Characterization of the Copper Chaperone Cox17 of *Saccharomyces cerevisiae*†

Chandra Srinivasan,‡ Matthew C. Posewitz,‡ Graham N. George,§ and Dennis R. Winge*,‡

University of Utah Health Sciences Center, Salt Lake City, Utah 84132, and Stanford Synchrotron Radiation Laboratory, Stanford University SLAC, P.O. Box 4393, Stanford, California 94309

Received February 20, 1998; Revised Manuscript Received April 6, 1998

ABSTRACT: Assembly of functional cytochrome oxidase in yeast requires Cox17, which has been postulated to deliver copper ions to the mitochondrion for insertion into the enzyme. This role for Cox17 is supported by the observation that it binds copper as a binuclear cuprous−thiolate cluster. X-ray absorption spectroscopy, together with UV−visible absorption and emission spectroscopy, indicates the presence of bound cuprous ions, trigonally coordinated by thiolate ligands. Analysis of the EXAFS shows three Cu−S bonds at 2.26 Å, plus a short Cu−Cu distance of 2.7 Å, indicating a binuclear cluster in Cox17. The cuprous−thiolate cluster in Cox17 is substantially more labile than structurally related clusters in metallothioneins.

The assembly of the respiration chain within the mitochondrion is dependent on a large number of genes. Over 30 complementation groups have been established for mutants exhibiting a deficiency of cytochrome oxidase (*1*). A number of genes encode subunits of the enzyme. Mammalian and yeast cytochrome oxidase complexes consists of 13 and at least 9 subunits, respectively (2−4). Some of the other gene products are critical for assembly of the oxidase, and others may be functional in cofactor delivery (*1*, *4*−*6*). The bovine enzyme contains two hemes, two distinct copper sites, one Zn(II), and one Mg(II) ion in addition to phospholipid constituents (*3*).

The complexity of the enzyme appears to require assessory proteins for assembly of the complex. Research on the assembly of cytochrome oxidase has focused on the yeast *Saccharomyces cerevisiae* (*1*, *4*−*6*). Two genes in *S. cerevisiae* have been shown to be important for incorporation of the Cu ions into the enzyme (*7*, *8*). Three Cu ions are inserted into cytochrome oxidase: two into subunit II, forming the binuclear Cu_A site; and a single Cu ion into subunit I, forming the binuclear Cu−heme a_3 reaction center (*3*). Since both subunits I and II are mitochondrially synthesized, Cu ion insertion must occur within the mitochondrion.

Cells harboring a mutant *COX17* were found to be respiratory deficient but able to express both mitochondrial and nuclear-encoded cytochrome oxidase subunits (*7*). The respiratory deficiency of the *cox17* mutant was suppressed by exogenous Cu(II), suggesting that Cox17 was important in Cu delivery to the mitochondrion (*7*). The point mutation in the *cox17* mutant was a Cys→Tyr substitution. Exogenous Cu(II) did not rescue a subset of other cytochrome oxidase mutants. The apparent defect in Cu delivery to cytochrome oxidase was not a general defect in Cu balance in the cell. The *cox17* mutant had normal superoxide dismutase activity (Sod1) and Fet3 ferro-oxidase activity (*7*). Sod1 and Fet3 require Cu for function.

Additional factors appear to be important for Cu ion insertion into cytochrome oxidase. The respiratory-deficient phenotype of the *cox17* point mutant was suppressed by high-copy *SCO1* and *SCO2* (*8*). However, high-copy *SCO1* exhibited only limited suppression in a *cox17* null strain. Sco1 was previously characterized as a cytochrome oxidase assembly factor localized within the inner mitochondrial membrane (*9*). Like *cox17* mutants, *sco1* mutants exhibit a specific defect in cytochrome oxidase (*8*). *sco1* mutants (null and point) cannot be suppressed by high exogenous Cu or high-copy *COX17*.

The *COX17* ORF is predicted to encode a 8057 dalton polypeptide. The localization of Cox17 in the cytosol and intermitochondrial membrane space led to the prediction that Cox17 may be a Cu shuttle protein important for the delivery of Cu ions to the mitochondrion for assembly of cytochrome oxidase (*10*). Yeast Cox17 contains seven cysteinyl residues of which six are conserved in the human Cox17 molecule (*11*). The presence a CCxC sequence motif is consistent with Cox17 being a copper binding protein.

Two other low molecular weight Cu shuttles have been described in yeast. Atx1 is the Cu chaperone involved in the delivery of Cu ions to the Golgi apparatus for biosynthesis of the Fet3 ferro-oxidase (*12*, *13*). The name Atx1 was derived from its antioxidant activity, which is the basis of its original discovery (*14*). Lys7 is important for providing Cu ions for the biosynthesis of the cytoplasmic Cu,Zn-superoxide dismutase (Sod1) (*15*). Cells lacking the Lys7

† This work was supported by Grant ES 03817 from the National Institutes of Environmental Health Sciences, NIH, to D.R.W. We acknowledge support from the National Institutes of Health (5P30-CA 42014) to the Biotechnology Core Facility for DNA synthesis. The Stanford Synchrotron Radiation Laboratory is funded by the Department of Energy, Office of Basic Energy Sciences. The Biotechnology Program is supported by the National Institutes of Health, Biomedical Research Technology Program, Division of Research Resources, and by the Department of Energy, Office of Biological and Environmental Research.

* To whom correspondence should be addressed. Telephone: 801-585-5103. Telefax: 801-585-5469. Email: dennis.winge@hsc.utah.edu.

‡ University of Utah Health Sciences Center.

§ Stanford Synchrotron Radiation Laboratory.

S0006-2960(98)00418-8 CCC: $15.00
Published on Web 05/02/1998

gene are devoid of Sod1 activity, resulting in the inability to biosynthesize lysine. Human homologues to Lys7, Atx1, and Cox17 have already been identified (*11*, *15*, *16*).

The discovery of intracellular Cu shuttles or transporters was expected as unbound Cu ions can catalyze Fenton chemistry, generating the highly reactive hydroxyl radical. The presence of multiple Cu shuttles yielding specific Cu delivery to subcellular locations raises intriguing questions about the mechanism of specific routing by these proteins and the mechanism of Cu ion insertion into the metalloenzymes. In this report, we demonstrate the existence of a labile, Cu(I) binuclear cluster in Cox17.

MATERIALS AND METHODS

Construction of the Synthetic COX17 Gene. A synthetic *COX17* construct was generated with codon bias optimized for expression in *S. cerevisiae* which also improved codon bias for bacterial expression. Polymerase chain reaction (PCR) using four overlapping oligonucleotides yielded a 220 base pair fragment which was digested with *Bam*HI and *Sal*I and cloned into a pBluescript vector. The presence of *COX17* was verified by restriction digestion and nucleotide sequencing. For *E. coli* expression of Cox17 as an amino-terminal GST fusion, the *Bam*HI/*Sal*I fragment from the pBluescript vector was excised and cloned into pGEX-4T-1. Standard culturing and transformation conditions were used.

Purification and Characterization of Cox17. *E. coli* strain BL21(DE3) harboring *COX17* in pGEX-4T-1 was induced with IPTG in cells cultured at 37 °C to an A_{600} of 0.6. Thirty minutes after the addition of IPTG, $CuSO_4$ was added to a final concentration of 1.4 mM, and the cells were allowed to grow for an additional 3 h. The cells were then pelleted by centrifugation and washed thoroughly with 0.25 M sucrose. The cell paste was suspended in phosphate-buffered saline (PBS) and 5 mM DTT and stored at −70 °C. The cells were lysed by freeze−thawing and repeated sonication. The cell lysate was centrifuged at 100000*g* at 4 °C for 30 min. The clarified lysate was mixed with Triton X-100 to a final concentration of 1% and filtered through a 0.45 μm filter prior to loading on a glutathione−Sepharose column equilibrated with PBS containing 5 mM DTT. After loading, the column was washed extensively with 10−15 column volumes of PBS with DTT, and then the fusion protein was eluted using 50 mM Tris-HCl buffer containing 20 mM glutathione at pH 8.0. The purity of the Cox17−GST fusion protein was verified by SDS−PAGE. After elution with glutathione, the fusion protein was digested with 10 units of thrombin overnight at room temperature. The cleaved Cox17 was separated from GST and unclipped fusion proteins by reapplying the digest over the glutathione−Sepharose column after dialysis. The flow-through from the column contained Cox17 which was further purified using a G-75 Sephadex gel filtration column.

Amino acid analysis was carried out after hydrolysis in 5.7 N HCl containing 0.1% phenol in vacuo at 110 °C. The analysis was performed on a Beckman 6300 analyzer. Purified Cox17 was found to have the expected mass by electrospray ionization mass spectrometry. The ESMS spectrum was recorded on a Fisons Instruments Trio 2000 spectrometer (VG Biotech).

Optical absorption spectroscopy was carried out on a Beckman DU 640 spectrometer. Ligand exchange reactions with bathocuproine disulfonate (BCS) were performed by moitoring the appearance of the Cu−BCS complex at 483 nm as a function of BCS concentration. The Cu−BCS complex was measured spectrophotometrically at 483 nm using a molar absorptivity of 12 250 (*17*). The copper concentration of the protein samples was fixed at 28 μM, and the samples were buffered with 20 mM Tris-HCl at pH 7.4. The copper concentration in the protein samples was measured using a Perkin-Elmer (AAnalyst 100) atomic absorption spectrophotometer. Luminescence was measured on a Perkin-Elmer 650-10s fluorometer with an excitation wavelength at 300 nm.

XAS measurements were carried out at the Stanford Synchrotron Radiation Laboratory with the SPEAR storage ring containing 55−100 mA at 3.0 GeV. Copper K-edge data were collected on beamline 7-3 using a Si(220) double-crystal monochromator, with an upstream vertical aperture of 1 mm, and a wiggler field of 1.8 T. Harmonic rejection was accomplished by detuning one monochromator crystal to approximately 50% off peak, and no specular optics were present in the beamline. The incident X-ray intensity was monitored using a nitrogen-filled ionization chamber, and X-ray absorption was measured as the X-ray Cu Kα fluorescence excitation spectrum using an array of 13 germanium intrinsic detectors (*18*). During data collection, samples were maintained at a temperature of approximately 10 K, using an Oxford Instruments liquid helium flow cryostat. Ten 30 min scans were accumulated, and the absorption of a copper metal foil was measured simultaneously by transmittance. The energy was calibrated with reference to the lowest energy inflection point of the copper foil, which was assumed to be 8980.3 eV. EXAFS data analysis was done using the EXAFSPAK suite of computer programs,[1] and the phase and amplitude functions were calculated using the ab initio code Feff version 7.02 (*19*, *20*).

RESULTS

A synthetic *COX17* gene was constructed with preferred yeast codons (*21*). The synthetic gene was cloned into expression vectors for *S. cerevisiae* and *E. coli*. Yeast expression of the synthetic untagged *COX17* was marginal. In contrast, cloning of the synthetic *COX17* gene into pGEX-4T-1 creating a *GST/COX17* hybrid gene resulted in good expression of the fusion protein in *E. coli*. Cox17 was purified from *E. coli* as a GST fusion using glutathione−Sepharose affinity chromatography. After elution with glutathione, the fusion protein was digested with thrombin to liberate the Cox17 from the hybrid. The eluent from the second glutathione−Sepharose column contained Cox17 and was further purified by gel filtration on Sephadex G-75. Cox17 recovered from gel filtration was greater than 90% pure by SDS−polyacrylamide gel electrophoresis. Chromatography of Cox17 on C_{18} RP-HPLC revealed a single proteinacious component eluting at 60% acetonitrile.

[1] The EXAFSPAK program suite was developed by one of the authors (G.N.G.) and is available on the SSRL Web site http://ssrl.slac.stanford.edu/exafspak.html or by application to G.N.G. in writing.

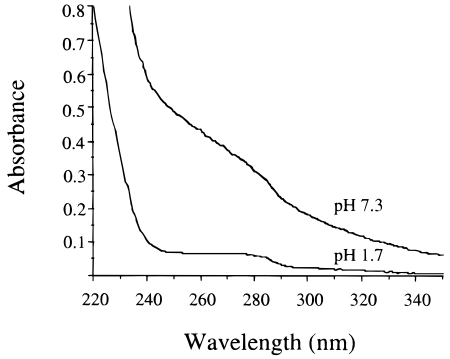

FIGURE 1: UV absorption spectra of CuCox17 (29 μM in protein) were recorded in 20 mM Tris-HCl containing 100 mM NaCl and 0.1% β-mercaptoethanol at pH 7.3 and at pH 1.7 with the addition of 1 N HCl.

Amino acid analysis of the purifed Cox17 revealed the expected composition of Cox17 with the N-terminal Gly-Ser appendage remaining from thrombin cleavage of the hybrid molecule. Electrospray mass spectrometry at low pH revealed a mass of 8197.3 amu. The expected mass of the cleaved Cox17 molecule is 8200.2 amu suggesting that the purified Cox17 does contain the dipeptide Gly-Ser appended to its N-terminus. Metal analysis revealed that Cox17 binds 2.0 $\pm$ 0.2 (3 independent isolates) Cu ions per molecule. The same Cu binding stoichiometry was observed for the undigested hybrid molecule. The CuCox17 complex is stable to high concentrations of thiolates as 20 mM GSH and 5 mM DTT are used during purification.

The ultraviolet absorption spectrum of CuCox17 revealed acid-labile transitions in the UV consistent with thiolate ligation (Figure 1). Human and yeast Cox17 share six conserved cysteinyl residues (*11*). Proteins exhibiting predominantly thiolate coordination of Cu ions show an envelope of S$-$Cu charge transfer bands centered around 260 nm (*21*). We compared CuCox17 to the well-studied Cys-rich, low molecular weight Cup1 metallothionein from yeast (*23*). Cup1 functions in Cu ion buffering by tenaciously binding Cu(I) ions within a heptacopper thiolate cluster (*24*). The UV transitions of CuCox17 are more pH-labile relative to CuCup1. Acidification to pH 5 reduces the absorbance at 255 nm of CuCox17 by 50%, whereas a pH of 2 only attenuates the absorbance of CuCup1 by 25%.

The CuCox17 complex is luminescent with emission around 570 nm. The observed luminescence is consistent with Cu(I) coordination in a solvent-shielded environment (Figure 2). The emission of monovalent metal ion complexes (Au, Ag, Cu) is attributed to trigonal coordination geometry (*25*). Similar emission is observed in the trigonally coordinated Cu(I) complexes of Cup1 and the Ace1 transcription factor (*21*). The relative quantum yield of CuCox17 is significantly lower than that of CuCup1, suggesting that the Cu center in Cox17 is more solvent-accessible than the buried heptacopper cluster in Cup1 (Figure 3).

The lability of the CuCox17 complex was also addressed using a ligand substitution reaction with bathocuproine disulfonate (BCS), a Cu(I)-specific chelator (*17*). Incubation of CuCox17 and CuCup1 at equimolar Cu levels with increasing concentration of BCS resulted in rapid displacement of Cox17-bound Cu(I), but minimal displacement of Cup1-bound Cu(I) (Figure 4). With a 16-fold molar excess

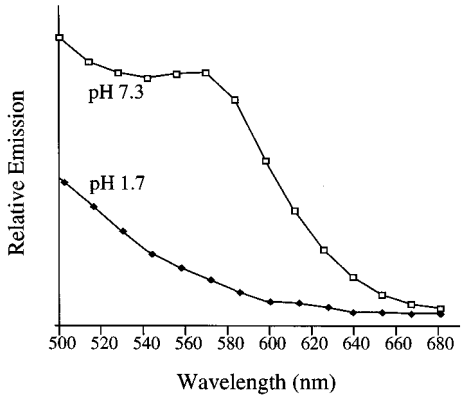

FIGURE 2: Cu(I) luminescence of CuCox17 at pH 7.3 and 1.7 was measured using 3.5 μM protein in 20 mM Tris, 100 mM NaCl, and 0.1% β-mercaptoethanol. The pH of the sample was lowered by the addition of 1 N HCl. The excitation wavelength was 300 nm, and the emission was varied from 500 to 680 nm with the emission maximum being around 570 nm.

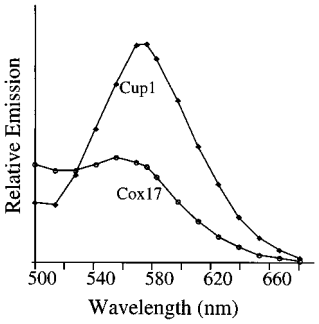

FIGURE 3: Cu(I) luminescence of CuCox17 and CuCup1 was measured using equivalent Cu(I) concentrations [7 μM Cu(I)]. Excitation was at 300 nm, and the emission was scanned from 500 to 680 nm with emission occurring near 570 nm.

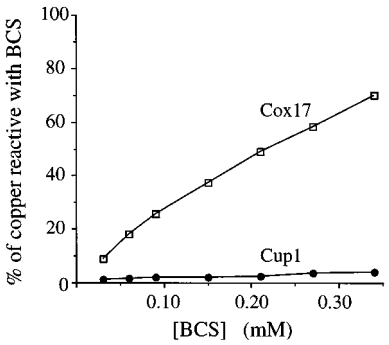

FIGURE 4: Reactivities of Cu(I) ions in complexes with Cox17 and Cup1 (28 μM copper in each case) in the presence of varying amounts of bathocuproine sulfonate (BCS) were measured by monitoring the absorbance of the CuBCS complex at 483 nm. After incubation with 0.34 mM BCS, 70% of the Cu(I) ions in Cox17 were transferred to BCS, whereas only 3.9% of the Cu(I) ions of Cup1 were present as CuBCS.

of BCS, nearly 70% of the Cox17-bound Cu(I) was displaced in comparison to less than 5% of the Cup1-bound Cu(I).

X-ray absorption near-edge spectroscopy was carried out to probe the structure of the Cu sites in Cox17. The copper K absorption near-edge showed a feature at 8983 eV consistent with a 1s$\rightarrow$4p transition of Cu(I) ions (*26*) (Figure 5). The energy and intensity of the near-edge feature relative to the absorption continuum are similar to those seen in trigonal Cu(I)$-$thiolate complexes (*26*, *27*). The X-ray absorption near-edge is shown for a Cu_4S_6 synthetic cluster,

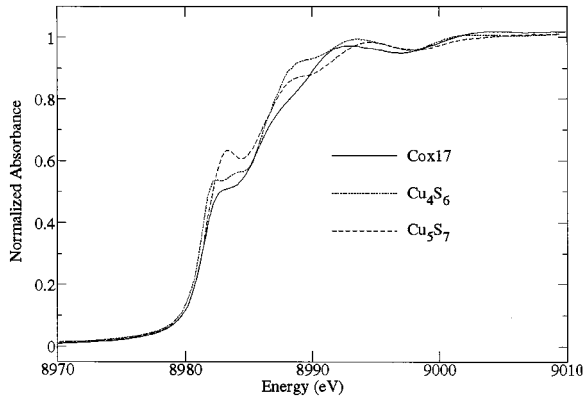

FIGURE 5: X-ray absorption edge spectrum of CuCox17 and two synthetic Cu(I)−thiolate cage clusters: $(Me_4N)_2[Cu_4(SPh)_6]$ or Cu_4S_6, which contains four trigonally bound Cu(I) ions, and $(Et_4N)[Cu_5(SBu^t)_6]$ or Cu_5S_6, which contains four trigonally bound Cu(I) ions and a single digonally bound Cu(I) ion.

$(Me_4N)_2[Cu_4(SPh)_6]$, containing exclusively trigonal Cu(I) coordination and a Cu_5S_6 synthetic cluster, $(Et_4N)[Cu_5(SBu^t)_6$, containing four trigonally bound Cu(I) ions and a single digonally bound Cu(I) ion (*28*, *29*). The edge peak is more prominent in a Cu(I) complex with purely digonal coordination (*26*). Four coordinate Cu(I) complexes have near-edge features above 8985 eV (*26*). The K-edge X-ray absorption of CuCox17 is consistent with Cu(I) coordination and suggestive of trigonal Cu(I) coordination.

The Cu K-edge EXAFS spectra and Fourier transform for CuCox17 are shown in Figure 6. The Fourier transform has two main peaks indicating that the EXAFS arises from two prominent interactions. The transform peak at 2.26 Å is due to first shell ligand backscattering. The 2.26 Å distance is consistent with trigonal Cu(I) coordination. Trigonal Cu(I)−thiolate complexes typically have Cu−S bond distances of 2.26−2.28 Å, whereas digonal Cu−S coordination gives distances closer to 2.16 Å (*28*, *29*). The inset of Figure 6 shows the transform of the $(Me_4N)_2[Cu_4(SPh)_6]$ synthetic cage cluster. The mean Cu−S bond distance in this tetracopper cluster with exclusive trigonal Cu coordination is 2.28 Å (*28*, *30*). Curve-fitting of the CuCox17 EXAFS was optimal with a Cu coordination number of 3, consistent with trigonal coordination geometry predicted by the X-ray edge feature and the mean Cu−S bond distance.

The outer shell interaction at 2.72 Å is best fit to a Cu−Cu backscattering interaction. A similar 2.74 Å peak is clearly seen in the tetracopper cage cluster (Figure 6 inset). The mean Cu−Cu distance in this cluster was found to be 2.74 ± 0.06 Å (*30*). The observation of a short 2.72 Å Cu−Cu distance is diagnostic of Cu cluster in Cox17. As expected, the best fit from EXAFS curve-fitting predicted a Cu−Cu coordination number of 1.

DISCUSSION

The candidate Cu ion shuttle for mitochondria is Cox17. In this report, we demonstrate that Cox17 is a Cu binding protein consisting of a labile, binuclear Cu(I)−thiolate cluster. The evidence for Cu(I) coordination is 2-fold. First, the emission of the CuCox17 complex is characteristic of Cu(I) coordination. Cu(II) complexes do not luminesce (*31*). Second, the X-ray absorption edge feature at 8983 eV is only seen in Cu(I) complexes. An initial report recently appeared on the purification of Cox17 from yeast. There was minimal Cu bound to the purified protein, but two Cu ions were reported to be bound after a reconstitution procedure (*10*).

Thiolate coordination in CuCox17 is suggested by the prominent, acid-labile transitions in the UV characteristic of S−Cu charge-transfer bands (*22*). Curve-fitting of the EXAFS of CuCox17 is best matched with the inclusion of sulfur scatterers. The observed mean Cu−ligand bond distance is within the expected range for Cu−S coordination.

The evidence for a binuclear cluster rests on the observed outer shell backscatter peak at 2.7 Å which is best fit as a short Cu−Cu interaction. A series of polycopper thiolate clusters have been structurally characterized with short 2.7−3 Å Cu−Cu distances (*28*). The nuclearity of the Cox17 cluster must be 2 as only two Cu(I) ions are bound.

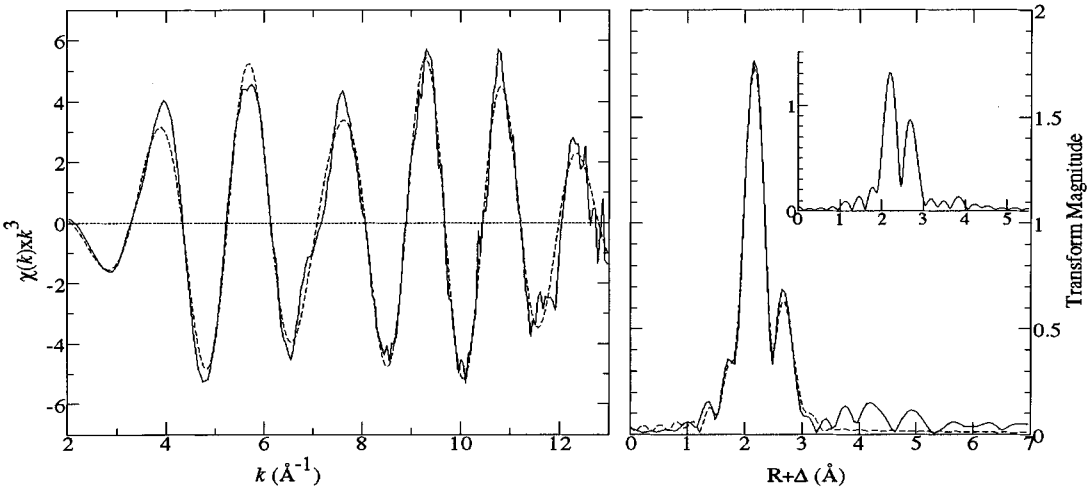

FIGURE 6: Cu K-edge EXAFS (left) and Fourier transform (right) of CuCox17. The solid lines show experimental data, and the broken lines show the results of least-squares refinement calculated with 3 Cu−S at 2.261(2) Å, with $\sigma^2 = 0.0047(1)$, and 1 Cu−Cu at 2.720(3) Å, with $\sigma^2 = 0.0061(3)$. The σ^2 values are the Debye−Waller factors, and the values in parentheses are the estimated standard deviations (precisions) obtained from the diagonal elements of the covariance matrix. We note that the accuracies will be somewhat greater than the precisions, being typically better than ±0.02 Å for interatomic distances and ±20% for coordination numbers and Debye−Waller factors. The inset shows the transform of the $(Me_4N)_2[Cu_4(SPh)_6]$ synthetic cage cluster.

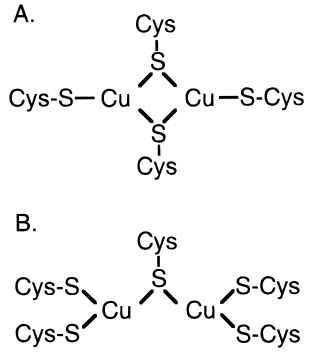

FIGURE 7: Candidate models for the binuclear Cu(I)−thiolate cluster in Cox17.

The coordination geometry of the binuclear cluster appears to be trigonal based on the intensity of the X-ray absorption edge feature, the observed emission, and the best fit of the Cu−S backscattering EXAFS data. Trigonal coordination of a binculear Cu(I) cluster would require 4−5 thiolates depending on the number of bridging thiolates. Two models for binuclear cluster are shown in Figure 7.

There are six conserved thiolates comparing the yeast and human Cox17 molecules, so either model is possible. The short Cu−Cu distance precludes a linear, singly bridging thiolate. A series of binuclear Cu(I) synthetic clusters have been characterized crystallographically that are relevant to the two candidate clusters in Cox17. Several binuclear synthetic clusters contain two bridging sulfur ligands; an additional cluster contains a singly bridging sulfur atom (32−34). The observed Cu−Cu distances in the doubly bridging complexes range from 2.68 to 3 Å, whereas the Cu−Cu distance in the singly bridged cluster is 3.3 Å (*33*, *35*). The 2.7 Å Cu−Cu distance in CuCox17 is consistent with the doubly bridging μ_2 model shown in Figure 7. The synthetic binuclear, doubly bridged Cu(I) complexes are not perfect models of the Cu(I) cluster in Cox17 as each Cu(I) in the synthetic clusters is bonded in distorted tetrahedral symmetry (*32*, *34*). The mean Cu−S bond distance (2.3−2.35 Å) is consistent with tetrahedral geometry, although each Cu(I) exhibits one long Cu−S bond (>2.4 Å) (*32*, *34*). Trigonal Cu(I)−thiolate complexes typically have shorter Cu−S bond distances in the range of 2.26−2.28 Å (*28*, *30*). The mean Cu−S distance of 2.261 Å in Cox17 and the near-edge X-ray absorption feature at 8983 eV are suggestive that the two Cu(I) ions in Cox17 are bound trigonally.

The candidate function of Cox17 is as a Cu shuttle delivering Cu ions to the mitochondrion for assembly of the cytochrome oxidase complex (*7*). Cytochrome oxidase contains two Cu centers; the Cu_A site is a binuclear, mixed valent site which is the acceptor site for an electron from reduced cytochrome *c*. The binuclear Cu_A site has two bridging thiolates (*3*). The second Cu site is the Cu_B center which is a binuclear Cu−heme a_3 center where oxygen is reduced to water.

It is curious that the mitochondrial Cu donor also consists of a binuclear center. However, the Cu_A binuclear site is distinct from the binuclear center in Cox17 in three regards. First, the Cu_A site is a delocalized, mixed valence center unlike the two Cu(I) sites in Cox17. Second, the Cu ions in the Cu_A site are in tetrahedral coordination, unlike the trigonal coordination in Cox17. Besides the two bridging thiolates in the Cu_A site, the other ligands in the Cu_A site for bovine and *Paracoccus* oxidases include two imidazole nitrogens, a methionine sulfur, and a peptidyl carbonyl (*3*, *35*). These residues are also critical for respiratory competency of the yeast oxidase (*36*). The unpaired electron is delocalized through the thiolate bridges. Third, the Cu−Cu distance in the Cu_A center is shorter at 2.5 Å (*3*, *37*). Similar mixed valent, binuclear centers with short 2.5 Å Cu−Cu distances are observed in the engineered binuclear Cu site in quinol oxidase and nitrous acid reductase (*38*, *39*). Whereas the short 2.5 Å Cu−Cu distance in the Cu_A site may result in limited, direct Cu−Cu bonding (*37*), the 2.7 Å Cu−Cu center in Cox17 is likely to be predominantly stabilized by μ-bridging thiolates and not direct Cu−Cu bonding.

It remains unclear how Cox17 is targeted to the mitochondrion. Cox17 is found in both the cytoplasm and intermitochondrial membrane (IM) space (*10*). Cox17 lacks a classical mitochondrial import sequence, so uptake may occur through the porous outer mitochondrial membrane or a pathway similar to that used by cytochrome *c* (*40*). The observation that a Cox17 fusion protein of 15 kDa remains predominantly in the cytoplasm (*10*) may arise from a size constraint in uptake or disruption of a docking interface. A mitochondrial receptor may exist that is accessible to Cox17 in the IM space. Beers et al. reported results suggesting that only a limited quantity of Cox17 can be taken up by mitochondria (*10*). One candidate receptor is Sco1 that is known to be critical for assembly of active cytochrome oxidase. Overexpression of *SCO1* can partially suppress the respiratory deficiency of a *cox17* null strain (*8*). Sco1 is an inner mitochondrial membrane protein (*9*). Alternatively, the CoxII subunit of cytochrome oxidase containing the Cu_A site may be the receptor site. This is the binding site of cytochrome *c* (*41*). The Cu_A site is only 8 Å below the surface of CoxII protruding into the IM space (*3*).

It remains to be resolved whether Cox17 is the direct or indirect Cu donor to both the Cu_A and Cu_B sites in cytochrome oxidase. The Cu_B site, unlike the Cu_A site, is deeply buried within the inner membrane of the bovine oxidase complex (*3*). The Cu_B is 13 Å below the plane of the inner membrane. It is conceivable that the inner membrane protein Sco1 is essential for Cu insertion in the Cu_B center. Additional studies with the *cox17* and *sco1* mutant cells should be informative in addressing these question.

The observed lability and solvent accessibility of the CuCox17 complex is consistent with its proposed role as a Cu(I) ion shuttle. Although CuCox17 is labile to ligand exchange reactions with the Cu(I) chelator BCS, the Cu(I) ions are stably bound to Cox17 in the presence of 20 mM glutathione. Cu ion shuttles would be expected to be kinetically labile for efficient Cu transfer to the target apoenzyme. This is in contrast to the observed thermodynamic stability of the Cu(I)-buffering metallothionein molecules. The heptacopper cluster in Cup1 is buried within the tertiary fold (*24*), and therefore the cluster is less susceptible to ligand exchange reactions and solvent quenching of emission. A doubly bridged binuclear center in CuCox17 would require only four thiolates. The presence of six conserved Cys residues in human and yeast Cox17 raises the possibility that the additional cysteinyl thiolates may be important in ligand

exchange reactions to move the Cu ions to the mitochondrial receptor sites.

ACKNOWLEDGMENT

We thank Dr. Alex Tzagoloff for helpful discussions. We are indebted to Martin J. George of SSRL for use of his data collection software, and to Ingrid Pickering of SSRL for assistance in Data Collection.

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BI980418Y