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Reactivity of ligand-swapped mutants of the SCO protein from *Bacillus subtilis*. Isomers of the CCH metal binding motif

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ABSTRACT

The *S*ynthesis of *C*ytochrome *O*xidase protein, or SCO protein, is required for the assembly of cytochrome *c* oxidase in many mitochondrial and bacterial respiratory chains. SCOs have been proposed to deliver copper to the Cu_A site of cytochrome *c* oxidase. We have reported that *Bacillus subtilis* SCO (i.e., *Bs*SCO) binds Cu(II) with high-affinity via a two-step process mediated by three conserved residues (i.e., two cysteines and one histidine, or the CCH motif). A remarkable feature in the reaction of reduced (i.e., di-thiol) *Bs*SCO with copper is that it does not generate any of the disulfide form of *Bs*SCO. This molecular aversion is proposed to be a consequence of a binding mechanism in which the initial copper complex of *Bs*SCO does not involve cysteine, but instead involves nitrogen ligands. We test this proposal here by constructing two isomers of *Bs*SCO in which the conserved copper binding residues (i.e., the CCH-motif) are retained, but their positions are altered. In these variants the two cysteines are exchanged with histidine, and both react transiently with copper (II) with distinct kinetic profiles. The reaction generates Cu(I) and the protein is oxidized to its disulfide form. EPR analysis supports a copper binding model in which cysteine, which is at the "histidine position" in the mutant, is part of an initial encounter complex with copper. When cysteine is the initial ligating residue an oxidation reaction ensues. In contrast initial binding to native *Bs*SCO uses nitrogen-based ligands, and thereby avoids the opportunity for thiol oxidation.

1. Introduction

Copper is a trace metal that is an essential component of many biological systems [1]. It is involved as a protein cofactor in ligand binding and electron transfer reactions that play central roles in cellular metabolism and signaling [2]. At the same time copper can promote redox stress by catalyzing non-specific redox reactions that may generate reactive molecules such as partially-reduced oxygen species [3]. Mechanisms have evolved within biological systems, therefore, to carefully control the levels of free copper ions. The current paradigm is that copper ions are delivered to their ultimate sites of action by specific protein molecules that serve to chaperone copper to its active site [4,5]. The concentration of "free" metals is maintained at a minimum so as to prevent any side reactions. Perhaps the best characterized of these metallochaperones is the *C*u *C*haperone for *S*uperoxide dismutase, or CCS protein, that has been shown to form a complex with apo-SOD to deliver copper [6].

Cytochrome *c* oxidase is a member of the heme-copper oxidase family of respiratory enzymes that catalyze the transfer of electrons from reduced cytochrome *c* to molecular oxygen [7]. The cytochrome *c* oxidase enzyme complex couples the energetically downhill passage of electrons from ferrocytochrome *c* to O_2, with the transport of protons across the lipid bilayer in which the protein complex resides [8]. The net result of this coupled reaction is the transduction of redox free energy into an ion gradient that can be used to drive other energy requiring processes. Much insight has been gained on the mechanism of the heme-copper O_2 reductase reaction by a combination of kinetic, spectroscopic and structural approaches. However, less is understood about the molecular requirements and mechanisms for assembly of the heme-copper protein complex. For example, mammalian mitochondrial cytochrome *c* oxidase is composed of thirteen protein subunits, five transition metals (3 copper ions, plus two heme iron ions) organized in three redox-active centers (i.e., Cu_A, cytochrome *a* and cytochrome a_3-Cu_B), non-redox active metal ions and tightly associated lipids. How these components are assembled has become a subject of considerable interest [9,10].

SCO was identified originally in yeast as an accessory protein required for the *S*ynthesis of *C*ytochrome *c* *O*xidase, or SCO [11]. A combination of genetic and biochemical approaches led to the view that SCO is involved in the assembly of the copper centers of the oxidase [12,13]. Studies using *Bacillus subtilis* demonstrate that SCO is specific for Cu_A assembly, and implies that a separate mechanism exists for the assembly of Cu_B

Abbreviations: *Bs*SCO, *Bacillus subtilis* SCO; CCH-*Bs*SCO, wild-type *Bs*SCO; HCC-*Bs*SCO, mutant *Bs*SCO with C45 swapped with H135; CHC-*Bs*SCO, mutant *Bs*SCO with C49 swapped with H135; BCS, bathocuproine disulfonate; DTDP, 4,4′ Dithiodipyridine; SOD, superoxide dismutase

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[14]. The Cox11 protein is found to be crucial for Cu_B assembly in some systems [15], although it is interesting to note that a homologue of Cox11 is not found in *B. subtilis*.

But what is the precise role of SCO in the assembly of Cu_A? The Cu_A center includes two copper ions that sit in close proximity to one another. They are held to the protein matrix by coordination to a number of specific amino acid residues. There are two histidine residues, one methionine, carbonyl oxygen from the protein backbone and two cysteine residues. The two cysteine thiolates each bridge between the two copper ions and originate from a -**CXXXC**- sequence within subunit II. The two copper ions of the Cu_A center function in a cooperative manner to accept electrons one at a time from ferrocytochrome *c* and transfer them to ferricytochrome *a* [16]. The Cu_A center oscillates between the +2, reduced state (i.e., each Cu ion is +1) and the +3, oxidized state (i.e., each copper ion is +1.5). The inclusion of two copper ions into a site containing two thiolate ligands makes for a potentially challenging assembly process. SCO proteins from mitochondria and bacterial systems have been shown to bind both Cu(II) and Cu(I) ions [13,17,18], and this has led to the proposal of a direct role for SCO in copper delivery to the Cu_A site. In addition, the overall fold of SCO places it in the thioredoxin family and this has led to a suggestion for a redox role for SCO in Cu_A assembly [19,20]. There is experimental evidence for a SCO homologue in *Thermus themermophilus* acting in disulfide exchange with the Cu_A site to maintain the cysteine residues in a competent state to bind copper [21]. Copper delivery to the reduced Cu_A site is facilitated by a separate protein (i.e., PCu(A)C). However, the universality of this mechanism is in question as there does not appear to be a homologue of the PCu(A)C protein in *B. subtilis*, nor in mitochondria and the PCu(A)C protein in *Rhodobacter* functions upstream of the SCO homologue in this system [22].

In this paper we explore the remarkable property of the *B. subtilis* version of SCO (i.e., *Bs*SCO) to bind Cu(II) in an aerobic environment without undergoing a copper-catalyzed thiol oxidation . Crystal structures of reduced (i.e., dithiol) and oxidized (i.e., disulfide) *Bs*SCO show that little structural change is required for an internal disulfide to form [23]. In addition, the formation of the intramolecular disulfide in oxidized *Bs*SCO confers little in the way of kinetic, or thermodynamic stability to the overall fold of the protein [24,25]. The Cu(II) complex with wild type *Bs*SCO exhibits tight binding (i.e., $K_D \sim 3.5$ pM) with two thiolates, one histidine and a water molecule as inner sphere ligands [18,26]. This complex is formed in a two-step process [27], and we have proposed that the initial encounter with copper does not involve thiol interaction [28]. In order to test this hypothesis further we have characterized isomeric variants of *Bs*SCO in which each of the thiols has been swapped with the conserved histidine residue. The nature of the reaction of *Bs*SCO with copper is changed in the two variants. A distinct intermediate is formed in each case that has differing degrees of stability. However, neither mutant version of *Bs*SCO goes on to form a stable complex with Cu(II). Both undergo a redox conversion generating Cu(I) and oxidized *Bs*SCO. Neither of these mutant forms of *Bs*SCO is functional in the expression of the cytochrome *c* oxidase complex in *Bacillus* cells indicating that the stable binding of Cu(II) by *Bs*SCO is a prerequisite for its function in vivo.

2. Materials and methods

Native and mutated versions of recombinant *Bs*SCO were expressed and purified as described previously [29]. Briefly, the soluble domain of *Bs*SCO is expressed in fusion with glutathione S-transferase in *Escherichia coli* and purified by affinity chromatography over glutathione Sepharose 4B resin (GE Healthcare Life Sciences). Cleavage of the fusion constructs was done using thrombin (Sigma). Thrombin was removed by the passage of the cleaved fusion protein over benzamidine-Sepharose 4 fast Flow (GE Healthcare Life Sciences). The purity of the *Bs*SCO samples was assessed by electrophoresis in 15% denaturing polyacrylamide gels under reducing conditions.

The concentrations of purified *Bs*SCO samples were routinely determined by the measurement of the absorbance at 280 nm. This value was converted to protein concentration using an extinction coefficient (i.e., 19.9 mM^{-1} cm^{-1}) calculated from the known sequence [30]. The thiol content of mutant and native *Bs*SCO was determined by reaction with 4,4′ dithiodipyridine (DTDP) [31]. The addition of excess DTDP to *Bs*SCO generates the thiopyridone product in proportion to the amount of thiol present in the sample. The amount of 4-thiopyridone product was quantified spectrophotometrically by measuring the absorbance at 324 nm and using an extinction coefficient of 18.8 mM^{-1} cm^{-1}. Determination of Cu(I) was done by the addition of excess bathocuproine disulfonate forming the BCS-Cu(I) complex that gives rise to a colored species with maximal absorbance at 483 nm having an extinction coefficient of 12.25 mM^{-1} cm^{-1} [32].

Structural depictions of wild-type *Bs*SCO (PDB ID: 1XZ0) and the two double mutants were made in PyMOL [33]. Structural models for the two mutant versions of *Bs*SCO were generated in Swiss-Model using wild type BsSCO as template [34]. Circular dichroism spectra were collected on an Applied Photophysics Chirascan instrument. Spectra recorded for secondary structural analyses were measured in a 0.1 mm cuvette to afford optimal light throughput in the short UV region. Analysis of the secondary structural content of *Bs*SCO was done with the CDNN program using the 23-spectrum basis set [35]. Thermal melting experiments were done in a 1 mm cuvette to accommodate the temperature probe. All samples for CD were diluted into 5 mM sodium phosphate buffer pH 7.0.

Absorbance spectra were recorded on an HP8452 diode-array spectrophotometer run by the Globalworks software package (OLIS, Inc.). Stopped-flow, rapid-scanning absorbance measurements were made using an RSM-1000 spectrometer (OLIS, Inc.) equipped with an OLIS-USA stopped-flow module and controlled by Globalworks software. Analysis of the multi-wavelength data sets was performed by a combination of singular value decomposition and iterative fitting to a mechanistic model. Singular value decomposition was used to obtain estimates of the number and forms of the spectral species as well as their observed rates of inter-conversion. These parameters were then refined in an iterative process using an explicit mechanistic model to arrive at a fit to the spectral/kinetic data set.

EPR spectra were recorded on a Bruker EMX spectrometer equipped with a high sensitivity cavity. Temperature was maintained at 77 K using a finger Dewar, or an ESR-900 flow cryostat (Oxford Instruments) operating with liquid N_2 as the cryogen. Samples were prepared by rapidly freezing in a cold ethanol bath maintained over a liquid N_2 reservoir. The frozen samples were then transferred to the cryostat. Spectral simulations were done using the SimFonia program from Bruker. The simulations yielded g-values along with nuclear and ligand hyperfine splitting values.

3. Results

3.1. Activity and stability characteristics of mutant versions of BsSCO

There are two loops in the structure of *Bs*SCO that include the copper binding ligands (see Fig. 1). Both of these loops lie outside of the canonical thioredoxin-core fold [23]. The first loop (residues I41 to T53), or 'Cys loop', includes two conserved cysteine residues (i.e., C45 and C49) in the sequence -**C**ETI**C**-. The second loop, or 'His-loop', starts at residue A122 and extends to residue S138 and contains a single, conserved histidine residue (i.e., H135) (see Fig. 1). We refer to these three copper binding residues as the CCH metal binding motif within SCO proteins. In this paper we focus on two double mutants of *Bs*SCO in which each of the conserved cysteine residues (i.e., C45 and C49) has been switched in the primary sequence with the histidine at position 135. The mutants are designated HCC-*Bs*SCO in which the cysteine

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