Intracellular copper routing: the role of copper chaperones

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Copper is required by all living systems. Cells have a variety of mechanisms to deal with this essential, yet toxic trace element. A recently discovered facet of homeostatic mechanisms is the protein-mediated, intracellular delivery of copper to target proteins. This routing is accomplished by a novel class of proteins, the 'copper chaperones'. They are a family of conserved proteins present in prokaryotes and eukaryotes, which suggests that copper chaperones are used throughout nature for intracellular copper routing.

TRANSITION METAL IONS, such as copper, iron, molybdenum and cobalt, play central roles in biology, largely because they are able to exist in multiple oxidation states *in vivo*. Copper exists as Cu^{2+} or Cu^{1+} under physiological conditions. Cells use copper as a structural element in regulatory proteins and harness the chemistry of this element in single-electron-transfer reactions. However, the redox property that makes copper an essential element of biological systems also contributes to its inherent toxicity. Redox cycling between Cu^{2+} and Cu^{1+} can catalyse the production of highly toxic hydroxyl radicals, with subsequent damage to lipids, proteins, DNA and other biomolecules[1].

A recently identified element for the management of cellular copper is the 'copper chaperone'[2]. These ubiquitous proteins have a critical biological function: to transport copper in the cytoplasm to the site of utilization by copper-dependent proteins. Consequently, copper chaperones prevent inappropriate copper interactions with other cellular components. These proteins have been identified in species ranging from prokaryotes through to humans. Based on studies in bacteria, yeast and mammalian cells, a unified model of copper chaperone function has begun to emerge.

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Chaperone function in a simple model system

The *cop* operon of the Gram-positive bacterium *Enterococcus hirae* plays a dominant role in copper homeostasis and consists of four genes, *copY, copZ*, *copA* and *copB* (Fig. 1a). Both the *copA* and *copB* genes encode copper pumps that belong to a subgroup of the P-type ATPase family of universal ATP-driven ion pumps[3]. Members of this subgroup transport transition metal ions and have been called CPx- or P_1-type ATPases[4,5]. CopA appears to be responsible for Cu^{1+} uptake when copper is limiting, whereas CopB secretes Cu^{1+} when it is in excess[6]. Expression of the *cop* operon is regulated by copper through the concerted action of the CopY repressor and CopZ, a copper-binding protein. When the cytoplasmic copper concentration rises, two Cu^{1+}CopZ molecules specifically deliver their copper to CopY to displace a structurally required Zn^{2+}, thereby releasing CopY from the DNA and inducing the *cop* operon[7]. This mode of regulation involving a small protein, a copper chaperone, to deliver the inducer to the repressor is new and so far unique.

A metallochaperone family

Members of a homologous family of metallochaperones, proteins that bind and transport metals specifically, have been described in humans (HAH1)[8], *C. elegans* (CUC-1)[9], *Arabidopsis* (CCH)[10], yeast (Atx1p)[11] and bacteria (CopZ, MerP)[12,13]. Surprisingly, it was found that the N-termini of cadmium-, silver- and copper-pumping CPx-type ATPases contain domains that are similar to Atx1p and CopZ. These domains occur in one or two copies in microbial enzymes through to six copies in the human Menkes and Wilson ATPases[5]. Furthermore, one or two of these domains are present in mercuric reductases from bacterial resistance systems[14]. MerP, the earliest of these proteins to be structurally described[13], is proposed to scavenge mercury in the periplasmic space and route it to the MerT transporter for uptake and subsequent detoxification by the cytoplasmic mercuric reductase. Clearly, a general transition, metal-binding domain – a type of 'modular' chaperone structure – has evolved, which can be tailored to function with different metal ions, either alone or as domains of large, specialized proteins. The role of these modules in some proteins is still undefined.

An open-faced β-sandwich for copper

Detailed structural studies have shown that members of this homologous family of chaperones have very similar folds. Nuclear magnetic resonance (NMR) has been used to determine the structures of metallated and apo CopZ (Ref. 12) and the mercury-binding homologue MerP (Ref. 13). Similarly, the structure of MNKr4, the fourth metal-binding domain of the Menkes ATPase, has been solved with silver in place of copper[15]. X-ray crystallography has been used to determine the structure of the yeast copper chaperone Atx1p (Ref. 16) with mercury bound in place of Cu^{1+}. These four proteins possess the same fold, consisting of four β strands forming an antiparallel β sheet, situated below two α helices. The structure of CopZ is highlighted in Fig. 2. This arrangement of secondary structure elements is characteristic of the ferredoxin-like proteins and is known as an 'open-faced β sandwich'[17]. In the metallochaperones and chaperone-like modules (CopZ, MerP, Atx1p, MNKr2 and 4) the metal-binding sequence motif, -CxxC-, occurs on the mobile loop between the first β strand and the first α helix.

More copper enzymes – more copper chaperones

In *Streptomyces* it was found that the protein MelC1 is required to transfer copper to apo-tyrosinase. MelC1 does not share sequence similarity with Atx1p, CopZ, and related chaperones, although it serves an analogous function and is

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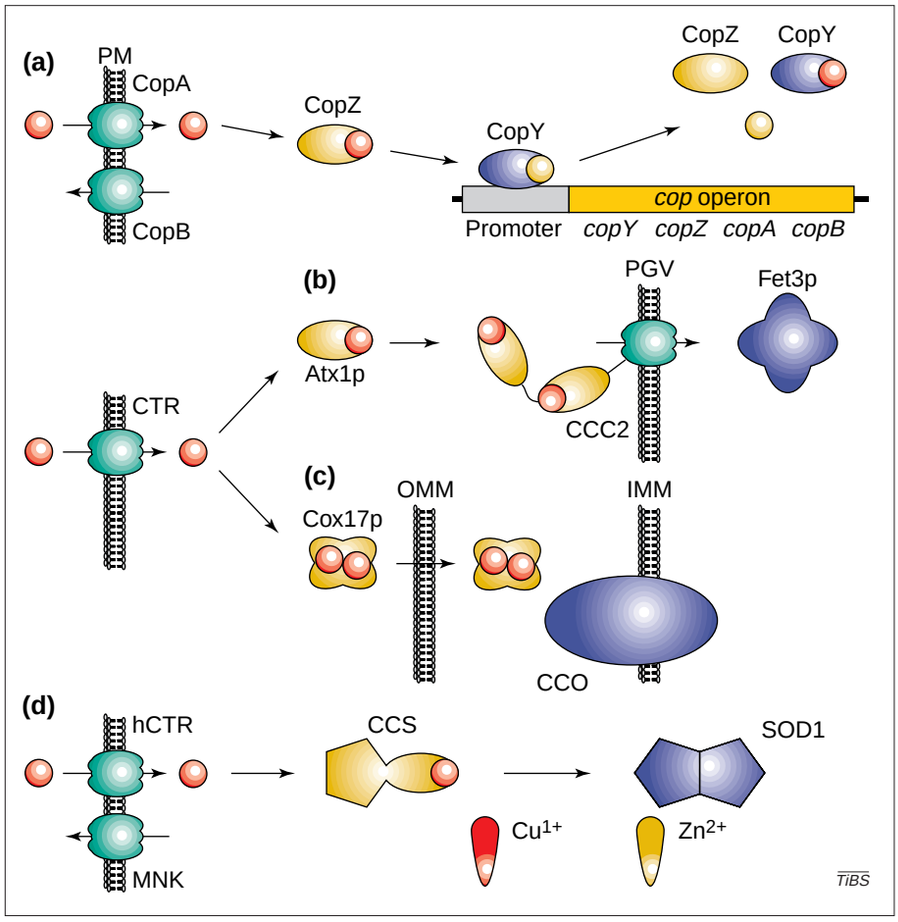

Figure 1

Copper chaperone function. **(a)** Copper homeostasis in *Enterococcus hirae* is effected by the proteins encoded by the *cop* operon. CopA, copper-import ATPase; CopB, Cu^{1+}-export ATPase; CopY, Cu^{1+}-responsive repressor; CopZ, chaperone for Cu^{1+} delivery to CopY. **(b)** The CTR protein family functions to transport copper ions into yeast cells. Atx1p delivers copper to the CPx-type ATPases resident in the *post*-Golgi apparatus for the maturation of Fet3p and other copper-dependent proteins. **(c)** Cox17p delivers copper to the mitochondrial intermembrane space for incorporation into cytochrome *c* oxidase (CCO). **(d)** hCTR, a human homologue of CTR, mediates copper-ion uptake in human cells. CCS delivers copper to cytoplasmic copper/zinc superoxide dismutase (SOD1).The function of CCS has been inferred from its sequence similarity to yeast proteins with known function and ability to complement *Lys7*$^{-}$ yeast cells. Abbreviations: IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; PM, plasma membrane; PGV, post Golgi vesicle.

thus a copper chaperone[18,19]. Additional types of copper chaperones were identified in eukaryotes, not unexpectedly in the light of the extensive compartmentalization of higher cells. Copper uptake in eukaryotes is accomplished by the copper transporter proteins (CTR)[20], whereas at least some prokaryotic cells rely on CPx-type ATPases for this process[5]. Recent advances have demonstrated that copper-dependent proteins in higher organisms can receive copper either during maturation in the Golgi, as in the case of ceruloplasmin[21], in the cytoplasm, as demonstrated for apo-superoxide dismutase (SOD1)[22] or in organellar locations, as observed for cytochrome *c* oxidase (CCO). Three distinct metallochaperones facilitating specific copper delivery have been described for these pathways in yeast (Fig. 1b,c) and human cells (Fig. 1d).

The yeast and human copper ATPases are distributed between the *trans*-Golgi and the plasma membrane. In the *trans*-Golgi they deliver copper to this compartment for incorporation into cuproenzymes. In both yeast and human cells, metallochaperones route copper to these ATPases. In yeast, Atx1p delivers copper to Ccc2p, the yeast CPx-type copper ATPase, for eventual incorporation into Fet3p (Ref. 23) (Fig. 1b). Fet3p is a plasma-membrane-bound multi-copper oxidase that resembles human ceruloplasmin. Fet3p, as well as ceruloplasmin, have been implicated in cellular iron uptake, revealing an interesting copper–iron connection[24].

The human Atx1p homologue, HAH1, can restore Fet3p function in yeast mutants lacking functional Atx1p (Ref. 22). From this observation it has been inferred that in human cells HAH1 mediates copper delivery to a *trans*-Golgi compartment, where copper is required for copper loading of ceruloplasmin[21]. As the human Menkes and Wilson ATPases can complement yeast *CCC2* knockout mutants, these human ATPases appear to fulfil similar roles to Ccc2p. This is supported by a corresponding localization of the Menkes and Wilson ATPases in the *trans*-Golgi network[25,26].

Superoxide dismutase 1 (SOD1) is a cytoplasmic copper/zinc protein that relies on the redox cycling of bound copper ions to detoxify superoxide radicals[27]. The 249-amino-acid protein Lys7p carries copper to SOD1 *in vivo* and thus functions as a copper chaperone[28]. Yeast strains lacking Lys7p are phenotypically identical to SOD1 mutants[22,29]. Lys7p co-localizes with SOD1 to the cytoplasm[22], consistent with its role as a copper chaperone for this enzyme. Furthermore, incorporation of ^{64}Cu into SOD1 is only observed in yeast cells expressing either Lys7p or CCS, the human homologue of Lys7p (Ref. 22) (Fig. 1d).

Sequence analysis of both Lys7p and CCS (Refs 30,31) reveals the presence of two domains. The first domain has sequence similarity to MerP, CopZ, Atx1p and HAH1, and is most likely involved in copper binding, whereas the second domain has 47% sequence identity with SOD1 (Ref. 32). The role of this second domain could be in the interaction of the chaperone and SOD1 (Ref. 32).

Copper transport to mitochondrial CCO is a cellular priority because of the protein's critical role in respiration. In yeast cells copper delivery to CCO is carried out by Cox17p (Ref. 33) (Fig. 1c). The assembly of CCO occurs on the inner mitochondrial membrane and all of the proteins required for the assembly process, including Cox17p, are found at the assembly site[34]. Cox17p is apparently required to carry copper through the mitochondrial outer membrane to apo-CCO in the mitochondrial intermembrane space. The route via which Cox17p crosses the outer mitochondrial membrane has not been delineated.

Cox17p, despite being of similar size, does not share significant sequence similarity with Atx1p or its homologues, an indication that Cox17p is unlikely to adopt a similar global fold. Indeed, X-ray absorption spectroscopy of Cox17p revealed the presence of two Cu^{1+} ions, trigonally coordinated by thiolate

ligands[35]. This is in contrast to the single copper-ion-binding sites identified in Atx1p (Ref. 2), CopZ (Ref. 12), MNKr2 (Ref. 36) and MNKr4 (Ref. 15). Clearly, widely different copper chaperones have evolved to deliver copper to specific locations. It is to be expected that other copper chaperones delivering copper to other cupro-enzymes or compartments, or both, will be discovered as our understanding of copper homeostasis advances.

A key-lock fit for copper chaperones and their target proteins?

Copper delivery by chaperones appears to be a specific process. For example, copper transfer from CopZ to CopY cannot be accomplished by MNKr2, even though it has a homologous structure and similar copper-binding attributes[7]. Clearly, the possession of a CopZ-like structure is not sufficient to promote Cu^{1+} transfer to CopY. This suggests that a 'key-and-lock' chaperone–target-protein interaction is involved in the copper-transfer process. It is also clear that the CopZ–CopY interaction is transitory[7]. A transitory interaction is consistent with the role of the copper chaperones, namely that of copper delivery and return to an apo-form for the repetition of the copper-delivery cycle.

Both Atx1p and CopZ possess multiple lysine residues that form a positively charged face on the protein surface[12,16]. Site-directed mutagenesis of Atx1p indicated that some of these lysines are required for the interaction with the copper ATPase, Ccc2p, suggesting that the docking involves electrostatic interactions[37]. Indeed, the structurally characterized fourth domain (MNKr4) of the Menkes ATPase contains an acidic face and a number of these acidic residues are conserved in Ccc2p (Ref. 16). More fully defined chaperone docking with target proteins will, however, require structural knowledge of both participating proteins and will have to await further study.

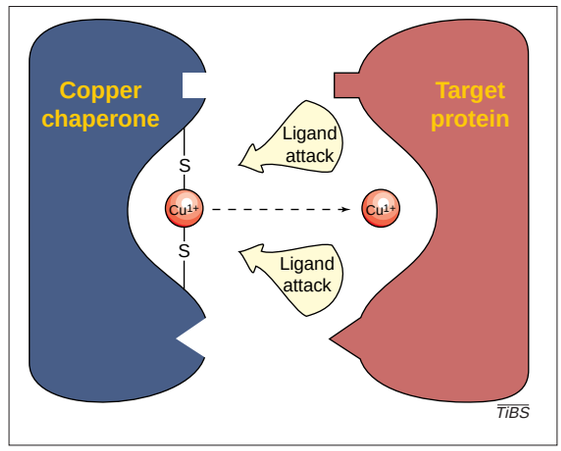

Figure 3

Model mechanism of copper chaperone and target protein interaction. The copper chaperones bind Cu^{1+} ions as a predominantly digonal complex (with a small percentage of trigonal species), with two cysteinyl sulfur ligands from the protein[40]. Ligand exchange or attack can be afforded by sulfur, nitrogen or oxygen atoms from amino acids in or near the metal-binding site of the target protein. In the case of proteins such as CopY and the CPx-ATPases the attacking elements are cysteinyl sulfurs. The attacking ligands are likely to be nitrogen or oxygen ligands in the case of SOD1 and cytochrome *c* oxidase.

A mechanism for copper transfer between the chaperones and their target proteins has not been determined experimentally. However, available data allow the development of a ligand-transfer model for the process. A model was proposed originally by Brown *et al.*[38] for mercury transfer from the MerP chaperone to the MerT transporter, and later by Pufahl *et al.*[2] for copper transfer by Atx1p. The Cu^{1+} complexes formed by the copper chaperones[2] are subject to attack by ligands in the recipient protein (Fig. 3). The attack is essentially a competitive exchange process. The exchange can be afforded by cysteinyl sulfurs in the target protein, in the case of proteins such as the CPx-ATPases, whereas the attacking ligand is likely to be nitrogen or oxygen in the case of SOD1 and cytochrome *c* oxidase. This exchange process is proposed to lead to the formation of a transitory, intermolecular chaperone–Cu^{1+}–receptor complex[2]. It is presumed that in further steps, the other ligands of the chaperone would also be exchanged by ligands of the receptor protein. Ultimately, the metal should be transferred to the more stable site. It is plausible that this exchange might involve a change in the oxidation state of the copper ion in some cases.

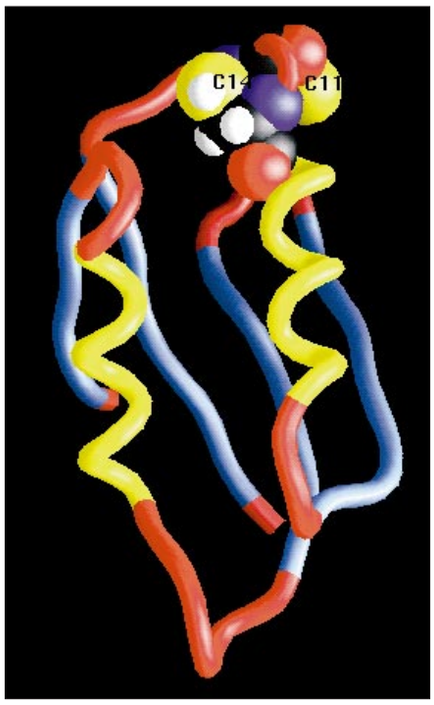

Figure 2

NMR structure of reduced CopZ. Schematic representation of the NMR-derived structure of reduced CopZ, displaying the conserved cysteine residues: α helix (yellow), β sheet (blue), loop or turn (red). The molecular model was prepared using GRASP (Ref. 39).

Conclusions

Copper chaperones fulfil the vital cellular function of copper transport to specific copper proteins. Such a function is necessary not only to protect the cell from the deleterious effects of free copper, but also to ensure that copper can reach its specific target protein. Deletion of a single copper chaperone appears to impair only copper delivery to its specific target without affecting the remaining copper proteins in the cell. This specific interaction seems to involve charged amino acid residues that are found clustered in patches on the surfaces of Atx1p and CopZ. Despite intense study of copper chaperones, there is no information as to where and how chaperones become loaded with copper. Do they scavenge it in the cytoplasm or pick it up at a specific site? Perhaps they interact with the cytoplasmic face of the CTR copper transporters. Considerably more functional and structural work will be required to understand such molecular aspects of copper chaperone function. The three-dimensional structure of the Atx1p- and CopZ-like domains has a broader role in metal transport than just the transfer of copper and has been utilized as a metal-binding 'module' in a wide variety of proteins for the transport of copper, silver, cadmium and mercury, highlighting the utility of this protein fold.

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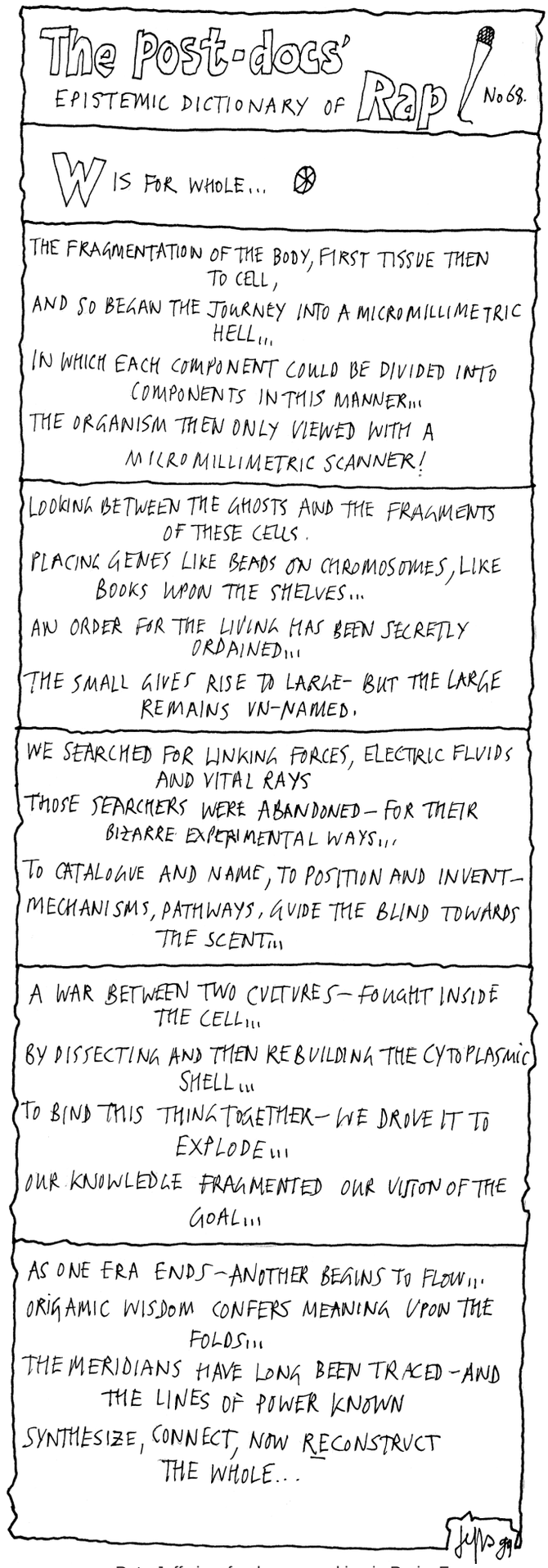

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