Yeast Cox17 Solution Structure and Copper(I) Binding*

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Cox17 is a 69-residue cysteine-rich, copper-binding protein that has been implicated in the delivery of copper to the CuA and CuB centers of cytochrome c oxidase via the copper-binding proteins Sco1 and Cox11, respectively. According to isothermal titration calorimetry experiments, fully reduced Cox17 binds one Cu(I) ion with a $K_d$ of $(6.15 \pm 5.83) \times 10^6$ M$^{-1}$. The solution structures of both apo and Cu(I)-loaded Cox17 reveal two α helices preceded by an extensive, unstructured N-terminal region. This region is reminiscent of intrinsically unfolded proteins. The two structures are very similar overall with residues in the copper-binding region becoming more ordered in Cu(I)-loaded Cox17. Based on the NMR data, the Cu(I) ion has been modeled as two-coordinate to the CuA and CuB centers of cytochrome c oxidase; ITC, isothermal titration calorimetry; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; rmsd, root mean square deviation; HSO4$^-$, heteronuclear single quantum coherence; AMS, 4-acetamido-4-maleimidostilbene-2,2'-disulfonic acid; EXAFS, extended x-ray absorption fine structure; NOESY, nuclear Overhauser effect spectroscopy; MES, 4-morpholineethanesulfonic acid; TOCSY, total correlation spectroscopy.

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The atomic coordinates and structure factors (codes 1U96 and 1U97) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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## References

1. The abbreviations used are: CeO, cytochrome c oxidase; ITC, isothermal titration calorimetry; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; rmsd, root mean square deviation; HSO$_4^-$, heteronuclear single quantum coherence; AMS, 4-acetamido-4-maleimidostilbene-2,2'-disulfonic acid; EXAFS, extended x-ray absorption fine structure; NOESY, nuclear Overhauser effect spectroscopy; MES, 4-morpholineethanesulfonic acid; TOCSY, total correlation spectroscopy.

2. CoO catalyzes the final step in eukaryotic respiration, the oxidation of reduced cytochrome c coupled to the reduction of dioxygen to two molecules of water (1). CoO is a multisubunit complex located in the mitochondrial inner membrane. Mammalian CoO comprises 13 subunits, of which 10 are nuclearily encoded and three are encoded by the mitochondrial genome. These three mitochondrial subunits, Cox1, Cox2, and Cox3, contain several essential cofactors (2). In the catalytic cycle, reduced cytochrome c transfers electrons to a dinuclear copper center, the CuA site, in Cox1. Electrons are then passed to a heme A cofactor in Cox1 and finally to the heterometallic heme A-CuB dioxygen-binding site in Cox1. Assembly of CoO is a complicated process involving import of the nuclearly encoded subunits to the mitochondrion, synthesis and incorporation of the two heme A groups, and delivery and insertion of the three copper ions (3). Knowledge of the latter process is important for understanding copper trafficking, especially in the context of recent research linking human diseases to deficiencies in copper metabolism (4, 5). Furthermore, formation of complex multinuclear copper cofactors such as Cu$_4$O$_2$ or the nitrous oxide reductase tetranuclear Cu$_2$ site (6) is not well understood.

3. Several proteins, including Cox17 (7), Sco1 (8), and Cox11 (9), have been implicated in loading of the CoO copper centers. Sco1 is involved in the assembly of the dinuclear CuA site (9, 10), whereas Cox11 is proposed to participate in loading the CuB site (11). Both receive copper directly from Cox17 (12). First identified in yeast, these proteins are highly conserved in other eukaryotes (13). Yeast Cox17 is a 69-residue protein localized to both the cytosol and mitochondrial intermembrane space (14). Yeast Sco1 is a 295-residue inner mitochondrial membrane protein comprising an N-terminal transmembrane segment and a C-terminal soluble domain (8). Yeast Cox11 is a 300-residue inner mitochondrial membrane protein, also composed of an N-terminal transmembrane segment and a C-terminal soluble domain (9, 10). Cox17 was originally proposed to shuttle copper between the cytosol and Sco1 (14). Recent data indicate that Cox17 tethered to the inner mitochondrial membrane is fully functional, however (15). In addition, mitochondrial copper levels are independent of the presence of Cox17 (16). These results suggest that trafficking copper ions between the cytosol and the mitochondrial intermembrane space is not a function of Cox17. Consistent with a role in CuA loading, some mutations in Cox17 lead to very low Cox2 levels (17). Although stable complexes between Cox17 and Sco1 or between Sco1 and Cox2 have not yet been reported, interactions between Sco1 and Cox2 have been detected by affinity chromatography and immunoprecipitation experiments (18), suggesting that metal transfer via transient complex formation is possible.

4. The copper binding properties of Cox17, Sco1, and Cox11 have been studied by spectroscopy and mutagenesis. Stoichiometries of two (14) or three (19) Cu(I) ions/recombinant yeast Cox17 monomer have been reported. Cox17 isolated directly from porcine intestine reportedly contains four Cu(I) ions (20). According to optical, luminescence (20, 21), and x-ray absorption (21) spectroscopic studies, Cox17 loaded with three or four copper ions contains a polynuclear cluster. Mutagenesis studies on yeast Cox17 indicate that three of seven cysteines, of which six are conserved, are required to produce active cytochrome c oxidase. These cysteines, Cys$^{23}$, Cys$^{24}$, and Cys$^{26}$, are found in a CCXC motif and are proposed to coordinate copper (19). The ~200-residue C-terminal soluble domain of Sco1 binds one Cu(I) ion (22, 23), and extended x-ray absorption fine
structure (EXAFS) data indicate the presence of one nitrogen and two sulfur ligands. These ligands include two cysteines in a conserved CXXC motif and a conserved histidine. Mutation of any one of these three residues abolishes copper binding by Sco1 and results in nonfunctional cytochrome c oxidase (22). The solution structure of apo Sco1 from Bacillus subtilis reveals that these ligands are derived from two flexible loop regions (24). The C-terminal soluble domain of Cox11 is a dimer and binds one Cu(I) monomer. EXAFS data indicate the presence of a dinuclear cluster with each Cu(I) ion ligated by three sulfurs (10, 25). The solution structure of dimeric Cu(I)-loaded Cox11 from Sinorhizobium meliloti suggests that two ligands derive from a CXC motif located on the side of an immunoglobulin-like β barrel structure, with the third ligand donated by the same CXC motif in the second monomer (25).

In contrast to Sco1 and Cox11, no structural data are available for Cox17. To understand the function of Cox17, knowledge of its three-dimensional structure and clarification of its copper binding stoichiometry are required. In particular, it is not known how Cox17 can recognize and interact with both Sco1 and Cox11, which have very different structures from one another (24, 25). It is also unclear how copper might be transferred from a polynuclear cluster in Cox17 to the mononuclear site in Sco1 or to the dinuclear site in Cox11 and subsequently to the dinuclear Cu$_2$ center or the mononuclear Cu$_9$ center. To address these issues, we have determined the solution structures of both the apoCox17 and Cu(I)-loaded (CuxCox17) forms of Cox17. In addition, we have used isotothermal titration calorimetry (ITC) to measure the association constant ($K$) and stoichiometry ($n$) of Cu(I) binding to Cox17.

**EXPERIMENTAL PROCEDURES**

**Protein Cloning and Expression—** Cox17 was cloned from yeast genomic DNA by PCR. The forward primer (5’-GGAATTCATATGACTGAACTCTAGAAGAAAGGGAGAACC-3’) was designed to include a 5’ NdeI restriction enzyme site, and the reverse primer (5’-CGGGGATCCCATATGAAGCCCGTGGATCCCGGAAAGC-3’) was designed to include a 3’ BamHI site. The PCR product was digested with NdeI/BamHI and inserted into the pET2a vector (Novagen). The resultant plasmid was then transformed into Escherichia coli Rosetta(DE3)-pLysS cells. Protein for ITC measurements was expressed in cells harboring pTY110 and pTY110/His/His. Protein for NMR structural characterization was expressed in cells harboring pTY110/His/His with T7 and pTY110/His/His with pLysS.

**Protein Purification—** The cells were lysed by freeze-thawing three times. Approximately 10 ml of 50 mM Tris-HCl, pH 8.0, 10% glycerol, and 0.2 mM tris(2-carboxyethyl)phosphine (TCEP) (buffer A) of harvested cells was added to the lysed cells. DNase I (−5 μg/ml) was then added, and the mixture was stirred at room temperature for 20–30 min. The lysate was clarified by ultracentrifugation at 25,000 × g for 2 h at 4 °C. After overnight dialysis against buffer A at 4 °C, the cell extract was applied to a DEAEX-Sepharose Fast Flow (Amersham Biosciences) column pre-equilibrated with buffer A and eluted with a 7.5 column volume 0.1–0.16 M potassium phosphate, pH 6.0, 10% glycerol, and 0.2 mM TCEP (buffer B), the sample was loaded onto a Bio-Scale CHT-I hydroxyapatite column (Bio-Rad) pre-equilibrated with buffer B. The protein was eluted with a 10 column volume 0.16 M potassium phosphate, pH 6.0, gradient, and concentrated, and further purified on a Hitach HiLoad 16/60 Superdex 75 (Amersham Biosciences) gel filtration column pre-equilibrated with buffer B. The purified Cox17 was then dialyzed into 20 mM potassium phosphate, pH 6.0, for storage at −80 °C. The identity of the protein was confirmed by N-terminal sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Protein Sciences Facility, University of Illinois at Urbana-Champaign). Both inductively coupled plasma-atomic emission spectroscopy using a Thermo Jarrell Ash Atomscan model 25 sequentially inductively coupled plasma spectrometer and atomic absorption spectroscopy using a PerkinElmer AAnalyst 700 indicated that Cox17 purified by this procedure contains no detectable copper ions.

**NMR Sample Preparation—** A 1 mM sample of purified Cox17 was first reduced by direct addition of 1 mM TCEP to a final concentration of 1 mM. The sample was then diazylized three times versus 20 mM potassium phosphate, pH 6.0, 1 mM TCEP and transferred to a room temperature Coy anaerobic chamber for three more cycles of dialysis versus 20 mM potassium phosphate, pH 6.0, to remove the TCEP. The final concentration of TCEP was estimated to be within the picomolar range. After the addition of D$_2$O to a final concentration of ~7% v/v, the sample was sealed inside a gas tight NMR tube (Wilmad) and removed from the chamber. Copper-loaded samples were prepared by dialyzing the samples into 100 mM potassium phosphate, pH 6.0, 100 mM NaCl during the final anaerobic dialysis cycle and then dialyzing in an anaerobic solution of 6.1 mM CuCl$_2$ in 1 mM NaCl and 10 mM HCl. $^{1}$H-$^{13}$C HSQC and aromatic $^{1}$H-$^{13}$C HSQC spectra were used to determine the end point of the titration. Samples in ~99% D$_2$O were prepared by lyophilizing the purified protein, reconstituting in 99.99% D$_2$O (Aldrich) inside the anaerobic chamber and sealing in a gas tight NMR tube.

**NMR Spectroscopy—** All of the NMR data were acquired at 25 °C on a Varian Inova NMR spectrometer equipped with a 13C/15N, 1H, 31P, 19F, and 15N triple resonance, xyz gradient probe. Assignment experiments were repeated under various conditions to include a 500 MHz Inova NMR spectrometer equipped with a 1H, 13C, 15N, and 19F triple resonance, xy gradient probe. Assignment experiments were acquired for the apoCox17 sample, and an HCCl1H/13C/1H-TOCSY experiment was additionally acquired for the CuCox17 sample. All of the NMR data processing and analysis was performed using Felix2000 software with a customized menu-driven interface.

**NMR Structure Calculations—** The structures were calculated with ARIA (version 1.2) in combination with CNS (28, 29). NOE restraints were obtained from three-dimensional $^{13}$C/15N-edited NOESY and three-dimensional aliphatic $^{13}$C/15N-edited NOE spectra. NOEs were calculated and assigned by Arcino software, which were checked manually throughout the refinement. A total of 27 phi and psi torsion angle restraints for apoCox17 and 25 phi and psi torsion angle restraints for CuCox17 were derived from an analysis of HN, Ca, Cβ, Cδ, and backbone $^{15}$N chemical shifts using TALOS (30). The torsion angle restraint bounds were set to three times the standard deviation of the calculated chemical shifts. These restraints were applied only for the helical regions predicted by TALOS. Extended backbone conformations were used as the starting models for the structure calculations. The default settings in the ARIA run.cns task file were used for the structure calculations except for the following parameters. The number of steps in the simulated annealing protocol was doubled for improved convergence, and the final force constants for the distance and torsion angle restraints were set to 50 kcal mol$^{-1}$ Å$^{-2}$ and 100 kcal mol$^{-1}$ rad$^{-2}$, respectively. Eighty structures were computed in the final iteration, of which 20 were chosen for further analysis. In addition, four possible copper binding schemes were modeled: a three-coordinate Cu(I) ion ligated by Cys$^{23}$, Cys$^{24}$, and Cys$^{26}$, and two three-coordinate models with ligation by Cys$^{23}$ and Cys$^{24}$, by Cys$^{23}$ and Cys$^{26}$, and by Cys$^{24}$ and Cys$^{26}$. The copper ion was modeled by appending it onto the protein sequence as an additional amino acid. Copper-sulfur bond restraints of 2.275 ± 0.05 and 2.159 ± 0.025 Å were applied to the three-coordinate and two-coordinate models, respectively. Cartesian molecular dynamics were employed for the structure calculations in which a Cu(I) ion was modeled. For apoCox17, CuCox17, and the four Cu(I)-bound models, the 20 structures determined to possess the lowest root mean square deviation (rmsd) from ideal covalent geometry were chosen for further analysis. These structures were analyzed with PROCHECK-NMR (31), and the figures were generated with MOLSCRIPT (32) and RASTER3D (33).

**ITC Measurements—** ITC experiments were performed using procedures similar to those described previously for Atox1 and the Wilson disease protein (34). Stock protein solutions of 100 μM Cox17 in 3 ml of...
chelated 100 mM MES, pH 6.5, 100 mM NaCl (buffer M) were incubated for 30 min on ice with 10 mM EDTA and either 1 mM TCEP or 100 mM dithiothreitol (DTT). The EDTA was then removed by two rounds of dialysis versus 500 ml of buffer M supplemented with either 1 mM TCEP or 100 mM DTT at 4 °C. At this point, the concentration of EDTA was estimated to be ~0.36 μM. The protein sample was then transferred to a room temperature Coy anaerobic chamber and dialyzed three times versus 500 ml of buffer M. The final concentrations of TCEP and DTT were estimated to be ~0.21 and ~21 mM, respectively. After this treatment, the residual copper content was determined by atomic absorption spectroscopy using a PerkinElmer Life Sciences AAnalyst 700 (detection limit for copper, 1.5 ppb). A standard curve was generated using a copper atomic absorption standard (Aldrich) diluted to 0.2, 0.5, 1, 2, 3, and 5 ppm. All of the samples and buffer M were found to contain ~25 ppb residual copper. A stock solution of 6.1 mM CuCl in 10 mM HCl and 1.0 M NaCl was prepared, and its concentration was determined by inductively coupled plasma optical emission spectroscopic analysis by Galbraith Laboratories (Knoxville, TN). Both protein and copper solutions were diluted with buffer M immediately before the ITC runs to 5–25 and 200–600 μM, respectively. The concentrations of the diluted Cox17 samples were determined using the Enhanced BCA protein assay (Pierce).

Protein samples were removed from the anaerobic chamber in a 2.5-ml Hamilton gas tight syringe and loaded into the calorimeter (MicroCal) sample cell under a constant pool of high grade pure argon. The Cu(I) solution was loaded into a 250-μl titration syringe in the anaerobic chamber and transferred to the calorimeter chamber under argon gas. For each experiment, an automated sequence of 20–30 injections of 5–10 μl of Cu(I) were applied to the protein in the sample cell with a 3–6-min pause between injections to allow for equilibration. The reaction solution was stirred at 400 rpm, and the temperature of the chamber was maintained at 25 °C working against a surrounding water bath temperature of 15 °C. All of the experiments were repeated at least three times with the same settings. The data were analyzed with the Origin 5.0 software package provided by MicroCal using a one-site binding model. A background correction was applied to each experiment, corresponding to the average of the last four injections and was consistent with a control experiment in which the Cu(I) solution was titrated into buffer alone. After subtraction of this heat of dilution, a nonlinear least squares method was used to obtain the best fit parameters for the number of binding sites, n, the association constant, K_a, and the change in enthalpy ΔH°. Two negative control experiments were used to address the specificity of copper binding (34). First, Mg(II) was titrated into 20 μM Cox17 using 300 μM MgCl_2 prepared in 10 mM HCl and 1 M NaCl. Second, Cu(I) was titrated into a 20–35 μM solution of lysozyme (14,296 Da; Research Organics). These control experiments were performed in triplicate under the same conditions used for the Cox17 experiments.

Quantitation of Free Cysteines—The redox state of Cox17 was determined by alkylation of the free cysteine residues with 4-acetamido-4-maleimidylstilbene-2,2′-disulfonic acid (AMS) (Molecular Probes), a reagent specific for reduced thiols. Five samples were prepared to determine the number of reduced cysteines, the accessibility of free cysteines, the efficiency of reducing agents, and the stability of reduced Cox17 to air oxidation: 1) as-isolated, purified Cox17, 2) Cox17 reduced with 100 mM DTT in the presence of 1 mM TCEP subsequently removed by anaerobic dialysis, 3) Cox17 reduced with 100 mM DTT subsequently removed by anaerobic dialysis, and 5) same treatment as in 4) followed by exposure to air for 20 min prior to AMS modification. For each reaction, 60 μl of 25 mM AMS, 6.25% SDS in buffer M were added to 100 μl of ~400 μM protein, yielding final concentrations of ~250 μM Cox17, 10 mM AMS, and 2% SDS. The reactions were shielded from light with aluminum foil and incubated at room temperature with vigorous stirring in the anaerobic chamber. After 2 h, the samples were dialyzed into water and analyzed by nonreducing SDS-PAGE on 15% Tris-glycine gels. For matrix-assisted laser desorption/ionization time-of-flight analysis (Mass Spectrometry Facility, University of Arizona and Protein Sciences Facility, University of Illinois at Urbana-Champaign), the derivatized proteins were diluted five times and submitted for analysis without reaction termination or further purification. Each conjugated AMS moiety increases the protein molecular mass by 490 Da. The number of reduced cysteines was also measured by using Ellman’s reagent and by a thiol and sulfide quantitation kit (Molecular Probes).

RESULTS AND DISCUSSION

Redox State of Cox17—To evaluate the method of preparing reduced Cox17 for Cu(I) binding titrations and to determine the availability of the seven cysteines, AMS modification was used. On nonreducing SDS-PAGE, all of the modified samples migrated at substantially higher molecular masses than unmodified Cox17, but mass spectrometry was necessary to quantify accurately the number of conjugated AMS moieties. Mass spectrometric analysis of as-isolated, purified Cox17 subjected to
AMS modification yielded a major peak at 7,914 Da corresponding to unmodified protein lacking the N-terminal methionine (predicted 7,925 Da) as well as a minor peak at 8,430 Da corresponding to the presence of one AMS moiety (predicted 7,925 Da) as well as a minor peak at 8,430 Da, suggesting that one cysteine remains partly reduced under the purification conditions. Samples reduced with either 1 mM TCEP or 100 mM DTT followed by reductant removal by anaerobic dialysis contained a spectrum of modified products ranging from 3 to 7 AMS units. For both reductants, peaks at corresponding to unmodified protein lacking the N-terminal methi-
somes on the three Cu(I) form of yeast Cox17 isolated by Winge and co-workers (19) indicate that the Cu(I) ions are arranged in a polynuclear cluster. Their samples exhibit optical transitions in the ultraviolet region and luminescence with an emission maximum of ~570 nm, both indicative of a solvent-shielded polycopper cluster. In addition, EXAFS data were best fit with three sulfur ligands at 2.25 Å and a Cu-Cu interaction at 2.7 Å, consistent with a polycopper cluster. The discrepancy in copper stoichiometry may be attributable to differences in how Cox17 is loaded with Cu(I). Winge and co-workers (19) typically add 1.4 mM CuSO₄ to E. coli cells after growth to an OD₆₀₀ of 0.1 but not before the addition of the NADH-generating system. This discrepancy is consistent with previous reports showing that the efficiency of maleimide labeling is significantly decreased by the presence of reductants (35). If SDS is not included in the modification reaction, only three of seven cysteines are modified regardless of the reductant and conditions used, implying that the other four cysteines are not easily accessible. Notably, quantitation by absorption spectroscopy using both Ellman’s reagent and a thiol detection kit led to an underestimated thiol content, a phenomenon also observed for the E. coli oxidoreductase DsbC (36).

**Binding of Cu(I) by Cox17—ITC data for Cu(I) binding to Cox17 were obtained using protocols developed in our laboratory specifically for analysis of Cu(I) binding to cysteine residues (34). The validity of this approach was tested by titrating Mg(II) into Cox17 (Fig. 1a). Specific Cu(I) binding was not observed in this control experiment or in a titration of Cu(I) into lysozyme. By contrast, we obtained interpretable and reproducible results for Cu(I) binding to Cox17. Data for Cox17 reduced with 1 mM TCEP were fit well with a one-site binding model (Fig. 1b) and parameters  a = 1.033 ± 0.032, Kₐ = (6.15 ± 5.83) × 10⁵ M⁻¹, and ΔH° = −6.0 ± 3.3 kcal mol⁻¹ (standard deviations from four measurements included). The value for ΔH° is apparent and includes contributions not only from Cu(I) binding but from associated events such as deprotonation of the cysteines and changes in the buffer ionization state. Using 100 mM DTT instead of 1 mM TCEP did not change the stoichiometry or thermodynamic parameters of Cu(I) binding to Cox17.

The most surprising finding from the ITC data is the measured stoichiometry of one Cu(I) ion per Cox17 monomer as compared with two (14), three (19), or four (20) Cu(I) ions as reported for other preparations. Extensive spectroscopic studies on the three Cu(I) form of yeast Cox17 isolated by Winge and co-workers (19) indicate that the Cu(I) ions are arranged in a polynuclear cluster. Their samples exhibit optical transitions in the ultraviolet region and luminescence with an emission maximum of ~570 nm, both indicative of a solvent-shielded polycopper cluster. In addition, EXAFS data were best fit with three sulfur ligands at 2.25 Å and a Cu-Cu interaction at 2.7 Å, consistent with a polycopper cluster. Luminescence is also observed for porcine Cox17 determined to contain four Cu(I) ions by mass spectrometric analysis (20). Similar optical features are not observed for our samples.

The discrepancy in copper stoichiometry may be attributable to differences in how Cox17 is loaded with Cu(I). Winge and co-workers (19, 21) typically added 1.4 mM CuSO₄ to E. coli cells expressing Cox17 1 h before harvest. For the ITC and NMR experiments, Cu(I) was added to the thoroughly demetallated apo protein. It could be that this apo protein is binding less than the full complement of Cu(I), but this possibility is unlikely because thiol quantitation clearly indicates that all seven cysteines are reduced and available for metal binding. Another
higher affinity binding sites via a slow kinetic rate constant is proposed to contain extremely limited free copper (39), suggesting that copper exchange is under kinetic, rather than thermodynamic, control (34). The situation may be the same for copper transfer from Cox17 to Sco1 and Cox11, but the Cu(I) binding affinities of these two target proteins have not yet been determined. Finally, it should be noted that a much higher affinity of $\text{CuSO}_4$ has an artificially high number of Cu(I) ions. Although roughly similar to the value of $K_\text{d}$ for the human copper chaperone Atox1 (34), the Cu(I) affinities of the yeast forms of Atox1 and the Wilson protein, Atx1 and Ccc2, estimated by competition with bathocuproine disulfonate (40) are much higher than those obtained for the human proteins by ITC measurements (34). The differences between the two methods remain to be resolved but could be related to interactions between the proteins and the competitor ligands.

**Structure Determination**—For both apoCox17 and CuCox17, 63 of the expected 65 backbone amide resonances and $\sim 90\%$ of all detectable carbon, nitrogen, and hydrogen nuclei were assigned. The missing amide resonances were for residues 1–2. All of the NOEs were assigned using an automated, iterative approach (28, 29). The ensemble of 20 apoCox17 structures is in excellent agreement with the experimental restraints, as indicated by low rmsd values with the input restraints, good geometry, and low atomic rmsds from the average structure in structurally ordered regions (Table I). Several copper binding models were analyzed for CuCox17 because the identities and coordination geometries of the ligating residues for Cox17 containing a single Cu(I) ion are not known. The best results were obtained for a two-coordinate model with the Cu(I) ion coordinated by Cys23 and Cys26 (Cys23/Cys26-Cu) (Table I). The 0.78 Å rmsd value for backbone atoms in ordered regions is significantly less than the value obtained for the models with coordination by Cys23 and Cys24 (Cys23/Cys24-Cu) (1.18 Å), by Cys24 and Cys26 (Cys24/Cys26-Cu) (1.30 Å), by Cys23, Cys24, and Cys26 (Cys23/Cys24/Cys26-Cu) (1.35 Å), and with no copper present (0.99 Å) (Table II). The two-coordinate model with the Cu(I) ion coordinated by Cys23 and Cys26 is also the model with the lowest percentage of residues in the disallowed regions (Table II).

**Overall Structure**—Both apoCox17 and CuCox17 consist of an $\sim 20$-residue unstructured N terminus followed by two a helices (Fig. 2, a and b). The two helices, identified by TALOS and confirmed by PROCHECK-NMR, span residues 27–39 and 48–60 in the apoCox17 structure and residues 28–39 and 48–59 in the CuCox17 structure. The structure of Cox17 differs from those of its target proteins, Sco1 comprises eight $\beta$ strands and four helices arranged in a thioredoxin-like fold (24), and
Cox11 exhibits a 10-stranded immunoglobulin-like \( \beta \) barrel structure (25). By contrast, members of the Atx1 family of copper chaperones and their target domains all exhibit a conserved \( \beta \alpha \beta \beta \alpha \beta \) fold (41, 42). Similarly, the copper chaperone for superoxide dismutase resembles its target enzyme copper, zinc superoxide dismutase (43, 44). For these proteins, the structural similarities promote complex formation and copper transfer (45). The structures of Cox17, Sco1, and Cox11 suggest that similarity between chaperones and target domains may be specific to these cytosolic systems rather than a general feature of metallochaperones.

The unstructured nature of the Cox17 N terminus as well as of \(-5\) residues at the C terminus is clear from \( ^{1}H-{^{15}}N \) NOE data (Fig. 2c) and may contribute to target recognition. A lack of structure in Cox17 was predicted by Punter and Glerum (17) based on the observation that Cox17 is unusually resistant to mutation, retaining function despite numerous nonconservative amino acid substitutions. This property, combined with a large number of charged residues and a relative lack of hydrophobic residues led to the proposal that Cox17 may be a natively unfolded or intrinsically unstructured protein (17). Such proteins are flexible, exhibit low levels of secondary structure, and are characterized by a large net charge (46). According to the NMR data, almost 40% of the total residues in Cox17 are unstructured (Fig. 2). In addition, Cox17 contains 18.8% glutamic acid residues and 15.5% lysine residues as compared with 6.2% and 5.7% in a typical protein (47). Thus, Cox17 has many characteristic features of an intrinsically unstructured protein. The structure is also consistent with the observation by ourselves and others (14, 21) that Cox17 consistently elutes from a gel filtration column with an apparent molecular mass approximately twice that expected.

Many intrinsically unfolded proteins adopt ordered structures in the presence of targets and ligands such as other proteins, nucleic acids, substrates, cofactors, and membranes (46). Unfolded domains are involved in transcriptional and

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**Fig. 2. Solution structure of Cox17.**

*a*, superposition of backbone atoms from the ensemble of 20 apoCox17 NMR structures. *b*, superposition of backbone atoms of the ensemble of 20 CuCox17 (Cys23/Cys26) NMR structures. *c*, heteronuclear \( ^{1}H-{^{15}}N \) NOEs plotted as a function of residue number for apoCox17 (black) and CuCox17 (red). Data from a three-point smoothing function for apoCox17 (black line) and CuCox17 (red line) are included. The gray line represents the threshold value of 0.6, above which residues are considered structured. Residues with overlapped resonances were omitted.
translational regulation, signal transduction, and membrane fusion and transport (48, 49). In many of these processes, the lack of structure confers a functional advantage in that one protein can recognize and bind to several different partners. Similarly, Cox17 has recently been shown to deliver copper to both Sco1 and Cox11 (12). Models of eukaryotic Sco1 (24) and Cox11 (25) indicate that the copper-binding site is located between two loops in Sco1 and on a β sheet in Cox11. It is likely that the unstructured regions of Cox17 adopt different structures upon interacting with Sco1 and Cox11, allowing it to transfer copper efficiently to both target proteins. The possibility that Cox17 interacts with its two targets via different surfaces is supported by the observation that mutation of Cys57 to a tyrosine impairs copper transfer to Sco1 but not to Cox11 (12).

Comparison of apoCox17 and CuCox17—The 1H-15N HSQC spectra of apoCox17 and CuCox17 show sizable chemical shift variations for residues 23–27, which comprise the copper-binding region (Fig. 3). The apo-Cox17 HSQC peaks residues slowly disappear, whereas the CuCox17 HSQC peaks for these residues slowly appear during a titration of 0.25–1 equivalent Cu(I) into the apo sample. The disappearance of the apo peaks and simultaneous appearance of the Cu peaks indicates that slow exchange is occurring. Upon conversion from the apo to the Cu(I) form, this region becomes more structured as evidenced by a decrease in the rmsd values for backbone atoms from 2.14 to 1.29 Å and for nonhydrogen atoms from 2.69 to 2.08 Å. No additional shifts in peak positions beyond the addition of one equivalent of Cu(I) are observed, consistent with the stoichiometry measured by ITC. Superposition of the two representative structures (Fig. 4a) and an analysis of the rmsd between them (Fig. 4b) indicate that the overall structures are very similar. In Fig. 4b, meaningful differences between apo and CuCox17 occur where the rmsd between the two representative structures is greater than the sum of the rmsds of the two ensembles of structures. There are slight differences for coordinating residue Cys26 and for Met58, which is close to the modeled Cu(I) ion (Fig. 4a).

The Copper-binding Site—The ITC and NMR data are most consistent with a single Cu(I) ion coordinated by Cys23 and Cys26. The S-Cu-S bond angle for the ensemble modeling this coordination is $\sim 164 \pm 8^\circ$, suggesting a close to linear geome-
try. In all conformers of this model, Cys26 adopts a similar position, whereas Cys23 is less ordered. A two-coordinate site is consistent with site-directed mutagenesis data. Mutant proteins in which any one of residues Cys23, Cys24, or Cys26 is replaced with serine retain the ability to bind copper but cannot activate CcO (19). The observation that only two cysteines are required for copper binding is consistent with the higher quality observed for the two-coordinate versus three-coordinate NMR models. It is unclear why all three cysteines are required for copper delivery, but a mutation near the unstructured N terminus could impair recognition of Sco1 or Cox11. Mutation of the other four cysteines, Cys16, Cys36, Cys47, and Cys57, does not interfere with copper binding (19), an observation explained by their distance from the copper-binding site (Fig. 5). Nevertheless, Cys23, Cys47, and Cys57 are important for function (17, 19), and Cys57 has been proposed to function specifically in recognition of Sco1 (12).

Coordination by two cysteines separated by two intervening residues is also observed for the Atx1 family of copper chaperones and their target domains, all of which contain a CXXC motif (13). Crystal and solution structures of Atx1 (50, 51), Atox1 (52), a bacterial homolog called CopZ (53, 54), and domains of their target copper transport ATPases (55–57) reveal that the metal-binding site is located on a surface-exposed loop between the first β strand and the first α helix in the βαβαββ fold, with the first cysteine derived from the loop and the second cysteine derived from the N terminus of the helix. Similarly, the copper-binding site in Cox17 is located on a loop with the second coordinating cysteine at the N terminus of a helix (Fig. 5). The exposed location of this site is consistent with a function in copper delivery to Sco1 and Cox11 and may also be important for acquiring copper within the mitochondrial intermembrane space.

The Atx1 chaperones contain a conserved lysine residue adjacent to the copper-binding site. This residue, which is essential for function (58), has been proposed to modulate copper transfer (50, 52). In the CuCox17 structure, conserved residue Lys30 is proximal to the copper-binding site and could counterbalance the negative charge because of the presence of three cysteines. In addition, several other positively charged residues, including Lys1, Lys31, and Arg33 (Fig. 5), are highly conserved. Models of yeast Sco1 (24) and human Cox11 (25) reveal a negatively charged surface patch near the copper-binding site. These conserved, positively charged residues in Cox17 might therefore play a role in target recognition and docking via complementary electrostatic surfaces. In support of this model, mutation of Arg33 to alanine or aspartic acid impairs Cox17 function (17). Notably, two glutamic acid residues that form the negatively charged region in the model of human Cox11 are not conserved in S. meliloti Cox11, consistent with the fact that Cox17 is only found in eukaryotes (25).

In sum, the copper binding properties and structure of Cox17 have been determined by a combination of ITC and NMR spectroscopy. Cox17 binds a single Cu(I) ion with an association constant of $10^6–10^7 \text{ M}^{-1}$. The solution structures of apoCox17 and CuCox17 reveal an unstructured N-terminal region followed by two α helices and several unstructured C-terminal residues. The Cu(I) binding site is best modeled as two-coordinate with ligation by Cys23 and Cys26. This site resembles that in the Atx1 family of copper chaperones and is well suited for copper transfer to the mononuclear and dinuclear centers in Sco1 and Cox11. Recognition of these two target proteins is probably facilitated by the unstructured regions of Cox17 and by the presence of several conserved, positively charged resi-
ues. Additional structural characterization will be critical to elucidating the molecular details of docking and copper transfer.

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