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Rapid Report

A Tetranuclear Cu(I) Cluster in the Metallochaperone Protein CopZ[†]

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ABSTRACT: Copper trafficking proteins and copper-sensitive regulators are often found to be able to bind multiple Cu(I) ions in the form of Cu(I) clusters. We have determined the high-resolution X-ray crystal structure of an Atx1-like copper chaperone protein from *Bacillus subtilis* containing a novel tetranuclear Cu(I) cluster. The identities and oxidation states of the cluster ions were established unambiguously by refinement of X-ray energy-dependent anomalous scattering factors. The $[Cu_4(S\text{-}Cys)_4(N\text{-}His)_2]$ cluster geometry provides new structural insights into not only the binding of multiple cuprous ions by metallochaperones but also protein-associated tetranuclear Cu(I) clusters, including those found in eukaryotic copper-responsive transcription factors.

Polynuclear cuprous−thiolate cluster proteins are found in a wide variety of organisms. Binuclear and trinuclear clusters are frequently found in enzymes catalyzing the oxidation of organic substrates or metal ions, while the μ_4-S-bridged tetranuclear Cu(I) cluster found in nitrous oxide reductase leads to the $2e^-/2H^+$ reduction of the greenhouse gas N_2O to give dinitrogen (*1*). Tetranuclear cluster proteins are also employed in nonenzymatic roles as metal sensors in biological systems. Among these are the Cu(I)-responsive transcription factors Ace1 and Mac1 from yeasts and fungi and the metal-responsive transcriptional activator MTF-1 from flies and mosquitos (*2*). All are believed to accommodate a Cu_4 cluster, yet thus far, none have been structurally characterized.

Despite being an essential nutrient, free copper is toxic, so the availability of free ions must be tightly regulated. The Atx1-like copper chaperone CopZ from the Gram-positive bacterium *Bacillus subtilis* functions as part of a complex cellular machinery for Cu(I) trafficking and detoxification. CopZ contains a MXCXXC metal-binding sequence motif (residues 11−16), and solution studies have revealed that uptake of Cu(I) by CopZ leads to protein dimerization and thence to discrete copper-bound forms of the protein involving up to at least three Cu(I) ions per dimer (*3*). Here, we present a high-resolution X-ray crystal structure of the four-copper CopZ dimer, $Cu_4(CopZ)_2$, and verify the arrangement of metal ions by refinement of anomalous scattering factors of the metal ions. The novel cluster geometry provides new structural insights into protein-associated tetranuclear Cu(I) clusters.

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Our attempts to crystallize a binuclear Cu(I)-bound form of the CopZ dimer $[Cu_2(CopZ)_2]$ under anaerobic conditions yielded crystals in space group $P2_1$ containing one CopZ dimer per asymmetric unit. X-ray fluorescence spectra revealed the crystal to contain copper only, and the X-ray anomalous scattering from the copper ions of the cluster was used to determine the crystal structure by SAD phasing methods. Surprisingly, however, not two but four heavy atom peaks per asymmetric unit were located, forming a tetranuclear cluster at the protein dimer interface. The refined crystal structure has an *R*-factor of 13.0% (R_{free} = 17.5%) at 1.5 Å resolution (for full details, see the Supporting Information). No excess, free copper was present in the solution preparation of the protein used for crystallization. Thus, for CopZ at least, it appears that the copper ion-bound form of the protein characterized in solution may not correspond to the thermodynamically most stable state observed in the crystal. This points to a rich variety of (and potential for interconversion between) Cu(I)-bound states in this metallochaperone.

The tetranuclear copper cluster in the refined crystal structure comprises two subsets of Cu(I) ions in different coordination environments (Figure 1). The outer Cu(I) ions (labeled 3 and 4) exhibit distorted trigonal coordination, while the inner ions (labeled 1 and 2) exhibited distorted digonal coordination.

Four cysteine residues (Cys13 and Cys16 from each of the two CopZ monomers of the dimer) are central to the formation of the cluster, whereby each acts as a ligand to an inner (digonal) and outer (trigonal) copper ion. Two histidine residues (His15 from each monomer) provide the remaining ligands to the trigonal Cu(I) ion sites. In addition, two water molecules (W1 and W2) move to points within 2.53 and 2.61 Å of the trigonal copper ions sites 3 and 4, respectively, imparting a partial tetrahedral character (see Table S2 of the Supporting Information). The Cu(I) ions in adjacent trigonal and diagonal sites lie at a distance of 2.57 Å, while the distance between the digonal sites is 2.74 Å. These distances, particularly the former, are shorter than the sum of the van der Waals radii of the ions, suggesting the presence of a true metal cluster. The average digonal Cu(I)−S distance is 2.21 (0.03) Å, a distance typical of digonal S−Cu−S coordination (*4*), while the average S−Cu−S angle is 144.0°, somewhat compressed relative to that in the yeast copper thionein crystal structure (*5*). This compression results from the approach of a third sulfur atom at a distance of 2.54 Å to the inner copper sites, imbuing them with a partial trigonal character. The average trigonal Cu(I)−S distance is 2.36 (0.06) Å, a value in keeping with those observed in similar copper sites in proteins in the Protein Data Bank (∼2.4 Å).

The Ser12 residues from each subunit form part of a second coordination sphere of the inner Cu(I) sites. The serine hydroxyl

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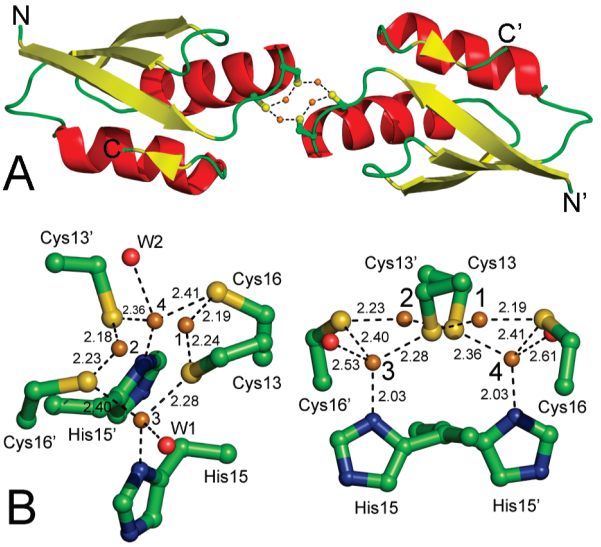

FIGURE 1: (A) Architecture of the $Cu_4(CopZ)_2$ complex. α-Helices are colored red and β-strands yellow. Cu(I) ions are colored orange. (B) Orthogonal views of the tetranuclear cluster. Residues contributing ligands to the copper ions of the cluster are shown, as are two coordinated ordered water molecules (W1 and W2) that form part of the coordination sphere of the outer copper sites.

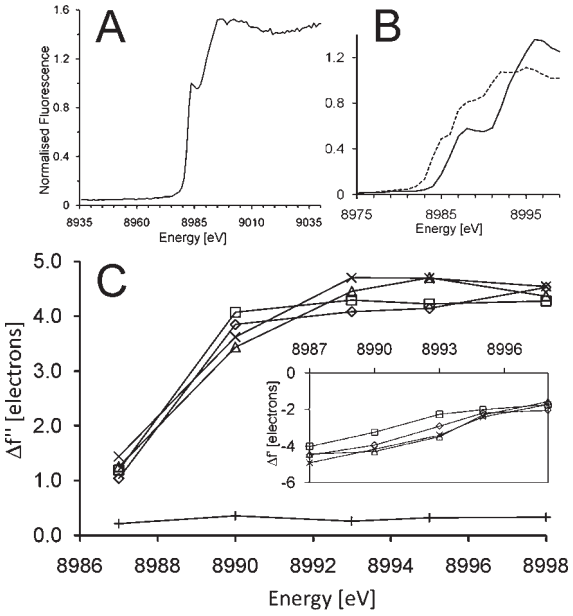

FIGURE 2: Analysis of the CopZ metal cluster. (A) X-ray fluorescence emission spectrum of a single $Cu_4(CopZ)_2$ crystal normalized to the value at 8984 eV. (B) Normalized edge spectra for model Cu(II) complexes, with S_4 (dashed line) and N_4 (solid line) equatorial ligand sets. Taken from ref *9*. (C) Refined anomalous scattering factors $\Delta f''$ and $\Delta f'$ (inset) for digonal, Cu1 (□) and Cu2 (◇), and trigonal, Cu3 (△) and Cu4 (×), copper sites. $\Delta f''$ scattering factors for sulfur are also given (+).

oxygen−Cu(I) distances are 2.92 and 3.04 Å for sites 1 and 2, respectively. In the same way, Tyr65 and Tyr65′ form part of a second coordination sphere to the outer Cu(I) sites with phenolic hydroxyl−Cu(I) distances of 3.39 and 3.46 Å for sites 3 and 4, respectively. The side chains of the methionine residue of each MXCXXC motif (Met11) point away from the cluster and insert into the core of the protein, making van der Waals contact with other hydrophobic residues, including the side chain of Tyr65. The methionine residue appears to contribute to local protein structural integrity and will therefore play an indirect role in copper binding. The coordinating histidine residues (His15 from each monomer) identified here as forming a direct ligand to the outer copper ions of the cluster are strongly conserved among copper chaperones from Gram-positive bacteria, including *Bacillus* and related species, and the *actinomycetalaes* (including streptomycetes and mycobacteria), and also occur in some Gram-negative proteobacteria, consistent with an important physiological function(s).

The inner Cu(I) ions of the tetranuclear cluster are buried at the CopZ dimer interface and shielded from interaction with solvent. The sulfur atoms of the four cysteine residues acting as ligands to the inner and outer sites are also buried. Luminescence in the ~600 nm region is often observed for protein-bound copper clusters and is indicative of the cluster being in a solvent-shielded environment (*6*, *7*). The observed solvent exposure of the cluster here is consistent with the lack of a luminescence signal associated with this complex (data not shown).

Given the sensitivity of Cu(I) to oxidation and the solvent exposure of the outer sites in the cluster, we were concerned about verifying that the cluster contained solely Cu(I) ions. The shoulder apparent in X-ray fluorescence spectra of $Cu_4(CopZ)_2$ crystals at ~8984 eV (Figure 2) arises from the 1s → 4p transition in digonally or trigonally coordinated Cu(I). However, this alone is insufficient for unambiguously determining the composition of the cluster, so X-ray energy-dependent anomalous scattering factors ($\Delta f''$ and $\Delta f'$) were refined for each metal ion in the X-ray energy range of 8987−8998 eV (*8*). This corresponds to the region where Cu(II) pre-edge features are typically observed (see Figure 2B for examples) (*9*). The refined $\Delta f''$ value of 0.3 electron for sulfur atoms refined as a single anomalous species was found to compare favorably with the tabulated value of 0.7 electron (*10*). The results for the cluster are consistent with scattering from copper ions alone, negating the possibility of adventitious binding of, for example, stray zinc at the more solvent-exposed trigonal cluster sites. Furthermore, the refined scattering factors suggest that all four copper sites are in the Cu(I) oxidation state, as no pre-edge features arising from tetrahedral Cu(II) are detected in the region of 8988−8990 eV.

The mononuclear copper-bound form of the human copper chaperone, Hah1, provides the only hitherto available structure of a dimeric copper chaperone of the Atx1 family (*11*). Structural alignment of a monomer of CopZ from $Cu_4(CopZ)_2$ with one from Hah1 (with which it shares 24% sequence identity) gave a root-mean-square deviation of 1.3 Å for all C_α atoms. However, despite obvious structural similarity between monomers of the two proteins, the packing of monomers in the two dimers is very different (Figure 3), reflecting the plasticity of the dimer interface. Nevertheless, this superposition also results in a positioning of the single Hah1 copper ion close (0.93 Å distance) to one of the inner ions of the CopZ tetranuclear cluster. Although our attempts to determine the structure of the $Cu_2(CopZ)_2$ form have not yet been successful, it is tempting to speculate that it is likely to feature the two "inner" Cu(I) ions (sites 1 and 2) from the $Cu_4(CopZ)_2$ structure. This is supported by the observation that these sites are fully occupied in the Cu_4 structure, whereas the two outer sites (3 and 4) have lower occupancies (see Table S3 of the Supporting Information).

The physiological relevance of the tetranuclear copper cluster form of CopZ described here is not clear. Certainly, it cannot be discounted that such forms may exist in vivo under conditions of copper stress. There are precedents for such a cluster among other

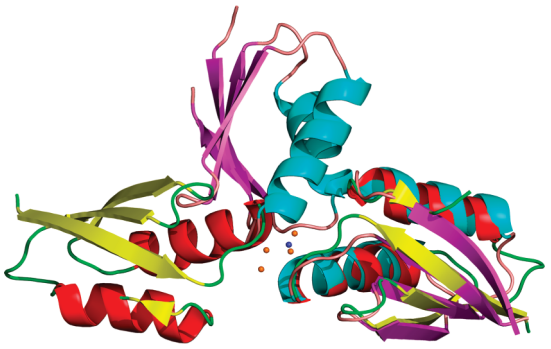

FIGURE 3: Comparison of the structures of dimeric Cu(I) chaperones. A superposition of the crystal structures of $Cu_4(CopZ)_2$ [red α-helices, yellow β-strands, and Cu(I) ions colored orange] and copper-bound, dimeric Hah1 [cyan α-helices, magenta β-strands, and Cu(I) ion colored blue] (*12*).

proteins involved in copper trafficking. For example, the eukaryotic copper chaperone Cox17, which directs Cu(I) for insertion into cytochrome *c* oxidase, binds a tetranuclear cuprous−thiolate cluster at its dimer interface (*12*). However, there is no sequence or structural similarity to CopZ, and the Cox17 cluster has been shown to be of the $[Cu_4(SR)_6]^{2-}$ variety. The high-resolution crystal structure of $Cu_4(CopZ)_2$ presented here is the first for a cluster of this type in a protein environment. The structure of the cluster is related to that reported for the $[Cu_4(SR)_6]^{2-}$ species in which an approximately tetrahedral core of Cu(I) ions is bound by bridging thiolates above each of the six edges (*13*). In the CopZ dimer, there are only four thiolates, so of the two remaining edge positions, one is shared by the pair of monodentate histidine nitrogens and the other is vacant (see Figure S2 of the Supporting Information). The "missing" bridging thiolate is partially offset by a long interaction between the inner Cu(I) ions (1 and 2) and Cys13 and Cys13′, respectively, imparting a trigonal character on these ions.

Copper metalloregulatory proteins in prokaryotes form either simple digonal, mononuclear, trigonal planar or binuclear Cu_2S_4 coordination complexes. Eukaryotic metal-responsive transcription factors bear cysteine-rich metal-binding sequences which differ in their cysteine content. These can contain six or more cysteines (yeast Ace1 and MTF-1 from fly and mosquito) or five cysteines and a histidine (e.g., Mac1 from yeast). Cluster structures based on $[Cu_4(SR)_6]^{2-}$ have been proposed for Ace1 (*14*) and the *Drosophila melanogaster* metal-responsive transcription factor dMTF-1 (*15*). The structure of the tetranuclear core of $Cu_4(CopZ)_2$ is perhaps most relevant to the clusters of other classes of eukaryotic copper-responsive transcription factors such as Mac1. The copper-binding motif of Mac1 contains five cysteines and a histidine that are functionally essential, and a $[Cu_4(S\text{-}Cys)_5(N\text{-}His)]$ structure was proposed for its cluster (*14*). Interestingly, EXAFS was unable to detect the histidine nitrogen ligand in Mac1 because it contributes a relatively small scattering signal compared to the cysteine sulfurs (*14*). Here, we were also unable to detect the histidine ligands by EXAFS. However, the close correlation of Cu−S and Cu···Cu distances between the crystallographic and EXAFS data indicates that the same $Cu_4(CopZ)_2$ species is present in solution and in the crystal (see the Supporting Information). The structure of $Cu_4(CopZ)_2$ presented herein presents new, intruiging insights into the flexibility of cuprous ion cluster binding by copper chaperones and copper-sensing proteins in general. The mixture of digonal and trigonal coordination of Cu(I) in the complex arises because of the insufficiency of thiolate ligands in CopZ required to form a more conventional $[Cu_4(SR)_6]^{2-}$ cluster species. The knowledge that recruitment of histidine residues may take place to allow a novel coordination of the tetranuclear cluster will open the door to possible re-interpretations of sequence data for putative metal binding regions in a wide range of proteins.

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SUPPORTING INFORMATION AVAILABLE

Experimental procedures, X-ray statistics and refined crystallographic parameters, $Cu_4(CopZ)_2$ cluster geometry, and Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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