Molecular Mechanism of Copper Transport in Wilson Disease

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Wilson disease is an autosomal recessive disorder of copper metabolism. The Wilson disease protein is a putative copper-transporting P-type ATPase, ATP7B, whose malfunction results in the toxic accumulation of copper in the liver and brain, causing the hepatic and/or neurological symptoms accompanying this disease. The cytosolic N-terminal domain (~70 kDa) of this ATPase comprises six heavy metal–associated domains, each of which contains the conserved metal-binding motif GMTCXXC. The N-terminal domain (Wilson disease copper-binding domain [WCBD]) has been expressed, purified, and characterized using various techniques. The WCBD binds six atoms of copper in the +1 oxidation state competitively, and with a greater affinity than all other metals. The copper atom is coordinated by two cysteines in a distorted linear geometry. Copper binds the WCBD in a cooperative manner and induces secondary and tertiary conformation changes. Zinc binding to the WCBD has also been characterized by circular dichroism spectroscopy and shown to produce conformational changes that are completely different from those induced by copper. The phosphorylation/nucleotide-binding domain of ATP7B has also been expressed and characterized and shown to be capable of binding ATP but lacking ATPase activity. A peptide corresponding to the sixth transmembrane domain of ATP7B has been constructed and shown to undergo secondary conformational changes upon binding a single atom of copper. Finally, a chimeric protein consisting of the WCBD and truncated ZntA, a zinc-transporting ATPase lacking the N-terminal domain, has been constructed and analyzed for metal ion selectivity. These results suggest that the core determines the metal ion specificity of P-type ATPases, and the N-terminal metal-binding domain may play a regulatory role. *Key words:* **ATP7B/ZntA chimera, copper trafficking, copper transport, copper-ATPase, copper binding, nucleotide-binding domain, phosphorylation domain, P-type ATPases, Wilson disease, Wilson disease gene.** *Environ Health Perspect* **110(suppl 5):695–698 (2002).**
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Wilson Disease

Wilson disease is a hereditary hepatic disease with neurological symptoms that was first described by Kinnear Wilson in 1912 (*1*). This disorder of copper metabolism is characterized by the toxic accumulation of copper in various tissues such as the liver, kidney, brain, and placenta due to the lack of biliary excretion of copper from the body (*2*). Elevated urinary copper levels are observed, due to the accumulation of copper in the kidneys, and impaired incorporation of copper into ceruloplasmin leads to lowered serum copper levels. Increased liver copper concentrations are due to the deficient biliary excretion of copper from the hepatocytes. Chelation and zinc therapy are two treatments used for Wilson disease. Chelators such as D-penicillamine (*3,4*) and trientine (*5,6*) are used to mobilize copper and facilitate its excretion from the body through urine. Zinc is used to prevent copper uptake from the intestine into portal circulation by inducing the synthesis of metallothionein (*7*). Metallothionein binds copper with high affinity and is subsequently eliminated in the feces as intestinal cells are sloughed off (*8,9*). Liver transplantation may be the only hope for patients with acute liver failure, which cannot be reversed with chelation or zinc therapy.

The Wilson Disease Gene and Its Expression

The Wilson disease gene *ATP7B* was localized to the q14.3 band of chromosome 13 and cloned by two independent groups in 1993 (*10,11*). The gene consists of 22 exons and encodes a copper-transporting P-type ATPase (ATP7B) belonging to and sharing many of the features of the cation-transporting P-type ATPase family (*12*). The Wilson disease protein is expressed mostly in the liver (*10*) and has been localized to the *trans*-Golgi network (TGN) by immunohistochemical studies (*13,14*). Such studies have also shown the trafficking of the ATPase from the TGN to cytoplasmic vesicles in response to an increase in copper concentration (*13,15*); this copper-dependent cycling of ATP7B probably accounts for the biliary excretion of copper from the liver and correlates well with the Wilson disease phenotype.

The Wilson Disease Copper-Transporting P-Type ATPase

Sequence analysis has identified the Wilson disease ATPase as a copper-transporting P-type ATPase (Figure 1). More specifically, the Wilson disease ATPase has several features distinguishing it from other members of the P-type ATPase family, classifying it as a CPx-type, type I, or heavy-metal P-type ATPase. Other members of the CPx-type ATPases are the bacterial copper (CopA) (*16*) and zinc (ZntA) (*17*) ATPases, and in humans, the Menkes disease copper-transporting ATPase (ATP7A) (*18–20*). The major difference between the CPx-type ATPases and other P-type ATPases is the presence of an additional pair of transmembrane helices and a cytoplasmic metal-binding domain at the N terminus. In addition to a pair of cysteines flanking the conserved proline residue in the transduction domain, the histidine and proline residues of the SEHPL sequence motif are highly conserved in heavy metal–transporting ATPases. The mutation of the conserved histidine residue of this motif, H1069Q, is one of the most common mutations found in Wilson disease (*21,22*). The involvement and the role of the SEHPL motif in the copper transport scenario are still not clear, although its importance is reflected in its conservation and its correspondence to a disease-causing mutation. Site-directed mutagenesis of the conserved histidine reveals that the motif is somehow involved in metal-ion–stimulated ATPase activity and phosphorylation of the transporter (*23,24*).

In addition to its ATP-driven copper transport role at the TGN where copper is incorporated into ceruloplasmin (*25,26*), the Wilson disease protein is also thought to be involved in the excretion of copper into bile at the canalicular membrane (*27*). The copper-stimulated trafficking of the transporter between the TGN and the canalicular membrane may involve the N terminus and is not clearly understood (*28*). It has been shown that the presence of at least one copper-binding domain close to the membrane channel is necessary for copper-induced redistribution of both the Wilson (*29*) and the Menkes disease transporter (*30*). Copper-induced conformational changes observed in the N-terminal copper-binding domain of the

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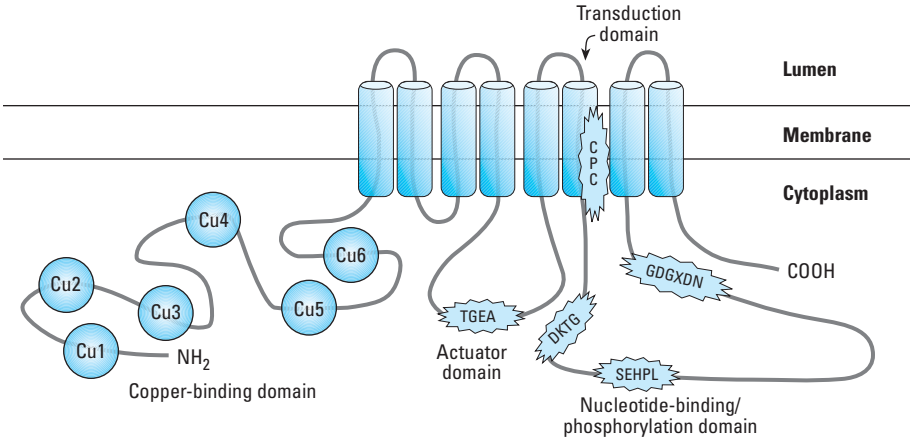

Figure 1. The Wilson disease protein is a copper-transporting CPx-type ATPase. The transporter consists of transmembrane, phosphorylation, nucleotide-binding, and actuator domains common to other P-type ATPases. Features unique to CPx-type ATPases include a large cytoplasmic metal-binding domain containing between one and six metal-binding motifs, a pair of N-terminal transmembrane helices, CPC, and the SEHPL motifs.

Wilson disease ATPase (WCBD) have been suggested as a mechanism behind this cellular trafficking. Protein–protein interactions or changes in the global conformation of the transporter may render a recognition site accessible to the components of the membrane-protein–sorting machinery and signal the protein to traffic between the TGN and the plasma membrane in a copper-dependent manner. Site-directed mutagenesis of residues in transmembrane helices 4 and 6 has implicated their involvement in the copper-dependent trafficking of the transporter. Tyrosine and dileucine motifs (*31,32*) in the Menkes disease transporter C terminus have also been suggested as recognition and *trans*-Golgi retention signals recognized by the vesicular trafficking machinery.

Characterization of Copper Binding to the WCBD

The WCBD has been the subject of intense study in our laboratory. This 70 kDa N-terminal domain encompassing all six metal-binding motifs has been expressed, purified, and shown to bind six atoms of copper in the +1 oxidation state. Using immobilized metal affinity chromatography, we have shown that the WCBD is able to bind different transition metals with varying affinities: Cu(II) > > Zn(II) > Ni(II) > Co(II) (*33*).

We employed competition ^{65}Zn blotting analysis to investigate the ability of the WCBD to bind copper and other transition metals (*33*). Of the transition metals tested, Cd(II), Au(III), and Hg(II) were able to successfully compete with zinc for binding to the domain. Copper was the strongest competitor and displayed a distinct cooperative binding mechanism not observed with the other transition metals.

Our X-ray absorption spectroscopy (XAS) studies of the WCBD containing substoichiometric amounts of copper have provided a wealth of detailed structural information regarding this domain (*34*). The X-ray absorption near edge structure spectra display a characteristic feature of the 1s to 4p transition of Cu(I) at 8,983 eV, verifying that copper bound to the WCBD is in the +1 state. The intensity of the transition at 8,983 eV, which is indicative of the geometry around the copper atom, is weaker than that of linear copper thiolate complexes but stronger than that of trigonal compounds. Extended X-ray absorption fine structure data show that the first coordination sphere consists of two sulfur atoms with a Cu–S distance of 2.17–2.19 Å. This is similar to the Cu–S bond distance observed in Menkes disease protein and falls between the distances observed for trigonal and linear Cu(I)–thiolate complexes (*35*). These observations suggest that the copper atom is coordinated by two cysteines in a distorted linear geometry.

Circular dichroism (CD) spectroscopy results show that copper binding induces conformational changes in the WCBD (*34*). The secondary structure region (200–270 nm) shows an increase in ellipticity upon binding of increasing amounts of copper, suggesting a stabilization of secondary structures relative to the apo state. The changes observed in the aromatic region (250–350 nm) were in agreement with those in the secondary structure region. The greatest changes in the spectra occur between the 2:1 and 4:1 copper-bound forms. The 2:1 and 4:1 copper-bound forms have very similar secondary structure but significantly different tertiary structure, which may reflect the cooperative nature of copper binding.

Characterization of Zinc Binding to the WCBD

Studies in this laboratory have also characterized the binding of zinc to the WCBD (*36*). The WCBD is able to bind six molar equivalents of zinc and undergo conformational changes that are completely different from those induced by copper binding. Our CD spectral analyses show that zinc binding is accompanied by an overall loss of secondary structure. This is further supported by our XAS data that indicate that the zinc-binding sites are occupied mostly by nitrogen and not sulfur atoms. Therefore, although the WCBD has the ability to bind several different metals, the different conformations induced by different metals may allow the transporter to differentiate between copper and other metals *in vivo*. To delineate the metal ion selectivity and to investigate whether this domain contributes to metal ion recognition by the transporter, a collaborative effort was undertaken to construct an ATP7B/ZntA chimeric protein (*37*).

Characterization of Metal Ion Selectivity of the Chimeric ATPase

ZntA is a CPx-type ATPase from *Escherichia coli*, which confers resistance to Pb(II), Cd(II), and Zn(II) (*38*). This protein has a single copy of the metal-binding motif, whereas ATP7B has six copies. Two chimeric proteins have been constructed in which the N-terminal of ZntA is replaced with either the entire N-terminal domain of ATP7B or just the sixth metal-binding motif of ATP7B (*37*). Both chimeras confer resistance to and display activity with Pb(II), Cd(II), and Zn(II), all of which are substrates of ZntA. There is no resistance or activity toward copper and silver, which are the substrates of ATP7B. Although the N-terminal domain of ZntA is not essential for its activity or selectivity toward a particular metal, it is required for full catalytic activity and cannot be replaced by the N-terminal domain of ATP7B. The results of this study suggest that the core of the P-type ATPase determines metal ion specificity and that the N-terminal plays a regulatory role, perhaps by interacting in a metal-ion–specific manner with the other parts of the transporter. Copper binding to the WCBD appears to elicit the conformational changes required to regulate the activity of ATP7B.

Core Elements within the Transduction Channel May Determine Substrate Specificity

Ca-ATPase (*39*), Na,K-ATPase (*40*), and H-ATPase (*41*) are three P-type ATPases for which a great deal of structural information is available. In these P-type ATPases, transmembrane domains M4, M5, and M6 form part of the channel and contain residues critical to

cation binding. In ATP7B, transmembrane domains TM6 and TM7 are predicted to correspond to M4 and M5 of P-type ATPases and form part of the channel (*42*). In a clever experiment that highlighted the central role of M4, Na,K-ATPase's cation-binding specificity was altered to that of H,K-ATPase by mutating residues within the channel (*43*). TM6 of ATP7B corresponds to M4 of Ca-ATPase, and both transmembrane domains contain a conserved proline residue found in all P-type ATPases. In the heavy metal ATPases, highly conserved cysteine residues flank this proline residue to form a CPC motif. Mammalian copper-transporting ATPases have an additional conserved cysteine, forming a CXXCPC motif. Site-directed mutagenesis studies of the cysteine residues in the CPC motif have revealed it to be essential for the copper transport function of the ATPase (*24,44*). The CPC motif is predicted to be one of the copper-binding sites in the channel.

Identification of Core Residues Involved in Metal Ion Binding and Specificity

To further characterize copper binding to the CPC motif, we constructed a peptide corresponding to residues from TM6 of ATP7B . Single C/S mutants of this peptide have also been synthesized. Preliminary CD results show that the peptide binds a single atom of copper and that copper binding induces secondary conformational changes in the peptide (*45*). Further studies in this area are aimed at the identification of other residues within the transduction channel that confer copper selectivity to ATP7B.

Characterization of the Phosphorylation/Nucleotide -Binding Domain

The second largest cytosolic domain of ATP7B, which encompasses the phosphorylation (P) subdomain, nucleotide-binding (N) subdomain, and the hinge region, has been expressed and purified in our laboratory and by others. In our laboratory, it has the ability to bind the fluorescent ATP analog TNP-ATP, but it has no ATPase activity (*46*). We speculate that this is may be due to the absence of other domains required for ATPase activity, in particular the actuator (A) domain. Mutational as well as structural analyses of other P-type ATPases suggest the involvement of the A domain in energy transduction and hydrolysis of the phosphoenzyme intermediate, formed during the catalytic cycle (*39*).

Gapped BLAST (basic local alignment search tool) alignment of Cu-ATPase and Ca-ATPase (*42*), together with the presence of highly conserved residues, suggests that the general mechanism and cation transport proposed for P-type ATPases likely apply to CPx-type heavy metal–transporting ATPases as well (*39,47*).

The sequence alignment of P-type and CPx-type ATPases reveals that ATP7B has large deletions in its A domain and also in its N domain. The P domain, however, is highly conserved. These observations give rise to a number of questions regarding how these differences in corresponding domains affect the mechanism of copper transport by ATP7B compared with the general mechanism proposed for P-type ATPases.

Proposed Mechanism for Copper Transport by the Wilson Cu-ATPase

Atox1, implicated as the metallochaperone for ATP7B, probably delivers copper ions to the WCBD (*48–50*). Atox1 itself has a copper-binding motif and is thought to specifically interact through complementary electrostatic surfaces with the copper-binding motifs and exchange copper (*3*). However, this may not be the only way by which the WCBD obtains its copper. Not all the copper-binding motifs found in the WCBD possess the complementary electrostatic patches necessary for interaction with Atox1 (*51*), and the list of other possible copper-binding proteins is growing. Preliminary metalloproteomic studies in our laboratory have identified a number of proteins, previously not classified as possessing any copper-binding activity. Many of these proteins themselves contain the CXXC motif or are associated in complexes with proteins that contain the CXXC motif (*52*). Interestingly, some of these proteins are also involved in the protein folding and disulfide bond isomerization pathways. Further investigation is required before any of these other candidates can be ruled out for delivery of copper to ATP7B.

On the basis of the degree of similarity between ATP7B and other P-type ATPases and structural/functional studies of this transporter, we can begin to form a model for the mechanism of copper transport by ATP7B based on the model proposed for classical P-type ATPases (*39,53*). The WCBD probably serves as the initial site for metal ion binding to the transporter. Specific interaction between the WCBD and its nucleotide binding/phosphorylation domain has been demonstrated, and binding of copper to the WCBD has been shown to dissociate this interaction (*54*). Although the WCBD has the ability to bind different metals, zinc binding studies seem to suggest that only the binding of copper induces the correct conformational changes necessary for the WCBD to dissociate from the other domain. This conformational change is tied to cytoplasmic copper concentrations, so it is possible for the copper-bound state of the WCBD to regulate the activity of the transporter. Copper binding to the WCBD may be what drives the transporter from an inactive or low activity state, where the cytoplasmic domains are all bound by low-copper WCBD, to an active state where the high-copper WCBD has released the other cytoplasmic domains.

Although the cytoplasmic domains of ATP7B and Ca-ATPase are very similar, there are large deletions and sequence alterations observed in the actuator domain and nucleotide-binding domains of ATP7B, which may allow for the specific interaction of the WCBD with these domains. After the dissociation of the domains, the mechanism of copper transport most likely progresses through the same E_1–E_2 intermediates that are proposed for other P-type ATPases (*39*).

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