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Stability of oxidized, reduced and copper bound forms of *Bacillus subtilis* Sco

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ABSTRACT

Sco is an accessory protein required for assembly of the Cu_A center of cytochrome *c* oxidase. Functions proposed for Sco include as a copper chaperone and as a thiol–disulfide exchange protein. Differential scanning calorimetry (DSC) is used here to assess the interaction between the *Bacillis subtilis* version of Sco (BsSco) and Cu(II). When BsSco binds Cu(II) its melting temperature increases by 23 °C, which corresponds to an equilibrium dissociation constant of 3.50 pM. In contrast BsSco exhibits a much weaker affinity for Cu(I) (K_D = 10 μM). BsSco–Cu(II) is stable over days indicating an extremely slow dissociation for BsSco–Cu(II). However, at high ionic strength in the presence of excess copper, BsSco–Cu(II) returns to its oxidized, disulfide-bonded state and loses its copper binding capacity with a half time of 100 s. DSC of BsSco at high ionic strength indicates an increase in stability of metal free, reduced BsSco combined with a small destabilization of BsSco–Cu(II). It is proposed that BsSco undergoes an ionic strength induced conformational change that promotes electron transfer from the thiol groups on BsSco to Cu(II) to effect copper release. Such a redox transformation could be an important aspect of the copper transfer role proposed for BsSco in Cu_A assembly.

Transition metal ions play diverse roles in biological systems. In many enzymes transition metal ions perform a critical job in catalysis and act as central organizing points for the overall structures [1]. In the mitochondrial respiratory chain the transition metals, iron and copper, function as specific catalysts of electron transfer (e.g., [2]) during oxidative phosphorylation. Transition metals also have the potentially dangerous capacity to act in free radical production [3] and as activators of oxidative stress [4]. The potential dangers of transition metal chemistry to biological molecules have been a driving force for the evolution of mechanisms to protect cells from these damaging reactions. For example, enzymes such as superoxide dismutase guard cells from reactive oxygen species that can be generated as byproducts of transition metal mediated redox reactions [5]. In this context the assembly of metalloproteins is an inherently dangerous process [6]. An extra layer of protection against aberrant assembly has evolved in the form of specific proteins that bind and chaperone metal ions to their active sites, and in the process maintain the reactive metals in a quiescent state and at such low concentrations [7,8] as to prevent unwanted side reactions. These metal ion delivery proteins have been termed metallochaperones for their protective role [9].

Cytochrome *c* oxidases have two copper centers (i.e., Cu_A and Cu_B) and two heme centers (i.e., cytochrome *a* and cytochrome a_3) that participate in the enzyme's overall redox reaction in which electrons are delivered from reduced cytochrome *c* to molecular oxygen [10]. Assembly of cytochrome *c* oxidase involves specific accessory proteins that aid in building the metal centers into a functional enzyme complex. There are two separate systems proposed for delivery of copper to the Cu_A and Cu>$_B$ centers. [11]. Sco is proposed to be involved specifically in the assembly of Cu_A [12], whereas Cox11 is involved with the assembly of Cu_B[13]. The Cu_A site is located in a water soluble domain of subunit II of the cytochrome *c* oxidase complex and contains two copper ions that are in very close proximity to one another, in a highly cooperative structure held together by bridging sulfur groups from two cysteine side chains [2]. In contrast Cu_B is located in a membrane embedded portion of subunit I close to the iron ion of cytochrome a_3. Cytochrome a_3 and Cu_B act together in the reaction catalyzed by cytochrome *c* oxidase to form the site of oxygen binding and reduction [2]. Separate mechanisms for assembly of Cu_A and Cu_B seem highly appropriate given their distinctive physical natures.

The assembly of mitochondrial cytochrome *c* oxidase involves the interplay of both mitochondrial-encoded and nuclear-encoded subunits along with the redox active metal centers mentioned above. It is estimated that more than 30 accessory proteins [14] are required in the assembly of mitochondrial cytochrome *c* oxidase in yeast. Copper must be delivered from outside the cell via the cytoplasm and ultimately to the inner mitochondrial membrane where the Cu_A center is located. Study of cytochrome *c* oxidase assembly in the aerobic bacterium *Bacillus subtilis* is based on the premise that the assembly mechanism will be simpler because *B. subtilis* expresses less

Abbreviations: BCS, bathocuproine disulfonate; BsSco, Sco1 homolog in *Bacillus subtilis*; DPDS, 4,4′-dipyridyl disulfide; DSC, differential scanning calorimetry; DTT, dithiothreitol; EPR, electron paramagnetic resonance

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complex homologs of the mitochondrial oxidase within a prokaryotic cellular architecture. The soluble domains of BsSco and the Cu_A domain of subunit II in cytochrome *c* oxidase are located on the outside of the *B. subtilis* plasma membrane and so the complication of delivering copper to an intracellular organelle as in eukaryotes is not required. In addition to the copper chaperone role considered for Sco, more general roles for Sco in copper homeostasis have been proposed from studies of copper transport in human cells [15]. The existence of bacterial species that do not express Cu_A, but still express a homolog of Sco, have led to proposals of alternative, redox-based roles for Sco [16,17].

We have shown that the *B. subtilis* gene *ypmQ* expresses a protein, BsSco[1], of high similarity to mitochondrial Sco1 from yeast, and that it is involved in the assembly of the Cu_A center in *B. subtilis* [18]. We reported the crystal structure of the soluble domain of BsSco for forms in which the conserved cysteine residues (i.e., -**C**XXX**C**-) are both reduced to a di-thiol state, or oxidized to a disulfide [19]. The thioredoxin core fold of BsSco led us to propose a redox role for BsSco in Cu_A assembly [20]. Despite much effort we were not able to stabilize a metal bound form of BsSco in the crystalline state. However, it has been demonstrated by spectroscopic and calorimetric approaches that BsSco in solution can bind Cu(II), Cu(I) and Ag(I) [20,21]. Solution structures for the Ni(II)- and Cu(I)- bound forms of human Sco1 agree on the assignment of inner sphere ligands to copper derived from EXAFS [22] and EPR studies [20,21]. The primary metal binding site for Sco proteins includes the two conserved cysteine residues at positions 45 and 49 along with the conserved histidine at position 135 (BsSco sequence numbers) [23,24].

Here we extend the measurement of BsSco's affinity for copper by quantifying the affinity of BsSco for Cu(I) and better estimating the high affinity binding constant for Cu(II). BsSco has over six orders of magnitude lower binding affinity for Cu(I) compared to its affinity for Cu(II). In phosphate buffer at neutral pH both the Cu(I) and Cu(II) complexes of BsSco are stable on a timescale of hours for BsSco–Cu(I), and days for BsSco–Cu(II). However, at high ionic strength in the presence of excess Cu(II) the BsSco–Cu(II) species undergoes a reaction with half-time of 100 s in which the Cu(II) bound form of BsSco is lost, and Cu(I) is generated along with oxidized BsSco. DSC studies of BsSco show an increase in stability of the metal free form of BsSco that may indicate an ionic strength dependent conformational change of the protein is mediating the redox activity of the copper-binding site. Conversion of the Cu(II) bound form of BsSco to BsSco–Cu(I) would enhance the ability of BsSco to participate in metal transfer with a target such as the apo-Cu_A site of cytochrome *c* oxidase.

1. Materials and methods

Recombinant BsSco was expressed and purified as described previously [25]. Purified BsSco was reduced by incubation with 2 mM DTT for 16 h at room temperature. DTT was then removed by five rounds of ultra-filtration using a 10 kDa filter and dilution with 25 mM phosphate buffer at pH 7.0. All buffer solutions used here were treated with Chelex resin (Sigma) to remove any contaminating metal ions. The extent of reduction of BsSco samples was assessed spectrophotometrically using the reagent 4,4′ dipyridyl disulfide that reacts with free thiols to release thiopyridone which has an extinction coefficient of 18.8 mM^{-1} cm^{-1} at 324 nm [26].

Titrations of reduced BsSco with Cu(I) were performed as follows. A solution of reduced BsSco was diluted into 25 mM sodium phosphate buffer pH 7.0 in an anaerobic fluorescence cuvette and equilibrated with Argon. Stock solutions of CuCl were prepared as described by Abajian et al. [27]. CuCl salt was placed in the side arm of a Thunberg flask that was filled with an appropriate volume of 10 mM HCl with 1 M NaCl. The contents of the flask were flushed with Argon prior to tipping the contents of the sidearm into solution. Concentrations of Cu(I) were verified by titration into excess bathocuproine disulfonate to form BCS–Cu(I) with an extinction coefficient of 12.25 mM^{-1} cm^{-1} at 483 nm [28].

In experiments on the stability of the BsSco–copper(II) complex copper was added to reduced BsSco as $CuCl_2$ in an amount sufficient to saturate the BsSco. The extent of formation of the BsSco–Cu(II) species was determined by the absorption intensity change at 352 nm upon addition of $CuCl_2$ to reduced BsSco. The difference extinction coefficient for BsSco–Cu(II) relative to free BsSco is 4.78 $mM^{-1}cm^{-1}$ at 352 nm [20]. Any excess $CuCl_2$ was then removed by gel filtration on G-25 Sephadex. In experiments with other metals we used the following salts: $AgNO_3$, $ZnCl_2$, $CoCl_2$, $CaCl_2$ and $NiCl_2$.

Differential scanning calorimetry was performed on a VP-DSC instrument from MicroCal (Northampton, MA). Samples for DSC were prepared as oxidized, reduced and reduced plus Cu(II) at a concentration of 15 μM protein. The DSC scans were run from 25 °C to 95 °C at a scan rate of 55 °C per h. All samples were thoroughly degassed and equilibrated at 18 °C prior to loading. Data acquisition and manipulation were done with the MicroCal software in the Origin 7.0 package. Calculation of binding constants from changes in melting temperatures requires measurement of the changes in enthalpy (ΔH_o) and heat capacity (ΔCp) of the protein in the absence of ligand along with melting temperatures in the absence and presence of ligand [29]. ΔCp was measured by taking the difference between the position of the baseline before and after the major transition and extrapolated to the position of the melting temperature. ΔH_o was measured as the area under the melting transition in the normalized DSC thermogram. Melting temperatures for each sample were the results of fits to a two-state transition model with a single transition. In the cases where a secondary transition was observed two independent processes were fitted.

Absorbance spectra were recorded on either a Cary 50 spectrophotometer or a Hewlett-Packard diode array (HP-8452A). Fluorescence data were recorded on a Fluorolog-3 spectrometer from Horiba Jobin-Yvon. Emission spectra were collected with excitation at 280 or 295 nm with excitation slits set at 1 nm and emission slits set at 2 nm. EPR spectra of BsSco were measured on a Bruker EMX spectrometer equipped with a high sensitivity cavity. The temperature of the sample was held at 77 K using liquid N_2-cooled finger Dewar. The microwave power was set at 2 mW with a modulation frequency of 100 kHz and modulation amplitude of 4 G.

2. Results

2.1. Titration of reduced BsSco with Cu(I)

BsSco has intrinsic fluorescence arising from aromatic amino acids in the protein's structure. When Cu(I) is added to reduced BsSco under anaerobic conditions the intrinsic fluorescence of BsSco is diminished in a copper concentration dependent manner. Fig. 1 shows a representative data set with a single binding site isotherm fit to the fluorescence change versus copper concentration. The average from three independent titrations yields a dissociation constant (K_D Cu(I)) of 10.8 ± 1.37 μM. For comparison an abbreviated data set is shown for a titration of BsSco with Cu(II). It is known from previous work that the interaction with Cu(II) is extremely tight (K_D Cu (II) ~ <50 nM) and that oxidized BsSco does not exhibit fluorescence quenching upon copper addition [20]. The arrow on the Cu(II) titration in Fig. 1is to indicate the breakpoint that occurs at approximately 14 μM $CuCl_2$ after which no further fluorescence change occurs. The breakpoint in the titration of BsSco with Cu(II) is consistent with tight binding of a BsSco–Cu(II) complex with 1:1 stoichiometry. To ensure that we are not observing Cu(II) binding to BsSco in the Cu(I) titrations both the absorbance and EPR spectra of the sample were measured during and after the completed titration. The fluorescence change observed upon Cu(I) binding is not