Expression, Purification, and Metal Binding Properties of the N-terminal Domain from the Wilson Disease Putative Copper-transporting ATPase (ATP7B)*

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The putative copper binding domain from the copper-transporting ATPase implicated in Wilson disease (ATP7B) has been expressed and purified as a fusion to glutathione S-transferase. Immobilized metal ion affinity chromatography revealed that the fusion protein is able to bind to columns charged with different transition metals with varying affinities as follows: Cu(II) > Zn(II) > Ni(II) > Co(II). The fusion protein did not bind to columns charged with Fe(II) or Fe(III). Competition Zn(II) blotting analysis showed that the domain is able to bind Zn(II) over a range of pH values from 6.5 to 9.0. Competition Zn(II) blotting showed that Cd(II), Hg(II), Mg(II), Mn(II), and Ni(II) relative to copper. Neutron activation analysis of the copper bound protein showed a copper-protein ratio of 6.5-7:1. Both Cu(II) and Cu(I) were found to have a higher affinity for the domain relative to Zn(II). In addition, a sharp, reproducible transition was only observed in competition experiments with copper, which may suggest that copper binding has some degree of cooperativity.

Copper is an essential trace element which forms an integral component of many developmentally important enzymes (1, 2). However, while trace amounts of copper are needed to sustain life, excess copper is extremely toxic. Although many aspects of copper transport and metabolism have been studied in the past, little is known about the specifics of intracellular copper trafficking. The cloning of the genes responsible for two major genetic disorders of copper metabolism in humans, Menkes disease (3-5) and Wilson disease (6, 7), has added an important genetic disorder of copper metabolism in humans, Menkes disease (3-5) and Wilson disease (6, 7) to the copper transport puzzle. Both genes have been found to have a higher affinity for the domain relative to Zn(II). In addition, a sharp, reproducible transition was only observed in competition experiments with copper, which may suggest that copper binding has some degree of cooperativity.

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The abbreviations used are: GST, glutathione S-transferase; WCBD, Wilson disease copper binding domain (residues 1–649); CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; BCS, bathocuproinedisulfonic acid; DTT, dithiothreitol; IMAC, immobilized metal ion affinity chromatography.

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applied to the column. The reaction was incubated at 22 °C for 30–45 min followed by elution of the purified domain in the same buffer. The purified metal binding domain obtained in this manner was then used in competition blotting experiments. Fusion protein, which was localized in inclusion bodies, was solubilized in 6 M urea and refolded essentially as described in Ref. 10 and subjected to glutathione affinity chromatography as described above. Eluted fusion protein was then subjected to anion exchange chromatography on a DEAE-Sepharose matrix equilibrated with elution buffer and eluted with 0.5 M NaCl.

**Immobilized Metal Ion Affinity Chromatography (IMAC)**—Samples of the fusion protein were dialyzed against IMAC buffer (20 mM NaH₂PO₄·H₂O, pH 7.0, 0.5 M NaCl) with or without 6 M urea and applied to chelating Sepharose fast flow columns charged with either Co(II), Ni(II), Zn(II) or Cu(II). Elution of the fusion protein was accomplished by lowering the pH to 6.0 or 4.0 or by the addition of chelators (EDTA, imidazole, or BCS) (see “Results and Discussion”). The ability of GST-WCBD to bind different metals was investigated using immobilized metal ion affinity chromatography. Samples of fusion protein were applied to columns charged with the indicated metal (Fig. 2) under non-denaturing, non-reducing conditions. GST alone was found to have some interactions with the metal columns under non-denaturing conditions and did not bind to the columns under denaturing conditions (data not shown). However, the results presented in Fig. 2 were very similar regardless of whether denaturing or non-denaturing conditions were used suggesting that the major metal binding interactions are from the WCBD. Specific binding of proteins with internal high affinity metal binding sites to IMAC columns under denaturing conditions has also been demonstrated for troponin T which contains four repeated metal binding domains as it is expressed in bacteria. Metal binding under denaturing conditions has been observed with the estrogen receptor DNA binding domain (2 zinc finger protein having 4 Cys in each finger) where low pH, urea, DTT, and chelating agents are needed to remove the bound metal.

**RESULTS AND DISCUSSION**

We have expressed the putative copper binding domain from the Wilson disease protein as a fusion to GST. Fig. 1 illustrates the results of a typical purification. Free WCBD is obtained by thrombin cleavage of the fusion protein while it is still bound to the glutathione affinity column. The free domain obtained by this method is approximately 80–90% pure, and its identity was confirmed by N-terminal sequence analysis. The fusion protein, which was found to be more stable, was obtained at greater than 90% purity using glutathione affinity chromatography and denaturing anion exchange chromatography. Treatment of the fusion protein eluted from the glutathione affinity column with 6 M urea in the absence of reducing agents with BCS gave rise to a reddish-orange color (λₘₐₓ = 480 nm) indicative of the Cu(I)(BCS)₂ complex (12). This suggests that the N-terminal metal binding domain may bind copper in the +1 oxidation state and that copper is being incorporated into the domain as it is expressed in bacteria. Metal binding under denaturing conditions has been observed with the estrogen receptor DNA binding domain (2 zinc finger protein having 4 Cys in each finger) where low pH, urea, DTT, and chelating agents are needed to remove the bound metal (13).

The ability of GST-WCBD to bind different metals was investigated using immobilized metal ion affinity chromatography. Samples of fusion protein were applied to columns charged with the indicated metal (Fig. 2) under non-denaturing, non-reducing conditions. GST alone was found to have some interactions with the metal columns under non-denaturing conditions and did not bind to the columns under denaturing conditions (data not shown). However, the results presented in Fig. 2 were very similar regardless of whether denaturing or non-denaturing conditions were used suggesting that the major metal binding interactions are from the WCBD. Specific binding of proteins with internal high affinity metal binding sites to IMAC columns under denaturing conditions has also been demonstrated for troponin T which contains four repeated metal binding motifs (14). The fusion protein was found to have varying affinities for columns charged with different transition metals. Based on the elution conditions, the order of affinity for the different metals was as follows: Cu(II) > Zn(II) > Ni(II) > Fe(III). No binding to columns charged with either Fe(II) or Fe(III) was observed. The varying affinities may be reflective of the inability of the metal binding sites of the domain to conform to the preferred ligation geometry of certain metals. It is interesting to note that the fusion protein could only be released from the Cu(II) column using the cuprous chelator BCS. Elution of the fusion protein was accompanied by the formation of the orange (λₘₐₓ = 480 nm) Cu(I)BCS₂ complex. This suggests that not only is the bound copper in the +1
A variety of metals, a competition $^{65}$Zn(II) blot was employed. At the upper end of the pH range tested, a decrease in the binding of Zn(II) to the domain is only observed for small amounts of Zn(II) under the same conditions. A significant amount of Zn(II) remains bound to the domain. 

"Experimental Procedures." GST alone did not bind appreciably to the domain. We have also observed binding of other metal ions to the domain. We have also observed binding of other transition metals to the domain. In particular, Cd(II), Au(III), and Hg(II) seem to have the highest affinities for the domain relative to Zn(II), whereas Mn(II) and Ni(II) had little or no affinity relative to zinc.$^2$ This is not surprising since Zn(II), Cd(II), and Hg(II) are in the same group and therefore have similar ligand geometries with iodide radii being the only difference. In contrast to the results found with IMAC, Fe(III) was able to act as a competitor in this experiment. This may result from an oxidation state, but that Cu(II) atoms may be reduced to Cu(I) upon binding to the domain.

The stoichiometry of copper binding to the domain was determined by neutron activation analysis using GST-WCBD which was refolded in the presence of copper. Samples were then dialyzed extensively against 1% formic acid to ensure the estimation of specifically bound copper only. Results showed a copper:protein ratio of 6.5–7.3:1. This suggests that each domain is responsible for binding one copper atom. The residues involved in metal ligation may reside in each domain. However, it is possible that amino acid residues outside of the domains may participate in metal ligation as well. In the case of the WCBD, there are six additional cysteine residues located in the regions between the metal binding domains. These residues may be involved in metal ligation or disulfide bridge formation.

To further investigate the metal binding properties of the domain a $^{65}$Zn(II) blotting assay was employed. This assay has previously been used to identify potential Zn(II)-binding proteins (11). Radioactive Zn(II) was chosen over copper because its longer half-life would facilitate a greater number of experiments, and it is more easily obtainable. Preliminary experiments have shown that the domain is able to bind Zn(II) in this assay and that pretreatment of the membranes with DTT was needed to observe Zn(II) binding (data not shown). The requirement of DTT pretreatment suggests that cysteine residues are directly involved in metal chelation and that a free sulfhydryl is required to chelate the metal. Following SDS-PAGE mixed disulfides may form due to the presence of β-mercaptoethanol in the loading buffer which is known to react with protein sulfhydryls (15, 16). DTT pretreatment would then be required to ensure the reduction of these disulfides. However, inclusion of DTT throughout the blotting experiment significantly reduced the amount of non-specific binding, most likely by chelating weekly bound metal atoms. This supports the finding that the GMTCXXC motif, strictly conserved in each of the metal binding domains of ATP7B as well as many bacterial heavy metal transporters, is crucial for binding (17, 18). The effect of pH on the binding of Zn(II) by the domain was investigated. Fig. 3 illustrates the results from such an analysis. Both the fusion protein and the free domain are able to bind Zn(II) over a range of pH values. The fusion protein appears to bind less zinc than the free domain because there is less of it blotted on the membrane. This was confirmed by staining the membranes post-autoradiography with Amido Black as described under "Experimental Procedures." GST alone did not bind appreciable amounts of Zn(II) under the same conditions. A significant decrease in the binding of Zn(II) to the domain is only observed at the upper end of the pH range tested.

To investigate the possibility that the domain is able to bind a variety of metals, a competition $^{65}$Zn(II) blot was employed. Fig. 4A summarizes the data obtained from such an analysis. Several metals were able to successfully compete with Zn(II) for binding to the domain. In particular, Cd(III), Au(III), and Hg(II) seem to have the highest affinities for the domain relative to Zn(II), whereas Mn(II) and Ni(II) had little or no affinity relative to zinc.$^2$ This is not surprising since Zn(II), Cd(II), and Hg(II) are in the same group and therefore have similar ligand geometries with iodide radii being the only difference. In contrast to the results found with IMAC, Fe(III) was able to act as a competitor in this experiment. This may result from an
inability of the protein to conform to the preferred ligation geometry of iron while it is bound to the column matrix. The reverse is probably true for Ni(II), which was unable to act as a competitor in the blotting experiments but bound to the domain tightly in the IMAC experiments. The binding of metal to the domain is specific since both Mg(II) and Ca(II) did not compete at all for Zn(II) binding.

Fig. 4B summarizes the results for competition blotting experiments involving copper as the competitor. At low concentrations, copper is able to decrease Zn(II) binding by about 30%. However, as the concentration is raised, the affinity for copper seems to increase rapidly. This pattern was only observed for copper and may suggest that copper ligation by the domain is to some degree cooperative. However, further experiments are needed before any conclusions about cooperativity can be made. The pattern is reproducible and is independent of whether copper is presented in the +1 or +2 oxidation state suggesting that the domain has similar affinities for both Cu(I) and Cu(II). The Menkes protein has recently been localized to the trans-Golgi network (19, 20) and has been shown to translocate to the plasma membrane under high copper concentrations (20). This translocation event could not be reproduced by adding Cd(II) or Zn(II). From these studies it has been hypothesized that the metal binding domain not only serves to ligate copper for translocation but also as a copper “sensor.” A similar control mechanism may be in operation for the Wilson disease protein. The domain could act as a copper sensor if the binding of multiple metal atoms is able to induce a conformational change in the domain. In this case copper is transported at low concentrations, but as the concentration of copper rises, the binding of additional metal atoms may lead to a conformational change in the domain. This change in conformation may then allow the Wilson disease protein to be translocated to another location, perhaps the canicular membrane, where it could help excrete excess copper into the bile.

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