Unresolved questions in human copper pump mechanisms

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Abstract. Copper (Cu) is an essential transition metal providing activity to key enzymes in the human body. To regulate the levels and avoid toxicity, cells have developed elaborate systems for loading these enzymes with Cu. Most Cu-dependent enzymes obtain the metal from the membrane-bound Cu pumps ATP7A/B in the Golgi network. ATP7A/B receives Cu from the cytoplasmic Cu chaperone Atox1 that acts as the cytoplasmic shuttle between the cell membrane Cu importer, Ctrl1 and ATP7A/B. Biological, genetic and structural efforts have provided a tremendous amount of information for how the proteins in this pathway work. Nonetheless, basic mechanistic-biophysical questions (such as how and where ATP7A/B receives Cu, how ATP7A/B conformational changes and domain-domain interactions facilitate Cu movement through the membrane, and, finally, how target polypeptides are loaded with Cu in the Golgi) remain elusive. In this perspective, unresolved inquiries regarding ATP7A/B mechanism will be highlighted. The answers are important from a fundamental view, since mechanistic aspects may be common to other metal transport systems, and for medical purposes, since many diseases appear related to Cu transport dysregulation.

Key words: copper transport, copper-chaperone, Wilson disease protein, Menke’s disease protein, ceruloplasmin, biophysical methods.

Copper (Cu) pumps of the human secretory pathway

Cu is found in the active sites of essential proteins that participate in cellular reactions such as respiration, antioxidant defense, neurotransmitter biosynthesis, connective-tissue biosynthesis and pigment formation (Harris, 2003; Huffman & O’Halloran, 2001; Puig & Thiele, 2002). The ability of Cu to oxidize/reduce (switch between Cu+ and Cu2+) allows Cu-containing proteins to play important roles as electron carriers and redox catalysts in living systems. To avoid Cu toxicity, the intracellular concentration of Cu is regulated via dedicated proteins that facilitate uptake, efflux as well as distribution to target Cu-dependent proteins and enzymes (Festa & Thiele, 2011; O’Halloran & Culotta, 2000; Robinson & Winge, 2010). In the human cytoplasm, after Cu has entered the cell via the Cu importer Ctrl1 (Ohrvik & Thiele, 2014), there are at least three independent Cu transport pathways. In the general pathway, conserved in most organisms, the 68-residue Cu chaperone Atox1 transports the metal to cytoplasmic metal-binding domains in ATP7A and ATP7B (also called Menke’s and Wilson disease proteins, respectively), two homologous multi-domain F13-type ATPases located in the trans-Golgi network (Fig. 1). Most Cu-dependent enzymes acquire Cu from ATP7A/B in the Golgi before reaching their final destination (e.g. blood clotting factors, tyrosinase, lysyl oxidase and ceruloplasmin) (Festa & Thiele, 2011; Koch et al. 1997; O’Halloran & Culotta, 2000; Robinson & Winge, 2010). Once transferred to ATP7A/B, the Cu ion is channeled to the lumen of the Golgi and loaded onto target Cu-dependent proteins. We recently reported that at least in vitro, in addition to ATP7A/B interactions, Atox1 can cross-react and exchange Cu with another cytoplasmic Cu chaperone, the Cu chaperone for superoxide dismutase, CCS, which delivers Cu specifically to SOD1 (Petzoldt et al. 2015). Thus, cross talk between cytoplasmic chaperones may be an unexplored mechanism that allows for efficient usage of cytoplasmic Cu ions.

ATP7A/B are elaborate multi-domain Cu pumps with eight membrane-spanning helices, an actuator (A) domain, as well as nucleotide-(N) and phosphorylation-(P) domains,
Metals, such as Zn, strongly individual ATP7B metal-binding domains can bind other soft metals are favored by sulfur ligands, as both Atox1 and the membrane channel.

Then is shuttled within the protein to Cu-binding sites in metal-binding domains of ATP7A/B and the metal does not confer intrinsic specificity to Cu ions, although soft metals are favored by sulfur ligands, as both Atox1 and individual ATP7B metal-binding domains can bind other metals, such as Zn, strongly in vitro (Niemiec et al. 2014). At normal cell conditions, however, metal-binding degeneracy is not a problem since metal transport is strictly governed by protein–protein interactions (Tottey et al. 2005, 2008).

**Moving Cu from chaperone to ATP7A/B**

It was originally assumed that Atox1 delivers Cu to one of the metal-binding domains of ATP7A/B and the metal then is shuttled within the protein to Cu-binding sites in the membrane channel. In vitro (Achila et al. 2006; Banci, 2006; Banci et al. 2008, 2009a, b; Pufahl et al. 1997; Wernimont et al. 2000) and in silico (Rodriguez-Granillo et al. 2010) work has shown that Cu transfer from Atox1 to metal-binding domains of ATP7A/B proceeds via Cu-bridged hetero-dimer complexes where the metal is shared between the two metal-binding sites (Fig. 2). Cu is thought to move from one protein to the other via ligand-exchange reactions involving tri-coordinated Cu–sulfur intermediates (Pufahl et al. 1997). All six domains of ATP7A/B can be loaded with Cu by Atox1 but only in some cases, have Cu-dependent protein–protein complexes been detected by NMR via slower tumbling times (Achila et al. 2006; Banci et al. 2005, 2008, 2009a, b). Based on affinity and NMR studies, Cu binding to an ATP7B metal-binding domain is favored over binding to Atox1 by a factor of 3–5 providing a shallow directional thermodynamic driving force. We found that upon mixing of Cu–Atox1 and the fourth metal-binding domain of ATP7B (WD4), a stable ternary complex assembled that was in equilibrium with substrates and products (Niemiec et al. 2012). In contrast, when mixing a two-domain construct of domains 5 and 6 in ATP7B (WD56) and Cu–Atox1, the protein–protein interaction was transient such that it did not survive size exclusion chromatography (SEC) but Cu transfer still took place (Nilsson et al. 2013).

For the Atox1 and WD4 pair, SEC in combination with titration calorimetry made possible thermodynamic analysis of the reaction in Fig. 2 and we identified that Atox1–Cu–WD4 hetero-protein complex formation is driven by favorable enthalpy and entropy changes, whereas the overall reaction, from Atox1 to WD4, relies on only enthalpy (Niemiec et al. 2012). In additional studies, involving protein engineering, we revealed that the first cysteine in each protein’s Cu binding motif was essential for hetero-protein complex formation but one of the second cysteines was not required. Thermodynamic analysis disclosed that the wild-type Cu site in the hetero-protein complex was dynamic (in agreement with positive entropy change, see above), involving entropy–enthalpy compensation (Niemiec et al. 2015). It remains unknown if the same mechanism and thermodynamic principles apply to the ATP7A/B hetero-protein complex formation.
apply to Atox1 interactions with the other five domains in ATP7B and when the target domain is surrounded by its natural domains within the full-length protein. Information on these (apparent) straightforward issues has been hampered by the difficulty to prepare large membrane proteins for biophysical studies.

The finding that the Cu chaperone in bacteria (that is homologous to Atox1) could bypass the single metal-binding domain of the bacterial P1b-type ATPase and instead deliver Cu directly to the membrane entry site (Gonzalez-Guerrero & Arguello, 2008) reinforced the idea that the human metal-binding domains played regulatory roles. Nonetheless, yeast complementation studies have shown that the presence of the human and yeast metal-binding domains, at least some domains in the case of the human protein, is essential for Cu transfer activity (Forbes et al. 1999; Morin et al. 2009). In 2011, the crystal structure of the bacterial ATP7A/B homolog Legionella pneumophila CopA was reported (Gourdon et al. 2011). Although the CopA structure was a breakthrough, there was no electron density resolved for its metal-binding domain (Gourdon et al. 2011). The CopA structure revealed a putative docking site for a chaperone, or an internal metal-binding domain, at the membrane entry site for Cu in the form of a kinked helix. Subsequent modeling studies have shown that the presence of the human and yeast metal-binding domains, at least some domains in the case of the human protein, is essential for Cu transfer activity (Forbes et al. 1999; Morin et al. 2009).

During the catalytic cycle, that requires ATP hydrolysis and ultimately results in Cu transfer to the lumen side of the membrane, ATP7A/B are likely to undergo significant conformational changes driven by domain–domain interactions (Lutsenko et al. 2007). Available predictions for how ATP7A/B works catalytically come from analogy with the calcium pump SERCA, for which structures of different enzymatic stages have been resolved (Bublitz et al. 2013). Since there are no structures of the arrangement of the six metal-binding domains within full-length ATP7A/B, it is unclear how these domains participate in the catalytic cycle. Interactions among the metal-binding domains of ATP7A/B are proposed to transmit signals long-range (Gourdon et al. 2012). In support, we found that even in a small construct of only domains 5 and 6 of ATP7B (WD56), minute variations in salt and pH conditions perturbed domain–domain relative fluctuations such that the efficiency of Atox1-mediated Cu delivery to these domains was modulated (Nilsson et al. 2013). This implies that local (temporal and spatial) fluctuations in the cellular environment may tune overall Cu pump activity via changes in domain–domain interactions.

For ATP7B, an interaction between the ATP-binding domain (N-domain) and a construct of the six metal-binding

Fig. 2. Top: Scheme of Cu transfer mechanism from Atox1 to the 4th metal-binding domain of ATP7B (WD4) indicating an intermediate hetero-protein complex in which the Cu ion is coordinated by cysteines in both proteins’ metal-binding loops. Bottom: Structural model of the Cu-dependent Atox1–WD4 hetero-protein complex.
domains of ATP7B was reported based on pull-down data (Tsivkovskii et al. 2001); this interaction was found to depend on the metal-loading status as well as on phosphorylation events and it was suggested to be an auto-inhibitory interaction (Hasan et al. 2012). A similar intra-protein interaction was reported for domains of a homologous bacterial Cu pump, also using pull down experiments with tagged proteins (Gonzalez-Guerrero et al. 2009). Since the bacterial homolog has only one metal-binding domain, one may speculate that the 6th metal-binding domain in ATP7B plays a key role in the interaction within the human protein, perhaps with the additional five metal-binding domains and the unstructured loop, unique to the human N-domain, fine-tuning the binding. However, our biophysical studies using 15N-labeled purified domains (N-domain mixed with four-domain construct, WDI-4 or with WD56) did not reveal any interaction for any protein pair (Åden and Wittung-Stafshede, unpublished), although we used high μM protein concentrations (Fig. 3). This suggests that the interaction identified by pull-down experiments depends sensitively on the environment, such as pH, salt and inter-domain interactions, or possibly on additional components present in the lysate. In a general sense, this result highlights the elusive nature of regulatory interactions; one must clearly test a range of conditions and constructs before making conclusions.

**Fate of Cu after reaching the lumern**

After Cu has passed the ATP7A/B channel to the lumen it is unclear how it is added onto target polypeptides. In ATP7A, a luminal loop has been identified that has Cu-binding capacity (Barry et al. 2011). In ATP7B, this loop is shorter but still has Cu-binding residues. Thus, this loop may be a transient site for the Cu ion before it is loaded on a target polypeptide. The mechanism of Cu loading onto target polypeptides is unknown; one possibility is that Cu becomes free in solution after leaving ATP7A/B and binding is simply driven by Cu–protein affinity.

Ceruloplasmin is a large six-domain ferroxidase, requiring six Cu ions for activity, which is Cu-loaded by ATP7B in the secretory pathway. Our *in vitro* studies of ceruloplasmin unfolding imply that metal binding must take place early on during protein folding in order for the polypeptide to fold into its functional form. If the polypeptide is allowed to fold without metals, then it misfolds into a dead end species that cannot bind Cu (Sedlak & Wittung-Stafsheden, 2007; Sedlak et al. 2008). Thus, appropriate timing of folding and binding events in the final step of the Cu transport cascade appears crucial. In the case of ceruloplasmin, this becomes important in the treatment of aceruloplasminemia (a condition where mutated ceruloplasmin it not loaded with Cu) since Cu supplementation will not rescue already misfolded apo-forms of ceruloplasmin.

**Interactions with other proteins and new functions**

In addition to internal domain–domain interactions, other proteins have also been proposed to regulate ATP7A/B activity. For example, the protein COMMD1 (Copper Metabolism Murr1 Domain) was recently shown to interact with the metal-binding domains of ATP7B and this appeared to regulate overall ATP7B stability via the ER degradation pathway (de Bie et al. 2007; Materia et al. 2012). The binding sites on neither protein nor the physical mechanism resulting in ER degradation (interaction causing protein destabilization or triggering of a cellular signal) are known. Surprisingly, COMMD1 was found to specifically bind Cu2+ *in vitro* and, therefore, it was speculated that the COMMD1–ATP7B interaction may be a way to eliminate oxidized (toxic) Cu from cells (Sarkar & Roberts, 2011).

The general idea has been that mammalian Cu transport protein levels are primarily regulated post-transcriptionally, such as via degradation pathways (Hasan & Lutsenko, 2012). To control for elevated Cu levels, ATP7A/B can redistribute reversibly from the Golgi to the plasma membrane to expel Cu and thereby protect against Cu toxicity (La Fontaine & Mercer, 2007). However, in 2008 it was reported that Atox1 had dual functionality and also acted as a Cu-dependent transcription factor (TF) that drives the expression of *Cd1* (cyclin D1), a protein involved in cell proliferation. A direct Cu-dependent interaction of GST-tagged Atox1 with a GAAAGA sequence in the promoter region of *Cld1* was demonstrated by an electrophoretic mobility shift assay (EMSA) (Itoh et al. 2008). Subsequently, this motif was found in the promoter regions of several other genes (Muller & Klomp, 2009). In support of playing a role in the nucleus, the sequence of Atox1 contains an apparent nuclear localization signal KKTKG within its C-terminal part and, although not discussed, in the initial discovery paper of Atox1 from 1999 (Hamza et al. 1999), immunofluorescence of HeLa cells indicated that Atox1 was distributed throughout the cell, including the nucleus. In our work, we have also detected Atox1 in the nuclei of HeLa cells (Fig. 4) although we find no binding to DNA duplexes harboring the target sequence *in vitro* (Kahra et al. 2015). The answer to this apparent paradox may be that Atox1 is an indirect TF working via interaction with another protein that contains the DNA-binding ability.

To search for new Atox1 partners, we performed a yeast two-hybrid screen (Hybrigenics; similar to (Rain et al. 2001)) using Atox1 as a LexA fusion bait toward a human placenta library of protein fragments (Wittung-Stafshede, unpublished); among 98 million possible interactions, we found 25 confident target proteins that interacted with Atox1 (Table 1). Of these interactions partners, at least six are known DNA/RNA-binding proteins. The results of this screen demonstrate that the Cu transport network is greater than what is currently known and, moreover, suggest that
Cu transport proteins (i.e. Atox1) likely have additional (yet unknown) partners and functions. Although only a screen that will require extensive follow up, these types of large-scale experiments that are available today are important in that they may identify new directions for future biophysical work.

**Outlook for Cu pump biophysics**

Many mutations in the genes that code for Cu transport proteins have been linked to human diseases. Mutations in ATP7A/B constitute the basis of Wilson and Menke’s...
diseases; missense mutations in almost all domains of ATP7B have been linked to Wilson disease with the most common mutation being H1069Q in the N-domain. Using a range of biophysical methods we could explain the underlying mechanism: the mutation did not affect domain stability or ATP-binding affinity; however, it made ATP bind in such a way that hydrolysis was hindered (Rodriguez-Granillo et al. 2008). In addition to direct genetic defects, Cu accumulation (either due to, or causing, Cu transport dysregulation) is often found in cancer tumors and upon neurodegeneration. Cu can bind specifically to the amyloidogenic proteins that are involved in Huntington’s, Parkinson’s and Alzheimer’s diseases; upon binding, amyloid formation is promoted in vitro (Faller et al. 2013), suggesting that Cu may be a causative agent of these diseases in vivo.

Another aspect is drug treatment and side effects. For the cancer drug cisplatin it has been reported that the drug hijacks Cu transport proteins as a possible mechanism to become exported out of cells. We showed that Atox1 can bind cisplatin together with Cu creating a di-metal site. Cisplatin binding causes Atox1 unfolding in vitro but prior to this, if a partner such as ATP7B is present, the cancer drug can be transferred further (Palm et al. 2011; Palm-Espling & Wittung-Stafshede, 2012; Palm-Espling et al. 2013, 2014). This may result in drug resistance but, considering the possibility of Atox1 entering the cell nucleus, Atox1 may in fact mediate the delivery of the drug to DNA.

Clearly, biophysical knowledge of mechanisms and regulation of Cu transport proteins may aid the development of new approaches to target disorders involving Cu transport proteins and imbalance in Cu metabolism. Biophysical studies of the mechanisms and proteins that facilitate human Cu transport may also provide predictions for how other metal transport systems work. Like in Cu transport proteins, the ferredoxin-like structural fold appears commonly used by zinc transport proteins (Lu & Fu, 2007; Lu et al. 2009), which is a group of metal transporters for which mechanistic and biophysical knowledge is severely lacking. Taken together, the unresolved questions described above emphasize the need for more careful biophysical studies and invite young biophysical scientists to enter this wide-open field.

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**Table 1. Results from a cDNA yeast two hybrid screen using Atox1 as bait (LexA fusion) and a human placenta RP6 library as prey (Hybrigenics). 98·2 million interactions were analyzed and 310 positive clones were fully processed. Detected interactions with the highest predicted biological scores (PBS) are listed below divided in four categories from A (the highest confidence rank) to D. There were two additional unknown proteins in B, and 12 additional proteins in the D category (three ubiquitin specific peptidases and nine unknown proteins) not listed here. No C scores were found. Several of the detected interaction partners have DNA/RNA binding capacity (bold name)**

<table>
<thead>
<tr>
<th>PBS</th>
<th>Target</th>
<th>Comment on target protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PTPRF</td>
<td>Protein tyrosine phosphatase, receptor-like; cell signaling, cancer</td>
</tr>
<tr>
<td>A</td>
<td>KIAA0947</td>
<td>Nuclear protein involved in RNA binding and regulation</td>
</tr>
<tr>
<td>A</td>
<td>ATP7B</td>
<td>Known Atox1 partner (positive control)</td>
</tr>
<tr>
<td>A</td>
<td>ATP7A</td>
<td>Known Atox1 partner (positive control)</td>
</tr>
<tr>
<td>B</td>
<td>DMT1</td>
<td>C-5 cytosine methyl transferase</td>
</tr>
<tr>
<td>B</td>
<td>CRELD2</td>
<td>Cysteine-rich with EFG-like Cu-binding domains</td>
</tr>
<tr>
<td>B</td>
<td>ZFHX3</td>
<td>TF, with zinc fingers</td>
</tr>
<tr>
<td>B</td>
<td>CPEB4</td>
<td>RNA-binding protein</td>
</tr>
<tr>
<td>D</td>
<td>LMCD1</td>
<td>Zinc-finger protein with LIM and cysteine-rich domains, regulator of transcription</td>
</tr>
<tr>
<td>D</td>
<td>PPM1A</td>
<td>Phosphatase; regulator of stress response, cell cycle control</td>
</tr>
<tr>
<td>D</td>
<td>TRIM26</td>
<td>Protein with tripartite motif (includes zinc-binding domains), DNA binding</td>
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Fig. 4. Former graduate student Maria Palm-Espoing made Fig. 1. The Swedish Research Council, the Wallenberg foundation, and Umeå University are acknowledged for financial support. This text was written during a sabbatical period at California Institute of Technology.

Conflict of interest
None.

References


