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Role of copper in folding and stability of cupredoxin-like copper-carrier protein CopC

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Abstract

CopC is a periplasmic copper carrier that, in contrast to cytoplasmic copper chaperones, has a β-barrel fold and two metal-binding sites distinct for Cu^{II} and Cu^{I}. The copper sites are located in each end of the molecule: the Cu^{I} site involves His and Met coordination whereas the Cu^{II} site consists of charged residues. To reveal biophysical properties of this protein, we have explored the effects of the cofactors on CopC unfolding *in vitro*. We demonstrate that Cu^{II} coordination affects both protein stability and unfolding pathway, whereas Cu^{I} has only a small effect on stability. Apo–CopC unfolds in a two-state reaction between pH 4 and 7.5 with maximal stability at pH 6. In contrast, Cu^{II}–CopC unfolds in a three-state reaction at pH $\geqslant$ 6 that involves a partly folded intermediate that retains Cu^{II}. This intermediate exhibits high thermal and chemical stability. Unique energetic and structural properties of different metalated CopC forms may help facilitate metal transport to many partners *in vivo*.

Keywords: Protein folding; Unfolding intermediate; Copper chaperone; Periplasm; Spectroscopy; Calorimetry

Copper is one of the most prevalent transition metals in living organisms; its biological function is intimately related to its redox properties. Many proteins that participate in cellular respiration, antioxidant defense, neurotransmitter biosynthesis, connective-tissue biosynthesis and pigment formation use copper as the prosthetic, active group [1–4]. Since free copper is toxic, copper homeostasis in living organisms is tightly controlled by subtle molecular mechanisms [5–7]. In eukaryotes, before cellular uptake *via* high-affinity copper transporters of the CTR family [4], Cu^{II} ions are reduced to Cu^{I}. During the past decade, an important class of proteins, termed copper chaperones, has been identified in the cytoplasm that binds Cu^{I} with Cys_2 coordination [4,8–11]. Recently, an atypical Cys_2His coordination was also found for Cu^{I} in a copper-chaperone from cyanobacteria [12,13]. These small, soluble proteins guide and protect the copper ions within the cell, delivering them to the appropriate functional protein receptors. P-type ATPases are membrane spanning receptor proteins with cytoplasmic metal-binding domains that transfer Cu^{I} through membranes from one cellular compartment to another. In humans, the Menkes and Wilson disease proteins are P-type ATPases involved in copper transport [4,9,14]; in an ATP-dependent process, they translocate copper from the cytoplasm into the Golgi network for loading into proteins. Solution NMR and crystal structures [15–22] have been reported for various metal-bound and/or apo-forms of cytoplasmic copper chaperones and metal-binding domains of P-type ATPases from a range of different organisms. In all structures, the proteins possess an αβ ferredoxin-like fold and a conserved MXCXXC motif.

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In addition to the cytoplasmic Cu^{I}-trafficking proteins, other proteins have recently been identified that are involved in bacterial resistance to high copper concentrations [23,24]. Since copper is particularly toxic to lower organisms such as bacteria, copper based bactericides are extensively used in agriculture to control plant pathogens. Copper resistant strains of *Pseudomonas syringae* pathovar *tomato* are characterized by a 35-kb plasmid that contains an operon (*cop*) encoding four proteins, CopABCD, acting under a copper inducible promoter requiring the regulatory genes, CopR and CopS, coded directly downstream from CopD [23–26]. This plasmid is able to confer copper resistance to the host strains of Gram-negative bacteria and protects the cells by sequestering the excess copper in the periplasm and in the outer membrane. Upon exposure to high copper concentrations, *P. syringae* cells turn blue and up to 12% of the dry weight of the cells may be copper [27]. CopA and CopC are soluble proteins present in the oxidizing environment of the periplasm, CopB and CopD appear to be membrane-bound copper pumps while CopS and CopR form part of copper sensing and gene induction systems. It is not yet clear how these proteins interact with each other to facilitate copper resistance.

The CopC protein is proposed to be a copper-carrier protein but in contrast to the cytoplasmic copper chaperones, it lacks cysteine residues and has two copper binding sites that are separated by 30 Å [25,26,28–30]. One metal site is specific for Cu^{I} and the other for Cu^{II}: the protein can be either simultaneously loaded with Cu^{I} and Cu^{II} or with one copper in either site. CopC has a β-sandwich structure comprised of β-strands arranged in a Greek Key topology similar to that of blue-copper proteins (*e.g.,* azurin) and domains of multi-copper oxidases (*e.g.,* ceruloplasmin) (Fig. 1). The suggested ligand environment based on EXAFS and NMR data is tetragonal $Cu^{I}(His)(Met)_3$ and tetrahedral $Cu^{II}(His)_2(Asp)(Glu)(OH_2)$ [28]. Whereas the Cu^{I} site is situated in a disordered loop near the C-terminus, the position of the Cu^{II} site is similar to that of the metal sites in blue-copper proteins. Solution and X-ray structures of apo- and various Cu^{I}/Cu^{II}-forms of CopC have been reported [27–30]. Although the core of the molecule is identical in all structures, the presence of metal affects the conformation of loop regions in both ends of the molecule. Based on crystal structures of the holo-forms at pH 4.6 and 7.5, it was suggested that the N-terminus is a Cu^{II} ligand at the higher but not the lower pH [27]. Notably, N-terminal Cu^{II} coordination has only been observed in a few proteins and peptides before (*e.g.,* albumins and neuromedins) [31–33]. The fundamental chemistry of copper binding and transfer in CopC was recently explored in depth *in vitro* [27]. CopC has high-affinity for both copper ions although the Cu^{II} site has the highest affinity ($\sim 10^{13}\ M^{-1}$). Inter-protein copper transfer reactions between sites, with or without involvement of copper-redox change, were demonstrated [27]. Thus, CopC may interact with different partner proteins *in vivo* and act as a "redox switch".

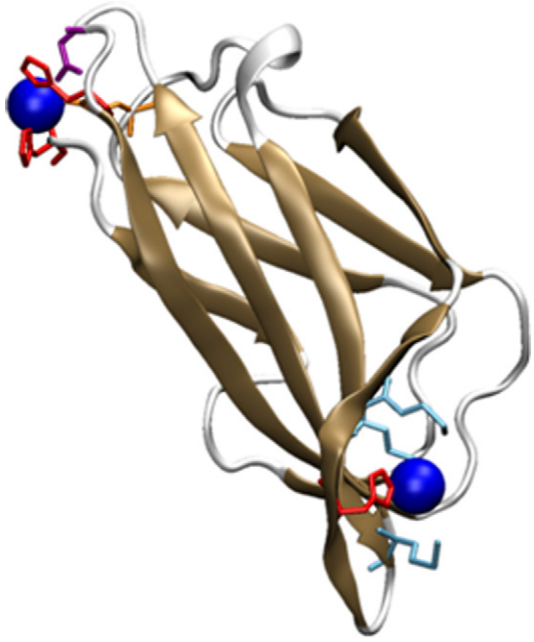

Fig. 1. Crystal structure of CopC (2C9Q) at pH 7.5 revealing the β-sandwich fold and the two copper sites. Cu(II) interacts with His1 (red), His91 (red), Glu27 (orange), Asp89 (purple) and the N-terminus (red) in a tetragonal environment forming a type-II copper site. Cu(I) interacts with a His48 (red) and several methionine (blue) sulfurs in a disordered loop.

To elucidate thermodynamic properties of CopC, and assess how they are linked to the occupation of the copper sites, we have here investigated CopC thermodynamic stability and unfolding as a function of copper loading and solvent conditions *in vitro*. We find that the two copper ions have different effects on the protein: whereas the Cu^{II} changes the unfolding mechanism and stabilizes an intermediate CopC species, the Cu^{I}-form behaves more like the apo-form. The diverging thermodynamic properties of various metalated forms of CopC may explain how this protein interacts with different partner proteins *in vivo* depending on copper levels and redox state.

Materials and methods

Protein expression and purification

Escherichia coli BL21(DE3) pLysS cells, transformed with the CopC gene cloned into the vector pAT2, were grown in LB media (37 °C) to an OD_{600nm} of 0.6 followed by protein expression induction with 0.4 mM IPTG. Cells were harvested after 6 h. Protein was extracted by freeze-thaw cycles into extraction buffer (20 mM Tris, pH 8.0, 1 mM PMSF, 1 mM EDTA). The cell lysate was then loaded on an anion exchange column (DEAE) pre-equilibrated with 20 mM Tris, 1 mM EDTA, pH 8.0. The flow-through fractions containing CopC were pooled and dialyzed against 20 mM MES, 1 mM EDTA, pH 6.0 and loaded onto a cation exchange column (SP) pre-equilibrated with the same buffer. CopC was eluted from the column with 20 mM MES, 1 mM EDTA, 0.5 M NaCl, pH 6.0 and then dialyzed into 20 mM Tris, pH 7.5. Purity was monitored by SDS–PAGE at each step. CopC was purified in its apo-form. Protein concentration was determined using $\varepsilon_{280nm} = 8700\ M^{-1}\ cm^{-1}$. The Cu^{II}-form is prepared by Cu^{II} ($CuCl_2$) addition (1 equivalent); the Cu^{I}-form is made by