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Differential affinity of BsSCO for Cu(II) and Cu(I) suggests a redox role in copper transfer to the Cu_A center of cytochrome *c* oxidase ☆

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ABSTRACT

SCO (synthesis of cytochrome *c* oxidase) proteins are involved in the assembly of the respiratory chain enzyme cytochrome *c* oxidase acting to assist in the assembly of the Cu_A center contained within subunit II of the oxidase complex. The Cu_A center receives electrons from the reductive substrate ferrocytochrome *c*, and passes them on to the cytochrome *a* center. Cytochrome *a* feeds electrons to the oxygen reaction site composed of cytochrome a_3 and Cu_B. Cu_A consists of two copper ions positioned within bonding distance and ligated by two histidine side chains, one methionine, a backbone carbonyl and two bridging cysteine residues. The complex structure and redox capacity of Cu_A present a potential assembly challenge. SCO proteins are members of the thioredoxin family which led to the early suggestion of a disulfide exchange function for SCO in Cu_A assembly, whereas the copper binding capacity of the *Bacillus subtilis* version of SCO (i.e., BsSCO) suggests a direct role for SCO proteins in copper transfer. We have characterized redox and copper exchange properties of apo- and metalated-BsSCO. The release of copper (II) from its complex with BsSCO is best achieved by reducing it to Cu(I). We propose a mechanism involving both disulfide and copper exchange between BsSCO and the apo-Cu_A site. This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

1. Introduction

Members of the heme-copper oxidase family include the cytochrome *c* oxidase sub-group (For reviews see [1,2]). Cytochrome *c* oxidases are multimeric integral membrane proteins that function to pass electrons from reduced cytochrome *c* to molecular oxygen. In the resulting reaction O_2 is reduced to two molecules of H_2O, four equivalents of ferrocytochrome *c* are consumed, four protons are consumed and four protons are pumped across the membrane. The oxygen reduction site consists of two closely grouped metal centers, cytochrome a_3 and Cu_B, that form the heart of the enzyme and are located in the membrane spanning regions of subunit I. A second heme-based center, cytochrome *a*, and a second copper center, Cu_A, function to mediate electron transfer from reduced cytochrome *c* to the oxygen reaction site. Cytochrome *a* is located adjacent to the cytochrome a_3-Cu_B center within the membrane spanning structure of subunit I, whereas Cu_A is located in a solvent exposed domain of subunit II. The solvent exposed domain of subunit II that houses Cu_A also supplies a binding site for cytochrome *c*. Cu_A is, therefore, perfectly situated to act as the initial electron acceptor from ferrocytochrome *c* and as an electronic gateway to the catalytic core at the cytochrome a_3-Cu_B center. Cu_A is composed of two copper ions poised in a mixed valence state such that the overall charge is +3 in the oxidized state (i.e. +1.5/Cu ion) and takes up one electron to go reduced with an overall charge of +2 (i.e. +1/ Cu ion). The two copper ions are bridged by two cysteine thiol side chains resulting in a redox and structurally complex structure (see Fig. 1). This paper will focus on some aspects of the role of the SCO protein in the assembly of the Cu_A center of cytochrome *c* oxidase in *Bacillus subtilis*.

In eukaryotic cells copper delivery to cytochrome *c* oxidase involves initial copper uptake into the cytosol via a specific plasma membrane transporter [3]. There are several copper binding proteins in the cytosolic space that are specific for delivery to different compartments. For example, the Cox17 protein ferries copper to the mitochondrion and SCO proteins function to facilitate delivery from Cox17 to the Cu_A site on subunit II of the oxidase. Although less is known about the requirements for Cu_B assembly the Cox11 protein is known to be required in some systems [4]. In gram positive bacteria such as *Bacillus subtilis* assembly occurs on the outer surface of the plasma membrane thus obviating the need for a specific transmembrane transporter and a cytosolic transfer protein. In *B. subtilis*, BsSCO appears to be the only protein factor directly linked to the assembly of the Cu_A center. The prokaryotic system, particularly *B. subtilis*, appears somewhat simpler (*c.f.*,[5]) in the assembly

Abbreviations: BCS, bathocuproine disulfonate; BsSCO, *Bacillus subtilis* homolog of SCO1; BsTRX, *Bacillus subtilis* homolog of thioredoxin; EcTRX, *Escherichia coli* thioredoxin; DTT, dithiothreitol; DTDP, 4,4′-dithiodipyridine; TCEP, Tris(2-carboxyethyl)phosphine

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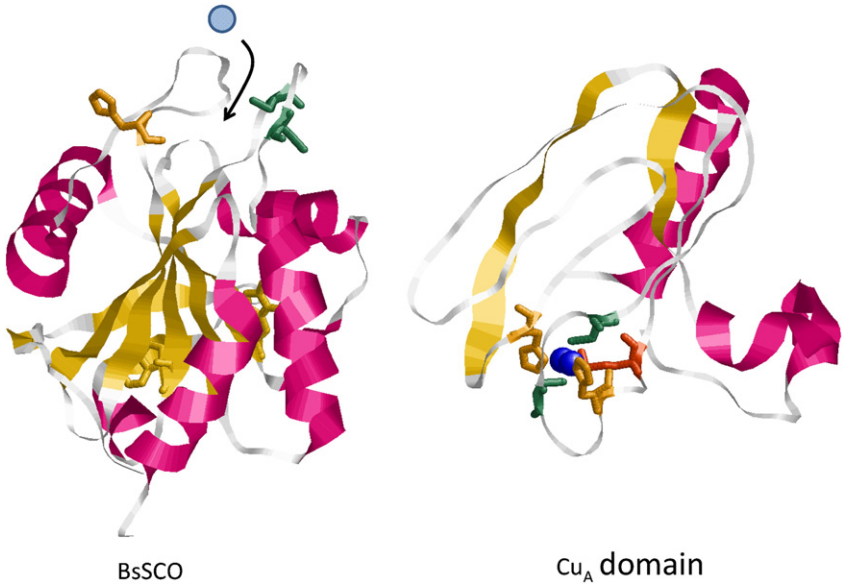


Fig. 1. Structures of BsSCO and the Cu_A-containing soluble domain of subunit II. The structures are shown as ribbon diagrams (BsSCO—PDB ID: 1XZO, subunit II—PDB ID: 3HB3) and the amino acid side chains that serve as metal ligands are shown in stick mode. The putative binding of copper to BsSCO for copper is represented by the blue sphere. It is clear that the relative position of the binding side chains must adjust and this adjustment is largely accommodated by the flexibility of the histidine loop.

of the heme-copper oxidases than its eukaryotic counterpart (For a review see [6]).

B. subtilis expresses two heme A containing respiratory oxidases that are members of the heme-copper oxidase family. One of these is a menaquinol oxidase and does not possess a Cu_A center, whereas the other is a cytochrome *c* oxidase with a typical dinuclear Cu_A center. BsSCO knockout strains of *B. subtilis* express wild type levels of menaquinol oxidase, but lack the cytochrome *c* oxidase. The BsSCO knockout phenotype can be recovered by supplying excess copper from the medium [7]. These observations allowed us to conclude that BsSCO is involved specifically in Cu_A assembly, and is not needed for Cu_B assembly. Due to the distinctive structure and molecular locations of the Cu_A and Cu_B centers in the oxidase, the use of distinct assembly mechanisms seems appropriate. However, the exact nature of BsSCO's involvement in Cu_A assembly is still open to question. We have gone on to express a soluble form of BsSCO that excludes the few amino acids from the primary structure that are covalently linked to lipid and serve to anchor the protein in the plasma membrane [8]. We have shown that BsSCO is capable of binding both Cu(I) and Cu(II) ions *in vitro*, but that Cu(II) is preferred by a wide margin [9]. Cu(II) binding to reduced apo-BsSCO occurs in a two step process [10] with an initial transient interaction that does not involve thiol ligation [11]. The BsSCO–Cu(II) transient intermediate relaxes to the final, stable complex with Cu(II) ligated by two cysteine thiols, a histidine nitrogen and one water molecule [9,12]. The equilibrium complex of Cu(II) with BsSCO is thermodynamically tight (K_D<3.5 pM) and kinetically inert (k_{off}<10^{-5} s^{-1}). Just how such a species would participate in the functional cycle of BsSCO is a question we attempt to pursue here. For, although it may be argued that BsSCO and SCO proteins in general do not function to deliver the Cu(II) ion *per se*, the avidity and stability of the BsSCO–Cu(II) species means that it will form and must dissociate for other roles of BsSCO to come into play. At high ionic strength the BsSCO–Cu(II) complex forms, but is not stable. In the presence of a second equivalent of copper a redox process spontaneously ensues generating Cu(I) and oxidized BsSCO [13]. The equilibrium complex of BsSCO with Cu(II) can be reduced by dithionite and the Cu(I) so generated is readily removed by BCS. We propose that binding of a second equivalent of copper in the Cu(I) state could trigger this redox process and result in both thiol and copper exchange between BsSCO and the apoCu_A center.

2. Materials and methods

Soluble BsSCO was expressed in *Escherichia coli* as a recombinant protein in fusion with glutathione S-transferase. The fusion protein was purified by glutathione-Sepharose affinity chromatography and the BsSCO fragment isolated as previously described [14]. The purity of the protein was assessed by SDS gel electrophoresis and the concentration of the samples determined by UV absorbance at 280 nm using an extinction coefficient of 19.4 mM^{-1} cm^{-1}. *Bacillus subtilis* thioredoxin (BsTRX) was cloned, expressed and purified as previously described [15]. The thiol level of BsSCO and BsTRX was routinely assessed by reaction with dithiodipyridine [16]. The presence of Cu(I) was assayed by complex formation with bathocuproine disulfonate (BCS) using an extinction coefficient of 12.25 mM^{-1} cm^{-1} at 483 nm [17].

Kinetic spectrophotometry was performed using an OLIS RSM 1000 spectrometer fitted with an OLIS two-syringe stopped flow. Spectra were recorded at intervals of 16 ms per spectrum. Data from these records were assessed by global fitting using the OLIS software package. The fitting procedure begins with singular value decomposition to estimate the number and form of the spectral intermediates in the dataset. A kinetic model is then selected and fit to the experimental data. A satisfactory fit is judged by the distribution of residuals between the fit and the data.

Steady-state fluorescence spectroscopy was performed on a Fluorolog-3 spectrometer from Horiba–Jobin–Yvon. Emission spectra were collected with excitation at 280 or 292 nm and with an excitation slit set at 1 nm and an emission slit set at 2 nm. Kinetic fluorescence measurements were done with the OLIS RSM 1000 spectrometer set up in fluorescence mode with 4 nm bandpasses for excitation and emission.

3. Results

3.1. The structural framework of BsSCO

The structure of BsSCO has the thioredoxin fold as its core feature. The ligands for copper binding include a pair of conserved cysteine residues that sit in loop 3 at the end of helix 1 and a conserved histidine residue about 100 amino acids to the C-terminus [18]. The conserved histidine is also contained in a loop that is highly flexible

(see Fig. 1) [19]. Ligation of copper by these three residues is best accommodated by structural adjustments of the highly flexible histidine loop. Although the structural adjustments necessary to bind copper are relatively small, and we have shown by UV-circular dichroism that barely discernable changes occur in the secondary structure upon copper binding [10], there is a profound effect on the overall stability of the protein structure (e.g., the melting temperature increases by 23 °C) when copper binds [13]. It appears that the binding site in reduced, apo-BsSCO is ready to accept copper and that no large structural realignments are required for copper to bind. However, the overall structure loses flexibility upon copper binding and the energetic stability increases. The soluble domain of Cu_A from subunit II of cytochrome *c* oxidase is also shown in Fig. 1. The structure containing the two bridging thiols of Cu_A is contained in a primary sequence that has two cysteines separated by three intervening residues, a primary structural feature that is shared with SCO proteins.

3.2. Kinetic studies of the binding and release of copper by BsSCO at high ionic strength

BsSCO binds Cu(II) in a two step process [10] that can be monitored by stopped flow absorbance spectroscopy (Fig. 2). Multi-wavelength analysis shows that the initial bimolecular reaction between BsSCO and copper generates an intermediate BsSCO–Cu(II) complex which then relaxes in a first order process to the equilibrium species. This two step process is illustrated here by absorbance vs. time profiles at 356 nm and 408 nm (Fig. 2B). The intermediate BsSCO–Cu(II) species has a red-shifted absorption maximum at 380 nm (Fig. 2C) with EPR properties consistent with two nitrogen and no sulfur ligands [11]. The equilibrium species has an absorption maximum at 352 nm (Fig. 2C) and EPR characteristics of a Cu(II) species ligated by two cysteine sulfurs, a histidine imidazole and a solvent water [9]. When this reaction is performed at high ionic strength ($\mu \geq 3$ M) and more than one equivalent of copper the BsSCO–Cu(II) complex is transient (Fig. 3A). Under conditions shown in Fig. 3 the 352 nm species of BsSCO appears maximally in a couple of seconds and then disappears over tens of seconds. Kinetic analysis shows that the BsSCO–Cu(II) species with absorbance maximum at 352 nm appears immediately without any evidence of the intervening 380 nm species observed at low ionic strength (Fig. 3B,C). When the Cu(I) specific reagent, BCS, is added following decay of the 352 nm species almost two equivalents of Cu(I) are recovered, whether the reaction is carried out anaerobically, or in air-equilibrated buffer. We conclude that the BsSCO–Cu(II) complex has undergone a redox transition generating Cu(I), and that the Cu(I) species is protected from reaction with O_2 by association with BsSCO. When BsSCO is recovered from this mixture it is found to be in its oxidized state with an internal disulfide present. We suppose that the conditions of high ionic strength along with a second equivalent of copper are hints as to the factors that activate metal exchange for the BsSCO–Cu(II) complex, which otherwise is very slow to release copper. In this light we have pursued further characterization of the redox properties of BsSCO.

3.3. Reaction of apo-BsSCO and BsTRX with small molecule reductants and oxidants

The structure of BsSCO has been determined in both its oxidized (i.e., disulfide) and reduced (i.e., dithiol) forms, and it is observed that the overall structure of BsSCO does not have to change much to accommodate intramolecular disulfide formation [18]. Oxidized apo-BsSCO is readily reduced to the dithiol state by the common disulfide reducing agents DTT and TCEP [9], and these rates are not accelerated in the presence of copper. The redox reaction can be monitored readily *in vitro* by intrinsic protein fluorescence as the

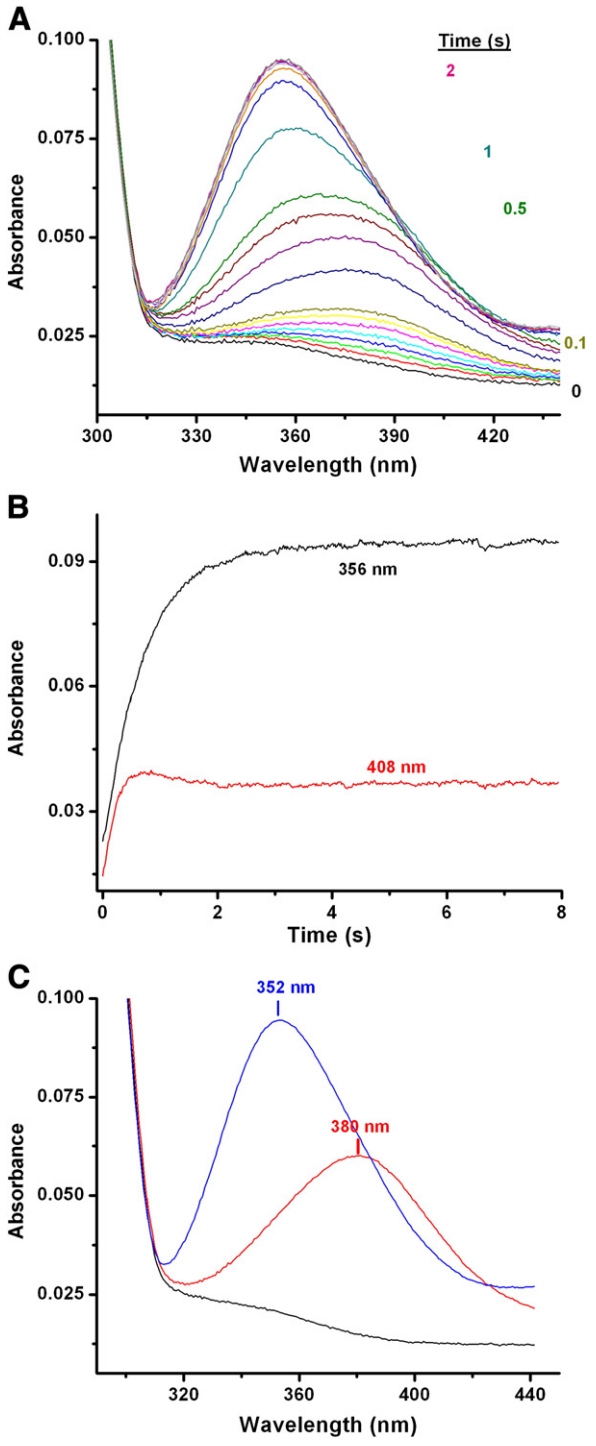


Fig. 2. Stopped-flow absorbance of BsSCO binding copper at low ionic strength. (A) Selected spectra from the time course observed for copper binding to BsSCO. The concentration of the reaction components were as follows, BsSCO 37.5 μM, $CuCl_2$ 75 μM and 50 mM sodium phosphate buffer pH 7.0. Spectra were collected at 16 ms intervals with the spectrum taken immediately after mixing assigned to zero time, the first set of spectra up to 0.1 s are shown, followed by spectra at 0.1 s intervals up to 0.5 s and then at 1 s intervals up to 8 s after mixing. (B) Two wavelengths versus time from the same data set in (A) are illustrated. The absorbance at 352 nm exhibits a continuous whereas there is a rise and fall in the absorbance at 408 nm that show two step nature of the process. (C) Global analysis of the three dimensional data set in panel A gives a best fit with a two-step process having observed first order rates of 4.3 s^{-1} and 1.45 s^{-1} and the spectral components illustrated here. Spectrum (i) is the initial species that gives rise to species (ii) with maximal absorbance at 380 nm and this evolves to species (iii) with maximum at 352 nm.

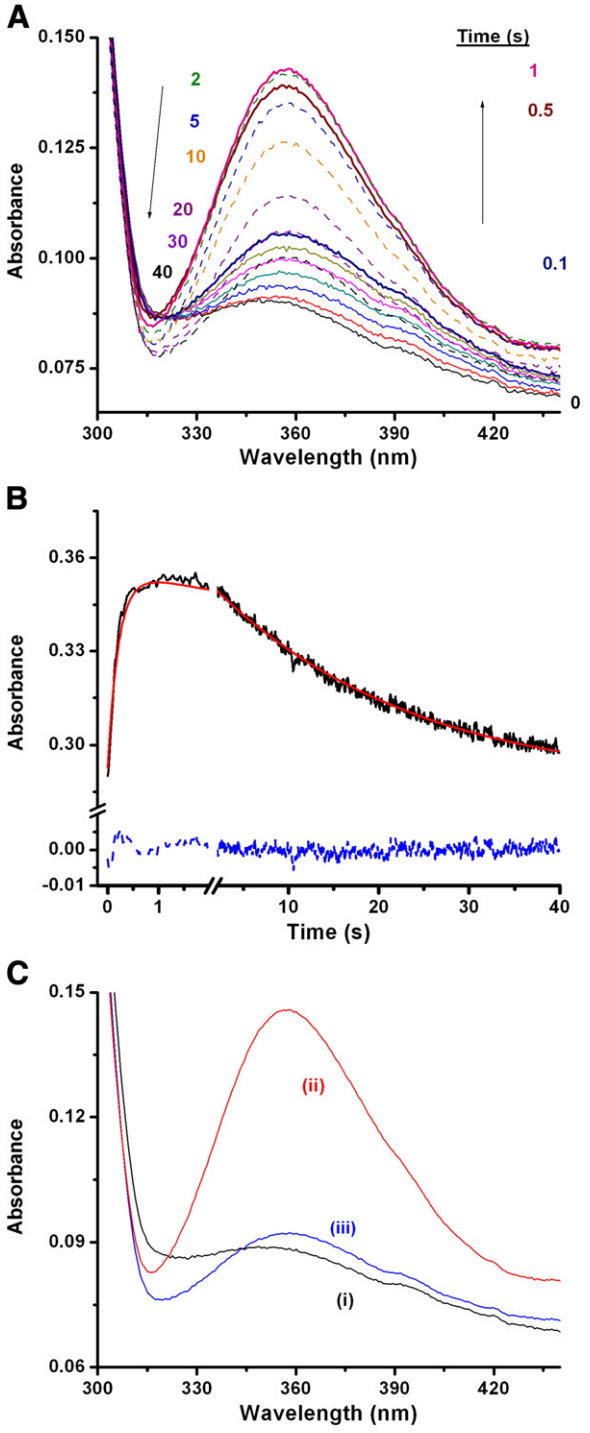


Fig. 3. Stopped-flow absorbance of BsSCO reacting with copper at high ionic strength. The conditions are the same as those in Fig. 2 except that the reaction medium contains 3 M NaCl. (A) Spectra were collected following mixing at 16 ms intervals. Selected spectra are shown at 16 ms intervals up to 0.1 s, at 0.1 s intervals up to 0.5 s. The intensity increase over the first second and then declines for the duration of the reaction. The collection time of the spectra are indicated in the figure. (B) The fit to a two step model. The solid black line is the overall kinetic data, the red line through this data set is the fit to a two-step model with apparent rates of 4.7 s^{-1} and 0.046 s^{-1}. The residual difference between the model and the fit are illustrated in blue along the $y=0$ position. (C) The three spectral components derived from the global fit. Species (i) is the initial species shown in black, the initial species gives rise to species (ii) in red with absorbance maximum at 352 nm and this decays to species (iii) in blue with decreased absorbance. There is no evidence for the appearance of the intermediate species observed at low ionic strength.

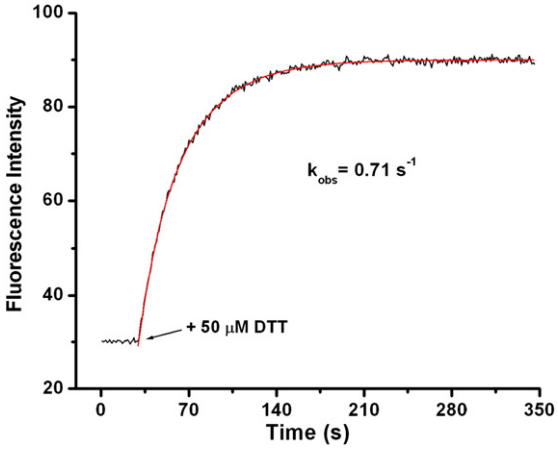


Fig. 4. Reduction of BsTRX by dithiothreitol. The reaction was monitored by intrinsic fluorescence with excitation at 292 nm and emission intensity measured at 342 nm. The concentration of thioredoxin is 16 μM in 50 mM sodium phosphate buffer pH 7.0. Reduction was initiated by addition of 50 μM dithiothreitol. The solid black line shows the fluorescence data and the red line through the data is the best fit using a single exponential model. The observed rate obtained under these conditions is indicated on the figure.

disulfide is an efficient quencher of tryptophan fluorescence [20]. For BsSCO the fluorescence increases by a factor of about 40% as the disulfide is reduced to the dithiol. For the canonical member of the thioredoxin family, *E. coli* thioredoxin (EcTRX), the intrinsic fluorescence increases by three-fold as the protein goes from oxidized to reduced [21]. The increase in fluorescence is due to the relief from quenching as the cystine moiety is reduced to the dithiol. The difference in efficiency of quenching between BsSCO and EcTRX reflects the distance between the tryptophan residues and the quenching disulfide. For comparison, we report here the reduction reaction of the *Bacillus subtilis* version of thioredoxin (*(i.e., BsTRX)*. The fluorescence of BsTRX increases in intensity by 3-fold as the protein is reduced by dithiothreitol (Fig. 4). The bimolecular reaction rate is marginally slower that that reported for ECTRX reduction and both are much more reactive with DTT than is BsSCO (see Table 1).

Reduced BsSCO exhibits kinetic stability *in vitro* with a half-time for auto-oxidation in air-equilibrated buffer of 32 h at 25 °C [9]. Reduced BsSCO is reactive with H_2O_2 and the reaction time course is followed here by the development of quenching of the intrinsic tryptophan fluorescence that occurs upon formation of cystine (Fig. 5). The reaction occurs as a single exponential decay with an observed rate that is proportional to the concentration of peroxide with a bimolecular rate constant of 8.14 M^{-1} s^{-1}. The reaction of reduced BsTRX with hydrogen peroxide is also measured by transient intrinsic fluorescence and the bimolecular rate constant is somewhat slower that that found for BsSCO (see Table 1) and both are much slower than a peroxiredoxin of the atypical, two cysteine group (i.e., PRX5) that reacts with hydrogen peroxide at a rate of 3×10^5 M^{-1} s^{-1}, and alkyl peroxides at rates approaching 10^7 M^{-1} s^{-1}[22].

Table 1
Rates of reaction for thioredoxin family proteins with small molecule reductants and oxidants.

Protein	Reductant		Oxidant	
	DTT ($M^{-1}s^{-1}$)	TCEP ($M^{-1}s^{-1}$)	O_2	H_2O_2 ($M^{-1}s^{-1}$)
BsSCO	20.1[a]	0.34[a]	6×10^{-6} s^{-1} [a]	8.14[b]
BsTRX	1.42×10^3 [b]	1.0[b]	slow[b]	3.33[b]
EcTRX	1.65×10^3 [c]	–	–	–
Peroxiredoxin	–	–	–	3×10^5 [d]

a—[8], b—here, c—[19], d—[20].

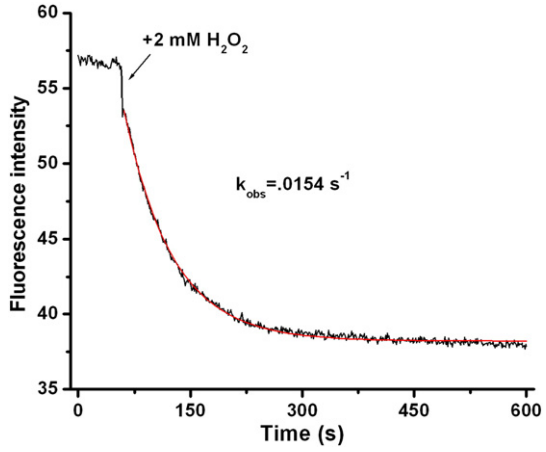


Fig. 5. Oxidation reaction of reduced apo-BsSCO with hydrogen peroxide. The concentration of BsSCO is 5.6 μM with a thiol content of 1.8 SH/BsSCO. The buffer is 50 mM sodium phosphate and the reaction is commenced by addition of 2 mM H_2O_2. The reaction was monitored by intrinsic fluorescence with excitation at 280 nm and emission intensity measured at 330 nm. The black trace is the data and the red line through is the single exponential fit. The observed rate for peroxide reacting with BsSCO is indicated on the figure.

3.4. Reduction of the copper site of BsSCO–Cu(II) and release of Cu(I)

Reduced apo-BsSCO is competent to bind metals and will form complexes with both Cu(II) and Cu(I) with unit stoichiometry. We have shown that BsSCO binds Cu(II) with extremely high affinity ($K_D \approx 3.5$ pM) compared to its affinity for Cu(I) ($K_D \approx 10$ μM) [10]. This drastic change in affinity of BsSCO for Cu(II) and Cu(I) ions raises the possibility of a redox dependent metal transfer mechanism. The Cu(II) complex of BsSCO is not reduced by dithiothreitol, but is readily reduced by dithionite. The kinetics of reduction of BsSCO–Cu(II) by dithionite is shown in Fig. 6. A sample of reduced, apo-BsSCO is first titrated with the desired amount of $CuCl_2$ to insure complete BsSCO–Cu(II) formation. The sample is then placed under an atmosphere of argon. A stock solution of sodium dithionite is prepared under argon and a microliter amount of dithionite is added to BsSCO–Cu(II) being stirred at 700 rpm as spectra are recorded at a rate of two per second. The concentration of dithionite in the reaction is determined by its absorbance at 314 nm using an extinction coefficient of 8 $mM^{-1}\,cm^{-1}$[23]. A set of spectra from the reduction are illustrated in Fig. 6A. The time course of the reaction is best measured at 400 nm as the absorbance from dithionite interferes with the peak absorbance of BsSCO–Cu(II) at 352 nm (Fig. 6B). The reduction process follows a simple single exponential decay with an observed rate proportional to the dithionite concentration with a value of $2.10 \pm 0.5 \times 10^3\ M^{-1}\,s^{-1}$. As a consequence of reduction and the relative affinity of BsSCO for Cu(II) and Cu(I) we reasoned that the equilibrium of the metal/BsSCO complex would readjust. We added BCS, a Cu(I) chelator, following dithionite reduction to probe for the presence of Cu(I). Addition of BCS to dithionite reduced BsSCO–Cu(I) gives a biphasic reaction for the formation of the BCS-Cu(I) complex (Fig. 6C). The two phases of the reaction are assigned to the presence of two populations of Cu(I) that which is bound to BsSCO and that free in solution. The ratio of the fast and slow reacting pools of Cu(I) reflect the equilibrium constant of BsSCO for Cu(I). We calculate an equilibrium dissociation constant of BsSCO for Cu(I) of 15 ± 5 μM that is in good agreement with that measured previously in a conventional titration [13]. At the end of this experiment all of the copper is transferred to BCS and BsSCO is in its apo form. This system is a model of the type of reduction induced copper transfer that could occur between BsSCO and the Cu_A site of cytochrome *c* oxidase.

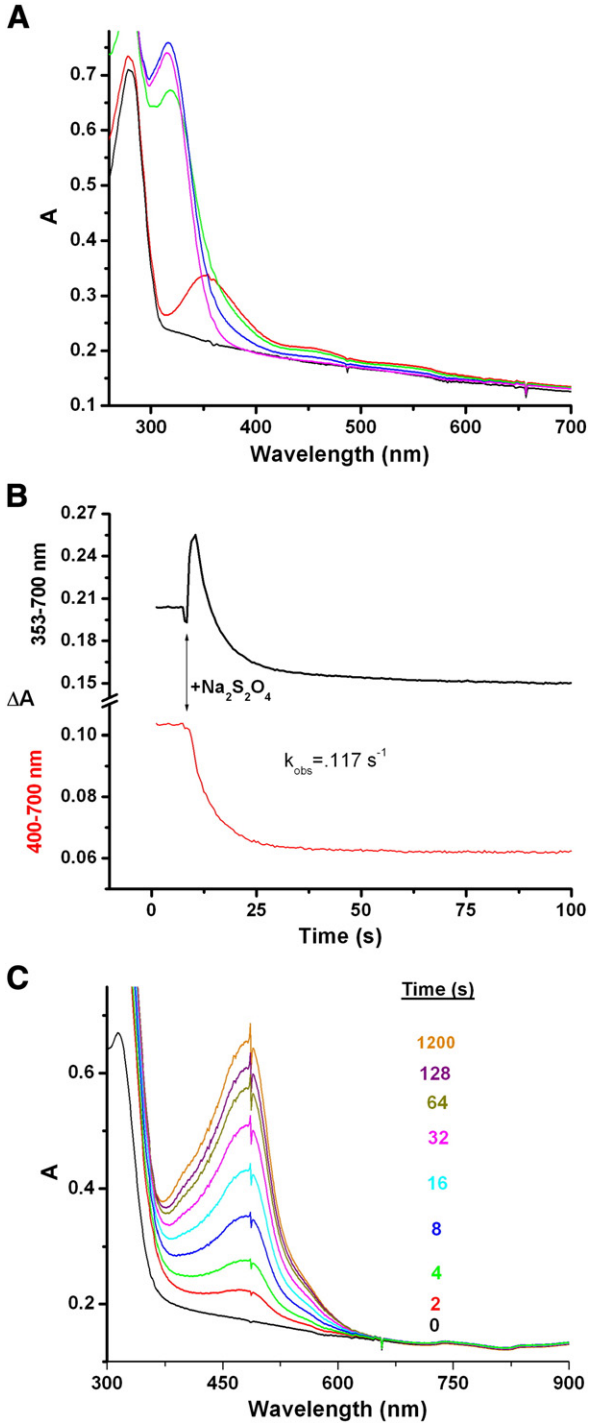


Fig. 6. Reduction of BsSCO–Cu(II) by sodium dithionite and transfer of Cu(I) to BCS. (A) Spectra taken during reduction of BsSCO–Cu(II) by sodium dithionite. The spectrum of 24 μM BsSCO in 50 mM sodium phosphate buffer (——), after addition of 45 μM $CuCl_2$ (——) and at various times after addition of 70 μM $Na_2S_2O_4$; (——) immediately, (——) s and (——) 40 s. (B) Time courses for the dithionite reduction reaction. The top panel shows the reaction recorded at 353 nm, the peak wavelength of the BsSCO–Cu(II) complex. The bottom panel shows the time course recorded at 400 nm that is within the first absorption band of the BsSCO–Cu(II) complex but removed from interference due to the addition of excess dithionite. In both cases the absorption at 700 nm is subtracted as reference. A single exponential fit to the data at 400 nm gives an observed rate of 0.117 s^{-1}. (C) Formation of BCS-Cu(I) from BsSCO–Cu(I). Spectra are shown before and following addition of 157 μM BCS. The recording times following the addition of BCS are shown on the Figure.

4. Discussion

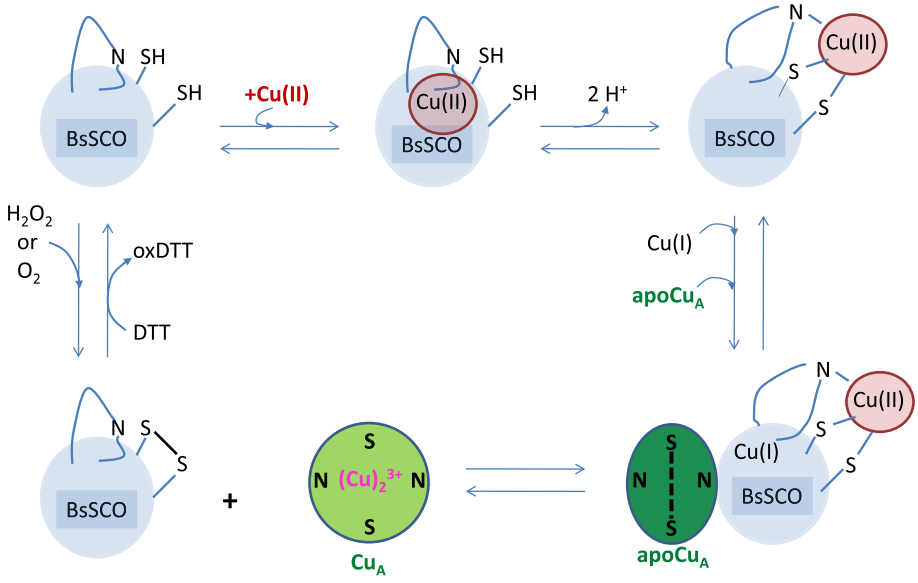
The structure of BsSCO places the protein in the thioredoxin family by virtue of its similar protein fold. As has been noted previously the thioredoxin fold has been used for the basis of a number of protein-based activities [24]. The similarity of BsSCO to thioredoxin led to the early proposal for BsSCO as a disulfide exchange protein [25]. The observation of both disulfide and dithiol forms of BsSCO with little structural alteration supports the notion of a redox role for BsSCO [18]. However, BsSCO is not very active in the standard thiol exchange assays used to characterize the classical thioredoxins, and its vestigial activity is not altered by mutation of the conserved cysteine residues to serine (Hill and Andrews, unpublished observation). However, thiol exchange activity has been demonstrated for SCO proteins from other prokaryotes (e.g., [26] and [27]). BsSCO is most closely related structurally to the peroxiredoxin members of the thiroedoxin family. However, as we illustrate here BsSCO's reaction with hydrogen peroxide is orders of magnitude slower than the values reported for peroxiredoxins. In general the dithiol state of BsSCO is quite stable as it also reacts slowly with molecular oxygen. The reaction of human thioredoxin with peroxide has recently been studied with respect to a role in oxidative signaling [28]. The reaction of human TRX with peroxide leads to formation of an intermolecular disulfide between the pair of redox active cysteine residues, although the rate of the reaction is not reported. We report here that the bimolecular reaction of BsSCO and BsTRX is similar with hydrogen peroxide and suggest that a role in redox signaling is unlikely via a reaction with hydrogen peroxide, but the oxidation reaction would certainly interfere with disulfide exchange, or metal binding by BsSCO. Addition of copper to proteins with vicinal sulfhydryls can act to speed attainment of disulfide formation. However, the BsSCO protein evades this oxidation process to form a copper complex with quantitative yield and high stability.

If BsSCO is to act in a copper transfer capacity the BsSCO–Cu(II) complex could be viewed as an impediment along the way because of its high stability and kinetic inertness. So to facilitate copper dissociation from the BsSCO–Cu(II) complex an additional feature must come into play. A factor in solution that is shown here to loosen the association of copper is ionic strength. We are not suggesting that ionic strength *per se* plays a direct role in copper transfer, but rather that the reactivity of BsSCO at high ionic strength may reflect an ionic engagement with its target molecule to affect the copper release process. The other mechanism to induce copper release that arises out of the differential stability of the Cu(II) and Cu(I) complexes is a redox mediated one. The Cu(I) complex is not only less stable than its Cu(II) counterpart but the release kinetics are favorable for copper transfer. Here we illustrate the principle by showing Cu(I) transfer to the specific reagent BCS.

A question that arises as to the role of Cu(II) complexes of SCO proteins concerns the nature of the redox milieu. It is known that the redox potential of the cytosolic space is reducing (e.g., [29]) and therefore it is reckoned to make sense that an inner mitochondrial membrane proteins such as SCO should bind Cu(I). In contrast, in gram positive bacteria the assembly of Cu_A is occurring on the external side of the plasma membrane in a space that is in redox equilibrium with the external medium and so is probably more oxidizing. In this instance it may be sensible for BsSCO to have evolved to prefer Cu(II). We know that BsSCO prefers Cu(II) over Cu(I) *in vitro* by more than 10^6-fold. However, the pertinent question in this regard for protein bound metal is whether the site is kinetically accessible by available reductants and oxidants, and thereby able to attain redox equilibrium with the surrounding medium. For example, the Cu_A site of cytochrome *c* oxidase has a high oxidation potential relative to the surrounding medium and yet it remains in a highly oxidized state *in vivo* [30]. Due to the specific electron transfer pathways into and out of the Cu_A site it is maintained in a state that is out of equilibrium with its strongly reducing environment. The redox potential of the environment may not itself be an absolute indicator of the redox state a given protein bound metal site might take up.

An additional feature of the assembly of Cu_A center is the fact that there are two coppers required in the final structure. BsSCO binds copper with high affinity in a 1:1 stoichiometry. We view it as unlikely that BsSCO would be delivering one copper at a time in two separate interactions. We suppose that a second equivalent of copper can associate with BsSCO or the BsSCO/apoCu_A complex and this second equivalent of copper functions to activate the BsSCO–Cu(II) complex for copper and redox transfer (Scheme 1). The redox activated copper transfer from BsSCO–Cu(II) that we observe at high ionic strength requires a second equivalent of copper and both copper ions are eventually captured by BCS as Cu(I).

The redox stimulated copper dissociation that we report here might also account for the difficulties with stabilizing the Cu(II)



Scheme 1. Copper binding and redox properties of BsSCO coupled to copper delivery to apo-Cu_A. BsSCO is depicted as blue oval with the three conserved copper ligands indicated. Apo-Cu_A is shown as a dark green oval with 4 out of the 6 copper ligands indicated, two imidazole nitrogens and two cysteine sulfurs. The two cysteines are shown as a disulfide in apo-Cu_A. Interaction between metalated, reduced BsSCO leads to metal and disulfide exchange to form Cu_A and oxidized, apo-BsSCO.

complex of BsSCO in structural studies. The Cu(II)–BsSCO complex could be subject to reduction by experimental conditions (e.g., X-ray irradiation, [31]) thereby generating the Cu(I) species, which then dissociates. This would leave behind apo-BsSCO in probably a mixture of redox states, and this was certainly our experience trying to collect X-ray data on the BsSCO–Cu(II) complex [18]. Our starting solution was at a concentration such that the complex was detectably red in color and the data set would return without a trace of metal in the binding site. In contrast, the same sample can sit for days at room temperature with no loss of intensity in the spectral properties of the BsSCO–Cu(II) metal center.

The high affinity binding of copper(II) by BsSCO serves to maximize the efficiency of copper capture and this presumably has advantages to the organism in environments where the amount of available copper is low. However, the kinetic stability of BsSCO–Cu(II) limits the capacity of this species as a copper transfer agent. We suggest that this kinetic inertness is overcome by reducing the Cu(II) ion *in situ* to Cu(I). This has the effect of inherently accelerating the ability of BsSCO for copper release. The mechanism outlined in Scheme 1 takes advantage of the high affinity of BsSCO–Cu(II) and a redox specific trigger to release copper ions in the reduced form. The redox-triggered release of copper also generates oxidized BsSCO as a byproduct. The re-reduction of oxidized BsSCO is shown in our scheme to be accomplished by DTT. However, this function is presumably performed *in vivo* by an additional accessory protein (e.g. another disulfide exchange protein [32]) or a small molecule reductant such as bacillithiol [33]. Such questions arise as to the function of our proposed cycle and we plan to address these in our future studies of the SCO-mediated system of Cu_A assembly.

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