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Copper binding traps the folded state of the SCO protein from *Bacillus subtilis*

Mark Lai, Katherine C. Yam, Diann Andrews, Bruce C. Hill *

Department of Biochemistry and the Protein Function Discovery Group, Queen's University, Kingston, ON, Canada K7L 3N6

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ABSTRACT

The SCO protein from the aerobic bacterium *Bacillus subtilis* (BsSCO) is involved in the assembly of the cytochrome *c* oxidase complex, and specifically with the Cu_A center. BsSCO has been proposed to play various roles in Cu_A assembly including, the direct delivery of copper ions to the Cu_A site, and/or maintaining the appropriate redox state of the cysteine ligands during formation of Cu_A. BsSCO binds copper in both Cu(II) and Cu(I) redox states, but has a million-fold higher affinity for Cu(II). As a prerequisite to kinetic studies, we measured equilibrium stability of oxidized, reduced and Cu(II)-bound BsSCO by chemical and thermal induced denaturation. Oxidized and reduced apo-BsSCO exhibit two-state behavior in both chemical- and thermal-induced unfolding. However, the Cu(II) complex of BsSCO is stable in up to nine molar urea. Thermal or guanidinium-induced unfolding of BsSCO-Cu(II) ensues only as the Cu(II) species is lost. The effect of copper (II) on the folding of BsSCO is complicated by a rapid redox reaction between copper and reduced, denatured BsSCO. When denatured apo-BsSCO is refolded in the presence of copper (II) some of the population is recovered as the BsSCO-Cu(II) complex and some is oxidized indicating that refolding and oxidation are competing processes. The proposed functional roles for BsSCO *in vivo* require that its cysteine residues are reduced and the presence of copper during folding may be detrimental to BsSCO attaining its functional state.

1. Introduction

Transition metal sites within proteins play numerous roles (e.g., [1]) as agents of catalysis and are particularly suited for redox reactions. The transition metal copper is involved in electron transfer proteins such as azurin [2] and in redox-based enzymes such as copper-containing nitrite reductase [3] and cytochrome *c* oxidase [4]. The respiratory enzyme complex cytochrome *c* oxidase has two different copper sites that are intimately involved in the oxidation of the substrate ferrocytochrome *c*, and the binding and reduction of molecular oxygen. One of the copper sites of cytochrome *c* oxidase is designated Cu_B and is composed of a single copper ion ligated to three histidine residues from subunit I of the protein complex. A fourth ligation site is open and provides a site for dioxygen binding between the Cu_B ion and the nearby iron ion of cytochrome a_3 (for review see [5]). The second copper site within cytochrome *c* oxidase is designated Cu_A. The Cu_A site is composed of two copper ions in a mixed-valence state such that in the oxidized center each Cu ion has a valence of +1.5 [6]. Reduction of Cu_A takes one electron and each ion is then cuprous (i.e., Cu^{1+}). The two ions of the Cu_A site are ligated by the side chains of two histidine residues, one methionine, the carbonyl oxygen of an aspartate residue and a pair of cysteine thiolates that bridge the two metal ions. Thus, a prerequisite for Cu_A site assembly over and above the proper protein sequence and fold is that the bridging cysteine residues must be in the reduced state (see Fig. 1).

The SCO[1] family of proteins includes the SCO1 and SCO2 species found in yeast [7] and human [8] mitochondria as well as a number of related proteins found in a variety of bacteria (e.g., [9]). Structural studies reveal a conserved thioredoxin fold (see Fig. 1) and a set of three invariant metal binding residues [10–12]. The metal binding residues include a pair of cysteines in the sequence – CXXXC – along with a histidine residue about 100 amino acids toward the C-terminus. We have shown that BsSCO binds Cu(II) with high affinity (K_D ~ 3.5 pM) and Cu(I) with lower affinity (K_D ~ 10 μM) [13]. The precise functional role of the SCO proteins has not been defined. The SCO proteins have been found to bind both Cu(I) and Cu(II) in 1:1 complexes and a direct role for SCO in copper transfer to apo-Cu_A has been proposed. The binding of copper to SCO has also been proposed to act as a switch in cell signaling [14]. Alternatively, the overall protein fold of SCO places it in the thioredoxin family [15] and the closest structural relative is a peroxiredoxin [16]. These observations suggest a redox role for SCO in a thiol/disulfide exchange interaction to maintain the cysteine residues of the Cu_A site in their reduced state. Experimental evidence from *Thermus thermophilus* shows the SCO homolog is able to reduce the disulfide form of apo-Cu_A to allow a second protein

Abbreviations: BCS, bathocuproine disulfonate; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine HCl; GST, glutathione S-transferase; SCO, accessory protein required for synthesis of cytochrome *c* oxidase; BsSCO, SCO1 homolog from *Bacillus subtilis*; UV-CD, circular dichroism recorded in the ultra-violet spectral region, (i.e., 180–260 nm)

* Corresponding author. Tel.: +1 613 533 6375; fax: +1 613 533 2497.
E-mail address: hillb@queensu.ca (B.C. Hill).

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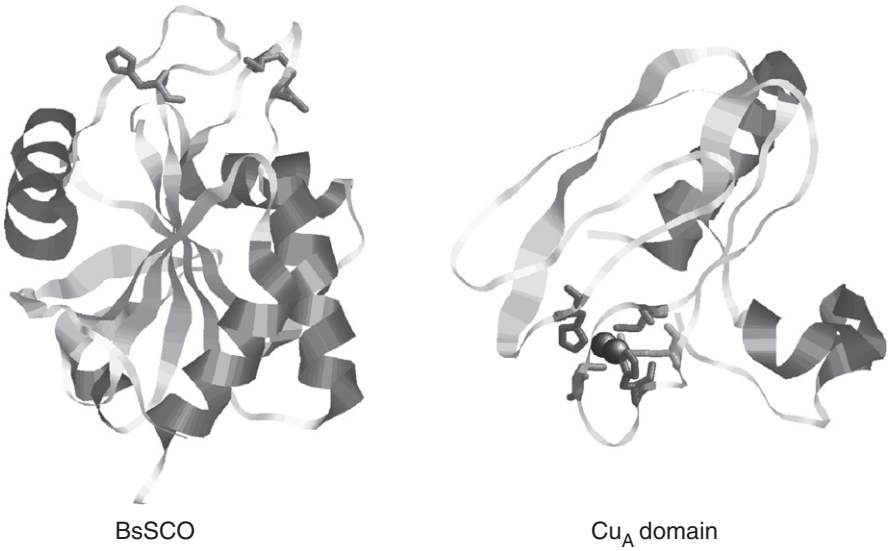

Fig. 1. The structures of BsSCO and the Cu_A domain of subunit II of cytochrome *c* oxidase. BsSCO (PDB ref. 1XZ0) is shown on the left with the protein backbone in ribbon presentation. The side chains of the putative copper binding residues (two Cys thiols and a His imidazole) are shown in stick mode. The soluble domain of subunit II of cytochrome *c* oxidase from *Paracoccus denitrificans* (PDB ref. 1AR1) is shown on the right. The two copper ions of the Cu_A center are shown in blue with the ligating side chains (two Cys, two His and one Met) shown in stick mode.

molecule (i.e., PCu_AC) to pass copper to the Cu_A site [17]. However, a homolog of PCu_AC is not present in *Bacillus subtilis* making the generality of this mechanism uncertain. Our view is that whether or not BsSCO functions as a metallochaperone it does have high affinity for copper and this interaction will be an important determinant of BsSCO functionality. We have therefore pursued here an understanding of the interaction of copper with BsSCO from the perspective of the effect of the metal on BsSCO folding and stability.

The interaction of proteins with metal cofactors is a common motif in protein-ligand interactions. However, there have been relatively few studies of how metal ions, and particularly transition metal ions, interact with proteins during the folding process [18,19]. Perhaps the best characterized example for copper/protein interactions is the electron transfer protein azurin. Studies of azurin folding in the presence of copper have shown that unfolded azurin binds copper and that copper can act to speed up attainment of the native fold [20–22]. We have shown here that the folding of apo-BsSCO occurs in a simple two-state manner in both its oxidized and reduced forms. The formation of the intramolecular disulfide in oxidized BsSCO has little effect on the equilibrium stability, or the kinetics of folding and unfolding. In contrast the effect of copper (II) binding on BsSCO stability is profound, and the BsSCO-Cu(II) complex is stable in up to nine molar urea. However, unfolded reduced apo-BsSCO undergoes rapid oxidation in the presence of copper. This is in stark contrast to folded BsSCO that interacts with copper to form a stable complex in which the two cysteine thiolates are metal ligands. When copper is included in the refolding reaction of BsSCO *in vitro* there is competition between oxidation and formation of the folded copper-bound state. These results may have implications for the timing of interaction of BsSCO with copper *in vivo* where retention of the reduced state of BsSCO is important for function in disulfide reduction or copper delivery.

2. Materials and methods

BsSCO was expressed as a GST-fusion protein, purified as a fusion protein, and the GST moiety cleaved by incubation with thrombin as described previously [23]. Glutathione used to purify the protein was removed by extensive dialysis. The purity of the preparations was assessed by polyacrylamide gel electrophoresis in the presence of SDS [24]. The concentration of BsSCO samples was determined by UV absorbance using an extinction coefficient of 19.4 $mM^{-1}\,cm^{-1}$ at 280 nm [25]. Fully oxidized BsSCO was generated by incubation of the purified protein in 3 M Gdn-HCl plus 1 mM hydrogen peroxide, or stoichiometric $CuCl_2$ in aerobic buffer for 1 h at 25 °C. Following the incubation period the sample was supplemented with 1 mM EDTA and 100 μM BCS and placed on a Sephadex G-25 column equilibrated with 50 mM sodium phosphate pH 7.0 for removal of the denaturant, oxidizing agents and chelators. The sample could then be concentrated by ultrafiltration, or used directly. Fully reduced BsSCO was produced by incubation of the protein in 3 M Gdn-HCl plus 1 mM DTT, 1 mM EDTA and 100 μM BCS. This sample was then placed on a Sephadex G-25 column equilibrated with 50 mM sodium phosphate pH 7.0 to remove DTT, Gdn-HCl and chelators. The redox status of purified apo-BsSCO was determined by reaction with the thiol reactive reagent 4,4′ dithiodipyridine which gives a colored thiopyridone product having an extinction coefficient of 18.8 $mM^{-1}\,cm^{-1}$ at 324 nm [26]. The samples used as oxidized BsSCO gave thiol counts of less 0.1 −SH per BsSCO, or 5% reduced, and the reduced BsSCO samples had ratios of better than 1.8 −SH per BsSCO, or 90% reduced.

The extent of copper (II) binding to BsSCO was determined by measuring the absorption at 352 nm using an extinction coefficient of 4.78 $mM^{-1}\,cm^{-1}$ [27]. Measurement of Cu(I) ions was done by complexation with BCS using an extinction coefficient of 12.25 $mM^{-1}\,cm^{-1}$ at 483 nm [28]. All buffers were treated with Chelex resin (Sigma) to remove any contaminating metal ions. Guanidine and urea containing buffers were prepared just before use as described [29].

UV–visible absorption spectra were measured with a Hewlett-Packard diode array spectrometer (HP-8452). Circular dichroism was measured with an Applied Photophysics Chirascan spectrometer equipped with a Peltier-controlled cuvette holder for temperature ramps. For thermal denaturation studies a thermometer was placed directly in contact with the sample solution. Samples were heated at a rate of 1 °C/min. CD spectra were collected at intervals of 1 nm with a bandpass of 1 nm and an integration time of 0.25 s. Analysis of the secondary structural content for BsSCO was done with the CDNN program [30] using the 23-spectrum basis set. Steady-state fluorescence was measured on a Jobin-Yvon Fluorolog Tau-3 spectrometer with excitation slit set at a bandpass of 1 nm and the emission slit at 2 nm. Stopped-flow fluorescence and absorbance were recorded on an OLIS RSM-1000 spectrometer. For fluorescence both