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Atx1-like chaperones and their cognate P-type ATPases: copper-binding and transfer

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Abstract Copper is an essential yet toxic metal ion. To satisfy cellular requirements, while, at the same time, minimizing toxicity, complex systems of copper trafficking have evolved in all cell types. The best conserved and most widely distributed of these involve Atx1-like chaperones and P_{1B}-type ATPase transporters. Here, we discuss current understanding of how these chaperones bind Cu(I) and transfer it to the Atx1-like N-terminal domains of their cognate transporter.

Keywords Copper trafficking · Chaperone · P-type ATPase · Copper transfer · Atx1 · CopZ

Introduction

The chemistry of copper

Copper, the 29th element of the periodic table, is abundant in the earth's crust at 55 ppm (Mason and Moore 1982). Its is present mainly as ores such as chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), cuprite (Cu_2O) and malachite [$Cu_2CO_3(OH)_2$)]. The relative ease with which the pure metal can be extracted from its ores led to the early use by man, first in the Far and Middle-East, and later in Europe, of copper for tools and weapons. The 'Copper Age' subsequently gave way to the 'Bronze Age', when techniques for alloying the metal with tin were developed, resulting in a harder and stronger metal (bronze). Although superseded by iron for tools and weapons, copper found applications in many other areas, including early printing plates, and the cladding of ships' hulls, which first exploited its cytotoxic properties in preventing the build up of weed (which could reduce the speed of ships significantly). The discovery of electricity and the fact that copper metal is second only to silver in terms of its conductivity properties led to the large scale use of copper in electrical devices, and this continues to be its most important application today.

In its ground state, copper has a valence electron configuration of $4s^1\ 3d^{10}$, and cation formation occurs by electron loss first from the 4*s* orbital, and then from the 3*d* orbitals. Copper compounds with oxidation states I, II and III are known, although the latter are rather unusual (Cotton et al. 1999). Cu(I) has a valence $3d^{10}$ configuration and therefore is diamagnetic. Being a soft Lewis acid, it has a tendency to bind to soft bases, including thiols, hydrides, alkyl groups, cyanide and phosphines. Simple Cu(I) compounds are most commonly linear, although trigonal and tetrahedral geometries are also common.

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Cu(I) complexes are colourless, unless the ligand is coloured or charge transfer bands arise from the complex formation. Cu(II) has a $3d^9$ configuration, is paramagnetic and, as an intermediate Lewis acid, forms complexes with a wider range of ligands than Cu(I), including sulphate and nitrate, as well as most of the ligands that Cu(I) binds to. It typically forms complexes with coordination numbers four, five and six, in which the Cu(II) adopts a square planar, pyramidal or tetragonally distorted octahedral arrangement. The absence of pure octahedral complexes is due to a Jahn–Teller distortion, which lifts the degeneracy of the octahedral d^9 e_g orbital subset.

Half reactions and standard redox potentials (versus NHE) of the Cu(I)/Cu(0) and Cu(II)/Cu(I) couples in aqueous solution are shown in Eqs. 1 and 2.

$$Cu(I)_{(aq)} + e^- \rightarrow Cu_{(s)} \quad E^0 = +0.52\,V. \tag{1}$$

$$Cu(II)_{(aq)} + e^- \rightarrow Cu(I)_{(aq)} \quad E^0 = +0.153\,V. \tag{2}$$

From these, it is clear that Cu(0) can be readily oxidized to Cu(I) and subsequently to Cu(II) by atmospheric oxygen (the potential for the O_2/H_2O couple is +0.81 V at neutral pH). Cu(I) is also susceptible to disproportionation to give Cu(II) and Cu(0), see Eq. 3.

$$2Cu(I)_{(aq)} \rightarrow Cu(II)_{(aq)} + Cu(s) \quad E^0 = +0.37\,V. \tag{3}$$

As a result of this, it is difficult to obtain stable solutions of Cu(I) ions. This can be achieved, however, by reacting, for example, Cu(I) halides or cyanides with excess of the halide/cyanide anion, leading to the formation of complex ions of Cu(I).

The biology of copper

Copper is a 'modern' bioelement

Copper is an extremely important trace metal in biological systems. The evolution of biological systems that utilize copper can be traced back to the period in which the earth's atmosphere became oxygenated some three billion years ago (Kaim and Rall 1996). Prior to this, Cu(II) would have been extremely scarce, and the insolubility of Cu(I) in aqueous solution at neutral pH would have severely limited the bioavailability of copper on the anaerobic earth. The oxygenation of the atmosphere would have reversed this, and at the same time severely limited the bioavailability of iron, which is highly insoluble in its oxidized Fe(III) state, and which, up to this point, had most likely dominated biological processes requiring redox-based catalysis. That copper is a 'modern' bioelement is consistent with the finding that the vast majority of copper containing proteins and enzymes are involved in the reactions of oxygen or small organic oxygen-containing compounds (Frausto da Silva and Williams 2001).

Copper metalloproteins

Many essential cellular processes require copper-containing proteins/enzymes. Copper centres in proteins generally occur as one of three main types (1, 2 or 3), and while other copper sites do exist, most are based on at least one of these. Type 1 copper centres are found in 'blue' copper proteins (e.g. azurin and plastocyanin) which function in electron transfer. The core of the centre is a copper ion trigonally ligated by two histidine nitrogen atoms and one cysteinyl sulfur (which results in an intense Cys-S to Cu(II) charge transfer band, giving a characteristic blue colour). There are usually more weakly coordinated axial ligands, with at least one typically being methionine. The variation in the identity of these axial ligands, and in the solvent accessibility of the copper binding sites within these proteins, tunes the copper redox potential by stabilizing the Cu(I) state to differing extents. As such, a wide range of potentials, from ~ +180 to +700 mV, is observed amongst blue copper proteins (Botuyan et al. 1996; Hart et al. 1996; Karlsson et al. 1997; Malmstrom and Leckner 1998).

Type 2 copper centres contain a copper ion coordinated by at least three histidine residues and usually has square planar or pyramidal

geometry. Examples of type 2-containing centres that occur in both prokaryotes and eukaryotes are the Cu_B centre of cytochrome *c* oxidase, which catalyses the four electron reduction of oxygen to water (Babcock and Wikstrom 1992; Garcia-Horsman et al. 1994), and the copper site of Cu,Zn superoxide dismutase (SOD) (Fridovich 1989), which is found in a wide range of eukaryotic and some prokaryotic cells. SOD protects the cell from oxidative damage by catalysing the disproportionation of the superoxide anion radical, $O_2^{-}\cdot$, into oxygen and hydrogen peroxide.

Type 3 copper centres contain two Cu(I) ions, each of which are ligated by three histidine residues. An example of a Type 3 copper centre is the dioxygen binding site in hemocyanin, the oxygen carrier in arthropods and molluscs (Volbeda and Hol 1989; Magnus et al. 1994; Cuff et al. 1998). Oxygen binds to the copper centre as a μ–η^2–η^2 bridge (Magnus et al. 1994; Fenton 1995), and in doing so becomes formally reduced to a peroxide ion, while the Cu(I) ions are oxidised to the +2 oxidation state.

An important class of copper containing enzymes, the multi-copper oxidases, contain all three types of copper centre. Ceruloplasmin, the largest source of copper in human plasma, and its yeast homologue Fet3 are examples of this class of protein, and both are involved in iron metabolism. The type 1 site appears to facilitate electron transfer from the Fe(II) substrate (Zaitseva et al. 1996; Machonkin and Solomon 2000) to a trinuclear copper cluster containing type 2 and 3 centres, which is the site of oxygen-binding and reduction (Hellman and Gitlin 2002).

Two important centres which cannot be categorized as type 1, 2 or 3 are the electron transfer Cu_A centres in cytochrome *c* oxidase and nitrous oxide reductase, and the catalytic Cu_Z centre in nitrous oxide reductase. Cu_A centres are highly delocalised dinuclear copper centres, with a formal charge on each copper ion of +1.5. Four ligands directly bind the Cu_A centre: two cysteine residues bridge the two copper ions and each copper ion is coordinated by a terminal histidine (Iwata et al. 1995; Tsukihara et al. 1995; Farrar et al. 1996). The Cu_Z centre in nitrous oxide reductase is a unique tetranuclear copper cluster bound by seven histidine residues and with an inorganic sulphide ligand which bridges all four copper ions (Brown et al. 2000; Rasmussen et al. 2000).

The importance of copper-containing enzymes is illustrated by Menkes' disease, a condition in which dietary copper cannot be distributed around the body from the site of absorption in the intestine, resulting in copper deficiency. This leads to a range of abnormalities including neurological, skeletal and pigmental, and normally death by the age of 3 years (Lutsenko and Petris 2002).

Cytotoxicity of copper

While copper is essential for many cellular functions, copper is also potentially highly toxic. This is largely due to the ability of copper to readily shuttle between +1 and +2 oxidation states. In the presence of hydrogen peroxide, Cu(I) can undergo Fenton-like chemistry, resulting in the production of the highly toxic hydroxyl radical (Halliwell and Gutteridge 1990; Koch et al. 1997). The resulting Cu(II) can be re-reduced by superoxide (or other cellular components, such as low-molecular weight thiol compounds), resulting in a Haber–Weiss-like cycle, see Eqs. 4–6.

$$Cu^{2+} + O_2^{-\cdot} \rightarrow Cu^{+} + O_2 \tag{4}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot} \tag{5}$$

$$O_2^{-\cdot} + H_2O_2 \rightarrow O_2 + OH^{-} + OH^{\cdot}. \tag{6}$$

Diseases in which copper accumulates illustrate the toxicity of the metal when in excess. Wilson's disease is an autosomal recessive genetic disorder in which copper accumulation occurs in the liver and brain (Brewer 2000), and has extremely severe effects due to copper-mediated damage. The condition is fatal unless treated to reduce the levels of 'free' copper. Recent advances in the treatment of Wilson's disease have led to the development of alternatives to the often toxic copper chelator penicillamine, including zinc therapy (which enhances expression of

intestinal metallotheonein which helps to prevent copper absorption) (Brewer 2006), trientine and tetrathiomolybdate (which are copper chelators with less severe side effects than penicillamine) (Murray et al. 2003; Brewer 2006).

Copper toxicity also likely results from the high affinity Cu(I) and Cu(II) exhibit for a range of protein sites (with cysteine, methionine and histidine side chains as potential ligands Koch et al. 1997), resulting in the displacement of native metal ions from active sites, as well as the misfolding of proteins.

Copper homeostatic mechanisms

The essential yet toxic nature of copper means that cells must have efficient homeostasis mechanisms that provide a sufficient supply of copper to copper-requiring enzymes, while at the same time preventing the accumulation of copper in a 'free' toxic form. An effective means of preventing the cytotoxic effects of cellular copper is to maintain it in a bound form that cannot participate in deleterious reactions. It has been known for a long time that, in eukaryotic cells, metallothioneins provide an important line of defense against toxic heavy metals (including copper) (Jensen et al. 1996; Tapia et al. 2004). These small, soluble, cysteine-rich proteins bind Cu(I) within multi-nuclear clusters. In situations in which the cellular concentration of Cu(I) (or other metals such as Cd(II) and Zn(II)) is dangerously high, metallothionein biosynthesis is induced so as to protect the cell from the cytotoxic effects of these metals (Dameron et al. 1991). Some bacterial cells also contain metallothioneins (Blindauer et al. 2002), but may also have alternative systems for dealing with toxic levels of heavy metal ions (Sun et al. 2002; Arnesano et al. 2003; Wernimont et al. 2003).

In addition to systems for rapidly chelating excess, toxic Cu(I), cells require other systems for the safe delivery of copper to copper-dependent enzymes. Over the last decade, several highly sophisticated systems of cellular copper transport have been discovered in both eukaryotic and prokaryotic cells (Labbe and Thiele 1999; Harrison et al. 2000; Rosenzweig 2001; Puig and Thiele 2002). In these copper trafficking systems, copper is transported across the cell membrane, and once inside the cell is bound by chaperone proteins, which transfer the copper specifically to the location in the cell where it is required. In some cases this is a copper requiring protein or enzyme, while in others it is another copper-trafficking protein, which further transports the copper, often across a membrane into a cell compartment, or out of the cell, if the copper concentration is in excess of the optimum level.

Saccharomyces cerevisae Atx1 was the first member to be identified Lin and Culotta (1995)] and the copper-transporting P-type ATPases with which they are associated. Members of these families are ubiquitous and highly conserved across all cell types. We focus on the copper-binding properties of these chaperones and the Atx1-like N-terminal soluble domains of the cognate ATPases, and the transfer of Cu(I) between them.

Atx1-like copper chaperones

Yeast Atx1 and human Hah1 (Atox1)

Saccharomyces cerevisae Atx1 is required for the transport of Cu(I) into the *trans*-Golgi network (TGN), the secretory compartment of the eukaryotic cell, which traffics proteins towards the cell membrane or beyond. Atx1 delivers copper to the Cu(I)-transporting P-type ATPase Ccc2, which transfers the metal ion across the membrane into the TGN. Here, copper is incorporated into the multi-copper oxidase Fet3, which is located in the cell membrane and is required for high-affinity iron uptake into the yeast cell [thus providing details of the link between copper and iron metabolism that has been known for many years Askwith et al. (1994)]. Atx1 deletion mutants show a deficiency in iron, due to the lack of copper incorporation into Fet3 (Lin et al. 1997).

Atx1 is a 72 amino acid residue polypeptide that adopts a $\beta\alpha\beta\beta\alpha\beta$-fold, in which the antiparallel β strands form a β-sheet, on which the two α helices are superimposed (an open-faced β-sandwich), see Fig. 1a (Rosenzweig et al. 1999; Arnesano et al. 2001b). The MXCXXC Cu(I)-binding motif is situated on a flexible solvent-exposed loop at the

beginning of the first α-helix. Details of the metal-binding site were revealed through the crystal structure of a Hg(II)-bound form of the protein (Rosenzweig et al. 1999) and NMR structures of apo- and Cu(I)-bound Atx1 (Arnesano et al. 2001b). These studies revealed that metal induced structural reorganization is limited to, or close to, the metal-binding motif. In particular, loop 1 becomes less solvent exposed and helix α1 extends by one turn upon Cu(I)-binding. The Cu(I) ion is bound in a trigonal environment, by Cys15 and Cys18 (of the motif) and by a third ligand which has not yet been identified (Pufahl et al. 1997; Arnesano et al. 2001b). EXAFS data fitted best to an all-sulphur coordination (Pufahl et al. 1997), suggesting that the third ligand might be a dithiothreitol (DTT) molecule from the Cu(I)-loading buffer. In contrast, Hg(II) is bound in a digonal, near linear environment by the two cysteines of Atx1, a geometry typical for Hg(II) (Rosenzweig et al. 1999). Also of significance is the proximity to the Cu(I) ion of the N_{ξ} atom of the side chain of Lys65 from loop 5. It is suggested that the positively charged lysine side chain might help to stabilize the net negative charge associated with Cu(I) bound by two cysteine thiolates (Arnesano et al. 2001b), and this residue is essential for efficient delivery of Cu(I) to Ccc2 (Portnoy et al. 1999).

The human Atx1 homologue, Hah1 (or Atox1) (Klomp et al. 1997), is a 68 residue polypeptide containing a MXCXXC Cu(I)-binding motif that can functionally substitute for Atx1 in a *atx1*Δ yeast mutant (Hung et al. 1998). It plays an essential role in human cells, delivering Cu(I) to the Cu(I)-transporting P-type ATPases ATP7A (Menkes protein) and ATP7B (Wilson protein) for transfer into the TGN (Hamza et al. 1999; Walker et al. 2002). Structural studies of Cu(I)-Hah1 revealed a $\beta\alpha\beta\beta\alpha\beta$-fold very similar to that of Atx1 (Wernimont et al. 2000; Anastassopoulou et al. 2004). The crystal structure of the Cu(I)-bound protein revealed it to be dimeric (Wernimont et al. 2000) (see Fig. 1b), with the Cu(I) in a distorted tetrahedral environment bound to three cysteine residues, with a fourth just beyond binding distance, but which is likely to be involved in secondary interactions which stabilise the Cu(I) geometry. The dimerization of Hah1 might be an artefact of crystallization. A solution NMR structure (Anastassopoulou et al. 2004) together with EXAFS studies (Ralle et al. 2003) have shown that in solution copper loaded Hah1 is monomeric and the Cu(I) ion adopts a linear geometry, but that it is capable of binding to a third exogenous ligand (Ralle et al. 2003). However, a study of Hah1 by surface plasmon resonance and in vivo FRET indicated the copper-mediated dimerization of the protein with a K_d in the micromolar range (Tanchou et al. 2004). The lysine residue close to the Cu(I)-binding motif of Atx1 is conserved in Hah1 (Lys60) but may not be as important as in Atx1 because it was found not to be essential for the efficient transfer of Cu(I) from Hah1 to Ccc2 in *atx1*-deficient yeast cells (Hung et al. 1998).

Bacterial Atx1-like proteins CopZ

Enteroccocus hirae CopZ

The molecular nature of copper metabolism in prokaryotes was first investigated in detail in the Gram-positive bacterium *Enteroccocus hirae* (see Solioz and Stoyanov 2003, and references therein). Copper metabolism in this organism is controlled by the products of the *cop* operon, which contains four genes: *copA* and *copB*, encoding two P-type ATPases; copZ, encoding an Atx1-like chaperone; and *copY*, encoding an unusual transcriptional regulator of the *cop* operon. Under conditions of normal copper concentration, CopY exists as a homodimer which binds to two distinct 28 basepair sequences in the promoter region of the *cop* operon, and inhibits transcription of the genes (Strausak and Solioz 1997). The DNA-binding conformation of CopY is stabilized by a Zn(II) ion bound by four cysteine residues in a tetrahedral environment (Cobine et al. 2002). In elevated copper, the *cop* operon is induced through binding of Cu(I) to CopY, resulting in the displacement of the Zn(II) ion, and thereby the conversion of CopY from a DNA-binding to a non-binding form (Cobine et al. 2002).

The 68 amino acid residue protein CopZ is required for the delivery of Cu(I) to CopY (Cobine et al. 1999). This is a quite different

interaction from that described for eukaryotic Atx1-like chaperones, which deliver Cu(I) to specific P-type ATPases. However, *E. hirae* CopZ has also been shown to interact with the P-type ATPase CopA. CopA is proposed to be involved in Cu(I)-uptake into the cell, and it is thought that the CopA–CopZ interaction results in Cu(I)-loading of the chaperone (Multhaup et al. 2001). Unusually, CopZ appears to be degraded at high levels of intracellular copper through a specific proteolitic pathway (Lu and Solioz 2001), leading to the proposal that high levels of CopZ may be toxic to the cell, although the mechanism of toxicity is not clear. *E. hirae* CopZ was the first bacterial copper chaperone to be structurally characterized (Wimmer et al. 1999), and it, too, adopts the ferredoxin-like $\beta\alpha\beta\beta\alpha\beta$-fold typical of this class of protein. The structure of the Cu(I)-bound form could not be solved due to signal broadening resulting from the likely dimerization of the protein.

Although studies of *E. hirae* copper metabolism have revealed many general principles of copper metabolism, it appears to be quite unusual: for example, homologues of CopY are found only in close relatives of *E. hirae*. More widespread are systems, which are more closely related to those of eukaryotes, in which a copper chaperone and a P-type ATPase are the key interacting partners. The Gram-positive bacterium *Bacillus subtilis* provides an example of this that has been very extensively characterized.

Bacillus subtilis CopZ

The *cop* operon of *B. subtilis* consists of only two genes, *copZ* and *copA*, encoding an Atx1-like copper chaperone and a Cu(I)-transporting P-type ATPase, respectively, under the control of a MerR-like regulator (CueR) (Gaballa et al. 2003). Inactivation of CopA led to enhanced sensitivity to environmental copper, indicating its major involvement in Cu(I)-export from the cell (Radford et al. 2003). Inactivation of CopZ also resulted in an increase in copper sensitivity, but also resulted in a significant decrease of cellular copper, implying that CopZ may acts as a cytoplasmic store of the metal ion under normal conditions (Radford et al. 2003). The structure of the 69 amino acid residue *B. subtilis* CopZ protein revealed the typical $\beta\alpha\beta\beta\alpha\beta$-fold, in which the predicted Cu(I)-binding loop showed significant disorder (in contrast to the structure of CopZ from *E. hirae*) (Banci et al. 2001b). Following the addition of Cu(I), the loop was found to be well ordered, and the structure confirmed the importance of the MXCXXC motif for Cu(I)-binding,see Fig. 1c. The structure of the Cu(I)-binding site is similar to that of Atx1, but lacks the lysine residue found in eukaryotic Atx1 family members. In its place is a tyrosine residue, which is not found so closely associated with the Cu(I)-site. In addition to the two coordinating cysteine residues, a third sulphur ligand was also present, likely from a molecule of DTT, which was used to maintain the apo-protein in the reduced state (Banci et al. 2001b, 2003d). Spectroscopic and bioanalytical studies of CopZ in the absence of DTT showed that *B. subtilis* CopZ dimerizes upon addition of Cu(I) (Kihlken et al. 2002) to form, initially a $(CopZ)_2Cu$ complex. On addition of further Cu(I), CopZ remains as a dimer, generating the complex $(CopZ)_2(Cu)_2$, with a likely dinuclear Cu(I) centre at the subunit interface. The dimeric form of CopZ can accommodate further Cu(I) ions, although the nature of such species is not yet clear (Kihlken et al. 2002).

Dimerization of CopZ was inhibited in the presence of a large (20-fold) excess of DTT, although data from analytical gel filtration indicated that, at 0.5 Cu(I)/CopZ, a major component of CopZ dimer remained (Kihlken et al. 2002). This is demonstrated more clearly in Fig. 2, which shows a plot of CD intensity at 265 nm as a function of Cu(I) added to CopZ. In the absence of DTT, intensity reaches a maximum at 0.5 Cu(I)/CopZ, indicating the formation of the $(CopZ)_2Cu$ complex, and subsequently decreases such that at a level of 1 Cu(I)/CopZ, it is significantly negative. In the presence of increasing concentrations of DTT, the initial intensity increase, reaching a maximum at 0.5 Cu(I)/CopZ, and indicating the formation of the $(CopZ)_2Cu$ complex, is not significantly affected by the presence of DTT. Only at a 20-fold excess does the initial part of the plot begin to move away from that measured in the absence of DTT. The

subsequent phase, however, is much more significantly affected by DTT, even at equimolar levels. At a 20-fold excess, intensity does not decrease at all, suggesting that, at a level of 1 Cu(I)/CopZ, excess DTT entirely abolished the formation of the $(CopZ)_2(Cu)_2$ complex. This is consistent with NMR structural studies and analytical gel filtrations studies that showed that, under these conditions, the protein is monomeric (Banci et al. 2001b; Kihlken et al. 2002).

Other low-molecular weight thiol compounds have recently been tested for their capacity to interfere with Cu(I)-binding to CopZ. Figure 3a, b show plots of CD intensity at 265 nm, and fluorescence intensity at 306 nm (due to the tyrosine residue of CopZ), respectively, as a function of Cu(I)/CopZ in the absence of exogenous thiols, and in the presence of a 20-fold excess of DTT, glutathione (GSH) and cysteine. Cu(I)-binding to CopZ causes a quench of the fluorescence, consistent with the close association of the Cu(I)-binding motif and Tyr65. In both the CD and fluorescence intensity plots data acquired in the absence and presence of thiols essentially overlay up to 0.5 Cu(I)/CopZ. Above this level, the data are quite distinct, indicating that thiols interfere in the subsequent binding of Cu(I) to CopZ in the order DTT > GSH ~cysteine.

Cyanobacterial ScAtx1

The cyanobacteria *Synechococcus* and *Synechocystis* transports Cu(I) into the cell cytoplasm via the P-type ATPase CtaA, and then into the thylakoid via a second P-type ATPase, PacS, for incorporation into plastocyanin (Phung et al. 1994; Tottey et al. 2001). An Atx1-like Cu(I) chaperone, ScAtx1, mediates the transfer between ATPase transporters. This 64 residue protein predictably adopts the typical $\beta\alpha\beta\beta\alpha\beta$-fold, but in the Cu(I)-bound form exhibits unusual coordination to the Cu(I) ion: in addition to cysteine ligation, the N2 atom of the imidazole ring of a histidine residue is also a ligand (Tottey et al. 2002; Banci et al. 2004a). Interestingly, the ligating histidine (His61) occurs at the same position as the conserved lysine residue of eukaryotic Atx1 proteins (Banci et al. 2004a).

The association state of ScAtx1 in solution is not yet clear. NMR correlation time data indicated the presence of a dimer, and a solution structure involving a symmetric dimer in which His61 of one subunit 'invades' the Cu(I)-binding site of the other subunit, was proposed. However, despite the correlation time data, the structural data were also consistent with a monomeric form of the protein featuring Cu(I) ligands Cys12, Cys15 and His61 from the same protein (Banci et al. 2004a).

Recently, it has been shown that substitution of His61 enhances the in vivo interaction between ScAtx1 and PacS but does not effect the interaction of Atx1 with CtaA (Borrelly et al. 2004). Therefore, it is proposed that His61 may be involved in controlling the dual actions of Atx1, ie switching it between removing copper from CtaA and donating copper to PacS.

Copper-transporting P-type ATPases

Atx1-like copper chaperones are associated with a copper-transporting P-type ATPase to which they usually deliver Cu(I) for export across a membrane (there are examples that have been mentioned above in which Cu(I) is received from the transporter after import across a membrane). P-type ATPases couple the hydrolysis of ATP to the transport of a substrate. The name is derived from the phosphorylation of a conserved aspartate residue in the conserved motif DKTGT during the reaction cycle (Pedersen and Carafoli 1987). This phosphorylation domain together with the 'actuator' and 'nucleotide-binding' domains are common elements of P-type ATPases. Copper-transporting P-type ATPases belong to the P_{1B}-subgroup of heavy metal-transporters, members of which have eight predicted transmembrane segments and are involved in the transport of Zn(II), Cd(II) and Pb(II), as well as Cu(I) and Ag(I) (Axelsen and Palmgren 1998). What marks out the heavy metal transporters as being distinct from other P-type ATPases is the presence of a CPX motif, located in a membrane spanning region, that is believed to be important for determining the metal ion substrate specificity

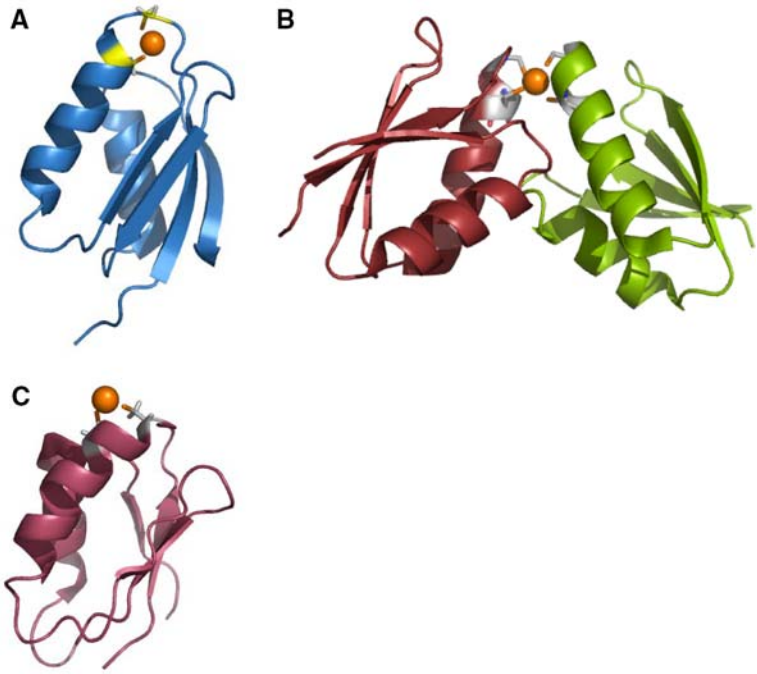

Fig. 1 Structures of Cu(I) bound Atx1-like copper chaperones. Structures of a, *S. cerevisae* Atx1 (pdb code: 1FD8); b, Human Hah1 (1FEE); and, c, *B. subtilis* CopZ (1P86) showing the typical $\beta\alpha\beta\beta\alpha\beta$-fold. Cu(I)-ligating cysteine residues are indicated in 'sticks' representation. All structure figures were rendered with Pymol (DeLano 2002) (www.pymol.org)

(Argüello 2003), and a variable number of usually N-terminal (but sometimes also C-terminal) metal binding domains. In Cu(I)-transporters, these often have significant primary sequence similarity to Atx1-like chaperones and number between 1 and 6: In *E. coli* CopA [involved in Cu(I)-export] there is one; in yeast Ccc2 [which acts with Atx1 to transport Cu(I) into the TGN for incorporation into Fet3] there are two; while the human Menkes and Wilson proteins each have six such domains. The function of these domains is not yet clear but they do not appear to be required for the transfer of copper across the membrane: in *E. coli*, the single N-terminal domain is not required for Cu(I) transport (Fan et al. 2001; Fan and Rosen 2002); in Menkes and Wilson proteins, the first four N-terminal domains appear to be important for the transfer of Cu(I) from Hah1 (see below), while the last two are key to the correct cellular localization of the proteins (Strausak et al. 1999; Voskoboinik et al. 1999; Cater et al. 2004). Under normal conditions both Menkes and Wilson proteins are found in the TGN where they deliver Cu(I) for insertion into copper-requiring proteins/enzymes, but under conditions of excess copper, they migrate to the plasma membrane, where they function in Cu(I) export. At least one N-terminal domain is required for correct copper trafficking.

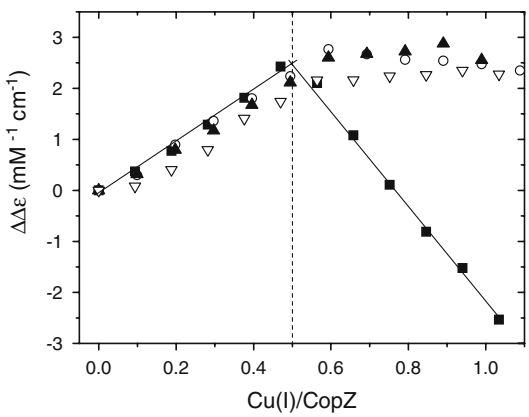

Fig. 2 Circular dichroism analysis of the effect of DTT on Cu(I)-binding to *B. subtilis* CopZ. Plot of change in CD intensity [$\Delta\Delta\varepsilon$ at 265 nm as a function of Cu(I) ions per CopZ, following the addition of 0–1 Cu(I)/CopZ (added as 0.09 molar equivalents) to CopZ (40 μM) in 100 mM MOPS buffer, pH 7.5, in the presence of no (*filled squares*), a 1:1 ratio (*open circles*), a 5:1 ratio (*filled triangles*) and a 20:1 ratio (*open triangles*) DTT. Pathlength 1 cm, temperature 25°C. Other experimental details as previously reported (Kihlken et al. 2002)

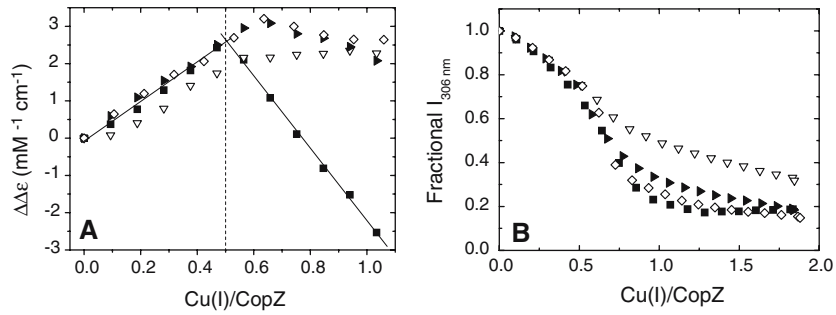

Fig. 3 Circular dichroism and fluorescence analysis of the effect of low-molecular weight thiols on Cu(I)-binding to *B. subtilis* CopZ. a Plot of change in CD intensity ($\Delta\Delta\varepsilon$) at 265 nm as a function of Cu(I) ions per CopZ, following the addition of 0–1 Cu(I)/CopZ (added as 0.09 molar equivalents) to CopZ (40 μM) in 100 mM MOPS buffer, pH 7.5, in the absence of low-molecular weight thiols (*filled squares*), and the presence of a 20:1 ratio of DTT (*open triangles*), glutathione (*filled triangles*), and cysteine (*open diamonds*). Pathlength 1 cm, temperature 25°C. Other experimental details as previously reported (Kihlken et al. 2002). b Plot of change in fractional fluorescence intensity at 306 nm as a function of Cu(I) ions per CopZ, following the addition of 0–1 Cu(I)/CopZ (added as 0.09 molar equivalents) to CopZ (25 μM) in 100 mM MOPS buffer, pH 7.5, in the absence of low-molecular weight thiols (*filled squares*), and the presence of a 20:1 ratio of DTT (*open triangles*), glutathione (*filled triangles*), and cysteine (*open diamonds*). Pathlength 1 cm, temperature 25°C. Fluorescence emission spectra were recorded using a Perkin-Elmer LS55 spectrophotometer at 25°C with excitation at 276 nm and excitation and emission slit widths of 8 nm

The first high-resolution structure of a P-type ATPase, that of a Ca(II)-tranporter, was published in 2000 (Toyoshima et al. 2000) and though the structure of a heavy metal transporter has not yet been solved, individual domains have been characterised, including N-terminal Atx1-like domains, the actuator domain (Sazinsky et al. 2006a), and the nucleotide binding domain (Dmitriev et al. 2006; Sazinsky et al. 2006b). Here, we focus on the Cu(I)-binding and structural properties of the Atx1-like domains.

The first structural characterization of an N-terminal metal binding domain was reported in 1998 for the fourth domain of the Menkes protein in the apo- and a Ag(I)-bound form (Gitschier et al. 1998). This first revealed the $\beta\alpha\beta\beta\alpha\beta$-fold that is common to these and the Atx1-like copper chaperones, and showed the metal ion to be bound in a digonal manner. Since then, domain 1 (DeSilva et al. 2005), 2 (Jones et al. 2003; Banci et al. 2004b) and 5 (Banci et al. 2005) of the protein, as well as domain 1 of yeast Ccc2 (Banci et al. 2001a) have also been structurally characterized. As expected, each of them exhibits the typical ferredoxin-like fold, with little structural change upon binding Cu(I) other than an ordering of the Cu(I)-binding loop. The coordination geometry at the metal ion is typically some way off diagonal (i.e. the bond angle is <180°), implying the interaction of a third ligand that could be derived from the protein or from the buffer.

Thus far, no structure is available for the entire six-domain section of either Menkes or Wilson proteins. A protein consisting of domains 5 and 6 of the Wilson protein was recently reported (Achila et al. 2006), which revealed two folded domains that are packed together with the nine residue linker sandwiched between them, see Fig. 4a. Each domain is capable of binding Cu(I), and at a level of one per domain, structural changes are confined to the metal-binding motif regions. This structure begins the process of defining the interaction between domains. Because the electrostatic charge distribution properties of the individual domains are variable, it is likely that they interact with one another in a specific and functionally significant way (Arnesano et al. 2002). CopA from *B. subtilis*, which interacts with CopZ in a Cu(I)-export pathway, is one of many bacterial P_{1B}-type ATPases that contains two N-terminal domains. The first domain was found to be unstable to unfolding, which precluded structural studies of the two domain protein (Banci et al. 2002). Substitution of a single residue, Ser46, for a valine yielded a stable protein, leading to a solution structure, see Fig. 4b

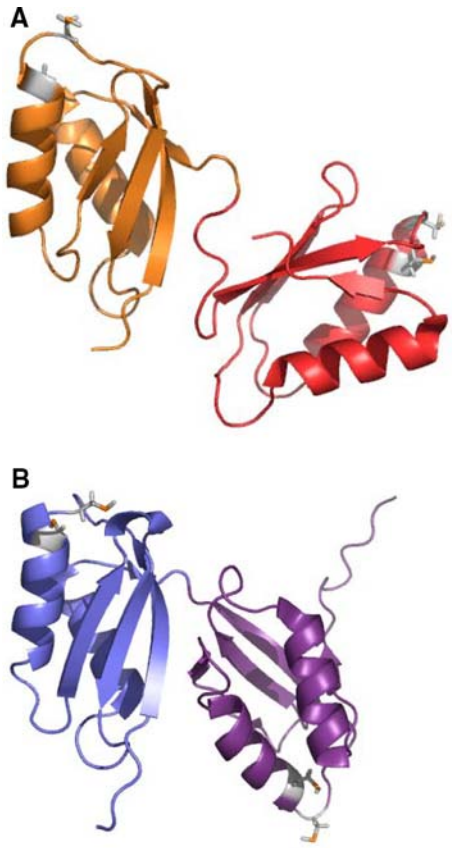

Fig. 4 Structures of P_{1B}-type ATPase N-terminal domains: domains 5 and 6 of Wilson protein and CopAab from *B. subtilis*. a Structures of domains 5 and 6 of the human Wilson protein (2EW9) and b *B. subtilis* S46V CopAab (1P6T). Cu(I)-binding site cysteine residues are indicated in 'sticks' representation

(Banci et al. 2003b, c). Each domain adopts a typical ferrodoxin-like fold and, because the interdomain linker is so short, the two domains are oppositely oriented such that their Cu(I)-binding motifs are located at opposite ends of the molecule. Recently, a wild-type version of the two N-terminal domains of *B. subtilis* CopA that is stable to unfolding has been generated and is currently being characterized (C. Singleton and N. E. Le Brun, unpublished data).

Cu(I)-binding studies with fusion proteins (with maltose binding protein) of the N-teminal domains of both Menkes and Wilson proteins demonstrated Cu(I)-binding in vivo and in vitro with a stoichiometry of one copper ion per metal binding domain (Lutsenko et al. 1997). However, a non-tagged version of the N-terminal domains of the Menkes protein was found only to bind four copper ions in vitro, indicating that only four out of the six predicted copper binding domains bind copper (Cobine et al. 2000). In the latter case, the Cu_4-species was found to be strongly luminescent, indicative of a solvent excluded multi-nuclear Cu(I) cluster. It is likely that Cu(I)-binding to such multi-domain proteins is highly complex and small changes in solution conditions may have a significant effect on the thermodynamics of binding, leading to quite different observations.

Copper transfer between copper chaperones and their cognate transporters

It is well established that Atx1-like copper chaperones interact directly with the N-terminal domains of specific P_{1B}-type ATPases to effect Cu(I) transfer between the two proteins (Lin et al. 1997; Pufahl et al. 1997; Larin et al. 1999; Portnoy et al. 1999; Huffman and O'Halloran 2000; Tottey et al. 2002; Walker et al. 2002; Radford et al. 2003). The Cu(I)-dependent interaction between Atx1 and the first N-terminal domain of Ccc2 was demonstrated directly by NMR chemical shift mapping and modelling experiments (Arnesano et al. 2001a, 2004), which revealed the formation of a complex (albeit a relatively weak one) between the proteins. Recently, the Cu(I)-mediated complex has been structurally characterised (Banci et al. 2006a), showing that the basic surface of Atx1 contacts an acidic surface of the N-terminal domain, thereby bringing the copper binding motifs of the two proteins in close proximity for copper exchange, see Fig. 5a. The coordination of the Cu(I) at an inter-protein site provides significant evidence in favour of the generally accepted mechanism of Cu(I) transfer between interacting partner proteins, shown in Fig. 5b, in which an intermediate complex featuring a three coordinate Cu(I), with thiolate ligands from both proteins is a central feature (Pufahl et al. 1997; Wernimont et al. 2000). Measurements of the relative affinities of the two proteins (expressed as an exchange

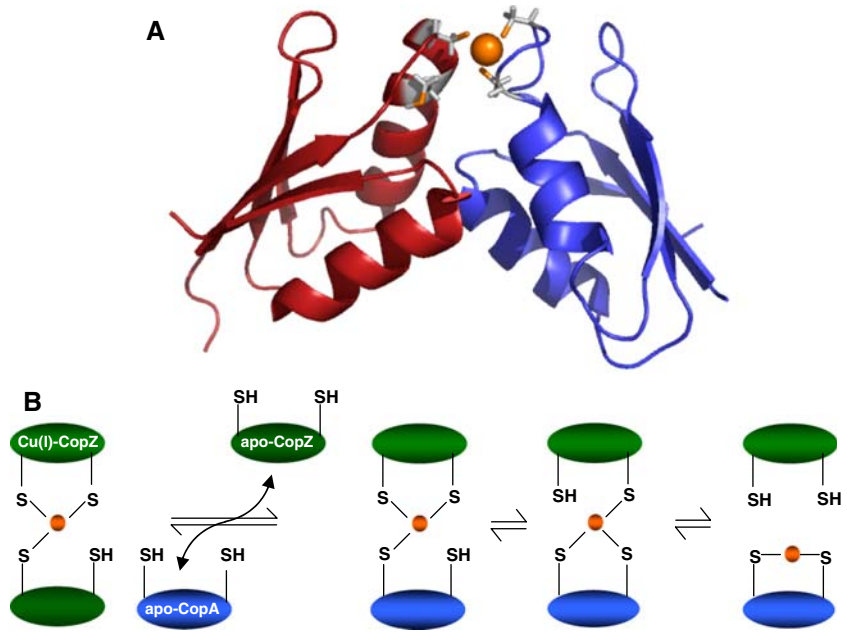

Fig. 5 Cu(I)-exchange between chaperone and transporter. a Structure of the Cu(I)-mediated complex between Atx1 and the first N-terminal domain of Ccc2 (2GGP) showing coordination of the Cu(I) by both Atx1 and Ccc2. b Proposed mechanism of Cu(I) transfer from a bacterial Atx1-like chaperone (*CopZ*) and its target transporter (*CopA*), based on that previously proposed for Atx1-Ccc2 (Pufahl et al. 1997; Wernimont et al. 2000)

equilibrium constant) indicated a rather shallow thermodynamic gradient for exchange (Huffman and O'Halloran 2000), leading to the proposal that the protein interaction serves to enhance the rate of Cu(I) transfer; that is, the process is under kinetic rather than thermodynamic control. Studies of *B. subtilis* CopZ and its interaction with the second N-terminal domain of CopA also revealed a weak complex formation, in this case mediated by the interaction of the acidic surface of CopZ with a basic patch on the CopA domain (Banci et al. 2003a). Cu(I)-mediated complex formation has also been detected between ScAtx1 and the N-terminal domain of PacS (Banci et al. 2006b).

The interaction of Hah1 with the six N-terminal domains of the Menkes and Wilson proteins has also been studied in some detail, and the roles of the individual domains are now becoming clearer. While the two domains closest to the first transmembrane segment of the protein (domains 5 and 6) are important for its Cu(I)-transport function and for its correct cellular localization, they do not interact with Hah1 (Achila et al. 2006); the first four domains are key for the interaction of the protein with Hah1, and therefore the transfer of Cu(I) (Larin et al. 1999; Van Dongen et al. 2004). There is evidence that domain 2 of the Wilson protein is the major site of interaction with Hah1 (Walker et al. 2004), although domain 4 is also able to form a complex with the chaperone (Achila et al. 2006), and to transfer the Cu(I) derived from Hah1 to domains 5 and 6. This work provides evidence for the vectorial transfer of Cu(I) along the N-terminal domains of the Wilson protein.

Concluding remarks

The necessity of maintaining cellular copper within a non-reactive environment while it is being trafficking around the cell or the body, from the point of entry to the point of utilization or exit, has led to the evolution of complex and elegant mechanisms that are remarkably well conserved from bacteria to humans. Atx1-like copper chaperones and their cognate P_{1B}-type ATPase transporters constitute the best conserved and most widespread of such systems. Cu(I) is bound extremely tightly to a solvent-exposed binding motif of the chaperone which facilitates transfer of the metal ion via a handover mechanism that avoids the release of the metal into solution. The redox potential of copper

bound to the chaperone (and subsequently) to the N-terminal domains of the transporter is likely to be extremely high, thus preventing redox chemistry from occurring at the bound Cu(I) ion.

The coordination environments of Cu(I) bound to chaperones appears to be somewhat different for eukaryotic and bacterial chaperones. The majority of evidence indicates that eukaryotic proteins are monomeric, but have a propensity to move from diagonal to trigonal coordination, an essential requirement for the transfer to the transporter. The bacterial proteins appear to have a greater tendency towards a trigonal coordination geometry that may be provided by dimerization of the protein, or by the coordination of an exogenous low-molecular weight thiol (such molecules are present in the cytoplasm). At low-copper loadings, *B. subtilis* CopZ dimers remain even in the presence of excess thiols. Thus, the transfer mechanism outlined above, which probably represents the common denominator of all transfer events, requires an additional step involving the exchange of the third Cu(I) ligand (supplied by a second chaperone molecule or by a small thiol molecule) for a cysteine thiolate of the target transporter N-terminal domain, see Fig. 5b (Banci et al. 2004a, 2006a, b).

The transfer reaction does not appear to be driven by a large thermodynamic gradient, and, indeed, under some circumstances some reversibility in the direction of transfer may be an important regulatory element. The Cu(I)-mediated chaperone-transporter interaction essentially catalyses the transfer reaction that would, in the absence of direct interaction, occur only slowly. This gated flow of Cu(I) seems to be the essence of how cells avoid toxic Cu(I) in solution.

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