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The affinity of yeast and bacterial SCO proteins for CU(I) and CU(II). A capture and release strategy for copper transfer

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ABSTRACT

SCO (*S*ynthesis of *C*ytochrome *c* *O*xidase) proteins are present in prokaryotic and eukaryotic cells, and are often required for efficient synthesis of the respiratory enzyme cytochrome *c* oxidase. The *Bacillus subtilis* version of SCO (*i.e.*, *Bs*SCO) has much greater affinity for Cu(II) than it does for Cu(I) (Davidson and Hill, 2009), and this has been contrasted to mitochondrial SCO proteins that are characterized as being specific for Cu(I) (Nittis, George and Winge, 2001). This differential affinity has been proposed to reflect the different physiological environments in which these two members of the SCO protein family reside. In this study the affinity of mitochondrial SCO1 from yeast is compared directly to that of *Bs*SCO *in vitro*. We find that the yeast SCO1 protein has similar preference for Cu(II) over Cu(I), as does *Bs*SCO. We propose a mechanism for SCO function which would involve high-affinity binding to capture Cu(II), and relatively weak binding of Cu(I) to facilitate copper transfer.

1. Introduction

A member of the heme-copper oxidase enzyme family is the common terminal enzyme of aerobic respiratory chains [1]. Members of the cytochrome *c* oxidase class are a sub-family of the heme-copper oxidases and are found in the inner mitochondrial membrane of eukaryotes and the plasma membrane of some aerobic prokaryotes [2]. Copper plays a prominent role in the activity of the cytochrome *c* oxidase complex. Copper functions as a redox shuttle in the dinuclear Cu_A center to receive electrons from the reducing substrate ferrocytochrome *c* and pass them on to the cytochrome *a* center. Electrons move from cytochrome *a* to the oxygen binding site that includes a second heme A group (*i.e.*, cytochrome a_3) and another copper ion (*i.e.*, Cu_B) arranged in a binuclear center, cytochrome a_3-Cu_B, where they cooperate to reduce molecular oxygen to water [3,4]. Cytochrome *a* and cytochrome a_3-Cu_B are contained in the membrane embedded subunit I. The Cu_A center is in the soluble domain of subunit II that is anchored to the membrane by two transmembrane α-helical segments. There is one member of the cytochrome *c* oxidase family expressed in the gram-positive bacterium *Bacillus subtilis*, and it is of the cytochrome caa_3 sub-class. Members of the cytochrome caa_3 sub-class have a cytochrome *c* domain fused into the canonical subunit II sequence at the C-terminal end. The cytochrome *c* domain resides adjacent to the Cu_A domain in the cytochrome caa_3 complex and is a highly efficient electron donor to the Cu_A site [5].

The assembly of cytochrome *c* oxidase is a complex process as it involves the coordination of a number of protein subunits and prosthetic groups that must be brought together to form an integral membrane structure [6]. The delivery of copper ions to this complex has received particular attention and the emerging view is that such oxygen-reactive, transition metal ions as copper follow a specified pathway involving a number of bound intermediates on the way to their sites of action [7]. The synthesis of cytochrome oxidase, or SCO, protein was originally recognized in yeast as an assembly factor specific for cytochrome *c* oxidase [8]. And further genetic work in yeast supports a role for the SCO protein in copper transfer to cytochrome *c* oxidase [9]. Studies using the aerobic bacterium *B. subtilis* showed that the bacterial version of SCO is required for Cu_A assembly, but not for assembly of the Cu_B center [10]. Structural studies of the SCO family of proteins recognize a shared thioredoxin core [11–13] with a pair of conserved cysteine residues in the sequence –**C**xxx**C**–, and these features led to the early suggestion that SCO proteins may act in a disulfide exchange capacity to keep the cysteine residues of Cu_A in a reduced state

Abbreviations: SCO, synthesis of cytochrome oxidase protein; BsSCO, SCO from *Bacillus subtilis*; ScSCO1, SCO1 from *Saccharomyces cerevisiae*; BCS, bathcuproine disulfonate; BCA, bicinchoninic acid; DTDP, 4,4′ dithiodipyridine

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appropriate for copper binding [14]. There is also a conserved histidine about one hundred amino acids toward the C-terminus in SCO sequences. The conserved cysteine pair and the conserved histidine are contained in two flexible loops that sit outside the thioredoxin core. The two loops come close enough in the folded structure to allow the three residues to act as inner sphere ligands in complexes with copper [15]. SCO has been proposed therefore to play a direct role in delivery of copper ions to the Cu_A site. SCO has been shown to bind both Cu(I) and Cu(II) ions *in vitro*. One current view holds that SCO has high affinity for Cu(I), and that Cu (I) is the most likely species to be involved in copper delivery (e.g., [16] and [17]). We have demonstrated that SCO from the aerobic bacterium *B. subtilis* (*i.e.*, *Bs*SCO) binds Cu(II) with a much higher affinity than Cu(I) [18]. Others have posited that both Cu(I) and Cu (II) complexes of SCO may be required for function [19].

In *B. subtilis* the metal binding site of *Bs*SCO is located on the outer surface of the plasma membrane and so, like the Cu_A domain of cytochrome *c* oxidase, is in equilibrium with the external medium. In contrast mitochondrial SCO proteins, along with the heme-copper oxidase complex, are anchored to the inner mitochondrial membrane. The topology is such that the metal binding site of SCO and Cu_A are exposed to the intermembrane space of the mitochondrion, and so in redox equilibrium with the cytosol. Thus, *Bs*SCO and *Sc*SCO1 reside in two different 'redox' environments. *Bs*SCO is in equilibrium with the oxidizing environment outside the cell, whereas mitochondrial SCO1 is in equilibrium with the reducing environment of the cytosol. Perhaps mitochondrial SCO proteins have evolved specificity to bind Cu(I), and aerobic bacterial SCO such as *Bs*SCO evolved to bind Cu(II). Such a scenario could explain the discrepancy reported in the literature for the metal ion preference of these two versions of SCO. In this report we have measured the metal binding affinity of yeast mitochondrial *Sc*SCO1 for Cu(II) and Cu(I) in both direct and competitive assays to compare its metal affinity to that of a bacterial form of SCO (*i.e.*, *Bs*SCO).

In direct binding assays we find that *Sc*SCO1 prefers Cu(II) over Cu(I) by a large margin similar to the difference reported for *Bs*SCO, and more than a factor of 10^6. Since the direct binding assays can be problematic we have explored these affinities in competition assays using both Cu(II) and Cu(I) specific ligands. These data are consistent with the direct binding assays in supporting the much higher affinity of *Sc*SCO1 for Cu(II). We conclude, therefore, that the mitochondrial and bacterial proteins exhibit a similar preference for Cu(II) over Cu(I), and that the proposition that a specific binding preference is a reflection of a different redox environment is not warranted.

*Sc*SCO1 also has an extra pair of cysteine residues that are in addition to the pair of conserved cysteine residues implicated in copper binding [20]. A BLAST search of the protein data base for the presence of this extra pair of cysteine residues finds numerous SCO proteins from the fungal kingdom with this feature. Since SCO proteins have been proposed to play a role in redox signaling [21] we pursued a further characterization of the extra cysteine pair in *Sc*SCO1 in comparison to the metal-binding thiol pair conserved in other SCO protein family members. We find that the extra cysteine pair does not have any direct contribution to high affinity copper binding and is reactive with thiol reagents. These features may make this cysteine pair more suitable as a sensor of the overall redox environment.

2. Materials and methods

Recombinant *Bs*SCO was expressed and purified as described previously [22]. Briefly, the soluble domain of *Bs*SCO is produced as a fusion protein with glutathione S-transferase and expressed in *Escherichia coli*. The fusion protein is isolated by passage of the soluble cell extract over a glutathione 4B Sepharose column. The fusion is cleaved on the column by addition of thrombin containing buffer followed by incubation at room temperature for 16 h. Thrombin is removed from the eluted *Bs*SCO by passage over benzamidine Sepharose 4 Fast Flow. The gene for *Sc*SCO1 was purchased from PlasmID (Harvard Medical School) in the shuttle vector pBY011. This was transformed into *E. coli* (DH5α) and then transferred into PGEX-4T3 (GE Healthcare). The soluble domain of *Sc*SCO1 is cloned in frame with glutathione S-transferase for expression in *E. coli*. The fusion protein was purified and cleavage tests run to determine the optimal concentration of thrombin and the time of incubation. The results showed complete cleavage of the fusion protein is achieved by incubation with 10 units of thrombin at 4 °C for two hours. This procedure yielded about 5 mg of purified *Sc*SCO1 per liter of *E. coli* culture. The purity of both *Bs*SCO and *Sc*SCO1 were assessed by SDS-polyacrylamide gel electrophoresis.

The concentrations of the purified proteins were determined by measuring their UV absorbance spectra. The extinction coefficient at 280 nm used for *Bs*SCO is $19.9\ mM^{-1}\ cm^{-1}$ and $29.0\ mM^{-1}\ cm^{-1}$ for *Sc*SCO1. These extinction coefficient were calculated from the primary sequence of each protein [23]. *Bs*SCO has two cysteine residues within its primary sequence, whereas *Sc*SCO1 has four cysteine residues. The thiol reactivity and content of each protein was determined routinely by assessing the time response and extent of reaction with the thiol reagent 4,4′ dithiodipyridine [24]. The amount of 4-thiopyridone product was determined spectrophotometrically using the extinction coefficient at 324 nm of $18.8\ mM^{-1}\ cm^{-1}$. Bathcuproine disulfonate (BCS) was used to measure the amount of Cu(I) using the extinction coefficient at 484 nm of $12.2\ mM^{-1}\ cm^{-1}$[25].

Absorbance spectra were collected on an HP8452 diode array spectrometer run under the Globalworks program (OLIS Inc.), or a Cary 50 spectrophotometer. Circular dichroism spectra were collected on an Applied Photophysics Chirascan spectrometer equipped with a temperature controlled cuvette holder. Temperature dependent CD spectra were measured using a 1 mm pathlength cuvette so as to immerse the temperature probe directly in the sample. A temperature ramp of 1 °C per minute was used. Thermal melts were analyzed by fitting the average CD intensity versus temperature to a sigmoidal curve to obtain an inflection point that is assigned as the melting temperature (T_M). Fluorescence spectra were measured on a Cary Eclipse spectrometer equipped with a temperature controlled, stirred cell holder.

Direct Cu(II) binding assays were performed by adding known amounts of copper to reduced, apo-SCO. Stock copper solutions were made in Chelex-treated, Milli-Q water and the concentration determined colorimetrically by forming the $(BCS)_2$-Cu(I) complex in the presence of excess reducing agent and BCS. Solutions of Cu (I) were prepared by addition of CuCl to a solution of Chelex-treated, Milli-Q water containing 10 mM HCl with 1 M NaCl [26]. The solution was prepared in a Thunberg flask with CuCl placed in the side arm. The contents of the side arm were tipped into the HCl, NaCl solution following exhaustive flushing with argon. Cu(I) titrations with SCO were performed by transferring argon-equilibrated aliquots of CuCl with a gas-tight syringe to an argon-equilibrated solution of SCO.

3. Results

3.1. ScSCO1 and BsSCO structures

The yeast SCO1 protein (*i.e.*, *Sc*SCO1) is an integral membrane protein that is anchored to the inner mitochondrial membrane by

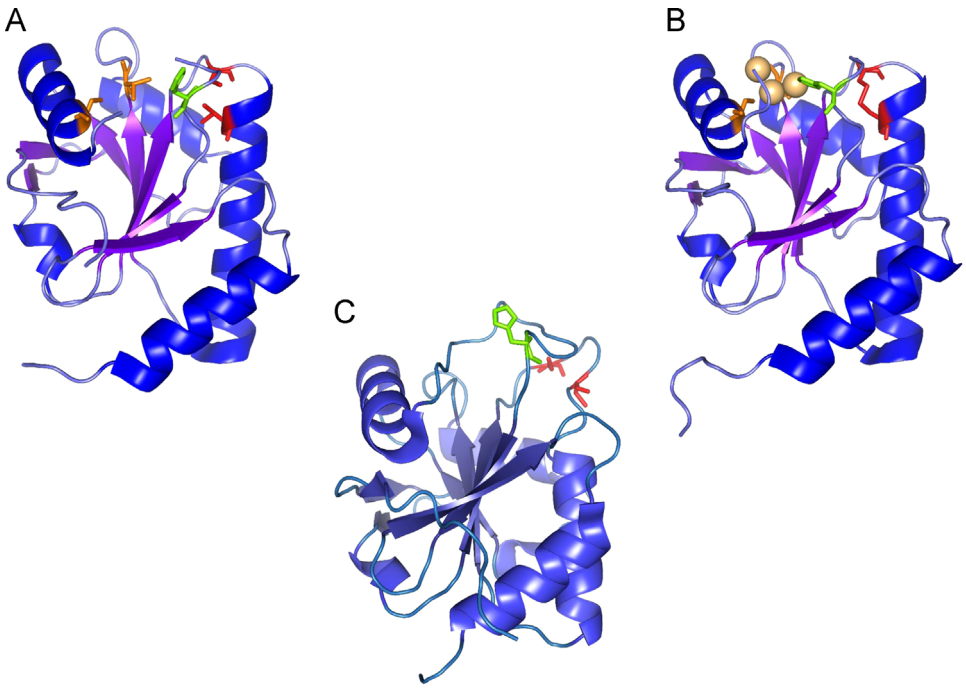


Fig. 1. Structures of *Sc*SCO1 compared with *Bs*SCO. The structures are shown as ribbon diagrams with residues of interest depicted in stick mode. The conserved cysteines are shown in red and the conserved histidine in green. The extra cysteines in *Sc*SCO1 are shown in orange. The copper ions in Cu-bound *Sc*SCO1 are presented as spheres. (a) apo-*Sc*SCO1 (PDB ID: 2B7K) (b) *Sc*SCO1 with bound copper (PDB ID: 2B7J) (c) apo-*Bs*SCO (PDB ID: 1XZO).

virtue of a single transmembrane α-helix predicted to form from residue 76 to residue 92. A mitochondrial targeting sequence precedes the membrane helical segment at the N-terminus. The mature protein sequence begins at glutamine residue 41 [27]. The majority of the *Sc*SCO1 structure consists of a soluble domain of approximately 200 amino acids (*i.e.*, residue 94–294) that follows the integral membrane portion. The soluble domain is located on the outside of the inner mitochondrial membrane (*i.e.*, in the intermembrane space), and contains the putative copper binding site. The structure of the soluble domain of *Sc*SCO1 (residues 111–279) has been determined in both apo- and copper-bound forms [13]. The structures of apo-*Sc*SCO1 and SCO from the gram-positive bacterium *B. subtilis* (*i.e.*, *Bs*SCO) are compared in Fig. 1. The three conserved residues (two cysteines and a histidine, or CCH-motif) proposed to play a major role in metal binding are highlighted in the *Sc*SCO1 structure (see Fig. 1a). The conserved cysteine pair in *Sc*SCO1 are located in a loop (*i.e.*, the cysteine loop) just adjacent to a major α-helix. In *Sc*SCO1 this loop is much shorter than in the corresponding *Bs*SCO structure (Fig. 1c), and the second conserved cysteine (*i.e.*, C152) actually sits within the adjacent α-helix in *Sc*SCO1.

The conserved cysteines that feature in members of the SCO family of proteins are separated by three amino acids (*i.e.*, –CXXXC–), and are followed by a histidine about 85 amino acids toward the C-terminus. These three residues (*i.e.*, the CCH motif) are required for binding copper and proposed to act as inner sphere ligands in the copper complex of yeast [16], human [28] and *B. subtilis* SCO proteins [15, 29]. There are an additional two cysteine residues in *Sc*SCO1 that are not found in the *Bacillus* or human homologs. A BLAST-p search of NCBI non-redundant protein sequences using the amino acid sequence bordered by the two non-conserved cysteine residues in *Sc*SCO1 (*i.e.*, Cys181 to Cys216) shows that the two cysteines are conserved in a number of lower eukaryotes from the acetomyces family of the fungi kingdom. We are interested in the role of this extra pair of cysteines (*vide infra*). The extra, non-conserved pair of cysteines in *Sc*SCO1 are shown as ligands to copper in the reported structure of the copper complex of *Sc*SCO1 (see Fig. 1b) [13]. This structure includes three forms of copper bound at slightly different positions that are proposed to represent distinct sub-populations of *Sc*SCO1 molecules in the crystal. The conserved cysteine pair are present in the disulfide, oxidized state in the copper complex of *Sc*SCO1.

We have purified the soluble domain of *Sc*SCO1 as expressed in *E. coli* (see Section 2). Circular dichroism studies in the UV region show that recombinant *Sc*SCO1 as purified adopts a stable, folded structure in solution (Fig. 2A). Analysis of the UV-CD spectrum of *Sc*SCO1 indicates a protein fold composed of a mixture of α-helix (~27%) and β-sheet (~20%) contributions, and a large component of random coil (~38%). This secondary structural composition for *Sc*SCO1 in solution is consistent with the high resolution structure observed in the crystal form of apo-*Sc*SCO1 (Fig. 1a) and is reminiscent of the UV-CD spectrum observed for *Bs*SCO [12]. The thermal melting response of *Sc*SCO1 as isolated is shown in Fig. 2B and reflects a stable, cooperatively-folded structure. The protein initially resists unfolding as the temperature increases above 25 °C, and then unfolds in a cooperative manner with an inflection point at about 52 °C consistent with melting of the protein's folded structure.

3.2. Redox reactivity of ScSCO1

*Sc*SCO1 possesses an additional two cysteines relative to many other eukaryotic (*e.g.*, human SCO1) and bacterial (*e.g.*, *B. subtilis* SCO) SCO homologs. As isolated *Sc*SCO1 takes a number of oxidation levels and we observe cysteine thiol counts ranging from close to zero (*i.e.*, fully oxidized) up to four equivalents per mole of *Sc*SCO1 (*i.e.*, fully reduced). Time courses for the reaction of two distinct oxidation levels of *Sc*SCO1 using the thiol-reagent DTDP are illustrated in Fig. 3. The extent of thiopyridone production in the reaction with DTDP is measured by its absorbance at 324 nm and indicates the amount of the thiol form of cysteine in the SCO protein sample, while the kinetic profile reports the reactivity of individual cysteine thiols. The time profile in trace (i) of Fig. 3 is from a *Sc*SCO1 sample that is nearly fully reduced. The final SH

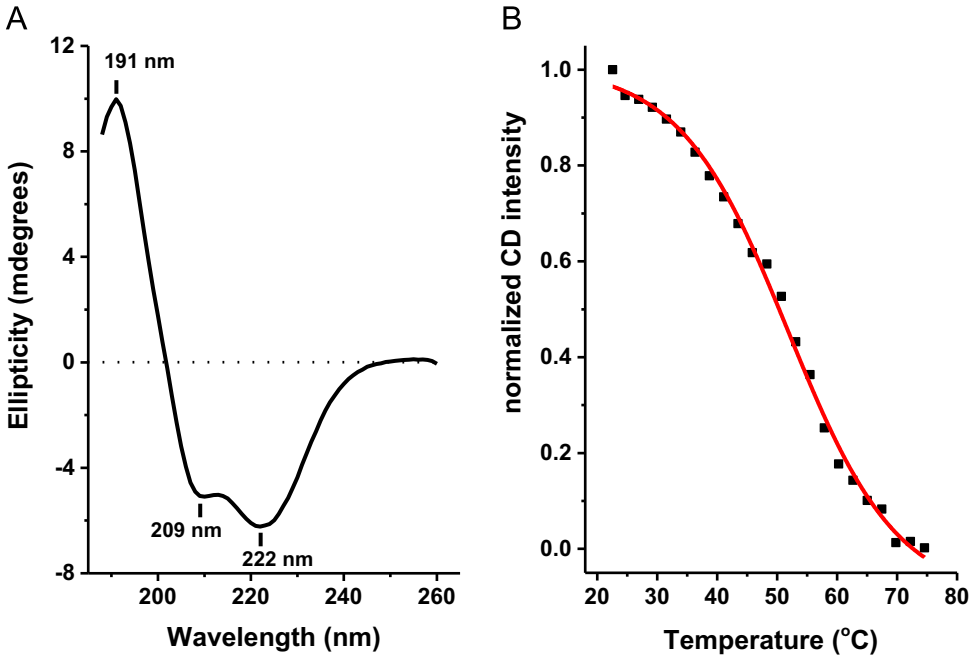


Fig. 2. Circular dichroism analysis of the structural integrity of recombinant *Sc*SCO1. (A) Far UV-CD spectrum of *Sc*SCO1. The protein is dissolved in 5 mM sodium phosphate buffer pH 7.0 at a concentration of 0.08 mg/ml with a thiol content of 0.2 per *Sc*SCO1 molecule. The pathlength of the cuvette is 1 mm, the temperature 20 °C and the bandwidth 2 nm. Data are collected at 1 nm intervals with an integration time of 0.25 s per point. (B) Thermal profile of *Sc*SCO1 stability. CD spectra were collected on the same sample using the same instrumental conditions detailed above. A temperature ramp was applied from 20 to 80 °C at a rate of 1 °C per minute. The experimental temperature was measured with a probe submersed in the sample. Spectra were recorded at 2 °C intervals and the average CD intensity, excluding wavelengths below 190 nm is plotted versus sample temperature. The fitted line is a sigmodal function used to determine the inflection point of the temperature response.

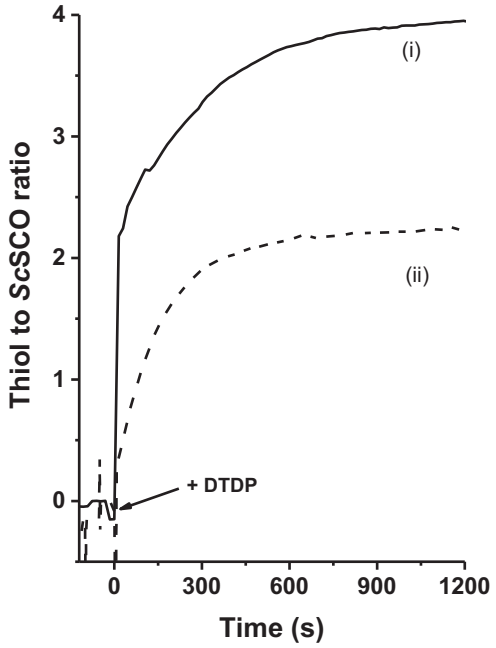


Fig. 3. Time courses of thiol reactivity for distinct redox levels of *Sc*SCO1. In trace (i) a sample of *Sc*SCO1 (final concentration = 12.0 μM) reacts with 100 μM DTDP. The reaction is initiated at time zero by mixing the contents of two sides of a split cuvette. The magnitude of the reaction is scaled to reflect the molar ratio of thiopyridone produced ($\epsilon_{324\ nm}$ mM^{-1} cm^{-1} = 18.8) to *Sc*SCO1 present. Trace (ii) is for a different sample of *Sc*SCO1 (final concentration = 9.96 μM). The reaction with 100 μM DTDP was initiated by tipping the split cuvette. The buffer is 50 mM sodium phosphate pH 7.0 and the temperature 20 °C in each case.

count of the sample in trace (i) is 3.96 mol of thiol per mole of *Sc*SCO1. This value approaches the limit of four consistent with fully reduced *Sc*SCO1. The sample illustrated in trace (ii) of Fig. 3 is partially reduced, and has a thiol count of 2.24 mol per mole of *Sc*SCO1 (Fig. 3). There is also a clear kinetic distinction between fast and slow reacting components that is evident in the nearly fully reduced sample in trace (i). The fast component of the reaction is complete in the four seconds required for mixing corresponding to a half-time of less than 2 s (*i.e.*, $k_{ob} \geq 0.35\ s^{-1}$). The extent of the fast phase consumes about one-half of the total reaction, and in the example shown in trace (i) corresponds to 2.13 thiols per *Sc*SCO1. The second phase proceeds more than 100-fold slower with an observed first order rate of $3.1 \times 10^{-3}\ s^{-1}$. In the partially reduced sample illustrated in trace (ii) the fast phase is largely absent (0.35 thiols per *Sc*SCO1) and the extent of the slow phase corresponds to two thiols per *Sc*SCO1. The slow phase proceeds with an observed first order rate of $5.8 \times 10^{-3}\ s^{-1}$. It is apparent that the four thiols fall into two groups of two thiols each based on their reactivity with DTDP. These two thiol pairs are also distinguished by their reactivity with copper (see below).

3.3. The reaction of ScSCO1 with CU(II)

*Sc*SCO1 reacts with Cu(II) to form a complex with UV-visible absorbance peaks at 364 nm, 470 nm and 600 nm (Fig. 4A). These features are similar to those reported previously for the absorbance spectrum of *Sc*SCO1 with Cu(II) [28], and similar to the optical absorption spectrum observed for *Bs*SCO with Cu(II) [29]. The formation of the copper complex coincides with a decline in the reactivity of *Sc*SCO1 with the thiol reagent DTDP (Fig. 4B). The fast phase of the thiol reaction observed with DTDP is almost completely abolished when *Sc*SCO1 forms the complex with Cu(II) (compare traces (i) and (ii) in Fig. 4B). This is consistent with the proposal that the two faster reacting cysteines in the DTDP-assay of fully reduced, apo-*Sc*SCO1 are the two conserved cysteines that are involved directly in copper ligation. In the copper (II) complex of *Sc*SCO1 the two ligating cysteines must be in slow exchange so as not to react with excess DTDP, and this is also consistent with their role as inner sphere ligands to copper. The rate of reaction of

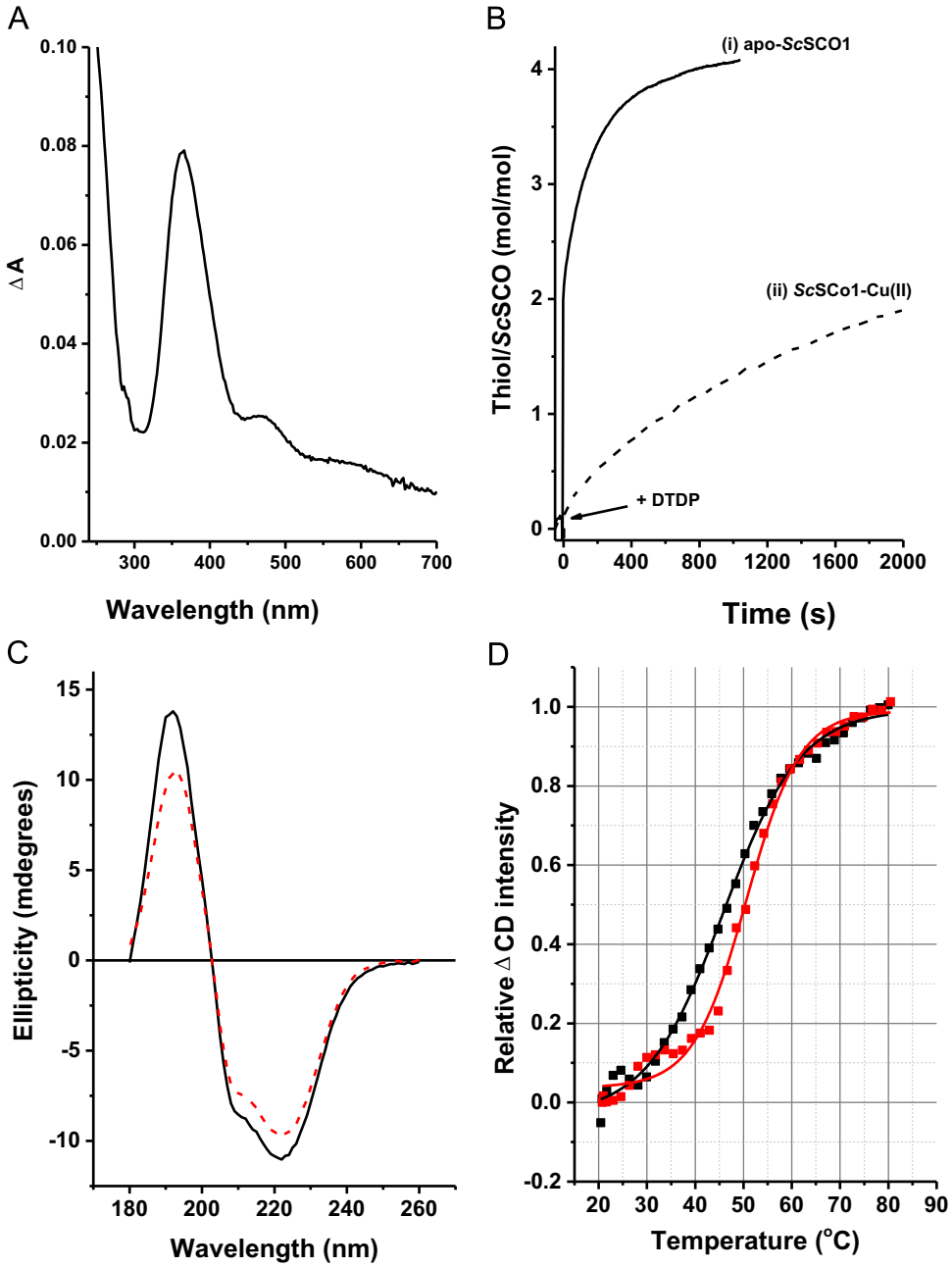


Fig. 4. Properties of the *Sc*SCO1-Cu(II) complex. (A) UV–visible absorbance spectrum of *Sc*SCO1-Cu(II). The concentration of *Sc*Sco1 is 18 μM and the thiol to protein ratio for this sample is 3.44. The sample is dissolved in 50 mM sodium phosphate pH 7.0 and the temperature is 20 °C. (B) The thiol reactivity changes upon copper (II) binding to *Sc*SCO1. The amount of reactive thiol was determined by the addition of excess DTDP and measuring the production of thiopyridone at 324 nm. The thiol to protein ratio was calculated using an extinction coefficient of 18.8 mM^{-1} cm^{-1} at 324 nm and 29 mM^{-1} cm^{-1} for *Sc*SCO1 at 278 nm. (C) Changes in the UV-CD spectrum of *Sc*SCo1 upon Cu(II) binding. The concentration of ScSCO1 is 1.38 mg/ml (*i.e.,* 60 μM) and the spectra are recorded in a 0.1 mm pathlength cuvette. The apo-protein (solid line) has a thiol to protein ratio of 3.6. The spectrum of Cu(II)-bound *Sc*SCO1 (dashed line) is obtained by titration with one equivalent of $CuCl_2$. (D) Thermal stabilities of reduced apo-*Sc*SCO1 and *Sc*SCO1-Cu(II). The two samples were obtained by diluting the samples in panel (C) 1:10 in 50 mM sodium phosphate buffer pH 7.0. Spectra were recorded from 260–190 nm in a 1 mm pathlength cell. The temperature was changed at a rate of 1 °C per min and spectra were collected at 2 °C intervals. The lines fit to the data are sigmoidal functions used to determine the inflection point of the response profile. Data for apo-*Sc*SCO1 are in black and for the Cu(II) complex of *Sc*SCO1 in red.

the second pair of slow-reacting cysteines decreases by a factor of five compared to reduced, apo-*Sc*SCO1 indicating that copper (II) binding to the conserved cysteine pair has also made the other two cysteine thiols less reactive.

Addition of one equivalent of copper (II) results in a small, but demonstrable shift in the UV-CD spectrum. The positive peak at 192 nm seen with reduced, apo-*Sc*Sco1 diminishes in intensity and shifts to 193 nm in the copper (II) complex. The zero-crossing point at 203 nm is unchanged in the copper complex as are the peak and shoulder positions at 222 nm and 209 nm. However, the intensity of the negative going peaks decline by about 15% (Fig. 4C). These changes presumably arise from a change in the secondary structure of the protein within the copper complex. Analysis of the UV-CD spectrum indicates a decline in α-helical content (20.5% in apo- *vs.* 17.3% in copper-bound) together with an increase in β-sheet content (27.7% in apo- *vs.* 33% in copper bound) on formation of the copper (II) complex. In addition variable temperature CD in the UV region reveals a mid-point temperature for thermal induced unfolding of 45.8 °C for reduced, apo-*Sc*SCO1 that increases to 50.6 °C in the copper (II) complex of *Sc*SCO1

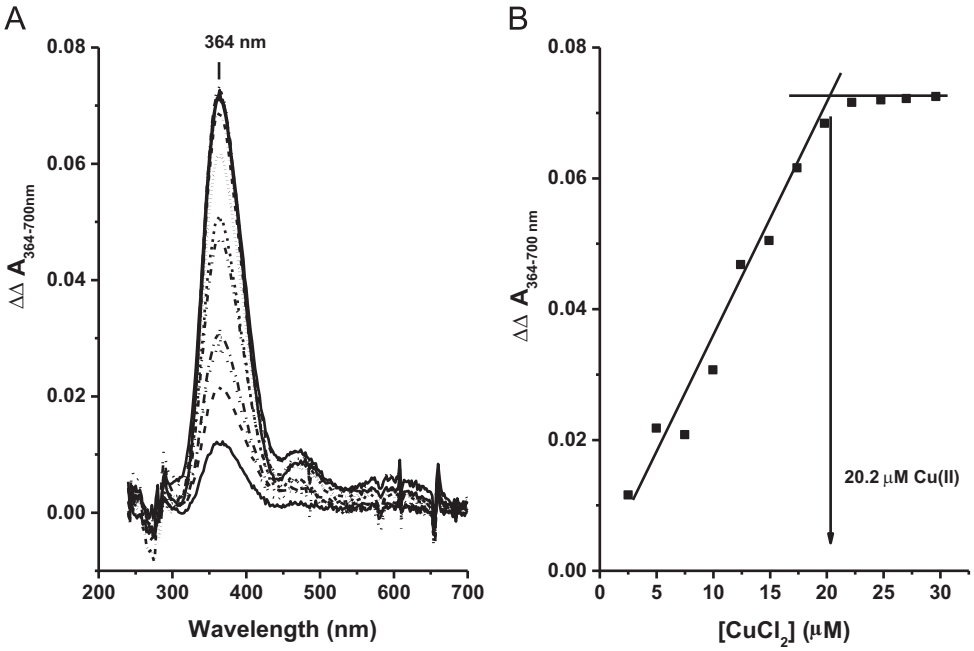


Fig. 5. Titration of *Sc*SCO1 with Cu(II). (A) Difference spectra generated by addition of $CuCl_2$ to apo-*Sc*SCO1. The concentration of apo-protein is 21.6 μM and spectra are shown at copper concentrations of 2.5 μM (——), 4.99 μM (– – –), 7.48 μM (● ● ●), 9.96 μM (– ● – ● – ●), 12.4 μM (– ● ● – ● ●), 14.9 μM (- - - -), 17.4 μM (●●●●), 19.8 μM (-●-●-●), 22.2 μM (——), 24.8 μM (– – –), 27 μM (● ● ●) and 29.6 μM (– ● – ● – ●). The buffer is 50 mM sodium phosphate pH 7.0 and the temperature 25 °C. (B) Absorbance intensity at 364 nm plotted versus $CuCl_2$ concentration. The saturation point was obtained at a copper concentration of 20.2 μM giving a Cu(II):*Sc*SCO1 ratio of 0.94 at saturation.

(Fig. 4D). In contrast, fully oxidized *Sc*SCO1 unfolds with a melting temperature of 53 °C (see Fig. 2B).

The affinity of Cu(II) binding with *Sc*SCO1 can be assessed by measuring the increase in intensity of the charge transfer transition in the UV as a function of the copper concentration. Fig. 5A shows a set of difference spectra using the spectrum of apo-*Sc*SCO1 as reference. The intensity of the spectrum increases in proportion to the copper concentration up to a limit of close to one copper ion per *Sc*SCO1 (Fig. 5B). The same distinct pattern can be observed by fluorescence spectroscopy, where the intrinsic fluorescence intensity from *Sc*SCO1 decreases in direct proportion to the copper concentration and abruptly stops upon reaching a limiting value. This type of binding pattern conforms to a tight binding regime where the equilibrium dissociation constant (*i.e., K*$_D$) is much less than the concentration of reactants. In this circumstance the equilibrium constant cannot be determined with high accuracy, but an upper limit can be assigned. We observe this same tight binding pattern with as little as 5 μM *Sc*SCO1 and conclude therefore that the K_D for Cu(II) binding by *Sc*SCO1 must be less than 0.5 μM. In such a tight binding reaction as observed here the stoichiometry of the complex is well determined. The average Cu(II):*Sc*SCO1 stoichiometry is measured to be 0.99 ± 0.083, and the difference extinction coefficient for *Sc*SCO1-Cu(II) is 4.03 ± 0.68 mM^{-1} cm^{-1} at 364 nm versus the apo-protein as reference.

3.4. The reaction of ScSCO1 with CU(I)

Binding assays of Cu(I) with SCO proteins are challenging because of the inherent oxygen sensitivity of the Cu(I) ion, and there is not an easily measurable UV–vis or EPR absorption associated with the SCO-Cu(I) complex. We have observed that that binding of Cu(I) to *Bs*SCO is accompanied by quenching of the intrinsic tryptophan fluorescence [18]. We report here a similar response of the intrinsic fluorescence of *Sc*SCO1 binding with Cu(I) (Fig. 6A). The fluorescence spectrum of the apo-*Sc*SCO1 is shown in trace (i) of Fig. 6A with an emission maximum at 326 nm. In the presence of saturating Cu(I) the intensity diminishes by about 40% and shifts to 333 nm. This change in fluorescence is measured as a function of Cu(I) concentration to assess the affinity of Cu(I) binding (Fig. 6B). The binding curve is well-fit by a rectangular hyperbola with an average equilibrium dissociation constant of 8.43 ± 2.0 μM from three independent titrations. We assess the absorbance spectrum throughout the titration to ensure that the Cu(II) complex is not generated (*i.e.,* ΔA at 364 nm). There is no evidence of the Cu(II)-*Sc*SCO1 complex in the data reported here.

3.5. Competitive copper binding assays

Direct binding assays such as those presented above have been criticized as an approach for measuring protein-metal complex stabilities (Xiao and Weld, 2010). Alternatively, competition assays offer an approach that allows for measuring protein/metal affinity over a wider range than direct approaches and are particularly relevant in the case of high affinity complexes. In other systems, for example measuring the high affinity for Cu(I) (*e.g.*, CopA [30], Cu, Zn-SOD [31]) the classical, tight-binding, colorimetric ligands for Cu(I) (*i.e.*, bathcuproine disulfonate or bicinchoninic acid) have been used as competitors. The absorbance signal for formation of the competing ligand/metal complex is used to monitor the progress of the equilibrium. In the case of *Sc*SCO1 an anaerobic solution of the small molecule ligand (e.g., BCS) is prepared in the presence and absence of reduced, apo-SCO, and the metal is then added. The anticipation is that where the protein has a much higher affinity than the competing ligand formation of the competing ligand complex will be delayed until the metal/protein complex is saturated and then the competing ligand/metal complex will appear. In the case where the protein has a much weaker affinity for the metal than the competing ligand the appearance of the metal/competing ligand complex will appear as though the protein is not present. In the case where the protein affinity is competitive with the ligand then the response will lie in between

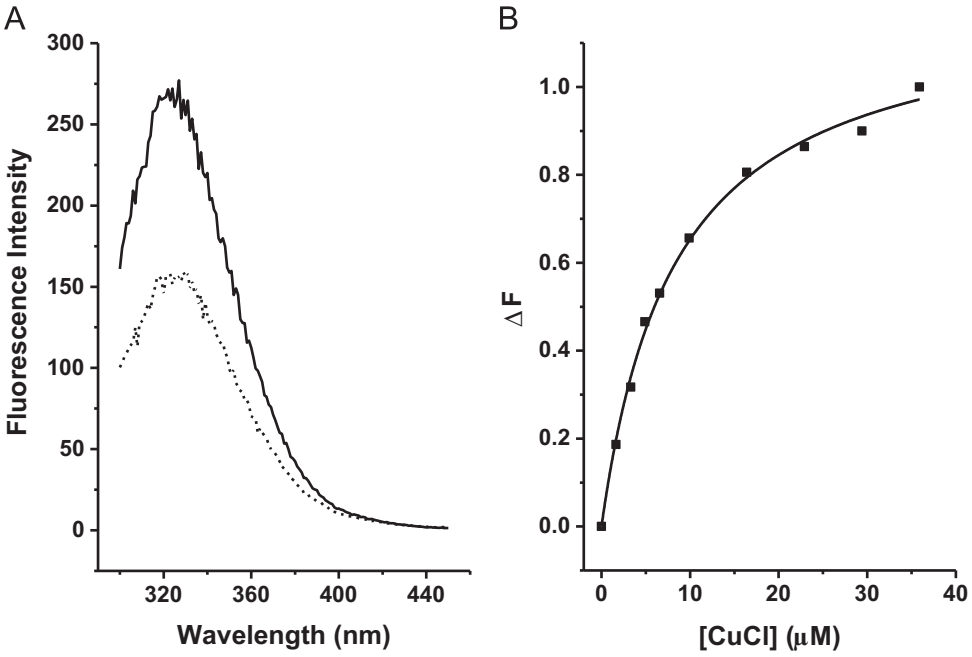


Fig. 6. Titration of *Sc*SCO1 with Cu(I). (A) Fluorescence spectra of reduced, apo-*Sc*SCO1 (–––) (6 μM) and in the presence of excess CuCl (- - - -) (36 μM). The sample was dissolved in 50 mM sodium phosphate buffer pH 7.0 and equilibrated with Ar in a gas tight fluorescence cuvette. Emission spectra were collected with excitation at 280 nm with an excitation slit of 1 nm and emission slit of 2 nm. The emission intensity is corrected for the inner filter effect in each case and for dilution after the addition of copper. The thiol determination for this sample was 3.7 thiols per *Sc*SCO1. (B) Relative fluorescence intensity versus CuCl concentration. The intensity is corrected at each copper concentration for the inner filter effect and dilution. The total intensity change is used to normalize the intensity change at each copper concentration. The concentration of *Sc*Sco1 is 6 μM, the buffer 50 mM sodium phosphate pH 7.0 and the temperature 25 °C. The wavelength for excitation is 280 nm with the slit set at 1 nm for excitation and 2 nm for emission. The protein sample and Cu(I) solution are equilibrated with Ar prior to the titration and Cu(I) additions are made with a gas tight syringe.

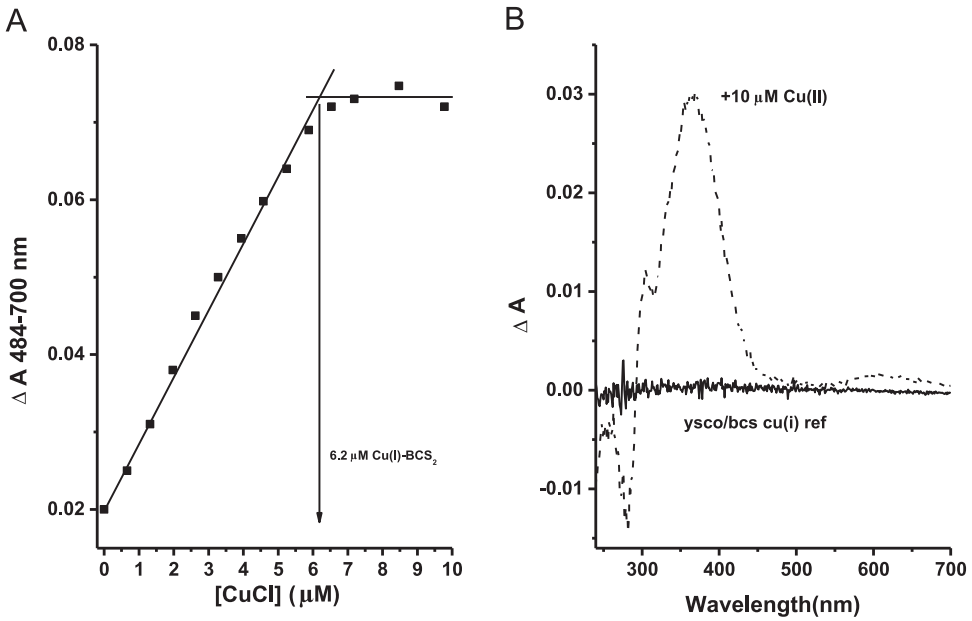


Fig. 7. Competition between *Sc*SCO1 and BCS for Cu(I). (A) A mixture of reduced apo-*Sc*SCO1 (12.3 μM) and BCS (12.5 μM) was made anaerobic by passing argon over the top of the stirring solution. An anaerobic solution of CuCl was prepared under argon in 10 mM HCl with 1 M NaCl in a Thunberg cell. Aliquots of the Cu(I) solution were transferred to the *Sc*SCO1/BCS mixture using a gas-tight syringe. Formation of the BCS-Cu(I) complex is monitored by measuring the increase in absorbance at 484 nm relative to a reference wavelength of 700 nm. The equivalency point is obtained at a copper concentration of 6.1 μM corresponding to 12.2 μM BCS. The titration was carried out in 50 mM sodium phosphate buffer pH 7.0 and the temperature was 25 °C. (B) Reactivity of *Sc*SCO1 in presence of $(BCS)_2$-Cu(I). The spectrophotometer was zeroed on the *Sc*SCO1/BCS mixture obtained at the end of Cu(I) titration (solid line). One addition of Cu(II) was made (i.e., 10 μM $CuCl_2$) to this mixture and the resulting spectrum recorded immediately (dashed line).

these two extremes. Fig. 7A shows a competition assay in which BCS is present along with reduced apo-*Sc*SCO1 as copper (I) is added to the mixture. There is no delay in the appearance of the $(BCS)_2$-Cu(I) complex and a sharp endpoint is reached at a concentration of Cu(I) corresponding to complete formation of the $(BCS)_2$-Cu(I) complex. We conclude that the affinity of *Sc*SCO1 for Cu(I) is much less than that of the BCS ligand. Since no evidence is obtained in this assay that *Sc*SCO1 is still capable of binding copper, the mixture of *Sc*SCO1, BCS and copper (I) was supplemented with slightly more than one equivalent of copper (II) (Fig. 7B). A

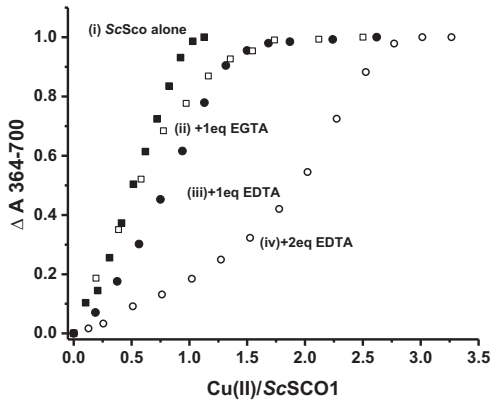


Fig. 8. Competition between *Sc*SCO1 and EGTA and EDTA for binding Cu(II). The equilibrium is followed by monitoring the absorbance increase at 364 nm as the *Sc*Sco1-Cu(II) complex accumulates as a function of increasing copper concentration. The absorbance is normalized to the total absorbance for ease of comparison. The *X*-axis is plotted as the ratio of added copper to *Sc*SCO1 concentration. The buffer is 50 mM sodium phosphate pH 7.0 and the temperature 25 °C. The symbols indicate the conditions as follow (■) apo-*Sc*SCO1 alone, (□), plus one equivalent of EGTA (8.9 μM *Sc*SCO1: 8.7 μM EGTA), (●) plus one equivalent EDTA (9.2 μM *Sc*SCO1: 8.7 μM EDTA) and (○) plus two and one-half equivalents EDTA (13.5 μM ScSCO1: 34.8 μM EDTA).

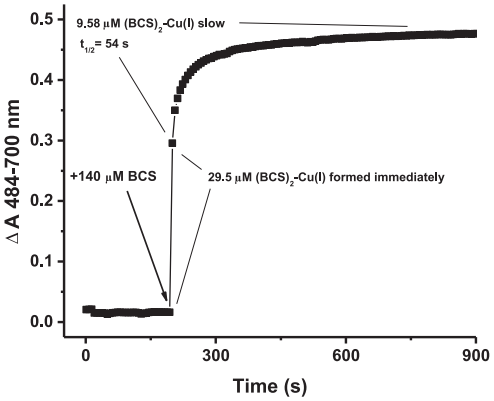


Fig. 9. Time course of $(BCS)_2$-Cu(I) formation following reduction of *Sc*SCO-Cu(II). Fully reduced, apo-*Sc*SCO (13.5 μM) was initially titrated to saturation with excess $CuCl_2$ (44 μM). The sample was flushed with argon and excess dithionite (125 μM) added to reduce the *Sc*SCO-Cu(II) complex, and any excess Cu(II), to Cu(I). The reactivity of Cu(I) was assessed by addition of excess BCS (140 μM). The reaction was measured at 484–700 nm subtracting the absorbance prior to addition of BCS. The buffer was 50 mM sodium phosphate pH 7.0 and the temperature 25 °C.

difference spectrum is generated upon addition of Cu(II) that forms immediately and represents nearly complete formation of *Sc*SCO1-Cu(II) demonstrating that under these conditions *Sc*SCO1 is still reactive with copper.

Competition assays can also be devised for Cu(II) binding to SCO. In this case we have used both EGTA ($K_D^{Cu(II)} = 2.5 \times 10^{-18}$) and EDTA ($K_D^{Cu(II)} = 2 \times 10^{-19}$) as potential competitors with *Sc*SCO1. The equilibrium for copper binding is monitored at 364 nm for formation *Sc*SCO1-Cu(II) (Fig. 8). Effective competition by the small molecule ligand will shift the appearance of the protein-metal complex to the right along the *x*-axis to higher copper concentrations. In the presence of one equivalent of EGTA the equilibrium progression for *Sc*SCO1 binding copper (II) is hardly altered, which is consistent with very tight binding of Cu(II) to *Sc*SCO1. In the presence of one equivalent of EDTA the binding of copper to *Sc*SCO1 is shifted further to the right consistent with the ten-fold higher affinity of EDTA for copper(II) over EGTA. An increase of EDTA to more than two-equivalent relative *Sc*SCO1 shifts the progress curve further to the right along the *x*-axis consistent with the expected concentration-dependent competition between *Sc*SCO1 and EDTA for Cu(II). A similar response is obtained when monitoring progress of copper (II) binding to *Bs*SCO in the presence of EDTA. We conclude that the binding of copper (II) to *Sc*SCO1 is extremely tight and the binding constant is similar to that for EDTA ($K_D \approx 10^{-18}$ M).

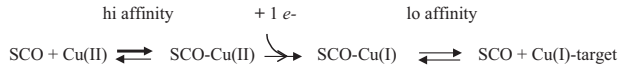
A variation on the Cu(I)-competition that we have used with *Bs*SCO is to first titrate apo-SCO with Cu(II), convert the Cu(II) to Cu (I) with excess reducing agent and then assess the position of the newly established Cu(I) equilibrium with excess BCS [32]. On addition of an excess of the Cu(I) chelator BCS we can observe the availability of the Cu(I) in this system (see Fig. 9). We observe a biphasic reaction with an initial immediate formation of the $(BCS)_2$-Cu(I) complex followed by a much slower development of the full reaction. If we assume the fast reacting Cu(I) represents the fraction that is free than the remainder is bound to *Sc*SCO1 and undergoes a relatively slow exchange with BCS ($t_{1/2} \sim 60$ s). In the data shown the ratio of copper to SCO is about 3:1 and the amount of freely available copper is high and reacts with BCS immediately. The remaining copper only reacts slowly and we presume this is from the dissociation of *Sc*SCO1-Cu(I) complex. In control experiments without *Sc*SCO1 the formation of $(BCS)_2$-Cu(I) is complete and instantaneous under these conditions. We can use these observations to calculate an equilibrium dissociation constant for the binding of Cu(I) to *Sc*SCO1. In four trials at different ratios of copper to *Sc*SCO1 we obtain an average K_D value of 9.18 ± 1.3 μM, consistent with the values obtained in direct titration of *Sc*SCO1 with Cu(I) reported above.

4. Discussion

Members of the SCO family of proteins are found in a wide variety of organisms and are often associated with efficient assembly of the cytochrome *c* oxidase complex [33]. Studies on purified *Bs*SCO have shown that it is able to bind one equivalent of copper in the cupric (*i.e.*, Cu(II)) oxidation state with extremely high affinity [18]. Direct spectroscopic binding assays show a tight binding form and allow only an upper limit for an estimate of the equilibrium dissociation constant (K_D«1 μM). Differential scanning calorimetry measures a substantial increase in the stability of *Bs*SCO upon binding one equivalent of Cu(II). Apo-*Bs*SCO has a melting temperature of 56 °C that shifts to 79 °C upon binding one equivalent of Cu(II). Such thermodynamic data yield an estimate of the equilibrium dissociation constant of the *Bs*SCO-Cu(II) complex of 3.5 pM. In contrast direct assay of Cu(I) binding to *Bs*SCO gives a K_D of approximately 10 μM for the *Bs*SCO-Cu(I) complex [18].

In eukaryotic organisms the SCO proteins are found as integral components of the inner mitochondrial membrane. Generally, they are anchored by a single transmembrane helix with the copper binding domain exposed to the intermembrane space. In the yeast *Saccharomyces cerevisiae* there are two types of SCO, known as SCO1 and SCO2. The two proteins are known to cooperate in copper homeostasis, but their exact roles in Cu_A assembly are not fully known [9]. Bacterial versions of SCO (*e.g.*, *Bs*SCO) are presumed to be homologs of the *Sc*SCO1 protein. EXAFS studies of *Sc*SCO1 reached the conclusion that it is a Cu(I) binding protein, and reported on the ligand field of this site [27]. However, a thermodynamic analysis of copper binding to *Sc*SCO1 has not been reported.

There is an apparent discrepancy in the literature on the copper redox species preferences of yeast mitochondrial and *Bacillus* SCO proteins with mitochondrial SCO viewed as being a Cu(I)-specific protein (e.g., [16]) and bacterial SCO having preference for Cu(II) over Cu(I) (e.g., [18]). One explanation for this difference would be that *Bs*SCO has high affinity for Cu(II) because it has evolved in an



Scheme 1. SCO captures copper as Cu(II) in a high affinity complex. This Cu(II) complex is reduced with addition of one electron to generate the Cu(I) complex. The low affinity SCO-Cu(I) can then release copper to its target apo-protein.

aerobic environment where Cu(II) is probably the dominant form of copper. In contrast, mitochondrial SCO proteins are exposed to the reducing environment of the cytoplasm and have only Cu (I) readily available [34,35]. In addition, the difficulties associated with determining protein/metal affinity constants has been recognized [36] and the characterization of ligands for competition assays reported [37].We sought therefore to compare the relative affinities of *Sc*SCO1 for Cu(I) and Cu(II) with the values we have obtained for *Bs*SCO. We find the relative affinities to be similar and that *Sc*SCO1 prefers Cu(II) over Cu(I) by orders of magnitude. However, it should be pointed out that high affinity binding has been reported for *Bs*SCO with Cu(I) although this work does not report on the affinity of *Bs*SCO for Cu(II) [19]. These authors show that *Bs*SCO and two mutants forms where the metal ligating histidine has been changed to methionine, or Seleno-methionine are all able to take Cu(I) from the $(BCS)_2$-Cu(I) complex. In our work we do not find that *Sc*SCO,or *Bs*SCO, is able to compete with BCS, or even with weaker Cu(I) ligands such as BCA or ferrozine [37], for Cu(I).

The determination of these affinities is an important parameter to obtain in order to consider how these proteins might function in cells. For example, if we accept that SCO has preference for Cu (I) with affinities for *Bs*SCO approaching 90 fM [19] and human SCO1 even tighter binding at 0.3 fM [17] then we are faced with another dilemma. Such high affinity in a simple binding mechanism and assuming a diffusion- limited formation rate constant can only be conferred by a very slow rate of dissociation. The half-life for the dissociation of such a high affinity SCO-Cu(I) complex ranges from about 1 day to 250 days. Of course the dissociation of such a complex could be facilitated by docking to its specific target, and such a mechanism has been proposed for metalation of the apo-Cu_A site in subunit II of cytochrome *c* oxidase (e.g., [35]).

Alternatively, if SCO proteins have strong binding with Cu(II), such as 3.5 pM reported for *Bs*SCO [38] and relatively weak binding with Cu(I) as reported here for *Sc*SCO1 then other scenarios can be considered. For example, a high affinity Cu(II) complex would allow SCO to harvest copper from relatively weakly bound, but abundant cellular pools of Cu(II) [34]. The stable Cu(II) complex of SCO would only release copper to its target upon the specific reduction of the Cu(II) ion to Cu(I) (see Scheme 1).

This would result in a redox mediated affinity change and subsequent transfer of copper to its target. Such a mechanism would require a specific means to reduce the SCO-Cu(II) complex to SCO-Cu(I). Whichever of these scenarios is more accurate depends critically on the relative affinities of these proteins for copper, and so their unambiguous determination will require further investigation.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.08.010.

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