for respiratory growth and has been implicated in the assembly of the Cu₉ site of cytochrome c oxidase. In the present study, we demonstrate that Cox11 is a copper-binding protein. The soluble C-terminal domain of Cox11 forms a dimer that coordinates one Cu(I) per monomer via three thiolate ligands. The two Cu(I) ions in the dimer exist in a binuclear cluster and appear to be ligated by three conserved Cys residues. Mutation of any of these Cys residues reduces Cu(I) binding and confers respiratory incompetence. Cytochrome c oxidase activity is reduced in these mutants. Thus, the residues important for Cu(I) binding correlate with in vivo function, suggesting that Cu(I) binding is important in Cox11 function.

Cytochrome c oxidase (CcO) is the terminal enzyme in the electron transport system, reducing oxygen to water and generating the proton gradient that drives ATP synthesis. Multiple subunits and several cofactors are necessary for catalytic activity, including two hemes a, a magnesium ion, a zinc ion, and three copper ions. Insertion of these cofactors and assembly of the CcO complex in the inner mitochondrial membrane, in some cases many Å units below the membrane surface, requires accessory proteins (for a general review, see Ref. 1). It has now become clear that the required metal ions cannot simply diffuse to the requisite compartment for insertion into the desired protein. In fact, a complex machinery of metal importers and chaperones has been discovered (2). The metallochaperone responsible for the delivery of copper to the mitochondrial Cox17 (3). Once there, it is postulated that Cox17 transfers copper to Sno1, the protein responsible for copper insertion into the binuclear Cu₉ site in Cox2 (4–6). It has been suggested that Cox11 plays a role in the delivery of copper to the Cu₉ site in Cox1 (7). Little is known about the mechanics of either process.

Saccharomyces cerevisiae COX11 is a nuclear gene that encodes a membrane-bound mitochondrial protein. Cox11 was first implicated in the CeO maturation process by Tzagoloff et al. (8). Cox11 is a metallochaperone for formation of the Cu B site of CcO. In the present study, we demonstrate that a soluble truncate of Cox11 is a copper protein, binding approximately one copper per polypeptide. Functional analysis of conserved amino acid residues showed that the Copper atom was bound by cysteine residues and that loss of copper binding correlated with loss-of-function in vivo. These studies support the postulate that Cox11 is a metallochaperone for formation of the Cu₉ site of CcO.

MATERIALS AND METHODS

Plasmids—The COX11 gene lacking the first 300 base pairs (bp) was amplified from S. cerevisiae genomic DNA by PCR. BamHI and SalI were added to the 5′- and 3′-ends, respectively. Unfavorable arginine, isoleucine, and leucine codons in the first 10 codons were corrected by replacement in the PCR primer for expression in Escherichia coli. The 609-bp fragment was cloned into pGEM-T (Promega) and sequenced. The entire COX11 gene was amplified from yeast genomic DNA, cloned into pGEM-T, and sequenced.

The 1.8-kb BamHI/SalI fragment was subcloned into pET32a (Novagen), creating pHCDW4 for expression of thioredoxin-Cox11 (TrxCox11) fusion protein. The entire COX11 gene, including 772-bp upstream and 100-bp downstream flanked by BamHI and HindIII sites, respectively, was amplified from yeast genomic DNA, cloned into pGEM-T, and sequenced. The 1.8-kb BamHI/HindIII fragment was subcloned into YEp351 to yield the high copy yeast expression vector, pHCDW3. Mutagenesis of COX11 was carried out using the QuickChange (Stratagene) site-directed mutagenesis kit on both pHCDW3 and pHCDW4. The resulting plasmids were sequenced and the BamHI/SalI and BamHI/HindIII fragments subcloned to ensure there were no undesirable mutations elsewhere in the vector.

Yeast Strains—S. cerevisiae strain BY4741/cex11 (mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 and its isogenic parent strain were purchased from Research Genetics. Cells were grown in YPD or synthetic medium lacking leucine that was supplemented with 2% glucose or 3% glycerol as the carbon source.

Purification of Cox11—E. coli strain BL21(DE3) harboring pHCDW4 was grown in LB-ampicillin to an OD₆₀₀ of 0.6–0.9 before induction with 0.3 mM IPTG for 4 h at 30 °C. If desired, CuSO₄ was added to a final concentration of 1.4 mM after 30 min of induction. The cells were...
harvested by centrifugation, washed with 0.25 M sucrose, and frozen at −70 °C. The cells were thawed, resuspended in lysis buffer (20 mM β-mercaptoethanol and 5 mM imidazole), and lysed by repeated sonication. The cell lysate was centrifuged at 100,000 g for 40 min. If copper was not added during growth, 450 μM CuSO4 was added. The supernatant was loaded onto a Ni-NTA (Qiagen) column equilibrated with lysis buffer. After loading, the column was washed with 10 column volumes of lysis buffer, and the protein was eluted with 250 mM imidazole in lysis buffer. Fractions containing Cox11 were filtered and loaded onto a Sephacryl S300 size exclusion column and eluted with PBS, 1 mM DTT. For generation of selenomethionine-labeled Cox11, E. coli BL21(DE3) harboring pH-19 (12), and the medium was supplemented with 60 mg/liter selenomethionine to prevent selenomethionine oxidation.

Protein/copper ratios are expressed as mol of copper per mol of polypeptide ± S.D. All samples were run in duplicate or triplicate, and n is the number of separate protein purifications analyzed.

### Table 1: Copper content of TrxCox11 variants

| Protein       | Cu/protein | n |
|---------------|------------|---|
| WT            | 1.23 ± 0.17 | 4 |
| C111A         | 0.44 ± 0.05 | 2 |
| M420L         | 0.46 ± 0.17 | 2 |
| M240L         | 1.11 ± 0.28 | 2 |
| C208A/C210A   | 0.31 ± 0.13 | 2 |
| C111A/C208A/C210A | 0.22 ± 0.04 | 2 |
| Dimer         | 1.02 ± 0.10 | 3 |

35 μM in order to produce absorbance scans within the linear range of the detector at 280 nm. Samples were centrifuged until sedimentation and chemical equilibrium were attained. Cells were scanned radially in continuous mode, with data resulting from 10 absorbance readings taken at 0.001-cm intervals. Equilibrium was confirmed by no change in scans taken at 4-h intervals. The value used for the partial specific volume, 0.731 ml/mg, was calculated from the amino acid sequence using the method of Ref. 13.

Various models describing the concentration distribution were fit to final absorbance versus radius data using non-linear least squares techniques and the analysis program NONLIN (14). NONLIN performs simultaneous non-linear least squares fits to one or more sets of ultracentrifuge data. This program can be used to determine molecular weights, association constants, and virial coefficients. In each case, the final fit resulted from the simultaneous fitting of up to four different concentration distributions. To avoid the problem of relative local min-
ima in the variance space, the fitting procedure was begun at many different initial points spanning the range of the parameters.

Measurements were carried out at the Stanford Synchrotron Radiation Laboratory with the SPEAR storage ring containing 60–100 mA at 3.0 GeV, on beamlines 9-3 and 7-3 operating with a wiggler field of 2 and 1.8 Tesla, respectively, and using a Si(220) double crystal monochromator. Beamline 9-3 is equipped with a rhodium-coated collimating mirror upstream of the monochromator, and a bent-cylindrical Rh-coated focusing mirror downstream of the monochromator. Harmonic rejection on 9-3 was accomplished by setting the cutoff energy of the focusing mirror to 11 keV, whereas on 7-3 harmonic rejection was achieved by detuning one monochromator crystal to ~50% of the peak value. The incident x-ray intensity was monitored using nitrogen-filled ionization chambers, and x-ray absorption was measured as the x-ray Kα fluorescence excitation spectrum using an array of thirty germanium intrinsic detectors (15). During data collection, samples were maintained at a temperature of ~10 K using a liquid helium flow cryostat. For each sample, between four and eight 35-min scans were accumulated, and the absorption of a standard copper metal foil was measured simultaneously by transmission. The energy was calibrated with reference to the lowest energy inflection point of the copper foil, which was assumed to be 8980.3 eV.

The extended x-ray absorption fine structure (EXAFS) oscillations $\chi$ (h) were quantitatively analyzed by curve-fitting using the EXAFSPAK suite of computer programs (ssrl.slac.stanford.edu/exafspak.html). Ab-initio theoretical phase and amplitude functions were calculated with the program FEFF (version 8.2) (16).

Mitochondrial Isolation and Oxidase Activity—Mitochondria were isolated from S. cerevisiae as described previously (17). Mitochondrial protein concentration was determined by the Bradford assay, and cytochrome c oxidase activities were measured as previously described (18).

Antibody Production and Western Analysis—Chicken antibodies were raised against recombinant Cox11 by Aves Laboratories, Inc. For Western analyses, proteins were separated on 12.5% acrylamide gels in Laemmli buffer and transferred to nitrocellulose. Blots were probed with antibodies against Cox11 (1:5000) or porin (1:15,000). Horseradish peroxidase-conjugated rabbit anti-chicken IgG was the secondary antibody. Proteins were visualized with ECL reagents (Amersham Biosciences, Inc. or Pierce) or 4-chloro-1-naphthol.

RESULTS

Cox11 Is a Copper-binding Protein—Cox11 is believed to facilitate copper incorporation into the CuB site of cytochrome c oxidase. One possible model for this function is for Cox11 to receive copper from Cox17 and insert it into Cox1. Consistent with its postulated role, Cox11 is localized to the mitochondrial membrane (8); the C terminus, which contains several conserved cysteines, methionines, and histidines capable of ligating copper, is believed to project into the intermembrane space.

to determine whether Cox11 binds copper, a soluble truncate lacking the mitochondrial targeting sequence and putative transmembrane helix was constructed. A hexahistidine-tagged version of truncated Cox11 expressed well but was insoluble under all conditions investigated (data not shown). To generate soluble protein, the 609-bp fragment encoding the truncate was inserted into the pET32a vector, generating a thioredoxin-tagged Cox11 fusion protein (Fig. 1). Purified and soluble TrxCox11 appears light brown when concentrated and is stable at 4°C in dilute solution. Atomic absorption spectroscopy showed that purified TrxCox11 bound ~1 copper atom per monomer of protein (Table I). The ultraviolet absorption spectrum exhibits a fairly broad absorption between 265 and 285 nm, consistent with the overlap of the aromatic protein residues and S-Cu charge transfer bands centered around 260 nm (19). TrxCox11 exhibits only very weak luminescence when excited at 300 nm, suggesting that the copper ion is not bound in a multicopper cluster in a solvent-shielded environment (data not shown).

Cox11 Is a Dimer—Several other mitochondrial copper proteins, including Cox1, exist as dimers or oligomers. To determine whether TrxCox11 was monomeric, purified protein was subjected to gel filtration chromatography (Fig. 2). TrxCox11 eluted primarily as a dimer, with a small fraction of protein consistently appearing as higher order aggregates. Concentration of the dimeric fractions and re-analysis by analytical gel filtration did not cause a change in the oligomeric state, suggesting that the higher order oligomers were nonspecific aggregates. Oligomerization was not dependent on copper, as purified apo-Cox11 also eluted at the molecular weight predicted for a dimer (data not shown). Furthermore, removal of the thioredoxin tag did not alter the aggregation state of TrxCox11. Incubation with thrombin to cleave the tag, followed by gel filtration analysis, generated a chromatogram consistent with the presence of monomeric thioredoxin and dimeric Cox11. When expressed as a control, thioredoxin migrated as a monomer on a sizing column and bound only 0.1 mol of copper per mol of protein ($n = 1$, data not shown).

A more quantitative analysis of the solution state of TrxCox11 was obtained by sedimentation equilibrium. The final fit used four data files of various concentrations. TrxCox11 was best described by a single species model returning a molecular mass of 80.6 ± 18 kDa, consistent with the calculated dimer molecular mass of 80.4 kDa. The data were well described by

![Fig. 4. X-ray absorption spectroscopy of Cox11. A, the near-edge spectra of TrxCox11 (a), Cox11 with the Trx tag removed (b), and selenomethionyl TrxCox11 (c). For comparison, B shows the near-edge spectra of Cox11 (a), Cox17 (b), and [Cu(SPH)₄]²⁻, a model tetracopper thiolate cluster with all trigonal Cu(I) ions (35) (c). The experimental data are the dashed lines, and the fits are the solid lines.](image-url)
this model as is evident by the randomly distributed residuals shown in Fig. 3. At the concentrations employed, the dimer was the sole species of TrxCox11 observed.

Cox11 Binds Copper with Three Sulfur-containing Ligands—X-ray absorption spectroscopy was performed to probe the coordination environment of the copper site of Cox11. The Cu-K absorption near-edge shows features characteristic of trigonal cuprous thiolate species (Fig. 4A). In particular, the intensity of the 8983 eV 1s → 4p feature is diagnostic of the coordination environment (20) and is very similar to that of CuCox17 and the trigonal [Cu₄(SPh)₆]²⁻–Cu(I)-thiolate complex (Fig. 4B). The edge of TrxCox11 dimer is essentially identical to the absorption edge obtained when all TrxCox11 aggregation states are pooled and analyzed. Moreover, removal of the thioredoxin tag by incubation with thrombin followed by gel-filtration chromatography caused no observable change in the copper site, as monitored by XAS (Fig. 4A).

The Cu-K-edge EXAFS spectra and their Fourier transforms (FT) of Cox11 are shown in Fig. 5, A and B, respectively. The EXAFS data for all preparations were very similar (Fig. 5). The transform shows two major peaks. The more intense peak (at about 2.25 Å) is attributable to Cu-S backscattering, whereas the other peak (at about 2.7 Å) is attributable to outer shell Cu-Cu backscattering. Curve-fitting analysis of the EXAFS spectra indicate three Cu-S ligands at 2.25 Å and one Cu-Cu at 2.71 Å (Table II). Attempts to fit the data using ligands other than sulfur yielded poor quality fits with physically unreasonable Debye-Waller factors.

Mutations That Abrogate Copper Binding Result in the Loss of Cytochrome c Oxidase Activity—The full sequence of yeast Cox11 contains five cysteines. Two of these cysteines are within the candidate mitochondrial targeting sequence and are not likely to be part of the mature protein. Thus, three cysteines and two methionines that are conserved between S. cerevisiae, Neurospora crassa, Arabidopsis thaliana, and Homo sapiens remain as possible copper ligands. Functional analysis of the conserved cysteine and methionine residues was probed by site-directed mutagenesis. Mutations were made in pHCDW3, and this high copy yeast expression vector was transformed into a H9004 cox11 strain. Transformants were tested for complementation of the inability of the H9004 cox11 strain to grow on the non-fermentable carbon source, glycerol. Cys-111, Cys-208, and Cys-210 were necessary for growth on glycerol as alanine substitutions at these positions failed to restore growth on glycerol medium (Fig. 6). Mitochondria isolated from the C111A, C208A, C210A, and C208A/C210A Cox11 mutant strains were isolated and found to lack CcO activity. Western analysis (Fig. 7) demonstrated that the failure of the Cys → Ala variants to complement the null phenotype was not caused by a lack of protein expression. Neither Met-224 nor Met-240 is necessary for respiration (Fig. 6).

Conserved Cysteines Bind the Cu(I) Ion—Site-directed mutagenesis was performed on pHCDW4 to determine which of the conserved cysteines and methionines in Cox11 participate in Cu(I) coordination. Because XAS data suggested that the Cu(I) ligands were sulfurs, nitrogen-donating histidines were ignored. The cysteines, including two that are located in a highly conserved Cys-Xaa-Cys motif, were mutated to alanines, whereas the conserved methionines were converted into leucines. For purification, the single mutations (C111A, M224L, and M240L), the double (C208A/C210A), and the triple (C111A/C208A/C210A) variants were generated. Substoichiometric amounts of copper were bound by the C111A mutant, the C208A/C210A variant, the C111A/C208A/C210A mutant, and the M224L variant (Table I). The mutants behaved similarly during purification and eluted as dimers on the gel filtra-
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Coordination numbers N, interatomic distances R are given in Å, Debye-Waller factors \( \sigma^2 \) (the mean-square deviations in interatomic distance) in Å², and the threshold energy shifts \( \Delta E \) are given in eV. The values in parentheses are the estimated S.D. obtained from the diagonal elements of the covariance matrix. The fit-error function \( F \) is defined as \( \frac{\sum|\exp(\frac{Z}{kT})-\exp(\frac{\Delta E}{kT})|^2}{\sum|\exp(\frac{\Delta E}{kT})|^2} \).

### Table II

| Sample        | Cu-S | Cu-Cu |
|---------------|------|-------|
|               | N    | R (Å) | \( \sigma^2 (\text{Å}^2) \) | N    | R (Å) | \( \sigma^2 (\text{Å}^2) \) | \( \Delta E \) (eV) | F    |
| TrxCox11      | 3    | 2.251 (1) | 0.0058 (1) | 1    | 2.710 (2) | 0.0046 (2) | -19.2 (8) | 0.251 |
| Cox11         | 3    | 2.245 (1) | 0.0047 (1) | 1    | 2.706 (2) | 0.0041 (2) | -19.2 (8) | 0.190 |
| SeMet Cox11   | 3    | 2.247 (1) | 0.0048 (1) | 1    | 2.702 (2) | 0.0044 (2) | -19.2 (8) | 0.169 |

**Fig. 6. Growth phenotype of COXI1 mutants.** High copy yeast expression plasmids carrying wild type or variant COXI1 alleles were transformed into the \( \Delta \)cox11 strain using the lithium acetate method. Transformants were grown in selective medium, diluted 10-fold serially, and spotted onto selective plates containing either glucose or glycerol as the carbon source.

**Fig. 7. Western analysis of Cox11 mutant proteins.** Mitochondria were isolated from \( \Delta \)cox11 cells harboring high copy expression plasmids bearing a wild type or variant COXI1 allele. 20 µg of protein was run on a 12.5% polyacrylamide gel and transferred to nitrocellulose for development. Cox11 and porin were visualized with specific antibodies. Porin is the loading control.

It has been proposed that Cox11 participates in the assembly of the Cuₙ chaperone. Cox11 was labeled with selenomethionine and XAS data were collected. Analysis of the Se-K-edge XAS was not possible because the high methionine content of the protein masked the contribution from Met-224, but the Cu-K-edge XAS showed no significant differences with the wild type, with no indications of Cu-Se backscattering. This result indicates that Met-224 is not coordinated to copper in Cox11.

### Discussion

It has been proposed that Cox11 participates in the assembly of the Cuₙ center of CcO (7). For this function, it is likely that Cox11 interacts with Cox17 to receive copper imported from the cytosol and inserts the copper ion into Cox1. In this study we show that, consistent with this role, Cox11 binds one Cu(I) ion per monomer. The soluble, truncated form of Cox11 bound copper tightly, as the metal was not lost during purification or dialysis in buffers containing β-mercaptoethanol and DTT. XAS shows that the Cu(I) ion is ligated by three sulfurs. These spectroscopic data suggested that methionines or cysteines, particularly two in a highly conserved Cys-Xaa-Cys motif, were probable Cu(I) ligands. Mutation of conserved cysteine residues (Cys-111, Cys-208, Cys-210) led to a significant loss of copper from the purified truncate. Mutation of one conserved methionine (Met-224) also led to a decrease in the copper-to-protein stoichiometry; however, subsequent spectroscopic analysis of selenomethionyl Cox11 showed no Se-Cu backscattering, suggesting that the copper ligands are three conserved cysteine residues.

In vivo, the full-length mutated COXI1 gene coding for each of the three conserved Cys → Ala substitution was unable to complement the growth phenotype of \( \Delta \)cox11 strains on glycerol. The Met → Leu variants showed wild type growth for both Met-224, which showed a decrease in copper binding when purified as a truncate, and Met-240, which bound stoichiometric amounts of copper. Hiser et al. (7) noted that substoichiometric amounts of \( R. \) sphaeroides Cox11 allowed normal CcO activity and spectroscopic characteristics. If M224L causes only a partial loss-of-function, one would not expect to see its effects in vivo. Thus, it is possible that Met-224 plays a role in copper transfer or stabilization of the copper site without binding in the inner coordination sphere.

The soluble truncate of Cox11 exists as a dimer when fusion-tagged or cleaved. This is consistent with its postulated role as the Cuₜ chaperone. CcO is a dimer in the crystal lattices and often in detergent-solubilized forms (21). If CcO is dimeric within the mitochondrial inner membrane, four coppers for the Cuₜ site and two coppers for the Cuₙ site are required. A single Cox11 dimer could reasonably be expected to deliver two copper atoms to a dimer of CcO. XAS shows a Cu-Cu interaction at 2.71 Å, although the copper-to-protein ratio is consistently 1:1. It is likely that the copper-binding site of Cox11 exists as a binuclear cluster at the interface of the dimerization domain, which may be useful for copper delivery. The absence of significant luminescence when excited at 300 nm suggests that the copper site is not a solvent-shielded cluster. Solvent exposure may be useful mechanistically, as the putative loading of copper from Cox17 to Cox11 may be transient in the relay to CcO, and the binding site must be accessible to the target protein. Additionally, although this site is solvent-exposed in the purified protein, the protein in vivo may be folded with the copper site facing the inner mitochondrial membrane for delivery to Cox1.

At present, the likely model for the copper site of Cox11 contains two trigonal copper atoms facing each other. It is unclear whether these ligands for each copper atom come from a single polypeptide, or if the ligation of a single copper atom is accomplished by a combination of cysteines from both polypeptides. One model is the trigonal sites in each monomer being closely juxtaposed giving a close 2.7 Å Cu-Cu distance. Precedence exists for two closely spaced trigonal sites (36).
copper transfer may require the monomeric state. An example of this is the copper transfer from the CCS metallochaperone to apo-superoxide dismutase (Sod1). Sod1 is a stable dimer, and yeast CCS (Lys7) is a monomer/dimer equilibrium (22), yet copper transfer from CCS to Sod1 occurs through a heterodimeric intermediate (23, 24). Similarly, Cox11 may contact both the mitochondrial copper shuttle and the target protein, forming a heterodimer first with its copper donor, Cox17, then with the ultimate acceptor, Cox1. Studies are in progress to elucidate whether Cox11 interacts directly with either Cox17 or Cox1.

It is possible that Cox11 requires other protein partners to be fully functional. The genome of the fission yeast Schizosaccharomyces pombe contains a COX11 homolog with a significant 5′-extension. Basic local alignment of that gene with the non-redundant data base shows that the decoded N terminus of the S. pombe Cox11 shares significant homology with the S. cerevisiae protein Rsm22. Rsm22 is a mitochondrial ribosome protein (25) that is necessary for respiratory growth (data not shown). Another known example of a fusion protein found in the genome of S. pombe is the Cox15/Yah1 fusion protein (11), which function in the biosynthesis of heme a (26). Cox15 and Yah1 are separate polypeptides in S. cerevisiae, but exist as a fusion protein in S. pombe. We suggest that, similar to Cox15/Yah1, Cox11 and Rsm22 function in concert to capture the nascent Cox1 polypeptide and, with the help of other proteins known to be important in the translation and assembly of Cox1-Cox3 (27–33), generate the mature Cox1 necessary for oxidative phosphorylation. Rather than occurring after assembly of the multisubunit complex, insertion of heme a and copper cofactors in Cox1 may occur co-translationally. Indeed, there is evidence that heme a insertion may occur prior to association of Cox1 with other subunits (34). The co-translationally inserted cofactors in Cox subunits may be a key component in the assembly of the active complex.

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