binding domain relative to the larger Rossmann fold is radically different in the three structures. This rotation of the zinc binding domain causes large changes in interatom distances in the different structures: up to a 10.8 Å difference between Sir2-Af2 peptide and Sir2-Af1-NAD, and up to a 14.3 Å difference between Sir2-Af2 peptide complex and SIRT2. Second, a loop that extends from the Rossmann fold domain and contacts NAD in the Sir2-Af1-NAD structure is disordered in the Sir2-Af2 peptide structure (indicated by a dotted line in the Figure). Since this loop is the most highly conserved stretch of amino acids in the Sir2 family, it is likely that the presence of NAD causes this loop to adopt a more ordered conformation. Finally, a different loop region that contains residues that contact the peptide substrate backbone and acetyl-lysine in Sir2-Af2 peptide (β7; called the FGE loop; see Figure) is shifted up to 8.7 Å to a more open conformation in the unliganded SIRT2 structure. The flexibility of the FGE loop is particularly interesting, since this loop seems to be central to the conformation of other parts of the molecule. In addition to making hydrogen bonds with the acetylated peptide, this loop has van der Waals contacts with helix α3, immediately adjacent to the NAD binding loop described above. There is also a salt bridge between Glu167 in the FGE loop and Lys159 in the zinc binding domain (in β6), and there is a connection to the putative active site via a hydrogen bond to His118. It is therefore tempting to speculate that the differences in conformation between the structures are all directly related to the presence of different bound substrates, and are mediated by the FGE loop. For example, one might imagine that binding of NAD to an unliganded enzyme would induce closure and/or ordering of the NAD binding loop, which would then cause the FGE loop to shift to the more closed conformation appropriate for peptide binding. Movement of the FGE loop could in turn explain the different positions of the zinc binding domain in the various structures.

Current models of the Sir2 reaction mechanism predict a nucleophilic attack on C1′ of the nicotinamide ribose by the carbonyl oxygen of the N-acetyl group of the substrate [8]. The proximity of the acetyl-lysine moiety to His118 in the Sir2-Af2 peptide structure could make the N-acetyl group a better nucleophile, in agreement with the proposed mechanism. An approximate location of the active site can be obtained by modeling the NAD structure from the Sir2-Af1-NAD crystal into the Sir2-Af2 peptide structure (see Figure). This picture of the active site is still somewhat imperfect because of steric clash between the N-acetyl group on the substrate and the 2′-hydroxyl group of NAD. Thus, it seems likely that the enzyme needs to sample even more conformations to form a productive enzyme-NAD-substrate ternary complex.

The idea that Sir2 enzymes undergo conformational movements during catalysis is appealing, since it might help explain how the energy expended by breakage of the C-N bond in NAD is used. What might such movement be doing? Conformational rearrangements may be crucial for some aspect of the deacetylation reaction mechanism, such as allowing tight binding to substrates or driving product release. It is also possible that the movement by the enzyme is doing work, allowing deacetylation to be coupled to another process that requires energy. Assembly of silencing complexes on chromatin could be one such process.

Though we now understand a great deal about the structural aspects of substrate binding by the Sir2 family of deacetylases, it remains to identify the in vivo targets of these enzymes. Given the evolutionary conservation of Sir2 family members, elucidation of their biological functions will undoubtedly uncover new roles for protein acetylation, be it on chromatin or elsewhere.

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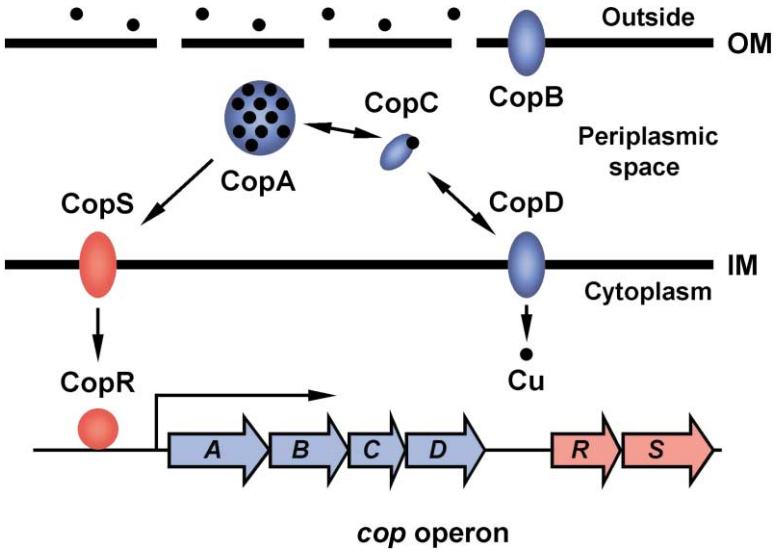
The ABCDs of Periplasmic Copper Trafficking

The structure of the CopC protein of *Pseudomonas syringae* pathovar *tomato* provides fascinating clues, not only to its role in the periplasmic space in copper resistance, but also to features important for copper trafficking and homeostasis that may be conserved in a variety of biological systems.

Copper is a transition metal essential for the activity of a wide variety of enzymes such as cytochrome c oxidase, Cu,Zn superoxide dismutase, and multicopper ferroxi-



A Model for Copper Homeostasis in *P. syringae*

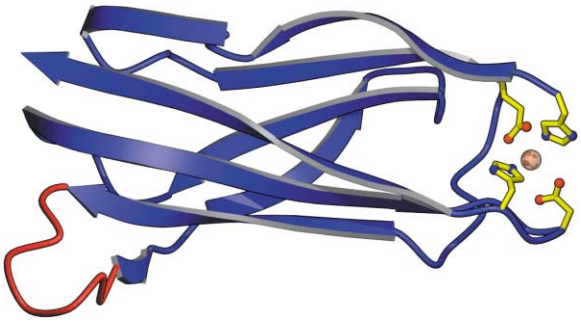
Extracellular copper reaches the periplasmic space through the porous outer membrane (OM). CopA, with strong homology to multicopper oxidases, could function in copper resistance either due to its high copper binding capacity or oxidase activity, or both. CopB and CopC also contribute to protection from copper toxicity. CopC may transfer copper from CopA to the inner membrane (IM) transporter CopD, where the copper is then imported into the cytoplasm. The inner membrane protein CopS is thought to sense excess copper in the periplasmic space, perhaps via interactions with CopA or CopC. The copper signal may be transmitted to CopR, which in turn induces the expression of the *copABCD* operon. The *copR* and *copS* genes, located downstream of the *copABCD* operon, are constitutively expressed. The precise role for these components in copper homeostasis, and the directionality of copper transfer by *P. syringae* CopC, is still an open question.

dases. However, the same redox properties that make copper so useful biochemically also underlie its potential as a powerful cytotoxin. Free copper ions participate in redox reactions that generate hydroxyl radicals, which are highly reactive species that cause lipid peroxidation, nucleic acid cleavage, and protein damage. As such, virtually all cells have developed sophisticated homeostatic mechanisms to tightly control copper uptake and its mobilization to appropriate target proteins and compartments. To ensure that sufficient copper is acquired to drive essential biochemical reactions yet prevent accumulation to levels that encourage harmful redox chemistry, both prokaryotic and eukaryotic cells homeostatically control copper via dedicated proteins that facilitate copper uptake, distribution, and efflux. These homeostatic mechanisms are dynamically regulated through cellular copper sensing mechanisms that operate at the level of gene transcription, protein stability, and trafficking.

Copper is transported across the eukaryotic plasma membrane by structurally conserved integral membrane proteins of the Ctr1 family, the expression of which in yeast cells is induced under conditions of copper scarcity and extinguished when copper is abundant [1]. A number of biochemical and genetic studies implicate cell surface metalloreductases in the reduction of copper to Cu(I), as a corequisite for its transport across the membrane by Ctr1. Furthermore, genetic and copper transport studies have demonstrated a requirement for conserved Ctr1 methionine residues in the second of three transmembrane domains (present as Met-X_3-Met) in the transport process. Moreover, under conditions of extreme copper limitation, extracellular methionine-rich sequences, present as Met-X-Met or Met-X_2-Met, are required for full activity of the Ctr1 transporter [2]. Consistent with the idea that Ctr1 proteins facilitate the uptake of Cu(I), these methionine thioether groups would be suitable ligands for guiding reduced copper into the cell. In prokaryotes, Cu(I) import is mediated by multimembrane-spanning P-type ATPases, such as CopA in *Enterococcus hirae* or CtaA in *Synechocystis* PCC 6803 [3], which harbor conserved extracellular copper binding motifs that make use of cysteine ligands.

While the majority of evidence suggests that Cu(I) is the form of the metal ion that is delivered to the cytoplasm in bacterial and eukaryotic cells, the propensity of Cu(I) to engage in redox chemistry in the presence of oxygen demands that the Cu(I) be escorted to specific cytosolic cupro-proteins and cellular organelles in a controlled yet kinetically labile manner. Bacterial and eukaryotic cytoplasmic copper trafficking is achieved through the use of small dedicated Cu(I) binding proteins called copper chaperones, which have specific delivery destinations that are mediated via highly specific protein-protein interactions. At the plasma membrane of the bacterium *E. hirae*, the CopZ copper chaperone interacts with the CopA P-type ATPase (which serves to import copper) via direct interactions with the CopA cytosolic copper binding domains. CopZ then directly delivers the metal to the transcriptional repressor CopY, thereby inactivating CopY repression, resulting in the expression of copper homeostasis genes.

In eukaryotes, copper distribution is mediated by at least three independent copper chaperones. In a manner somewhat analogous to the *E. hirae* CopZ chaperone, the eukaryotic Atx1 (or Atox1 in mammals) metallochaperone mediates copper delivery to proteins localized in the secretory pathway, via direct interactions with the cytosolic metal binding modules of secretory compartment integral membrane copper transporting P-type ATPases. The Cu(I) is thought to be transferred through a series of ligand exchange reactions involving two- and three-coordinate intermediates between CX_2C cysteine motifs from both proteins. Genetic and structural studies suggest that charged surfaces in both partners facilitate the correct and specific alignment for these interactions [4, 5]. The CCS copper chaperone inserts copper into the active site of Cu,Zn superoxide dismutase, by direct docking with a Cu,Zn SOD monomer. The third known eukaryotic copper chaperone, Cox17, targets copper



Model for the CopC-Cu(II) Three-Dimensional Structure

Using the coordinates from the structure of apoCopC by Arnesano et al., a structural model was derived with a copper atom modeled into the presumptive Cu(II) binding site. The side chains of Glu27, Asp89, and His91 were rotated in silico to facilitate tetragonal coordination of Cu(II). Due to the relative disorder of the amino-terminal CopC residues in the structural data, His1 was repositioned to provide the fourth ligand for the Cu(II) site. Therefore, this is a hypothetical model of a potential Cu(II)-CopC structure. The loop containing the methionine-rich region is depicted in red, and the side chains of the four putative Cu(II) ligands are shown in yellow. (The structure was modeled with Povscript software, with generous assistance from Dr. Daniel Peisach).

to mitochondrial cytochrome c oxidase that, while the precise biochemical mechanism remains to be clarified, is likely to involve copper hand-offs to at least one additional downstream protein before reaching the copper binding cytochrome oxidase subunits [1, 4, 5].

The import and delivery of Cu(I) within the confines of the bacterial and eukaryotic cytosol, a highly reducing environment, is strongly dependent on the use of Cu(I) transporters and chaperones. This general mechanism is useful and important for environments rich in glutathione or other reductants, but how is copper handled in cellular compartments that are highly oxidizing, where copper is expected to predominantly exist in the Cu(II) state? One such environment, the periplasmic space, is a center for disulfide bond formation, yet the highly oxidizing environment could pose a serious threat if redox active metals such as copper are freely engaged in redox chemistry that generates reactive oxygen species. Important clues are beginning to emerge from the elucidation of the structure of the periplasmic copper binding protein CopC from *Pseudomonas syringae*, reported in this issue of *Structure*.

The use of copper compounds for plant pathogen control has led to the selection of copper-resistant pathogens such as the *P. syringae* strain isolated from infected tomatoes in California several decades ago. This Gram-negative bacterium harbors a 35 kb plasmid with a copper-inducible operon (*copABCD*) consisting of four genes (see first Figure) [6]. The *CopA* gene encodes a methionine-rich periplasmic protein capable of binding up to 11 copper atoms, and shares homology with multicopper oxidases such as ceruloplasmin in human serum or the Fet3 ferroxidase involved in high-affinity iron uptake in yeast. Whether CopA functions in copper resistance due to its high copper binding capacity, its multicopper oxidase activity, or both, is not yet clear. The CopB protein, also rich in methionine residues, is an integral membrane protein of the outer membrane, for which no direct evidence exists for copper binding. The CopC protein, reminiscent of the copper chaperones, is a small soluble protein found in the periplasmic space that has been shown to bind one atom of copper. Finally, the CopD protein, which contains several putative transmembrane domains, is thought to be localized to the *P. syringae* inner membrane. The results of genetic studies support the notion that the periplasmic proteins CopA and CopC, and perhaps the outer membrane protein CopB, confer copper resistance, while CopC and CopD could function in copper transport into *P. syringae* cells [6]. By extension, then, these observations suggest that the mechanism of copper resistance in *P. syringae* is at least heavily dependent on copper sequestration and accumulation in the periplasm and outer membrane. Indeed, *P. syringae* cells exposed to high concentrations of copper turn blue, accumulate copper in the periplasmic space, and have been found to accumulate as much as 12% of their dry weight in copper.

Arnesano et al. have solved the three-dimensional solution structure of the *P. syringae* apoCopC protein [7]. The NMR data and electronic spectroscopy of the current work shows that CopC binds one copper atom per molecule. This implies that unlike CopA, which binds 11 copper atoms, CopC functions not to sequester copper, but instead may play a role in copper trafficking. Titration of apoCopC with $CuSO_4$ indicated an affinity constant of $1.3 \pm 0.5 \times 10^4\ M^{-1}$ for copper (II) binding to CopC. The EPR spectrum of $Cu(II)_1$-CopC predicts the copper (II) atom is ligated with nitrogen and oxygen donor atoms in a tetragonal coordination geometry. Four residues that are highly conserved in the CopC family—His1, His91, Glu27, and Asp89—are likely to form this type II copper site (see second Figure for a Cu(II)-CopC model). NMR dispersion results support the presence of a water molecule 2.75 Å from the copper atom. While mobility studies indicate a monomeric form for both apo and Cu(II)-loaded CopC [7], analytical gel filtration experiments with a potential CopC homolog in *Escherichia coli*, PcoC, suggest that the protein oligomerizes upon copper binding [8]. Taken together, the low affinity of CopC for copper and the predicted solvent-exposed location of the metal are consistent with a role for CopC in copper mobilization in the periplasmic space.

While the structure of apoCopC is the first of its class to be determined, it does possess structural features characteristic of proteins from other classes. CopC is comprised of nine β strands that fold into a Greek key β barrel motif similar to the cupredoxin fold shared by blue copper proteins and multicopper oxidases. Unlike these other similar classes of proteins, however, CopC is missing an amino-terminal β strand, placing the amino terminus at the opposite end of the barrel in comparison to cupredoxins, and resulting in a distinct positioning of the putative Cu(II) ligands of CopC. Furthermore, in place of the helical regions found in these other classes of CopC homologous proteins, CopC possesses a disordered loop that contains an MX_2MXHX_2M motif (see second Figure, shown in red). Upon addition of more than one equivalent of copper to apoCopC, spectral variations occurred in the region of the β barrel containing

the methionine-rich motif [7], suggesting the possibility of structural rearrangements due to copper binding to this region. Isotopic labeling studies of the conserved methionine residues in the *E. coli* CopC homolog were consistent with a copper-methionine distance of 3.5–3.9 Å [8]. Interestingly, the recently determined crystal structure of the *E. coli* multicopper oxidase CueO shows the presence of a methionine-rich α helix blocking access to the type I copper site. It has been proposed that this methionine region, in concert with a disordered portion of the CueO protein (residues 380–403), also rich in methionines and histidines, may be involved in copper binding that could lead to ordering and formation of a substrate-specific binding site [9].

Both site-directed mutagenesis data for the yeast and mammalian Ctr1 high-affinity copper transporters, and the conservation of methionine-rich regions in other prokaryotic and eukaryotic proteins involved in copper homeostasis, implicate a critical role for these regions in the function of their respective proteins [2]. One may tentatively suggest a similar function for the methionine-rich region in the CopC protein, perhaps serving as an additional copper binding site. It is possible that upon copper binding to the type II site, conformational changes occur that, when disseminated throughout CopC, order the previously disordered methionine-rich loop, and thus facilitate its interaction with an additional copper atom or a target protein for metal exchange. Interestingly, Gram-positive bacteria, which lack a periplasmic space, express a CopCD fusion protein, providing an intriguing clue that CopC may functionally and directly interact with CopD to facilitate copper transfer. While no experimental data exist to support a model where CopC would interact with two proteins, the type II copper site of CopC could donate or receive copper from one protein, perhaps the multicopper oxidase-like protein CopA, and the methionine-rich region could transfer or receive the metal from another protein, such as the inner membrane protein CopD. Two distinct copper binding sites have been proposed for the copper chaperone for Sod1 (CCS) [10]. CCS contains three clearly defined domains. The amino-terminal domain I, similar in structure to the Atx1 (Atox1) metallochaperone, contains a CX_2C motif involved in copper coordination. The small carboxy-terminal region (domain III), which appears unstructured in the three-dimensional structure of CCS, contains an essential CXC motif that becomes structured in the CCS-Sod1 heterodimer. A model for CCS copper transfer from the donor protein to the partner protein Sod1 has been proposed. The CX_2C cysteine residues from domain I would acquire the copper from a donor protein and transfer it to the CxC cysteine residues in domain III. Finally, domain III would translocate the metal to the active site of Sod1 through specific docking via a series of ionic interactions [10]. This model, and the previous demonstration of interactions between the Atx1 and CopZ copper chaperones and their cognate copper transporter molecules, imply that the presence of highly charged clusters of amino acids on the surface of CopC, evident from the structure presented by Arnesano et al., could also facilitate protein-protein interactions that are important for copper transfer. The elegant structural data on CopC will provide a platform for dissecting the precise roles for CopC in bacterial copper homeostasis, and is certain to stimulate further investigations into how proteins in distinct biochemical environments function in copper delivery and exchange.

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