A COMPETITION STUDY ON COPPER-BINDING AFFINITY OF SCO PROTEIN

By

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Abstract

Cytochrome c oxidase catalyzes the reduction of molecular oxygen to water and contributes to the electrochemical gradient by translocating protons across the membrane. The SCO protein (for Synthesis of Cytochrome c Oxidase) is proposed to be an important assembly factor in biogenesis of the oxidase. Particularly, SCO has been demonstrated to function as a metallochaperone that receives copper ions from an upstream copper source in the cell and subsequently delivers them to the CuA centre in subunit II of cytochrome c oxidase. However, the SCO protein binds copper ions tightly and forms a stable complex in vitro that is extremely difficult to dissociate. Direct titration and differential scanning calorimetry, for example, have demonstrated a tight binding between SCO and Cu(II). Nonetheless, the reported dissociation constant $K_D$ falls in a wide range from 65 nM to 3.5 pM. In this study, binding affinities of Bacillus subtilis SCO (BsSCO) for both Cu(II) and Cu(I) ions were quantitatively estimated via competition with various copper ion ligands and chelators. Ethylenediamine tetraacetic acid (EDTA) was used as a competitor to BsSCO. In this case, BsSCO is able to compete with the chelator for copper-binding, which binds Cu(II) with $K_D \sim 3.1 \times 10^{-16}$ M (i.e., 0.31 fM). Estimation of binding affinity via competition provides a different perspective of quantifying the interaction between the SCO protein and copper ions. The tight binding (i.e., in the fM range) between the Cu(II) and the SCO protein suggests that Cu(II) ion is unlikely to be the oxidation form during the transfer from SCO to the CuA site. Strong Cu(I) ligands, such as ferrozine (Fz), were introduced in quantification of the Cu(I) binding affinity of the
SCO protein. Competition with Fz for Cu(I) binding supports a relatively weak interaction between BsSCO and Cu(I) ions, which characterized with $K_D \sim 10 \ \mu$M. Therefore, we propose that a stable BsSCO-Cu(II) complex could be reduced to BsSCO-Cu(I) in order for the SCO protein to fulfill a copper chaperone role in vivo that delivers and releases its copper ion to the Cu$_A$ site of cytochrome c oxidase.
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List of Abbreviations

BCA: bicinechoninic acid
BCS: bathocuproine disulfonate
CcO or COX: cytochrome c oxidase
Da: Dalton (kDa, kilo Dalton)
DSC: differential scanning calorimetry
DTDP: dithiodypridine
DTT: dithiothreitol
EDTA: ethylenediamine tetraacetic acid
EGTA: ethyleneglycol tetraacetic acid
EPR: electron paramagnetic resonance
Fz: ferrozine
Gdn-HCl: guanidine hydrochloride
GST: glutathione s-transferase
ITC: isothermal titration calorimetry
NTA: nitrilotriacetic acid
PBS: phosphate buffered saline (solution)
SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis

SCO Nomenclature:

Apo-SCO: SCO protein with two (Bacillus) or four (Saccharomyces) reactive thiol groups but no bound copper ion
BsSCO: SCO protein from *Bacillus subtilis*

Cu(I)-SCO: Cu(I) bound SCO protein

Cu(II)-SCO: Cu(II) bound SCO protein

HsSCO1: SCO1 protein from *Homo sapiens*

Oxidized SCO: SCO protein with disulfide bond formed between copper-binding cysteine residues

SCO: Synthesis of cytochrome c oxidase

ScSCO1: SCO1 protein from *Saccharomyces cerevisiae*

ScSCO2: SCO2 protein from *Saccharomyces cerevisiae*
Chapter 1

INTRODUCTION

Metal ions are partners of various enzymes, and they serve as substrates, cofactors, prosthetic groups and even catalytic centres in different biological systems. Cytochrome c oxidase (CcO), a metal-coordinated enzyme complex, requires the incorporation of two heme A groups and three copper ions for its catalytic activity\(^1\). This enzyme catalyzes the reduction of di-oxygen to generate water molecules \textit{via} the concomitant oxidation of cytochrome c, which donates electrons to the oxidase as the primary source\(^2\). CcO is, therefore, an integral membrane oxygen reductase located in the prokaryotic plasma membrane, or in the inner mitochondrial membrane of eukaryotes\(^3,4\).

CcO is a member of the heme-copper protein family and an important enzyme present across all kingdoms of living organisms\(^3\). This enzyme is also known as complex IV (the terminal component) in the electron transport chain of organisms that employ oxygen as the final electron acceptor. While the enzyme facilitates the redox reaction, the free energy of water formation is utilized by the CcO to translocate protons across the membrane (plasma or inner mitochondrial), thereby contributing to the trans-membrane proton gradient that is used by ATPase (complex V) to synthesize ATP\(^2\). Indeed, CcO is a vital component in basic energy metabolism for oxygen-dependent organisms. Not only does the survival of eukaryotes such as yeast and humans rely on the proper function of
the oxidase, this enzyme is also essential in aerobic prokaryotes, for example the
gram-positive *Bacillus subtilis*.

Because CcO is a hetero-oligomeric enzyme complex\(^1\), assembly of the
functional protein is a complicated process. Firstly, CcO assembly certainly
includes multiple steps with a number of discrete and stable intermediates. The
three largest subunits (I, II, and III) form the functional core of CcO\(^1\). These three
subunits are highly conserved in prokaryotes and eukaryotic cells\(^5\). Subunits I, II,
and III are, in fact, encoded in the mitochondrial genome of eukaryotes,
consistent with the endosymbiotic origin of mitochondria\(^5\). As a result, the
assembly process involves four subunits in prokaryotes\(^6\) (the three core subunits
plus one variable subunit) to as many as thirteen in eukaryotes \(^7\) (the three core
subunits plus ten additional subunits). In yeast, for example, there are nine
accessory subunits that, along with the three core subunits, make up a twelve-
subunit complex of CcO. These ancillary subunits are encoded in the nuclear
genome of the eukaryotic species, synthesized in the cytoplasm, and
subsequently imported into the mitochondria\(^8\). For eukaryotic organisms, CcO
subunits from both mitochondrial and nuclear origins have to come together to
successfully form a functional enzyme complex. Consequently, the coordination
of nuclear and mitochondrial genomes is also a key aspect of CcO assembly.
CcO biogenesis involves regulation at different cellular levels including
transcription, translation, post-translational modification, and compartmental
import\(^9,10\).
Figure 1. The catalytic core of eukaryotic cytochrome c oxidase. Only the membrane-embedded subunits I and II are shown in the cartoon presentation, which incorporate all the heme groups and copper ions of the enzyme. A cytochrome c molecule is bound to subunit II in the hydrophilic inter-mitochondrial space. The red arrow indicates the flow of electrons from the docking cytochrome c to the Cu₄ centre. Electrons are ultimately passed to the cytochrome a₃-Cu₅ hetero-binuclear centre where one oxygen molecule is reduced to two water molecules during each reaction cycle. The blue arrow illustrates one of the possible paths of protons that are translocated across the membrane during the redox reaction. For a complete reaction cycle, four electrons are received from cytochrome c, four protons are transferred to water molecules, and four protons are translocated across the membrane. This figure is modified from Belevich et al.¹¹.
The complexity of CcO assembly arises from its requirement for metal cofactors such as heme groups and copper ions. Even for prokaryotic CcO’s that possess only four subunits, complexity still remains due to the requirement for metal ion incorporation into multiple redox active centres. In order for the metalloenzyme to function properly, well-coordinated interactions between CcO and its metal ions are crucial. Eukaryotic CcO requires the coordination of two heme A groups and three copper ions in order to catalyze the redox reaction described above\(^1\). All redox cofactors of the enzyme exist within the evolutionarily conserved subunit I and II\(^5\). There are two copper-ion-based redox centres in CcO, Cu\(_A\) and Cu\(_B\), and they are located in subunits II and I, respectively\(^1,12\). The Cu\(_A\) site is a bi-metallic centre\(i.e.,\) two copper ions\(^\)\(^12\) whereas the Cu\(_B\) centre contains only one copper ion\(^6\). In addition, the oxidase also recruits as cofactors one magnesium ion in subunit II on the interface between subunits I and II (See Figure 1), one zinc ion, and one sodium ion into its structure \(^1,6\). However functional roles of these metal cofactors remain unknown. As a result, locations of zinc and sodium ions within the oxidase are not illustrated in Figure 1.

The redox reaction begins with the electrostatic interaction between cytochrome \(c\) and subunit II at a specific binding site on the surface of the subunit\(^13\). Electron flow initiates from the docked cytochrome \(c\) molecule to the Cu\(_A\) centre, resulting the concurrent oxidation of the heme \(C\) prosthetic group in cytochrome \(c\) (illustrated in Figure 1). The subsequent internal electron transfer in the oxidase proceeds in four steps\(^4\). Electrons move from the Cu\(_A\) centre in subunit II to cytochrome \(a\) in subunit I, and are eventually passed to the cytochrome \(a\)_3/Cu\(_B\) hetero-binuclear centre where di-oxygen is split and reduced.
to water. For one complete reaction cycle that reduces one molecule of molecular oxygen to two molecules of water, cytochrome c turns over four times in order to transfer four electrons to CcO. In eukaryotic cells, a concomitant translocation of four protons across the inner mitochondrial membrane contributes to the proton gradient (see Figure 1). A similar proton gradient is seen in bacteria, but it is formed across the plasma membrane. The Cu$_A$ centre is, therefore, critical in the initial steps of the CcO catalytic cycle.

It has been proposed originally that CcO acquires copper ions spontaneously from its surrounding environment during assembly$^5$. However, it has been shown that there is less than one free copper ion on average in a cell that is bound to aqueous small molecules and that is readily available for cellular metabolism$^{14}$. This observation is consistent with the destructive redox activities of biological copper ions, for example the Fenton chemistry of Cu(II) and Cu(I) ions reacting with oxygen species. Indeed, reactive free copper ions within a cellular environment are prone to generate oxidative damage$^{15}$. Therefore, it seems rational for the cell to strictly regulate its copper content. Overall, the cell has a great number of ancillary proteins to contain the production of oxidative damage (e.g., Copper Chaperone for Superoxide dismutase, CCS). In the case of CcO assembly, these ancillary proteins are not necessarily directly associated with the catalytic function of the oxidase. This is also consistent with the observation that biogenesis of the CcO is by no means a process of self-association. Instead, a set of factors called copper chaperones supports copper ion incorporation of the metalloenzyme$^8$. 
Copper chaperones can bind and release copper ions with proper control mechanisms which limit their reactivity in aqueous solution as free ions. Assembly and folding of CcO components require these copper-binding factors that transport and insert copper cofactors into the enzyme. In fact, a number of accessory proteins of CcO had been proposed to be capable of delivering the metal ion to the CcO\textsuperscript{16,17}. In this light, it becomes evident that the large numbers of assembly proteins that are associated with CcO have many important biological roles to fulfill \textit{in vivo}. Not only do these accessory proteins facilitate assembly of CcO, they also could be involved in chemical control of metal ions and in maintenance of balanced intracellular redox equilibrium. While some thirty complementation groups have been identified in yeast alone for assembly of the CcO complex\textsuperscript{16}, there are few of them that have been intensively studied. Thus, this research project focuses on one of the chaperone-like proteins that plays a crucial role in CcO assembly. Particularly, the copper-binding protein SCO, short for Synthesis of Cytochrome c Oxidase, is proposed to escort copper ions to the Cu\textsubscript{A} centre of subunit II.
Figure 2. Proposed role of the SCO protein in CcO assembly. COX17, COX11, and SCO are all assembly factors of CcO. COX17 has been proposed to bind Cu(I) ions within the inter-mitochondrial space, and subsequently deliver the copper ions to COX11 and SCO. COX11 has been linked to maturation of the CuB centre. The SCO protein has been postulated to insert copper ion(s) to the CuA centre while subunit II folds into its native conformation. Only subunit I is shown in the background, yet subunit II has been proposed to join an existing I/IV/V sub-complex during assembly. This figure is modified from Cobine et al.\textsuperscript{18}.

The expression of the SCO protein has been linked to the accumulation of subunit I and II of CcO during assembly\textsuperscript{19}. Therefore, SCO is proposed to be one
of the assembly factors in CcO biogenesis. The current view of the primary function of the SCO protein is that it acts as a copper chaperone, receiving and delivering copper ion(s) to the bi-metallic CuA site located in subunit II of CcO\(^\text{20}\). In binding with copper ion(s), the SCO protein is proposed to function as a potential copper ion shuttle and an escort protein. Its requirement for assembly of CcO have been experimentally confirmed by mutagenesis studies \textit{in vitro}\(^\text{19,21,22}\). Despite the fact that transcription and translation of CcO subunit II does not require SCO, the expressed subunits are rapidly degraded in the absence of the protein\(^\text{19}\). Studies have suggested that the family of SCO proteins is likely to be involved in the assembly of the copper centres of CcO\(^\text{22}\), however, the role as a copper ion chaperone is still matter of debate.

This research project investigates one of the favorite proposed physiological functions of SCO as a copper-binding protein. The exact nature of the bound copper ion in SCO \textit{in vivo} is still unclear. In biological systems copper ions can exist in two oxidation states: Cu(I) (reduced) and Cu(II) (oxidized). When the bi-metallic CuA centre is in the reduced form, the oxidation state of the two copper ions is Cu(I). In oxidized CuA the overall oxidation state is +3, each copper ion is +1.5 and the dinuclear site is in a mixed-valence state. By far, the most detailed studies of the SCO protein are centred on its ability to bind copper ions \textit{in vitro}. The metal binding properties of SCO have been characterized previously\(^\text{23-25}\). SCO proteins have been shown to bind to either Cu(I) or Cu(II) in a 1:1 stoichiometry \textit{in vitro}\(^\text{25,26}\). BsSCO has been shown to preferentially bind to Cu(II) with a substantially higher affinity than for Cu(I)\(^\text{25}\). Unlike its eukaryotic homologs, which preferentially bind Cu(I)\(^\text{27}\), BsSCO binds Cu(II) more tightly than
any other metal ion\textsuperscript{24}. However, the details of copper ion interaction with SCO proteins have not been fully determined. Taken together, BsSCO binds Cu(I) and Cu(II) ions with distinct affinities. We, therefore, hypothesize that this difference in binding affinity could promote the release of copper ion(s) from the SCO-Cu complex, completing a copper chaperone role of the SCO protein in maturation of the Cu\textsubscript{A} site.

There are several goals that this research study aims to achieve. The first objective of the project is to study the copper-binding properties of the BsSCO protein \textit{in vitro}. The SCO protein is conserved in the spectrum of living organisms. The family of SCO proteins comprises members implicated in the formation of the Cu\textsubscript{A} centre. Studies of CcO assembly in bacteria have provided additional model systems for elucidating the functions of SCO, taking advantage of a relatively less sophisticated assembly mechanism for CcO. Therefore, the research concentrates on quantitatively estimating the binding affinities of SCO for both Cu(I) and Cu(II) ions. In this work, competition assays, along with copper ligands of well-characterized copper-binding affinities, are employed to evaluate the interaction between the SCO protein and copper ions. Moreover, there are discrepancies in the literature with respect to copper-binding affinities of SCO proteins for Cu(I) and Cu(II). Therefore, this project also assess the discrepancies in estimated dissociation constants of SCO-Cu(II) and SCO-Cu(I) complexes, which were determined \textit{via} different approaches (\textit{e.g.}, Isothermal Titration Calorimetry (ITC) and Differential Scanning Calorimetry (DSC)).

Another objective of this work is to identify and characterize the relevant players that are critical to SCO-dependent metallation of subunit II. Also, we are
interested in the mechanism in which copper ions are incorporated into the CcO during assembly of the enzyme complex. Therefore, our study aims to provide a workable model of Cu₄ centre assembly such that we could better understand the nature of the binding interaction between the SCO protein and copper ion(s). Last but not the least, this study focuses on SCO proteins from two model organisms: the prokaryotic *Bacillus subtilis* and the eukaryotic *Saccharomyces cerevisiae*. The chaperone role of the SCO protein has been proposed in both model organisms in which SCO proteins receive copper ions from an upstream copper ion source and subsequently deliver them to the Cu₄ centre of the subunit II of the cytochrome *c oxidase*\textsuperscript{13,22}. There are certainly biological differences in SCO proteins from *Bacillus* and *Saccharomyces*. In order to identify any biochemical difference (e.g., copper-binding properties) in *BsSCO* and *Saccharomyces cerevisiae* SCO (ScSCO1) proteins, this research study compares the SCO proteins from the two model organisms.
Chapter 2

LITERATURE REVIEW

2.1 Copper Trafficking and Distribution Pathways

Biological systems exploit metal ions for their chemical reactivity and properties that organic molecules may not necessarily possess. Copper ions provide biomolecules such as proteins with redox catalytic potential and diverse functions. As a result, copper ions in both Cu(I) and Cu(II) oxidation states are widely employed by living organisms in their metabolic processes\(^\text{18}\). On the other hand, biological available copper ions present a dilemma to the cell. They are considered toxic because readily available free copper ions inside a cell that are bound to small molecules or in aqua-complex forms may generate reactive oxygen species\(^\text{15}\). Copper toxicity may lead to oxidative stress and ultimately causes damage to the cell\(^\text{14}\). Not surprisingly, cells monitor internal copper trafficking and maintain strict regulation of copper ion concentration\(^\text{28}\).
Figure 3. Pathways of copper ion trafficking within an eukaryotic cell. Two of the three proposed copper chaperones, COX17 and CCS are shown along with the respective protein targets, SCO proteins and cytosolic Cu/Zn superoxide dismutase (SOD1). SCO1 and SCO2 proteins receive copper ions from COX17 and deliver them to cytochrome c oxidases in mitochondria. The third proposed copper chaperone, Atox1 delivers copper ions to the Wilson and Menkes copper transporting ATPases located in the endoplasmic reticulum. The mechanisms regulating initial copper ion uptake into the cell via the membrane transporter Ctr1 and the subsequent distribution of the metal ion to each chaperone remain unclear. This figure is modified from Bartnikas and Gitlin29.
The cell membrane constitutes an initial barrier to the entry of extracellular copper ions (see Figure 3). The charged, hydrophilic, copper ions are impermeable to cell membranes, and thus they are actively imported into eukaryotic cells via a specialized protein family of copper ion transporters (Ctr1) as shown in Figure 3. The Ctr1 protein is the only high-affinity eukaryotic transporter specific for copper ion import that is well characterized. In addition, the high-affinity copper ion uptake system is supplemented by a number of low-affinity transporters that work a similar fashion as the Ctr1 (e.g., Ctr2 and Ctr4). The copper uptake system in prokaryotic species is not currently well characterized, and a homologue of the family of Ctr proteins has not been identified in any prokaryotic cell.

Similar to other transition metal ions, copper has a number of oxidation states. Cu(II) ions are water-soluble and the dominant form in nature. Cu(I) ions are not on their own water-soluble. However, copper ions have been proposed to enter eukaryotic cells in the reduced Cu(I) form, whereas extra-cellular copper ions are mostly in the more stable oxidized Cu(II) form. Most likely, the Cu(II) ion is reduced to Cu(I) by a membrane-bound reductase as it is transported into the cell via the Ctr1 transporters. Since a homologous copper transport protein has not been found in prokaryotes, it is possible that both Cu(II) and Cu(I) ions are present in the space between the cell wall and the plasma membrane.

To take advantage of the redox properties of the Cu(I)/Cu(II) couple in aqueous systems suitable ligands must be available. Both Cu(II) and Cu(I) oxidation states are employed by proteins and enzymes in biological systems with the protein serving as the multi-dentate ligand to the copper ion. Once
copper ions are inside the cell, a class of proteins known as copper chaperones can bind to the imported ions within the cellular environment, protecting the cell from potential oxidative damage caused by copper ions. Copper chaperones also have the ability to release metal ions to provide adequate copper supply for downstream target proteins. In both prokaryotic and eukaryotic cells, specific and conserved copper chaperones have been reported. How copper chaperones acquire their metal ions from the Ctr1 proteins is an open issue. This transfer of copper ions is possibly achieved through a direct interaction. However, an intermediate-dependent mechanism has also been proposed to ferry copper ions between the Ctr1 and various downstream chaperones.

There are three conserved chaperone-mediated delivery pathways that distribute copper ions to the desired destinations in eukaryotic cells (see Figure 3). The cytosol, the endoplasmic reticulum, as well as the mitochondria are the major compartments that require a supply of copper ions. Although there is no direct evidence, several cytosol-localized proteins are proposed to shuttle copper ions from the cell membrane to internal cellular compartments such as the inter-membrane space of mitochondria. Among various metalloenzymes, CcO represents the major requirement for copper ions in mitochondria, and even in the cell as a whole.

The pathway by which mitochondrial CcO acquires copper ions has been elucidated (see Figures 2 and 3). At least three proteins have been implicated to function in the delivery of copper ions to the copper centres of the oxidase. In the pathway that delivers copper ions to the mitochondrion, it is unclear how the COX17 protein receives its copper ions. The copper-binding protein has been
proposed initially as a copper chaperone that receives copper ions from the Ctr1 protein and subsequently passes them on to the downstream copper-binding proteins, COX11 and SCO. However, COX17 has not been demonstrated to escort copper ions from the cytosol to the mitochondrion. Consequently, the role of COX17 as a copper chaperone that delivers copper ions from the cytosol into the mitochondrion has been challenged. Although the COX17-Cu(I) complex has been observed in the cytosol\(^{37}\), other studies show that COX17 needs not to leave the inter-mitochondrial space to function in the implicated copper delivery pathway\(^{38}\). In other words, COX17 may shuttle copper ions only within the mitochondrion. This is consistent with tethering studies in which COX17 is altered so as to be anchored to the inner mitochondrial membrane, and yet proper assembly of CcO is observed\(^{39}\). These observations have thus challenged the possibility that COX17 functions as a cytoplasmic chaperone for mitochondrial copper ion uptake. Moreover, it has been demonstrated that any copper-bound complex of COX17 cannot be transported into the mitochondrion\(^{40}\). Alternatively, yeast COX17 enters the mitochondrion through the TOM channel in a reduced, apo form by an oxidative folding mechanism catalyzed by the Mia40-Erv1 system, suggesting that the chaperone binds copper ions in its destined compartment\(^{40}\).

Finally, the proposed assembly factor SCO delivers copper ion(s) to the Cu\(_A\) centre of CcO. At the same time, COX11 has been suggested to escort one copper ion to the Cu\(_B\) centre in subunit I\(^{41}\) (see Figure 2). Nonetheless, much about the COX11 is still unclear.

CcO belongs to the heme-copper oxidase family, wherein two heme A groups and three copper ions are coordinated by various amino acid residue
ligands within subunit I and II\textsuperscript{1}. Two of the three copper ions are recruited into the Cu\textsubscript{A} site of CcO, which is exposed in inter mitochondrial space, or the space between the cell wall and the plasma membrane in gram-positive bacteria. Both copper ions are in the Cu(I) form when the binuclear site is reduced\textsuperscript{12}. Different from the SCO protein, the Cu\textsubscript{A} motif shares sequence homologies with the blue copper proteins, azurin and plastocyanin\textsuperscript{12}. The Cu\textsubscript{A} motif also has the characteristic β-structure of blue copper proteins, the so-called cupredoxin fold. However, the Cu\textsubscript{A} site binds copper ions in a similar coordination to the SCO protein. The ligands of the two copper ions are two histidine residues, one methionine sulfur, one backbone carbonyl oxygen from a glutamate residue, and two cysteine thiolates\textsuperscript{4}. Therefore, SCO and Cu\textsubscript{A} evolve from different protein fold families, yet they both use histidine and cysteine residues to coordinate copper ions. In addition, the Cu\textsubscript{B} centre has the third copper ion and is buried within the membrane (see Figure 2)\textsuperscript{41}. A cytochrome a\textsubscript{3} prosthetic group forms a hetero-di-nuclear centre with the closely located Cu\textsubscript{B} site where reduction of oxygen to water occurs\textsuperscript{41}.

2.2 The SCO protein and its Homologues

The first discovered member of SCO in the protein family is from S. cerevisiae (i.e., ScSCO1). The yeast SCO protein is a nuclear-encoded gene product from the model eukaryotic organism\textsuperscript{19}. Native ScSCO1 is an integral membrane protein that is anchored in the inner-mitochondrial membrane of the yeast cell via a single trans-membrane α-helical segment of the protein\textsuperscript{42}. In contrast, the native SCO protein from the gram-positive B. subtilis is anchored to the membrane via covalently attached lipid\textsuperscript{43}. ScSCO1 has been shown to be
involved in assembly of the subunits I and II of CcO\textsuperscript{19,42,44}. In fact, ScSCO1 was originally identified as a high-copy suppressor in yeast strains in which COX17 was inactivated. COX17-knockout strains of yeast can be complemented by overexpression of ScSCO1\textsuperscript{45}. However, the reverse complementation does not occur. COX17 overexpression cannot complement yeast SCO1 knockouts\textsuperscript{45}. Furthermore, mutagenesis has shown that ScSCO1 functions downstream of COX17. Mutational studies have also demonstrated that changes in ScSCO1 amino acid sequence result in degradation of CcO subunits\textsuperscript{46} (subunit I and II in particular). Without the SCO1 protein, the eukaryotic CcO subunit II cannot be properly assembled, ultimately resulting in respiratory deficient cells that are unable to metabolize oxygen as the terminal electron acceptor\textsuperscript{47}.

SCO proteins are evolutionarily highly conserved, and homologs of ScSCO1 are found across the kingdoms of living organisms where they are also required for assembly and proper function of cytochrome c oxidases\textsuperscript{20}. Other members of the SCO protein family are identified by their sequence and structural similarity to ScSCO1. The prokaryotic B. subtilis, for example, expresses a homologous protein of ScSCO1, referred to as BsSCO (see Figure 4). The DNA of the gram-positive bacterium encodes a homolog of ScSCO1 from the ypmQ gene\textsuperscript{43}. The expression of BsSCO has been shown to be essential and mandatory for assembly of the Bacillus oxidase that contains a Cu\textsubscript{A} centre\textsuperscript{43}. Deletion of the ypmQ gene results in loss of activity of the oxidase. Nonetheless, the exclusive role of the SCO protein in copper ion delivery to the Cu\textsubscript{A} centre has been challenged by the finding that certain bacteria express an apparent SCO homolog (with sequence and structural similarities) yet do not express any
oxidase that contains Cu₄ centre\textsuperscript{20}. The oxidase from *Neisseria*, for example, does not contain a Cu₄ domain yet a SCO homolog has been identified in this species. Although a Cu₄ moiety could be present in alternative terminal oxidoreductases (e.g., nitrous oxide reductase\textsuperscript{12}, the relationship between the SCO protein and these enzymes remains unknown.
Figure 4. Ribbon representation of BsSCO. The crystal structure of BsSCO shows the secondary structure, including eight β-sheets (red) and four α-helices (cyan). The three conserved copper-binding cysteine (yellow) and histidine (blue) residues are labeled along with the C- and N-terminal ends. PDB#: 1ON4.
The SCO protein family is characterized by a conserved motif, consisting of a pair of copper-binding cysteine residues in a CxxxCp sequence where X can be any amino acid (see Figure 4). Structural characterization of the SCO protein across different organisms has shown that its sequence contains the conserved motif. In addition to the reduced di-thiol state, the two conserved cysteine residues can form an intra-molecular disulfide bond. It has been shown that only the reduced, di-thiol form of the SCO protein is able to bind a copper ion, whereas the oxidized form cannot form a complex with either Cu(I) or Cu(II).

Noticeably, the conserved CxxxCp motif in SCO proteins is shared with the sequence found in the Cuₐ site in which copper-binding is also achieved by adjacent cysteine residues. Despite this similar coordination in the ligation of copper ions between cysteine-containing motifs from SCO and Cuₐ, their core structures are largely distinct and thus possibly evolve from different origins.

In addition to the cysteine pair from the CxxxCp motif, there is a conserved histidine residue located approximately one hundred amino acid residues downstream towards the C-terminus of the SCO protein. (The exact number varies among species.) Three-dimensionally close to the cysteine pair in the folded tertiary structure (see Figure 4), this histidine residue has been demonstrated to function in copper binding as well. In yeast, for example, the His239 residue works in concert with the two conserved cysteine residues to bind one molar equivalent of copper ion. Mutation of any of the three residues of SCO compromises the protein’s ability to assist CcO assembly, although less stable copper-binding of the SCO-Cu complex from His mutants could still be observed in vitro.
Another key structural feature of the SCO protein is that its overall fold places it in thioredoxin protein family (see Figure 5). Thioredoxin proteins catalyze redox exchange reactions within the cell. The physiological role of thioredoxin is likely to assist in the maintenance of disulfide bonds within cellular proteins in the correct arrangement for native protein folding and in buffering the redox state of the cell. Thioredoxin and members of the thioredoxin family are found throughout the spectrum of living organisms, and function in a variety of redox reaction, e.g., disulfide bonds reduction, peroxide detoxification via thioperoxidases etc.\textsuperscript{50}. Proteins of this family have a characteristic thioredoxin fold, which consists of a central β-sheet structure surrounded by several α-helices. The highly conserved core functional motif undergoes redox chemistry as part of their specific catalytic mechanism. The redox-active CXXC motif lies on the N-terminus of an α-helix (i.e., α-helix-2) as shown in Figure 5, possessing the redox activity \textit{in vivo} and converting disulfides into reduced cysteine residues in substrate proteins.
Figure 5. Stereoview of the active sites in SCO, thioredoxin (TRX), and peroxidase (PRX). The conserved cysteine pair in the CxxxC motif of SCO is essentially in positions identical to the one in TRX and the cysteine and Thr pair in PRX. The α–helices (blue) and β–sheets (red) near the active sites of SCO, TRX, and PRX also align with each other three-dimensionally. This structural alignment holds true for nearly all members of the thioredoxin family currently deposited in the Protein Data Bank. The Figure is modified from Williams et al. 51.
2.3 Proposed Functions of the SCO Protein

One of the proposed roles for the SCO protein, rather than as a copper ion transporter, is that it functions as a redox catalyst. This concept was introduced because of the similarity between the SCO protein sub-family and thiol-disulfide reductases as well as peroxidases (see Figure 5) (both from the thioredoxin family). Indeed, it has been widely speculated that proteins that possess the thioredoxin fold are likely to catalyze disulfide redox exchange reactions in vivo. The SCO protein possesses a thioredoxin fold in its core structure, consisting of a combination of α helices and β sheets. The crystal structure of BsSCO has been shown to be similar to the thioredoxin proteins (see Figures 4 and 5). The fold of SCO is thioredoxin-like, consisting of a central β-sheet surrounded by α-helices as illustrated in Figures 4 and 5. Consequently, the structural similarity of the SCO protein to the thioredoxin protein family suggests a redox role for SCO in place of, or in addition to its role as a copper chaperone.

Other possible roles of the SCO protein in vivo have been proposed besides the copper ion delivery role in CcO assembly. For example, the cysteine residues are suggested to be involved in the redox preparation of the Cu₄ site while the protein may not necessarily bind copper ion(s) in vivo. Indeed, the disulfide bridge formed between the copper-binding cysteine residues can readily undergo redox reactions by strong reducing agents such as DTT or TCEP. The SCO protein has also been implied in sensing and reacting with peroxides within the cell. Or, the SCO protein could be involved in the maintenance of chemical control for copper ions within the cell and the balance of intracellular redox
equilibrium\textsuperscript{55}. However, these different proposals on the function of the SCO protein require further investigation.

The role of the SCO protein as a copper chaperone is questioned by the failure to obtain protein crystals with either Cu(I) or Cu(II) ions\textsuperscript{52,56}. Co-crystallization of BsSCO with Cu(II) has been unsuccessful, although it can be argued that the lack of observed copper binding is due to potential crystallization problems or due to adventitious copper binding rather than a functional necessity. To illustrate, the reported crystal structure of BsSCO does not contain any bound copper ion but contains both the thiol (reduced) and disulfide (oxidized) conformers\textsuperscript{52}, reinforcing a redox-switching role for BsSCO. Furthermore, efforts to co-crystallize other members of the SCO protein family with Cu(I) or Cu(II) ion have failed thus far\textsuperscript{48,52,56}. Human SCO1 protein crystal structure has been solved without any bound copper ion. Consequently, the lack of a bound copper ion to the conserved site in various SCO crystal structures from different species has led to the proposal that the SCO protein is not a copper chaperone that is able to bind and release copper ions to its target. Noticeably, a research group has solved the yeast SCO1 protein (\textit{i.e.} ScSCO1) structure with bound copper ions\textsuperscript{48}. However, the bound copper ions are not found at the proposed, conserved copper-binding site. Three Cu(II) ions are bound to a different pair of cysteine residues than those identified by conservation as copper ligands.

Despite all these proposals and possibilities suggested, spectroscopic findings and mutational studies suggest the potential biological role of SCO is as a copper chaperone to deliver copper ions to Cu\textsubscript{A}\textsuperscript{57,58}. A SCO-Cu complex has not been captured by X-ray crystallography, but it has been widely documented
by other methods and studies. For example, BsSCO knockout strains can regain functional CcO expression by supplementing the growth medium with high copper ion concentration\textsuperscript{43}. In other words, functional oxidases from \textit{B. subtilis} can be assembled in the absence of BsSCO only when high copper ion content is provided. This observation supports the potential role of the SCO protein as a copper chaperone. In fact, SCO proteins have been categorized as a family of metal-binding proteins. BsSCO has been shown to bind Cu(I), Cu(II) and Ag(I) ions with 1:1 stoichiometry\textsuperscript{57,58}. This equal-molar stoichiometry is consistent with the studies that show a stable BsSCO-Cu(II) complex\textsuperscript{25}. Moreover, spectroscopic studies have shown that yeast and human SCO proteins can bind one molar equivalent of the copper ion in either Cu(I) or Cu(II) oxidation state\textsuperscript{59}. This observation also supports the proposed role of the SCO protein as a copper ion shuttle, delivering copper ions to the Cu\textsubscript{A} site of CcO.

There are dissimilarities between the family of SCO proteins and that of thioredoxin proteins. Firstly, the conserved cysteine-containing motifs from the two protein families are positioned in different sequence locations. The catalytic cysteine residues in thioredoxin are located on an \(\alpha\)-helix, buried relatively within the hydrophobic core of the protein, whereas the two copper-binding cysteine residues from the SCO protein are on a relatively exposed and flexible loop region (see Figure 6). In addition, proline residues, which are considered helix-breaker, are found in close proximity to these cysteine residues in SCO. Moreover, the thioredoxin and other related protein families with a thioredoxin-fold contain a conserved \textit{cis}-proline residue that is absolutely required for its function\textsuperscript{60,61}. However, this proline residue is not found in SCO proteins. Taken
together, structure and function relationships suggest that while SCO proteins may have overall homology to the thioredoxin family, the SCO proteins have evolved an ability to bind copper ions and become escort factors for CcO biogenesis.

Certain organisms express two or more SCO proteins with great sequence and structural similarities, yet they are proposed to function differently\textsuperscript{62,63}. It is shown that yeast SCO2 protein (i.e., ScSCO2) can partially complement a COX17 knockout only if supplemented with extra copper ions in the growth medium. Interestingly, yeast SCO2 cannot complement a SCO1 knockout, suggesting the two SCO forms in yeast have non-overlapping functions within the cell\textsuperscript{45,64}. As a result, the family of SCO proteins has also been proposed to directly insert both of the copper ions that are found in the Cu\textsubscript{A} centre\textsuperscript{45,59}. Although ScSCO2 is likely to be involved in copper incorporation into the Cu\textsubscript{A} site, the detailed function of yeast SCO2 is unclear. Similar studies have been done for human SCO1 and SCO2\textsuperscript{65}. Mutations in human SCO2 lead to distinct pathological phenotypes compared to SCO1 mutants, yet the oxygen-metabolizing function of the human CcO is only partially affected by a SCO2 knockout.

2.4 Copper-binding Properties of the SCO Protein

\textit{Bs}SCO and ScSCO1 are homologous proteins that bind copper ions via the same conserved cysteine and histidine residues. However, \textit{Bs}SCO and ScSCO1 are distinct in that \textit{Bs}SCO binds Cu(II) in preference to Cu(I)\textsuperscript{24}, whereas ScSCO1 has been reported as a Cu(I)-binding protein\textsuperscript{27}. As illustrated in Table 1, the relatively high dissociation constants of \textit{Bs}SCO for Cu(II) ion has been
deduced by DSC ($K_D \sim 3.5 \times 10^{-12}$ M)$^{24}$ and ITC measurements ($K_D \sim 6.5 \times 10^{-8}$ M)$^{25}$. Noticeably, the equilibrium binding affinity estimated by ITC should be considered as an upper limit (i.e., the affinity could be much higher). In other words, the binding affinity of BsSCO for the Cu(II) ion falls in the range between $10^7$ ITC and $10^{11}$ M$^{-1}$ (DSC). In contrast, compared to the tight binding of BsSCO for Cu(II) ion, the SCO protein binds Cu(I) with a $K_D$ in the micro-molar range$^{24}$ (see Table 1). Noticeably, tight binding has been reported between eukaryotic SCO and Cu(I) ions (see Table 1). For example, the human SCO1 protein has been reported to bind Cu(I) ions extremely tightly, comparable to the dissociation constant of BsSCO-Cu(II) complex. Shown in Table 1, one research group has demonstrate a tight-binding between BsSCO and Cu(I) with an apparent $K_D$ in the pico-molar range (i.e., $\sim 10^{-13}$ M). There are, therefore, discrepancies in quantifying the binding affinity of the SCO protein for copper ions.

<table>
<thead>
<tr>
<th>Species</th>
<th>Oxidation State</th>
<th>Cu(II)</th>
<th>Cu(I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BsSCO</td>
<td>$K_D \sim 3.5 \times 10^{-12}$ M (DSC)$^{24}$</td>
<td>$K_D \sim 6.5 \times 10^{-8}$ M (ITC)$^{25}$</td>
<td>$K_D \sim 10.8 \times 10^{-6}$ M (titration)$^{24}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_D \sim 10^{-13}$ M (competition)$^{23}$</td>
</tr>
<tr>
<td>ScSCO1</td>
<td>No data available</td>
<td></td>
<td>No data available</td>
</tr>
<tr>
<td>Human SCO1</td>
<td>No data available</td>
<td></td>
<td>$K_D \sim 3.1 \times 10^{-15}$ M$^{66}$</td>
</tr>
</tbody>
</table>

Table 1. Binding affinities of SCO proteins for copper ions. Binding affinities are expressed as dissociation constants, $K_D$, of SCO-Cu complexes. ScSCO1 and human SCO1 have been shown as copper-binding proteins, however binding affinities have not been quantitatively estimated except for the human SCO1-Cu(I) complex.

There are certainly biological differences between Bacillus and the more complicated eukaryotic yeast with compartmentalized internal organelles to account for the difference in binding affinities of the SCO protein for Cu(II) and
Cu(I) ions. BsSCO is localized to the space between the cell wall and the plasma membrane of the gram-positive bacterium while ScSCO1 is compartmentalized in the inter-mitochondrial space and anchored in the inner mitochondrial membrane. The cell wall presents little in the way of a diffusional barrier to copper ions but is mainly for physical support. Although the mechanism of copper ion trafficking is not fully understood in prokaryotic cells, it is likely for BsSCO to have access to Cu(II) ions as in contrast to eukaryotic SCO proteins. In other words, BsSCO would have access to both Cu(I) and Cu(II) ions without the assistance from any upstream copper ion source. In agreement with this concept, BsSCO has been shown to bind to both Cu(I) and Cu(II) ions in vitro\textsuperscript{25}. In addition, no prokaryotic homolog of COX17 has been identified (\textit{i.e.}, as the upstream copper source of prokaryotic SCO). Within the inter-mitochondrial space, Cu(I) is expected to be the predominant form under a relatively more reducing condition. As such, copper ions are not considered to be available as Cu(II) within eukaryotic cells. In addition, these ions (\textit{i.e.}, Cu(I) or Cu(II)) are thought to be bound to proteins at virtually all times.

Another feature that could contribute to the difference in binding affinities of BsSCO and ScSCO1 for copper ions is a structural one. The three conserved amino acid residues are critical in copper-binding of the SCO protein. However, as shown in Figure 6 the two conserved cysteine and the histidine residues from BsSCO are located on two different flexible loops. In contrast, the eukaryotic SCO proteins such as ScSCO1 and human SCO1 possess only one flexible loop that is able to rotate freely without much steric hindrance (see Figure 6). This difference in the degree of flexibility between BsSCO and ScSCO1 may account
for the difference in binding affinities. Moreover, the coordination chemistry of Cu(I) and Cu(II) ions is often distinct. The reduced Cu(I) ion prefers sulphur donor ligands such as cysteine or methionine, yet Cu(II) forms stable coordination with nitrogen donors such as histidine or oxygen donors such as glutamate or aspartate. The binding of Cu(I) with the SCO protein, therefore, exhibits triagonal coordination by the three conserved residues, whereas Cu(II) binding includes a possible water molecule as the fourth ligand.

Figure 6. Putative copper-binding residues in SCO protein structures. The copper-binding sites of SCO proteins from Bacillus (BsSCO), yeast (ScSCO1), and human (HsSCO1) are shown along with labeled putative copper-binding residues. This figure is modified from Abajian and Rosenzweig.

3.1 Protein Expression and Purification

Recombinant *B. subtilis* SCO (*BsSCO*) protein was expressed as a water-soluble fragment of the full-length native *BsSCO*. Primers used in the construction of the soluble *BsSCO* were reported previously\(^{57}\). The shortened DNA sequence of the native protein was inserted into the pDA295 plasmid vector, giving a glutathione-S-transferase (GST) – *BsSCO* fusion construct. The fusion protein was expressed in *E. coli* BL21 (DE3) cell strains. The transformation protocol was followed from early studies\(^ {43}\). The *E. coli* cells were streaked onto LB (ampicillin) plates for overnight incubation. Bacterial cultures from the overnight incubation were allowed to grow in four 1.4 L Fernbach flasks that each contained 700 mL of LB media (pH 7.6) and antibiotic ampicillin (100 µg/mL). The cell density was constantly monitored by measuring optical absorbance at 600 nm. A reference reading was taken of just the growth media prior to inoculation. When the optical density at 600 nm increased to between 0.6 and 0.8, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to each of the flasks to a final concentration of 1 mM. The *E. coli* cell cultures were harvested three hours, following induction, by centrifugation (32,600 x g). The pelleted cells were re-suspended in phosphate buffered saline (PBS) at pH 7.4 with 1 mM ethylenediamine tetraacetic acid (EDTA). Finally, 80 mL of the re-suspended cell extracts were split into two 50 mL conical tubes and frozen in storage freezer -80 °C.
Frozen cells (stored in 50 mL conical tubes) were thawed at room temperature and then incubated with DTT (final concentration 5 mM), lysozyme (1 mg/mL), DNase and RNase (both 37.5 mg/mL) for 30 minutes at 4 °C. The lysed and digested cell extracts were later divided into four tubes, each of which contained 20 mL of the extract. Each tube was subsequently mixed with 10 mL of 4% Triton X-100 (final concentration 1%), followed by another incubation period of 1 hour at 4 °C. Finally, the cell extract was centrifuged at 32,600 x g for 20 minutes in a Beckman J6 centrifuge that employed a JA-25.5 rotor. The supernatant was harvested and the pellet discarded. The pH of the supernatant was adjusted to 6.4 by additions of sodium hydroxide (100 mM).

The supernatant (approximately 160 mL) was loaded onto a 9 mL Glutathione Sepharose 4B Fast Flow column (Amersham) at a rate of 1.5 mL/minute. The column was subsequently washed with approximately 30 mL of PBS at pH 7.7 at the same flow rate, removing proteins that were bound non-specifically to the column. To allow the cleavage of the fusion protein, two hundred units of the thrombin protease (Sigma) diluted in PBS pH 7.7 were added to the column at a rate of 1.5 mL/minute. The chromatography set-up was left at room temperature (~24 °C) for 16 hours to achieve complete cleavage of the fusion protein by the protease. The cleaved target protein, BsSCO, was subsequently eluted and collected in PBS pH 7.7. Residual thrombin units were removed from the eluents that contained the SCO protein by incubation with Benzamidine Sepharose 4 Fast Flow resin (Amersham) for 1 hour at 4 °C, and then by elution in PBS pH 7.7. The final purified protein was exchanged for sodium phosphate buffer pH 7.0 and was concentrated by centrifugation at 1,500
x g in a Beckman J6/M centrifuge that accommodated Amicon Ultra-15 concentrators (10,000 MW cut-off). The purity of the target protein was assessed by SDS-PAGE.

The homologous SCO protein from S. cerevisiae was expressed and purified in a similar fashion as to recombinant BsSCO. However, the soluble domain of the ScSCO1 protein was cloned into a different expression vector — pJR1284. Moreover, the ScSCO1- and BsSCO-GST fusion proteins appeared to possess distinct sensitivities towards thrombin proteolysis. As a result, the GST moiety was removed from the fusion with ScSCO1 protein using less thrombin units (approximately 50 units) for a shorter incubation time (< 1h).

3.2 Preparation of Reduced Apo-SO Proteins

Due to the presence of excess DTT during protein purification, samples were predominantly reduced (i.e., thiol count close to two). If a purified BsSCO or ScSCO1 sample had a relatively low thiol count, then the oxidized sample was mixed with Gdn-HCl (final concentration 3 M), DTT (2 mM), BCS (50 µM), and EDTA (250 µM). The mixture was incubated overnight at room temperature. Subsequently, centrifugal ultrafiltration was performed to remove the non-protein reagents at 1,500 x g with an Amicon Ultra-15 concentrators (10,000 MW cut-off). A free thiol count (see later) was performed to evaluate the redox state of the DTT-reduced protein sample (thiol count close to two was ideal for further copper-binding experiments). All buffers used after the initial purification of SCO proteins were treated with Chelex resin (Sigma) to remove any trace of contaminant metal ions.

3.3 Assessment of Reactive Thiol Groups
Quantification of the protein concentration was accomplished spectrophotometrically using an established extinction coefficient for BsSCO at 280 nm of 19,180 M$^{-1}$cm$^{-1}$. The molar absorptivity for ScSCO1 at 280 nm was 29,000 M$^{-1}$cm$^{-1}$. The spectrum of the sodium phosphate buffer alone was used as reference and subtracted from spectra of the SCO protein.

Protein samples were assessed for the presence of reduced thiol groups from the cysteine residues in SCO sequence by their reactivity with dithiodipyridine (DTDP) (Bio-RAD). A split cuvette was filled with 780 µL sodium phosphate buffer (pH 7.0) on one side (i.e., the protein side). The other side of the split cuvette was filled with 798 µL of the buffer (i.e., the buffer side). Reference UV absorbance spectra were recorded before addition of 20 µL of the concentrated protein stock to the protein side of the cuvette, achieving a total volume of 800 µL and a 1:40 dilution of the protein. After the addition of the SCO protein, UV spectra were recorded/scanned. DTDP (2 µL, 40mM) was subsequently added to the buffer side of the cuvette to make up a total volume of 800 µL. Several spectral scans (from 220 nm to 700 nm) were recorded after the addition of DTDP until a stable reading was obtained. The split cuvette was then sealed at the top by parafilm and solutions from both sides of the cell were evenly mixed by inversion. After mixing the protein sample with DTDP, another set of spectra was recorded. The spectroscopic focus was shifted from 280 nm to 324 nm due to the appearance of the thiopyridone species, which was the product from the reaction of reduced thiol groups with DTDP.

DTDP molecules react with reduced thiol groups in 1:1 stoichiometry. Therefore, the absorbance band centred at 324 nm corresponds to the thiol
concentration. The molar absorptivity of 4-thiopyridone (\(i.e., \epsilon_{324}\)) was 18,800 M\(^{-1}\)cm\(^{-1}\). In order to determine the number of reactive thiol groups on the protein, a ratio was calculated between the spectra intensities at 280 nm and at 324 nm after the reaction is complete. The expected number for a BsSCO sample with completely reduced cysteine residues is two. The same method was employed for evaluating the redox state of the thiol groups from the yeast SCO protein. Since there are four reactive cysteine residues in ScSCO1 the thiol count is expected to be four for the completely reduced protein.

### 3.4 Competition Between SCO and Chelators for Cu(II) Ions

A stock solution of 3.3 mM CuCl\(_2\) was prepared. The concentration of the copper ion was carefully measured by converting Cu(II) to Cu(I) with excess sodium dithionite and subsequently measuring the absorption intensity of BCS-Cu(I) complex at 484 nm (\(\epsilon_{484} = 13,000\) M\(^{-1}\)cm\(^{-1}\)). For copper-binding experiments, small aliquots of the Cu(II) stock solution were diluted (final concentration 10 µM) with sodium phosphate buffer (50 mM, pH 7.0) in a 1 cm path length cuvette. Spectra of the phosphate buffer and the Cu(II) solution were scanned separately as references. One molar equivalent amount of EDTA (\(i.e., 10\) µM) was titrated in several additions into the diluted copper solution while the UV spectra of the sample were constantly monitored and recorded. The mixture of Cu(II) and EDTA was left at room temperature for several minutes to allow the binding of the two species to reach an equilibrium. The EDTA-Cu(II) complex did not have distinguishable absorption properties within the scanned wavelength range (\(i.e., 220-700\) nm), however its formation could be assessed by electron paramagnetic resonance (EPR). Aliquots of concentrated BsSCO stock were
titrated into the solution of EDTA-Cu(II) to observe the degree of competition of the two “ligands” for Cu(II) ions. The extent of Cu(II) binding to BsSCO was determined by measuring the absorption at 352 nm. When Cu(II) was bound to the BsSCO protein, the complex had a characteristic absorbance centred at 352 nm (ε = 4,780 M⁻¹cm⁻¹)²⁵ (362 nm for ScSCO1-Cu(II)). All absorbance spectra were measured on a Hewlett-Packard HP-8452A diode array spectrophotometer.

In experiments in which the SCO protein competed for Cu(II) binding with ligands EGTA and NTA, the set-up was the same except the competitive ligand EDTA was simply replaced by EGTA or NTA. BsSCO was subsequently added into the solutions as a metal ion competitor for Cu(II) ions.

3.5 Competition Between SCO and Copper Ligands for Cu(I) Ions

Because Cu(I) readily reacts with oxygen generating Cu(II) and partially reduced O₂, all Cu(I) experiments were conducted under anaerobic conditions. Solutions of Cu(I) were prepared in a Thunberg flask under an Argon atmosphere. Transfers of Cu(I) into the Argon equilibrated protein solution were completed by using suitable gas-tight syringes. In parallel experiments, Cu(I) ions in aqueous solution were prepared by reducing Cu(II) with sodium dithionite.

To characterize the binding affinity of SCO protein for Cu(I), BCS and ferrozine (Fz) were used as the competing ligands against apo-SCO proteins. BCS and Fz form coloured complexes with Cu(I) ions of known binding affinity. Titrations were performed in the presence and absence of the SCO protein to determine its relative affinity for Cu(I).

Because binding of Cu(I) to SCO proteins does not yield observable electronic absorption spectrum nor an observable change in far-UV CD, the
competition was monitored by the formation of the ligand-Cu(I) complex. The presence of Cu(I) was assessed by complex formation with BCS using an extinction coefficient of 12,250 M⁻¹cm⁻¹ at 483 nm. The extent of Cu(I) binding to Fz was determined by measuring the absorption at 480 nm with an extinction coefficient of 4,320 M⁻¹cm⁻¹. Cu(I) ion binding to the SCO protein was also measured by the degree of quenching of the intrinsic fluorescence of the protein.

### 3.6 Intrinsic Tryptophan Fluorescence

Fluorescence data were recorded using a Horiba Jobin-Yvon Fluorolog-3 fluorescence spectrometer, with an excitation slit set at 1 nm and an emission slit set at 2 nm. The excitation wavelength was 280 nm for all experiments, while emission spectra were scanned from 300 nm to 450 nm in 1 nm increments.

### 3.7 Circular Dichroism Spectroscopy

Circular dichroism spectra were measured using a Chirascan spectrometer (Applied Photophysics) and a digital-subtractive circular dichroism module. Spectra of the BsSCO samples (final concentration 10 µM) in 10 mM phosphate buffer (pH 7.0) were scanned from 260 nm to 180 nm in 1 nm wavelength increments using a 0.1 mm path-length quartz cuvette (Hellma). For denaturing experiments, the SCO protein was mixed with urea at varying concentrations (0 M to 8 M) and equilibrated for 1 h at room temperature. For thermal scanning of the sample, spectra were recorded at regular intervals as the temperature increased. The temperature ramp was set at a rate of 1 °C/minute climbing from 25 °C to 90°C. Due to the physical nature of the particular CD apparatus, the maximal temperature obtained was 80 °C, which was sufficient to denature the protein sample.
Chapter 4

RESULTS

4.1 Purification of BsSCO and ScSCO1

Quantitative characterization of biomolecules such as a protein of interest requires its successful biosynthesis and purification. The GST-SCO fusion protein was expressed in *E. coli*, purified from the cytosolic extract, and the linker between GST and SCO moieties was cleaved by thrombin protease treatment to give soluble recombinant SCO. Purification was evaluated at each stage by SDS-PAGE to ensure proper expression, and the purity of the final product (see Figure 7 for BsSCO and Figure 8 for ScSCO1).

An average yield of approximately 10 mg per 2.8 L cell culture media was routinely obtained for BsSCO. A Coomassie blue stained gel of fractions from a preparation of recombinant BsSCO is illustrated in Figure 7. IPTG induction during the culturing period led to an accumulation of an intense protein band in the cell extract, with an apparent molecular weight of 46,000 shown in Lane 2 (cell extract, *i.e.*, EX) in Figure 7, corresponding to the GST-BsSCO fusion protein. The cell extract that contained the fusion proteins was passed over the GST affinity column, and the pass-through collected (*i.e.*, PT). The band corresponding to the GST-BsSCO fusion is greatly diminished in the pass through (Figure 7, Lane 3 as indicated by the red arrows) indicating good binding between the GST domain and the affinity resin. The isolated fusion protein was subsequently cleaved on the column by incubation with thrombin for 16 hours. After the proteolytic process, the soluble domain of the SCO protein elutes from
the glutathione-affinity column (Figure 7, Lane 4). Following the cleavage period, the bound GST motif was washed off from the column by including glutathione in the buffer (Figure 7, Lane 5). By the end of the thrombin cleavage stage, two sample fractions are obtained that corresponded to the BsSCO and the GST domains with apparent molecular weights of 19,000 and 24,000, respectively. The relative positions on the gel and molecular weights of the proteins were consistent with previous studies\textsuperscript{57}. The final purified fraction of SCO was further treated with benzamidine-linked Sepharose (\textit{i.e.,} to remove thrombin) and by centrifugal ultrafiltration (Figure 7, Lane 6). The concentration of stock SCO protein was estimated by UV-spectrophotometry at the end of each preparation. The absorbance peak at 280 nm was used to determine protein concentration ($\epsilon_{\text{BsSCO}} = 19,200 \text{ M}^{-1}\text{cm}^{-1}$; $\epsilon_{\text{ScSCO1}} = 29,000 \text{ M}^{-1}\text{cm}^{-1}$).
Figure 7. Purification of BsSCO. The samples were treated with 10% sodium dodecyl sulfate (SDS), 0.3 M Tris-HCl pH 8.0 and bromophenol blue. The mixtures were heated at 37°C for one hour prior to loading. Lane 1 (ladder): low range protein standards (BioRad) corresponding to molecular weights of 15 000, 27 000, 35 000, and 55 000. Lane 2 (cell extract): post-induction cell extracts combined from four independently grown cell cultures containing the fusion protein (1.6 µg). Lane 3 (pass through): elution with buffer pH 7.7 containing a small fraction of the fusion protein (1.2 µg). Lane 4 (thrombin treatment): elution with thrombin protease (Sigma, 200 units) containing SCO (3.4 µg) and other unidentified protein bands. Lane 5: elution with glutathione following thrombin treatment containing the GST moiety (11 µg). Lane 6: final BsSCO sample after thrombin removal and centrifugal concentration (1.4 µg).

The preparation for ScSCO1 was performed in a similar fashion as the BsSCO purification. However, the average yield of ScSCO1 per 2.8 L cell culture media was less than 5 mg. The low yield of eukaryotic SCO is due to the greater sensitivity of the ScSCO1 domain to unintended thrombin proteolysis. As shown in Figure 8, fractions from each stage of ScSCO1 purification were collected. The GST motif has the same molecular weight and it falls in the same position on the SDS-PAGE gel relative to the standard ladder (Figure 8, Lane 4). The recombinant ScSCO1 was longer in the primary structure (approximately 50
amino acid residues) and was heavier in overall molecular weight (approximately 22,000). Lane 5 in Figure 8 indicates that the purity of the concentrated ScSCO1 is good.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Samples</th>
<th>Mol. Wt. Stds. (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ladder</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>EX</td>
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</tr>
<tr>
<td>3</td>
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<td>5</td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>(0.5µg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.2µg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.4µg)</td>
<td></td>
</tr>
</tbody>
</table>

_Figure 8. Purification of ScSCO1._ The samples were treated with 10% sodium dodecyl sulfate (SDS), 0.3 M Tris-HCl pH 8.0 and bromophenol blue. The mixtures were heated at 37°C for one hour prior to loading. Lane 1 (ladder): low range protein standards (BioRad) corresponding to molecular weights of 15 000, 27 000, 35 000, and 55 000. Lane 2 (cell extract): post-induction cell extracts combined from four independently grown cell cultures containing the fusion protein (2.4 µg). Lane 3 (pass through): elution with buffer pH 7.7 containing a small fraction of the fusion protein (0.5 µg). Lane 4: elution with glutathione following thrombin treatment containing GST (0.2 µg). Lane 5: final ScSCO1 sample (0.4 µg) after thrombin removal and centrifugal concentration.

**4.2 Evaluation of the Redox State of Cysteine Residues**

An important characteristic of the SCO protein is the redox state of its thiol groups, which are expected to be important determinants of the protein’s functional status. The primary sequence of the protein contains two conserved copper-binding cysteines that are three residues apart. They are able to form a
disulfide bond, completely depriving the SCO protein of its ability to bind copper ions\textsuperscript{52}. Only SCO proteins with two reactive thiol groups can bind copper ions in stable complexes. Therefore the thiol count is important in evaluating if a sample is suitable for further copper-binding and competition experiments. Because this study focuses on characterizing the copper-binding properties of BsSCO and ScSCO1, the redox states of the SCO proteins were assessed by evaluating the number of their reactive thiol groups prior to any binding experiments.

DTDP is the key reagent in the assay since a thiopyridone product is formed when DTDP reacts with a thiol group. The thiopyridone product has a characteristic UV spectrum centred at 324 nm ($\epsilon = 18,800 \text{ M}^{-1} \text{cm}^{-1}$)\textsuperscript{69}. Therefore, the change in absorbance at 324 nm is used to determine the concentration of thiol groups available for reaction in the presence of an excess of DTDP. The difference between the reference UV absorbance of DTDP and the spectra of the thiopyridone product at 324 nm indicates the concentration of reactive thiol groups in the mixture. The ratio of the concentration of the thiopyridone over the concentration of the tested SCO sample gives the thiol count, or SH count. While the thiopyridone product has a characteristic UV absorbance peak at 324 nm, BsSCO has a signature UV absorbance peak due to its aromatic amino acid content, primarily tryptophan and tyrosine residues in the protein ($\epsilon_{280} = 19,200 \text{ M}^{-1} \text{cm}^{-1}$). Reduced BsSCO should yield two moles of thiol per mole of the protein. Such samples were used as “reduced” protein in subsequent experiments. Completely reduced and oxidized samples of apo-BsSCO should ideally have two and zero free thiol groups, respectively. Based on calculations of the UV spectral data, a typical reduced sample had an SH count of 1.8 – 2.0, whereas
the same value for an oxidized sample was 0 – 0.2. A typical spectrophotometric determination of protein free thiol groups is illustrated in Figure 9. The thiol count was consistent with the Cu(II) binding to BsSCO. For example, a thiol count of 1.8 implied that about 90% of Cu(II) were bound to the protein. For copper-binding related experiments, a thiol count of 1.8 was set to be the acceptable lower limit for SCO protein sample.

Figure 9. Evaluation of the redox states of thiol groups by DTDP. Left) UV absorption spectra of 40 µM 4-thiopyrdone (solid line), 40 µM DTDP (dashed line), and 40 µM mercaptan (dotted line) in aqueous buffer at pH 7.0. Right) Two-step reaction of mercaptan with DTDP yields one equivalent of 4-thiopyridone in each step. The molar absorptivity (ε) refer to λ = 324 nm. Figure is modified from Riener et al.69

The redox state of purified protein was not identical between preparations, with some preparations yielding higher numbers of reduced thiol groups per mole of protein than others. Purified BsSCO protein eluted from the column varied in
the degree of reduction, from zero reactive thiol group to two free thiol groups per protein. This variation could be due to many factors such as the contamination of the solutes, from handling, and the exposure to oxidizing reagents such as oxygen in the air. In order to recycle SCO proteins with low numbers of free thiol groups, oxidized samples were treated with DTT, EDTA, and BCS. With treatment by the strong reducing agent and the metal chelators, the number of thiol groups per mole protein in the reduced state increased significantly and could be thus used again in copper-binding experiments (data not shown).

4.3 Copper-binding by BsSCO and ScSCO1

Upon the introduction of Cu(II) ion into a reduced apo-BsSCO sample (10 µM), a new UV spectra feature is observed that has a prominent absorbance peak at 352 nm (ε = 4,780 M⁻¹cm⁻¹). The absorbance peak at 352 nm results from the interaction between the two cysteine residues of the SCO protein with a Cu(II) ion. In addition, the characteristic spectrum of the BsSCO-Cu(II) complex also has a shoulder at 452 nm (secondary) and a separate peak at 552 nm (tertiary)²⁵.

In addition to apo-SCO as the concentration of the Cu(II) ion increases, the absorbance intensity at 352 nm increases proportionally and eventually reaches a plateau. The absorbance peak at 352 nm increases proportionally with the amount of Cu(II) ions added until equal molar amounts of BsSCO and Cu(II) ions are present. Further addition of copper beyond one copper ion per BsSCO molecule yields no further spectral change. Reaction stoichiometry is inferred by extrapolating the two linear segments of the graph (total ligand added vs. total acceptor) to their point of intersection, which represents the endpoint of the titration. This pattern is consistent with previous studies that showed a 1:1
binding stoichiometry. The concentration of Cu(II) and the absorbance intensity of the Cu(II)-BsSCO were used to plot a binding curve with the total concentration of the Cu(II) ion on the x-axis, and the absorbance intensity on the y-axis.

Figure 10. Binding curve of BsSCO and Cu(II) ions. Cu(II) ions were titrated into a solution of BsSCO while the absorbance was monitored from 220-700 nm. The ratio of the total Cu(II) concentration to the protein concentration is plotted against the absorbance at 352 nm, indicating the formation of BsSCO-Cu(II) complex. For every addition of Cu(II), there is a proportional increase in absorbance until approximately one molar equivalent of copper ions added. The increment abruptly stops, indicating that the interaction between BsSCO and Cu(II) ions is saturated.

A similar titration of Cu(II) ions with ScSCO1 was performed as that described above with BsSCO (see Figure 11). The yeast SCO1 protein is able to bind to Cu(II) ions. The ScSCO1-Cu(II) complex has an absorbance peak centred at 360 nm (data not shown). The binding curve of ScSCO1 and Cu(II) ions is
plotted in Figure 11. Tight binding is observed between ScSCO1 and Cu(II) ions with a sharp break point. Such behavior indicates a binding affinity of less than 1 µM, but without further studies a better estimate cannot be made of how tightly the yeast SCO protein binds Cu(II) ions.

![Figure 11. Binding curve of ScSCO1 and Cu(II) ions.](image)

Cu(II) ions were titrated into a solution of ScSCO1 while the absorbance was monitored from 220-700 nm. The ratio of the total Cu(II) concentration to the protein concentration is plotted against the absorbance at 360 nm, indicating the formation of ScSCO1-Cu(II) complex. For every addition of Cu(II), there is a proportional increase in absorbance until approximately one molar equivalent of copper ions added (i.e., ~ 20 µM). The increment abruptly stops, indicating that the interaction between BsSCO and Cu(II) ions is saturated.

4.4 Cu(II) Competition: BsSCO with EDTA, EGTA, and NTA
As mentioned previously, the SCO protein has such high affinity for Cu(II) ions that traditional titration experiments are unable to quantitatively assess the affinity. Therefore, competition for Cu(II)-binding is performed between BsSCO and other copper ion ligands, or chelators such as ethylene diamine tetraacetic acid (EDTA). EDTA is a hexadentate chelator that binds copper ions through its two amines and four carboxylates moieties. It adopts an octahedral geometry to form strong complexes with Cu(II) ions. Because of its high denticity, the chelator has a high affinity for copper ions (i.e., $K_D \sim 3.1 \times 10^{-16}$ M) (see Table 2).

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_D$ for Cu(II)-ligand (M)</th>
<th>Effect on BsSCO-Cu(II) formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>$3.1 \times 10^{-16}$ (i.e., 0.31 fM)$^{70}$</td>
<td>an initial lag phase</td>
</tr>
<tr>
<td>EGTA</td>
<td>$2.5 \times 10^{-14}$ (i.e., 25 fM)$^{70}$</td>
<td>half of the BsSCO-Cu(II) formed at one molar equivalent of Cu(II) ions</td>
</tr>
<tr>
<td>NTA</td>
<td>$5 \times 10^{-13}$ (i.e., 50 pM)$^{71}$</td>
<td>Observed copper ion exchange</td>
</tr>
</tbody>
</table>

Table 2. Binding affinities of copper chelators for Cu(II) ions.

The binding curve of BsSCO and Cu(II) ions in the absence and presence of one molar equivalent of EDTA is shown in Figure 12. In this particular competition, EDTA was first mixed to an apo-BsSCO sample. The introduction of EDTA to BsSCO has no apparent effect on the UV absorbance across the spectrum of the protein (i.e., 220 nm – 700 nm) (data not shown). Aliquots of Cu(II) ions were subsequently titrated into the mixture of equal molar BsSCO and EDTA. BsSCO and EDTA in the solution thus compete for Cu(II) binding. However, titrating Cu(II) ions into BsSCO and EDTA mixture did not reveal a proportional increase in absorbance at 352 nm until one molar equivalent of Cu(II) ions was added to the BsSCO/EDTA mixture. We interpret this lag phase of
BsSCO-Cu(II) formation is due to the chelating effect of EDTA. In other words, BsSCO-Cu(II) complex was not formed until all EDTA has been bound to available copper ions in the system. This observation is consistent with the fact that EDTA has a reported higher Cu(II) binding affinity than that of BsSCO. With similar experimental set-ups, more EDTA had been added to the system to increase the molar concentration of the chelator, favoring the binding of EDTA with the Cu(II) ion. As expected, the lag phase in the appearance of the peak at 352 nm was elongated due to the increased concentration of EDTA (data not shown). This observed lag phase in Figure 12 supports the conclusion that EDTA competes with BsSCO for Cu(II) binding.
Figure 12. Competition for Cu(II) ions between EDTA and BsSCO. The binding curve of BsSCO and Cu(II) ions is shown in black squares. At one molar equivalent of Cu(II) ions and BsSCO, the absorbance intensity at 352 nm abruptly stops increasing, indicating that the interaction between Cu(II) and BsSCO is saturated. The binding curve of BsSCO and Cu(II) ions in the presence of one molar equivalent of EDTA is shown in red dots. The increment of absorbance intensity at 352 nm is delayed initially and reaches a plateau until approximately two molar equivalent of Cu(II) ions are added into the system.

In order to better estimate the binding affinity of BsSCO for Cu(II) ions, a ligand or chelator with lower binding affinity than EDTA for Cu(II) was needed. Ethylene glycol tetraacetic acid (EGTA) is a weaker Cu(II) chelator than EDTA and the EGTA-Cu(II) complex has an established dissociation constant of $2.5 \times 10^{-14}$ M$^{70,71}$. Thus, EGTA has a relatively 100-fold lower affinity for Cu(II) ions.
than that of EDTA. Similar to EDTA, however, EGTA does not have any distinct absorbance feature in the measureable UV range (i.e., 220 – 700 nm).

Figure 13 compares the binding curves of BsSCO and Cu(II) ions in the absence and presence of EGTA. In the absence of the chelator, tight binding behavior is exhibited between BsSCO and copper ions. Each addition of the copper ion leads to an equal increase in absorbance at 352 nm indicating the formation of BsSCO-Cu(II). At a ratio of one Cu(II) to BsSCO the increase in absorbance at 352 nm abruptly stops and the interaction is presumably saturated. In the presence of EGTA the formation of BsSCO-Cu(II) is delayed presumably due to formation of the EGTA-Cu(II) complex. At a ratio of one copper ion to BsSCO the reaction is approximately half-complete in the presence of one molar equivalent of EGTA. Such a result implies that EGTA and BsSCO have roughly equal affinities for Cu(II) ions (i.e., 25 fM). It is important to note that this conclusion assumes that the system attains equilibrium under the conditions used in all competition experiments.
Figure 13. Competition for Cu(II) ions between EGTA and BsSCO. The binding curve of BsSCO and Cu(II) ions is shown in black squares. At one molar equivalent of Cu(II) ions and BsSCO, the absorbance intensity at 352 nm abruptly stops increasing, indicating that the interaction between Cu(II) and BsSCO is saturated. The binding curve of BsSCO and Cu(II) ions in the presence of one molar equivalent of EGTA is shown in red dots. The increment of absorbance intensity at 352 nm is delayed and reaches a plateau until approximately two molar equivalent of Cu(II) ions are added into the system. At a ratio of one equivalent of copper ions to BsSCO, the absorbance intensity is approximately half of the maximal value obtained in the absence of EGTA, indicating that BsSCO equally competes for Cu(II) ions with the chelator.

In previous competition experiments, Cu(II) ions were titrated into a mixture of apo-BsSCO and competitor chelators with fixed, known molar concentrations. In order to assess the equilibrium status of the BsSCO/EDTA or the BsSCO/EGTA mixtures, the degree of copper ion exchange from each complex was measured. Firstly, titrations of BsSCO into a mixture of EDTA-Cu(II) complex were performed (see Figure 15), while the concentration of the protein
was increased upon each addition in order to achieve effective competition. The molar concentration of Cu(II) ions was kept in a 1:1 ratio to that of EDTA. Titration of BsSCO into a preformed EDTA-Cu(II) complex shows no evidence of BsSCO-Cu(II) formation (see Figure 15). Thus the conclusion is that EDTA does not give up Cu(II) ions easily. As illustrated in Figure 15, even in the presence of 4-fold molar excess of BsSCO over EDTA-Cu(II), the BsSCO-Cu(II) complex is still not observed to form. It is suggested that this observation is due to the extremely slow dissociation of the EDTA-Cu(II) complex. A parallel titration was also performed by adding EDTA into a solution of preformed BsSCO-Cu(II) and there is no measurable copper exchange from BsSCO to EDTA as judged by the absorbance intensity at 352 nm of the BsSCO-Cu(II) complex (data not shown). The conclusion is that BsSCO-Cu(II) does not give up Cu(II) easily, and there is a strong and stable interaction between BsSCO and Cu(II) ions, which is consistent with previous titration and calorimetry studies.

The equilibrium status of the BsSCO/EGTA mixture from the previous competition experiment was also evaluated. In these experiments BsSCO is titrated into a preformed EGTA-Cu(II) complex. Similar outcome is observed when EDTA was replaced by EGTA. Over the course of various incubation time (i.e., from 1 h to 10 h), no BsSCO-Cu(II) formation is observed (data not shown). Furthermore, this lack of exchange of copper ions is observed up to 10-fold in excess of the protein (data not shown). A similar result is obtained when EGTA is added to a preformed BsSCO-Cu(II) complex. The absorbance intensity at 352 nm of the BsSCO-Cu(II) species is unchanged upon additions of EGTA (up to 100-fold molar excess and over a period of 24 h) (data not shown). These
observations indicate that whichever Cu(II) bound species is formed (i.e., EGTA-Cu(II) or BsSCO-Cu(II)) it is inert to exchange copper ions. In other words, the dissociation rates of EGTA-Cu(II) and BsSCO-Cu(II) complexes are extremely slow. To illustrate, if the BsSCO-Cu(II) complex and EGTA did not exchange copper ions within 10 h, then the half-time for dissociation must be longer which corresponds to a $K_{\text{off}}$ of at least $2 \times 10^{-5}$ s$^{-1}$. This number is consistent with the estimations determined from previous kinetic studies$^{24}$. In the parallel experiment, no copper exchange was observed from the preformed EGTA-Cu(II) complex to BsSCO for at least 12 h (data not shown).

A third competitive ligand was selected for a Cu(II) binding experiment. Nitrilotriacetic acid (NTA) is also a copper chelating agent and forms coordination compounds with Cu(II) ions$^{70}$. Moreover, NTA is a tripodal tetra-dentate tri-anionic ligand, and it binds Cu(II) ions in a 1:1 ratio. NTA has a much lower binding affinity for Cu(II) ions than EDTA$^{70}$ (i.e., $K_D \sim 5 \times 10^{-13}$ M, see Table 2). Aliquots of BsSCO were titrated into a solution of the NTA-Cu(II) complex. As the concentration of SCO increases, absorbance at 352 nm is monitored (see Figure 14). The binding curve in Figure 15 shows the formation of the BsSCO-Cu(II) complex as measured at 352 nm in the presence of one molar equivalent of NTA. There is, indeed, formation of BsSCO-Cu(II) when BsSCO is titrated into a solution of the NTA-Cu(II) complex. Moreover, copper ion exchange is still observed when the concentration of NTA is increased up to 10-fold in excess to Cu(II) ions (data not shown). In comparison, this copper ion exchange is not observed when BsSCO is added to the preformed EDTA-Cu(II) complex as
discussed previously. In fact, no signal was observed at 352 nm when BsSCO is added to either preformed EDTA-Cu(II) or EGTA-Cu(II) complex.

Figure 14. Formation of BsSCO-Cu(II) in the presence of NTA. Equal molar amounts of Cu(II) and NTA were mixed and it was assumed that NTA-Cu(II) complex was formed. Aliquots of BsSCO (in colors) were titrated into the preformed NTA-Cu(II) complex as indicated by the growth of the absorbance peak at 280 nm ($\varepsilon_{\text{BsSCO-Cu(II)}} = 19,180 \text{ cm}^{-1}\text{M}^{-1}$). The appearance of the absorbance peak at 352 nm indicates the formation of the BsSCO-Cu(II) complex in the presence of the competing ligand NTA.
Figure 15. Copper ion exchange between BsSCO and NTA and EDTA. Aliquots of BsSCO were titrated into preformed NTA-Cu(II) (black squares) and EDTA-Cu(II) (red dots) complexes. The absorbance peak at 352 nm was monitored while the protein was added to the mixture. Copper ion exchange is observed between NTA-Cu(II) and BsSCO, yet the formation of BsSCO-Cu(II) is not observed in the presence of EDTA.

4.5 Cu(I) Competition: BsSCO with BCS and Fz

The SCO protein is a copper-binding protein, and is able to bind both Cu(II) and Cu(I) ions. In the CuA centre of cytochrome c oxidase, both copper ions are in the Cu(I) form when the site is reduced. Therefore, the characterization of Cu(I) binding by the SCO protein is of interest. In this project, the aim is to quantitatively determine the Cu(I) binding affinity of the SCO protein by using competition experiments. Bathocuproine disulfonate (BCS) is a derivative of phenanthroline and forms a BCS-Cu(I) complex that has absorbance peak at 484
nm. The transfer of copper ions from BsSCO to BCS was monitored in the following way. The BsSCO complex with Cu(II) was first made with copper ions added in an equal amount to the BsSCO concentration. Then the Cu(II) was reduced to Cu(I) by addition of an excess of sodium dithionite (see Figure 16A). It is observed that the band at 352 nm disappears indicating that the preformed BsSCO-Cu(II) is reduced upon addition of dithionite. Sodium dithionite has the added feature of reducing any oxygen present in the system. This chemical species also has absorbance at 308 nm that disappears on oxidation. The presence of absorbance at 308 nm in Figure 16A indicates excess dithionite (~0.1 mM) is present. When BCS is added to the system the BCS-Cu(I) complex is observed to form over time (see Figure 16B). The time course of BCS-Cu(I) formation has two phases. The initial fast phase has a half-time of approximately 6 s and results in formation of about one-half of the total copper ion available. The second half of the BCS-Cu(I) complex forms about ten times more slowly. At the end of the experiment all the copper ion present in the system is in the form of a BCS-Cu(I) complex. It is proposed that the biphasic kinetics of BCS-Cu(I) formation arises from two pools of Cu(I)-free and protein bound. The values of the concentrations of these species can be obtained from UV spectroscopic data, and can be used to calculate an equilibrium binding constant for BsSCO-Cu(I). The total BsSCO concentration is 25 µM and if it is assumed that an equal amount of copper ions is added one half of them is free and half is bound. Therefore,

\[ K_D = [\text{BsSCO}][\text{Cu(I)}] / [\text{BsSCO-Cu(I)}] = 12.5 \, \mu\text{M} \]
<table>
<thead>
<tr>
<th>Species</th>
<th>$\beta_2$ value for Cu(I)-(ligand)$_2$ (M$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batho cuproine disulfonate (BCS)</td>
<td>$10^{19.8}$</td>
</tr>
<tr>
<td>Bicinichoninic acid (BCA)</td>
<td>$10^{17.2}$</td>
</tr>
<tr>
<td>Ferrozine (Fz)</td>
<td>$10^{15.1}$</td>
</tr>
</tbody>
</table>

Table 3. Binding affinities of copper ligands for Cu(I). BCS, BCA, and Fz bind Cu(I) ions in 2:1 ratio. Thus their binding affinities are expressed as formation constants, $\beta_2$ values, in M$^{-2}$.

Figure 16. Reaction of BsSCO-Cu(II) by sodium dithionite (Na$_2$S$_2$O$_4$) and transfer of Cu(I) to BCS. Above) Spectra taken during reduction of BsSCO-Cu(II) by sodium dithionite. The spectrum of 24 µM BsSCO in 50 M sodium phosphate buffer (black), after addition of 45 µM CuCl$_2$ (red) and at various times after addition of 70 µM Na$_2$S$_2$O$_4$; (green) immediately, (blue) 10 s and (pink) 40 s. Below) Formation of BCS-Cu(I) from BsSCO-Cu(II). Spectra are shown before and following addition of 157 M BCS. The recording times following the addition of BCS are shown on the Figure. This figure is modified from Hill and Andrews$^{70, 72}$. 
In order to verify the results using BCS as a competitor, another Cu(I) ligand, ferrozine (Fz), was used as a competing ligand. Fz was chosen because it has a relatively lower affinity for Cu(I) as compared to BCS and BCA. A titration of ferrozine on its own with Cu(I) ions is shown in Figure 17A. Formation of the Fz-Cu(I) complex is indicated by increase in spectrometric signal at 470 nm. The binding curve of Fz shows tight binding with a linear increase in absorbance up to the limiting value. The breakpoint is observed at a ferrozine to copper ion ratio of 2 keeping with the reported stoichiometry of Fz:Cu(I) complex. Ferrozine has a documented formation constant $\beta_2$ value of $10^{15.1}$ M$^{-2}$, which is lower (i.e., weaker) than Cu(I) binding by BCS. The titration Fz with Cu(I) was repeated in the presence of one equivalent of BsSCO (see Figure 17B). The presence of BsSCO did not alter the apparent affinity of Fz for Cu(I) consistent with a much weaker binding of Cu(I) by the protein. Competitive titration was also performed in the reverse direction, in which apo-BsSCO was introduced to a pre-formed Fz-Cu(I) complex (data not shown). The intensity of the absorption at 470 nm due to the formation of the Fz-Cu(I) complex was not significantly affected in the presence of up to 10-fold in excess molar equivalent of BsSCO.
Figure 17. Binding curves of ferrozine (Fz) in the absence and presence of BsSCO. Above) Binding curve of Fz and Cu(I) ion. Aliquots of ferrozine were titrated into 25 µM of CuCl. The formation of Fz-Cu(I) complex was monitored by the appearance of an absorbance peak at 470 nm. The increase abruptly stops at approximately 1.8 molar equivalent of Cu(I) indicating the binding of Fz is saturated with the copper ion. Below) Formation of Fz-Cu(I) complex in the presence of BsSCO. Aliquots of ferrozine were titrated into a solution of 25 µM preformed BsSCO-Cu(I) complex. The formation of Fz-Cu(I) complex was monitored by the appearance of an absorbance peak at 470 nm.
Chapter 5

DISCUSSION

5.1 Competition Between BsSCO and Copper-binding Ligands

One of the goals of this binding study is to obtain an adequate thermodynamic description of the interaction between the SCO protein and copper ions. The association of an acceptor (i.e., the SCO protein) with a ligand (i.e., copper ion) to form a complex is often accompanied by perturbation of the spectral characteristics of one of the reactants. Spectroscopic titrations usually involve the measurement of a spectral parameter as a function of the total concentration of the ligand added. In Cu(II) binding experiment, the formation of the BsSCO-Cu(II) complex was monitored spectrometrically since the charge transfer between the SCO protein and Cu(II) creates a strong absorption signal at 352 nm. On the other hand, Cu(I) ions are spectrometrically silent and BsSCO-Cu(I) does not produce any distinct, measurable, spectroscopic peak. Alternatively, the absorption peaks that are resulted from Cu(I) ligands and the copper ions are investigated in Cu(I) competition assays.

In order for the SCO protein to fulfill a chaperone-like role in copper ion delivery to the Cuₐ centre of CcO, such as illustrated in Figure 2, it must achieve several important functions. The abilities to bind copper ions, to recognize the target site, and to release copper ions to the Cuₐ site are essential for the SCO protein as a proposed copper chaperone. For copper ion delivery to Cuₐ, SCO must first be able to bind to copper ions. In prokaryotes, such as B. subtilis, there is only one cellular membrane, within which integral membrane proteins,
including BsSCO, reside. Therefore, BsSCO has access to environmental copper ions since the cell wall offers no barrier to copper ion diffusion. In other words, BsSCO is able to bind copper ions in either Cu(I) or Cu(II) oxidation states without difficulty. This is consistent with the fact that there is no upstream copper source for the SCO protein found in prokaryotic species so far, i.e., such as a homologue of COX17. However, in the case of eukaryotic SCO proteins, where copper ions must be imported into the internal cellular compartments, it is proposed that copper chaperone COX17 acts as a shuttle to deliver copper ions to SCO\textsuperscript{37}. In fact, the interaction between COX17 and SCO has been demonstrated both \textit{in vitro} and \textit{in vivo}\textsuperscript{26,73}. The SCO protein binds the copper ions supplied by COX17 and recognizes the subsequent target molecule (i.e., apo-subunit II). Copper binding to BsSCO has been reported by multiple groups\textsuperscript{57-59}. Binding of copper ions has also been reported for homologs of \textit{Bacillus} SCO, including human SCO1 and yeast SCO1\textsuperscript{47,59}. The copper-binding experiments in this study are consistent with previous results, showing the SCO protein is able to bind one molar equivalent of copper ions in either Cu(I) or Cu(II) oxidation forms. Furthermore, previous studies clearly show that BsSCO is required in the assembly of CcO as the BsSCO knockout of \textit{B. subtilis} is deficient in CcO\textsuperscript{43}. The requirement of BsSCO in assembly of CcO, along with the ability to bind both Cu(II) and Cu(I) ions, make BsSCO an ideal candidate to serve as a copper trafficking protein that delivers copper ions to the mixed-valence Cu\textsubscript{A} centre.

The final step of copper ion transfer would be the requirement for release of the bound copper ion from SCO, allowing its subsequent uptake to the target
site. If the SCO protein functions as a copper chaperone in CcO assembly, then the SCO-Cu complex should dissociate relative easily and in a short period of time. However, the primary difficulty associated with this determination is the tight binding between BsSCO and Cu(II). SCO and Cu(II) form a very stable complex \textit{in vitro}, and it takes a long time for Cu(II) to dissociate from the protein\textsuperscript{74}. This slow release is a major determinant of the reported $K_D$ of 3.5 pM for the interaction of BsSCO with Cu(II) estimated \textit{via} DSC\textsuperscript{24}. The DSC determination of the binding constant is not necessarily an absolute value, as some scan rate dependence of the interaction was observed during the experiment. The $K_D$ determined as 3.5 pM, therefore, should be taken as an upper limit for the interaction, despite the fact that this is a very tight binding constant. Tight binding of pico-molar magnitude is reasonable for a copper binding protein, as other metal binding protein also have affinities reported in this range or even higher\textsuperscript{71}. However, the slow exchange rate of the BsSCO-Cu(II) complex disfavors the SCO protein as a copper chaperone. Moreover, previous studies of \textit{Bacillus} SCO have shown a 1:1 stoichiometry of BsSCO-Cu(II) binding with a $K_D$ of 65 nM\textsuperscript{24}. This investigation suggests that further characterization of this binding constant would likely require competitive binding experiments, for competition assay could reveal the true interactions between an acceptor such as the SCO protein and its ligand (\textit{i.e.}, the copper ion). The estimated $K_D$ of 65 nM does not necessarily provide a definitive picture of the BsSCO-Cu(II) interaction. Indeed, for a tight-binding protein such as SCO, typical direction titrations have a limit in terms of quantifying the binding affinity.
Competition-binding assays are employed in this study, introducing several strong ligands with well-characterized binding affinities for copper ions. Since the dissociation of the copper ion from a SCO-Cu complex is one of the keys in elucidating the physiological function of the SCO protein, this project focuses on the release mechanism of the copper ion from SCO. The results obtained from competition studies with EDTA, EGTA, and NTA for Cu(II) ions are consistent with previous work\textsuperscript{24,25,72}. BsSCO binds to Cu(II) tightly and the complex does not exchange the copper ions for hours.

Quantitative studies of Cu(I) binding in proteins are scarce. This is probably because of the intrinsic instability of aqueous free Cu(I), especially when present at significant concentration in aqueous buffer. Moreover, relatively weak binding (\textit{i.e.}, SCO-Cu(I)) presents a different challenge for detection and quantification. The change in the spectroscopic signal that arises from the interaction between Cu(I) ion and its ligands must be large enough to be measured accurately. In particular, high-affinity interaction characterization requires small amounts of protein and ligand, but the sensitivity of detection may then become limiting. Since the binding of Cu(I) to SCO proteins is not directly apparent, the observable signals produced by the binding of Cu(II) provide an analog for what is considered the more physiological binding of Cu(I). Eukaryotic SCO proteins are integral components of the inner mitochondrial membrane and prefer to bind Cu(I). The human SCO1 protein, for example, has a reported $K_D$ that is competitive to that of BCS for Cu(I)\textsuperscript{26}. In contrast, BsSCO binds Cu(I) with an relatively weak affinity (\textit{i.e.}, $K_D \sim 10 \, \mu M$) despite the utilization of the same conserved metal binding motifs as found in all SCO family proteins.
Since the Cu\textsubscript{A} site has two copper ions, it is hypothesized that the ability of SCO proteins to bind to Cu(II) and Cu(I) would facilitate the establishment of the mixed valence observed in the Cu\textsubscript{A} centre. Cu(I) binding preference exhibited by eukaryotic SCO is consistent with the reducing environment in the inter mitochondrial space, whereas Cu(II)-binding by BsSCO can be associated with a non-reducing environment outside of the plasma membrane of the gram-positive bacterium. BsSCO is also capable of binding Cu(I), however with a much lower affinity reported than that is observed for Cu(II) ions\textsuperscript{24}. The underlying physical understanding of the difference in metal ion preference for mitochondrial SCO proteins and for BsSCO is not complete. It is hypothesized that the biological differences in the surrounding environment of SCO proteins are reflected in their biochemical and molecular aspects. Reliable quantification of copper-protein interactions would underpin the molecular understanding of how SCO proteins bind and release copper ions. It is also a key to understanding the molecular basis of the assembly of the oxidase. The dissociation of the SCO-Cu complex has been demonstrated in a reduction experiment where BsSCO-Cu(II) is reduced to BsSCO-Cu(I)\textsuperscript{24}. The available copper ions, dissociated from the BsSCO-Cu(I) complex, are captured by the strong Cu(I) ligand BCS. The relatively weak interaction between SCO and Cu(I) suggests a possible mechanism of copper ion release. A stable SCO-Cu(II) complex that does not exchange copper ions easily can be reduced by a strong reducing agent (e.g., dithionite) to SCO-Cu(I), which releases the copper ion readily in a physiological relevant content. Experimental conditions \textit{in vitro} may not be directly indicative of physiological situations experienced within the cell, they do provide the first
evidence of conditions required, and may provide an indication of how SCO proteins interact with CcO in vivo.

In this study, Cu(I) affinities of SCO were estimated by introducing a Cu(I) ligand, ferrozine (Fz). The formation constant of the probe complex Fz-Cu(I) used for the evaluation was determined by direct titration. Extension of Cu(I) probes from the stronger ligands BCS and BCA to the weaker ones such as Fz allows quantitative analysis of relatively weak Cu(I) binding site in the SCO protein. Our data show that, even with a relatively weak Cu(I) affinity, Fz forms a complex with Cu(I) ions despite the presence of the competing BsSCO. This observation indicates that the affinity of Fz for Cu(I) may be outside of the range of the Cu(I) affinity of BsSCO. Nonetheless, establishment and implementation of BCS and Fz as a pair of complementary Cu(I) affinity probes has unified the scattered data to a single, workable, affinity scale. Noticeably, one research group suggests that the affinity of SCO for Cu(I) falls in the range of BCA, which has a $\beta_2$ value of $10^{-17}$ M$^{-2}$. However the results obtained from competition studies with Fz suggest otherwise. In this particular study, the researchers show that BsSCO is able to take the Cu(I) ion dissociated from the BCA-Cu(I) complex. Since BCA has a greater formation constant than Fz (see Table 3), the expected outcome of competition between Fz and BsSCO for Cu(I) would be that the presence of the protein competes with Fz and thus delays the formation of the Fz-Cu(I) complex. In other words, if SCO has a much higher Cu(I) affinity similar to BCA, the formation of the Fz-Cu(I) complex, as indicated by an absorption signal at 470 nm, should be delayed. SCO, in this case, is expect to act as a competitor for Cu(I) in
the system. However, this competition with BCA for Cu(I) is not observed in our competition studies.

The NMR structure of BsSCO has been solved, suggesting different binding geometry and ligands for Cu(I) and Cu(II) ions. Consistent with previous studies, conserved copper-binding cysteine and histidine residues are critical in SCO function. More importantly, other additional ligands such as water are also involved in binding. Because the coordination of Cu(I) and Cu(II) ions are distinct in SCO, the ligands and binding geometry may change upon the interaction with the two different copper ions. This change in binding geometry leads to a possible more compact and rigid SCO-Cu(II) complex than SCO-Cu(I). Taken together, these differences in binding ligands of the SCO protein, binding geometry, and the binding site conformation of the SCO protein could contribute to the variation in the binding affinities of SCO for Cu(I) and for Cu(II).

5.2 Comparison Between BsSCO and ScSCO1

Structural studies have revealed that a thioredoxin-like fold is well conserved among SCO protein. This fold topology consists of four strands of β-sheets and three flanking α helices and it belongs to a subset of the thioredoxin superfamily. Both BsSCO and ScSCO1 possess a common thioredoxin-like fold at the core of the two proteins’ three-dimensional structures. The sequence and structural similarities suggest that this thioredoxin region has been conserved or has converged in evolution to interact with a cysteine residue in thiol/disulfide-containing substrates (e.g., CuA site of CcO). However, the thioredoxin fold conserved in the SCO protein has not been linked to the proposed copper-binding function of the protein. Instead, the presence of the thioredoxin-like fold in
SCO leads researchers to speculate that redox properties are one of the central functions of SCO proteins. Indeed, other possible physiological functions of the SCO protein have been suggested, rather than being directly involved in copper insertion to the Cu_A centre of the oxidase. For all the structural information available, the conserved thioredoxin fold in found in SCO proteins from various species across the spectrum of living organisms.

There are several unique features in eukaryotic SCO proteins. For example, a β-hairpin structure is located in the extended, solvent exposed loop connecting a helix and a β-strand in eukaryotic SCO proteins. This structure contains the copper-binding His residue that is fundamental in the SCO function. Moreover, yeast SCO proteins possess an extra pair of cysteine residues that is located in close proximity to the conserved copper-binding site. The two extra cysteine residues are absent in other eukaryotic species outside the kingdom of Fungi. In addition, cysteine residues are replaced by small, hydrophobic, amino acid residues in the SCO protein from *B. subtilis*.

*BsSCO* shows structural properties similar to those found in eukaryotic SCO proteins. *Bacillus* SCO has a greater structural flexibility than ScSCO1 in the surrounding of the copper-binding site. Specific amino acid substitutions in critical points of the thioredoxin-like fold in prokaryotes can largely affect the structural flexibility of the copper-binding region of SCO proteins. Consequently, the copper-binding affinity of prokaryotic SCO may differ. It is possible that for SCO protein-protein interaction may induce conformational changes of the metal-binding site, thus promoting the copper ion release to the Cu_A site.

**5.3 Spectroscopic Characterization of BsSCO and ScSCO1**
Quantitative investigations of binding affinity and interaction stoichiometry require reactant preparations of high purity. In particular, *in vitro* characterization of binding affinity requires the purity of the protein of interest. However, *in vitro* experiments also demand a relatively large amount of proteins. Since this study has concentrated on aerobic *B. subtilis* as the model organism to work on, the SCO protein from the prokaryotic species is transformed and over-expressed in *E. coli*. In order to isolate the SCO protein after the expression stage, a fusion protein of GST–SCO is constructed. Lane 2 in Figure 7 and 8 show the proper expression of the fusion protein, which corresponds to a molecular weight of approximately 46,000. The GST and the SCO moieties of the fusion protein are linked via a designed short linker that contains a cleavage site for thrombin protease. Figure 7 demonstrates that the quality of the preparation for the *BsSCO* protein is high. Both quantity and purity are satisfactory for further binding experiments.

The fusion protein of GST and yeast SCO protein is constructed and expressed in a similar fashion. Figure 8 shows the preparation of the eukaryotic protein. The average yield of ScSCO1 is lower than that of *BsSCO*. The eukaryotic SCO protein is relatively less stable under proteolysis compared to *BsSCO*. As a result, less thrombin and shorter incubation time are carried out to separate GST and SCO moieties. There are possibly several factors that affect the stability and therefore the yield of the final yeast product.

One of the objectives of this project is to study eukaryotic SCO proteins in order to compare any similarity and to identify any difference between prokaryotic and eukaryotic SCO proteins. A tight-binding between ScSCO1 and Cu(II) is
observed from direct titration experiments as illustrated in Figure 11. This observation is consistent with the shared structural and functional similarities between BsSCO and ScSCO1. The key copper-binding residues as well as the local (i.e., the copper site) and overall three-dimensional shape of the SCO protein have been conserved in evolution. As a result, SCO homologues are expected to be able to interact with copper ions for function (or at least part of the function). However, a strong interaction between ScSCO1 and Cu(II) ions indicates that Cu(II) may not dissociate from the ScSCO1-Cu(II) complex easily and the process could be slow. Moreover, direct titration is unable to quantify a tight-binding interaction such as ScSCO1 and Cu(II). As a result, the next step would be to set up competition for estimating the binding affinity of ScSCO1 for Cu(II). It has been shown that the yeast SCO protein is able to bind with both Cu(I) and Cu(II) ions. Quantification of Cu(I) binding can also be achieved via competition studies with well-characterized Cu(I) ligands such as BCS.
Chapter 6

SUMMARY AND CONCLUSIONS

The protein family of SCO, short for Synthesis of Cytochrome c Oxidase, is a metal-binding protein that binds one stoichiometric copper ion via its conserved cysteine and histidine residues. This family of proteins is conserved across aerobic organisms and has been shown to be essential in accumulation of cytochrome c oxidase subunits and in proper assembly of the Cuₐ centre. Without SCO, functional oxidase cannot be assembled. However, the mechanism by which SCO functions has not been fully understood. Two possible functional roles of the SCO protein have been elucidated. Firstly, the copper-binding protein is capable of fulfilling a role in vivo as a copper chaperone that receives and delivers copper ions into the binuclear copper site of the subunit II of the CcO complex. SCO can also function as a redox catalyst in order to maintain the reduced state of the cysteine side chain in the Cuₐ centre by a thiol exchange mechanism.

In favor of the role as a copper chaperone, the SCO protein has been found to bind copper in both oxidation states Cu(I) and Cu(II) and to form stable complexes. Nonetheless, the mechanism of the copper ion release from the SCO protein is largely unclear. This study focuses on the ability of the SCO protein to bind copper ions, in either Cu(I) or Cu(II) oxidation form, and also concentrates in the subsequent release of the copper ions from the binding site. The aim of the project is to quantitatively describe the interaction between SCO and copper ions. Based on the competitive binding experiments, we observe that EDTA and EGTA
are able to compete with BsSCO for Cu(II) ions. However, neither the protein or these copper ion chelators release Cu(II) ions easily, compromising the equilibrium of the competition system. On the other hand, SCO binds to Cu(I) more weakly than to Cu(II) and the release of Cu(I) from BsSCO is relatively fast. Therefore, the reduction of Cu(II) to Cu(I) may promote an increase in the rate of dissociation of bound copper ion from the SCO protein, fulfilling a possible copper delivery role. The in vivo implication is that it is unlikely for SCO to deliver Cu(II) into the oxidase because of the tight binding and relatively high affinity for the copper ion. As a result, Cu(I) is more likely to be the form that is involved during the transfer from SCO to the Cu_A centre.

Estimation of binding affinity can help us to understand how copper ions bind with and dissociate from the SCO protein. In a bigger picture, it can contribute to understanding the assembly of the oxidase enzyme complex, particularly the maturation of the Cu_A centre. For future direction, competition experiments can be continued. Different competitors or ligands, such as small copper binding peptides, could be used. Similar competition experiments can be continued on yeast SCO protein. For example, experiments can be designed to monitor the formation of BCS-Cu(I) complex in the presence of ScSCO1. Furthermore, competition can be performed between yeast and Bacillus SCO proteins for copper ion such that a relative binding affinity can be estimated.
Reference List


