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BIOCHEMICAL BASIS OF REGULATION OF HUMAN COPPER-TRANSPORTING ATPASES

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Summary

Copper is essential for cell metabolism as a cofactor of key metabolic enzymes. The biosynthetic incorporation of copper into secreted and plasma membrane-bound proteins requires activity of the copper-transporting ATPases (Cu-ATPases) ATP7A and ATP7B. The Cu-ATPases also export excess copper from the cell and thus critically contribute to the homeostatic control of copper. The trafficking of Cu-ATPases from the *trans*-Golgi network to endocytic vesicles in response to various signals allows for the balance between the biosynthetic and copper exporting functions of these transporters. Although significant progress has been made towards understanding the biochemical characteristics of human Cu-ATPase, the mechanisms that control their function and intracellular localization remain poorly understood. In this review, we summarize current information on structural features and functional properties of ATP7A and ATP7B. We also describe sequence motifs unique for each Cu-ATPase and speculate about their role in regulating ATP7A and ATP7B activity and trafficking.

Keywords

ATP7A; ATP7B; copper; P-type ATPase; Atox1

Introduction

The human Cu-ATPases ATP7A and ATP7B are essential for intracellular copper homeostasis. The Cu-ATPases use the energy of ATP hydrolysis to transport copper from the cytosol into the secretory pathway and thus supply the metal for subsequent biosynthetic incorporation into various copper-dependent enzymes. ATP7A is required for formation of functional tyrosinase (1), peptidyl- α -monooxygenase (2), lysyl oxidase (3), and possibly some other enzymes (4), while ATP7B is essential for the biosynthesis of holo-ceruloplasmin, a copper-dependent ferroxidase (5). In addition to their biosynthetic role, human Cu-ATPases participate in the export of excess copper from the cells. Overexpression of ATP7A in transgenic animals is associated with the decrease of copper content in tissues, which is particularly apparent in the heart and the brain (6). The essential role of ATP7A in copper export from intestinal epithelium is best illustrated by the phenotype of Menkes disease. In this lethal human disorder, the

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functional ATP7A is lost due to various mutations in the corresponding gene, resulting in greatly impaired export of copper from the enterocytes (7–9).

In hepatocytes, a copper exporting role belongs to another copper-transporting ATPase, ATP7B (10). Liver is the major organ of copper homeostasis in the body and is involved in removal of excess copper (11). Copper is exported from the liver into the bile and then to the feces in a process that requires the activity of ATP7B. Genetic inactivation of ATP7B results in accumulation of copper in the liver and a severe human disorder, Wilson disease. The disease is characterized by a spectrum of liver pathologies ranging from hepatitis and cirrhosis to liver failure (12). In both Menkes disease and Wilson disease, the lack of functional Cu-ATPase is also associated with the disrupted delivery of copper to the secretory pathway. The lack of copper incorporation into ceruloplasmin in Wilson disease is utilized as a biochemical marker for diagnosing the disease. In Menkes disease, the deficiency of active copper-dependent enzymes, for example lysyl oxidase, greatly contributes to the severity of the disease phenotype (13).

Two functions of human copper-transporting ATPases can be described as biosynthetic (the delivery of copper to the secretory pathway for metallation of cuproenzymes) and homeostatic (the export of excess copper from the cell). These two functions are associated with the distinct intracellular targeting of the transporters (Figure 1). The localization in the *trans*-Golgi network (TGN), which is observed for both ATP7A and ATP7B under low copper conditions, reflects their role in the delivery of copper to copper-dependent enzymes. Such enzymes as tyrosinase, peptidyl- α -monooxygenase, and ceruloplasmin have been shown to co-localize with Cu-ATPases in the TGN and require the ATPase-mediated copper transport for formation of holo-enzyme (1).

It is not known whether the metallation of cuproenzymes occurs only in the TGN or if small quantities of Cu-ATPases are also present along the secretory pathway for re-metallation of secreted enzymes, if the latter loses copper. Such a scenario is possible in the case of ATP7A, since this Cu-ATPase (unlike ATP7B) can migrate towards the basolateral membrane in the same direction as secreted proteins (see below). In fact, in the rat parotid acinar cell ATP7A is found not only in the TGN (predominant localization), but also in immature and mature secretory granules (14), where it may participate in copper delivery to peptidyl- α -monooxygenase and/or other copper-binding proteins.

The second function of Cu-ATPases - the export of copper from the cell for further utilization in the blood, milk, or for removal into the bile - requires trafficking of Cu-ATPases from the TGN to vesicles (Figure 1). This re-localization occurs in response to copper elevation, hormone release, or other signaling and developmental events (15–18). It is thought that in response to these signals the Cu-ATPases sequester copper into the vesicles. The vesicles then fuse with the membrane releasing copper into the extracellular milieu (18–20). Therefore, the regulation of intracellular localization of Cu-ATPases represents the key mechanism that determines whether the Cu-ATPases perform their homeostatic or biosynthetic function at a given moment.

Another level of regulation of copper transport must exist in cells where both Cu-ATPases are simultaneously co-expressed. While certain cells have only one Cu-ATPase (for example, ATP7B in hepatocytes), a number of cells and tissues (such as brain, mammary gland, and placenta) express both ATP7A and ATP7B. In this latter case, it is not known whether a preference exists in the distribution of copper between ATP7A and ATP7B and whether or not the same mechanisms regulate the Cu-ATPases function. Recent data from several laboratories suggest that two human Cu-ATPases differ in their enzymatic characteristics, trafficking properties, interacting partners, and regulation (see below for details). It is also clear that unique

sequence elements are present in the structure of two human copper pumps that may contribute to their distinct properties. In this review we summarize what is currently known about structure, function, and regulation of ATP7A and ATP7B, and speculate about possible contribution of unique sequence elements in the Cu-ATPase to their activity and regulation.

1. ATP7A and ATP7B are representatives of the P_{1B}-family of ion-transporting ATPases

At the biochemical level, the function of Cu-ATPases is to translocate copper across the membrane from the cytosol into the lumen of appropriate intracellular compartment (either TGN or vesicles). The vectorial copper translocation across the membranes is driven by the hydrolysis of ATP; the number of copper ions transported per one hydrolyzed ATP is currently unknown. Both ATP7A and ATP7B belong to the P_{1B}-subfamily of the P-type ATPases. This subgroup contains over 100 members (see <http://www.patbase.kvl.dk/IB.html>) and is characterized by distinct 8 transmembrane segment topology, the presence of the metal-binding sites at the N- and/or C-termini of the molecule, as well as characteristic sequence motifs (see below for details; for recent review see (21)). Similarly to all members of the P-type ATPase family, the human Cu-ATPases hydrolyze ATP with the formation of a transient acyl-phosphate intermediate (Figure 2). Phosphorylation takes place at the invariant Asp residue in the signature motif DKTG (Figure 2A). The reaction requires the transfer of copper from the cytosol to the intra-membrane portion of the transporter; while the release of copper to the opposite side of the membrane is accompanied by dephosphorylation (22,23). Cu-ATPases can also be phosphorylated by inorganic phosphate at the same aspartate residue within the DKTG motif. This reaction is reverse to the dephosphorylation step and is inhibited by copper binding to the intra-membrane site(s) from the luminal milieu (24). Comparing the ability of Cu-ATPase to be phosphorylated from ATP or Pi and the effect of copper on this reaction may serve as a tool for determining the conformational state of the enzyme.

During the catalytic cycle the Cu-ATPases are likely to undergo significant conformational changes (23). By analogy with other P-type ATPases, the binding of copper from the cytosolic side is thought to take place when the protein is present in the so-called E1-state, which is characterized by high affinity of the intramembrane sites for the transported ion. The measurements of copper-dependence of catalytic phosphorylation yielded apparent affinity of these sites for copper in the range of 0.7–2.5 μ M (25,26). Copper binding to the intra-membrane sites is associated with the transfer of γ -phosphate of ATP to the Cu-ATPase and transient stabilization of the phosphorylated state of the protein E1P (Figure 2B). In this state, access to intra-membrane sites from the cytosol is blocked and copper is sequestered in the “occluded” form. Subsequently, the Cu-ATPase undergoes conformational change to the E2P state, and the affinity for copper is decreased. It is thought that in this state copper is released from the transporter and is taken up by an acceptor protein. Intermediate proteins are not required for copper transfer from the transporter to acceptors (27), however interaction between ATP7A and SOD3, a putative target protein of ATP7A activity, has been reported (4). This latter observation suggests that the donor-acceptor interaction, although not obligatory, may facilitate metal transfer to the copper-requiring enzymes in the secretory pathway. After copper is released, the Cu-ATPase dephosphorylates (E2 state) and then undergoes conformational transition into a high-affinity state, E1, for initiation of the next transport cycle.

2. Functional activity of human Cu-ATPases is coupled to their ability to traffic

The conformational changes, that take place when copper is bound to and released from the transport site(s), appear to be intimately linked to the ability of the Cu-ATPase to traffic between the intracellular compartments. Mutation of the invariant sequence motif TGE>AAA stabilizes the protein in the E2P-like state and also triggers the redistribution of Cu-ATPases from the TGN to the vesicles mimicking the response of the transporter to elevated copper (28,29). Additional mutation of catalytic Asp to Glu in the background of the TGE>AAA

mutant of ATP7A abolishes this effect and disrupts the trafficking from TGN under either low or high copper conditions. Similar inhibition of trafficking is observed when catalytic Asp is mutated to Glu in the wild-type background. Consequently, it has been proposed that the copper-dependent exit of Cu-ATPases from the TGN “requires a catalytically active enzyme and is associated with formation of the phosphorylated catalytic intermediate”(28).

Subsequent experiments utilizing the TGE>AAA mutant demonstrated that this mutant is hypersensitive to copper and traffics even when all known copper-binding sites necessary for catalytic activity and phosphorylation are inactivated (29). Altogether, these studies pointed to the presence of additional copper binding sites in Cu-ATPases that may act as copper sensors and be involved in the initiation of trafficking. We hypothesize that such sites are present in the luminal loops of the transporter (described below) and that stabilization of a copper-bound conformation of Cu-ATPase (that may resemble the E2P state) could be critical for the ability of Cu-ATPases to relocate from the TGN to vesicles.

3. Domain organization of human Cu-ATPases

The molecular architecture of human Cu-ATPase is shown in Figure 2A. Both ATP7A and ATP7B have 8 predicted transmembrane segments (TMS) that form a copper translocation pathway. The highly conserved residues CPC in TMS6 contribute to the intramembrane copper-binding site(s). The experimental data obtained for prokaryotic copper-transporting ATPases, the disease-causing mutations, and the modeling of TMS1-6 using homology with Ca^{2+} -ATPase suggest that other possible candidates for copper coordination are residues YN in TMS7 and MxxxS in TMS8 (30) (Figure 2A). Mutation of the corresponding residues in Cu-ATPase CopA from *A.fulgidus* disrupts copper-dependent phosphorylation of CopA in the presence of ATP, while phosphorylation by inorganic phosphate remains intact in all mutants except Y682S (30). These observations strongly support the role of corresponding residues in copper coordination within the membrane (although in the absence of high-resolution structure indirect conformational effects of mutations cannot be fully excluded). Consistent with this conclusion, the M1356V mutation in the MxxxS motif of mouse Cu-ATPase ATP7B disrupts the copper-transport activity of the protein (31). The mutation is associated with significant pathological changes in mutant animals (the so-called “toxic milk” mice phenotype). It should be noted that more recently described “Jackson toxic milk mouse” has similar phenotype, but a different mutation in *Atp7b* – a Gly712Asp substitution in TMS2 (32).

The transmembrane segments of Cu-ATPases are connected by loops of different length, which are fairly short at the luminal side of the transporters. The major bulk of Cu-ATPases and their key functional domains (the N-terminal domain, the ATP-binding domain, the A-domain, and the C-terminal tail) are all cytosolic (Figure 2A). The N-terminal domain contains six copper-binding sites and serves as a regulatory center of human Cu-ATPases (Figure 2A, for details see below). The ATP-binding domain and the A-domain are essential for enzymatic activity of ATP7A and ATP7B and contain sequence motifs common to all P-type ATPases. Beyond these common sequence motifs, the Cu-ATPases show little primary sequence similarity to other well-known ion-pumps, such as Ca^{2+} -ATPase or Na^+ , K^+ -ATPase. Nevertheless, the three-dimensional fold of their ATP-binding domain and the A-domain is very similar to the corresponding domains of other P-type ATPases. This has been experimentally demonstrated by the recently determined solution structure for the nucleotide-binding domain of ATP7B (33) (Figure 2B) and by crystal structures of the ATP-binding domain and A-domain for bacterial Cu-ATPase CopA (34,35).

ATP7A and ATP7B share significant structural homology with each other, particularly in the core portion of their molecules, which is common to all P-type ATPases. At the same time, there are several regions in which ATP7A and ATP7B are quite different, while their orthologues display high sequence conservation in the same regions. It is tempting to speculate

that the structural variability between ATP7A and ATP7B may contribute to their differences in enzymatic activity, regulation, and/or trafficking to different membranes in polarized cells. In addition to amino-acid variations (for example, in the C-termini of ATP7A and ATP7B), the structural differences between human Cu-ATPases are manifested by the presence of well-defined inserts containing sequences unique for each ATPase. Such inserts are observed in the N-terminal domain, in the first luminal loop, and within the nucleotide-binding domain of ATP7A and ATP7B.

4. ATP7A and ATP7B have distinct functional properties

Current data demonstrate that two human Cu-ATPases, when expressed in the same cell or tissue, have distinct physiological roles and different response to regulators. For example, in polarized Jeg-3 cells derived from placental trophoblasts, ATP7A and ATP7B mRNA show different response to treatment with insulin (increase and decrease, respectively) and corresponding proteins appear to have different trafficking properties (36). Similarly, the *in vitro* experiments indicate that the functional characteristics of human Cu-ATPases, ATP7A and ATP7B, are distinct, perhaps contributing to their specific physiological roles. When compared under identical conditions *in vitro*, the membrane-bound ATP7A shows faster phosphorylation from ATP and it also dephosphorylates more rapidly (25). This observation suggests that the ATP7A turnover and copper transport rates are probably higher. Although transport rates have not been compared directly for ATP7A and ATP7B, it appears that ATP7B is indeed a slower transporter (31,37,38).

Which structural features are responsible for the distinct rates of phosphorylation and dephosphorylation of ATP7A and ATP7B? The role of the N-terminal domain in regulating enzyme turnover has been suggested for bacterial Cu-ATPases (39,40), however, the step of the cycle which is affected by the N-terminus remains in dispute. For human ATP7A, it was shown that simultaneous mutation of all metal binding sites within the N-terminal domain slows the rate of ATP7A dephosphorylation (23). This effect could be due to dysregulation of conformational transitions and/or inefficient delivery of copper to the transport site(s) when cytosolic metal-binding sites are mutated. Consistent with the role of the N-terminal domain in conformational transitions, the CxxC>AxxA mutation within MBD6 of ATP7B results in the apparent shift of the E1–E2 equilibrium towards E1; the shift is evident from the increase in the apparent affinity of intra-membrane sites of ATP7B for copper (41). These results support the notion that the MBDs that are closest to the trans-membrane portion of Cu-ATPase may modulate the affinity of intra-membrane sites for copper by regulating conformational transitions of the enzyme. The sites unique for human Cu-ATPases (MBD1–4) are not involved in regulation of affinity for copper, but appear to play an auto-inhibitory role via inter-domain interactions (see section 7 for details, (41)).

5. The P_{1B}-ATPases have a characteristic transmembrane hairpin TMS1,2

Six trans-membrane segments (TMS3–8) in the membrane portion of Cu-ATPases have equivalents in the structure of other P-type ATPases; while the first transmembrane hairpin (TMS1,2) is unique for the P_{1B}-ATPases and is not found in other P-type pumps. TMS1,2 is directly linked to the large N-terminal copper-binding domain via TMS1. The sequence of the TMS1,2 hairpin is not conserved in various Cu-ATPases, therefore this hairpin is unlikely to play a direct role in copper coordination within the membrane during copper occlusion and phosphorylation steps. At the same time, the hairpin is important for the Cu-ATPase folding or function as evidenced by the “toxic milk” phenotype resulting from the Gly712Asp substitution in TMS2 (32). The TMS1,2 hairpin also contains a number of residues that can contribute to metal translocation across the membrane by guiding copper towards or from the intramembrane transport sites (Figure 3A). The comparison of the TMS1,2 hairpins for two human Cu-ATPases show that both ATP7A and ATP7B have a pair of Cys residues

conspicuously positioned next to each other in the plane of the membrane (Figure 3A, B). This location suggests their possible involvement in either metal coordination or in forming a disulfide bridge. It is interesting that the treatment of ATP7B with the copper chelating reagent bathocuproine disulphonate (BCS) in the absence of disulfide-reducing reagents results in irreversible inactivation of the transporter, while the same treatment in the presence of reductant permits reactivation of Cu-ATPase by subsequent addition of copper (our observation). Whether or not this effect is due to oxidation of a cysteine pair within TMS1,2 remains to be determined.

Another interesting feature of the first hairpin is a cluster of Met residues at the luminal end of the TMS1 and TMS2 (Figure 3), which can also be found in many, although not all, Cu-ATPases (see the P-type ATPase database at <http://www.patbase.kvl.dk/IB.html>). The highly conserved Met is also present at the luminal end of TMS3 of copper-specific P_{1B}-ATPases, while in the P_{1B}-ATPases transporting the divalent Cd²⁺ and Zn²⁺, this position is frequently taken by an acidic residue. Copper binds to the N-terminal cytosolic sites of human Cu-ATPases in the reduced Cu(I) form (42,43) and it is thought that copper is translocated across the membrane in that form. The Met residues are appropriate ligands for coordinating reduced copper; therefore it is tempting to speculate that the Met cluster formed by the luminal ends of TMS1, TMS2, and TMS3 may contribute to the vestibule region where copper exits the transporters.

6. Does the extra-cellular loop TMS1–2 play a role in regulation of Cu-ATPases?

The unique functional feature of eukaryotic Cu-ATPases that distinguishes them from bacterial copper pumps is their direct involvement in the biosynthesis of copper-dependent enzymes. This process takes place in the lumen of *trans*-Golgi network, i.e. at the site of copper release from the transporters. It has been previously reported that the biosynthesis of secreted copper-containing enzymes, such as ceruloplasmin, correlates well with the rate of copper incorporation into these proteins (44). Although the rate of apo-protein production and secretion is not affected by copper levels, the amount of holo-protein is greatly diminished when copper is limiting (45). The tight link between the copper transporter activity and the biosynthesis of copper-dependent enzymes is also emphasized by correlation in their expression levels and developmental co-regulation (for example, see (2,46)). These observations raise interesting questions as to whether the rate of copper release from Cu-ATPases is coupled to subsequent copper transfer to the acceptor proteins and how the rate of copper release might be regulated.

In the catalytic cycle of Cu-ATPases, copper release is intimately linked to dephosphorylation (Figure 2B). Therefore characterizing factors that affect the dephosphorylation step may provide some insight into the regulation of copper release. It is interesting, that under identical conditions *in vitro* ATP7A shows 6-fold faster dephosphorylation compared to ATP7B (25). Given high sequence similarity of their trans-membrane portions it seems plausible that the difference in the rate of dephosphorylation of these two Cu-ATPases (and/or copper release) could be associated with the structural variations in their luminal loop(s).

The precise borders of transmembrane segments in Cu-ATPases are not known, however predictions can be made using hydropathy profiles. These predictions suggest that the most significant difference between ATP7A and ATP7B in their luminal portion is observed in the extracellular loop connecting TMS1 and TMS2. In ATP7B this region is 10–15 residues long, while the same loop in ATP7A contains an insert with additional 17 amino-acid residues (Figure 3). The sequence insert is highly enriched in Met and His residues (total 8). The presence of several copper-coordinating residues in close proximity to each other suggest that TMS1,2 loop of ATP7A may contain extra copper-binding site(s) to accept copper when it is released from the trans-membrane portion of the transporters.

The presence of several potential copper coordinating residues in the TMS1,2 also raises the possibility that this hairpin may serve as the “luminal copper sensor”. As we discussed above, the transport activity of Cu-ATPases has been linked to their ability to traffic from the TGN in response to elevated copper (28,37,47) and it parallels the biosynthesis of copper-dependent enzymes. It seems plausible that trafficking can be initiated when the amount of copper transported into the secretory pathway exceeds the biosynthetic needs of a cell. Under these circumstances, some copper may remain bound to the intra-membrane transport site locking the Cu-ATPase in the E2P state and, presumably, making it suitable for trafficking.

Alternatively, copper may leave the transport site(s) and stay in the luminal Met-rich vestibule or bind to the TMS1,2 loop. In this case, stabilization of a specific copper-bound state, rather than formation of phosphorylated intermediate, would serve as a signal for the initiation of Cu-ATPase trafficking from the TGN. TMS3 was shown to be involved in the retention of ATP7A within the TGN (48). The TMS1,2 hairpin is connected to TMS3 by a short intracellular loop and it is easy to imagine that the copper-induced changes in the TMS1,2 may also affect TMS3 and decrease the TGN retention.

The proposed models are speculative, but testable. If indeed the TMS1,2 hairpin plays a role in copper sensing and trafficking, it is most likely not the only contributor to this complex process. As we discuss in other sections, both the N-terminal and C-terminal domain are involved in determining the intracellular localization of human Cu-ATPases; in addition, several interacting proteins have been identified that may contribute to Cu-ATPase targeting and trafficking (49–51).

7. The diverse roles of the metal-binding sites within the N-terminal domain of Cu-ATPases

The N-terminal domain of copper transporting ATPases consists of 6 repetitive sequences (Figure 2A) that are characterized by an invariant GMxCxxC motif. Each of these sequences fold into individual domains and bind single copper ion in the reduced Cu(I) form via two cysteine residues. The role of individual metal-binding domains (MBDs) has been a subject of intense investigations (38,41,52–57) and a consensus has begun to emerge. It seems firmly established that the very N-terminal metal-binding sites, MBD1–4, are not required for transport function. In fact, the deletion of these MBDs in ATP7B does not alter the apparent affinity of the transporter for copper but stimulates copper-dependent catalytic phosphorylation (41). This result is consistent with the inhibitory role of this region in regulating the activity of Cu-ATPase. Such regulation is likely to be mediated through the domain-domain interactions within the transporter. The interactions between the N-terminal domain and the ATP-binding domain of ATP7B, which are weakened by copper binding to the former, were experimentally demonstrated (58).

Although MBDs1–4 are not essential for the transport activity of Cu-ATPases, their regulatory role is important and may contribute to a fine-tuned regulation of ATPase trafficking. An interesting disease-causing mutation in ATP7A has been described recently by Paulsen and colleagues (59). These authors investigated a large frame-shift deletion in ATP7A identified in a Menkes disease patient with unusually mild symptoms and long survival. They provided evidence for reinitiation of protein translation, which they predicted to produce ATP7A with MBD1–4 missing but MBD5 and MBD6 retained. The corresponding ATP7A variant had some copper transport activity, as revealed by yeast complementation assay (in this assay, copper transport to ferroxidase Fet3 in the secretory pathway is first disrupted by the deletion of yeast Cu-ATPase Ccc2p, thus leaving yeast iron-deficient. The transport is then restored and the phenotype is corrected by heterologous expression of human Cu-ATPase). In addition to transport activity, the truncated ATP7A variant was able to traffic from the TGN in response to elevated copper. In fact, the truncated ATP7A appeared more sensitive to intracellular copper levels. Compared to the full-length ATP7A, the retention of the truncated ATP7A in Golgi

compartment required treatment of cells with higher concentrations of copper chelator (59). This observation could be similar to findings in ATP7B, where the deletion of MBD1,4 appears to facilitate the delivery of copper to the membrane portion of the transporter (41).

As we discussed above, the role of MBD5 and MBD6 seem to influence the affinity of the intra-membrane copper-binding site(s), most likely by shifting the E1–E2 equilibrium upon copper binding/dissociation. Copper binding to isolated MBD5,6 of ATP7B does not significantly alter their structure (60) and only small rearrangements are detected in proximity to the metal binding site (described below). Nevertheless, mutations of metal-coordinating Cys to Ala in a single MBD6 alter the apparent affinity of intra-membrane site(s) for copper (see for example, (41)). These observations suggest that the metal-binding site of MBD6 is located in close proximity to (and perhaps interacts directly with) the other domains of ATP7B (Figure 2A), thus influencing conformational changes of the entire protein and the affinity of intramembrane sites. The best candidate for such interactions would be the A-domain, which plays a key role in conformational transitions in other P-type ATPases, however, the direct experimental evidence for such interaction is still missing.

8. Structure of individual N-terminal MBDs

Solution structures for all six metal binding domains of Cu-ATPases (mostly ATP7A) have now been published (57,60–65). Each domain consists of four β -strands and two α -helices folded into a stable $\beta\alpha\beta\alpha\beta$ “ferredoxin-like” structure (see Figure 2A for MBD5 and 6 of ATP7B). The structures of the individual domains are quite similar. The third metal binding domain, MBD3, which has the greatest amount of sequence variation, has some notable differences in the metal binding region (discussed below), but retains the same general fold. In all MBDs, the conserved GM(T/H)CxxC copper binding motif lies at the surface of the domain in the β 1- α 1 loop, with the second copper-coordinating cysteine at the beginning of the α 1 helix (Figure 4A).

Apo-structures of MBDs show that this loop is flexible and relatively unstructured (Figure 4B). Upon binding of Cu(I), the loop acquires rigidity with the copper atom bound between the two cysteine residues in a linear coordinate environment. These structural data are in accordance with the results of X-ray absorption spectroscopy that have suggested a S-Cu(I)-S coordinating geometry with S-Cu(I) bond lengths of 2.2–2.3 Å (43,66,67). After copper binds, only the β 1- α 1 loop and the N-terminus of the α 1 helix demonstrate significant rearrangement (Figure 4B); the rest of the domain remains largely unperturbed (62–64,68,69).

In addition to Cys residues directly involved in copper coordination, several other amino acids contribute to copper binding environment within individual MBD. Mutations of the “X” residues in the CxxC motif have been shown to alter the flexibility of the metal binding loop (68). The loop is situated near a core of hydrophobic residues, which provide a stable association between the β 1 strand and the α 1 helix regardless of whether or not copper is bound. Four conserved residues contribute to this core – Met14 in the β 1- α 1 loop, Ile21 in the α 1 helix, Leu38 in the β 2- β 3 loop, and Phe66 in the α 2- β 4 loop. In all N-terminal MBDs, except MBD3, Phe66 is conserved. A Phe-Ile substitution in MBD2 does not alter the apparent off-rate of copper in the presence of the copper chelator diethyldithiocarbamic acid, DDC (65), while a Phe-Ala substitution in MBD1 appears to confer greater flexibility on the overall protein (68). These data suggest that while this residue may not directly influence copper binding or retention, it could be important for maintaining the compact structure of the domain.

The third metal binding domain of ATP7A has the most unique sequence of the six domains, as well as having lower affinity for copper in solution (61). In MBD3 the residue Phe66 is substituted by a Pro residue (Figure 4B), however the hydrophobic pocket is maintained by the presence of a Tyr at position 69. The α 2 helix and the β 4 strand are shorter, generating an

extended $\alpha 2$ - $\beta 4$ loop that is unique to MBD3. Also unlike other domains, the apo-form of MBD3 features an extended $\alpha 1$ helix which incorporates both metal coordinating cysteines (Figure 4A). To bind copper, the helix must be unwound in order for the coordinating sulfur groups to form a linear geometry with the bound Cu(I) molecule. As the structures of individual MBDs were solved in absence of the rest of the protein, it is possible that in the full-length Cu-ATPase such unwinding occurs as the result of interactions of MBD3 with other MBDs (or perhaps with the copper chaperone, see below) making this domain more suitable for copper binding. Recent studies characterizing MBDs in the context of the entire N-terminal domain of ATP7B found little differences between individual metal-binding sites, including MBD3, in their ability to bind copper (70). However, the accessibility of cysteine residues to labeling with Cys-directed probe differed between MBDs, with MBD3 showing very little labeling (70).

9. Structural changes within N-terminal domain are likely to occur within linker regions

Although the solution structures of individual metal binding domains are now available, how the N-terminal MBDs are packed in the full-length protein remains unknown. An increasing body of data point to a higher order structure within the N-terminal domain that is influenced by copper binding. Although XAS of individual MBDs shows a dominant two-coordinate copper binding environment, a third ligand can contribute to copper coordination (71). In solution, such ligands could be provided by reducing reagents present in the buffers, including DTT, glutathione, or TCEP (43). In a fully folded protein with multiple copper binding sites, tight packing of metal binding domains may bring additional residues in contact with bound copper or bring copper from two neighboring sites in close proximity to each other. Indeed, XAS analysis of the N-terminal domain with 4–5 bound Cu atoms demonstrates copper-copper distances of 2.6 Å, indicative of metal-binding sites proximity (43).

The structural rigidity of individual MBDs implies that significant conformational changes observed in the entire N-terminal region of Cu-ATPases upon copper binding (66) take place in the loops connecting these individual domains. This can be demonstrated using limited tryptic hydrolysis of the N-terminal domain of ATP7B (as described in (67)), when cleavage takes place between MBDs and different patterns of peptides are generated depending on whether the N-terminus is in the apo- or copper bound form. For example, binding of copper to MBD2 of ATP7B was shown to result in the exposure of the loops flanking MBD4 and their increased susceptibility to proteolytic cleavage (67). It is possible that slight conformational changes in MBDs upon copper binding are amplified through the linker regions, leading to larger scale changes of the entire N-terminal domain. Such structural rearrangements within the linker regions are likely to contribute to copper-dependent regulatory events, which require the N-terminal domain. For example, changes in the structure of linkers may affect the inter-domain interactions, result in exposure of sites for the kinase-mediated phosphorylation, and/or modulate interactions with regulatory proteins acting on Cu-ATPases (see section 15).

The loops connecting N-terminal MBD vary in length (Figure 5). In addition, the N-terminus of human ATP7B has an extension of 63 amino acid residues, which is critical for proper trafficking of ATP7B in polarized hepatocytes (72). The ATP7B construct lacking MBD1–4, but containing the extension, is targeted and traffics normally towards apical membrane. In contrast, the ATP7B variant lacking MBD1–4 and the extension constitutively traffics to the basolateral plasma membrane (72). Interestingly, the sheep orthologue of ATP7B (sATP7B) has two hepatic forms: one “human-like” and another with the alternate N-terminus, which represents longer extension. In non-polarized CHO-K1 cells both forms of sATP7B are similarly located in the TGN and both redistribute to a vesicular compartment in response to elevated copper(73). How different N-terminal extensions modulate localization and trafficking of sATP7B in polarized cells has not yet been determined.

ATP7A lacks the very N-terminal extension, but has a long (91 amino-acid residues) insert between the first and second MBDs (Figure 5), whereas ATP7B has a much shorter MBD1,2 linker (13 residues). Secondary structure predictions for both the N-terminal extension of ATP7B and the ATP7A MBD1,2 insert suggest that these regions are folded and thus may represent small recognition domains for protein-protein interactions that are unique for the corresponding Cu-ATPases.

Although specific length of connecting loops varies, the N-terminal domains of ATP7A and ATP7B share a common motif where the linker between MBDs 4 and 5 is much longer than the one between MBD5 and 6 (Figure 5). This observation and the presence of only one or two MBDs in bacterial Cu-ATPase suggest that MBD5,6 may represent an autonomous sub-domain within the N-terminus of Cu-ATPases. This hypothesis is supported by a recent solution structure of MBD5,6 from ATP7B, which revealed tight packing of two MBDs against each other (60). The structure also showed that both MBDs retain the expected ferredoxin-like fold, but their metal binding sites are located at the distal surfaces of the structure (Figure 2A), ruling out direct interaction between the sites and inter-domain copper transfer (60). This structural arrangement and the ability of recombinant human MBD4 to transfer copper to MBD5 (60) are also consistent with the idea that independently folded MBD1–4 may play a regulatory role in gating access of copper to domains 5 and 6.

The N-terminal domains of both ATP7A and ATP7B contain cysteine residues in addition to the twelve Cys directly involved in copper coordination. Circular dichroism data demonstrate a lack of disulfides in copper loaded N-ATP7B, suggesting that these additional cysteines exist as free thiols (66). Recently, both ATP7A and ATP7B were shown to be glutathionylated in a cell at their N-terminal domain (50). Whether “extra” cysteine residues represent sites of regulatory glutathionylation or whether this modification affects copper-coordinating residues remains to be established.

10. Atox1 interactions with the N-Terminal domain of Cu-ATPases

The redox potential of copper makes it a useful cofactor for many enzymes, but also requires copper to be sequestered at all times to avoid oxidative damage. In yeast, it has been demonstrated that almost no copper exists free in solution, instead all copper is bound to proteins or small molecules such as glutathione (74). When copper enters the cell through the transporter Ctr1, it is thought to immediately pass to another molecule. The job of specifically distributing copper to intracellular compartments and proteins is attributed to a family of proteins known as copper chaperones (Figure 1). The cytosolic protein Atox1 (HAH1) has been shown to play a key role in the delivery of copper to Cu-ATPases. The deletion of the *Atox1* gene in mice leads to both intracellular copper accumulation as well as a decrease in the activity of secreted copper-dependent enzymes (75), indicative of the diminished Cu-ATPase transport activity. In yeast, the Atox1 ortholog Atx1 has been shown to facilitate function of Ccc2, the P-type ATPase, which transports copper into the late Golgi compartment (76). The evolutionary conservation of functional interactions between Atox1 and Cu-ATPases emphasizes the role of the chaperone in regulating the Cu-ATPase function.

Structurally, Atox1 is a 68-amino acid residues protein that has the $\beta\alpha\beta\beta\alpha\beta$ fold and a single MxCxxC copper binding motif common with the N-terminal MBDs of Cu-ATPases (77). Similarly to the N-terminal MBDs, Atox1 binds copper with a linear, two-coordinate geometry. X-ray absorption data also demonstrate that recruitment of a third ligand by a copper-bound Atox1 is possible and results in a three-coordinate adduct (78). These data are highly suggestive of a transfer mechanism where copper moves from Atox1 to a metal-binding domain of Cu-ATPase through a three-coordinate intermediate (77). The critical role of copper-binding to CxxC motif for the formation of the chaperone-target adduct was directly illustrated by yeast two-hybrid studies (79) and by recent NMR studies (80). The crystal structure of Atox1,

showing the molecule as a homo-dimer situated around a single copper molecule, provided a glimpse of how such copper-transfer intermediate may look (77). In solution, the intermediate is transient and cannot be visualized (63).

11. Atox1-mediated copper transfer

In vitro, Cu-Atox1 has been demonstrated to transfer copper directly to the N-terminal domain of ATP7B in a dose dependent fashion (60,61,63,70,81). All six sites can be filled in this manner, though a significant excess of Atox1 (5–50 fold over the N-terminal domain of ATP7B) is needed to do so. Real-time PCR measurements suggest that in HepG2 cells Atox1 is present in excess to ATP7B (the Atox1 mRNA is 3–4 fold more abundant than ATP7B mRNA, our unpublished data), however the ratio at the protein level has not been determined. It may be that the process of copper transfer by Atox1 is further facilitated by some other proteins or by protein modifications. Recent yeast two-hybrid and immunoprecipitation studies demonstrated interaction between immunophilin FKBP52 and Atox1 that was enhanced in a copper-supplemented medium (82). Furthermore, over-expression of FKBP52 in HEK293 cells increased copper efflux similarly to over-expression of ATP7B.

It is not clear how the interaction between FKBP52 and Atox1 would stimulate copper efflux. FKBP52 is a multi-domain protein with peptidyl-prolyl *cis/trans* isomerase activity (83). It may interact with the Leu-Pro motif in Atox1 (82) and stabilize an Atox1 conformation that favors copper transfer to ATP7B. FKBP52 also contains a calmodulin binding domain, as well as tetratricopeptide repeat domains which bind Hsp90; as such, the protein may be capable of mediating interactions with a larger complex of proteins necessary for ATPase trafficking. Most of the immunophilin research has focused on the protein's roles in steroid hormone regulation and neuroprotection. Whether FKBP52 links these processes to copper homeostasis is unknown, but certainly intriguing. A better understanding of FKBP52 function in copper homeostasis would be very useful.

Transfer of copper by Cu-Atox1 stimulates catalytic phosphorylation of ATP7B, with a dose-dependent response similar to that of free copper (81). Individually, the N-terminal MBDs have similar copper association constants that are equivalent or lower than that of Atox1 (70). However, the ability of MBDs to accept copper from Atox1 in the presence of other domains differs (84). When Cu-Atox1 is presented to the N-terminal domain of ATP7B *in vitro*, the copper is preferentially transferred to the MBD2 (67). Such selectivity is not seen with free copper or when copper is presented in the complex with glutathione. The preference for one MBD over the others is also lost when the N-terminal domains with all but one mutated MBDs are compared for their ability to receive copper from Atox1 (70). This result suggests that the inter-domain interactions mediated through functional N-terminal metal-binding loops could be essential for precise and ordered transfer of copper from Atox1 to the intra-membrane sites of the transporter. Indeed, mutating the cysteines in MBD2 prevents Cu-Atox1 from stimulating catalytic activity of the ATPase, though the mutant can still be stimulated by free copper at the wild-type level (67). It appears that MBD2 may act as an entryway for copper into the transporter or that the copper occupancy of MBD2 serves as a conformational switch allowing copper transfer to other MBDs.

It is currently not clear whether better exposure or other characteristics of MBD2 make it a site of preferential copper transfer from Atox1. When presented as individual domains, MBD4 has a stronger affinity for Cu-Atox1 than does MBD2 (60), but in the fully folded protein MBD4 may not be sufficiently exposed and may only become available after transfer of the first copper to MBD2. It has been also proposed that the charged surface exposed residues may contribute to chaperone-target recognition. Atox1 has a considerable positively charged surface region featuring a number of lysine residues in proximity to the metal binding loop. Negatively charged regions of several MBDs may be complimentary (67), however current data does not

support the idea of surface complementarity being a driving force for selective Atox1-MBD recognition. Rather, the complementary surface charge distribution may be an important factor in packing of the MBDs within the N-terminal domain. The observation that the distribution of charges on the surface of MBDs of ATP7A and ATP7B appear to follow the same pattern (85,86) is consistent with this idea.

For ATP7A, little is known about the copper transfer process, though themes similar to those obtained for Atox1-ATP7B interactions are emerging. In solution, Cu-Atox1 presented with MBDs 4–6 of ATP7A demonstrated a preference for donating copper to MBD4 (87), although MBD5 and 6 of ATP7A can also accept copper from Cu-Atox1 (62,63) at variance with ATP7B. MBD3 of ATP7A is poorly metallated by Cu-Atox1 in solution, likely due to the unusual organization of its metal-binding site (see above). As we discuss above, domain-domain interactions may perturb the structure of MBD3 to make it more accessible to copper binding. Alternatively, the interaction with Atox1 may affect the conformation of this domain when it is present in the full protein and allow for copper transfer to occur.

12. The role of Atox1 in the regulation of Cu-ATPases in a cell

Genetic deletion of Atox1 results in an impaired copper efflux from the cell consistent with the disrupted delivery of copper to Cu-ATPases and diminished transport activity (75). In addition, Atox1 deletion alters the time- and copper-dependence of ATP7A exit from the TGN (more copper and longer times are needed to elicit the same trafficking of ATP7A in the *Atox1*^{-/-} cells compared to control (88)). These observations suggest that although Atox1 is not indispensable for trafficking response, it may facilitate trafficking of ATP7A through protein-protein interaction either with the transporter itself or with trafficking machinery. Alternatively, and perhaps more likely, the diminished delivery of copper to the Cu-ATPase due to the lack of the chaperone slows down transport activity of ATP7A. This would result in a slower accumulation of copper in the lumen of TGN, lower level of copper incorporation into copper-dependent enzymes, such as tyrosinase, and the delayed trafficking response if the signal for ATP7A re-localization is originated in the lumen (see above for details on proposed luminal copper signaling).

In vitro studies suggest that in addition to copper transfer and concomitant stimulation of Cu-ATPase activity, Atox1 may play an additional role by decreasing copper occupancy of the N-terminal domain when copper is low. In its apo-form, Atox1 can remove copper from 4 or 5 although not all 6 sites in the N-terminal domain and down-regulate the enzyme by about 50% (81). Regulation of copper occupancy of the regulatory metal-binding sites in Cu-ATPases may not only affect the transport activity but also contribute to intracellular trafficking of Cu-ATPases. In a cell, human Cu-ATPases are thought to be present in at least three distinct locations: in the TGN, in recycling vesicles, and, transiently, at the plasma membrane (Figure 1). Copper stimulates exit of Cu-ATPase from the TGN and trafficking towards vesicles and eventually to the plasma membrane, while decrease in copper concentration results in the return of Cu-ATPase from plasma membrane/vesicles to the TGN. As was shown for ATP7A, when intracellular copper levels are constantly high, Cu-ATPases do not return to TGN but became targeted to the pool of recycling vesicles, presumably to continue copper export via the plasma membrane (89). What mechanism determines the recruitment of Cu-ATPases to this pool of vesicles rather than their return to the TGN? Since this process is copper-dependent it is tempting to speculate that the copper occupancy of the N-terminal domain could be a contributing factor. It could be that in high copper Atox1 maintains the regulatory sites in the N-terminal domain in a metal-bound form, which is necessary for targeting or retention in the vesicles. When intracellular copper becomes low, apo-Atox1 may remove copper from the regulatory copper site facilitating transition to the state suitable for return to the TGN (Figure 1).

13. The ATP-binding domain of human Cu-ATPases

The ATP-binding domain located between TM6 and TM7 is composed of two independently folded parts: the nucleotide-binding domain (N-domain), which is involved in coordination of the adenine moiety, and the phosphorylation domain (P-domain), which contains residues directly involved in catalytic reaction, including invariant Asp residue in the DKTG motif (Figure 6, marked in red). The P-domain is highly conserved among all P-type ATPases. The structure of this domain has been determined as a result of efforts to characterize the ATP-binding region of the bacterial Cu-ATPase CopA (35). The P-domain consists of a six-stranded parallel β -sheet sandwiched between six α -helices, three on each side of the sheet (Figure 6). The invariant Asp residue in the DKTGT is positioned close to the hinge region connecting the P-domain and N-domain and faces the nucleotide binding domain (Figure 6). Another loop, containing a set of residues important for catalysis, GDGXND, is located at the edge of the crevice marking the interface between the N- and P-domains (35).

In contrast to the P-domain, which is conserved in all P-type ATPases, the N-domain of Cu-ATPases has a distinct sequence and a characteristic set of residue involved in ATP coordination (Figure 6). The ATP-binding environment in ATP7B is formed by the residues H1069, G1099, G1101, I1102, G1149, and N1150 of ATP7B, which are conserved in the P_{1B}-ATPase subfamily (33). The three-dimensional fold of the N-domain is preserved in the entire P-type ATPases family despite the lack of primary sequence conservation indicating that overall domain architecture is essential for the function of these transporters. The N-domain of ATP7B consists of a six-stranded beta-sheet with two adjacent alpha-helical hairpins (33). In ATP7A and ATP7B, the N-domain also contains a structural element, inserted between the β 3 and β 4 strands of its core structure, which is absent in Cu-ATPase from bacteria, yeast and flies. In the solution structure of the N-domain of ATP7B this region is mostly unfolded and flexible (Figure 2A), however it is possible that in the full-length Cu-ATPase, in the presence of other domains it may adopt a defined structure. Although the sequence of the insert is conserved among ATP7A and ATP7B orthologues, respectively, there is no sequence similarity in this region when ATP7A and ATP7B are compared. The insert is not required for ATP-binding by the N-domain (33) and the location of this insert at the protein surface facing the cytosol suggests possible regulatory functions, such as involvement in regulatory inter-domain or protein-protein interactions.

14. The role of the DxxK motif in regulating the N- and P-domain movements

The DxxK motif is present in both ATP7A and ATP7B and is located at the very end of the N-domain and the beginning of the flexible linker connecting the N- and P-domains. Mutations of either ¹²³⁰D or ¹²³³K in this motif in ATP7A result in a significant decrease of ATP7A copper transport activity (37). At the same time, the D1230A mutant shows markedly increased affinity for ATP and can undergo catalytic phosphorylation and turnover (37). Therefore, the loss of transport activity in this mutant appears to be due to uncoupling between the catalytic and transport activity of ATP7A. Alternatively, or in addition, the marked decrease in copper transport for this mutant may be caused by a significant shift in conformation of ATP7A towards E1P form as was earlier observed for the D>A or D>N substitutions of the equivalent aspartate residue in some other P-type ATPases (90). Overall, the position of DxxK in a flexible linker connecting the N- and P-domain and the effect of D1230 mutation on ATP binding and transport activity suggest an important role for this Asp residue in regulating movement of the domains upon ATP binding.

15. Mechanisms that control intracellular distribution of Cu-ATPases

A large body of work has been published characterizing intracellular behavior of overexpressed ATP7A and ATP7B in model cell system (for review see (91)), however detailed analysis of the endogenous proteins has only recently begun to emerge. In enterocytes in copper-depleted

conditions, the majority of endogenous ATP7A is concentrated in the perinuclear region and co-localizes with syntaxin 6, a t-SNARE that functions in post-TGN trafficking (19). Following treatment with as little as 1 μ M copper, about 40% of ATP7A re-localizes to small vesicles close to the basolateral membrane. At the same time, the amount of ATP7A at the plasma membrane increases approximately 3-fold. However, the plasma membrane amounts represent only 8–10% of total ATP7A, i.e. ATP7A remains mostly intracellular (a similar result was observed for higher copper concentrations) (19). Consequently, it has been proposed that the increase in intracellular copper leads to sorting of ATP7A from a post TGN compartment into vesicles, rather than directly to the plasma membrane. The vesicles accumulate excess copper and then release their content into the basolateral milieu by exocytosis (19). During this process ATP7A appears at the plasma membrane and is then rapidly endocytosed. When cytoplasmic copper levels fall, ATP7A is retrieved from vesicles (and the plasma membrane) and is delivered back to the post-TGN compartment. A similar model for Cu-ATPases trafficking has been suggested based on experiments using transgenic mice over-expressing ATP7A (20) and for intracellular trafficking of ATP7B in polarized HepG2 cells (92).

The protein machinery that controls the various steps of ATP7A and ATP7B trafficking is unknown. Several proteins involved in specific interactions with ATP7A and ATP7B have been identified (12,49–51,93). However, the contribution of these proteins to the exit of ATP7A and ATP7B from the TGN, to the retention in the vesicles, endocytosis, and the return of Cu-ATPases to the TGN remains unknown. In addition, ATP7A and ATP7B were shown to be phosphorylated by an uncharacterized kinase(s) in response to elevated copper (94,95). The phosphorylation involves Ser residues (95,96) and requires the presence of functional N-terminal domain. In ATP7B, the disease-causing mutation in the N-terminal domain G591D that disrupts interaction with Atox1 (97), also abolishes copper-dependent kinase-mediated phosphorylation (94). (The mutation is located within MBD6 and the disruption of interaction between mutant ATP7B and Atox1 suggests the overall effect of this mutation on protein folding). Similarly, the lack of copper-dependent phosphorylation was observed in ATP7A mutant, in which all N-terminal MBDs were mutagenized (CxxC to SxxS) (95). These results suggest a role for the N-terminal domain in the kinase-mediated response. However, it remains to be determined whether the N-terminal domain interacts directly with the kinase and whether this interaction is facilitated by copper.

The level of phosphorylation of ATP7B correlates with the intracellular localization of the transporter, therefore it is possible that phosphorylation occurs after Cu-ATPases leave the TGN. In this case, phosphorylation might be required to target/retain the transporter in vesicles, where it functions to export copper. Consistent with this idea, the decrease in copper concentration following copper removal from the growth medium is accompanied by dephosphorylation of ATP7B and the return of transporter to the TGN (94). Currently there is no information of what kinases or phosphatases are involved in the regulation of Cu-ATPases, although *in vitro*, partial dephosphorylation can be achieved using λ -phosphatase (94).

Recent studies indicate that the N-terminal domain contributes significantly to directing ATP7B to appropriate vesicles in polarized cells. As described above, the deletion of the very N-terminal segment of ATP7B (residues 1–63) along with MBD1–4 results in relocation of ATP7B towards the basolateral membrane in vesicles (i.e. the correct apical targeting is disrupted) (72). Furthermore, this response takes place even in the absence of elevated copper. Addition of first 63 amino-acid N-terminal amino-acid residues to the truncated ATP7B restores both the copper-dependent targeting and correct polarity upon trafficking, even in the absence of MBD1–4 (72). It seems likely that this very N-terminal segment of ATP7B is essential for interaction with the machinery that determines apical delivery of ATP7B. How this segment regulates copper dependence is unclear. It could be that it contributes to the auto-inhibitory function of the N-terminal domain and therefore deletion or mutations within this

domain may activate the ATPase transport function and increase its apparent sensitivity to copper.

Another region that may contribute to determining the destination of Cu-ATPase after leaving the TGN is located between MBD6 and TMS1. Studies of chimeric molecules, in which the corresponding region of ATP7B replaced the N-terminus of ATP7A, showed the ATP7B-like trafficking behavior of chimera in CHO cells. It was suggested that this region may contain a targeting signal that directs the Cu-ATPase to appropriate cellular destination (91). The chimera construct has not been yet analyzed in polarized cells, and it would be interesting to determine the respective contributions of different regions of the N-terminal domain (the very N-terminal segment and the segment between MBD6 and TMS1) to the apical versus basolateral trafficking.

16. The role of the C-terminal tail in protein stability and intracellular localization of Cu-ATPases

The C-termini of ATP7A and ATP7B are less characterized than other domains of Cu-ATPases, however the available data strongly suggest their important role in stability and regulation of copper pumps. Deletion of the entire C-terminal tail in ATP7B has a deleterious effect on protein stability (98) suggesting that the C-terminus is likely to interact with other protein regions promoting their folding and/or protecting these regions from intracellular proteases. Similarly, a frameshift mutation in ATP7A, which results in a truncation of a portion of C-terminus at the position 1451 is associated with a marked decrease in protein stability and disease phenotype (99).

In addition to its structural role, the C-termini of human Cu-ATPase contribute to the trafficking of the transporters. The C-terminus of ATP7A contains the di-leucine motif L1487L1488, which was shown to be essential for steady-state localization of the protein within the TGN (100–102). Substitution of these two leucine residues for alanines is associated with the shift of intracellular distribution of ATP7A from the perinuclear region towards the vesicles and the plasma membrane (100) and slower endocytosis of ATP7A from the plasma membrane (101). Altogether, the results suggested that the L1487L1488 motif is essential and sufficient for retrieval of ATP7A from the plasma membrane, but is not sufficient to drive the ATP7A return to the TGN.

The C-terminus of ATP7B contains the tri-leucine motif, LLL^{1454–1456}, which by analogy with ATP7A was predicted to act as an endocytic retrieval signal. The LLL>AAA mutation causes ATP7B to constitutively localize to vesicles when ATP7B is expressed in CHO cells (103). The significance of this result is not entirely clear. Since ATP7B is not trapped at the plasma membrane it seems unlikely that the endocytic step is disrupted by substitution of leucines for alanines. Thus, the tri-leucine motif could be involved in returning ATP7B from vesicles to the TGN. Alternatively alterations within the C-terminal tail may alter protein conformation making it more sensitive to copper concentration, thus mimicking response to elevated copper.

Conclusions

Human Cu-ATPases ATP7A and ATP7B are complex molecular machines that have a multifaceted role in copper homeostasis. They regulate intracellular copper concentration, export copper out of cells for further utilization or removal, and critically contribute to the biosynthesis of copper-dependent enzymes. At the molecular level, the activity of ATP7A and ATP7B is modulated through conformational changes and alterations in the inter-domain interactions. In addition, unique segments of the transporters appear to contribute to the regulation of Cu-ATPases targeting and trafficking. Although significant progress has been

made in understanding the ligand-binding properties of Cu-ATPases, much remains to be learned about precise intracellular mechanisms that regulate their function.

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References

1. Petris MJ, Strausak D, Mercer JF. *Hum Mol Genet* 2000;9(19):2845–2851. [PubMed: 11092760]
2. Steveson TC, Ciccotosto GD, Ma XM, Mueller GP, Mains RE, Eipper BA. *Endocrinology* 2003;144(1):188–200. [PubMed: 12488345]
3. Tchapanian EH, Uriu-Adams JY, Keen CL, Mitchell AE, Rucker RB. *Arch Biochem Biophys* 2000;379(1):71–77. [PubMed: 10864443]
4. Qin Z, Itoh S, Jeney V, Ushio-Fukai M, Fukai T. *Faseb J* 2006;20(2):334–336. [PubMed: 16371425]
5. Terada K, Nakako T, Yang XL, Iida M, Aiba N, Minamiya Y, Nakai M, Sakaki T, Miura N, Sugiyama T. *J Biol Chem* 1998;273(3):1815–1820. [PubMed: 9430732]
6. Ke BX, Llanos RM, Wright M, Deal Y, Mercer JF. *Am J Physiol Regul Integr Comp Physiol* 2006;290(5):R1460–1467. [PubMed: 16397091]
7. Kaler SG. *Am J Clin Nutr* 1998;67(5 Suppl):1029S–1034S. [PubMed: 9587147]
8. Kodama H, Murata Y. *Pediatr Int* 1999;41(4):430–435. [PubMed: 10453200]
9. Tumer Z, Horn N. *J Inherit Metab Dis* 1998;21(5):604–612. [PubMed: 9728340]
10. Tao TY, Gitlin JD. *Hepatology* 2003;37(6):1241–1247. [PubMed: 12773998]
11. Linder MC, Wooten L, Cerveza P, Cotton S, Shulze R, Lomeli N. *Am J Clin Nutr* 1998;67(5 Suppl):965S–971S. [PubMed: 9587137]
12. Das SK, Ray K. *Nat Clin Pract Neurol* 2006;2(9):482–493. [PubMed: 16932613]
13. Royce PM, Steinmann B. *Pediatr Res* 1990;28(2):137–141. [PubMed: 1975662]
14. D'Amico F, Skarmoutsou E, Sanfilippo S, Camakaris J. *Acta Histochem* 2005;107(5):373–378. [PubMed: 16185750]
15. Petris MJ, Mercer JF, Culvenor JG, Lockhart P, Gleeson PA, Camakaris J. *Embo J* 1996;15(22):6084–6095. [PubMed: 8947031]
16. Schlieff ML, Craig AM, Gitlin JD. *J Neurosci* 2005;25(1):239–246. [PubMed: 15634787]
17. Kelleher SL, Lonnerdal B. *Am J Physiol Regul Integr Comp Physiol* 2006;291(4):R1181–1191. [PubMed: 16741141]
18. Niciu MJ, Ma XM, El Meskini R, Ronnett GV, Mains RE, Eipper BA. *Neuroscience* 2006;139(3):947–964. [PubMed: 16549268]
19. Nyasae L, Bustos R, Braiterman L, Eipper B, Hubbard A. *Am J Physiol Gastrointest Liver Physiol*. 2006
20. Monty JF, Llanos RM, Mercer JF, Kramer DR. *J Nutr* 2005;135(12):2762–2766. [PubMed: 16317117]
21. Arguello JM, Eren E, Gonzalez-Guerrero M. *Biometals*. 2007
22. Tsivkovskii R, Eisses JF, Kaplan JH, Lutsenko S. *J Biol Chem* 2002;277(2):976–983. [PubMed: 11677246]
23. Voskoboinik I, Mar J, Strausak D, Camakaris J. *J Biol Chem* 2001;276(30):28620–28627. [PubMed: 11373292]
24. Tsivkovskii R, Efremov RG, Lutsenko S. *J Biol Chem* 2003;278(15):13302–13308. [PubMed: 12551905]
25. Barnes N, Tsivkovskii R, Tsivkovskaia N, Lutsenko S. *J Biol Chem* 2005;280(10):9640–9645. [PubMed: 15634671]
26. Hung YH, Layton MJ, Voskoboinik I, Mercer JF, Camakaris J. *Biochem J* 2007;401(2):569–579. [PubMed: 17009961]

27. El Meskini R, Culotta VC, Mains RE, Eipper BA. *J Biol Chem* 2003;278(14):12278–12284. [PubMed: 12529325]
28. Petris MJ, Voskoboinik I, Cater M, Smith K, Kim BE, Llanos RM, Strausak D, Camakaris J, Mercer JF. *J Biol Chem* 2002;277(48):46736–46742. [PubMed: 12228238]
29. Cater MA, La Fontaine S, Mercer JF. *Biochem J* 2007;401(1):143–153. [PubMed: 16939419]
30. Mandal AK, Yang Y, Kertesz TM, Arguello JM. *J Biol Chem* 2004;279(52):54802–54807. [PubMed: 15494391]
31. Voskoboinik I, Greenough M, La Fontaine S, Mercer JF, Camakaris J. *Biochem Biophys Res Commun* 2001;281(4):966–970. [PubMed: 11237756]
32. Coronado V, Nanji M, Cox DW. *Mamm Genome* 2001;12(10):793–795. [PubMed: 11668395]
33. Dmitriev O, Tsvikovskii R, Abildgaard F, Morgan CT, Markley JL, Lutsenko S. *Proc Natl Acad Sci U S A* 2006;103(14):5302–5307. [PubMed: 16567646]
34. Sazinsky MH, Agarwal S, Arguello JM, Rosenzweig AC. *Biochemistry* 2006;45(33):9949–9955. [PubMed: 16906753]
35. Sazinsky MH, Mandal AK, Arguello JM, Rosenzweig AC. *J Biol Chem* 2006;281(16):11161–11166. [PubMed: 16495228]
36. Hardman B, Michalczyk A, Greenough M, Camakaris J, Mercer JF, Ackland ML. *Biochem J*. 2006
37. Voskoboinik I, Mar J, Camakaris J. *Biochem Biophys Res Commun* 2003;301(2):488–494. [PubMed: 12565888]
38. Voskoboinik I, Strausak D, Greenough M, Brooks H, Petris M, Smith S, Mercer JF, Camakaris J. *J Biol Chem* 1999;274(31):22008–22012. [PubMed: 10419525]
39. Mandal AK, Arguello JM. *Biochemistry* 2003;42(37):11040–11047. [PubMed: 12974640]
40. Rice WJ, Kovalishin A, Stokes DL. *Biochem Biophys Res Commun* 2006;348(1):124–131. [PubMed: 16876128]
41. Huster D, Lutsenko S. *J Biol Chem* 2003;278(34):32212–32218. [PubMed: 12794172]
42. Sarkar B. *J Inorg Biochem* 2000;79(1–4):187–191. [PubMed: 10830865]
43. Ralle M, Lutsenko S, Blackburn NJ. *J Inorg Biochem* 2004;98(5):765–774. [PubMed: 15134922]
44. Sato M, Gitlin JD. *J Biol Chem* 1991;266(8):5128–5134. [PubMed: 2002050]
45. Gitlin JD, Schroeder JJ, Lee-Ambrose LM, Cousins RJ. *Biochem J* 1992;282 (Pt 3):835–839. [PubMed: 1554368]
46. Lockhart PJ, Mercer JF. *Gene* 1999;236(2):251–257. [PubMed: 10452945]
47. Forbes JR, Cox DW. *Hum Mol Genet* 2000;9(13):1927–1935. [PubMed: 10942420]
48. Francis MJ, Jones EE, Levy ER, Ponnambalam S, Chelly J, Monaco AP. *Hum Mol Genet* 1998;7(8):1245–1252. [PubMed: 9668166]
49. Tao TY, Liu F, Klomp L, Wijmenga C, Gitlin JD. *J Biol Chem* 2003;278(43):41593–41596. [PubMed: 12968035]
50. Lim CM, Cater MA, Mercer JF, La Fontaine S. *Biochem Biophys Res Commun* 2006;348(2):428–436. [PubMed: 16884690]
51. Lim CM, Cater MA, Mercer JF, La Fontaine S. *J Biol Chem* 2006;281(20):14006–14014. [PubMed: 16554302]
52. Strausak D, La Fontaine S, Hill J, Firth SD, Lockhart PJ, Mercer JF. *J Biol Chem* 1999;274(16):11170–11177. [PubMed: 10196202]
53. Cater MA, Forbes J, La Fontaine S, Cox D, Mercer JF. *Biochem J* 2004;380(Pt 3):805–813. [PubMed: 14998371]
54. Forbes JR, Hsi G, Cox DW. *J Biol Chem* 1999;274(18):12408–12413. [PubMed: 10212214]
55. Payne AS, Gitlin JD. *J Biol Chem* 1998;273(6):3765–3770. [PubMed: 9452509]
56. Iida M, Terada K, Sambongi Y, Wakabayashi T, Miura N, Koyama K, Futai M, Sugiyama T. *FEBS Lett* 1998;428(3):281–285. [PubMed: 9654149]
57. Goodyer ID, Jones EE, Monaco AP, Francis MJ. *Hum Mol Genet* 1999;8(8):1473–1478. [PubMed: 10400994]
58. Tsvikovskii R, MacArthur BC, Lutsenko S. *J Biol Chem* 2001;276(3):2234–2242. [PubMed: 11053407]

59. Paulsen M, Lund C, Akram Z, Winther JR, Horn N, Moller LB. *Am J Hum Genet* 2006;79(2):214–229. [PubMed: 16826513]
60. Achila D, Banci L, Bertini I, Bunce J, Ciofi-Baffoni S, Huffman DL. *Proc Natl Acad Sci U S A* 2006;103(15):5729–5734. [PubMed: 16571664]
61. Banci L, Bertini I, Cantini F, DellaMalva N, Herrmann T, Rosato A, Wuthrich K. *J Biol Chem* 2006;281(39):29141–29147. [PubMed: 16873374]
62. Banci L, Bertini I, Cantini F, Migliardi M, Rosato A, Wang S. *J Mol Biol* 2005;352(2):409–417. [PubMed: 16083905]
63. Banci L, Bertini I, Ciofi-Baffoni S, Chasapis CT, Hadjiliadis N, Rosato A. *Febs J* 2005;272(3):865–871. [PubMed: 15670166]
64. Banci L, Bertini I, Del Conte R, D'Onofrio M, Rosato A. *Biochemistry* 2004;43(12):3396–3403. [PubMed: 15035611]
65. Jones CE, Daly NL, Cobine PA, Craik DJ, Dameron CT. *J Struct Biol* 2003;143(3):209–218. [PubMed: 14572476]
66. DiDonato M, Hsu HF, Narindrasorasak S, Que L Jr, Sarkar B. *Biochemistry* 2000;39(7):1890–1896. [PubMed: 10677240]
67. Walker JM, Huster D, Ralle M, Morgan CT, Blackburn NJ, Lutsenko S. *J Biol Chem* 2004;279(15):15376–15384. [PubMed: 14754885]
68. DeSilva TM, Veglia G, Opella SJ. *Proteins* 2005;61(4):1038–1049. [PubMed: 16211579]
69. Gitschier J, Moffat B, Reilly D, Wood WI, Fairbrother WJ. *Nat Struct Biol* 1998;5(1):47–54. [PubMed: 9437429]
70. Yatsunyk LA, Rosenzweig AC. *J Biol Chem*. 2007
71. Cobine PA, George GN, Winzor DJ, Harrison MD, Moghaddas S, Dameron CT. *Biochemistry* 2000;39(23):6857–6863. [PubMed: 10841766]
72. Guo Y, Nyasae L, Braiterman LT, Hubbard AL. *Am J Physiol Gastrointest Liver Physiol* 2005;289(5):G904–916. [PubMed: 15994426]
73. Lockhart PJ, Mercer JF. *Eur J Cell Biol* 2001;80(5):349–357. [PubMed: 11432725]
74. Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. *Science* 1999;284(5415):805–808. [PubMed: 10221913]
75. Hamza I, Faisst A, Prohaska J, Chen J, Gruss P, Gitlin JD. *Proc Natl Acad Sci U S A* 2001;98(12):6848–6852. [PubMed: 11391006]
76. Pufahl RA, Singer CP, Peariso KL, Lin SJ, Schmidt PJ, Fahrni CJ, Culotta VC, Penner-Hahn JE, O'Halloran TV. *Science* 1997;278(5339):853–856. [PubMed: 9346482]
77. Wernimont AK, Huffman DL, Lamb AL, O'Halloran TV, Rosenzweig AC. *Nat Struct Biol* 2000;7(9):766–771. [PubMed: 10966647]
78. Ralle M, Lutsenko S, Blackburn NJ. *J Biol Chem* 2003;278(25):23163–23170. [PubMed: 12686548]
79. Larin D, Mekios C, Das K, Ross B, Yang AS, Gilliam TC. *J Biol Chem* 1999;274(40):28497–28504. [PubMed: 10497213]
80. Banci L, Bertini I, Cantini F, Felli IC, Gonnelli L, Hadjiliadis N, Pierattelli R, Rosato A, Voulgaris P. *Nat Chem Biol* 2006;2(7):367–368. [PubMed: 16732294]
81. Walker JM, Tsivkovskii R, Lutsenko S. *J Biol Chem* 2002;277(31):27953–27959. [PubMed: 12029094]
82. Sanokawa-Akakura R, Dai H, Akakura S, Weinstein D, Fajardo JE, Lang SE, Wadsworth S, Siekierka J, Birge RB. *J Biol Chem* 2004;279(27):27845–27848. [PubMed: 15133031]
83. Davies TH, Sanchez ER. *Int J Biochem Cell Biol* 2005;37(1):42–47. [PubMed: 15381148]
84. Wernimont AK, Yatsunyk LA, Rosenzweig AC. *J Biol Chem* 2004;279(13):12269–12276. [PubMed: 14709553]
85. Arnesano F, Banci L, Bertini I, Ciofi-Baffoni S, Molteni E, Huffman DL, O'Halloran TV. *Genome Res* 2002;12(2):255–271. [PubMed: 11827945]
86. Huffman DL, O'Halloran TV. *Annu Rev Biochem* 2001;70:677–701. [PubMed: 11395420]
87. Banci L, Bertini I, Cantini F, Chasapis CT, Hadjiliadis N, Rosato A. *J Biol Chem* 2005;280(46):38259–38263. [PubMed: 16172131]

88. Hamza I, Prohaska J, Gitlin JD. *Proc Natl Acad Sci U S A* 2003;100(3):1215–1220. [PubMed: 12538877]
89. Pase L, Voskoboinik I, Greenough M, Camakaris J. *Biochem J* 2004;378(Pt 3):1031–1037. [PubMed: 14640979]
90. McIntosh DB, Clausen JD, Woolley DG, MacLennan DH, Vilsen B, Andersen JP. *J Biol Chem* 2004;279(31):32515–32523. [PubMed: 15133025]
91. Mercer JF, Barnes N, Stevenson J, Strausak D, Llanos RM. *Biometals* 2003;16(1):175–184. [PubMed: 12572677]
92. Bartee MY, Lutsenko S. *Biometals*. 2007
93. Stephenson SE, Dubach D, Lim CM, Mercer JF, La Fontaine S. *J Biol Chem* 2005;280(39):33270–33279. [PubMed: 16051599]
94. Vanderwerf SM, Cooper MJ, Stetsenko IV, Lutsenko S. *J Biol Chem* 2001;276(39):36289–36294. [PubMed: 11470780]
95. Voskoboinik I, Fernando R, Veldhuis N, Hannan KM, Marmy-Conus N, Pearson RB, Camakaris J. *Biochem Biophys Res Commun* 2003;303(1):337–342. [PubMed: 12646208]
96. Vanderwerf SM, Lutsenko S. *Biochem Soc Trans* 2002;30(4):739–741. [PubMed: 12196182]
97. Hamza I, Schaefer M, Klomp LW, Gitlin JD. *Proc Natl Acad Sci U S A* 1999;96(23):13363–13368. [PubMed: 10557326]
98. Hsi G, Cullen LM, Moira Glerum D, Cox DW. *Genomics* 2004;83(3):473–481. [PubMed: 14962673]
99. Dagenais SL, Adam AN, Innis JW, Glover TW. *Am J Hum Genet* 2001;69 (2):420–427. [PubMed: 11431706]
100. Petris MJ, Camakaris J, Greenough M, LaFontaine S, Mercer JF. *Hum Mol Genet* 1998;7(13):2063–2071. [PubMed: 9817923]
101. Francis MJ, Jones EE, Levy ER, Martin RL, Ponnambalam S, Monaco AP. *J Cell Sci* 1999;112 (Pt 11):1721–1732. [PubMed: 10318764]
102. Lane C, Petris MJ, Benmerah A, Greenough M, Camakaris J. *Biometals* 2004;17(1):87–98. [PubMed: 14977365]
103. Cater MA, La Fontaine S, Shield K, Deal Y, Mercer JF. *Gastroenterology* 2006;130(2):493–506. [PubMed: 16472602]

Figure 1. The dual role of copper-transporting ATPase ATP7B in hepatocyte

Copper enters the cell from the basolateral membrane via high-affinity copper transporter Ctr1 and is delivered to various cell targets with the help of copper chaperones. Atox1 transfers copper to ATP7B located in the TGN. ATP7B transports copper into the lumen of TGN, where copper is incorporated into ceruloplasmin (CP), which is subsequently excreted into the blood. When copper is elevated (red arrow), ATP7B traffics to vesicles. Vesicles filled with copper fuse with the apical (canalicular) membrane, copper is exported, and ATP7B is rapidly endocytosed. When copper is decreased ATP7B returns back to the TGN. It is possible that Atox1 regulates both copper delivery to ATP7B when copper is high and copper removal from ATP7B when copper is low.

Figure 2. Trans-membrane organization and catalytic cycle of human Cu-ATPases

(A) Cartoon illustrating the major functional domains of Cu-ATPases. The N-terminal domain contains six copper-binding MBDs (MBD1–6). The transmembrane portion has eight TMS; the position of residues predicted to be involved in copper coordination (CPC, YN, and MxxS) is indicated. The A-domain may link changes in the N-terminal domain with those in the ATP-binding domain and in the transmembrane portion. The ATP-binding domain consists of the P-domain and the N-domain. The domains of ATP7B for which structure has been experimentally determined are indicated by dashed circles and corresponding structures are shown. Two Leu residues in the C-terminal tails required for endocytosis and/or return to TGN are indicated by “LL”. (B) The simplified catalytic cycle of human Cu-ATPases. Two major conformational states associated with high affinity for ATP and Cu (E1) and lower affinity for these ligands (E2) as well as phosphorylated intermediates (E1-Pi-Cu and E2-Pi-Cu) are shown

Figure 4. Metal binding pockets of N-terminal MBD2 and MBD3

(A) Comparison of the overall structure for the “typical” N-terminal domain, MBD2, and MBD3 of ATP7A according to (61,64). The corresponding secondary structure elements in two MBDs are indicated by the same color, the position of CxxC motif is indicated by red colors and letters. (B) The details of the metal binding pockets of N-terminal MBD2 and MBD3 in apo (left) and Cu-loaded (right) forms. Copper binding pocket of MBD2 of ATP7A (61) may be considered representative of the other MBDs of ATP7A and ATP7B. In MBD2, the loop containing GMxCxxC motif is flanked internally by the conserved Phe66. Copper binding to this region is associated with a small conformational shift (left). In the isolated MBD3 of ATP7A (58), Tyr69 performs the function of Phe66 and a larger rearrangement is necessary

to coordinate copper. Copper coordinating cysteines are shown in green; copper is shown as a blue sphere. Letter R in the circles indicates side-chains non-coordinating amino-acid residues; they are shown to emphasize the rearrangement of the backbone upon copper binding

Figure 5. Schematic of N-Terminal domains of ATP7A (A) and ATP7B (B)

The length of inter-domain loops (in amino acid residues) is indicated. Loop is defined as the segment between folded 72-aa MBDs. (i) The loop between metal binding sites 1 and 2 of ATP7A is predicted to have a structure similar to the other metal binding sites, but lacks copper-coordinating residues. (ii) Metal binding sites 5 and 6 have been shown to be necessary for proper trafficking of ATP7A (87). (iii) The very N-Terminal region of ATP7A is also essential for proper trafficking and membrane targeting (69). (iv) MBS 2 has been shown to be the primary site of copper transfer from Atox1 (64). (v) MBS 4 has a higher affinity for Atox1 than does MBS2, and also has been shown capable of transferring copper to MBSs 5 and 6 (57). However, in rat WNDP, this MBD lack CxxC motif and is not capable of copper binding. (vi) MBDs 5 and 6 are essential for copper transport, and have been shown to regulate affinity of the Intra-membrane copper binding site (40). (vii) Location of the G591D mutation, a disease causing mutation that disrupts Atox1 interactions with the N-Terminal domain.

Figure 6. The ATP-binding domain of Cu-ATPases (based on the structure of CopA (35))
The invariant residues in proximity to the adenine moiety in the N-domain are indicated by the yellow color. The invariant Asp in the DKTG motif and the GDGxND sequence are marked in red. The position of residue equivalent to Asp1230 of ATP7A in the DxxK motif is in blue.