

Human Sco1 and Sco2 Function as Copper-binding Proteins*

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Yih-Chern Horng^{‡1}, Scot C. Leary^{§‡1,2}, Paul A. Cobine[‡], Fiona B. J. Young[§], Graham N. George[¶], Eric A. Shoubridge^{§3}, and Dennis R. Winge^{‡4}

From the [‡]Departments of Medicine and Biochemistry, University of Utah Health Sciences Center, Salt Lake City, Utah 84132, the

[§]Department of Human Genetics and Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada, and

[¶]University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E2, Canada

The function of human Sco1 and Sco2 is shown to be dependent on copper ion binding. Expression of soluble domains of human Sco1 and Sco2 either in bacteria or the yeast cytoplasm resulted in the recovery of copper-containing proteins. The metallation of human Sco1, but not Sco2, when expressed in the yeast cytoplasm is dependent on the co-expression of human Cox17. Two conserved cysteines and a histidyl residue, known to be important for both copper binding and *in vivo* function in yeast Sco1, are also critical for *in vivo* function of human Sco1 and Sco2. Human and yeast Sco proteins can bind either a single Cu(I) or Cu(II) ion. The Cu(II) site yields S-Cu(II) charge transfer transitions that are not bleached by weak reductants or chelators. The Cu(I) site exhibits trigonal geometry, whereas the Cu(II) site resembles a type II Cu(II) site with a higher coordination number. To identify additional potential ligands for the Cu(II) site, a series of mutant proteins with substitutions in conserved residues in the vicinity of the Cu(I) site were examined. Mutation of several conserved carboxylates did not alter either *in vivo* function or the presence of the Cu(II) chromophore. In contrast, replacement of Asp²³⁸ in human or yeast Sco1 abrogated the Cu(II) visible transitions and in yeast Sco1 attenuated Cu(II), but not Cu(I), binding. Both the mutant yeast and human proteins were nonfunctional, suggesting the importance of this aspartate for normal function. Taken together, these data suggest that both Cu(I) and Cu(II) binding are critical for normal Sco function.

Cytochrome *c* oxidase (CcO)⁵ is the terminal enzyme of the energy-transducing, electron transfer chain within the mitochondrial inner membrane. Mammalian CcO consists of 13 polypeptide subunits, three of which (CoxI–CoxIII) are encoded by the mitochondrial genome and the remaining 10 of which are encoded by the nuclear genome (1, 2). An additional 30 proteins are believed to be required for the assembly of the CcO complex (3). A number of these accessory proteins are important in the processing and translation of *COXI–COXIII* mRNA transcripts,

in membrane insertion of subunits, and in either the synthesis or delivery of cofactors. The cofactors in CcO include two copper sites (Cu_A and Cu_B), two heme A moieties, and a magnesium and zinc ion (4).

The Cu_A site is a binuclear, mixed valent copper center localized in the CoxII subunit, whereas the Cu_B site consists of a single copper ion within a Cu-heme A binuclear center in the CoxI subunit (4). The CoxI and CoxII subunits are synthesized within the mitochondrion, so copper site insertion must occur during insertion of the nascent polypeptides into the inner membrane.

Several proteins, including Cox11, Cox17, Cox19, Cox23, and Sco1, have been implicated in the assembly of the copper centers in CcO in yeast and all have human homologs (5). Cox17 is a soluble copper metallochaperone within the mitochondrial intermembrane space (IMS). Sco1 and Cox11 are inner membrane proteins tethered by a single transmembrane helix and are implicated in the assembly of the Cu_A and Cu_B centers, respectively (6, 7). Both proteins are copper-binding proteins, and mutations that abrogate Cu(I) binding attenuate *in vivo* assembly of CcO (8–11). Recently, we demonstrated that Cox17 is capable of donating Cu(I) to both Sco1 and Cox11 (12). The prediction is that copper ions are only transiently bound to Sco1 and Cox11 in yeast prior to donation to CoxII and CoxI, respectively. Yeast Sco1 was shown to form a transient complex with CoxII (13).

Assembly of CcO in mammalian cells has additional components, since two distinct Sco-like molecules are involved in the assembly process. Mutations in both human genes, *SCO1* and *SCO2*, have been identified, and these result in respiratory chain deficiency associated with CcO assembly defects (14–16). Although yeast also has a second Sco protein, designated Sco2, it has no function in CcO assembly (17). The human Sco1 and Sco2 molecules share the Cu(I) binding motif of yeast Sco1; however, nothing is currently known about the copper binding by human Sco1, whereas limited data exist on copper binding by human Sco2 (18, 19). Neither human Sco1 nor Sco2 is able to rescue the respiratory defect of yeast *sco1* null cells (20). However, a chimera containing the N-terminal segment of yeast Sco1 fused to the C-terminal segment of human Sco1 is functional in *sco1* null cells (20). Two lines of evidence suggest that both human Sco1 and Sco2 proteins probably function in copper metallation of CcO. First, overexpression of *COX17* partially rescues the CcO deficiency of *SCO2*, but not *SCO1*, patient cells (21). Second, the CcO deficiency in *SCO1* and *SCO2* patient cell lines is partially suppressed by the addition of exogenous copper to the culture medium (18, 21, 22).

It is unclear why mammalian cells require two distinct Sco molecules for CcO maturation. Patients with mutations in *SCO2* have a clinical presentation that is distinct from that of *SCO1* patients (15, 16, 23). *SCO2* mutations are associated with neonatal encephalocardiomyopathy, whereas *SCO1* patients present with neonatal hepatic failure and ketoacidotic coma. The distinctive clinical presentation is not a result of tissue-specific expression of the two genes, since *SCO1* and *SCO2* are ubiquitously expressed and exhibit a similar expression pattern in dif-

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¹ These two authors contributed equally to this work.

² Recipient of a post-doctoral fellowship from CIHR.

³ An International Scholar of the Howard Hughes Medical Institute and a senior investigator of CIHR. To whom correspondence may be addressed: Dept. of Human Genetics and Montreal Neurological Institute, McGill University, 3801 University St., Rm. 660, Montreal, Quebec H3A 2B4, Canada. Tel.: 514-398-1997; Fax: 514-398-1509; E-mail: eric@ericpc.mni.mcgill.ca.

⁴ To whom correspondence may be addressed. Tel.: 801-585-5103; Fax: 801-585-5469; E-mail: dennis.winge@hsc.utah.edu.

⁵ The abbreviations used are: CcO, cytochrome *c* oxidase; IMS, intermembrane space; NTA, nitrilotriacetic acid; DTT, dithiothreitol; WT, wild type.

Copper Binding to Human Sco1 and Sco2

ferent human tissues (14). Recent studies with immortalized fibroblasts from *SCO1* and *SCO2* patients suggest that Sco1 and Sco2 have non-overlapping but cooperative functions in CcO assembly (21). The evidence for this conclusion is that the CcO assembly pathway is blocked at a similar step in both *SCO1* and *SCO2* patient cell lines and that over-expression of either gene in the reciprocal patient cell line resulted in a dominant negative effect on CcO activity (21).

Although Sco proteins are reported to be competent to bind copper ions, this function was questioned in a recent report on the crystal structure of human Sco1 (24). Sco1 crystallized without bound copper, and the three residues implicated in Cu(I) binding to yeast Sco1 were not in optimal configuration for trigonal Cu(I) binding (24). The authors suggested that Sco1 is not a copper metallochaperone essential for metal transfer to CoxII but stated that the protein may have a critical role in redox sensing. In this study, we analyze the copper binding properties of human Sco1 and Sco2 and report that copper binding appears to be important for the function of each protein. We report that both proteins have the ability to bind either Cu(I) or Cu(II) and that Cu(II) binding may be an important facet of function.

MATERIALS AND METHODS

Yeast Strains and Human Cell Lines—All yeast strains used were in W303 background (MAT a, *ade2-1*, *his3-1,15*, *leu2,3,112*, *trp1-1*, *ura3-1*). The strain *cox17Δ-SCO2/COX17* for the yeast cytosolic assay was generated by transforming *cox17* with pRS303-*MET25-SCO2/COX17* (25). The *cox17-SCO2/COX17* strain was cultured on plates or in liquid in complete medium lacking uracil for pYEF2 selection or lacking leucine for pRS315 selection. *sco1Δ* cells were cultured on plates or in liquid in complete medium lacking tryptophan for TNDW4(*SCO1*) selection (9). Cells were cultured with glucose, raffinose, or galactose as carbon sources as described before. DNA transformations were performed using a lithium acetate protocol. Primary cell lines from control *SCO1* and *SCO2* patient skin fibroblasts were immortalized and cultured as previously described (21).

Plasmids—hSCO1 (lacking the first 333 bp) or hSCO2 (lacking the first 234 bp) amplified by PCR was cloned into pHis-parallel 2 to generate pHis-hSCO1 or pHis-hSCO2 by adding BamHI and SalI restriction sites to the 5'- and 3'-ends, respectively. The same hSCO fragment fused to a 5' sequence encoding a poly-His purification tag was amplified from pHis-hSCO1 or pHis-hSCO2 by PCR with NotI and EcoRI restriction sites added to the 5'- and 3'-ends, respectively. The truncated *SCO1* genes were subcloned into pYEF2 containing the *GAL1* promoter and *CYC1* terminator for transformation of *cox17Δ-SCO2/COX17* cells. The plasmid is designated YEp-*GAL1*-hSCO1 or YEp-*GAL1*-hSCO2. Mutagenesis of SCO genes was carried out using the QuikChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA) on both pHis-hSCO1 and pTNDW4 (yeast *SCO1*). The resulting *SCO1* mutants were sequenced, and those in pTNDW4 were subcloned to ensure there were no mutations in the plasmid backbones. The construction of YCp-*MET25-hCOX17* and pRS303-*MET25-SCO2/COX17* was similar to the construction of YCp-*MET25-COX17* and YCp-*MET25-SCO2/COX17* described previously. The Sco2-Cox17 fusion contains the 5,104 residues of Sco2, including the N-terminal mitochondrial import sequence and transmembrane domain of Sco2 fused to Cox17. The correct sequences of all plasmids generated in this study were confirmed by sequencing.

Wild type hSCO1 and hSCO2 cloned into the gateway-modified retroviral expression vector pLXSH or pBABE were used as templates to generate specific point mutants using the QuikChangeTM site-directed

mutagenesis kit (Stratagene). The fidelity of all resultant constructs was confirmed by sequencing prior to their use in expression studies.

Protein Purification—Recombinant human and yeast Sco and mutant Sco were purified from BL21 (DE3) transformants harboring pHis-hSCO or pTNDW2 (His-tagged *SCO1* or mutant *SCO1*) as described previously by Nittis *et al.* (9). Nickel-NTA Superflow (Qiagen) was used for the purification of the His-tagged Sco proteins. Yeast transformants with YEp-*GAL1*-hSCO were cultured in raffinose medium to an A_{600} of 0.6. Galactose was then added to induce expression of the His-tagged Sco. Cells were harvested after 5 h for preparation of lysates by use of a French press and subsequent Ni²⁺-NTA purification from clarified samples.

UV-visible Electronic Absorption and Electronic Paramagnetic Resonance Spectroscopies—Absorption spectra of a sample in a cuvette with a path length of 1 cm were recorded with a Beckman DU640 spectrophotometer. X-band EPR spectra were obtained on a 9-GHz Bruker EMX spectrometer. All samples were run at 77 K in a liquid nitrogen finger Dewar. Spin quantitation was determined relative to a 0.5 mM CuEDTA standard.

Assays—The copper and nickel concentration of the protein samples was measured using a PerkinElmer Life Sciences (AAAnalyst 100) atomic absorption spectrophotometer or a PerkinElmer Optima (3100XL) ICP spectrometer. A bathocuproine sulfonate assay was used to determine the Cu(I) content of the protein samples. The appearance of a Cu(bathocuproine sulfonate)₂ complex was measured by monitoring the absorbance at 483 nm using a molar extinction coefficient of 12,250 cm⁻¹ M⁻¹. Protein was quantified by amino acid analysis after hydrolysis in 5.7 N HCl at 110 °C *in vacuo* on a Beckman 6300 analyzer. Oxygen consumption assays were performed on a YSI 5300A biological oxygen monitor. The rate of oxygen consumption of each strain was calculated based on the linear portion. Each strain was assayed repeatedly two or three times to get the average rate.

Immunoblot Analysis—Protein (10–50 μg) from the mitochondrial fraction was electrophoresed on a 15% SDS-polyacrylamide gel system and transferred to nitrocellulose (Bio-Rad). Membranes were blocked in 1× phosphate-buffered saline (50 mM Na₂PO₄, 100 mM NaCl, pH 7.0), 0.01% Tween 20, and 10% milk solution prior to detection with appropriate antibodies and visualization with Pierce chemiluminescence reagents using a horseradish peroxidase-conjugated secondary antibody. Antiserum to porin (Por1) was from Molecular Probes, Inc. (Eugene, OR). Rabbit anti-Sco1 antiserum was generated as described previously (9).

Immortalized human fibroblasts were differentially permeabilized using digitonin in order to generate mitochondrially enriched fractions (26) and subsequently solubilized in phosphate-buffered saline containing 1.5% lauryl maltoside supplemented with complete protease inhibitor mixture (Roche Applied Science) as previously described (21). Equal amounts of protein were fractionated on 15% SDS-PAGE gels and transferred to nitrocellulose. Membranes were blotted with polyclonal antisera raised against human Sco1 (21) and Sco2 (18) and a monoclonal anti-porin antibody (Calbiochem). Following incubation with the relevant secondary antibody, immunoreactive proteins were detected by luminol-enhanced chemiluminescence (Pierce).

Miscellaneous—Phoenix amphotropic cells (Dr. G. Nolan, Stanford University) were used to transiently produce and package all individual human cDNA constructs of interest. Subsequent infection and selection of fibroblast cell lines were essentially as previously described (21). Protein concentration (27), CcO (28), and citrate synthase (29) activities were measured as described elsewhere.

RESULTS

Recombinant Human Sco1 and Sco2 Expression—To study the biochemical properties of the globular domains of human Sco1 and Sco2, truncates were engineered lacking only the N-terminal mitochondrial targeting sequence and single transmembrane helix (Fig. 1). Soluble domains of each protein were used for our studies to avoid the difficulties of purifying and working with membrane proteins. Soluble domains of human SCO1 and SCO2 fused to a 5' poly-His tag were expressed in *Escherichia coli* BL21 (DE3) cells for the purpose of purification by Ni²⁺-NTA chromatography and subsequent characterization of the two proteins. Protein recovered after imidazole elution was found to contain bound copper by atomic absorption spectroscopy. Each protein fraction was subsequently chromatographed on gel filtration to recover homogeneous Sco1 and Sco2. Both proteins eluted from gel filtration in fractions corresponding to monomeric molecules. The copper content of hSco1 and hSco2 was 1.0 and 0.8 mol eq, respectively. Dialysis of the copper-containing proteins overnight in 1 mM EDTA resulted in only a slight depletion of bound copper, with human Sco1 and Sco2 each retaining 0.8 mol eq of bound copper (TABLE ONE). No metal ions other than low levels of Ni(II), derived from the Ni²⁺-NTA resin used in the purification, were detected using ICP spectroscopy. Samples dialyzed against EDTA were depleted of the minimal levels of Ni(II). Cleavage of the poly-His tag followed by subsequent purification of the non-tagged protein retained 80% of the bound copper. Thus, the copper ions bound to the fusion protein were associated with the Sco1 protein and not the His purification tag. The copper ions were stably associated with both Sco proteins.

Absorption spectroscopy of purified Sco1 revealed a chromophore in the visible region of the spectrum with maxima at 360 and 480 nm (Fig. 2A). The visible absorption bands resembled the S-Cu(II) charge transfer bands of nitrosocyanin (30, 31), and the presence of bound Cu(II) in human Sco1 as purified was confirmed by EPR (Fig. 3A). Integration of the EPR signal revealed a Cu(II) content of 0.3 mol eq. Titration of human Sco1 with the Cu(I) chromogenic chelator bathocuproine sulfonate revealed a Cu(I) content of 0.7 mol eq. The stoichiometry in

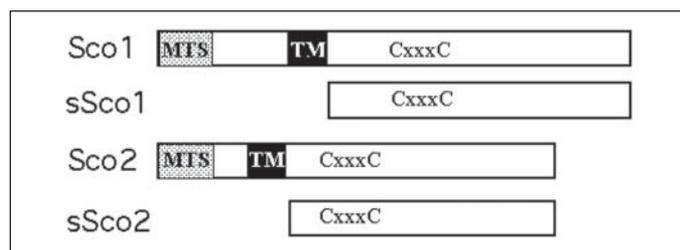


FIGURE 1. Diagram of the protein sequences of human Sco1 and Sco2. The positions of the mitochondrial target sequences (MTS), transmembrane helices (TM), and essential CXXX motif are indicated. The soluble fragments used in the present study are shown as sSco1 and sSco2. The truncates lack only the mitochondrial target sequence and transmembrane helix.

combination with EPR spectroscopy suggests that each monomeric Sco1 molecule contains either a bound Cu(I) or a bound Cu(II). The fraction of bound Cu(I) versus Cu(II) was similar in multiple isolates of hSco1. To determine the binding stability of the two valence states of copper, hSco1 dialyzed overnight with 1 mM EDTA revealed a Cu(II) content of 0.17 mol eq, whereas the total copper content dropped to 0.78 mol eq. Although the Cu(II) content was reduced 2-fold by the EDTA dialysis treatment, the chromophore content was unchanged. The dialysis result is consistent with the EPR spectrum that forms two g_z values with different hyperfine splittings, indicating the presence of two Cu(II) species in human Sco1 (Fig. 3A). Although human Sco1 contains two Cu(II) species, only one generates the chromophore. Two distinct Cu(II) species were also observed in the Sco1 protein from *Bacillus subtilis* (32).

Incubation of hSco1 with 1 mM DTT overnight reduced the Cu(II) content from 0.35 to 0.24 mol eq, but the chromophore content was unchanged (TABLE ONE). Thus, the Cu(II) species that is responsible for the visible chromophore is stable to dialysis in 1 mM EDTA and 1 mM DTT. The EPR spectrum of the CuSco1 sample after dialysis in EDTA and DTT is shown in Fig. 3B. This spectrum is characterized by only four hyperfine splittings of the g_z component of the Cu(II) signal. This pattern of hyperfine splitting is consistent with a single Cu(II) species that gives rise to the visible absorption bands.

Prolonged incubation of Sco1 at 4 °C did not alter the abundance of the Cu(II) chromophore. As mentioned, the chromophore was not bleached by 1 mM DTT but was abolished by dithionite. After bleaching by 2 mM dithionite followed by dialysis in buffer, the copper content was reduced by an increment corresponding to the quantity of copper originally present as Cu(II). Thus, upon reduction of Cu(II), the Cu(I) is unable to stably associate with the Sco1. Analogous experiments with human Sco2 found that the Cu(I) and Cu(II) adducts were indistinguishable from those of human Sco1. Collectively, these data argue that the unique function of each human Sco in the metallation of CcO is not attributable to differences in their copper-binding properties.

The EPR spectrum of Cu(II)Sco1 (Fig. 3B) shows features that are typical of ¹⁴N ligand hyperfine interaction (a three-line pattern from the $I = 1$ nucleus superimposed on a four-line pattern from the $I = 3/2$ ^{63,65}Cu hyperfine) that is expected from a histidine nitrogen ligation of the metal. This is illustrated by the inset in Fig. 3B, which shows the second derivative of the $g_x - g_y$ region of the Cu(II) EPR spectrum, with clear structure attributable to ¹⁴N ligand hyperfine to at least one such ligand. A more complete analysis of the EPR spectroscopy of Cu(II)Sco1, including multifrequency measurements will be presented in a subsequent publication.

Previously, we reported the presence of Cu(I) in yeast Sco1, but only a percentage of the copper was reactive with the Cu(I) chelator bathocuproine sulfonate (9). To determine whether yeast Sco1 also contained bound Cu(II), the soluble domain of yeast Sco1 was recombinantly expressed and purified as described previously (9). The bound copper

TABLE ONE

Copper binding stoichiometry of human Sco1 and Sco2 purified from bacteria

The Sco proteins were purified by a combination of Ni²⁺-NTA chromatography and gel filtration. Copper and protein were quantified by atomic absorption spectrometry and amino acid analysis, respectively.

	Copper/protein	Cu(II)/protein	Dialysis (1 mM EDTA)		Dialysis (1 mM DTT), Cu(II)/protein
			Copper/protein	Cu(II)/protein	
WT human Sco1	0.95 ± 0.03	0.35 ± 0.08	0.78 ± 0.05	0.17 ± 0.02	0.24 ± 0.04
D259A human Sco1	1.13 ± 0.26	0.17 ± 0.02	0.12 ± 0.05	0.10 ± 0.03	ND ^a
H260A human Sco1	0.66 ± 0.09	0.34 ± 0.07	0.28 ± 0.06	ND	ND
WT human Sco2	0.79 ± 0.06	0.35 ± 0.01	0.75 ± 0.07	0.34 ± 0.08	0.32 ± 0.03

^a ND, not detectable.

Copper Binding to Human Sco1 and Sco2

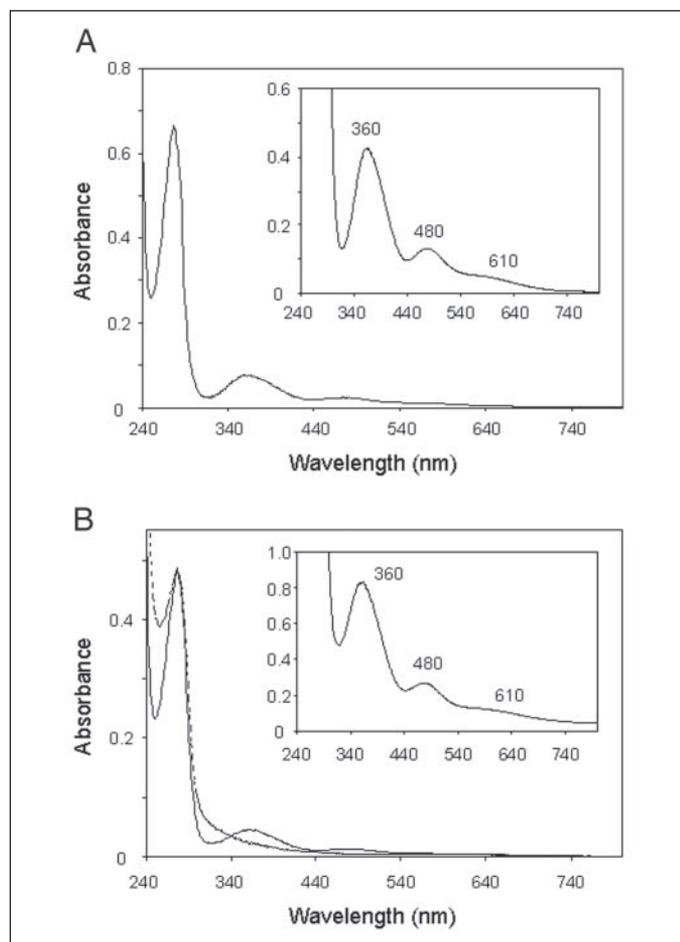


FIGURE 2. Absorption spectroscopy of purified human and yeast Sco1 proteins. *A*, absorption spectrum of human Sco1 (23.4 μM protein) purified by Ni^{2+} -NTA and gel filtration. The inset shows the S-Cu(II) charge transfer bands of a 129 μM sample. *B*, absorption spectrum of yeast Sco1 (14 μM protein). The inset shows the S-Cu(II) charge transfer bands of a 250 μM sample. The dotted line shows the absorption spectrum of the D238A mutant protein.

content was 0.7 mol eq of copper (TABLE TWO). Quantitation of the Cu(II) content by integration of Cu(II) EPR signal revealed 0.3 mol eq of bound Cu(II). Dialysis of the yeast CuSco1 overnight with 1 mM EDTA and 1 mM DTT revealed 0.65 mol eq of bound copper after dialysis and a Cu(II) content of 0.27 mol eq. Thus, binding of both Cu(I) and Cu(II) was stable in yeast Sco1. Absorption spectroscopy of the yeast CuSco1 revealed visible absorption bands at 360 and 480 nm, similar to those observed with the human Sco proteins (Fig. 2*B*). EPR spectroscopy of yeast CuSco1 revealed the presence of two distinct Cu(II) species (Fig. 3*A*), and, like human Sco1, dialysis against 1 mM EDTA and 1 mM DTT yielded a single Cu(II) species that results in the visible absorption bands (Fig. 3*B*).

Purification of Human CuSco1 and CuSco2 from Yeast—As a second test of copper binding, we expressed the soluble domains of *SCO1* and *SCO2* as His tag fusions in the yeast cytoplasm as described previously (12). Each soluble domain lacked only the mitochondrial targeting sequence and single transmembrane helix. The fusion genes were placed under the control of the *GAL1* promoter and expressed from a high copy YEp plasmid. The absence of the mitochondrial targeting sequence resulted in expression in the yeast cytoplasm. Expression of the proteins in the cytoplasm was important, since the yeast cytoplasm is a highly competitive environment for Cu(I) due to the abundant Cup1 and Crs5 metallothionein molecules and, as a result, contains essentially

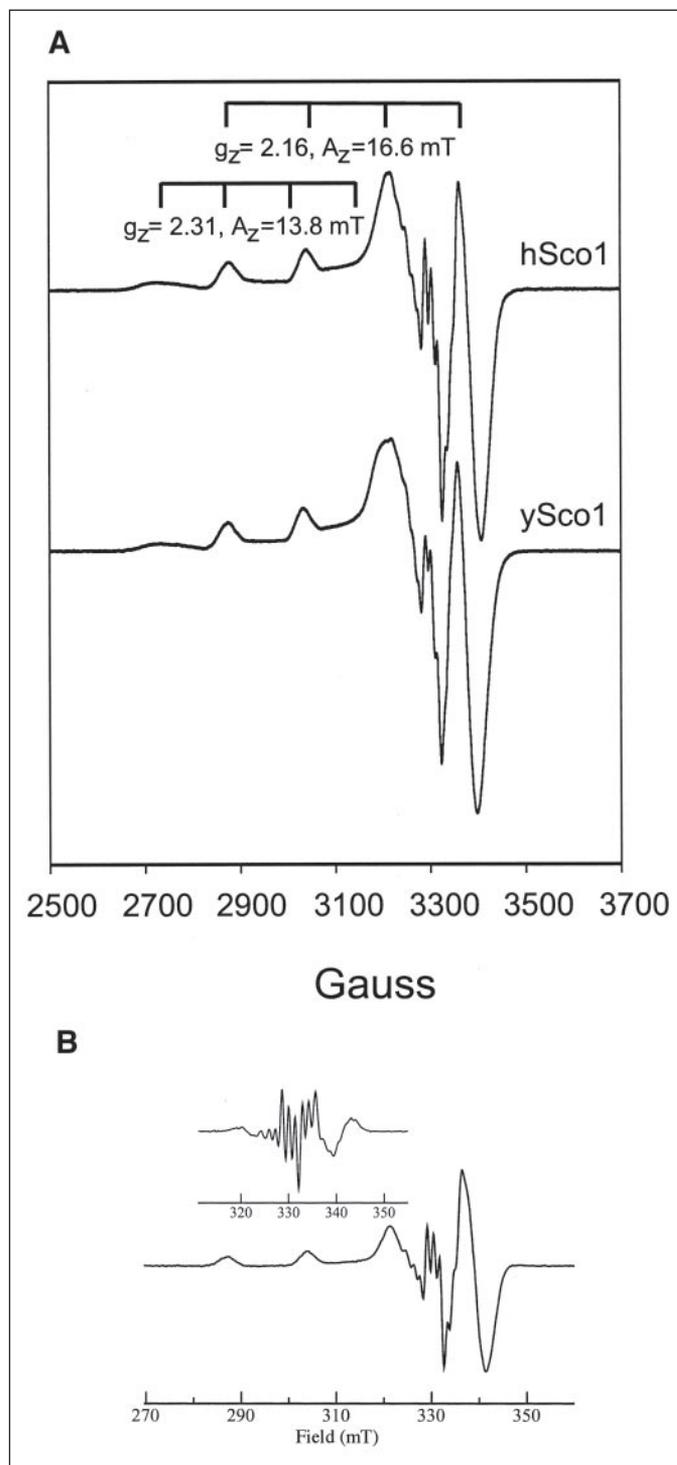


FIGURE 3. EPR spectrum of human and yeast Sco1 samples. *A*, EPR spectrum of human and yeast Sco1 proteins as isolated. The concentrations of the human and yeast proteins were 2.3 and 2.4 mM, respectively. Shown are the hyperfine splittings of the g_z components. The presence of two g_z components suggests the presence of two distinct Cu(II) species. *B*, the spectrum of the human Sco1 sample (0.67 mM) dialyzed against 1 mM EDTA and 1 mM DTT is shown. EPR spectra were recorded at 9.429 GHz with a power of 19.8 milliwatts with a modulation amplitude of 4 G.

no free copper (33). The presence of bound copper in the Sco proteins purified from the cytoplasm is therefore an indication of their copper binding avidity. Human Sco1 and Sco2 were purified from the yeast cytoplasm of cells cultured in synthetic complete medium with no supplementation of exogenous copper salts. Purification was accomplished

TABLE TWO

Copper binding stoichiometry of yeast Sco1 purified from bacteria

Yeast Sco1 was purified by a combination of Ni²⁺-NTA chromatography and gel filtration. Copper was quantified by atomic absorption spectrometry, and protein was quantified by amino acid analysis.

Yeast Sco1	Copper/protein	Cu(II)/protein	Dialysis with 1 mM EDTA, 1 mM DTT	
			Copper/protein	Cu(II)/protein
WT	0.72 ± 0.01	0.29 ± 0.04	0.65 ± 0.03	0.27 ± 0.02
D238A	1.51 ± 0.66	1.16 ± 0.45	0.95 ± 0.23	ND ^a
D238E	0.97	0.46	0.67	0.02
H239A	1.20	0.72	0.31	ND

^a ND, not detectable.

by Ni²⁺-NTA chromatography followed by gel filtration. Human Sco1 was recovered with a bound copper content of 0.3 mol eq, whereas human Sco2 revealed a bound copper content of 0.9 mol eq (TABLE THREE). Human Sco1 and Sco2 purified from the yeast cytoplasm contained both Cu(I) and Cu(II). The visible absorption bands arising from S-Cu(II) charge transfer bands were similar to those of the Cu(II) complexes isolated from recombinant proteins expressed in bacteria.

The low copper content of human Sco1 was consistent with inefficient copper binding of yeast Sco1 expressed as a soluble domain in the yeast cytoplasm of *cox17Δ* cells as described previously (12). Maximal copper binding to yeast Sco1 required the co-expression of yeast Cox17 (12). To determine whether the presence of Cox17 was an important determinant of copper binding to human Sco1, human *SCO1* and human *COX17* were co-expressed as soluble proteins in the yeast cytoplasm. Overexpression of *COX17* results in considerable Cox17 protein in the cytoplasm. The presence of Cox17 resulted in an increase in the copper content of Sco1 from 0.3 to 1.0 mol eq, similar to what we observed previously with the yeast proteins (12) (TABLE THREE). The co-expression of human Cox17 and Sco2 did not alter the copper stoichiometry of human Sco2. Thus, human Sco2 was efficiently copper-loaded in the yeast cytoplasm in the absence of human Cox17, whereas the loading of Sco1 was Cox17-dependent. The copper metallation of human Sco2 cannot be mediated by yeast Cox17, since the cells contained only a Sco2/Cox17 fusion that remains tethered to the mitochondrial inner membrane (25). Plausible scenarios addressed under "Discussion" may contribute to the metallation of human Sco2 in the yeast cytoplasm.

Mutational Analysis of Yeast Sco1—The presence of stably associated Cu(I) and Cu(II) in Sco1 and Sco2 samples suggests that both valence states of copper in Sco proteins may be physiologically functional. The availability of the crystal structure of human Sco1 permitted identification of conserved residues in the vicinity of the Cu(I) site. The Cu(II)-S charge transfer bands arise from cysteinyl thiolates, so the Cu(I) and Cu(II) sites must be in close juxtaposition. We targeted conserved residues in the vicinity of the Cu(I) site to find mutations that abrogated Cu(II) binding. The Cu(II) site in nitrosocyanin, which exhibits visible absorption bands similar to Sco1 and Sco2, is a five-coordinate site formed by two histidyl ligands, one cysteine, one carboxylate, and a solvent ligand. Thus, we anticipate that one or two additional ligands may be present besides the two Cys and His residues that are important for Cu(I) binding. Most of the conserved residues in the vicinity of the Cu(I) site are carboxylates, so the mutational screen focused on these conserved carboxylates as potential Cu(II) ligands (Fig. 4).

We initially tested single mutations in yeast *SCO1* to evaluate the functionality of the selected carboxylates Asp¹⁵⁰, Asp¹⁸², Asp¹⁸⁶, Asp¹⁹⁷, Asp²³⁴, and Asp²³⁸. Three additional conserved residues that were mutated included Arg¹⁸⁵, Tyr¹⁹⁴, and Gln²³³. Yeast *SCO1* alleles mutated at these codons were assessed in *sco1Δ* cells for glycerol growth, which requires a functional CcO complex. The only mutants

TABLE THREE

Copper binding stoichiometry of human Sco1 and Sco2 purified from the yeast cytoplasm of cells overexpressing human SCO1 or SCO2 in the presence and absence of co-overexpression of human COX17

The Sco proteins were purified by a combination of Ni²⁺-NTA chromatography and gel filtration. Copper was quantified by atomic absorption spectrometry, and protein was quantified by amino acid analysis.

Proteins expressed	Copper/protein (mean)
Human Sco1	0.29 ± 0.07
Human Sco1 + human Cox17	1.02 ± 0.06
Human Sco2	0.88 ± 0.01
Human Sco2 + human Cox17	0.87 ± 0.01

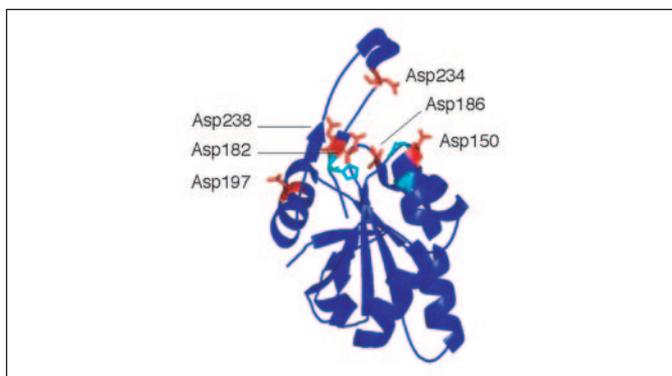


FIGURE 4. Structure of the human Sco1 protein with residues shown that were mutated in the present study. The copper binding Cys and His residues are depicted by sticks colored cyan. The aspartate residues mutated are shown in red.

that failed to propagate on glycerol medium were the D186A and D238A variants (Fig. 5A). These two mutants were the only variants that also failed to restore oxygen consumption to *sco1Δ* cells (Fig. 5B). Yeast harboring a double mutant consisting of D150K and D182K substitutions showed 35% of WT oxygen consumption (data not shown). Sco1 was nonfunctional with Asp¹⁸⁶ converted to Ala but was functional if the residue was a glutamate. The nonfunctional Asp¹⁸⁶ variant was an unstable protein and was therefore not pursued any further. In contrast, the D238A mutant was only partially attenuated in protein level (Fig. 5C). However, since the mutant alleles were expressed from a high copy plasmid, the protein level of the D238A was considerably higher than the level of Sco1 in WT cells. Replacing Asp²³⁸ with a glutamate yielded a functional Sco1 molecule, but oxygen consumption of cells harboring this mutant Sco1 was only about half of WT levels (Fig. 5B).

Several mutant *SCO1* variants were expressed in *E. coli* to determine the effects of the substitutions on copper binding. Asp¹⁵⁰ lies between the two critical Cys residues (Cys¹⁴⁸ and Cys¹⁵²) for Cu(I) binding. A double mutant containing D150K and D182K substitutions was purified and found to exhibit wild type Cu(I) and Cu(II) binding and was not further pursued (data not shown). In contrast, the D238A mutant was isolated with 1.5 mol eq of copper bound, but upon dialysis in 1 mM

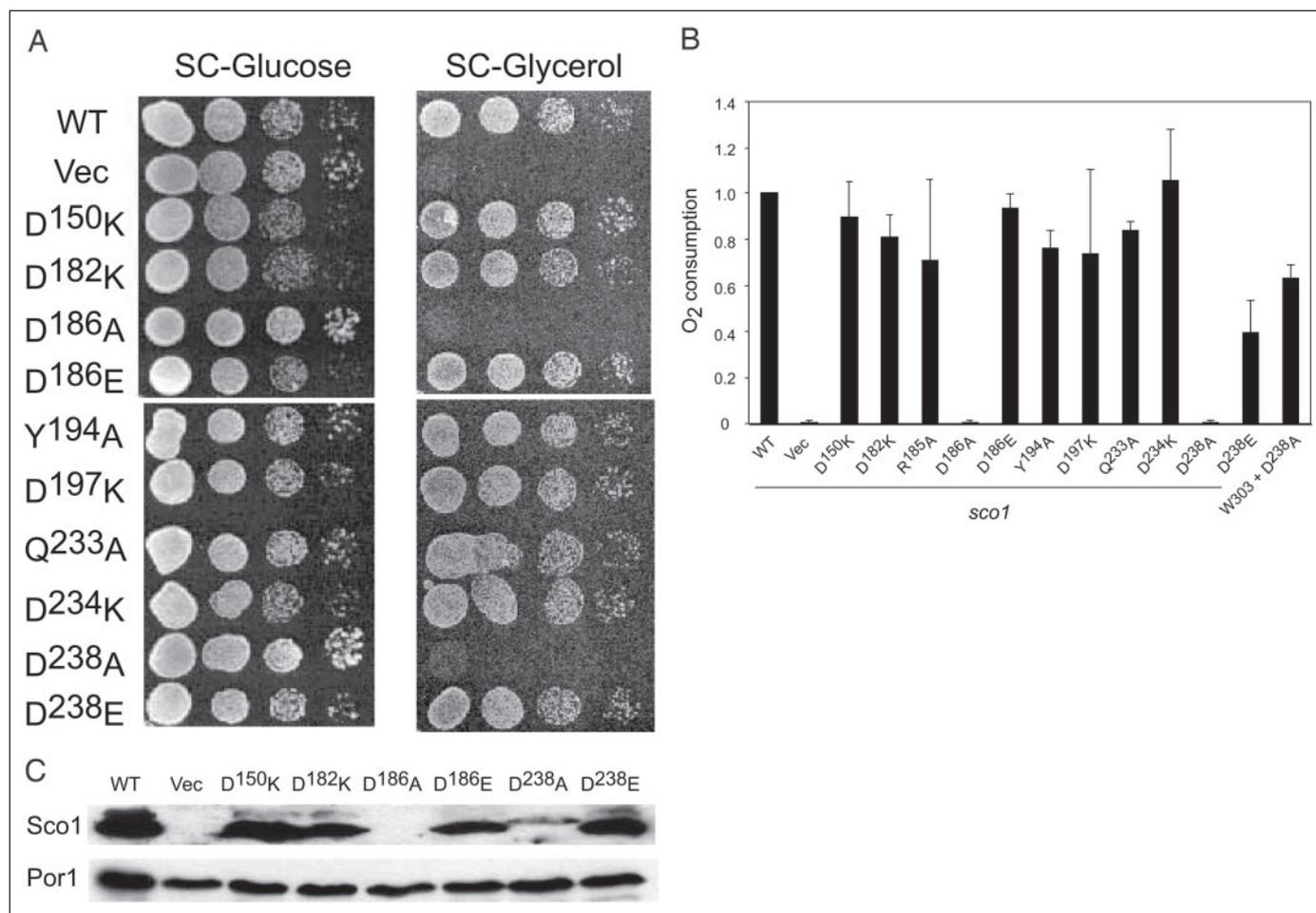


FIGURE 5. **Functional analysis of yeast *SCO1* mutants.** *A*, *sco1* Δ cells transformed with either wild type *SCO1* or mutant alleles were plated on glucose- or glycerol-containing growth medium. Serial dilutions are shown. *B*, oxygen consumption of the transformants mentioned in *A* cultured on glucose-containing medium. *C*, Western analysis of Sco1 and mutant Sco1 molecules in *sco1* Δ cells cultured in glucose-containing medium. Western analysis of Por1 was used as a loading control.

EDTA and DTT the copper content fell to 1 mol eq (TABLE TWO). The D238A mutant showed no visible S-Cu(II) transitions, and no Cu(II) was observed by EPR after dialysis in 1 mM EDTA/DTT (TABLE TWO, Fig. 2*B*) despite the fact that the mutant protein showed a circular dichroism spectrum similar to the WT protein, suggesting that both proteins were similarly folded (data not shown). Replacing Asp²³⁸ with a glutamate instead of an alanine resulted in recovery of the chromophore in the partially active protein. However, the bulk of the Cu(II) in the D238E mutant protein was lost, and the chromophore was largely depleted by EDTA dialysis (TABLE TWO). Overexpression of the D238A mutant Sco1 in WT cells showed a modest dominant negative effect on CcO activity (Fig. 5*B*). Asp²³⁸ is adjacent to His²³⁹, a residue important in Cu(I) binding (9). To determine whether His²³⁹ is important for the stability of the Cu(II) chromophore, a H239A mutant of yeast *SCO1* was purified from *E. coli*. Consistent with our previous results, this mutant showed decreased copper binding (9), and the residual CuSco1 did not show any S-Cu(II) visible transitions. The Cu(II) associated with the yeast H239A protein was also lost upon dialysis.

Mutational Analysis of Human Sco1 and Sco2—Residues in human Sco1 and Sco2 that correspond to the Cu(I) binding Cys and His residues as well as the two carboxylates in yeast Sco1 found to be important for physiological function were mutated to codons encoding Ala. The mutant genes were transduced into immortalized *SCO1* and *SCO2* patient fibroblasts as previously described (21). Overexpression of wild type *SCO1* and *SCO2* rescued the CcO deficiency in the respective

patient fibroblasts (21) (Fig. 6, *A* and *B*). However, alanine substitutions of the two Cu(I)-ligating Cys residues that correspond to yeast Cys¹⁴⁸ and Cys¹⁵² in either human Sco1 or Sco2 abrogated function, and fibroblasts overexpressing these mutant proteins showed no restoration of CcO activity (Fig. 6, *A* and *B*). The lack of functional complementation was not attributable to lack of expression of either mutant protein (data not shown). In fact, overexpression of the mutant Sco2 in either patient background resulted in a dominant negative phenotype in which the residual CcO activity was further attenuated. Likewise, a H239A substitution (using yeast numbering) in either human Sco1 or Sco2 compromised function. The inactivity of mutant Sco1 and Sco2 molecules with substitutions in residues corresponding to yeast Cu(I) binding residues suggests that copper binding is important for the function of both human Sco1 and Sco2.

Asp to Ala substitutions were engineered at Asp codons that correspond to Asp mutants tested in yeast *SCO1* (see above), and all Sco variants were overexpressed in both *SCO1* and *SCO2* patient backgrounds. Immunoblot analysis revealed that all mutants were stably expressed severalfold above control levels (Fig. 6*C*). However, only overexpression of the D186A mutant human Sco1 resulted in partial rescue of the CcO deficiency in *SCO1* patient fibroblasts. None of the remaining Asp to Ala Sco variants were able to restore CcO activity in either patient background (Fig. 6, *A* and *B*), and all were generally compromised to a similar extent with respect to wild type function.

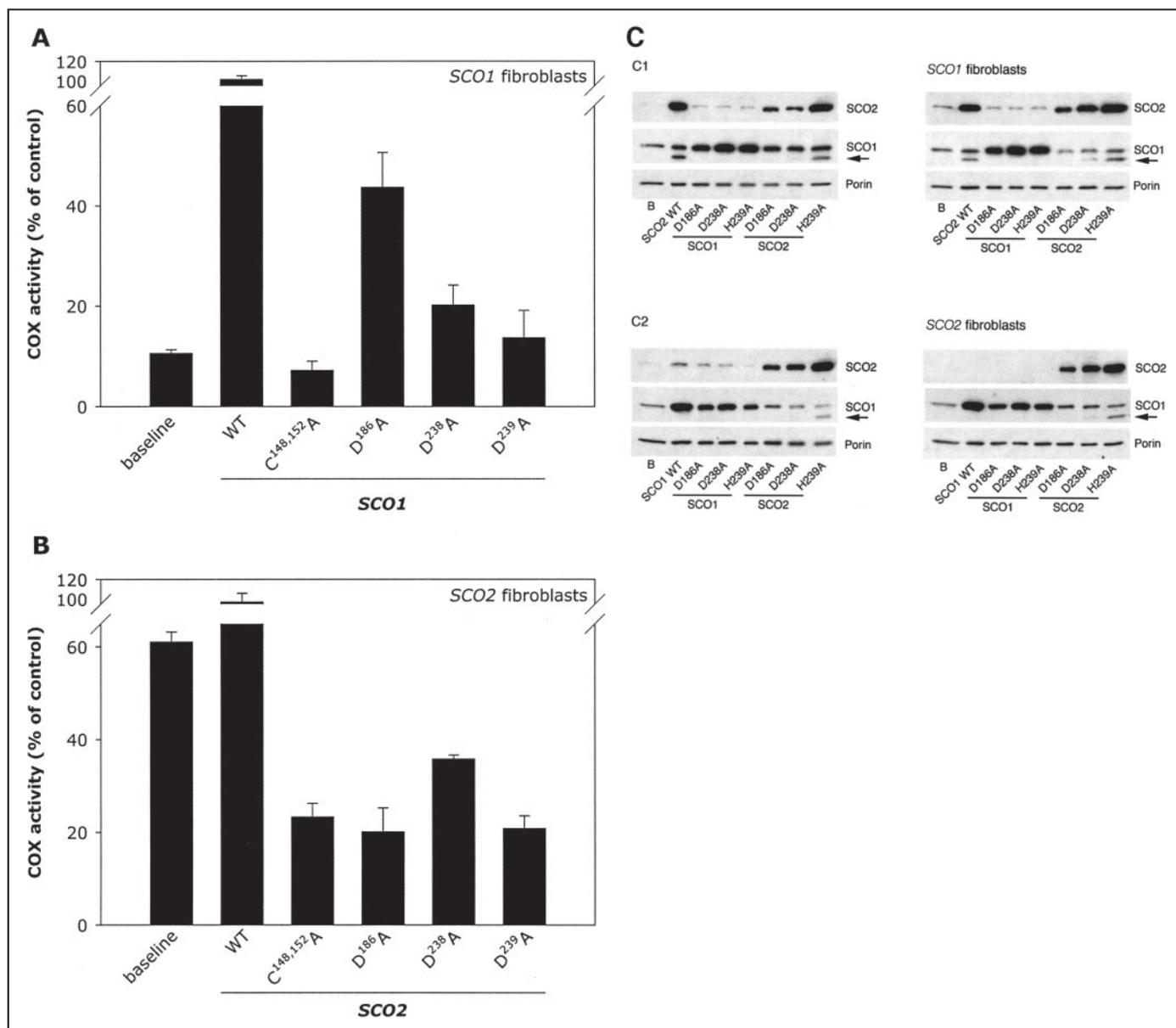


FIGURE 6. Complementation analysis in *SCO1* and *SCO2* patient fibroblasts transduced with a panel of retroviral expression vectors expressing either WT or mutant Sco variants. A and B, CcO activity, expressed as a percentage of control ($n = 3$), in untransduced *SCO1* and *SCO2* fibroblasts (baseline) and in those overexpressing the wild type and variant forms of the Sco proteins. C, immunoblot analysis of expression of Sco1 and Sco2 in fibroblast lines from two controls (C1 and C2) and *SCO1* and *SCO2* patients in untransduced cells (B) and in cells overexpressing the WT and variant forms of the Sco proteins. Porin served as an internal loading control. The arrow in *Sco1* panel reflects residual anti-Sco2 immunoreactivity and is not attributable to a nonspecific band arising from incubation in the Sco1 antisera. For all panels, the numbering of the residues for the human Sco proteins corresponds to that of yeast Sco1.

Previously, human *SCO1* and *SCO2* were shown to exert a dominant negative effect when expressed in the reciprocal patient background. Overexpression of each wild type SCO in the reciprocal patient background resulted in a 2-fold diminution in CcO activity (Fig. 7); however, the various *SCO1* mutants tested above are far less pronounced in their dominant negative effects on CcO activity upon their overexpression in *SCO2* patient fibroblasts. In contrast, the mutant *SCO2* variants resemble WT *SCO2* in their dominant negative effects in *SCO1* fibroblasts. Collectively, these data are consistent with the two Sco proteins interacting with each other and suggest that Sco1, but not Sco2, requires bound copper in order for this interaction to occur.

Two mutant variants of human *SCO1* were expressed in bacteria for characterization of the purified proteins. The D238A mutant of Sco1

was purified from *E. coli* as a copper-binding protein with a copper stoichiometry of 1.1 mol eq (TABLE ONE). Although 0.2 mol eq of Cu(II) was present as detected by EPR, the protein exhibited no visible transitions, and the Cu(II) content was lost upon EDTA dialysis. The Cu(II) EPR signal was similar to that of the yeast D238A mutant and was distinct from the two Cu(II) species seen in the WT protein. The human mutant differed from the yeast mutant in that much of the bound Cu(II) was also removed by EDTA dialysis.

The H239A mutant of human Sco1 was purified from *E. coli* as a copper-binding protein with a copper stoichiometry of 0.7 mol eq. The protein as isolated exhibited weak S-Cu(II) visible transitions when compared with the WT Sco1. However, the chromophore was lost upon EDTA dialysis, and no residual Cu(II) was observed. These data suggest that the inability of these mutants to functionally com-

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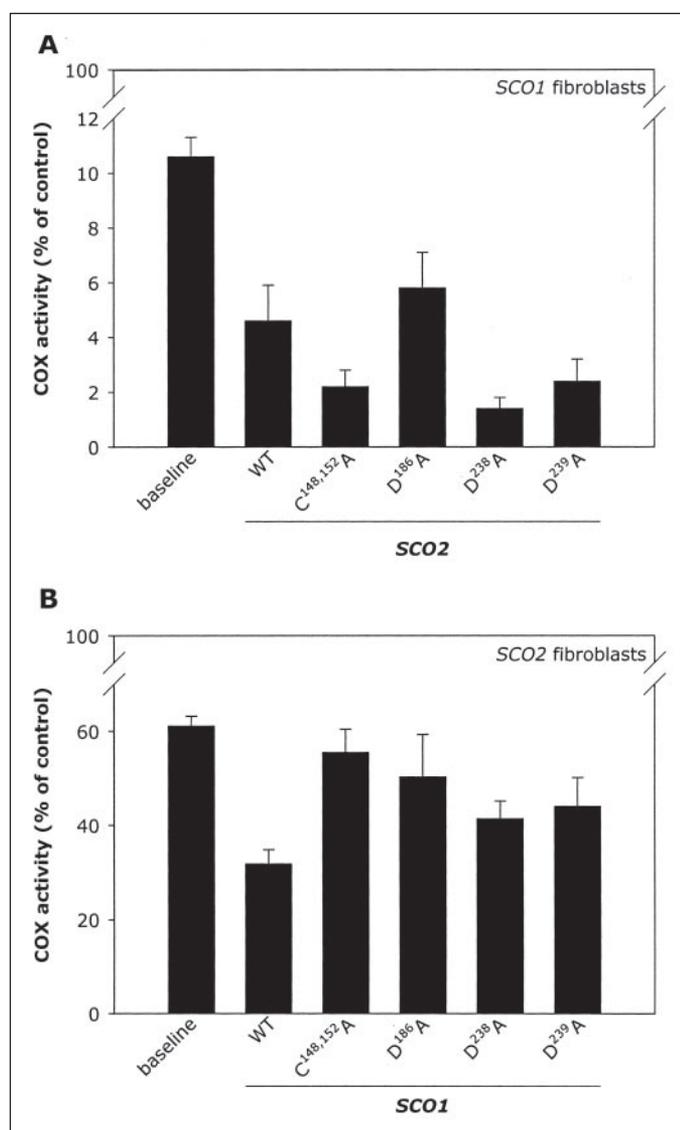


FIGURE 7. Functional analysis of dominant negative effects in *SCO1* and *SCO2* patient fibroblasts transduced with a panel of WT and mutant Sco variants. A and B, CcO activity, expressed as a percentage of control ($n = 3$), in untransduced (baseline) and transduced patient fibroblasts. For both panels, the numbering of the residues for the human Sco proteins corresponds to that of yeast Sco1.

plement the CcO deficiency upon overexpression is a direct consequence of their failure to stably bind Cu(II). It is unlikely that the mutations alter the homodimeric state of Sco1 and Sco2 within the mitochondrial inner membrane, since oligomerization is dependent on the transmembrane domain.

DISCUSSION

This study firmly establishes that both human Sco1 and Sco2 are copper-binding proteins and that the function of each is dependent on copper ion binding. Four lines of evidence support specific copper ion binding to human Sco1 and Sco2. First, expression of the soluble domains of human *SCO1* or *SCO2* in bacteria resulted in the recovery of Cu-proteins in which the bound copper was stable to dialysis against 1 mM EDTA and DTT. Second, expression of the soluble domains of either Sco1 or Sco2 in the yeast cytoplasm, which is a highly chelating environment, yielded copper-binding proteins in both cases. The copper metallation of Sco1 was dependent on the co-expression of human Cox17, whereas copper metallation of Sco2

was independent of Cox17. Third, mutational analysis of human *SCO1* and *SCO2* suggests that both proteins require the cysteinyl and histidyl residues for *in vivo* function. Overexpression of the mutant Sco proteins with the copper-binding Cys and His residues replaced by alanines failed to rescue the CcO deficiency of either *SCO1* or *SCO2* patient fibroblasts. Fourth, the series of mutants analyzed in the present study show that copper binding to Sco1 is important for the candidate interaction with Sco2 based on the dominant negative effect on residual CcO activity.

Previous evidence suggested that human Sco2 bound copper. The soluble domain of human Sco2 purified from *E. coli* was reported to bind a single copper ion (18, 19). A human Sco2 mutant lacking the two conserved cysteinyl residues bound only residual copper (18). However, recombinant Sco1 was reported to be devoid of bound copper (24).

The purported function of Sco proteins is the copper metallation of the Cu_A site of CoxII in CcO assembly. If Sco1/Sco2 proteins donate copper to CoxII, one may question why the Sco proteins stably bind copper. Studies to elucidate the copper binding affinities of other copper metallochaperones have yielded conflicting data, with some studies suggesting very tenacious binding (34, 35). Although a metallochaperone may tenaciously bind copper ions, the transient interactions with target proteins may facilitate copper ion transfer. Thus, the stable association of copper ions with human Sco1 and Sco2 does not disqualify them as metallochaperones.

The Cox17 dependence for copper loading of human Sco1 is consistent with data on yeast Sco1 suggesting that Cox17 is the physiological donor of Cu(I). The normal physiological site for this transfer is within the mitochondrial IMS as Cox17 is a soluble protein within the IMS, and the copper-binding domain of Sco1 projects into the IMS. The surprising result is that copper loading of Sco2 is independent of Cox17 in the yeast cytoplasm. Human Sco2 may be metallated by a cytosolic Cu(I) pool such as CuGSH; however, this observation does not imply that copper loading of Sco2 within the IMS is independent of Cox17. An intriguing parallel is the copper metallation of yeast *versus* human Sod1. Whereas copper metallation of yeast Sod1 is strictly dependent on the Ccs1 metallochaperone, metallation of human Sod1 expressed in yeast can occur in the absence of Ccs1 (36). CuGSH is capable of donating Cu(I) to human Sod1 but not yeast Sod1. Human Sco2 may be similar to human Sod1 in having the ability to be metallated by CuGSH or another copper complex within the yeast cytoplasm.

The present study clearly shows that the Sco proteins can stably bind either Cu(I) or Cu(II). The purified yeast and human Sco proteins exist as monomers, so the single metal binding site can accommodate either valent state of copper. The Cu(I) site in yeast Sco1 was previously shown to be a three-coordinate site with two cysteinyl and one histidyl ligands (9). In the structure of human apo-Sco1, the two cysteines (Cys¹⁶⁹ and Cys¹⁷³, human Sco1 numbering) exist in a protruding loop region, and the His²⁶⁰ is spatially nearby (24). Although backbone changes would be needed if the apo-conformer was to coordinate Cu(I) in planar trigonal geometry, apoproteins frequently exhibit conformations slightly altered from the metallo-conformer (37, 38). The Cu(II) site that yields the S-Cu(II) charge transfer transitions in the visible spectral region is likely to have additional donor atoms. The red Cu(II) protein nitrosocyanin binds Cu(II) in a square pyramidal geometry using two N(His) ligands, and single S(Cys), O(Glu), and solvent ligands (30). The visible transitions arise from S(Cys)-to-Cu charge-transfer transitions dominated by σ rather than π donor interactions (31). The red copper site in nitrosocyanin is distinct from blue Cu(II) sites in having a longer S-Cu bond distance, resulting in less covalence. These features arise from the

increased coordination number of the red Cu(II) site and the associated axial water (31). The similar Cu(II) absorption transitions in nitrosocyanin and Sco1/Sco2 suggest that the chromophoric Cu(II) site in Sco1/Sco2 is also dominated by S σ -Cu transitions and may be five-coordinate with an axial solvent molecule. This prediction is substantiated by the hyperfine splitting constant (A) of the g_z component, which is consistent with a type 2 Cu(II) site with square planar or square pyramidal geometries. EPR simulations of the rhombic Cu(II) species suggest that at least one ligand is a nitrogen, which we propose arises from His²⁶⁰ in human Sco1.

To identify additional potential ligands for the Cu(II) site in Sco proteins, we analyzed a series of mutant proteins with substitutions in conserved residues in the vicinity of the Cu(I) site. Mutation of several conserved carboxylates in this region did not alter *in vivo* function or the presence of the Cu(II) chromophore. In contrast, replacement of Asp²³⁸ in human or yeast Sco1 abrogated the Cu(II) visible transitions, and in yeast Sco1 it attenuated Cu(II), but not Cu(I), binding. Both the mutant yeast and human proteins were nonfunctional, suggesting the importance of this aspartate for normal function. The role of the conserved Asp in eukaryotic Sco proteins could serve either as a fourth ligand for Cu(II), as a constraint on the orientation of the adjacent histidyl ligand, or as a residue that interfaces with a target protein. It seems unlikely that the aspartate is an essential residue for orientation of the adjacent histidine, since Cu(I) binding is normal in the D238A yeast mutant Sco1. The present studies suggest that Asp²³⁸ is an important residue in stabilizing the Cu(II) site in yeast Sco1 and that this is crucial for normal Sco function. The Cu(II) site that is reducible persists in the D238A mutant, suggesting that it is dispensable for function.

The significance of the Cu(II) site in Sco is intriguing. Sco1 binds only a single metal ion, whereas the Cu_A site is a binuclear, mixed valent copper site. Thus, one attractive scenario is that Sco proteins transfer both a Cu(I) and Cu(II) ion to form the mixed valent Cu_A site. Two other functions have been postulated for Sco proteins. First, Sco1 was recently suggested to function as a redox switch, in which oxidation of Cu(I) to Cu(II) induces release of the Cu(II) ion, thereby permitting the two thiolates to participate in a peroxidase reaction (24); however, the present study demonstrates that Cu(II) is stably bound, so a role for Sco proteins as peroxidases is not compelling. Second, the structural resemblance of Sco1 to peroxiredoxins and thioredoxins raised the possibility that Sco proteins may function as thiol:disulfide oxidoreductases (39). Sco proteins were suggested to facilitate disulfide exchange reactions with the thiolates in the Cu_A center of CoxII. However, we show here that the cysteines and the spatially close histidine in eukaryotic Sco proteins are important for copper ion coordination. Although copper coordination does not rule out a secondary role as a thiol:disulfide oxidoreductase, it makes it less likely.

Two distinct Cu(II) centers form in human and yeast Sco proteins. Only one Cu(II) species contributes to the S-Cu(II) charge transfer bands, and the second Cu(II) species is readily reduced by DTT and removed by chelators. It is not clear whether the reducible Cu(II) site uses some of the same ligands as the chromophoric Cu(II) center. Reduction of the Cu(II) center responsible for the visible transitions by dithionite labilizes the ion rather than converting the Cu(I) to stable trigonal coordination analogous to the physiological Cu(I) complex. This property in Cu(II) coordination is more consistent with Sco proteins being Cu(I)/Cu(II) donors rather than Cu(II) metalloenzymes. Although a thiol:disulfide oxidoreductase function is expected to exist within the IMS, since numerous proteins in this

mitochondrial compartment have disulfide bonds, we do not expect the Sco proteins to fulfill this role.

If Cox17 is the physiological copper donor to eukaryotic Sco proteins, the question arises how the Cu(II) site in Sco protein is generated, since only Cu(I) is observed in Cox17 (40). Incubation of Sco1 protein for prolonged periods of time did not change the Cu(I)/Cu(II) ratio, suggesting that neither yeast nor human Sco1 proteins can catalyze the oxidation of bound Cu(I) by themselves. Since Cu(II) was found to be associated with yeast and human Sco proteins expressed in either yeast or bacteria, the prediction is that either a common cellular component facilitates the oxidation or formation of Cu(II) occurs during the folding and formation of the Cu-Sco complex.

The necessity for two Sco proteins in Cu_A site formation in mammals remains unclear. It is unlikely that Sco1 and Sco2 in humans each transfer one unique valence state of copper to CoxII, since we observed no preference of human Sco1 or Sco2 for Cu(II) coordination. One attractive hypothesis is that the two proteins work as a unit to transfer two copper equivalents to form the Cu_A site in a single step. Although the soluble domains of each pure protein existed as monomers in our studies, the full-length proteins are homodimers *in vivo* (13) and are tethered to the IM by a transmembrane helix. The dominant negative effects observed in patient fibroblasts overexpressing the reciprocal Sco protein are consistent with an interaction physiologically. It is conceivable that a Sco1-Sco2 complex may induce one protein to catalyze the oxidation of a Cu(I) to Cu(II), enabling the complex to simultaneously transfer both Cu(I) and Cu(II), or alternatively an additional assembly factor may be required for Cu(I) oxidation. Future studies on the Sco1-Sco2 complex will address this prediction.

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