Yeast Sco1, a protein essential for cytochrome c oxidase function is a Cu(I) binding protein

Thalia Nittis‡, Graham N. George§, and Dennis R. Winge‡*  

‡ University of Utah Health Sciences Center, Salt Lake City, Utah 84132  
§ Stanford Synchrotron Radiation Laboratory, Stanford Linear Accelerator Center,  
2575 Sand Hill Road, Menlo Park, California 94025.

* To whom correspondence should be addressed  
801-585-5103 telephone  
801-585-5469 fax  
dennis.winge@hsc.utah.edu

Running Title: Sco1 is a copper-binding protein
**Abbreviations and Textual Footnotes**

This work was supported by a grant ES 03817 from the National Institutes of Environmental Health Sciences, NIH to D.R.W. The Stanford Synchrotron Radiation Laboratory is funded by the Department of Energy, Offices of Basic Energy Sciences and Biological and Environmental Research, and the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program.

EXAFS: Extended x-ray absorption fine structure, BCS: bathocuproine sulfonate;
GST: glutathione S-transferase; COX: cytochrome c oxidase; PMS: post-mitochondrial supernatant
Abstract

Sco1 is a conserved, essential protein, which has been implicated in the delivery of copper to cytochrome $c$ oxidase, the last enzyme of the electron transport chain. In this study, we show for the first time that the purified C-terminal domain of yeast Sco1 binds one Cu(I) per monomer. X-ray absorption spectroscopy suggests that the Cu(I) is ligated via three ligands, and we show that two cysteines present in a conserved motif, CxxxC, and a conserved histidine are involved in Cu(I) ligation. Mutation of any one of the conserved residues in Sco1 expressed in yeast abrogates the function of Sco1 resulting in a non-functional cytochrome $c$ oxidase complex. Thus, the function of Sco1 correlates with Cu(I) binding. Data obtained from size-exclusion chromatography experiments with mitochondrial lysates suggest that full-length Sco1 may be oligomeric in vivo.
Cytochrome c oxidase (COX) is the terminal enzyme of the respiratory chain, embedded in the inner mitochondrial membrane of all eukaryotes and the plasma membrane of some prokaryotes. During aerobic growth, COX catalyzes the transfer of electrons from cytochrome c to oxygen. Electron flow through COX is coupled to proton transfer across the inner mitochondrial membrane. This results in creation of an electrochemical proton gradient, providing energy for ATP synthesis. COX is a multisubunit complex, consisting of 2-4 subunits in bacteria, 12 in yeast and 13 in mammals [1-3]. In eukaryotes, the 3 largest subunits are encoded by mitochondrial genes (COX1, COX2 and COX3) and form the catalytic core. The remaining subunits are encoded by nuclear genes and must be imported into the mitochondrion. Several of these subunits are small, and consist of a single helix situated on the periphery of the enzyme.

In addition to the genes encoding the COX subunits, a large number of other nuclear genes are required for the proper assembly and function of this enzyme [4]. The products of these genes include factors involved in processing and translation of the subunit mRNAs, chaperones that assist in the assembly process, and proteins involved in synthesis or delivery of cofactors. COX contains several cofactors, including two copper (Cu) sites (CuA and CuB), two heme groups (hemes \( a \) and \( a_3 \)), one magnesium and one zinc ion. Electrons from reduced cytochrome c first enter the bimetallic CuA site located within the C-terminal globular, hydrophilic domain of subunit 2 in the inter-membrane space. The electron from the reduced CuA center is transferred to heme \( a \) and subsequently to the binuclear heme \( a_3-CuB \) site in Cox1. The CuB site is buried 13 Å below the inner membrane surface [5]. Since both Cox1 and Cox2 are synthesized inside the mitochondria, the 3 Cu atoms have to be imported from the cytoplasm. So far, 3 proteins (Cox17, Sco1 and Cox11) have been implicated in Cu atom delivery and insertion into COX.

*S. cerevisiae* lacking Cox17 have all the phenotypes associated with COX-deficient pet mutants. The cells cannot respire, have no COX enzymatic activity, and lack hemes \( a \) and \( a_3 \) [6]. These deficiencies are corrected by adding excess Cu(II) to the growth media. Since Sod1, a
copper-requiring enzyme in the cytoplasm, is functional, the role of Cox17 is specific to
cytochrome c oxidase and/or mitochondria. Cox17 is a small protein (8 kD) found in both the
cytosol and mitochondrial inter-membrane space [7], consistent with a role in shuttling Cu ions
between these 2 compartments. Furthermore, purified Cox17 binds 3 Cu(I) ions per monomer in
a polycopper thiolate cluster [8]. The CuCox17 conformer exists in a dimer/tetramer equilibrium.

Sco1 was first implicated in Cu ion delivery by the observation that cox17 mutant cells
could be suppressed by overexpression of either SCO1 or the homologous SCO2 gene [9]. Both
Sco1 and Sco2 are localized to the inner mitochondrial membrane, and Δsco1 cells are respiratory
deficient [9,10]. Excess Cu(II), overexpression of either COX17 or SCO2, cannot correct the
sco1-associated deficiency. Moreover, in the absence of Sco1, both Cox1 and Cox2 subunits are
unstable and degraded [11,12]. Sco1 and Sco2 contain a potential metal binding motif, CxxxC,
and both cysteines in yeast Sco1 are essential for function [13]. Sco1 and Sco2 are conserved in
humans [14], and lack of either protein results in COX deficiency and death in infants within a
few months of birth [15-17]. All known patients are compound heterozygotes, with one mutant
allele always being a null allele. Interestingly, the second allele contains a missense mutation
falling either near the CxxxC motif or a conserved histidine in the C-terminal end.

It has been proposed that Cox17 may deliver Cu atoms to Sco1, which in turn transfers
them to COX [6]. Specifically, Sco1 seems to be involved in Cu ion delivery to Cox2, but not
Cox1. Mattatall et al. (2000) showed that the Bacillus subtilis Sco1 homologue, YpmQ,
suppresses expression of cytochrome c oxidase, but not a second oxidase, menaquinol oxidase,
which only contains a CuB center. Interestingly, Cox2 contains a metal binding motif (CxExC)
similar to that in Sco1, and this motif provides 3 of the 6 ligands for binuclear CuA site formation.
Cu ion insertion into Cox1 may require another protein, the product of COX11. Cox11 appears
to be necessary for formation of the CuB center in the Rhodobacter sphaeroides Cox1 subunit
[18].
In this study we provide further evidence that Sco1 is involved in insertion of Cu ions into COX. We purified a soluble truncated form of Sco1, and show that it binds one Cu(I) atom per monomer. X-ray absorption spectroscopy suggests that the Cu(I) ion is coordinated via 3 ligands. Site-directed mutagenesis of potential ligands and subsequent purification of the mutant proteins, implicate the 2 cysteines of the conserved CxxxC motif and a conserved histidine to be involved in Cu(I) ligation. Cu coordination correlates with \textit{in vivo} Sco1 function.

\textbf{MATERIALS AND METHODS}

\textit{Plasmids} - The \textit{SCO1} gene lacking the first 285 base pairs (bp) was amplified from \textit{S. cerevisiae} genomic DNA by PCR. \textit{BamHI} and \textit{SalI} were added to the 5’ and 3’ ends, respectively, and the 603 bp PCR product was cloned into pGEM-T (Promega) and sequenced. The \textit{BamHI}/\textit{SalI} fragment was cloned into both the GST-fusion expression vector pGEX-4T-1 (Pharmacia), creating pTNDW1, and the His-fusion expression vector pHis-Parallel2, a derivative of pET22B (Novagen) [19], creating pTNDW2. An untagged version of \textit{SCO1} lacking the first 285 bp was also created for purification. PCR amplification from genomic DNA was used to add \textit{NdeI} and \textit{BamHI} sites at the 5’ and 3’ ends, respectively, and an AUG start site just 3’ to the \textit{NdeI} site on the 5’ end. The PCR product was cloned into pGEM-T and sequenced. The \textit{NdeI}/\textit{BamHI} fragment was subcloned into pAED4, a derivative of the T7-based expression vector pET-3a [20], to create pTNDW3. The entire \textit{SCO1} gene, including 347 bp upstream and 378 bp downstream, was amplified from yeast genomic DNA, cloned into pGEM-T, and sequenced. The 1613 bp fragment, flanked by added 5’ \textit{BamHI} and 3’ \textit{KpnI} sites, was subcloned into YEp424 to yield a high copy yeast expression vector (pTNDW4). Mutagenesis of \textit{SCO1} was carried out using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) on both pTNDW2 and pTNDW4. The resulting \textit{SCO1} mutants were sequenced and those in pTNDW4 were subcloned to ensure there were no mutations in the plasmid backbones.
**Yeast strains** - W303Δsco1 (MATα ade2-1 his3-11,15 leu2-3,112 ura3-1 sco1::URA3) was constructed with a one-step gene replacement method [21]. The URA3 gene was PCR amplified from pRS406 (YIp). The primers used consisted of 40 nucleotides (nts) of sequence identical to the left and right genomic sequence flanking SCO1, followed by 20 nts of sequence specific for URA3. The PCR product was transformed into wild-type aW303, and transformants that grew on media lacking uracil were further tested for inability to grow on media containing glycerol. The gene knockout was verified by southern analysis. The resulting strain was crossed to αW303, and diploids were selected, sporulated, and tetrads dissected to confirm a 2:2 segregation of the Δsco1 phenotype with the URA3 marker. Strains JM22 (MATa his4 op1 rho+) and VC36 (MATa his4 op1 rho+ cox2-) were a generous gift from Robert Poyton (Boulder, CO) [22]. Strains NB40-36A (MATα lys2 arg8::hisG ura3-52 leu2-3,112 rho+), HM4 (MATα lys2 arg8::hisG ura3-52 leu2-3,112 rho+ Cox2-100), NB80 (MATα lys2 arg8::hisG ura3-52 leu2-3,112 his3-deltaHindIII rho+), NB40-3C (MATα lys2 arg8::hisG ura3-52 leu2-3,112 his3-deltaHindIII rho+ cox2-62) were a kind gift from Thomas Fox (Ithaca, NY) [23].

**Purification of Sco1 - E. coli** strain BL21(DE3) harboring either pTNDW1 (GST-tagged), pTNDW2 (His-tagged) or pTNDW3 (untagged), was grown to an OD₆₀₀nm of 0.6-0.8 prior to induction with 0.3 mM IPTG for 3 h. Thirty min after the addition of IPTG, CuSO₄ was added to a final concentration of 1.4 mM. The cells were harvested by centrifugation, washed with 0.25 M sucrose, and frozen at -70°C. The cells were thawed, resuspended in lysis buffer, and lysed by repeated sonication. The cell lysate was centrifuged at 100,000g at 4°C for 40 min. The supernatant was filtered through a 0.45 µm filter, and then loaded onto the appropriate column equilibrated with lysis buffer. Lysis buffer was 2x phosphate-buffered saline (PBS) [24], with 1 mM dithiothreitol (DTT) for GST-Sco1; 2x PBS with 10 mM 2-mercaptoethanol and 10 mM imidazole for His-Sco1; and 20 mM Tris-HCl, pH 7.8 with 1 mM DTT for untagged Sco1. The columns used were glutathione-Sepharose (Pharmacia) for GST-Sco1; Nickel-NTA Superflow (Qiagen) for His-Sco1; and for untagged Sco1 we first used a HiPrep 16/10 DEAE column.
followed by a G-75 Superdex 26/60 column (Pharmacia). After loading, the columns were washed with at least 6 column volumes of the appropriate lysis buffer (for His-Sco1, the concentration of imidazole was increased to 20 mM). GST-Sco1 was eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0); His-Sco1 was eluted with 250 mM imidazole in lysis buffer; and untagged Sco1 was eluted from DEAE with a 600 ml 0–0.5 M NaCl gradient. Buffer for the G-75 column was 50 mM potassium phosphate, 100 mM NaCl, 1 mM DTT, pH 7.0. Fractions containing Sco1 were pooled and concentrated with ultrafiltration devices (Vivascience). His- and GST-tagged proteins were dialyzed in 2x PBS with 1 mM DTT using Slide-A-Lyzer cassettes with a 3,350 Da cutoff (Pierce). GST was cleaved with thrombin protease (Amersham Pharmacia Biotech), while the poly-His tag was cleaved with rTEV protease (Gibco Life Technologies).

**Analyses** - Protein was quantified by amino acid analysis after hydrolysis in 5.7N HCl containing 0.1% phenol *in vacuo* at 110°C. The analysis was performed on a Beckman 6300 analyzer. The copper concentration of the protein was measured using a Perkin-Elmer (AAnalyst 100) atomic absorption spectrophotometer. Luminescence was measured on a Perkin Elmer 650-10S fluorimeter with excitation set at 300 nm. Ligand exchange was measured by bathocuproine disulfonate (BCS) competition studies. The appearance of a Cu(BCS)$_2$ complex was measured by monitoring the absorbance at 483 nm with respect to time using a molar extinction coefficient of 12,250 [8]. Circular dichroism spectroscopy measurements were carried out in a Jasco J-715 spectrophotometer.

X-ray absorption spectroscopic measurements were carried out at the Stanford Synchrotron Radiation Laboratory with the SPEAR storage ring containing 60-100 mA at 3.0 GeV, on beamlines 7-3 and 9-3 using Si(220) double crystal monochromators, and with wiggler fields of 1.8 and 2T, respectively. Beamline 9-3 is equipped with a Rh-coated collimating mirror upstream of the monochromator and a bent-cylindrical Rh-coated focusing mirror downstream of the monochromator. Harmonic rejection was accomplished by setting the cutoff energy of the
focusing mirror to 14 keV. On beamline 7-3, which contains no focusing optics, harmonic rejection was accomplished by detuning one monochromator crystal to approximately 50% off-peak, and the energy resolution was optimized by using an upstream vertical aperture of 1 mm. In both cases, the incident X-ray intensity was monitored using a nitrogen-filled ionization chamber and X-ray absorption was measured as the Cu K $\alpha$ fluorescence excitation spectrum using an array of thirteen (on beamline 7-3) and thirty (on beamline 9-3) germanium intrinsic detectors. During data collection, samples were maintained at temperature of approximately 10°K using an Oxford Instruments liquid helium flow cryostat. For each sample between six and sixteen 35 min scans were accumulated, and the absorption of a standard copper metal foil was measured simultaneously by transmittance. The X-ray energy was calibrated with reference to the lowest energy inflection of the foil, which was assumed to be 8980.3 eV. The extended X-ray absorption fine structure (EXAFS) oscillations $c(k)$ were quantitatively analyzed by curve-fitting using the EXAFSPAK suite of computer programs (http://ssrl.slac.stanford.edu/exafspak.html), using ab-initio theoretical phase and amplitude functions calculated using the program FEFF V 8.2 [25,26]. Two synthetic copper model compounds were used, a trigonal $[\text{Cu}_4(\text{SPh})_6]^{2-}$ cluster [27] and a digonal $[\text{Cu}($SC$_{10}$H$_{12}$)$_2]^{2-}$ compound, generously provided by Jim Penner-Hahn [28].

Mitochondrial isolation and oxidase activity - Mitochondria were isolated from $S$. cerevisiae as described previously [29]. Mitochondrial protein concentration was determined by the Bradford assay, and cytochrome $c$ oxidase activities were performed as described [30].

Size-exclusion chromatography of mitochondrial lysates - Purified mitochondria were pelleted and resuspended at 5-10 mg/ml in size-exclusion buffer (50 mM potassium phosphate, 100 mM NaCl, 1 mM DTT, pH 7.0) containing either 0.5% Triton X-100, 2.2% Triton X-100 or 1% cholate. After vortexing briefly, lysates were clarified by centrifugation at 100,000g at 4°C for 30 min. Lysates were filtered through 0.45 μm syringe filters, and loaded onto a Sephacryl S300 column (Pharmacia), equilibrated with the same buffer used for lysis. Column fractions were analyzed by SDS-PAGE, followed by immunoblotting and autoradiography, as described below.
Antibody production and western analysis - Two New Zealand white rabbits (Western Oregon Rabbitry) were injected subcutaneously with 2 mg of purified GST-Sco1 emulsified in Freund’s complete adjuvant (Difco Laboratories). After 4 weeks the rabbits were boosted with 2 mg of protein mixed with Freund’s incomplete adjuvant, and serum was obtained 12 days later by final bleeding. For western analyses, proteins were separated on 16% acrylamide gels in the Laemmli buffer system, and transferred to nitrocellulose (Bio-Rad Laboratories). Blots were probed with antibodies against Sco1 (1:5000-1000), Cox2 or porin (Molecular Probes). As secondary antibody, horseradish-peroxidase-conjugated donkey anti-rabbit antibody or goat anti-mouse antibody (Amersham Pharmacia Biotech) was used at a 1:3000 dilution. Proteins were visualized with ECL reagents (Amersham Pharmacia Biotech) followed by autoradiography. Protein bands were quantified with Quantity One software (Bio-Rad Laboratories).

RESULTS

Sco1 is a copper binding protein - Sco1 is thought to function in facilitating copper transfer from Cox17 to Cox2. One model suggests that Sco1 interacts physically with both proteins, accepting Cu ions from Cox17 and inserting them into the CuA site in Cox2. The C-terminal domain of Sco1 is believed to protrude into the mitochondrial inter-membrane space, analogous to the domain of Cox2 containing the CuA center, and it contains a potential metal-binding CxxxC motif [7]. These 2 conserved cysteine residues in Sco1 are essential for function [13]. Thus, it is possible that Sco1 binds Cu atoms via these 2 cysteines. To test this model, SC01 from S. cerevisiae was cloned into 3 different expression vectors and purified from E. coli. The first 95 residues, including the putative mitochondrial targeting sequence and the transmembrane domain, were deleted in order to produce a soluble protein. Three different vectors were used to produce untagged Sco1, and Sco1 tagged at its N-terminus with either GST or a poly-His peptide. Two
different tags were to ensure the tag did not alter the physical properties of the protein. All 3 constructs expressed well in *E. coli*, with protein yields higher than 3 mg per liter of culture. Each protein preparation showed a single component by SDS-PAGE, and showed the expected amino acid composition after acid hydrolysis.

Atomic absorption spectroscopy showed that purified Sco1 bound approximately 1 Cu atom per monomer. GST-Sco1 bound 1.41 (n=1) and His-Sco1 bound 1.12 ± 0.32 (n=6) Cu ions per polypeptide. Cleavage and removal of the purification tags resulted in a small loss in Cu ion binding. The cleaved GST-Sco1 protein bound 0.81 ± 0.13 (n=2), while the cleaved His-Sco1 protein bound 0.80 ± 0.12 (n=2). The untagged protein reproducibly bound less copper, but multiple chromatographic steps were required for its purification. To determine whether the purified C-terminal domain of Sco1 exists as a monomer or oligomer, the protein was subjected to analytical gel filtration on Sephadex G-75. All 3 Sco1 proteins eluted in fractions equivalent to monomeric species (Fig. 1).

A comparison of CuCox17 and CuSco1 suggests these proteins have inequivalent copper centers. Unlike CuCox17 [8], CuSco1 did not luminesce when excited at 300 nm. Luminescence is characteristic of proteins containing solvent-shielded polycuprous centers. The lack of luminescence in CuSco1 is suggestive that the Sco1 does not contain a polycopper thiolate center. Also, in comparison to Cox17, the Cu ions in Sco1 were less reactive with competing ligands, such as the Cu(I) specific chelator bathocuproine disulfonate (BCS) (Fig. 2) [8]. The CuSco1 complex was only half as reactive as CuCox17 complex in a ligand exchange reaction with BCS. However, both CuSco1 and CuCox17 were more reactive compared to the CuCup1 metallothionein complex, which contains a buried heptacopper cluster.

*Sco1 binds Cu(I) via three ligands* - X-ray absorption spectroscopy was carried out to probe the structure of the copper site in the different Sco1 variants (untagged versus cleaved from purification tags). Figure 3a compares the Cu K near-edge spectra of the three Sco1 variants, and shows that the spectra are very similar. Cuprous compounds have characteristic near-edge
spectra that can be used as an indicator of geometry [31,32,32a]. For digonally coordinated Cu(I) with linear geometry, the degenerate $4p_{x,y}$ orbitals are non-bonding and retain pure p character, leading to an intense dipole-allowed ($\Delta l = \pm 1$) $1s\rightarrow4p$ transition at around 8983 eV in the near-edge spectrum [31]. For trigonally coordinated Cu(I) the degeneracy is lifted and s-p mixing occurs, leading to a reduction in dipole-allowed intensity of the 8983 eV peak [31]. Figure 3 also compares the protein near-edge spectra with those from two cuprous thiolate complexes, the trigonal $[\text{Cu}_4(\text{SPh})_6]^2^-$, and the digonal $[\text{Cu(}\text{SC}_{10}\text{H}_{12})_2]^{2^-}$ [27,28,32,33]. It can be seen that the intensity of the 8983 eV peak in all the Sco1 spectra resembles that of the trigonal, and not the digonal species. Thus, Cu(I) exhibits clear trigonal coordination in the truncated Sco1 either purified as a fusion protein or as an untagged molecule. Interestingly, the rising portion of the edge jump is shifted to higher energy by several eV in the Sco1 spectra relative to the trigonal thiolate model (i.e. 8989 eV in Sco1 vs. about 8986.5 eV for $[\text{Cu}_4(\text{SPh})_6]^2^-$), indicating that Sco1 and the model compound have somewhat different metal coordination environments.

The Cu K-edge EXAFS spectra and Fourier transform for CuSco1 samples are shown in Figures 3b and 3c. The Fourier transform consists of a single peak indicating that the EXAFS arises from one prominent interaction. The EXAFS data for the 3 different protein preparations are very similar. In all cases, the best fit of the EXAFS was with 2 Cu-S and 1 Cu-N, although the fits for 3 Cu-S or 2 Cu-N and 1 Cu-S were inferior as judged by the higher error value of the fitting routine (Table I). One can rule out the 2 Cu-N, 1 Cu-S coordination complex based on an unreasonable Debye-Waller factor for this fit and a higher fit error. Although EXAFS cannot discriminate between N and O as ligands, it is unlikely an oxygen serves as a ligand for Cu(I). Thus, of the N/O pair, a His imidazole nitrogen is the most likely Cu(I) ligand. The ~2 Å EXAFS of Cu-N is almost in-phase with 2.25 Å Cu-S EXAFS over the the $k$-range of our data, which means that the Fourier transform peaks overlap, and that Cu-S and Cu-N at these particular distances are difficult to distinguish. The presence of a putative histidine ligand is expected to yield outer shell scattering with transform peaks at ~3 and 4 Å arising from the
imidazole ring atoms, but such outer shell EXAFS would be concealed by the noise of our data. Thus, EXAFS analysis suggests, but cannot conclusively prove, a ligation complex involving 2 Cu-S and 1 Cu-N. The shift of the rising portion of the near-edge supports this hypothesis, as the near-edge of a sulfur ligated species would be expected to be shifted to lower energy relative to a nitrogen ligated species.

X-ray absorption spectroscopy of the uncleaved GST-Sco1 fusion protein revealed a difference in the Fourier transform of the Cu EXAFS. The cleaved and uncleaved GST-Sco1 fusions molecules showed a common first shell scatter peak at 2.25 Å, however, the GST-Sco1 Cu(I) complex showed a significant outer shell scatter peak that fit as a Cu-Cu interaction at 2.71 Å (data not shown). GST is an obligate dimer and could thus bring Cu(I) centers in 2 adjacent Sco1 molecules in close proximity. This outer shell peak was lost when the GST moiety was removed.

The CxxxC motif and His239 are involved in Cu(I) binding. Yeast Sco1 contains 4 Cys residues, only two of which (C148 and C152) are known to be important for function [13,34]. Site-directed mutagenesis was carried out to determine which of these Cys residues are involved in Cu(I) ligation. Since X-ray absorption spectroscopy predicts Sco1 to bind Cu(I) via at least two sulfur ligands, the Cys residues were mutated to alanines in two combinations, and the mutant proteins were purified as His-tagged molecules. Atomic absorption spectroscopy of the mutant proteins showed that two essential cysteines, C148 and C152, are involved in Cu(I) ligation, whereas the non-essential C181 and C216 residues are not. The purified His-Sco1 C148,152A double mutant protein contained 0.14 ± 0.04 (n=2) Cu(I) per monomer, whereas the His-Sco1 C181,216A double mutant protein contained 0.89 (n=1) Cu(I) per monomer, similar to the wild-type protein. The purified wild-type and mutant proteins showed similar ellipticities in the far ultraviolet circular dichroism spectrum consistent with a folded conformer (data not shown).
Functional analysis of the Cys residues was probed by mutagenesis. Mutations were made in pTNDW4, a high copy yeast plasmid carrying full-length SCO1 under the control of its own promoter and terminator. Plasmids carrying wild-type or mutant sco1 alleles were transformed into a Δsco1 strain, and tested for complementation on glycerol/ethanol (SC-EG) containing media (Fig 4a). Consistent with previously published results [34], C148 and C152 were necessary for growth on non-fermentable carbon sources, while C181 and C216 were not. Even the C181,216A double mutant showed no growth defect on glycerol medium. Mitochondria isolated from each of these strains were tested for COX activity as described previously [30]. COX enzyme activity in the mutant strain harboring the Cu-binding, C181,216A Sco1 was identical to that of the wild-type protein, while mutant strain containing the non-Cu binding, C148,152A mutant Sco1 was devoid of COX activity. Thus, Sco1 function correlates with Cu(I) binding.

EXAFS suggested the third ligand may be an oxygen or nitrogen group. An Asp residue is conserved within the essential CxDxC motif. Cox2, the putative target of Sco1, contains glutamic acid in the corresponding position, and that residue is involved in Cu ion ligation within the binuclear Cuₐ site [3,35,36]. Mutation of D150 in yeast Sco1 is non-essential, since the D150A Sco1 transformant grew on SC-EG-containing media and exhibited wild-type cytochrome c oxidase activity.

His239 is an essential residue in yeast Sco1 and the Sco1 homolog of Bacillus subtilis [34,37]. To confirm this result, H239 was mutated and tested for complementation of the Δsco1 strain. Cells harboring the H239A Sco1 were respiratory incompetent (Fig 4a). The His239Ala mutant protein bound 0.14 ± 0.1 (n=2) Cu per protein. The reduced Cu content of the His mutant corroborates the EXAFS curve-fitting data suggesting that His239 is the third Cu(I) ligand.

All yeast transformants were plated on SC-EG media supplemented with increasing amounts of Cu(II). Excess Cu(II) failed to rescue the defective cells (data not shown). Figure 4b
shows that mutant Sco1 molecules were stably expressed and imported into the mitochondria. Furthermore, Cox2 levels were diminished in cells expressing non-functional Sco1 protein. Lode et al. (2000) also showed that Cox2 levels decreased when C148, C152 and H239 were mutated.

**Sco1 exists within a complex in vivo** - The Sco1 truncate described above was monomeric. To evaluate full-length Sco1, detergent-solubilized mitochondrial lysates were subjected to size exclusion chromatography and column fractions analyzed by SDS-PAGE and western blotting. When mitochondria from wild-type cells were lysed in either 0.5% or 2.2% Triton X-100 and proteins fractionated on a Sephacryl S300 column, Sco1 eluted in fractions corresponding to a molecular mass larger than 200 kDa (Fig 5, panel 2). The same fractions containing Sco1 also contained Cox2 (Fig 5, panel 1). Presumably Cox2 was still part of the entire COX complex, since COX retained its activity in these lysates (data not shown) and COX monomers would be expected to elute at this size [38,39]. This result suggested that Sco1 and Cox2 could be interacting physically in vivo, or that Sco1 was in a complex independent of COX, and the two complexes coincidentally co-eluted. To test the first hypothesis, mitochondrial lysates from strains in which Cox2 was not expressed were analyzed under the same conditions. The absence of just one COX subunit can lead to instability and degradation of the holoenzyme [39-41]. The results obtained showed that the size of Sco1 was unaffected in the absence of Cox2 (Fig 5, panel 3), suggesting that these two proteins were not in the same complex. This result does not exclude a transient interaction between these proteins. In fact, Lode et al. (2000) demonstrated that Sco1 and Cox2 interact with each other by affinity chromatography and co-immunoprecipitation experiments. Sco1 protein levels were unaltered in the strain lacking Cox2. Sco1 eluted in the similar fractions in mitochondrial lysates from cells lacking either Cox17 or Cox11 suggesting that these proteins are not in stable complex with Sco1 (Fig. 5 panels 4 and 5).

To determine whether Sco1 needs to be functional to assemble into the complex, lysates from Δsco1 cells harboring pTNDW4 C148,152A Sco1 or pTNDW4 H239A Sco1 were analyzed by size exclusion chromatography. The data obtained suggest that function is not required for
assembly of Sco1 into the complex (Fig 5, panels 6 and 7). Sco1 was expressed from a high copy 
plasmid in these two strains. Interestingly, all of the Sco1 protein detected in these experiments 
existed within the complex. Sco1 levels were increased approximately 3-fold in purified whole 
mitochondria when SCO1 was present on the plasmid yet no low molecular weight Sco1 was 
observed.

Detergent binding to hydrophobic proteins alters their mass and mobility on gel filtration 
columns. The nonionic detergent Triton X-100 has a monomeric mass of 625 Da and forms 
micelles containing 75-165 detergent molecules depending on experimental conditions [43]. 
Inclusion of this detergent in lysates and buffers may result in micelles entrapping several 
proteins that normally do not interact. To demonstrate that the complex containing Sco1 was not 
an artifact due to Triton X-100, a different detergent was used in similar experiments. Cholate 
was chosen because of its small micelle aggregation number (4) and its smaller size (409 Da). In 
the presence of cholate, Sco1 eluted later consistent with a smaller particle size (Fig. 6). The 
apparent mass of the cholate-solubilized Sco1 is larger than that expected for a simple cholate 
micelle with a single Sco1. Thus, Sco1 is likely present in a protein complex that may or may not 
contain other proteins.

DISCUSSION

Sco1 is believed to participate in the metallation of the CuA site in Cox2 [6]. Sco1 is 
proposed to accept Cu(I) from Cox17, a candidate metallochaperone that shuttles between the 
cytoplasm and the mitochondrion, and subsequently to insert the Cu ion into Cox2. To fulfill 
this role, Sco1 must be capable of binding copper. In this study, we show for the first time that 
purified Sco1 binds one Cu(I) atom per monomer. A truncated, soluble form of Sco1 was 
purified either as an untagged molecule or with one of two different tags fused to its N-terminus. 
All 3 protein preparations bind Cu(I). The Cu(I) ion appears to be tightly associated and could
not be stripped by extensive dialysis of CuSco1 samples in buffer containing DTT. X-ray absorption spectroscopy clearly suggests that the Cu(I) atom is coordinated via three ligands.

X-ray absorption spectroscopy, combined with previous functional studies [13] led to the prediction that the conserved cysteines in the CxxxC motif (C148, C152) were likely Cu(I) ligand candidates in Sco1. Purified His tagged-Sco1 C148,152A bound significantly less Cu(I) than the wild-type protein (Cu:protein ratio of 0.14 vs. 1.12), suggesting that these two residues are involved in Cu(I) ligation. The lack of any appreciable change in the far UV circular dichroism ellipticity suggests the double mutation did not induce major structural changes. C148 and C152 are functionally important residues ([13] and Fig. 4a). This suggests that Cu(I) binding is essential for in vivo Sco1 function.

The His239Ala mutant protein bound reduced levels of Cu also (Cu:protein ratio of 0.14) consistent with His239 being the third ligand. All Sco1 proteins contain a histidine residue corresponding to yeast His239. Curve-fitting analysis of the EXAFS of CuSco1 did not conclusively discriminate between a 3 Cu-S and a 2 Cu-S, 1 Cu-N coordination. The Cu K near-edge absorption was slightly shifted to higher energies than that expected for purely thiolate coordination, which is consistent with the presence of one nitrogen ligand. The combination of the EXAFS data and the reduced Cu content of the His239Ala mutant Sco1 suggest that the single Cu(I) ion in Sco1 is coordinated by Cys148, Cys152 and His239. Ample precidence exists for trigonal planar Cu(I) coordination with two thiolates and a nitrogen in small inorganic complexes [44,45].

Size-exclusion chromatography on detergent-solubilized mitochondria revealed that Sco1 eluted in the fractions higher in molecular weight than that expected for a monomeric protein. Even with cholate with its low aggregation number, the apparent size of Sco1 is consistent with a larger complex. Sco1 may be oligomeric or in association with other mitochondrial proteins. Although detergents may entrap proteins within micelles that normally do not interact, the narrow size distribution of the Sco1-containing fractions is inconsistent with non-specific
entrapment. Non-functional Sco1 (mutants Sco1 C148,152A and Sco1 H239A) migrate through the column in a similar manner to wild-type Sco1. Thus, copper binding and assembly into the complex are likely to be independent of each other.

We show clearly that Sco1 is not associated with Cox2 in the cytochrome c oxidase complex. Also, Sco1 does not appear in stable association with Cox11 as deletion of Cox11 did not alter the elution size of Sco1. Likewise, Sco1 is not stably associated with Cox17. Western blotting of the detergent solubilized elution fractions with anti-Cox17 did not reveal co-chromatography of Cox17 and Sco1.

The gel filtration data with detergent-solubilized mitochondria is consistent with full-length Sco1 being oligomeric. When expressing Sco1 from a high copy yeast plasmid, we observed that a fraction of the protein remained in the cytoplasm. Size-exclusion chromatography of cytoplasmic fractions in the presence of Triton revealed that Sco1 was assembled into a complex. Because the purified Sco1 truncate is monomeric, oligomerization most probably involves the N-terminal portion of the protein. We favor a model where full-length Sco1 is an oligomeric protein under normal conditions, although the possibility exists that the in vivo Sco1 complex may contain other proteins.

The significance of Sco1 oligomerization in vivo is unclear. Cox17 the candidate Cu(I) donor to Sco1 exists as an oligomer within the mitochondrial intermembrane space [8]. Since oligomerization of Cox17 is important for its function [8], the possibility exists that Cu(I) ions are donated from the Cox17 tetramer to the Sco1 oligomer. The Cu(I) ions in the Cox17 oligomer are clustered, so the possibility remains that the in vivo Sco1 oligomer may also have Cu(I) sites clustered or in close proximity. The candidate receptor of Sco1-bound Cu(I) is believed to be the binuclear CuA site of Cox2. Two Cu ions may be donated by a Sco1 dimer to form the binuclear CuA center. Sco1 oligomerization may also be significant as its receptor, cytochrome c oxidase, is believed to exist in vivo as a dimer complex with each monomeric units containing 12-13 subunits [39,46]. If metallation of the CuA center occurs after assembly of the dimeric COX complex,
Sco1 oligomerization may permit metallation of the two CuA centers in the dimer. Further studies are in progress to test these models.

Acknowledgements We thank members of our laboratory, especially Drs. Daren N. Heaton and Heather Carr, for many helpful discussions. We also thank Garth Garrison for excellent technical assistance. We acknowledge support from the National Institutes of Health (5P30-CA 42014) to the Biotechnology Core Facility for DNA synthesis and to the DNA Sequencing Facility at the University of Utah for DNA sequencing.

REFERENCES


Table I

Curve Fitting Results of Cu K Edge EXAFS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cu-S</th>
<th>Cu-N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>R (Å)</td>
</tr>
<tr>
<td>His-Sco1 Cleaved</td>
<td>3</td>
<td>2.248 (4)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.251 (4)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.259 (2)</td>
</tr>
<tr>
<td>GST-Sco1 Cleaved</td>
<td>3</td>
<td>2.247 (4)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.251 (2)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.259 (2)</td>
</tr>
<tr>
<td>Untagged</td>
<td>3</td>
<td>2.239 (3)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.264 (4)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.299 (5)</td>
</tr>
</tbody>
</table>

N is the coordination number, R is the mean interatomic distance, $\sigma^2$ the mean square deviation in R, and $\chi$ is the fit error function. The fit error is defined as $[\Sigma (\chi_{\text{exp}} - \chi_{\text{calc}})^2 k^6 / \Sigma \chi_{\text{calc}} k^6 (\sigma_{\text{calc}}^2 k^6)]^{1/2}$. N and $\sigma^2$ exhibit a high degree of mutual correlation in the refinement, and because of this the fits shown are "best integer fits" where N values were held fixed at integer values and the other parameters refined. The values given in parentheses are estimated standard deviations (precisions) obtained from the diagonal elements of the covariance matrix. We note that the accuracies will be related to, but larger than these values, and can be assumed to be close to ± 0.02 Å for bond-lengths.
**Figure Legends**

Figure 1. Elution profile of His-Sco1 on an analytical gel filtration column. A G-75 Superdex 26/60 (Pharmacia) column was calibrated with molecular weight standards (thin line): bovine serum albumin (BSA, 66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12 kDa). Purified His-Sco1 wild-type (WT, bold line) and His-Sco1 C148,152,181,216A (dashed line) were chromatographed under the same conditions. The predicted mass for monomeric His-Sco1 is 26,232 Da.

Figure 2. Ligand exchange reactions of Cu(I)-containing proteins with the Cu(I) chelator bathocuproine sulfonate (BCS). The concentration of Cu(I) in each sample was 39 µM. The concentration of BCS was 300 µM. The formation of the Cu(BCS)_2 complex was measured at 483 nm with respect to time using a molar extinction coefficient of 12,250.

Figure 3. X-ray absorption spectroscopy of purified Sco1. XAS near-edge spectra (A), EXAFS (B), and Cu-S phase-corrected EXAFS Fourier transforms (C) for 3 different constructs. Panel A also compares the near-edge spectra of trigonally and digonally coordinated model compounds (the broken lines shows the untagged Sco1 spectrum again for closer comparison with the trigonal compound). With the EXAFS (B) and the Fourier transforms (C), the solid lines are the experimental data, and the dashed lines are the best fit using the parameters in given in Table 1.

Figure 4. *In vivo* characterization of Sco1 mutant proteins. High copy yeast expression plasmids carrying either WT or mutant sco1 alleles were transformed into a Δsco1 strain. Transformants were grown in selective medium, diluted 10-fold serially, and spotted onto selective plates containing either glucose or glycerol/ethanol (A). Mitochondria were isolated.
from each transformed strain grown in selective glucose-containing medium, and 10 µg protein run on 16% acrylamide gels (B). Sco1 and Cox2 were visualized after western blotting of mitochondrial proteins with specific antibodies. Porin was used as a loading control.

Figure 5. Size-exclusion chromatography of mitochondrial lysates. Mitochondria were isolated from each strain indicated on the left-hand side of the figure. Approximately 5-10 mg mitochondrial proteins were solubilized with 0.5% Triton X-100, and the lysates clarified by centrifugation. Filtered lysates were applied to a Sephacryl S300 column from Pharmacia. The column had been previously calibrated with molecular weight standards: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (66 kDa), and cytochrome c (12 kDa). Three ml column fractions were collected and analyzed by SDS-PAGE followed by immunoblotting and ECL detection. Antibodies against Cox2 (panel 1) or Sco1 (panels 2-7) were used.

Figure 6. Size-exclusion chromatography of overexpressed Sco1 solubilized with different detergents. Mitochondria were purified from a Δsco1 strain harboring pTNDW4 (WT). Mitochondria were lysed with either 0.5% Triton X-100 or 1% cholate, and the lysates analyzed on the Sephacryl S300 column as described.
Fig 2

% Cu(I) removal by BCS

Cox17

Sco1

Cup1

time (sec)
Fig 4a
WT  Vec  148  150  152  181  216  239  239

Sco1

Cox2

Porin

Fig 4b
Fraction # | Mol. Mass (kDa)  
--- | ---
32 | 669
34 | 440
36 | 232
38 | 158
40 | 32
42 | 44
44 | 669
46 | 440
48 | 232
50 | 158
52 | 32
54 | 44
56 | 669
58 | 440

Fig 5
Strain: Δsco1 + pTNDW4

<table>
<thead>
<tr>
<th>Fraction #</th>
<th>Mol. mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>232</td>
</tr>
<tr>
<td>50</td>
<td>158</td>
</tr>
<tr>
<td>52</td>
<td>66</td>
</tr>
<tr>
<td>54</td>
<td>12</td>
</tr>
<tr>
<td>56</td>
<td>48</td>
</tr>
<tr>
<td>58</td>
<td>50</td>
</tr>
<tr>
<td>60</td>
<td>52</td>
</tr>
<tr>
<td>62</td>
<td>54</td>
</tr>
<tr>
<td>64</td>
<td>56</td>
</tr>
<tr>
<td>66</td>
<td>58</td>
</tr>
<tr>
<td>68</td>
<td>60</td>
</tr>
<tr>
<td>70</td>
<td>62</td>
</tr>
<tr>
<td>72</td>
<td>64</td>
</tr>
<tr>
<td>74</td>
<td>66</td>
</tr>
<tr>
<td>76</td>
<td>68</td>
</tr>
<tr>
<td>78</td>
<td>70</td>
</tr>
</tbody>
</table>

- Triton X-100
- Cholate

Fig 6
Yeast Sco1, a protein essential for cytochrome c oxidase function, binds copper via conserved cysteinyl residues and forms oligomers in vivo
Thalia Nittis, Graham George and Dennis Winge

J. Biol. Chem. published online September 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107077200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2001/09/06/jbc.M107077200.citation.full.html#ref-list-1