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). 65Zn(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 65Zn(II) blotting showed that Cd(II), Hg(II), and other metals charged with different transition metals with varying affinities as follows: Cu(II) > Zn(II) > Ni(II) > Co(II). The competition protein did not bind to columns charged with Fe(II) or Fe(III), 65Zn(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 65Zn(II) blotting showed that Cd(II), Hg(II), Au(III), and Fe(III) can successfully compete with Zn(II), at comparable concentrations, for binding to the domain. In contrast, the domain had little or no affinity for Cu(II), Mg(II), Mn(II), and Ni(II) relative to copper. Neutron activation analysis of the copper bound to the domain showed a copper:protein ratio of 6.5–7.3:1. 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 new dimension and a new perspective to the copper transport puzzle. Both genes have bacterially expressed and purified this domain as a fusion to GST and investigated its metal binding properties.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of the highest purity available and all buffers were argon-purged for at least 20 min unless otherwise stated. 65ZnCl₂ (2.95 μCi/ml, 12.2 mg of zinc/ml) was obtained from Amersham Life Sciences. Tetrakis(acetonitrile)copper(I) hexafluorophosphate was obtained from Aldrich Chemical Co.

Construction of pGEX-WCBD Expression Vector—The 2-kilobase cDNA encoding the WCBD (residues 1–649) was produced in two fragments from total human liver RNA using reverse transcriptase-polymerase chain reaction. Primers were designed to create a BamHI restriction site at the 5′ end and a SalI site at the 3′ end. The following primers were used: 5′-TATCGGATCCATGCCTGAGCAGGAG-3′ and 5′-AACCTTTAAATCTCCAGGTTG-3′; 3′ end; 5′-ACTTGC-TGACATCAGCTCCATATCCCATTTTG-3′ and 5′-GGAATGCATTTG-TAACTTTAAAATTCCCAGGTGG-3′. The sequence of the constructs was confirmed by dideoxy sequencing and cloned into the GST fusion expression vector pGEX-4-T-2 (Pharmacia Biotech Inc.) to create the vector pGEX-WCBD.

Expression and Purification—GST-WCBD was expressed in the Escherichia coli strain BL21(DE3) at midlog phase of bacterial growth (A₉₀₀ of 0.6–0.8) by induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. The bacteria were lysed by two cycles of freeze/thaw in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml lysozyme). The lysate was centrifuged at 118,000 × g for 45 min. The soluble fraction was applied to a glutathione Sepharose 4B affinity matrix and eluted with 20 mM Tris-HCl, pH 8.0, 0.6 M urea, 75 mM NaCl, 1 mM EDTA, 1 mM DTT (elution buffer). When required, the column-bound fusion protein was digested with thrombin to release the WCBD alone in the following manner: 10 units of thrombin/mg of fusion protein was added to 1 bed volume of phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) and allowed to incubate for 2 h at 25 °C. EDTA (1 mM) and EGTA (1 mM) were added to the elution buffer to displace the WCBD from the GST matrix. The flowthrough was collected and concentrated for use in further experiments.

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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.
Metal Binding Properties of the Copper Binding Domain from ATP7B

Fig. 1. Summary of typical purification of WCBD and GST-WCBD. GST-WCBD was expressed and purified as described under "Experimental Procedures." A, purified WCBD eluted from glutathione-Sepharose-4B affinity chromatography was digested with thrombin on the column as described under "Experimental Procedures." B, GST-WCBD eluted with urea from glutathione affinity resin following solubilization and refolding from inclusion bodies. C, purified GST-WCBD eluted from DEAE-Sephacel anion exchange resin.

applied to the column. The reaction was incubated at 22 °C for 30–45 min following 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-Sephacel 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, pH 7.0, 0.5 M NaCl) with or without 6 M urea and 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-Sephacel matrix equilibrated with elution buffer and eluted with 0.5 M NaCl.

Fig. 2. Comparison of GST-WCBD binding to various metal columns in IMAC under non-denaturing conditions. Samples of GST-WCBD were dialyzed against IMAC buffer and applied to chelating Sepharose FF resin charged with the indicated metal. Various elution conditions appear across the top of the gel. The metals are arranged in order of increasing strength of binding. No binding was observed to columns charged with iron. Note that elution from the Cu(II) column is achieved only though the use of cuprous chelator BCS. Very similar results were obtained using denaturing conditions.

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) >> Co(II). 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(II)BCS2 complex. This suggests that not only is the bound copper in the +1...
 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 65Zn(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 ligate 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 nonspecific binding, most likely by chelating any Zn(II) in the sample and ascorbate (data not shown).

The effect of pH on the binding of Zn(II) was investigated. Fig. 3 presents as the CuCl₂ complex while Cu(I) was presented as tetrakis(acetonitrile) copper(I) hexafluorophosphate. The Cu(I) complex was prepared in an argon-purged solution of 2% acetonitrile. Similar results were obtained when Cu(I) was generated in vitro using CuCl₂ and ascorbate (data not shown).

The competition of Cu(I) binding to the domain was performed as described under “Experimental Procedures.” In each case the final 65Zn(II) concentration is 30 μCi (~10 μM). A, competition of 65Zn(II) binding with various transition metals. Each competitor metal was added as the chloride salt at the indicated concentration. Successful competition resulted in a decreased signal relative to the control. Ni(II) and Mn(II) showed little or no affinity for the domain relative to Zn(II). Mg(II) and Cd(II) showed no affinity for the domain relative to zinc. B, competition of 65Zn(II) binding with Cu(II) (●) and Cu(I) (○). Cu(I) was presented as the CuCl₂ complex while Cu(I) was presented as tetrakis(acetonitrile)copper(I) hexafluorophosphate. The Cu(I) complex was prepared in an argon-purged solution of 2% acetonitrile. Similar results were obtained when Cu(I) was generated in vitro using CuCl₂ and ascorbate (data not shown).


during the review of this manuscript a paper was published describing the metal binding properties of the MBP-fused metal domains of the Wilson and Menkes proteins (21). The authors report results similar to those in this paper. However, we have used a wider range of metals and two independent techniques to assess metal specificity of the domain. We have also observed binding of other transition metals to the domain.
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 affinity for copper 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.

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