Characterization of the Cytochrome c Oxidase Assembly Factor Cox19 of Saccharomyces cerevisiae*

Kevin Rigby1, Limei Zhang1, Paul A. Cobine1, Graham N. George1, and Dennis R. Winge1

Received for publication, October 27, 2006, and in revised form, January 17, 2007 Published, JBC Papers in Press, January 19, 2007, DOI 10.1074/jbc.M610082200

From the 1Departments of Medicine and Biochemistry, University of Utah Health Sciences Center, Salt Lake City, Utah 84132 and the 2University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E2, Canada

Cox19 is an important accessory protein in the assembly of cytochrome c oxidase in yeast. The protein is functional when tethered to the mitochondrial inner membrane, suggesting its functional role within the intermembrane space. Cox19 resembles Cox17 in having a twin CX3C sequence motif that adopts a helical hairpin in Cox17. The function of Cox17 appears to be a Cu(I) donor protein in the assembly of the copper centers in cytochrome c oxidase. Cox19 also resembles Cox17 in its ability to coordinate Cu(I). Recombinant Cox19 binds 1 mol eq of Cu(I) per monomer and exists as a dimeric protein. Cox19 isolated from the mitochondrial intermembrane space contains variable quantities of copper, suggesting that Cu(I) binding may be a transient property. Cysteinyl residues important for Cu(I) binding are also shown to be important for the in vivo function of Cox19. Thus, a correlation exists in the ability to bind Cu(I) and in vivo function.

Cytochrome c oxidase (CcO)2 is the terminal enzyme of the energy-transducing respiratory chain in mitochondria of eukaryotes and certain prokaryotes. The eukaryotic enzyme catalyzes the reduction of molecular oxygen and couples this reaction with proton translocation across the inner membrane of mitochondria to generate the membrane potential necessary for ATP synthesis. Eukaryotic CcO consists of 12 or 13 subunits, with the three core enzyme subunits (Cox1–Cox3) being encoded by the mitochondrial genome. The catalytic cofactors in CcO include two modified heme a moieties and three copper ions (1). One metal site, designated heme a3-CuB, is deeply buried within the Cox1 subunit. The other two copper ions exist in the binuclear CuA center within Cox2.

Assembly of CcO requires a myriad of steps, including the assembly of subunits translated on cytoplasmic and mitochondrial ribosomes, modification of protoheme to heme a, and the delivery and subsequent insertion of this moiety, along with that of copper into the nascent enzyme complex (2, 3). A number of accessory factors, including Cox11, Cox17, Cox19, Cox23, and Sco1, are known to be important or have been implicated in the copper metallation of CcO (4, 5). Copper insertion into nascent Cox1 and Cox2 chains occurs in the IMS, since the accessory molecules are localized within this compartment. Two proteins, Cox11 and Sco1, are associated with the inner membrane, although each has a soluble domain protruding into the IMS. Cox11 and Sco1 mediate the copper metallation of the CuB and CuA sites in Cox1 and Cox2, respectively (3, 5).

Cox17, Cox19, and Cox23 are members of a conserved family of proteins containing a twin CX3C structural motif that adopts a helical hairpin conformation in Cox17 and the structural subunit Cox12 (6–8). These proteins have similar spacing of the four cysteinyl residues that form the two disulfide bonds in apoCox17 and apoCox12 (1, 8).

Cox17 is a key copper metallochaperone within the IMS, acting as the donor of Cu(I) to both Sco1 and Cox11 in yeast (9). Although Cox17 does not form a stable interaction with either Sco1 or Cox11, Cox17 appears to use distinct interfaces to transfer Cu(I) to each target protein. Cox17 can exist in two distinct Cu(I) conformers. One conformer consists of a single Cu(I) coordinated to a monomeric protein stabilized by two disulfide bonds (10). Coordination of a single Cu(I) ion in Cox17 requires isomerization of the disulfides such that one disulfide consisting of Cys26/Cys57 is converted to a Cys24/Cys57 disulfide prior to Cu(I) binding (8). A second Cu(I) conformer is an oligomeric protein complex coordinating a polycopper-thiolate cluster (11). The polycopper protein is in a dimer/tetramer equilibrium, with the polycopper cluster probably existing at the dimer interface (11). The tetracopper cluster conformer necessitates that multiple cysteine residues be in the reduced thiolate state. Three of six conserved cysteines are present in a C25CXC36 sequence motif. Only those three cysteines are essential for in vivo function (12). A mutant form of Cox17 lacking the remaining three conserved cysteines is functional, suggesting that Cox17 is functional without either of the two disulfides in the twin CX3C structural motif. The single copper is digonally coordinated by Cys23 and Cys26, yet the polycopper cluster requires at a minimum the three essential cysteines. The essentiality of Cys24 is consistent with Cox17 existing in a polycopper configuration within the IMS.
Cox19 resembles Cox17 in being a soluble protein within the mitochondrial IMS, although the protein is also found in the cytoplasm when overexpressed (6). Cells lacking Cox19 are respiratory deficient and have reduced CcO activity. Unlike cox17Δ cells, the respiratory defect of cox19Δ cells is not reversed by the addition of exogenous copper salts. The structural similarities of Cox19 with Cox17 suggested that Cox19 may also function in the copper metallation of CcO. Here Cox19 is reported to resemble Cox17 in its ability to coordinate Cu(I). Cysteine residues important for in vivo Cox19 function are important in Cu(I) coordination.

MATERIALS AND METHODS

Yeast Strains/Growth Medium—A BY4742 wild-type strain (Mat-α, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) and an isogenic cox19Δ strain (Mat-α, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, cox19:kanMX4) obtained from Research Genetics were used for all experiments. Yeast plate cultures were grown on synthetic complete medium lacking uracil to ensure maintenance of plasmids and utilized either 2% glucose (SC Glu) or a 2% glycerol, 2% lactate (SC Gly/Lac) mix as carbon sources. Yeast strains cultured in liquid medium were pregrown in synthetic complete medium lacking uracil containing 2% glucose and lacking uracil (SC –Ura,Glu) and inoculated into rich medium (yeast extract and peptone) containing 2% galactose (YPGal) for growth to early stationary phase.

Yeast Vectors—Four versions of COX19 were cloned into a yeast low copy vector (YCP pRS316) under the control of the regulatable MET25 promoter and containing the CYCI terminator. COX19-Myc and COX19-Strep were generated by PCR. The 5’ oligonucleotides contained a 5’ BamHI site, whereas the 3’ oligonucleotides removed the stop codon and added coding sequence for either a 3’ Myc tag (LEQKLISEEDL) or Strep-tag (SQHSPQFEK), and a 3’ Sall site. Yeast genomic DNA was used as a template for PCR. PCR products were then cloned into pRS316 as BamHI/SalI fragments. Sequencing was used to confirm cloning products.

All mutants were generated by site-directed mutagenesis using the Stratagene QuikChange kit. H26A, H26M, C30A, C40A, C52A, C62A, C3062A-Myc, and C4052A mutants were generated in the COX19-Myc construct, whereas the H26A/C30A, C3062A-Strep, Quad, and R63T mutants were generated in the COX19-Strep construct.

The IM-tethered Cox19 constructs were created by fusing the first 104 codons of SCO2 to the existing COX19-Myc and COX19-Strep, as was done previously with Cox17 (13). The resulting fusion proteins, termed IM-Cox19-Myc and IM-Cox19-Strep, contain the N-terminal mitochondrial import sequence and transmembrane domain of Sco2 fused to Cox19-Myc and Cox19-Strep. IM-tethered Cox19 mutants, including the H26A/C30A, C3062A-Strep, Quad, and R63T mutants, were engineered and verified by sequencing. All yeast DNA transformations were performed using a lithium acetate protocol.

Bacterial COX19-Strep expression vectors were constructed using PCR. The 5’ oligonucleotide was constructed to include a 5’ Ncol site as well as optimize the first 20 codons for Escherichia coli expression. The 3’ oligonucleotide contained sequence for removing the stop codon, encoding a short SGS linker followed by the 8-residue Strep-tag II sequence (WSHPQFEK), and a 3’ BamHI site. S. cerevisiae genomic DNA was used as a PCR template. The PCR product was digested with NcoI/BamHI and cloned into the pET9d vector. Cloning was confirmed by DNA sequencing.

Protein Purification—pET9d expression vectors containing COX19-Strep alleles were transformed into competent BL21 (pLysS) E. coli. Transformants were pregrown at 37 °C to A600 = 0.6, induced by the addition of the 0.45 mm isoprpyl-1-thio-β-d-galactopyranoside, and incubated for an additional 4 h at 30 °C. For purification of copper-loaded protein, CuSO4 was added to the culture medium, to a final concentration of 1 mM, just prior to induction. Cells were then washed and resuspended in 50 mM Tris, 100 mM NaCl, pH 8.0, buffer. Cells were lysed by freeze-thawing followed by repeated sonication in the above buffer with or without 1 mM DTT added. Lysate was cleared by centrifugation at 50,000 × g for 30 min at 4 °C. Supernatant was then loaded onto a 10-ml Strep-Tactin column (Qiagen), preequilibrated in 50 mM Tris, pH 8.0, buffer with or without 1 mM DTT. The column was then washed three times: once with 40 ml of wash buffer (50 mM Tris, 100 mM NaCl, pH 8.0), once with 40 ml of high salt buffer (50 mM Tris, 500 mM NaCl, pH 8.0), and again with 40 ml of original wash buffer. Purified protein was then eluted off with 40 ml of elution buffer (50 mM Tris, 100 mM NaCl, pH 8.0, 1 mM desthiobiotin). The purified protein was concentrated to the desired volume in a 5000 Da cut-off Vivaspin 20 (VivaScience) spin column. Flow-through, washes 1–3, and elution fractions were analyzed by SDS-PAGE on a 15% polyacrylamide gel and visualized by Coomassie staining. Approximately 1.6 mg of Cox19 was isolated per liter of culture.

Copper concentrations were determined for the same fractions by using a PerkinElmer Life Sciences AAAnalyst 100 atomic absorption spectrophotometer. Pure, concentrated protein was subjected to 12-h dialysis in wash buffer with and without 1 mM DTT at 4 °C using a Slide-A-Lyzer dialysis cassette with a 3500 Da cut-off membrane (Pierce).

For reconstitution studies, apoCox19-Strep was denatured and reduced in 6 M guanidinium HCl, 100 mM DTT for 1 h at room temperature. The protein was desalted into 50 mM Tris, 100 mM NaCl, pH 7.4, using Bio-Gel P-6 resin (Bio-Rad). Cysteine reduction was verified by quantifying reduced thiols by the DTDP assay described above. Emission from 450 to 700 nm was monitored upon titration with Cu(I)-acetonitrile with excitation and slit widths set as previously described (30).

Protein was quantified by amino acid analysis on a Hitachi L-8800 analyzer after hydrolysis in 5.7 N HCl containing 0.1% phenol in vacuo at 110 °C. Metal concentrations were measured on a PerkinElmer Optima 3100XL inductively coupled plasma optical emission spectrometer. Quantitation of sulfhydryl groups was performed with 2,2’-dithiodipyridine as described previously (14). The quantity of titratable Cu(I) in CuCox19-Strep was measured by competition with bathocuproine sulfonate. Appearance of Cu(bathocuproine sulfonate), (ε = 12,250) was measured by monitoring absorbance at 483 nm over a 15-min period. All bathocuproine sulfonate competition experiments were performed in the absence of DTT. EPR was
used to quantify the amount of Cu(II) in samples. Quantification was based on Cu(II)-EDTA standards.

Spectroscopy—CuCox19-Strep purified in 1 mM DTT was dialyzed in 50 mM Tris, 100 mM NaCl, pH 8.0, and scanned from 240 to 600 nm on a Beckman DU640 UV-visible spectrophotometer. Luminescence of apoCox19-Strep and CuCox19-Strep was measured on a PerkinElmer Life Sciences LS 55 spectrometer after excitation of 300 nm. Emission was monitored from 450 to 700 nm, with excitation and emission slit widths set at 5 and 20 nm, respectively.

X-ray Absorption Spectroscopy Data Collection—X-ray absorption spectroscopy measurements were conducted at the Stanford Synchrotron Radiation Laboratory with the SPEAR 3 storage ring containing 80–100 mA at 3.0 GeV. Copper K-edge data were collected using beamline 9-3 with a wiggler field of 2 teslas and employing an Si(220) double-crystal monochromator. Beamline 9-3 is equipped with a rhodium-coated vertical collimating mirror upstream of the monochromator and a bent cylindrical focusing mirror (also rhodium-coated) downstream of the monochromator. Harmonic rejection was accomplished by setting the energy cut-off angle of the mirrors to 12 keV. The incident and transmitted x-ray intensities were monitored using nitrogen-filled ionization chambers, and x-ray absorption was measured as the copper K-α fluorescence excitation spectrum using an array of 30 germanium detectors (15). During data collection, samples were maintained at a temperature of ~10 K using an Oxford instruments liquid helium flow cryostat. For each sample, three or four 35-min scans were accumulated, and the energy was calibrated by reference to the absorption of a standard copper metal foil measured simultaneously with each scan, assuming a lowest energy inflection point of the copper foil to be 8980.3 eV.

The extended x-ray absorption fine structure (EXAFS) oscillations (k) were quantitatively analyzed by curve fitting using the EXAFSPAK suite of computer programs. *Ab initio* theoretical phase and amplitude functions were calculated using the program FEFF version 8.2 (16). No smoothing, filtering, or related operations were performed on the data.

Size Exclusion Chromatography—Analytical size exclusion chromatography was utilized to evaluate the quaternary state of apoCox19-Strep and CuCox19-Strep using a 10/30 G-75 Superdex column equilibrated with 50 mM Tris, 100 mM NaCl, pH 8.0, with or without 1 mM DTT. Data were recorded using an Oxford instruments liquid helium flow cryostat. For each sample, three or four 35-min scans were accumulated, and the energy was calibrated by reference to the absorption of a standard copper metal foil measured simultaneously with each scan, assuming a lowest energy inflection point of the copper foil to be 8980.3 eV.

The extended x-ray absorption fine structure (EXAFS) oscillations (k) were quantitatively analyzed by curve fitting using the EXAFSPAK suite of computer programs. *Ab initio* theoretical phase and amplitude functions were calculated using the program FEFF version 8.2 (16). No smoothing, filtering, or related operations were performed on the data.

Size Exclusion Chromatography—Analytical size exclusion chromatography was utilized to evaluate the quaternary state of apoCox19-Strep and CuCox19-Strep using a 10/30 G-75 Superdex column equilibrated with 50 mM Tris, 100 mM NaCl, pH 8.0, with or without 1 mM DTT. Data were recorded using an Oxford instruments liquid helium flow cryostat. For each sample, three or four 35-min scans were accumulated, and the energy was calibrated by reference to the absorption of a standard copper metal foil measured simultaneously with each scan, assuming a lowest energy inflection point of the copper foil to be 8980.3 eV.

The extended x-ray absorption fine structure (EXAFS) oscillations (k) were quantitatively analyzed by curve fitting using the EXAFSPAK suite of computer programs. *Ab initio* theoretical phase and amplitude functions were calculated using the program FEFF version 8.2 (16). No smoothing, filtering, or related operations were performed on the data.

Size Exclusion Chromatography—Analytical size exclusion chromatography was utilized to evaluate the quaternary state of apoCox19-Strep and CuCox19-Strep using a 10/30 G-75 Superdex column equilibrated with 50 mM Tris, 100 mM NaCl, pH 8.0, with or without 1 mM DTT. Data were recorded using an Oxford instruments liquid helium flow cryostat. For each sample, three or four 35-min scans were accumulated, and the energy was calibrated by reference to the absorption of a standard copper metal foil measured simultaneously with each scan, assuming a lowest energy inflection point of the copper foil to be 8980.3 eV.

The extended x-ray absorption fine structure (EXAFS) oscillations (k) were quantitatively analyzed by curve fitting using the EXAFSPAK suite of computer programs. *Ab initio* theoretical phase and amplitude functions were calculated using the program FEFF version 8.2 (16). No smoothing, filtering, or related operations were performed on the data.

Characterization of Cox19 of *S. cerevisiae*—Mitochondria were isolated according to the method described previously (17) in the presence of 1 mM phenylmethysulfonyl fluoride. Mitochondrial copper protein concentrations were quantified by Bradford assay (18). CoC enzymatic activity in isolated mitochondria (5–10 µg of protein) was quantified by monitoring oxidation of 32 µM reduced equine heart cytochrome c at 550 nm in 40 mM KH2PO4, pH 6.7, 0.5% Tween 80.

Immunoblotting Analysis—50 µg of mitochondrial or cytosolic protein was loaded onto a 15% polyacrylamide gel, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. Membranes were probed with the indicated primary antibody and visualized with ECL reagents (Pierce), following incubation with a horseradish peroxidase-conjugated secondary antibody. Anti-Myc antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-Strep antibody was obtained from IBA. Antisera to the mitochondrial OM porin (Por1) and cytosolic phosphoglycerol kinase (Pgk1) were obtained from Molecular Probes, Inc. (Eugene, OR).

RESULTS

Recombinant Cox19 was expressed in *E. coli* as a fusion protein with an 8-residue C-terminal Strep-tag II extension. CuSO4 was added to cultures during the isopropyl 1-thio-β-d-galactopyranoside induction phase. Purification of the fusion protein on Strep-Tactin beads with DTT-containing buffers revealed pH-sensitive UV absorbance in the 250 nm region (Fig. 2A) and luminescence near 570 nm with excitation at 300 nm (Fig. 2B). These spectral properties are consistent with Cu(I)-thiolate coordination as seen in CuCox17. The
emission is abolished by treatment with the strong Cu(I) chelator KCN.

ApoCox19 was generated by denaturation of the protein in guanidinium HCl. After reduction of the thiolates with 100 mM DTT, a fully reduced apoCox19 molecule was recovered as determined by DTNB analysis. Titration of apoCox19 with Cu(I)-acetonitrile resulted in Cu(I)Cox19 formation as judged by luminescence. Emission was maximal with 1 mol eq of Cu(I) added per monomer (Fig. 3). The addition of Cu(I) in excess of 1 mol eq resulted in diminution in the observed emission. Samples titrated with excess Cu(I) followed by desalting retained only 1 mol eq of Cu(I) and had an emission yield of a sample with only 1 mol eq of Cu(I) added. Attenuation in emission by excess copper is observed in other Cu(I)-thiolate cluster proteins, including AceI and the metallothionein Cup1 (20). Binding of excess Cu(I) is believed to disrupt the structure of each protein, thereby increasing solvent accessibility to the Cu(I)-thiolate bonds.

Both CuCox19 and apoCox19 exist as a stable dimeric species (Fig. 4A). The dimeric state of apoCox19 and CuCox19 was confirmed by sedimentation equilibrium (data not shown). In excess of 0.5 mM apoCox19 and CuCox19 show tetrameric and higher order oligomeric species (Fig. 4B).

X-ray absorption spectroscopy was used to provide structural information around the Cu center in CuCox19. The copper K-edge x-ray absorption near edge spectrum of CuCox19 showed a feature at 8983 eV diagnostic of a 1s→4p Cu(I) transition (21) (Fig. 5A). Four coordinate Cu(I) complexes have near edge features above 8985 eV, and thus the presence of quaternary Cu(I) coordination in CuCox19 is ruled out (21). The x-ray absorption near edge spectra for (Cu₄(SPh)₆)₄− , containing a trigonal Cu(I) coordination and (Cu(SC₁₀H₁₂)₂)₂− with a digonal Cu(I) coordination are also included in Fig. 5A for comparison. The energy and intensity of the near edge features in absorption.
The nuclearity of the polycopper center in Cox19 is unclear. The best fit is achieved by including three copper atoms with two shorter Cu–Cu interactions at 2.66 Å and one longer Cu–Cu interaction at 2.86 Å (Table 1). It is noted that this fit led to a significantly larger Debye Waller factor for both Cu–Cu interactions compared with that of tetranuclear copper thiolate clusters in other copper-binding proteins reported (24, 25). However, a fit of three copper atoms is inconsistent with the mean stoichiometry of one Cu(I) ion per dimer. A fit with only one copper in the scattering shell arising from a binuclear site increased the F factor from 13.9 to 16.9%.

Recombinant Cox19 can be isolated as a zinc protein when the protein is expressed in cultures supplemented with exogenous ZnSO4. The protein was isolated as a stable dimeric species with a single Zn(II) bound per monomer and the protein had four titratable thiolates as determined by DTNB analysis.

To assess the metal binding status of Cox19 in yeast, attempts were made to purify Strep-tagged Cox19 from the IMS using Strep-Tactin affinity chromatography. In multiple isolates of IMS Cox19, six isolates contained bound copper (range 0.1–0.7 mol eq/monomer), whereas four isolates contained <0.1 mol eq. Copper was the only metal ion observed by inductively coupled plasma optical emission analysis. The source of the observed variability was not identified but did not appear to correlate with cell growth phase or growth medium. The redox state of the thiolates does also not appear to be a significant variable in the amount of recovered bound copper. The recovered protein contained titratable thiolates, suggesting that the protein was largely in the reduced state within the IMS.

Cells lacking Cox19 are respiratory deficient (6) and are unable to propagate on medium containing nonfermentable carbon sources (Fig. 6A). However, these cells grow normally in medium containing the fermentable glucose. Growth of cox19Δ cells on glycerol/lactate medium is restored in vector-borne COX19 transformants (Fig. 6A). Cox19 fusions with either C-terminal Strep or Myc tags are functional in conferring glycerol/lactate growth (Fig. 6A; Strep fusion not shown). The Myc-tagged and Strep-tagged Cox19 molecules are localized solely within the mitochondrion (Fig. 6B). Quantitation of CcO activity revealed that cells with either Cox19 fusions contained only 50% of wild-type CcO activity (data not shown). CcO activity in cox19Δ cells was less than 10% of WT levels. Transformants of WT cells with either Cox19 fusion showed a weak dominant negative effect on CcO activity but no effect on glycerol/lactate growth.

We demonstrated previously that Cox17 was also functional when tethered to the mitochondrial IM through the Sco2 transmembrane segment (13). A Cox19 fusion protein was generated with a segment of Sco2 encoding its mitochondrial targeting sequence and transmembrane domain. The Cu(I) binding domain of Sco2 that projects into the IMS was replaced with the Cox19 molecular. cox19Δ cells containing the IM-tethered Cox19 chimera are able to propagate on glycerol/lactate medium (Fig. 6A), although cells harboring the chimeric Cox19 contained only 50% of CcO activity of transformants with either Myc- or Strep-tagged Cox19. The
Characterization of Cox19 of S. cerevisiae

IM-tethered Cox19 chimera was also localized within the mitochondrion, and no cleavage product was observed with the Myc-tagged chimera (Fig. 6B), suggesting that the chimeric protein was functional itself.

Yeast lacking Cox19 or Cox17 have normal mitochondrial levels of copper, zinc, and iron. Cells lacking both Cox19 and Cox17 also have normal mitochondrial metal levels (data not shown), suggesting that the two proteins have no function in modulating mitochondrial metal levels.

As mentioned, Cox19 contains four conserved thiolates present in the twin CX_9C structural motif (Fig. 7A). The four thiolates in apoCox17 and Cox12 exist in two disulfide bonds stabilizing the helical hairpin structure (8) (model shown in Table 2). The distal pair of cysteines (removed from the Cu(I) binding center) in Cox17 is not essential for physiological function (12). To determine whether the cysteines in Cox19 are functionally significant, mutational analysis was carried out converting individual cysteines to alanines and engineering two double mutants, replacing both distal and proximal Cys pairs with alanine residues.

Yeast cells harboring mutant COX19 alleles with Cys → Ala substitutions at codons 40, 52, and 62 are respiratory competent. However, cox19Δ cells with a mutant COX19 containing a C30A substitution was growth-compromised on glycerol/lactate (Fig. 7B). The double C40A,C52A mutant of Cox19 is functional, whereas the double C30A,C62A mutant is more severely compromised than the single C30A mutant Cox19. As expected, the quadruple Cys mutant is nonfunctional (Fig. 7C).

Adjacent to the functionally important Cys30 is a highly conserved sequence motif of FPLDH (Fig. 7A). Since histidine is a candidate Cu(I) ligand, a H26A mutant allele of Cox19 was evaluated for in vivo function (Fig. 7B). cox19Δ cells containing the H26A mutant Cox19 are able to propagate on glycerol/lactate medium, although a double H26A,C30A mutant is growth-compromised (Fig. 7C).

The single Cys or His mutants of Cox19 were stably expressed in yeast, whereas the C30A,C62A and H26A,C30A double mutants as well as the quadruple Cys mutant that were growth-compromised on glycerol/lactate medium failed to accumulate in mitochondria. Cox19 uptake into the mitochondrial IMS is dependent on the Mia40 import pathway (26). IMS protein import mediated by Mia40 occurs through transient intermolecular disulfide bonds (27). The low accumulation of the double Cys and quadruple Cys mutants may attenuate IMS import. To bypass the Mia40 import pathway, mutant Sco2/Cox19 fusion proteins were engineered for the C30A,C62A and H26A,C30A double substitutions in addition to the quadruple Cys mutant. Although the protein level of each mutant chimera was comparable with that of the WT IM-tethered Cox19 chimera, the mutant IM-tethered chimeras were nonfunctional in promoting glycerol/lactate growth (Fig. 7C) or O2 activity (Fig. 7D).

**FIGURE 5.** X-ray absorption spectroscopy of CuCox19. A, comparison of the copper K x-ray absorption near edge spectra of CuCox19, Cox17, the trigonally coordinated \([\text{Cu}_{3}(\text{SR})_{6}]^{2+}\), and the digonally coordinated \([\text{Cu}(\text{SR})_{2}]^{+}\).

B, EXAFS oscillations (B) and EXAFS Fourier transforms (C) of CuCox19 and Cox17. Solid lines, experimental data; broken lines, best fits obtained with the parameters given in Table 1. All Fourier transforms are phase-corrected for Cu-S back-scattering.
The original cox19 mutation that led to the cloning of COX19 encoded a protein with an R63T substitution near Cys62 (6). This mutant Cox19 was nonfunctional even when expressed as an IM-tethered Cox19 fusion. The mutant fusion protein was stably expressed (Fig. 8, A and B).

The nonfunctional Cox19 mutant proteins were evaluated for copper binding after purification from E. coli. The C30A single Cox19 mutant as well as the C30A,C62A and H26A,C30A double mutants were compromised in Cu(I) binding (Table 2). The two nonfunctional double mutants had the most severe attenuation in Cu(I) binding. As expected, no Cu(I) binding was observed in the quadruple Cys mutant.

Copper reconstitution studies were carried out with certain mutants (Table 2). Mutants showing low recovered copper following purification also showed diminished stoichiometry of maximal luminescence as well as reduced measured stoichiometry.

**DISCUSSION**

Cox17 and Cox19 are structurally related proteins with a conserved twin CX9C sequence motif. Like Cox17, Cox19 is functional when tethered to the mitochondrial IMS. This result suggests that their functional role is within the mitochondrial IMS. Both proteins are capable of Cu(I)-thiolate coordination, and cysteinyl residues important for their physiological function in the assembly of CcO are important in Cu(I) coordination. In addition, both proteins have an ability to oligomerize to dimeric and tetrameric states. Whereas Cox19 exists in a stable dimer, Cox17 can also exist as a monomer. Each of these features will be separately discussed.

The family of twin CX9C proteins appear to be IMS functional proteins. Import of Cox17 and Cox19 into the IMS is mediated by Mia40 in a process involving transient intermolecular disulfide bonding (27). Cox19 was shown previously to accumulate within the cytoplasm when overexpressed (6), although presently we find the Strep- and Myc-tagged fusion Cox19 to be localized exclusively within the IMS. Limited accumulation of Cox19 and Cox17 within the yeast cytoplasm may arise from limitations in the Mia40 import pathway.

Cox17 and Cox19 share an ability to bind Cu(I) in thiolate coordination. Whereas Cox17 has two additional conserved cysteinyl residues besides the four in the twin CX9C motif, Cox19 contains only the four motif Cys residues. Thus, Cu(I) thiolate coordination in Cox19 must involve motif Cys residues. Only the proximal Cys pair (Cys30 and Cys62) are functionally important in Cox19, since a double substitution of the distal pair (C40A,C52A) results in a functional molecule. In Cox17, only one of the proximal pair cysteines (Cys26) is functionally important (12). Although a C57Y Cox17 mutant is non-functional, it can bind Cu(I) and coordinates it in thiolate coordination.

**TABLE 1**

List of EXAFS curve fitting results for CuCox19

| Cu-S | Cu-N/O | Cu-Cu |
|------|--------|-------|
| N    | R      | σ²    | N    | R      | σ²    | N    | R      | σ²    |
| 3    | 2.257  | 0.0079| 1    | 2.655  | 0.0082| 3    | 2.261  | 0.0077| 1     | 2.664  | 0.0103| 1    | 2.861  | 0.0113|
|      | 2.256  | 0.0052| 1.976| 0.0112| 0.169 | 2.642| 0.0086| 0.190 |

$N$, the coordination number; $R$, the interatomic distance between the absorber and back-scatter; $σ^2$, Debye-Waller factor (the mean square deviation in interatomic distance); Error, the fitting error, defined as $\sum k^2(\chi_{\text{expt}}^2 - \chi_{\text{calcd}}^2)/\sum k^2\chi_{\text{expt}}^2$. The numbers in parentheses are the estimated S.D. precision. The best fit for the sample is highlighted in boldface type.
A functional, a C57A mutant protein is functional. The Cox19 Cys^{30} residue, corresponding to the Cox17 Cys^{26}, is the only single Cox19 Cys mutant that is partially compromised in its in vivo function. The single C30A Cox19 mutant is also weakly compromised in Cu(I) binding, whereas the C30A,C62A double mutant is more severely compromised in both in vivo function and Cu(I) binding. Thus, a correlation exists between in vivo function and Cu(I) binding.

His^{26} within the conserved FPLDH motif appears to correlate in function and Cu(I) binding status. Although His^{26} may be predicted to be a Cu(I) ligand, EXAFS analysis of the protein does not support that assignment. An imidazole-Cu(I) bond would be expected closer to 2 Å, and imidazole carbons may be expected to contribute to back-scattering in the 4 Å region of the Fourier transform. The absence of these features is consistent with His^{26} not being a direct Cu(I) ligand. His^{26} may have a structural role in stabilizing the Cu(I) thiolate center.

EXAFS analysis gives a number of important clues to the structure at the copper center of Cox19. The energy and intensity of copper K-edge near edge features and Cu–S bond length are useful in determining coordination number, whereas the Cu–Cu distance and intensity helps to interpret the nuclearity of the cluster. The x-ray absorption spectroscopy data support the presence of a polynuclear Cu(I) thiolate cluster with a trigonal coordination for each copper atom in the cluster. The observed short Cu–Cu distance suggests the presence of multiple bridging sulfurs in the cluster, and this is consistent with our mutational analysis. The Cu(I) coordination sphere may involve Cys^{30} and Cys^{62}. Since Cox19 is an obligate dimer, the Cu(I) center may be influenced by its dimeric structure.

FIGURE 7. Mutational analysis. A, schematic diagram of Cox19 and mutants engineered. B and C, growth test of mutant strains. BY4742 cox19Δ strains containing mutant alleles of COX19 expressed from low copy plasmids were assessed for growth on glucose and glycerol/lactate. D, CcO enzymatic activities of mutant strains. Mitochondria isolated from the mutant strains were assessed for CcO activity.
The nuclearity of the copper cluster in Cox19 remains unclear. If the single Cu(I) in each monomer is in close juxta- position to the Cu(I) ion in the second monomer, a thiol- bridged binuclear Cu-Cu center may form. The CuCox19 sample used for EXAFS analysis was millimolar in concen- tration, and Cox19 oligomerizes beyond dimeric species at this concentration. A tetranuclear Cu(I) cluster may exist within a higher oligomer of Cox19. Due to the oscillation cancellation effect of the EXAFS component from two Cu_{II}/H18528/H18528/H18528/Cu interactions, we could not draw a solid conclusion on the nuclearity of the copper cluster by EXAFS analysis alone at the current resolution of the experiment. Future experiments will attempt to address the nuclearity of the Cu(I) center at lower protein concentrations requiring more synchrotron beam time.

Two lines of evidence suggest that Cu(I) binding to a dimeric Cox19 is physiologically relevant. First, attempts to purify Strep-tagged Cox19 from the IMS resulted in variable results concerning whether the recovered protein contained bound Cu. No isolate of IMS Cox19-Strep contained Zn(II), and some isolates contained bound copper. The recovered protein contained titratable thiolates, suggesting that the protein was largely in the reduced state within the IMS. Purification of Strep-tagged Cox17 from the IMS yielded protein with only limited quantities of bound copper (0.1–0.2 mol eq). If Cox19 has a copper shuttle role analogous to Cox17, Cu(I) binding may only be transient. Second, the lack of glycerol growth observed with the C30A,C62A double mutant was weakly sup- pressed by high exogenous copper. This weak copper suppres- sion is not observed in cofx19A cells.

One curious aspect of Cox17 and Cox19 is that both genes lie within a segment of chromosome XII that is duplicated on the opposite side of the centromere. The S. cerevisiae genome has many duplicated chromosomal regions that account for up to 16% of the yeast proteome, and it has been proposed that these have arisen from an ancient duplication of the entire genome (8). Within the limited repeat, Cox19 occupies a gene position analogous to that of Cox12, a subunit of CcO that also contains a twin C_{X}9C motif. The significance of this duplication is unclear. The bovine equivalent of Cox12, CoxVIB, contains two disulfide bonds in the crystal structure (1), although neither disulfide is essential for Cox12 function.3

We demonstrated previously that Cox17 is competent in Cu(I) transfer to both Sco1 and Cox11 for metallation of CuA and CuB sites in Cox2 and Cox1, respectively (9). If Cox17 dis- tributes Cu(I) to both Sco1 and Cox11, the major question concerns the role of Cox19. One attractive scenario is that Cox17 and Cox19 both contribute to CuA site formation. Since the CuA site is a mixed valent binuclear copper center, it is conceiv- able that CuA site formation is mediated by two Cu transfer reactions, one of which involves Cu(II).

We showed that Sco1 is able to bind either Cu(I) or Cu(II) and that the Cu(II) conformer exhibits a S-Cu(II) charge trans- fer absorption band (29). Sco1 may transfer both a Cu(I) and Cu(II) ion successively to form the mixed valent CuX site. We have demonstrated that Cox17 transfers only Cu(I) to Sco1.

3 K. Rigby, P. A. Cobine, H. S. Carr, and D. R. Winge, unpublished observations.
Future studies will address whether Cox19 is able to generate the S-Cu(II) chromophore in Sco1.

Acknowledgment—We acknowledge the generous assistance and advice of Dr. Alex Tzagoloff in these studies.

REFERENCES

1. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Hakashima, R., Yaono, R., and Yoshikawa, S. (1995) Science 269, 1069–1074
2. Herrmann, J. M., and Funes, S. (2005) Gene (Amst.) 354, 43–52
3. Barrientos, A., Barros, M. H., Valnot, I., Rotig, A., Rustin, P., and Tzagoloff, A. (2006) Gene (Amst.) 354, 43–52
4. Carr, H. S., and Winge, D. R. (2003) Acc. Chem. Res. 36, 309–316
5. Khalimonchuk, O., and Rodel, G. (2005) Mitochondrion 5, 363–388
6. Nobrega, M. P., Bandeira, S. C. B., Beers, J., and Tzagoloff, A. (2002) J. Biol. Chem. 277, 40206–40211
7. Barros, M. H., Johnson, A., and Tzagoloff, A. (2004) J. Biol. Chem. 279, 31943–31947
8. Arnesano, F., Balatri, E., Banci, L., Bertini, I., and Winge, D. R. (2005) Structure 13, 713–722
9. Horng, Y. C., Cobine, P. A., Maxfield, A. B., Carr, H. S., and Winge, D. R. (2004) J. Biol. Chem. 279, 35334–35340
10. Abajian, C., Yatsunyk, L. A., Ramirez, B. E., and Rosenzweig, A. C. (2004) J. Biol. Chem. 279, 53584–53592
11. Heaton, D. N., George, G. N., Garrison, G., and Winge, D. R. (2001) Biochemistry 40, 743–751
12. Heaton, D., Nittis, T., Srinivasan, C., and Winge, D. R. (2000) J. Biol. Chem. 275, 37582–37587
13. Maxfield, A. B., Heaton, D. N., and Winge, D. R. (2004) J. Biol. Chem. 279, 5072–5080
14. Grassetti, D. R., and Murray, J. F. (1967) Arch. Biochem. Biophys. 119, 41–49
15. Cramer, S. P., Tench, O., Yocum, M., and George, G. N. (1988) Nucl. Instrum. Methods Phys. Res. Sect. A 266, 586–591
16. Rehr, J. J., Albers, R. C., and Lill, R. (1992) Methods Cell Biol. 65, 37–51
17. Bradford, N. M. (1976) Anal. Biochem. 59, 248–254
18. Changela, A., Chen, K., Xue, Y., Holschen, J., O’Halloran, T. V., and Mondragon, A. (2003) Science 301, 1383–1387
19. Winge, D. R., Gradén, J. A., Posewitz, M. C., Martin, L. J., Jensen, L. T., and Simon, J. R. (1997) J. Bioinorg. Chem. 2, 2–10
20. Kau, L.-S., Spira-Solomon, D. J., Penner-Hahn, J. E., Hodgson, K. O., and Solomon, E. I. (1987) J. Am. Chem. Soc. 109, 6433–6442
21. Dance, I. G. (1986) Polyhedron 5, 1037–1104
22. Bowmaker, G. A., Clark, G. R., Seadon, K. J., and Dance, I. G. (1984) Polyhedron 3, 535–544
23. Horng, Y.-C., Leary, S. C., Cobine, P. A., Young, F. B. J., George, G. N., and Winge, D. R. (2005) Biochemistry 41, 6469–6476
24. Xiao, Z., Loughlin, F., George, G. N., Howlett, G. J., and Wedd, A. G. (2004) J. Am. Chem. Soc. 126, 3081–3090
25. Rissler, M., Wiedemann, N., Pfannschmidt, S., Gabriel, K., Guiard, B., Pfanner, N., and Chacinska, A. (2005) J. Mol. Biol. 353, 485–492
26. Mesecke, N., Terziyska, N., Kozany, C., Baumann, F., Neupert, W., Hell, K., and Herrmann, J. M. (2005) Cell 121, 1059–1069
27. Terziyska, N., Lutz, T., Kozany, C., Mokranjac, D., Mesecke, N., Neupert, W., Herrmann, J. M., and Hell, K. (2005) FEBS Lett. 579, 179–184
28. Horng, Y.-C., Leary, S. C., Cobine, P. A., Young, F. B. J., George, G. N., Shoubridge, E. A., and Winge, D. R. (2005) J. Biol. Chem. 280, 34113–34122
29. Dameron, C. T., Winge, D. R., George, G. N., Bansone, M., Hu, S., and Hamer, D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6127–6131