THE INTERACTION BETWEEN THE SCO PROTEIN FROM
BACILLUS SUBTILIS AND COPPER

By

YUEYANG LAI

A thesis submitted to the Department of Biochemistry
In conformity with the requirements for
the degree of Master of Science

Queen’s University
Kingston, Ontario, Canada
(December, 2010)

Copyright ©Yueyang Lai, 2010
Abstract

Members of the Sco protein family have been proposed to function in the assembly of cytochrome $c$ oxidase in the respiratory chain of all aerobic life forms. The Sco protein in *Bacillus subtilis*, BsSco, is characterized for its folding/unfolding behavior in the presence or absence of Cu(II) in this study. The folding/unfolding of apo-BsSco is investigated by CD and fluorescence spectroscopies. BsSco follows an apparent two-state mechanism in both folding and unfolding processes. The two apo forms of BsSco, reduced and oxidized, exhibit similar equilibrium stabilities suggesting that the formation of an intramolecular disulfide in oxidized apo-BsSco does not add to BsSco’s overall stability. In contrast, Cu(II) binding to reduced apo-BsSco results in extreme stabilization and resistance to unfolding in urea. However, when Cu(II) is present with unfolded, reduced apo-BsSco, the protein is rapidly oxidized. Another widely used denaturant, GdnHCl, is able to unfold Cu(II)-BsSco by allowing the loss of Cu(II) from the metal/protein complex. When the presence of Cu(II)-BsSco complex and the protein’s folded state are monitored simultaneously, the unfolding of Cu(II)-bound BsSco occurs coincidently with Cu(II) dissociation. We suggest that the loss of Cu(II) binding and the loss of BsSco’s native conformation are coincident, which leads to the conclusion that Cu(II)-BsSco does not unfold until it forfeits Cu(II). The kinetics of folding/unfolding of reduced, oxidized and Cu(II) bound BsSco are explored by stopped-flow fluorescence spectroscopy. The rate constants at which the two apo forms of BsSco fold and unfold are measured and plotted versus denaturant concentration. Reduced and oxidized forms of apo-BsSco are similar in folding and unfolding kinetics. Cu(II)-involved refolding
kinetics of BsSco show that Cu(II) is able to accelerate the rate of refolding. However, the involvement of Cu(II) in the refolding process results in two competing processes: oxidation and Cu(II) binding. Which process predominates depends on the refolding rate which further depends on the denaturant concentration. This study has provided direct evidence for metal-involved stabilization of BsSco which is beneficial to efficiently fulfill its copper trafficking duty in a cellular environment.
Acknowledgements

The author would like to thank Diann Andrews for her generous help and support all through this project. The author also expresses sincere appreciation to supervisor Dr. Bruce C. Hill.
# Table of Contents

Abstract................................................................................................................................. ii
Acknowledgements ................................................................................................................ iv
Table of Contents.................................................................................................................... iv
List of Figures.......................................................................................................................... vii
List of Abbreviations............................................................................................................... ix
Chapter 1 Introduction........................................................................................................... 1
Chapter 2 Literature review.................................................................................................... 8
  2.1 Cytochrome c oxidase and the Sco proteins................................................................. 8
  2.2 Protein Folding............................................................................................................... 14
Chapter 3 Methods and Materials......................................................................................... 16
  3.1 Protein expression and purification............................................................................... 16
  3.2 Free thiol groups count of purified BsSco.................................................................... 17
  3.3 Preparation of reduced, oxidized and Cu(II)-BsSco..................................................... 18
  3.4 Equilibrium unfolding experiments monitored by CD spectrometry........................ 19
  3.5 Equilibrium unfolding experiments monitored by fluorescence spectroscopy......... 20
  3.6 Kinetic refolding/unfolding experiments monitored by stopped-flow fluorescence spectroscopy............................................................................................................ 20
Chapter 4 Results.................................................................................................................. 22
  4.1 Protein purification and preliminary sample characterization.................................... 22
  4.2 Equilibrium unfolding experiments............................................................................ 25
  4.3 Equilibrium unfolding curves...................................................................................... 29
  4.4 Unfolding profiles of Cu(II)-BsSco............................................................................. 34
  4.5 Kinetics of refolding and unfolding of BsSco............................................................... 43
Chapter 5 Discussion.............................................................................................................. 56
  5.1 Metal-assisted folding and BsSco................................................................................... 56
  5.2 Folding and unfolding thermodynamics of BsSco....................................................... 57
  5.3 Folding and unfolding thermodynamics of Cu(II)-BsSco.............................................. 60
  5.4 Copper oxidation of thiol groups.................................................................................. 63
  5.5 Folding kinetics of BsSco.............................................................................................. 67
  5.6 BsSco and its role in the assembly of the Cu$_A$ center of cytochrome c oxidase........ 67
Chapter 6 Summary and conclusions................................................................................... 70
List of Figures

Figure 1: Electron transport catalyzed by cytochrome c oxidase in the respiratory chain of eukaryotes.................................................................9
Figure 2: Proposed copper delivery chain to cytochrome c oxidase in mammals........11
Figure 3: Structural comparison between the soluble domain of ySco1 and BsSso........13
Figure 4: SDS-PAGE of BsSco at different purification stages..............................23
Figure 5: Absorption spectra of reduced apo- and Cu(II)-BsSco..................................24
Figure 6: CD spectra of apo-BsSco in 0 M and 6 M urea.........................................26
Figure 7: Thermal denaturation of apo-BsSco..........................................................27
Figure 8: Fluorescence spectra overlay of apo-BsSco in urea of varying concentration...28
Figure 9: Equilibrium unfolding profile of reduced and oxidized apo-BsSco denatured by Gdn using CD as a monitoring tool.................................................................30
Figure 10: Equilibrium unfolding profile of reduced and oxidized apo-BsSco denatured by urea using CD as a monitoring tool.................................................................31
Figure 11: Equilibrium unfolding curves of reduced and oxidized apo-BsSco denatured by GdnHCl using fluorescence as a monitoring tool......................................................32
Figure 12: Equilibrium unfolding curves of reduced and oxidized apo-BsSco denatured by urea using fluorescence as a monitoring tool......................................................33
Figure 13A: Reduced apo-BsSco unfolds in urea......................................................36
Figure 13B: Cu(II)-BsSco is resistant to denaturation in urea.....................................37
Figure 14A: Reduced apo-BsSco unfolds in GdnHCl..............................................37
Figure 14B: Cu(II)-BsSco unfolds in GdnHCl..........................................................38
Figure 15A: Kinetics of Cu(II)-BsSco denaturation by GdnHCl..................................39
Figure 15B: Kinetics of Cu(II)-BsSco denaturation by urea.....................................40
Figure 16: Loss of structure and loss of conformation are coincident..........................41
Figure 17: Equilibrium unfolding curve of apo (reduced and oxidized) and Cu(II) bound BsSco denatured by urea using CD as a monitoring tool.............................................42
Figure 18A: Unfolding kinetics of oxidized apo-BsSco at 6.4 M urea............................44
Figure 18B: Time course of unfolding kinetics of oxidized apo-BsSco at 6.4 M urea ..........45
Figure 19A: Refolding kinetics of oxidized apo-BsSco at 1.8 M urea............................46
Figure 19B: Time course of refolding kinetics of oxidized apo-BsSco at 1.8 M urea ........47
Figure 20: Chevron plots of reduced and oxidized apo-BsSco.....................................48
Figure 21A: Refolding kinetics of reduced apo-BsSco in the presence of Cu(II) at 0.5 M urea....51
Figure 21B: Time course of refolding kinetics of reduced apo-BsSco in the presence of Cu(II) at
0.5 M urea......................................................................................................................52
Figure 22A: Refolding kinetics of reduced apo-BsSco in the presence of Cu(II) at 1.5 M urea....53
Figure 22B: Time course of refolding kinetics of reduced apo-BsSco in the presence of Cu(II) at
1.5 M urea......................................................................................................................54
Figure 23: Reduced apo-BsSco refolding with Cu(II) results in oxidation.........................55
Figure 24: Ribbon representation of BsSco showing the two external loops of BsSco containing
the residues involved in metal binding...........................................................................69
Diagram 1: Primary sequence of full-length BsSco.......................................................16
Scheme 1: Sequential binding of BsSco to Cu(II)..........................................................61
Scheme 2: Proposed mechanism of Cu(II) oxidation of BsSco........................................63
Scheme 3: Refolding rate-dependent oxidation by Cu(II) of reduced apo-BsSco undergoing
refolding..........................................................................................................................66
List of Abbreviations

ITC: Isothermal titration calorimetry
DSC: Differential scanning calorimetry
PBS: phosphate buffered saline solution
GST: Glutathione S-transferase
MW: Molecular weight
PMSF: Phenylmethylsulphonyl fluoride
DTT: Dithiothreitol
EDTA: Ethylenediamine tetraacetic acid
DPDS: Diphenyldisulfide
CD: Circular dichroism
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
GdnHCl: Guandine Hydrochloride
SH count: free thiols count
BCS: Bathocuproinedisulfonate

Sco Nomenclature:
ySco1: yeast Sco protein
BsSco: Sco protein in *Bacillus subtilis*
Reduced apo-BsSco: apo-BsSco with two free thiols at C45 and C49
Oxidized apo-BsSco: apo-BsSco with a disulfide formed between C45 and C49
Cu(II)-BsSco: Cu(II) bound BsSco
Chapter 1
Introduction

Cytochrome c oxidase is a large protein complex involved in the respiratory chain of all aerobic life forms (1). The assembly of mitochondrial cytochrome c oxidase is an extremely complex process that involves ten to thirteen subunits of both mitochondrial and nuclear origin, and requires assistance of over thirty accessory proteins (1-2). The complexity of cytochrome c oxidase assembly also originates from its requirement for copper insertion into the redox active centers of cytochrome c oxidase (2). The pathway by which cytochrome c oxidase acquires copper has been elusive. Copper delivery to cytochrome c oxidase must be mediated by copper trafficking proteins (3). Although specific proteins have been implicated in the assembly of the copper centers of cytochrome c oxidase, their roles including as a copper chaperone are still being debated.

The Sco protein family comprises members proposed to function in the assembly of the binuclear Cuₐ center of cytochrome c oxidase (1). The first member of this family, yeast Sco1 (ySco1), was originally identified as a high-copy suppressor of a COX17 mutation in yeast (4). The function of COX17 is to shuttle copper to the intermembrane space of mitochondria (4-6). Other members of the Sco protein family are identified by their similarity to ySco1. Structural characterization of the Sco proteins across different organisms (yeast, human, bacteria) has shown that the Sco proteins contain a conserved CXXXCP sequence motif and a conserved histidine located about one hundred amino acids toward the C-terminus from the conserved cysteines (7-10). A number of spectroscopic studies have shown that yeast and human Sco proteins can bind one
equivalent of both Cu(I) and Cu(II) (11). The binding of Cu(I) with Sco exhibits trigonal coordination by three residues (the two conserved cysteines and a histidine) whereas Cu(II) binding includes a water molecule as the fourth ligand (7-9). Mutational studies have shown that Sco1 may function downstream of COX17 in the copper delivery chain and mutations in Sco1 result in cytochrome c oxidase deficiency (3,4). The location of Sco proteins in mammals and yeast being in the inner mitochondrial membrane, along with spectroscopic findings and mutational studies suggest the potential role of Sco1 as a copper chaperone that ultimately delivers copper to CuA. However, structural studies of human Sco1 show that it exhibits a peroxiredoxin fold similar to that of the thioredoxin proteins, therefore supporting a redox role for Sco (9). The role of Sco as a copper chaperone is also questioned by the inability of human Sco1 to co-crystallize with Cu(I) (9). Moreover, the exclusive role of Sco in CuA assembly is challenged by the finding that some bacteria express an apparent Sco homolog but do not express a protein containing CuA (12).

Studies of cytochrome c oxidase assembly in bacteria have provided additional model systems for elucidating the functions of Sco, taking advantage of the less sophisticated assembly mechanism of cytochrome c oxidase and simpler copper uptake pathways in the periplasm of bacteria. The gram-positive bacterium Bacillus subtilis encodes a homolog of yeast Sco1 from the ypmQ gene (BsSco) (13). BsSco is a membrane protein containing a lipid for membrane anchorage. Deletion of the ypmQ gene results in loss of activity of B. subtilis cytochrome c oxidase containing the CuA site, but not the menaquinol oxidase containing the CuB site, suggesting that BsSco is required for
cytochrome c oxidase assembly similar to its eukaryotic counterparts (13). BsSco has been shown to preferentially bind Cu(II) with a substantially higher affinity than for Cu(I), consistent with the non-reducing environment of B. subtilis cytochrome c oxidase (14). However, the crystal structure of BsSco does not contain bound copper but contains both the thiol and disulfide conformers (disulfide-bonded between C45 and C49), reinforcing a redox-switching role for BsSco (10). The disulfide bridge formed between C45 and C49 is readily reducible by reducing agents such as DTT or TCEP (tris(2-carboxyethyl)phosphine). The redox role of BsSco is further consolidated by its structural similarity to the thioredoxin protein family (10). A recent study focusing on the mechanism of Cu₅₆ assembly in another bacterium Thermus thermophilus, employing transition metal NMR (nuclear magnetic resonance), concluded that the Sco protein in T. thermophilus acts as an exclusive thiol-disulfide reductase in Cu₅₆ assembly which serves in the reductive preparation of the Cu₅₆ cysteine residues (15). The proper copper chaperone for Cu₅₆ is another protein PCu₅₆C in T. thermophilus. Unfortunately, we have been unable to identify a homolog of PCu₅₆C in B. subtilis, and there has been no identification of eukaryotic homologs of PCu₅₆C so far.

The metal binding properties of BsSco have been characterized previously (16). BsSco has been shown to bind Cu(II), Cu(I) and Ag(I) with 1:1 stoichiometry in each case (16). The metal binding ligands include the two conserved cysteine residues (C45 and C49) along with the conserved histidine (H135) (16). Unlike its eukaryotic homologs, which preferentially bind Cu(I), BsSco binds Cu(II) with a binding affinity many orders of magnitude higher than any other metal ions (14, 16-17). ITC and DSC experiments
have provided quantitative measurements of the binding between BsSco and Cu(II). ITC estimated an upper limit of the equilibrium dissociation constant ($K_D$) to be 65 nM and DSC determined the $K_D$ to be 3.5 pM, whereas BsSco binds Cu(I) with a $K_D$ in the micromolar range (14,16). The Cu(II) bound form of BsSco is also more stable than the apo-BsSco of both thiol and disulfide forms (reduced and oxidized). Cu(II)-BsSco has a melting temperature 23 °C higher than apo-BsSco and the Cu(II)-BsSco complex is stable for days (14).

Another approach that can quantitatively characterize the degree of thermodynamic stability (equilibrium stability) conferred by copper is that of protein folding. This type of experiment also allows an understanding of how metals affect protein folding both thermodynamically and kinetically and a delineation of the folding mechanism of a protein. The potential effects of metal binding on protein folding are exemplified by azurin, a blue-copper protein involved as an electron transfer protein in denitrification and respiration (18). Azurin requires copper as a cofactor and metals play an important role in azurin’s folding. The fastest way for azurin to fold and form the functional complex is to bind its cofactor, Cu(II), in its unfolded form first and then start folding. Cu(II) and Zn(II) have been shown to significantly increase the equilibrium stability and the rate of folding of azurin (18). The rationale underlying the effect of metals on protein folding is that the metal ion can act as a nucleus that directs folding by local formation of structures around the metal binding site.
BsSco is another ideal candidate to study the connection between metal/protein interaction and protein conformation. One can study how metals affect BsSco’s folding by monitoring both the thermodynamics and kinetics of the folding/unfolding of BsSco in the absence and presence of copper. BsSco binds Cu(II) with an extremely high affinity (14). Previous preliminary studies have provided clues that BsSco may comply with the two-state model (unpublished data). The unfolding of BsSco, using GdnHCl as a denaturant, exhibits an apparent unfolding transition. If copper were to stabilize BsSco, this transition should shift to a higher concentration range of the denaturant for Cu(II)-BsSco in an equilibrium unfolding experiment. To compare the folding/unfolding behavior of apo forms of BsSco and Cu(II)-BsSco, equilibrium unfolding experiments are able to yield thermodynamic parameters (e.g., unfolding free energy) associated with protein folding that are useful for comparison. Kinetic experiments provide another way to examine the stability of different forms of BsSco.

In this body of work, the purified soluble portion of BsSco was subjected to both equilibrium and kinetic refolding/unfolding experiments. The major objectives are as follows:

a) To test the compliance of BsSco with the two-state model of protein folding.

b) To determine the difference in equilibrium stability among different forms of BsSco

c) To determine the difference in refolding/unfolding kinetics among different forms of BsSco.
In order to achieve these objectives, three forms of BsSco are investigated: reduced, oxidized and Cu(II) bound. Optimal conditions to unfold BsSco \textit{in vitro} are first screened by equilibrium unfolding experiments employing several denaturing conditions (GdnHCl, urea and temperature). The compliance of BsSco with the two-state model is evaluated and the unfolding profiles of the three forms of BsSco are determined for two chemical denaturants (GdnHCl and urea). The folding of BsSco follows a two-state mechanism. The equilibrium stability of oxidized apo-BsSco is NOT significantly different than reduced apo-BsSco. Cu(II) binding to reduced apo-BsSco leads to extreme stabilization and resistance to unfolding in urea. However, GdnHCl is able to unfold Cu(II)-BsSco by allowing the loss of Cu(II)-binding \textit{via} high ionic strength-activated Cu(II) oxidation. Cu(II) oxidation of BsSco is also observed in the case of reduced, apo-BsSco in its unfolded form, which is susceptible to copper catalyzed oxidation rather than forming a stable complex with the metal. Kinetic experiments are performed using stopped-flow fluorescence spectroscopy to measure rates of folding and unfolding for all three forms of BsSco. The refolding/unfolding kinetics of reduced and oxidized apo-BsSco are similar. However, Cu(II) binding significantly increases the refolding rates of BsSco. Nevertheless, Cu(II) binding to reduced apo-BsSco undergoing refolding adds another layer of complexity. The presence of Cu(II) in a refolding environment oxidizes BsSco, leading to a competition between two processes: Cu(II)-catalyzed oxidation and refolding. Which one gains predominance depends on the refolding rate which further depends on the concentration of denaturant. Higher denaturant concentrations give lower refolding rates which in turn lead to a kinetic advantage for the oxidation process. Such findings
implicate an importance to protect BsSco from Cu(II) in its environment while new BsSco molecules are being made and folded in the cell. The effect of Cu(II) on the stability of BsSco can be described as: Cu(II) can either stabilize BsSco through binding or oxidize BsSco through Cu(II)-mediated oxidation.
Chapter 2

Literature review

2.1 Cytochrome c oxidase and the Sco proteins

Cytochrome c oxidase is a large protein complex that functions in the final link of the respiratory chain of all aerobic organisms (1, 19). It is also known as complex IV in the respiratory chain and catalyzes electron transfer from cytochrome c to molecular oxygen, generating water as the final product. Four electrons are transferred from four molecules of cytochrome c through the redox centers of cytochrome c oxidase to one molecular oxygen, generating two molecules of water. A concomitant translocation of four protons across the inner mitochondrial membrane contributes to the proton gradient which is utilized by ATP synthase to generate ATP (Fig. 1) (1, 19-21). The assembly of cytochrome c oxidase is a complex process. Cytochrome c oxidase and other members of the heme-copper oxidase family are composed of many subunits of both nuclear and mitochondrial origins with the addition of two heme groups, three copper ions (Fig. 1), all of which require assistance of up to thirty accessory proteins (1, 21-25). The number of subunits that make up the heme-copper oxidases varies in different organisms, from thirteen in mammals, 10-12 in yeast to 3-4 in bacteria (1, 23). Three subunits (I-III) form the functional core in all of the heme-copper oxidases from eukaryotes to bacteria (21, 26). The complexity of the assembly of cytochrome c oxidase also arises from its requirement for heme group and copper ion insertion. The two copper-based redox centers of cytochrome c oxidase, CuA and CuB, are located in subunits II and I, respectively (21, 27). These two copper centers require insertion of three copper ions.
Cu_A is a dinuclear center requiring two copper ions whereas Cu_B is a mononuclear center requiring one copper ion (1, 23-25, 28).

Figure 1: Electron transport catalyzed by cytochrome c oxidase in the respiratory chain of eukaryotes. Electron flow is indicated by thin blue arrows. Electrons are transferred from cytochrome c through the two copper centers and two heme centers of cytochrome c oxidase to molecular oxygen. Protons are transported across the inner mitochondrial membrane and indicated by thick black arrow. Water is generated as the final product.

The question of how cytochrome c oxidase acquires copper ions has been of great interest to researchers (2, 22, 29). It had been taken for granted that cytochrome c oxidase spontaneously acquires copper ions until some accessory proteins of cytochrome c oxidase had been proposed to be capable of transporting metals ions (1-4, 30-32).
possibility of spontaneous acquisition of copper by cytochrome c oxidase is contradicted by the fact that free copper ions within a cellular environment are prone to generate oxidative damage (33-35). Others have reported that there is less than one free copper ion on average in a cell (36). It has been proposed, therefore, that copper insertion into cytochrome c oxidase must be accomplished by a class of proteins known as copper chaperones (proteins that transport metal to specific partners) (30-32). Copper ions are delivered to the redox centers of cytochrome c oxidase by these copper chaperones. At least three proteins have been implicated to function in the delivery of copper ions to the copper centers of cytochrome c oxidase (5-6, 11, 37-38). COX17 is proposed as an upstream copper chaperone which relays copper ions to downstream receivers, which are proposed to be COX11 and the Sco proteins (6, 38). COX11 has been suggested to deliver copper to the CuB center (37-38). The Sco proteins, Sco1 and Sco2, have been proposed to directly transport two copper ions to the CuA center (6, 39-42). The proposed copper delivery chain to cytochrome c oxidase in mammals is illustrated in Figure 2. COX17 receives copper from the integral membrane protein, copper transporter Ctr1. COX17 passes copper downstream to the copper chaperone Sco which delivers copper to the CuA center of cytochrome c oxidase. COX11 receives copper from COX17 and delivers it to the CuB center of cytochrome c oxidase. Although direct evidence has NOT been obtained to define the exact roles of COX11 and the Sco proteins, they have all been experimentally confirmed by mutagenesis to be required in the assembly of the corresponding copper centers (13, 37-38, 39-40).
The Sco protein family consists of proteins implicated in the formation of the Cuₐ center (3, 6-11, 39-41). These proteins are membrane proteins that are anchored either to the inner-membrane of mitochondria in eukaryotes, or the cell membrane in prokaryotes (7-11, 39-40). In eukaryotes, the mitochondrial location in the cell makes them capable of serving as a copper chaperone to receive copper ions from COX17 which moves freely in the cytosol. Sco1 was first discovered in yeast (i.e., ySco1) where researchers found Sco1 as a high copy suppressor of COX17 mutations (42). Homologs of ySco1 are found across several organisms (yeast, human, bacteria) where they are also shown to be required for the assembly of cytochrome c oxidase (11, 13, 41). The Sco proteins are
conserved among all organisms. They are characterized by two conserved metal binding motifs, a $CXXXCP$ motif and a histidine (7-10). The two metal binding motifs contribute to their high binding affinity for metal ions, especially copper (7-10). The key structural feature of the Sco protein family is its striking similarity to the core structure of the thioredoxin protein family (7-10). Thioredoxins are present in all organisms and function in reduction of other proteins through cysteine thiol-disulfide exchange (43-46). An additional structural feature of the Sco protein family is the flexible loops external to the core thioredoxin structure. In ySco1, a flexible loop structure anchors the conserved histidine residue (H239) where it works in concert with the two conserved cysteine residues located in the junction between $\alpha2$ and $\beta3$ to bind one equivalent of Cu(I) (7).

The homolog of ySco1 in *B. subtilis* has attracted the interest of many researchers because the less sophisticated structure of cytochrome c oxidase in bacteria implies a simpler assembly process that may be advantageous to study the role of the Sco protein. The homolog of Sco1 in *B. subtilis*, BsSco, has also been shown to be a mandatory assembly factor for cytochrome c oxidase (13). While eukaryotic Sco proteins prefer to bind Cu(I), BsSco binds Cu(II) with an extremely high binding affinity ($K_D = 3.5$ pM) (14), which is supportive of its potential role as a copper chaperone. BsSco is a small membrane protein anchored to the cell membrane by a lipid (10, 14). In contrast to ySco1 which contains only one flexible loop, the two conserved metal binding motifs of BsSco ($C_{45}XXXC_{49}$ and $H_{135}$) are located on two flexible loops (Fig. 3) (14). The crystal structure of BsSco has been shown to be similar to the thioredoxin proteins (14). However, co-crystallization of BsSco with Cu(II) has been unsuccessful (14). In fact, efforts to co-
crystallize the Sco proteins with copper have all failed thus far (An incorrect structure of ySco1 co-crystallized with Cu(II) was reported in 7) (7-10). The role of the Sco protein as copper chaperone is also disfavored by its structural similarity to the thioredoxin proteins.

![Figure 3: Structural comparison between the soluble domain of ySco1 and BsSso. Panel A, ribbon representation of ySco1. Panel B, ribbon representation of BsSco (Panel A adapted from 7).](image)

The discovery of another protein in *Thermus thermophilus*, PCuA C (periplasmic Cuₐ center copper chaperone), also challenges the copper trafficking duty of the Sco proteins (15). Researchers have claimed that PCuA C is the correct copper chaperone for Cuₐ center assembly whereas the Sco protein in *T. thermophilus* functions in thiol-disulfide exchange (similar to thioredoxin) which keeps the cysteine residues of the Cuₐ center in a
reduced state (15). However, a homolog of PCu₇C is not found in B. subtilis. The specific role of the Sco proteins is still under debate.

2.2 Protein Folding

The structure-function paradigm has been accepted for decades. Most enzymes still require correct three-dimensional structure to function despite the discovery of IDPs (intrinsically disordered proteins) (47). Accumulation of misfolded proteins in vivo leads to diseases such as Alzheimer’s disease and mad cow disease (48-49). The folding of proteins in vivo starts co-translationally and may require the assistance of molecular chaperones (50-51). Reversible unfolding of proteins in vitro has lent a tool to study the nature of protein folding, allowing researchers to explore the most basic characteristics of folding for individuals proteins. These characteristics include the rate of folding, unfolding free energy, unfolding transition and compliance with the two-state mechanism. Other aims of implementing in vitro folding/unfolding experiments are based on the objective to elucidate the effect of different factors, other than extreme conditions which are normally not present in a cellular environment, on the folding of a protein. For example, the effect of metal interactions on protein folding is one of these factors, and it has rarely been studied.

Metals are partners of many proteins and serve as substrates, cofactors, prosthetic groups and catalytic centers. In order to function, a well coordinated interaction between the protein and the metal is crucial. It may be disastrous if a protein unfolds as it interacts with its metal partner. In contrast, it brings advantages if a protein becomes more stable
as it interacts with its metal partner. Azurin, a blue copper protein which catalyzes electron transfer in denitrification/respiration chains, is a classic example (18). Azurin requires Cu(II) to function. Cu(II) binding increases its thermodynamic stability by 5.7 kcal mol\(^{-1}\) (18). The assembly of azurin also benefits from Cu(II) binding as Cu(II) binding before folding of azurin is the fastest way towards holo-protein formation. The effect of Cu(II) binding is well defined in this case and it can be applied to other proteins such as BsSco.
Chapter 3
Methods and Materials

3.1 Protein expression and purification

*Escherichia coli* BL21(DE3) cells transformed with pDA395 containing the gene of the GST-fusion protein (soluble portion of BsSco, primary sequence of full-length BsSco is illustrated in Diagram 1) were streaked onto LB(amp) plates for overnight incubation. The transformation protocol was reported previously (13). Cells from an overnight incubation were inoculated into four equivalents of 0.7L LB media in Fernbach flasks containing 100 µg/mL ampicillin. Bacterial cells were allowed to grow to reach an optical density of 0.6-0.8 at which point IPTG was added to each of the flasks to a final concentration of 1 mM to induce protein expression for 3 hours. Cell cultures were harvested by centrifugation and resuspended in PBS buffer (pH 7.4) with 1 mM EDTA and 100 mM PMSF. Resuspended cell extracts were split into two 50 mL conical tubes and frozen at -80 °C.

![Diagram 1: Primary sequence of full-length BsSco. Post-translational modification is indicated by black arrow in which the cleavage of the first 18 residues is followed by an attachment of a diacyl glycerol moiety to cysteine (52). Our purified soluble domain of BsSco is indicated in blue and includes two additional residues in the brackets. Key binding residues for Cu(II) are indicated in red.](image)
Frozen cells were thawed and incubated on ice for 30 minutes with DTT (final concentration = 5 mM), lysozyme (1 mg/mL), DNase and RNase (both 37.5 mg/mL). The incubated cell extract was divided into four tubes, each of which contained 20 mL of extract, and was mixed with 10 mL of 4% Triton X-100, followed by another incubation period of 1 hour at 4 °C. Centrifugation at 16000 rpm in Beckman J6 centrifuge employing a JA-25.50 rotor was used to harvest the supernatant. The pellet was discarded. The supernatant was adjusted to pH 6.4 by addition of sodium dihydrogen phosphate (100 mM). The supernatant was loaded onto a 9 mL Glutathione Sepharose 4 Fast Flow column (Amersham) at a rate of 1.5 mL/minute. The column was washed with PBS (pH 7.7) to remove proteins that did not bind to the column. Thrombin (200 units) was added to the column and incubated for 16 hours at room temperature to allow the cleavage of fusion protein. PBS (pH 7.7) was used to elute the soluble BsSco. The eluants were pooled and subjected to Benzamidine Sepharose treatment to remove excess thrombin. The final purified protein was exchanged for sodium phosphate buffer (pH 7.0) and concentrated by centrifugation at 2200 rpm in a Beckham JA-25.50 rotor employing Amicon Ultra-15 concentrators (10000 MW cut off). The purity was checked by SDS-PAGE.

3.2 Free thiol groups count of purified BsSco

A split cuvette was filled with sodium phosphate buffer (pH 7.0) to record a reference absorption spectrum. Purified BsSco was diluted 50-fold in sodium phosphate buffer (pH 7.0) in a total volume of 1 mL in one side of the split cuvette. The other side
of the split cuvette was filled with 999 µL of buffer. An absorption spectrum was measured for the protein. The spectrum of the buffer alone was used as reference and subtracted from the spectrum of the protein. DPDS (1 µL, 40 mM) was added to the buffer side of the split cuvette to a total volume of 1mL. A spectrum was recorded for DPDS. The split cuvette was then sealed by parafilm and the two sides were mixed by inverting. DPDS reacts with free thiol group with 1:1 stoichiometry. Another spectrum was measured after mixing. A difference spectrum was made between the two spectra before and after mixing that gives an absorbance band centered at 324nm corresponding to the thiol group concentration. The spectrum of the protein minus the buffer is used to determine the concentration of BsSco and then to compute the average number of free thiol groups per protein molecule. The extinction coefficient of the thiopyridone that is the product of DPDS reacting with the thiol group of cysteine is 18.8 mM⁻¹ cm⁻¹ at 324 nm. The extinction coefficient of BsSco at 280 nm is 19.2 mM⁻¹ cm⁻¹.

3.3 Preparation of reduced, oxidized and Cu(II)-BsSco

Some samples were purified reduced (an SH count close to 2). If not, a purified BsSco sample (10 mg/mL) was mixed with GdnHCl (final concentration=3 M), DTT (2 mM) and EDTA (250 µM). The sample was left overnight at room temperature. Centrifugation at 2200 rpm in a swinging bucket rotor employing Amicon Ultra-15 concentrators (10000 MW cut off) was used to wash away non-protein reagents. A free thiol count was employed to determine the redox state of the sample (an SH count close to two is ideal).
Oxidized apo-BsSco was prepared by denaturing the protein with 3 M GdnHCl and chelating any residual metal ions by 250 μM EDTA. An overnight incubation was required to let the denatured sample go spontaneously oxidized. The redox state of the sample was confirmed by a by free thiol count (an SH count close to zero is ideal).

Cu(II)-BsSco was prepared by adding a sufficient volume of 200 mM CuCl$_2$ to reduced, apo-BsSco to allow 1:1 binding. A slight excess of Cu(II) ions is allowable. An alternative is to titrate reduced apo-BsSco sample with CuCl$_2$ solution until there is no further change at 352nm on the absorption spectrum.

3.4 Equilibrium unfolding experiments monitored by CD spectrometry

CD spectra were taken on a Chirascan Spectrometer (Applied Photophyscis). Samples were prepared in 5 mM sodium phosphate buffer, pH 7.0. For CD deconvolution, a reduced sample was prepared at 1 mg/mL. CD spectra were measured from 180-260 nm in 1nm increments using either a 1 mm or 0.1 mm pathlength quartz cuvette (Hellma). For chemical denaturation experiments, reduced, oxidized and Cu(II)-BsSco samples were prepared at 0.1 mg/mL in urea or GdnHCl at varying concentrations. CD spectra were recorded from 200-260nm in 1nm increments using a 1 mm pathlength quartz cuvette (Hellma). For thermal denaturation, reduced apo-BsSco samples were prepared at 0.1 mg/mL. Temperature ramping was set at a step of 1 °C/min climbing from 25 to 90 °C. Spectra were recorded at regular intervals as the temperature increased.
3.5 Equilibrium unfolding experiments monitored by fluorescence spectroscopy

Fluorescence data were recorded using a Horiba Jobin-Yvon Fluorolog-3 fluorescence spectrometer, with 1 nm excitation bandpass and a 2 nm emission bandpass. The excitation wavelength was 280 nm for all experiments. Emission spectra were scanned from 300 to 450 nm in 1 nm increments. For chemical denaturation, reduced and oxidized apo-BsSco samples were prepared at 0.15 mg/mL in urea or GdnHCl at varying concentrations. Samples were measured in 1 cm quartz cuvette with constant stirring.

3.6 Kinetic refolding/unfolding experiments monitored by stopped-flow fluorescence spectroscopy

Stopped-flow fluorescence experiments were implemented using an OLIS RSM-1000 spectrometer. A different scanning range for emission spectra was employed (290 to 420 nm) for kinetic spectroscopy. Reduced or oxidized apo-BsSco samples were prepared at a concentration of 5 mg/mL (250 μM). Protein samples were prepared either native in 50mM sodium phosphate buffer or unfolded in the same buffer but with 5 M urea. For unfolding kinetic experiments, native protein samples were mixed in a one to ten ratio with a series of unfolding buffers in the OLIS stopped flow mixer. For refolding experiments, the unfolded protein sample was mixed with a series of refolding buffers of varying concentrations of urea (urea concentration < 2.5 M). The change in fluorescence signal caused by refolding or unfolding was recorded as a function of time.

For Cu(II)-involved refolding experiments, reduced apo-BsSco samples were prepared unfolded in 5 M urea. The samples were mixed with refolding buffer containing
a stoichiometric amount of Cu(II) to allow Cu(II) binding while BsSco is refolding. The change in fluorescence signal caused by refolding and Cu(II) binding was recorded as a function of time.
Chapter 4

Results

4.1 Protein purification and preliminary sample characterization

The protein used in this project is the soluble portion of BsSco with its lipid binding site removed. The GST-tagged fusion protein was expressed in *E. coli*, purified from a cytosolic extract and cleaved by protease treatment to yield the final soluble product. A yield of 16-25 mg per 2.8 L cell culture was routinely obtained. Purification was followed at each stage by SDS-PAGE to ensure proper expression, correct flow and purity of the product (Fig. 4). Our protein of interest was induced by IPTG during the culturing period (lanes 2-5). The fusion protein was cleaved on the column by incubation with thrombin for 16 hours. The soluble domain was eluted (lanes 6 and 7) following the cleavage period. GST was then eluted from the column by washing with glutathione-containing buffer (lanes 8 and 9). The final purified protein was obtained after benzamidine treatment (*i.e.*, to remove thrombin) and centrifugal concentration (lane 10). An apparent MW of 21000 was calculated from the SDS-PAGE which is consistent with previously reported results (53).

An important characteristic of BsSco is its redox state. The primary sequence of BsSco contains two cysteines located three residues apart. They can form a disulfide bond, completely depriving BsSco of its ability to bind metal ions. The redox state of BsSco can be determined by a count of free thiol groups. DPDS will form a thiopyridone product in reaction with free thiol groups, the thiopyridone product has a characteristic peak at 324 nm (extinction coefficient = 18.8 mM$^{-1}$cm$^{-1}$) (13, 16). BsSco has a peak at
280 nm (extinction coefficient = 19.2 mM\(^{-1}\)cm\(^{-1}\)) (13, 16). The difference at 324 nm after addition of 20 mM DPDS to the purified BsSco provides a way of estimating how many

![SDS-PAGE of BsSco at different purification stages.](image)

**Figure 4**: SDS-PAGE of BsSco at different purification stages. The samples were treated with 10% SDS, 0.3M Tris-HCl pH 8.0 and bromophenol blue. The mixtures were heated at 37 °C for one hour prior to loading. Lane 1, protein standards, lanes 2-5, post-induction cell extracts from four independently grown cell cultures (20 µg), lane 6-7, pooled fractions before benzamidine treatment in duplicates (6 µg and 3.2 µg), lanes 8-9, Elution with glutathione following thrombin treatment containing GST in duplicates (10 µg), lane 10, final BsSco sample after thrombin removal and centrifugal concentration (6 µg).

free thiol groups on average (0-2) a particular sample has per BsSco molecule. A typical reduced sample has an SH count of 1.8-2.0, whereas the same value for an oxidized sample is 0-0.2. Completely reduced and oxidized samples of apo-BsSco will ideally have an SH count of two and zero, respectively.
Figure 5: Absorption spectra of reduced apo- and Cu(II)-BsSco. Reduced apo-BsSco was prepared at a concentration of 0.8 mg/mL in 50 mM sodium phosphate buffer pH 7.0. The same sample was mixed with CuCl$_2$ (200 µM) to allow Cu(II) binding. Reduced apo- and Cu(II)-BsSco are represented by solid line (——) and dotted line (------), respectively. Cu(II) binding results in a prominent peak at 352 nm.

Only BsSco molecules with two free thiol groups can bind Cu(II) in a stable complex, therefore the free SH count is important to evaluate if a sample is be used for Cu(II) binding-related experiments. Cu(II) binding to BsSco alone has a characteristic spectrum (Fig. 5). It has a prominent peak at 352 nm (extinction coefficient = 4.78 mM$^{-1}$ cm$^{-1}$) (17), along with bands at 452 nm and 552 nm (peak at 552 nm not shown in Fig. 5). These bands appear upon addition of Cu(II) to reduced apo-BsSco. The percentage of Cu(II) binding can be calculated by subtracting the absorbance of the two spectra at 352nm.
nm and comparing the difference with the absorbance intensity of apo-BsSco at 280 nm. The SH count and the degree of Cu(II) binding should be consistent, e.g., an SH count of 1.8 should give a degree of Cu(II) binding of 90%. For Cu(II) binding-related experiments, 80% Cu(II) binding is the lower limit that we accepted for proceeding with the sample.

4.2 Equilibrium unfolding experiments

Our purified apo-BsSco has native secondary structure as confirmed by CD spectrometry (Fig. 6). Deconvolution of the CD data gives an estimate of the proportion of secondary structures: α-helix 27.6%, β-sheet 38.5% and random coil 36.6%. This estimation is consistent with previously published x-ray data and indicates that the soluble apo-BsSco used here has a folded structure (10). Denaturing of the native protein results in unfolding. A urea concentration of 6 M is sufficient to unfold the protein, resulting in flattening of the CD spectrum around the 220 nm area. The signal drop around 220 nm signifies substantial loss of α-helical structure. However, the CD signal change before 200 nm is not accessible in the presence of urea. A full deconvolution of the denatured state of BsSco cannot be obtained under these conditions.

An alternative to chemical denaturation of proteins is to induce unfolding by temperature. In contrast to urea or GdnHCl induced unfolding, thermal denaturation gives a more interpretable CD spectrum. The spectrum can be deconvoluted to estimate the structure of denatured protein. “Unfolded” BsSco still maintains residual structure as the
Figure 6: CD spectra of apo-BsSco in 0 M and 6 M urea. Reduced apo-BsSco samples were prepared at a concentration of 0.1mg/mL (50 µM) in 50 mM phosphate buffer, pH 7.0. CD spectra were recorded from 180-260 nm in the UV range, cuvette pathlength = 1 mm. Native apo-BsSco and denatured (6 M urea) apo-BsSco are represented by solid(——) and dotted(-----) lines, respectively.

Content of β-sheet increases while the α-helical content almost disappears (see Discussion). Thermal denaturation also provides evidence that the folding/unfolding transition of BsSco follows an apparent two-state mechanism (Fig. 7). Denaturation of apo-BsSco at increasing temperatures from 25-90 °C yields a set of gradually
transforming spectra with an isosbestic point at 215 nm. Such spectral behavior supports a two-state mechanism for unfolding.

**Figure 7:** Thermal denaturation of apo-BsSco. Apo-BsSco samples were prepared at a concentration of 0.1 mg/mL (50 μM) in 50 mM phosphate buffer, pH 7.0. CD spectra were scanned from 180-260 nm in 1 nm increments in the UV range, cuvette pathlength = 1 mm. Native apo-BsSco was subjected to temperatures from 25-90 °C. The CD spectra are shown at the indicated temperatures (selected spectra).
Fluorescence spectroscopy was used to confirm the CD data in an equilibrium unfolding context. The sensitivity of fluorescence spectroscopy is advantageous for rapid kinetic studies which are a focus of this work. In equilibrium studies, fluorescence spectra of apo-BsSco at varying urea concentrations change in shape. The native spectrum is high in intensity and emits at a maximum of 329 nm (Fig. 8). At the highest urea concentration

Figure 8: Fluorescence spectra overlay of apo-BsSco in urea of varying concentrations. Apo-BsSco samples were prepared at a concentration of 0.15 mg/mL (15 μM) in 50 mM phosphate buffer, pH 7.0. Samples were denatured by urea of varying concentrations from 0-8 M (indicated in the graph) and monitored by fluorescence, cuvette pathlength = 1 cm.
(8 M), the intensity is diminished and the peak is at 347 nm. An unusual feature of these spectra is a shoulder at 310 nm that is higher than the Raman peak from the solvent water. This feature is not present in the denatured protein and may arise from tyrosine residues. BsSco has six tyrosines and two tryptophans. Tyrosine’s fluorescence may be independently evident on the spectrum instead of transferring to and enhancing the tryptophan fluorescence.

4.3 Equilibrium unfolding curves

Equilibrium unfolding profiles can be generated by compiling and normalizing the raw data to indicate the fraction unfolded taking the assumption that the unfolding transition of BsSco is complete at the highest denaturant concentration used in these experiments. Eight unfolding profiles were generated from different combinations of denaturants and monitoring tools: CD or fluorescence combined with GdnHCl or urea (Fig. 9-12), for two different forms of apo-BsSco, reduced and oxidized. The unfolding processes driven by urea or GdnHCl are all characterized by a sharp unfolding transition in support of the two-state model. GdnHCl unfolding of apo-BsSco commences at around 1.0 M, followed by a steep transition to the unfolded end of the profile where the transition is complete at around 2.0 M. The unfolding transition for urea denaturation is from 2-4 M. It is clear that urea is a weaker denaturant than GdnHCl.

The unfolding profiles of reduced and oxidized apo-BsSco are similar. However, there is a consistent difference in the unfolding transition of the two redox states of BsSco at which oxidized apo-BsSco starts to unfold at a higher denaturant concentration than
Figure 9: Equilibrium unfolding profile of reduced and oxidized apo-BsSco denatured by Gdn using CD as a monitoring tool. Protein samples were prepared at a concentration of 0.1 mg/mL in 50 mM sodium phosphate buffer, pH 7.0. Samples were denatured by GdnHCl of varying concentrations from 0-7 M and monitored by CD, cuvette pathlength = 1 mm. CD data at 222 nm were normalized and converted to fraction unfolded. Reduced and oxidized apo-BsSco are represented by blue squares (■) and red diamonds (♦), respectively.

reduced apo-BsSco. This difference is not large in the two unfolding profiles generated from CD data (Fig. 9 and 10), and the same pattern is observed in Figures 11 and 12 which are generated from fluorescence data. The fluorescence spectra have better signal to noise than the CD data and allow for application of non-linear curve fitting (Fig. 11-12)
to extract the equilibrium parameters of unfolding (54). The equation for fitting is shown as follows:

\[ f = \frac{(a_1 + b_1([\text{denaturant}]) + (a_2 + b_2([\text{denaturant}])) \exp((\Delta G_{u} - m_{eq}([\text{denaturant}])/RT))}{1 + \exp((\Delta G_{u} - m_{eq}([\text{denaturant}])/RT))} \]  

Figure 10: Equilibrium unfolding profile of reduced and oxidized apo-BsSco denatured by urea using CD as a monitoring tool. Protein samples were prepared at a concentration of 0.1 mg/mL in 50 mM sodium phosphate buffer, pH 7.0. Samples were denatured by urea of varying concentrations from 0-9 M and monitored by CD, cuvette pathlength = 1 mm. CD data at 222 nm were normalized and converted to fraction unfolded. Reduced and oxidized apo-BsSco are represented by blue squares (■) and red diamonds (♦), respectively.
where $\Delta G_u$ is the free energy of unfolding in the absence of denaturant, $m_{eq}$ is the equilibrium $m$-value and accounts for steepness of the unfolding transition, $a_1$ and $a_2$ are the fluorescence signals of the ideally folded and unfolded state in the absence of any denaturing conditions, and $b_1$ and $b_2$ are the changes in the signals of the folded and unfolded states as function of denaturant in the pre- and post-transition regions.

Figure 11: Equilibrium unfolding curves of reduced and oxidized apo-BsSco denatured by GdnHCl using fluorescence as a monitoring tool. Protein samples were prepared at a concentration of 0.15 mg/mL in 50 mM sodium phosphate buffer, pH 7.0. Samples were denatured by GdnHCl of varying concentrations from 0-7 M and monitored by CD, cuvette pathlength = 1 mm. Fluorescence data (peak wavelength) were normalized and converted to fraction unfolded. Reduced and oxidized apo-BsSco are represented by blue squares (■) and red diamonds (◆), respectively. Curve fitting is done by Microsoft Excel and the fits are indicated on the graph as dotted lines in the respective colors. Fitting parameters are shown for each trace.
Equilibrium unfolding curves of reduced and oxidized apo-BsSco denatured by urea using fluorescence as a monitoring tool. Protein samples were prepared at a concentration of 0.15 mg/mL in 50 mM sodium phosphate buffer, pH 7.0. Samples were denatured by GdnHCl of varying concentrations from 0-9 M and monitored by CD, cuvette pathlength = 1 mm. Fluorescence data (peak wavelength) were normalized and converted to fraction unfolded. Reduced and oxidized apo-BsSco are represented by blue squares (■) and red diamonds (♦), respectively. Curve fitting is done by Microsoft excel. Fitting parameters are shown for each trace.

\[ \Delta G_u = 9.0 \text{ kcal/mol} \quad a_1 = 0.87 \quad b_1 = 0.02 \]
\[ m_{eq} = 2.93 \text{ kcal/mol/M} \quad a_2 = 0.002 \quad b_2 = 0.05 \]

\[ \Delta G_u = 11.9 \text{ kcal/mol} \quad a_1 = 0.86 \quad b_1 = 0.02 \]
\[ m_{eq} = 3.5 \text{ kcal/mol/M} \quad a_2 = -0.001 \quad b_2 = 0.04 \]

Figure 12: Equilibrium unfolding curves of reduced and oxidized apo-BsSco denatured by urea using fluorescence as a monitoring tool. Protein samples were prepared at a concentration of 0.15 mg/mL in 50 mM sodium phosphate buffer, pH 7.0. Samples were denatured by GdnHCl of varying concentrations from 0-9 M and monitored by CD, cuvette pathlength = 1 mm. Fluorescence data (peak wavelength) were normalized and converted to fraction unfolded. Reduced and oxidized apo-BsSco are represented by blue squares (■) and red diamonds (♦), respectively. Curve fitting is done by Microsoft excel. Fitting parameters are shown for each trace.

Curve fitting of the two datasets obtained from fluorescence data explains the apparent difference on the unfolding profiles. Free energy values (\(\Delta G_u\)) extracted by curve fitting shows an average of 8.95 ± 0.23 kcal/mol for reduced apo-BsSco compared to 10.6 ± 2.1 kcal/mol for oxidized apo-BsSco. Greater unfolding free energy results in greater resistance to unfolding of oxidized apo-BsSco compared to reduced apo-BsSco.
However, statistical comparison reveals a p value of 0.47 which means the $\Delta G_u$ values for reduced and oxidized apo-BsSco is not significantly different.

### 4.4 Unfolding profiles of Cu(II)-BsSco

The major interest of this work is to explore the effect of Cu(II) on folding/unfolding of BsSco. Similar experiments reported for apo-BsSco were repeated for Cu(II)-BsSco. Cu(II)-BsSco simply does NOT unfold in urea up to 9.6 M urea (Fig. 13B). Reduced apo-BsSco unfolds as the urea concentration rises from 0 to 9 M (Fig. 13A), whereas Cu(II)-BsSco maintains its native structure in 9.6 M urea which is close to the highest urea concentration possible (10 M). Cu(II)-BsSco also stays structured in 9.6 M urea at pH 4.0. We propose that the binding of Cu(II) causes a global stabilization that is resistant to unfolding.

In contrast to urea, another denaturant GdnHCl, does induce unfolding of Cu(II)-BsSco (Fig. 14A and B). GdnHCl and urea are both widely used denaturants, but the high ionic strength of GdnHCl is distinctive from urea. This leads to the idea that the high ionic strength of GdnHCl may be important for unfolding. We have encountered high salt-activated oxidation of BsSco before. We soon realized that the protein samples following GdnHCl unfolding had lost their Cu(II) (confirmed by absorption spectrometry), and had an SH count close to zero (e.g., SH = 0.16). This suggests that the Cu(II)-BsSco samples have undergone oxidation while Cu(II) is lost in concentrated GdnHCl. Actually, from previous studies, we know that BsSco can be oxidized by Cu(II). Cu(II)-BsSco loses Cu(II) binding in 6 M NaCl as Cu(II) gets converted to Cu(I),
Figure 13A: Reduced apo-BsSco unfolds in urea. Protein samples were prepared at a concentration of 0.12 mg/mL for the apo protein and 0.3 mg/mL for Cu(II) bound protein in 50mM sodium phosphate buffer. CD spectra were recorded from 200-260 nm at a step of 1 nm, cuvette pathlength = 1 mm. Reduced apo-BsSco unfolds as the urea concentration (indicated in the graph) increase from 0-9 M.
Figure 1B: Cu(II)-BsSco is resistant to urea denaturation. Protein samples were prepared at a concentration of 0.12 mg/mL for the apo protein and 0.3 mg/mL for Cu(II) bound protein in 50mM sodium phosphate buffer. CD spectra were recorded from 200-260 nm at a step of 1 nm, cuvette pathlength = 1 mm. Urea concentration increases from 0-9 M urea. Cu(II)-BsSco remains folded regardless of urea concentration (indicated in the graph).
and Cu(I) dissociates from BsSco leaving BsSco oxidized (14). We have discovered that reduced apo-BsSco in its unfolded form is also subject to copper catalyzed oxidation.

Figure 14A: Reduced apo-BsSco unfolds in GdnHCl. Protein samples were prepared at a concentration of 0.1 mg/mL for the apo protein and 0.18 mg/mL for Cu(II) bound protein in 50 mM phosphate buffer, pH 7.0. CD spectra were recorded from 200-260 nm at step of 1 nm, cuvette pathlength = 1mm. Reduced apo-BsSco unfolds in GdnHCl as the GdnHCl concentration (indicated in the graph) increases.
This is confirmed by SH count after addition of trace amount of Cu(II) (5 μM) to reduced apo-BsSco (200 μM) sample (an SH count close to 0 compared to an SH count of 1.8

Figure 14B: Cu(II)-BsSco unfolds in GdnHCl. Protein samples were prepared at a concentration of 0.1 mg/mL for the apo protein and 0.18 mg/mL for Cu(II) bound protein in 50 mM phosphate buffer, pH 7.0. CD spectra were recorded from 200-260 nm at step of 1 nm, cuvette pathlength = 1mm. Cu(II)-BsSco unfolds in GdnHCl as the GdnHCl concentration (indicated in the graph) increases.
before addition of Cu(II)). However, apart from oxidation, it is reasonable to postulate that BsSco bound with Cu(II) (instead of getting oxidized by Cu(II)) does NOT unfold until it releases Cu(II), that is the loss of Cu(II) and the loss of structure are coincident.

Figure 15A: Kinetics of Cu(II)-BsSco denaturation by GdnHCl. Cu(II) bound protein samples were denatured by both GdnHCl and urea. The loss of structure and loss of Cu(II) binding were simultaneously monitored by CD in a 1 mm pathlength cuvette for a period of time (100 mins) at 222 nm and 352 nm, respectively. CD spectra were taken at 0 time (blue trace) and 100 mins (pink trace). The inset shows the difference in Cu(II) signal at 352 nm.
Further comparison of the stability of Cu(II)-BsSco in urea and GdnHCl shows that Cu(II)-BsSco is kinetically stable in urea, but unstable in GdnHCl (Fig. 15 A and B). The samples in either GdnHCl or urea are bound with Cu(II) at time zero as judged by peaks in the visible region of the CD spectra. After 100 minutes, the visible region of the CD spectrum of the sample in GdnHCl becomes less intense (inset of panel A) and this is accompanied by a substantial signal drop at 220 nm which reflects the loss of structure. In

Figure 15B: Kinetics of Cu(II)-BsSco denaturation by urea. Cu(II) bound protein samples were denatured by both GdnHCl and urea. The loss of structure and loss of Cu(II) binding were simultaneously monitored by CD in a 1 mm pathlength cuvette for a period of time (2 hrs) at 222 nm and 352 nm, respectively. CD spectra were taken at 0 time (blue trace) and 2 hrs (pink trace). The inset shows the difference in Cu(II) signal at 352 nm.
contrast the visible and UV CD spectra of the sample in urea are unchanged after 100 minutes. This finding also supports our hypothesis that the loss of Cu(II) binding and the loss the structure are coincident.

Time courses of the signal loss in the UV and visible CD are illustrated in Figure 16.

The signal change in the UV region at 222 nm and in the visible region at 352 nm are

![Graph showing changes in ellipticity at 222 nm and 352 nm over time](image)

**Figure 16:** Loss of structure and loss of conformation are coincident. Cu(II)-BsSco was prepared at 1 mg/mL in 50mM phosphate, pH 7.0. The sample was denatured by 2M GdnHCl over a period of 100 minutes to allow gradual loss of Cu(II) binding. CD spectra were recorded from 200-500 nm (cuvette pathlength = 1 mm) so that the conformation signal (222 nm) and Cu(II) binding signal (352 nm) can be monitored simultaneously. The change at 222 nm and 352 nm were plotted as a function of time. Change in conformation signal (■) is plotted on the left Y axis. Change in copper signal (♦) is plotted on the right axis.
coincident. Since unfolding of apo-BsSco is rapid and complete under these conditions it is proposed that copper binding prevents BsSco unfolding and that protein denaturation only proceeds at the rate of copper dissociation.

The equilibrium unfolding profile of Cu(II)-BsSco looks flat and only the pre-transition region is present on the graph when compared to the unfolding curves of

![Graph](image)

*Figure 17: Equilibrium unfolding curve of apo (reduced and oxidized) and Cu(II) bound BsSco denatured by urea using CD as a monitoring tool. Protein samples were prepared at 0.1 mg/mL in 50 mM phosphate buffer, pH 7.0. Samples were denatured by urea of varying concentrations and monitored by CD, cuvette pathlength = 1mm. CD data at 222 nm were normalized and converted to fraction unfolded. Reduced, oxidized and Cu(II) bound BsSco are represented by blue squares(■), red diamonds (♦) and purple circles (●), respectively.*
reduced and oxidized apo-BsSco (Fig.17). Therefore, we cannot calculate an unfolding free energy for Cu(II)-BsSco in urea. But we can surmise that the unfolding free energy for Cu(II)-BsSco is large compared to apo-BsSco.

4.5 Kinetics of refolding and unfolding of BsSco

The rapid kinetics of refolding/unfolding of BsSco were investigated by stopped-flow equipped fluorescence spectroscopy using urea as the denaturant. The purpose of these experiments is to measure the rate constants at which different forms of BsSco refold and unfold. For this type of kinetic experiment, protein samples are prepared at a relatively high concentration (5 mg/mL is the lower limit) to ensure sufficient signal strength and sensitivity. Protein samples were excited at 280 nm, fluorescence emission spectra were recorded at a time interval of 16 ms starting from immediately after mixing with appropriate buffers for unfolding or refolding.

The kinetics of unfolding and refolding of BsSco are studied in different experiments with different protein samples. For unfolding of BsSco, the protein samples are prepared in native conditions and mixed with denaturing buffer (1:10 mixing ratio) containing a high concentration of urea (e.g., 10 M) to allow unfolding. The process of unfolding is monitored by fluorescence spectroscopy during the time period of denaturation (Fig. 18A). The example illustrated here is the unfolding of oxidized apo-BsSco in 6.4 M urea. The first spectrum recorded immediately after mixing is a typical spectrum (Fig. 18A, red) for native BsSco with high fluorescence intensity at a maximum of 330 nm. The following spectra become less intense and the peak shifts to longer
wavelengths as the protein sample ages. The unfolding of this particular oxidized sample is finished within one second as judged by Figure 18B. Global curve fitting of the intensity change over the entire spectrum gives a rate constant for unfolding at 6.4 M urea,

![Fluorescence spectra during the time period of unfolding](image)

**Figure 18A:** Unfolding kinetics of oxidized apo-BsSco at 6.4 M urea. Oxidized apo-BsSco was prepared at a concentration of 5.5 mg/mL in 50 mM sodium phosphate buffer, pH 7.0. The protein sample was mixed (1:10, final [BsSco] = 0.55 mg/ml) with denaturing buffer, allowing unfolding to occur in 6.4 M urea. Fluorescence spectra during the time period of unfolding (for clarification purposes, selected spectra at a time interval of 32 ms are shown). The first spectrum record immediately after mixing is represented in red, subsequent spectra are at 16 ms intervals.
$k_u = 4.8 \text{ s}^{-1}$. The fitting also produces two spectra corresponding to the folded and unfolded forms, supporting a two-state mechanism for unfolding of BsSco.

Figure 18B: Time course of unfolding kinetics of oxidized apo-BsSco at 6.4 M urea. Oxidized apo-BsSco was prepared at a concentration of 5.5 mg/mL in 50 mM sodium phosphate buffer, pH 7.0. The protein sample was mixed (1:10, final [BsSco] = 0.55 mg/ml) with denaturing buffer, allowing unfolding to occur in 6.4 M urea.

For studying the refolding of BsSco, a protein sample is prepared in urea-containing (5 M) sodium phosphate buffer. The unfolded protein sample is mixed with refolding buffer containing no urea, allowing refolding of the unfolded protein to occur. An example shown here to illustrate the refolding of oxidized apo-BsSco is in 1.8M urea
(Fig.19A). The first spectrum recorded (Fig. 19A, red) is a typical spectrum of unfolded BsSco with low fluorescence intensity and a maximum at 347 nm. The spectra following mixing become more intense and the peak shifts to a shorter wavelength as the unfolded

Figure 19A: Refolding kinetics of oxidized apo-BsSco at 1.8 M urea. Oxidized apo-BsSco was prepared at a concentration of 8 mg/mL in 50 mM sodium phosphate buffer, pH 7.0. The protein sample was mixed (1:10, final [BsSco] = 0.8 mg/ml) with native buffer, allowing refolding to occur in 1.8 M urea. Fluorescence spectra during the time period of refolding (time interval = 32 ms). The first spectrum is recorded immediately after mixing and is represented in red. Subsequent spectra are recorded at 16 ms intervals.
protein sample ages. The refolding process is finished within 1 s as judged by Figure 19B.

Global curve fitting of the intensity change of the entire spectra over time gives a rate

![Graph showing refolding kinetics](image)

*Figure 19B: Time course of refolding kinetics of oxidized apo-BsSco at 1.8 M urea. Oxidized apo-BsSco was prepared at a concentration of 8 mg/mL in 50 mM sodium phosphate buffer, pH 7.0. The protein sample was mixed (1:10, final [BsSco] = 0.8 mg/ml) with native buffer, allowing refolding to occur in 1.8 M urea.*

constant for refolding at 1.8 M urea, $k_f = 1.6 \text{ s}^{-1}$. The fitting also produces two spectra corresponding to the folded and unfolded forms, supporting a two-state mechanism for refolding of BsSco.
All observed rate constants obtained in the kinetic refolding/unfolding experiments for reduced and oxidized apo-BsSco are plotted on a common axis versus final denaturant concentration (Fig. 20). This graph is referred to as a Chevron plot which lays out the relationship between rate constants and denaturant concentration. The rate of folding and unfolding is related to the driving force (i.e., denaturant concentration) in a simple two-state system. Therefore, one may anticipate the two arms on these plots will be

\[
\begin{align*}
A &= 3.47 \\
m_f &= -0.94 \text{ kcal/mol/M} \\
B &= -5.11 \\
m_u &= 0.57 \text{ kcal/mol/M}
\end{align*}
\]

Figure 20: Chevron plots of reduced and oxidized apo-BsSco. Rate constants were obtained from kinetic refolding and unfolding experiments of BsSco. The rate constants were converted to natural logarithmic scale and plotted against the urea concentration. Reduced and oxidized apo-BsSco are represented by blue squares (■) and red diamonds (◇), respectively. Global curve fitting is done by Microsoft excel and indicated on the graph as dotted lines in the respective colors.
represented by a straight line. In the Chevron plot, lower urea concentrations correspond with higher refolding rate constants for the refolding half and higher urea concentrations correspond with higher unfolding rate constants. Comparison of these two Chevron plots shows that the kinetics of refolding and unfolding of reduced and oxidized apo-BsSco are similar. Curve fitting can also be performed with the Chevron plot using equation (2) described in 54 (Fig. 20):

$$\ln(k_{obs}) = \ln[\exp(A + \frac{m_f[\text{denaturant}]}{RT} + \exp(B + \frac{m_u[\text{denaturant}]}{RT}))]$$ (2)

where $m_f$ and $m_u$ accounts for the slopes of the two halves of the Chevron plot, measured in kcal/mol/M. A and B are the natural logarithm values of the folding and unfolding rate constants in the absence of denaturant, respectively.

Curve fitting of the Chevron plots estimates the rate constant values in the absence of denaturant. It is clear that the refolding rate for reduced apo-BsSco (i.e., 30.3 s$^{-1}$) is slightly slower than that of oxidized apo-BsSco (i.e., 32.1 s$^{-1}$). However, the unfolding rate constant of reduced apo-BsSco (i.e., 1.0 x 10$^{-4}$ s$^{-1}$) is less than that of oxidized apo-BsSco (i.e., 6.0 x 10$^{-3}$ s$^{-1}$). The slight differences in refolding and unfolding rate constants between reduced and oxidized forms of apo-BsSco is consistent with results obtained from equilibrium unfolding experiments, which implies that the extra disulfide bond of oxidized apo-BsSco does not significantly alter the folding/unfolding kinetics of BsSco.

The extreme stability against urea denaturation for Cu(II)-BsSco implies a low rate of unfolding. We observe no change in the spectrum of Cu(II)-BsSco in 9.6 M urea over
two hours. Therefore, the rate of unfolding BsSco-Cu(II) is less than $10^{-5}$ s$^{-1}$ in 9.6 M urea and eliminates the possibility of doing kinetic unfolding experiments for Cu(II)-BsSco. However, it is possible to proceed with an reduced apo-BsSco sample to observe the effect of Cu(II) on refolding. In this context, it is expected that Cu(II) binding, which substantially stabilizes BsSco, should increase the rate of refolding.

A Cu(II)-involved refolding experiment is illustrated at 0.5 M urea in Figure 21A. Reduced apo-BsSco in urea-containing (5 M) sodium phosphate buffer is mixed with Cu(II) containing buffer with no urea, allowing the refolding and Cu(II) binding to occur. In this context, refolding should precede Cu(II) binding. It has been reported previously that Cu(II) binding to native BsSco results in a decrease in fluorescence intensity, but without a shift in the wavelength maximum (14, 17). Therefore, in this particular refolding experiment, the increase in fluorescence intensity caused by refolding may be opposed by a fluorescence decrease due to Cu(II) binding. The initial spectrum observed after mixing has relatively high fluorescence intensity and a peak wavelength of 330 nm (Fig. 21A, red). This is consistent with refolding of the protein sample in the dead-time ($t_{1/2} < 2$ ms) of the stopped-flow mixer. As time proceeds the fluorescence intensity declines at an observed rate of 20.6 s$^{-1}$ while retaining an emission maximum at 330 nm. Refolding is followed by Cu(II) binding, resulting in a significant drop in fluorescence intensity without a peak shift. Therefore, Figure 21A reflects a two step process with initial refolding at a rate too fast to be monitored (i.e., $k_{obs} > 350$ s$^{-1}$) followed by Cu(II) binding at a rate of 20.6 s$^{-1}$ obtained from the curve fitting of Figure 21B.
Figure 21A: Refolding kinetics of reduced apo-BsSco in the presence of Cu(II) at 0.5 M urea. Reduced apo-BsSco was prepared unfolded at a concentration of 7 mg/mL (stock concentration) in 50 mM sodium phosphate buffer containing urea (5 M), pH 7.0. The protein sample was mixed with refolding buffer containing 50 μM Cu(II), allowing refolding and Cu(II) binding to occur in 0.5 M urea. Selected spectra during the time period of refolding (time interval = 32 ms). The first spectrum record at time zero and is represented in red.

Raising the urea concentration should slow refolding and perhaps allow the refolding event to be observed directly in the presence of copper. The refolding process in the presence of Cu(II) at 1.6 M urea is shown in Figure 22A. Again the initial spectrum following mixing has relatively high fluorescence intensity with the maximum at 330 nm indicating that refolding is still too fast to observe under these conditions. The phase of
Figure 21B: Time course of refolding kinetics of reduced apo-BsSco in the presence of Cu(II) at 0.5 M urea. Reduced apo-BsSco was prepared unfolded at a concentration of 7 mg/mL (stock concentration) in 50 mM sodium phosphate buffer containing urea (5 M), pH 7.0. The protein sample was mixed with refolding buffer containing 50 μM Cu(II), allowing refolding and Cu(II) binding to occur in 0.5 M urea.

The declining fluorescence intensity proceeds at an observed rate of 3.4 s⁻¹ obtained from the curve fitting of Figure 22B.

The difference in the observed rate ascribed to Cu(II) binding during refolding leads to the idea that another process apart from refolding and Cu(II) binding occurs in this experiment. We know that denatured BsSco undergoes copper catalyzed oxidation and this would be competing with Cu(II) binding as the urea concentration increases from 0.5
Figure 22A: Refolding kinetics of reduced apo-BsSco in the presence of Cu(II) at 1.6 M urea. Reduced apo-BsSco was prepared unfolded at a concentration of 7 mg/mL (stock concentration) in 50 mM sodium phosphate buffer containing urea (5 M), pH 7.0. The protein sample was mixed with refolding buffer containing 50 μM Cu(II), allowing refolding and Cu(II) binding to occur simultaneously at 1.6 M urea. Panel A, selected spectra during the time period of refolding (time interval = 32 ms). The first spectrum recorded immediately after mixing is represented in red.

To test this hypothesis, the extent of Cu(II) binding during refolding is measured by the extent of formation of the copper complex. The same reduced protein sample was split into a number of samples, and each was refolded with Cu(II) at different concentrations of urea (Fig. 23). The extent of Cu(II) binding to refolding BsSco in 0.5 M urea is 50% (red, Fig. 23) compared to native reduced apo-BsSco (pink, Fig. 23). Higher urea concentrations lead to further loss of Cu(II) binding (blue to dark green, Fig.
Reduced apo-BsSco was prepared unfolded at a concentration of 7 mg/mL (stock concentration) in 50 mM sodium phosphate buffer containing urea (5 M), pH 7.0. The protein sample was mixed with refolding buffer containing 50 µM Cu(II), allowing refolding and Cu(II) binding to occur simultaneously at 1.6 M urea.

23). One may ask if it is the presence of urea that prevents Cu(II) binding or leads to BsSco oxidation. To answer this question, Cu(II) was added to a native sample of reduced apo-BsSco in 1.5 M urea (green, Fig. 23). This sample gives almost the same Cu(II) binding signal compared to the Cu(II) bound sample in buffer without urea (pink, Fig. 23). Therefore, it is proposed that Cu(II) oxidizes reduced apo-BsSco while it’s refolding.
Figure 23: Reduced apo-BsSco refolding with Cu(II) results in oxidation. Different reduced apo-BsSco samples were prepared from the same stock of protein. Each sample was prepared to have a concentration of 1 mg/mL in sodium phosphate buffer, pH 7.0. One sample was recorded for absorption spectrum directly (cyan). A second sample was mixed with Cu(II) (pink). Other samples were mixed with Cu(II) while refolding at varying concentrations of urea (0.5-2.5 M as indicated). A last sample was mixed with Cu(II) in 1.5 M urea but not in a refolding context (green).
Chapter 5
Discussion

5.1 Metal-assisted folding and BsSco

The folding of proteins has been extensively studied. Many proteins require a particular three-dimensional structure to function. The folding of newly synthesized proteins in vivo starts co-translationally and with the assistance of molecular chaperones. Most purified proteins have stable structures in vitro that can be unfolded by various treatments including chemical denaturants, temperature, and extremes of pH.

Many proteins also use metals as cofactors and catalysts to function. It is only natural to propose that a metal’s interaction with a specific protein will influence its folding. For example, metal-requiring proteins may go through two pathways to form the functional holo-protein after they have been synthesized: binding metal before or after folding. A classic example is azurin which requires Cu(II) as a cofactor. The fastest pathway for functional azurin assembly is to bind Cu(II) before folding (18). Metal binding before folding takes milliseconds and the subsequent folding takes a similar amount of time. However, binding Cu(II) after folding takes minutes to finish the assembly of holo-azurin. It is proposed that metal binding before folding serves as a catalytic nucleus that directs the pathway towards the folded form, and significantly increases the rate of functional azurin assembly (18). How metalloproteins can acquire metals before folding has been enigmatic. Metallochaperones are potential candidates that could fill this role.
The value of BsSco as a tool to study the effect of metals on the folding of proteins relies on its high binding affinity for Cu(II). Although the exact role of BsSco has not been elucidated, its interaction with Cu(II) has been well documented. Single-domain proteins are ideal for studying folding behavior in the context of metal interaction. The effect of metals on azurin, which is also a single-domain protein, has been well studied and it has provided convincing evidence that Cu(II) is able to thermodynamically stabilize azurin and kinetically facilitate the formation of holo-azurin. The basic hypothesis is that the folding of BsSco will be accelerated in the presence of Cu(II), due to Cu(II) binding to unfolded BsSco or an intermediate state that appears on the folding pathway.

5.2 Folding and unfolding thermodynamics of apo-BsSco

CD spectrometry is an efficient and convenient method to monitor the secondary structure and to observe structural changes in proteins. Deconvolution of the UV-CD spectrum allows an estimate of the proportion of secondary structural elements present in a particular protein sample (55). For example, our CD analysis of a sample of native, reduced apo-BsSco has a secondary structure distribution as follows: $\alpha$-helix 27.6%, $\beta$-sheet 36.5% and random coil 36.6%. This estimation is consistent with previously reported X-ray data. In 6 M urea, there is a considerable change in the CD spectrum of reduced apo-BsSco, representing a loss in secondary structure. This concentration of urea ($i.e.$, 6 M) is far beyond the point at which the unfolding transition is complete. Nevertheless it is of interest to ask the question: does “unfolded” protein retain residual
structure. The UV-CD spectra of proteins that have undergone chemical denaturant-mediated (GdnHCl and urea) unfolding cannot be deconvoluted with confidence due to the inaccessibility of the CD spectrum below 210 nm in the UV region. However, thermal denaturation allows access to the entire UV-CD spectrum and hence deconvolution of the CD spectrum of the denatured protein. Such analysis of thermally denatured BsSco shows that “unfolded” protein still retains a considerable amount of secondary structure. There is a decrease in α-helical content (reduced to 6.2%) accompanied by an increase in random coil (increased to 52.1%) along with an increase in β-sheet content (increased to 42.5%).

Previous studies have revealed that about 20% of β-strands are present in proteins fully “unfolded” by GdnHCl in which β-strands should be broken (55). Researchers conducting these studies have suggested that the denatured proteins contain an ensemble of residual secondary structures, partly unstructured content and completely disordered structures. Comparison of our data to previously reported results shows that the unfolded structure of BsSco is most similar to thioredoxin proteins in which α-helical content remains 6.1%. β-sheet content increases to 34.6% along with an increase in random coil to 42.1% (56).

Many small globular single-domain proteins follow a two-state mechanism in their folding behavior, which means there are only two thermodynamically stable species in the folding transition, the unfolded form and the folded form (57-59). The view that BsSco is also a two-state protein is supported by the chemical unfolding profiles of apo-BsSco obtained here. A single sharp transition is present in all of the unfolding profiles whether monitored by UV-CD or fluorescence. In addition, the thermal denaturation of
apo-BsSco shows an isosbestic point in the spectra collected over the entire denaturation range which also supports a two-state mechanism.

Oxidized apo-BsSco has an extra disulfide bond compared to reduced apo-BsSco, where the extra disulfide bond should lend extra stability to the oxidized protein. Previous experiments have explored the relationship between the added stability due to the presence of a disulfide bond and the length separating the two cysteine residues in the primary sequence (60-62). The further the two cysteine residues are separated the more stability that is conferred. In addition, extra stability from the presence of a disulfide bond also depends on cysteine’s position in relation to the domain structure of the protein. For example, inter-domain disulfide bonds give more structural stability than intra-domain disulfide bonds. In our case, the cysteine residues forming the disulfide bond in apo-BsSco are only three residues apart (C65 and C69) and they are on the same structural unit. This length of separation predicts there will be little effect, if any, on the equilibrium stability of BsSco. Our results reveal that the unfolding profiles of reduced and oxidized apo-BsSco exhibit a consistent difference in which oxidized apo-BsSco is more resistant to unfolding than reduced apo-BsSco. However, a statistical comparison of the unfolding free energy values gives a p value 0.47 (significance cut off p=0.05), thus denying statistical significance to the difference in equilibrium stability of the two forms. This outcome is consistent with the short distance between the two cysteines within BsSco’s primary sequence.
5.3 Folding and unfolding thermodynamics of Cu(II)-BsSco

Cu(II)-BsSco is resistant to unfolding in urea, however Cu(II)-BsSco does unfold in GdnHCl. We ascribe this difference to the high ionic strength of GdnHCl compared to urea. We have reported that high ionic strength activates a redox exchange process in which Cu(I) is released from Cu(II)-BsSco and BsSco’s thiols are oxidized to a disulfide (14). We propose that Cu(II)-BsSco is resistant to denaturation as long as Cu(II) stays bound. This might be explained by the structural constraint placed by Cu(II) binding that blocks the initial step in the whole unfolding process. The two external loops are held together by Cu(II) interaction with the binding ligands. We suspect that, if BsSco were to unfold, the two loops must be flexible enough to aid the disassembly of the core structure. If not, the core structure of BsSco cannot be unfolded. This hypothesis can be tested by linking the two external loops using methods other than Cu(II) binding. Site-directed mutagenesis creating a cysteine pair capable of forming a disulfide bond between the two loops is a potential experimental design. If this version of BsSco is resistant to unfolding, it would support our hypothesis.

We have done thermal denaturation of BsSco-Cu(II) by DSC and shown a 23 °C temperature shift in the melting temperature (14). It might be possible to understand the kinetic relationship between unfolding and copper dissociation by using rapid temperature jump to observe if Cu(II)-BsSco unfolds before Cu(II) dissociates.
5.4 Copper oxidation of thiol groups

Copper oxidation of thiol groups is well documented (63-65). Nevertheless the exact mechanism of the oxidation process has remained elusive. Although BsSco binds and forms a complex with Cu(II) using its two thiol groups, copper-mediated oxidation of thiol groups is a more common phenomenon (65-66). We propose that binding between BsSco and Cu(II) is a sequential process initiated by the interaction of Cu(II) with histidine first, followed by addition of the two thiol groups as inner sphere ligands (Scheme 1). This is supported by the outcome from two previous experiments. Firstly, the BsScoH135A mutant in which the ligating histidine has been changed to alanine,
binds Cu(II) only transiently (67). Formation of a complex with Cu(II) occurs over a short period time (several minutes) and this is followed by Cu(I) dissociation and oxidation of the thiol groups. Secondly, it is shown here that only trace amounts of Cu(II) are able to oxidize unfolded reduced apo-BsSco. We propose that, in unfolded BsSco, the protein has lost its structural integrity and hence the reaction sequence leading to a stable copper complex is not followed. Therefore, Cu(II) interacts with the thiol groups without the involvement of histidine. In this case, copper oxidation of thiol groups occurs in a catalytic manner to oxidize the entire BsSco sample.

We have encountered another form of copper oxidation in this work, the high ionic strength-activated loss of Cu(II) binding from BsSco. This phenomenon is actually a process of copper-mediated oxidation of BsSco’s thiol groups. The proposed mechaism of Cu(II) oxidiation of reduced apo-BsSco is illustrated in Scheme 2. In this process, high ionic strength activates a process in which bound Cu(II) is reduced to Cu(I). In the presence of the Cu(I) chelator BCS, Cu(I) dissociates from the protein. The thiol groups are oxidized to corresponding radicals, two of which react to form a disulfide. The underlying mechanism as to how high ionic strength can trigger this process is unknown. But because high ionic strength is not normally present in a cellular environment, this concern remains less biologically significant. However, the opportunity for unfolded reduced apo-BsSco to encounter copper that would lead to thiol oxidation does have some biological significance. In contrast to azurin, which binds Cu(II) in its unfolded state, binding of reduced apo-BsSco in its unfolded state leads through the process of Cu(II)-
mediated oxidation of BsSco and switches to its disulfide form. Therefore, if BsSco were to function as a copper chaperone, acquiring copper before folding is problematic.

Scheme 2: Proposed mechanism of Cu(II) oxidation of BsSco.

5.5 Folding kinetics of BsSco

In Figure 20, the Chevron plot of reduced and oxidized apo-BsSco are compared. In the unfolding half, the rate constants increase as the urea concentration increases. The refolding rate constants decline as the urea concentration increases in the refolding half. Comparison of the two Chevron plots shows that reduced and oxidized apo-BsSco behave similarly from a kinetic point of view. However, a second look at the overlay of the two plots shows that, in the refolding half, the rate constant of oxidized apo-BsSco is slightly larger than reduced apo-BsSco at almost any measured urea concentration. There is less of a distinctive difference between the rates of unfolding observed for oxidized and
reduced states of apo-BsSco. The slight difference in equilibrium stability conferred by the presence of the intramolecular disulfide in apo-BsSco is recapitulated in the rates observed for folding and unfolding. Our Chevron plot starts at 1 M urea in the refolding half. In fact, urea concentrations lower than 1 M were also attempted (0.5, 0.8 M). Nevertheless we did not observe a refolding process at these urea concentrations, which means the first spectrum recorded immediately after mixing gives a completely folded shape. For the refolding transition to go unobserved at 0.5 and 0.8 M urea the refolding process must be complete in the “dead-time”, or mixing time (i.e., < 1.5 ms) of the stopped flow. This implies a rate of refolding of greater than 600 s\(^{-1}\). The folding kinetics of Cu(II)-BsSco cannot be directly observed. In order to implement the kinetic experiments on Cu(II)-BsSco, we need to prepare the protein samples folded or unfolded. It is impossible to prepare an unfolded Cu(II)-BsSco sample because 1), Cu(II)-BsSco does not unfold in urea, 2), if we prepared unfolded reduced apo-BsSco, then add Cu(II), the protein goes oxidized (see Results section 4.4).

An alternative to performing this type of experiment on Cu(II)-BsSco is to investigate the effect of Cu(II) on refolding of reduced apo-BsSco. We have shown in equilibrium unfolding experiments that Cu(II) confers extreme stability upon BsSco. It is of interest to explore how Cu(II) stabilizes BsSco from a kinetic sense. It is natural to propose that Cu(II) should speed up folding and slow down unfolding of BsSco. By following this hypothesis, we allowed Cu(II) binding to occur while reduced apo-BsSco is refolding.
The reaction profile of Cu(II)-involved refolding exhibits two phases. The first phase is an instantaneous shift to 330 nm followed by a second phase of decreasing fluorescence intensity. We ascribe the first phase to extremely fast refolding with Cu(II) at 0.5 M urea. The interesting feature of Figure 21 is the presence of the second phase, which is a Cu(II) binding process as judged by a substantial drop at about 330 nm. From this we can tell that the refolding process is finished in less than 3 ms and Cu(II) binding occurs during or after refolding. A higher urea concentration of 1.6 M was attempted in the presence of Cu(II) in the hope that a higher urea concentration would slow refolding. However, the refolding process is still too fast to observe (see Figure 22) and Cu(II) binding is still evident. Rate extraction from these two datasets gives apparent rates for Cu(II) binding of 20.6 s\(^{-1}\) at 0.5 M urea and 3.6 s\(^{-1}\) at 1.6 M urea. It is a sequential process, so the rate of Cu(II) binding cannot occur faster than refolding. This is true for our samples as the refolding rate constants are 7.4 and 12.2 s\(^{-1}\) for reduced and oxidized apo-BsSco at 1.6 M urea, respectively. As the refolding process is slower at 1.6 M urea this allows for greater opportunity for the competing process of oxidation to occur.

We measured the extent of formation of BsSco-Cu(II) along with the SH count of the samples from these experiments. The yield of BsSco-Cu(II) decreases at slower refolding rates and in all cases the SH count is close to zero. The refolded protein that is not present as the Cu(II) complex has, therefore, undergone oxidation. Therefore, we reason that Cu(II) binding during the refolding of reduced apo-BsSco triggers a second competing process, Cu(II)-catalyzed oxidation of BsSco. Cu(II) binding and oxidation are competing with each other until all protein molecules are bound with Cu(II) or
oxidized to its disulfide form. Which process predominates depends on the refolding rate. It is proposed that the faster refolding, the more protein is Cu(II) bound and the less protein is oxidized (Scheme 3). This proposal is supported by the last experiment in this project: the formation of Cu(II) complex is monitored directly by absorption spectrometry in the context of refolding. In Figure 23, refolding with Cu(II) at only 0.5 M urea results in a loss of 50% Cu(II) binding. Higher urea concentration further reduces Cu(II) complex formation. One may suggest that the presence of urea is the major cause of the diminished Cu(II) binding. This is proven wrong by adding Cu(II) directly to a non-refolding, native reduced apo-BsSco in 1.5 M urea (BsSco is folded in 1.5 M urea), at which the formation of Cu(II) complex at 1.5 M urea is only slightly diminished compared to the native protein.

The phenomenon of Cu(II) oxidation of refolding BsSco suggests that, although we have experimentally determined BsSco to be a two-state protein, the folding intermediates of BsSco may exist long enough to interact with Cu(II).

Scheme 3: Refolding rate-dependent oxidation by Cu(II) of reduced apo-BsSco undergoing refolding.
5.6 BsSco and its role in the assembly of the Cu$_A$ center of cytochrome c oxidase

BsSco, a member of the Sco protein family found in the aerobic bacterium B. subtilis, differs from its eukaryotic counterparts. Eukaryotic Sco proteins are integral components of the inner mitochondrial membrane and prefer to bind Cu(I) (11). In contrast, BsSco prefers to bind Cu(II) with an extremely high binding affinity (14) despite the utilization of the same conserved metal binding motifs (CXXXC and histidine) as found in eukaryotic Sco proteins. It has been suggested that Cu(I) binding preference exhibited by eukaryotic Sco is consistent with the reducing environment in the cytoplasm (8, 11, 68), whereas the Cu(II) binding preference of BsSco is consistent with the 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 than is found for Cu(II) (14, 54). Furthermore, previously reported results clearly show that BsSco is required in the assembly of cytochrome c oxidase as the BsSco knockout strain of B. subtilis is deficient in cytochrome c oxidase (13). The requirement of BsSco in the assembly of cytochrome c oxidase, along with the ability to bind both Cu(II) and Cu(I), make BsSco an ideal candidate to serve as a copper trafficking protein that delivers copper ions to the mixed-valence Cu$_A$ center. The underlying physical understanding of the difference in metal preference for mitochondrial Sco proteins and BsSco is not complete. Mitochondrial Sco proteins from yeast and human bind Cu(II) but their relative binding affinities have not been reported.

Structurally, BsSco’s proposed role as a copper chaperone is challenged by its sequence similarity to the thioredoxin protein family (10). This suggests BsSco can fulfill
a redox role in the assembly of cytochrome c oxidase. Previously discovered characteristics of BsSco also include thiol/disulfide switching between the two cysteine residues of BsSco, further consolidating BsSco’s role as a disulfide/thiol reductase to maintain the Cu_A center’s cysteine residues reduced. Although BsSco interacts with Cu(II) with high affinity, comparison of BsSco to the copper chaperone proteins reveals that many metallochaperone proteins contain a conserved heavy metal binding motif MXCXXC and an “open-faced β-sandwich” global fold which are not present in BsSco (69). Co-crystallization of BsSco with Cu(II) also has been unsuccessful. Researchers at the Magnetic Resonance Center in Florence have claimed definitively that, in *T. thermophilus*, the correct copper chaperone for the assembly of cytochrome c oxidase is the protein PCu_A_C (15). The Sco homolog in *T. thermophilus* serves as a reductase for the reductive preparation of the Cu_A center. However, searches of the *B. subtilis* genome fail to identify a PCu_A_C homolog. Structural similarity of BsSco to the thioredoxin family is limited to the core structure. BsSco possesses two loops external to the thioredoxin core that contain the metal binding motifs (Fig. 24 adapted from 3 and 10). The two loops, which are not present in the thioredoxin proteins, are ideal to reach out and bind metal ions. At last, a very simple and obvious question is, if BsSco is not a copper chaperone, what prevents it from binding Cu(II) if BsSco has such a high bind affinity for Cu(II)? Formation of the Cu(II) bound form would presumably block BsSco’s thiol exchange activity.

We propose that it is still an open question as to whether BsSco is able to serve as a copper chaperone. It is also possible that BsSco can serve a dual role in the assembly of
cytochrome c oxidase by both delivering copper and reducing equivalents to maintain the cysteines of Cuₐ in reduced state. We don’t have definitive answers to finalize the functionality of BsSco at this point. Further experiments are needed to answer this question.

Figure 24: Ribbon representation of BsSco showing the two external loops of BsSco containing the residues involved in metal binding (highlighted in the red box). (Figure adapted from 3)
Chapter 6

Summary and conclusions

BsSco is an accessory protein involved in the assembly of cytochrome c oxidase in *B. subtilis*. While the exact role of BsSco has not been elucidated, copper delivery and redox exchange are two possible roles for BsSco. As a member of the Sco protein family, BsSco is capable of fulfilling a role as a copper chaperone to deliver Cu(II) to the Cuₐ center of cytochrome c oxidase through its tight binding with Cu(II). It can also fulfill a redox role to maintain the reduced state of the Cuₐ center through its transformation from the reduced form to the disulfide, oxidized form. Further researches are needed to finalize the function of BsSco.

This study focuses on the folding/unfolding behavior of BsSco in the context of metal interaction. We exploited the effect of Cu(II) on folding/unfolding BsSco. The extra disulfide bond of apo-BsSco does NOT significantly increase thermodynamic stability. However, Cu(II)-BsSco is entirely resistant to denaturation in as long as the Cu(II)-BsSco complex is stable. High ionic strength disassembles the complex through Cu(II)-mediated oxidation, leading to Cu(II) dissociation and oxidation of BsSco. Kinetic investigation of refolding/unfolding of BsSco shows that refolding/unfolding kinetics of reduced and oxidized apo-BsSco are similar. Cu(II) facilitates folding of BsSco. Nevertheless, refolding BsSco in its reduced form is prone to be oxidized by Cu(II). These findings have provided evidence for metal stabilization of proteins. They also suggest that, as a binding partner of BsSco, Cu(II) acts as a two-edged sword that can
either stabilize the native state of BsSco, or oxidize BsSco and thereby cause its inactivation.
Reference


