N-terminal Domains of Human Copper-transporting Adenosine Triphosphatases (the Wilson’s and Menkes Disease Proteins) Bind Copper Selectively in Vivo and in Vitro with Stoichiometry of One Copper Per Metal-binding Repeat*

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N-terminal domains of the Wilson’s and Menkes disease proteins (N-WND and N-MNK) were overexpressed in a soluble form in Escherichia coli as fusions with maltose-binding protein, purified, and their metal-binding properties were characterized. Both N-MNK and N-WND bind copper specifically as indicated by the results of metal-chelate chromatography, direct copper-binding measurements, and chemical modification of Cys residues in the presence of different heavy metals.

When E. coli cells are grown in the presence of copper, N-MNK and N-WND bind copper in vivo with stoichiometry of 5–6 nmol of copper/nmol of protein. Copper released from the copper-N-MNK and copper-N-WND complexes reacts with the Cu(I)-selective chelator biocinchoninic acid in the absence of reducing agents. This suggests that in proteins, it is bound in reduced Cu(I) form, in agreement with the spectroscopic properties of the copper-bound domains.

Copper bound to the domains in vivo or in vitro specifically protects the N-MNK and N-WND against labeling with the cysteine-directed probe; this indicates that Cys residues in the repetitive motifs GMTCCXXXIE are involved in coordination of copper.

Direct involvement of the N-terminal domains in the binding of copper suggests their important role in copper-dependent functions of human copper-transporting adenosine triphosphatases (Wilson’s and Menkes disease proteins).

All living organisms require copper for continued growth and development. Copper is an essential component of cell metabolism serving as a cofactor for numerous enzymes, such as cytochrome oxidase, superoxide dismutase, and dopamine-

hydroxylase. Disruption of copper transport across the cell membrane causes a number of diseases, which differ in their severity, frequency, and susceptibility to treatment. Among them are Menkes disease, a neurodegenerative disease, caused by copper deficiency in a number of tissues (1, 2), and Wilson’s disease, a hepatic disease, often with neurodegeneration symptoms, caused by excessive accumulation of copper in liver cells (2, 3). The genes affected in Menkes and Wilson’s disease patients were identified recently (4–9), and this led to the discovery of human copper-transporting adenosine triphosphatases (ATPases).¹

Menkes and Wilson’s disease genes were shown to encode very similar proteins (54% of their amino acid residues are identical) with significant homology to the cation-transporting P-type ATPases (4–9). Based on the symptoms of the Menkes and Wilson’s diseases, the MNK and WND proteins were proposed to be copper-specific pumps. Analysis of their amino acid sequences also revealed that the WND and MNK proteins have distinctive structural features, suggesting unique mechanistic steps in the process of binding and translocation of copper (10).

The most striking and intriguing feature in the structure of both human copper-transporting ATPases is the presence of six repetitive sequences of approximately 70 amino acid residues at the N terminus of the molecule (Fig. 1). Each of these repeats contains a GMTCXXXIE sequence motif, which was earlier found in the structure of mercury-binding proteins and in bacterial Cd-ATPase (11–14). This motif was immediately noted in the structural analysis of human copper-ATPases, and a role of the N terminus in the binding of heavy metals was proposed (4–6, 8, 13). However, the involvement of the N-terminal domain in specific recognition of heavy metals and its precise role in the functioning of human copper-ATPase remain unknown. The stoichiometry of heavy-metal binding, the oxidation state of bound copper, and residues involved in heavy-metal coordination were also not characterized.

Metal-binding repeats of the WND and MNK proteins belong to a continuous sequence of about 600 amino acid residues in the N-terminal portions of the WND and MNK molecules. This segment is separated from the “core,” ATPase, portion of the molecule (Fig. 1), and it seems plausible that these long characteristic sequences form independently folded domains. Such independently folded domains of proteins have been overexpressed in Escherichia coli and purified, and their structural and functional properties have been successfully characterized in a number of cases (for an example, see Ref. 15).

Here we report the overexpression of the N-terminal domains of the Menkes and Wilson’s disease proteins in soluble form and the purification and functional characterization of these domains.

¹ The abbreviations used are: ATPase, adenosine triphosphatase; MBP, maltose-binding protein; Na-P, sodium phosphate; IAA, iminodiacetic acid agarose; BCA, biocinchoninic acid; CPM, 7-diethylamino-3-(4′-maleimidyphenyl)-4-methylcoumarin.
Expression of Heavy-metal Binding Domains of the WND and MNK Proteins—To create fusion proteins of the N-WND and N-MNK with the maltose-binding protein (MBP), segments corresponding to 64–1868 base pairs of the WND cDNA and 1–1856 base pairs of the MNK cDNA were amplified by polymerase chain reaction and then incorporated into pMAL-c2 vector (New England Biolabs) at the 3' end of coding sequence for the maltose-binding protein (MBP), after the factor Xa cleavage site. Stop codons were engineered at the 3'-end of coding sequence after the factor Xa cleavage site and between the two fractions was analyzed by gel electrophoresis and confirmed by sequencing of the protein inserts to ensure the lack of repetitive sequences in the N-terminal region. Phosphorylated Asp residue in the ATP-binding domain; arrow, approximate position of the C terminus of the expressed N-terminal domains.

EXPERIMENTAL PROCEDURES

Expression of Heavy-metal Binding Domains of the WND and MNK Proteins—The involvement of cysteine residues in coordination of copper was demonstrated by the ability of different heavy metals to protect cysteine residues in the metal-binding domains against labeling with the cysteine-directed fluorescent reagent 7-diethylamino-3-(4'-maleimidylphenoxy)-4-methylcoumarin (CPM, Molecular Probes). For these experiments, purified N-WND and MNK proteins were incubated in the presence of increasing concentrations of different heavy metals (see legend for Fig. 7) and then pulse-labeled with a 10–20 molar excess of CPM with respect to protein for 1 min in the dark. The reaction was stopped by the addition of Laemmli sample buffer containing 1% β-mercaptoethanol, and proteins were run on a 10% Laemmli gel. Differences in the fluorescence were monitored under UV light.

Stoichiometry of Copper Binding—The stoichiometry of copper binding and the oxidation state of bound copper were determined by a bicinchoninic acid (BCA)-based assay (19). Protein-copper complexes were quickly disrupted by acid denaturation, and the released copper was detected with BCA as described (19) in the absence or presence of reductants (1 mM ascorbate). BCA is highly sensitive and specific for Cu(I), which rapidly forms an intense purple complex with this reagent (ε = 7.7 × 10^5 nm). If copper is present in solution in Cu(II) form, the reducing agent (1 mM ascorbate) has to be added to the incubation medium for copper-BCA complex to be formed. The lack of effect of ascorbic acid indicated that copper released from the protein was in the Cu(I) state. A difference in the absorbance of copper-BCA complex at 562 nm in the presence and absence of ascorbate points to the Cu(II) form of released cation.

This procedure has been used to measure the in vivo and in vitro binding of copper to the domains. To determine the amount of copper incorporated into the fusion proteins in vivo, cells were grown in the presence or absence of 0.5 mM CuCl₂. The N-MNK and N-WND were purified, and their copper content was determined with BCA (see above). To estimate the amount of copper bound to the protein in vitro, purified copper-free fusion proteins at a concentration of 0.1–0.2 mg/ml were mixed with CuCl₂ (at approximate molar ratio of 20 μmol of copper per 1 μmol of protein) in the presence or absence of freshly prepared 1 mM ascorbate. After 10–15 min incubation at room temperature, unbound copper was removed by overnight dialysis at 4 °C, and bound copper was measured with BCA in the presence of 1 mM ascorbate.

RESULTS

Expression of the N-terminal Domains of Human Copper-transporting ATPases in Soluble Form—Our initial attempt to express N-WND and N-MNK proteins in E. coli led to the accumulation of protein in inclusion bodies under all experimental conditions we tested. N-WND or N-MNK proteins in a water-soluble and functional form, we used a new approach, which was reported to produce a dramatic increase in solubility of eucaryotic proteins produced in E. coli (20). This approach requires cotransformation of E. coli cells with two plasmids: one containing the coding sequence for the protein of interest, and the other is a thioredoxin-encoding plasmid. The expression of high levels of thioredoxin seems to change the redox potential of E. coli cells, mimicking the environment of eucaryotic cells: this in turn facilitates proper folding of eucaryotic proteins. The cation binding specificity of N-WND and N-MNK fusion proteins was determined by metal-chelate chromatography on iminodiacetic acid agarose (IAA-resin, Sigma) equilibrated with different heavy metals. IAA-resin was extensively washed with Na-P buffer, pH 7.5, and then equilibrated with 5–10 volumes of the same buffer containing chloride salt of different metals at a final concentration of 1 mol/l. Excess heavy metals were removed by several washes with Na-P buffer, and then proteins in Na-P buffer were added to the resin (the experiments were done either by a centrifugation method or on mini-columns made from pipette tips). 10–15 μg of total protein in 100 μl of Na-P buffer were added to 25 μl of IAA-resin. Binding was allowed to proceed for 5–10 min at room temperature. Bound and unbound proteins were separated after a quick centrifugation step (5 min at 10,000 × g, minifuge, Fisher), and unbound protein was saved for further analysis. Resin then was washed with 200 μl of Na-P buffer, and bound protein was eluted with 100 μl of 10 mM EDTA in Na-P buffer. Both eluted and unbound fractions were concentrated by centrifugation in the SpeedVac centrifuge or using the Centricon 30 units. Proteins were separated by 10% Laemmli gels (18), and intensities of the Coomassie-stained bands were compared by densitometry.

Involvement of Cysteine Residues—The expression of human copper-transporting ATPases (Wilson's and Menkes disease proteins). Repetitive sequences in the N-terminal region are depicted by boxes and numbered; P, position of the phosphorylated Asp residue in the ATP-binding domain; arrow, approximate position of the C terminus of the expressed N-terminal domains.
proteins and increases their solubility (20). We were successful in using this system for both N-WND and N-MNK metal-binding domains, and after induction with isopropyl-β-D-thiogalactopyranoside, we observed a significant level of expression of both fusion proteins (Fig. 2A). Analysis of the distribution of the expressed protein between water-soluble and insoluble fractions demonstrated that about 40–60% of the fusion proteins were in the soluble fraction (Fig. 2B). We also determined that the presence of 0.5 mM CuCl₂ in the growth medium did not affect the level of expression or solubility of either fusion proteins (Fig. 2). Proteins were purified by affinity chromatography on amylose column, yielding about 1–2 mg of purified proteins from 1 liter of cell culture.

**N-WND and N-MNK Domains Bind Copper Selectively in Vitro**—To determine cation-binding specificity of the purified N-WND and N-MNK domains, we used metal-chelate chromatography on IAA, equilibrated with different heavy metals (see “Experimental Procedures” for details). Our preliminary experiments showed that MBP does not bind to any of the metal-chelate columns we tested; thus, we used the entire fusion proteins for these experiments.

Both N-WND and N-MNK fusion proteins display high selectivity toward copper-equilibrated resin, as shown in Fig. 3. No binding was observed for cadmium- and cobalt-equilibrated columns, and no protein was bound to heavy metal-free resin or to the resin equilibrated with manganese (Fig. 3). Minor binding to the zinc-equilibrated resin was seen for both N-WND and N-MNK.

These experiments indicated that purified N-MNK and N-WND proteins specifically bind copper in vitro, when copper is added to these proteins in a partially chelated Cu(II) form. In cells, however, copper will likely be presented to the N-WND and N-MNK domains in a reduced form. To test whether the N-terminal domains of human copper-ATPases can bind copper under reducing conditions, we compared the incorporation of copper into the N-WND and N-MNK domains in a reduced form. To test whether the N-terminal domains of human copper-ATPases can bind copper under reducing conditions, we compared the incorporation of copper into the N-WND and N-MNK domains in the presence and absence of a reducing agent. The purified N-MNK and N-WND-proteins were incubated with the 20 molar excess of CuCl₂ in the presence and absence of 1 mM ascorbate. The unbound copper was removed by overnight dialysis, and copper bound to the proteins was measured using the BCA-based assay as described under “Experimental Procedures.” Our data demonstrate that both N-WND and N-MNK not only bind Cu(I) but that there is, in fact, an increase in the level of incorporation of copper into both domains when reducing reagent is present (Fig. 4).

**In Vivo, Copper Binds to the N-WND and N-MNK in Cu(I) Form with the Stoichiometry of One Copper Per Metal-binding Repeat**—To confirm that the N-MNK and N-WND proteins bind copper in vivo, we grew *E. coli* cells and induced expression of the proteins in medium containing 0.5 mM copper. The N-MNK and N-WND fusions were isolated from these and control (no copper present in the growth medium) cells, and the amount of copper bound to the proteins was determined using the BCA-based assay.

Fig. 5 illustrates that the N-MNK and N-WND proteins obtained from copper-treated cells did bind this heavy metal, whereas no copper binding was observed for control proteins. The stoichiometry of copper binding to the N-WND and the N-MNK domains was calculated in three independent experi-

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**Fig. 2.** A, expression of the N-MNK-MBP and N-WND-MBP fusions in the absence or presence of copper in the media. B, distribution of the expressed N-MNK fusion protein between soluble and insoluble fractions after coexpression with thioredoxin. Arrow, position of the N-MNK and N-WND fusion proteins.

**Fig. 3.** Binding of expressed N-WND and N-MNK fusions to IAA agarose-resin, equilibrated with different metal ions. Fusion proteins were loaded on the columns, washed with 10 volumes of buffer, and then eluted with 10 mM EDTA. Bound and nonbound material was analyzed by gel electrophoresis. Densitometry of Coomassie-stained bands was used to determine the amount of bound proteins. MBP, obtained after cleavage of the fusions with factor Xa, by itself did not bind to any of these columns.
Copper-containing N-WND and N-MNK proteins do not absorb in the 600–800 nm wavelength range typical for Cu(II)-protein complexes (21), even when copper was added in the form of CuCl₂ (data not shown). This suggested that the oxidation state of copper was changed upon binding. To confirm this hypothesis, we used BCA. BCA is highly sensitive and specific for Cu(I), which rapidly forms an intense purple complex with this reagent (19). The presence of reducing agents, such as ascorbic acid, is required for a similar complex to be formed with Cu(II) (Fig. 6). Thus, we purified the N-MNK and the N-WND domains, which have copper incorporated in vivo, and compared the amount of BCA-copper complex formed following protein denaturation in the presence and absence of 1 mM ascorbic acid. Fig. 6 illustrates that the amount of copper-BCA complex was not significantly affected by the presence of the reducing agent. This result indicates that copper bound to the N-WND and N-MNK proteins was already in the reduced Cu(I) form. In these experiments, we observed, however, a difference between the rate of the BCA-copper complex formation for the N-WND and N-MNK domains. Copper released from the N-MNK domain formed a complex with BCA rapidly, with or without reducing agent present. In the same experiments, copper released from the WND domain quickly formed the BCA-copper complex in the presence of ascorbic acid, whereas without ascorbate, longer incubation times were required for the same complex to be formed. (Long incubation of control Cu(II) solution with BCA did not lead to the formation of the copper-BCA complex, which still required the presence of the ascorbate.)

Involvement of Cysteine Residues in Coordination of Copper—Each putative metal-binding repeat in the N-WND and N-MNK proteins contains two invariant cysteine residues, which are obvious candidates for interactions with heavy metals. To examine whether cysteine residues play a role in heavy-metal binding and to confirm the selectivity of the domain toward copper, we compared the ability of different heavy metals to protect cysteine residues in the “metal-binding motifs”
against labeling with the cysteine-directed fluorescent CPM; the idea was that cysteine residues involved in coordination of a heavy metal would be less reactive toward the CPM. (MBP does not have cysteine residues in its sequence). Fig. 7 shows that incubation of the N-WND domain with copper indeed prevents labeling of cysteine residues with fluorescent probe, whereas the presence of cadmium was without effect on labeling, in agreement with the results obtained with metal-chelate chromatography. The identical results were obtained for the N-MNK-domain (data not shown).

Involvement of Cys residues in coordination of copper, which was incorporated into the N-terminal domains in vivo, has also been tested. CPM labeling of N-WND and N-MNK, purified from control cells and cells grown in the presence of copper, was compared. As shown in Fig. 8, copper-free N-WND and N-MNK were readily modified with the cysteine-directed probe, whereas almost no labeling was seen in the copper-bound proteins. Thus, binding of copper to the N-WND and N-MNK domains in vitro and in vivo significantly decreases the availability of the Cys residues for modification with the Cys-directed probe, indicating that cysteines are probably directly involved in the coordination of copper.

**DISCUSSION**

Here we report functional characterization of the purified N-terminal domains of the Wilson’s and Menkes disease proteins. We demonstrate that both N-MNK and N-WND readily and selectively bind copper in vivo and in vitro. This is based on a series of experiments, including direct measurements of copper incorporation in vivo, and results of metal-chelate chromatography for the purified proteins and fluorescent labeling of the cysteines in the presence of different heavy metals. Each of the six metal-binding repeats in the N terminus of the copper-ATPases appears to coordinate copper, which binds to both N-WND and N-MNK in its reduced form.

Both N-MNK and N-WND show high selectivity toward copper. This property is in agreement with the proposed physiological role for the Menkes and Wilson’s disease proteins as copper-specific ion pumps. Our initial studies of heavy-metal specificity of the N-MNK and N-WND proteins, which were carried out following denaturation and refolding of the expressed N-WND and N-MNK, also demonstrated selectivity toward copper. However, refolded N-WND and N-MNK proteins showed significantly less discrimination between different heavy metals, because they were bound equally well to the zinc and copper-chelate columns (22). These data point to the important role of the well-preserved three-dimensional structure of the domains for selectivity toward copper. It is quite possible that the large number of heavy metal-binding repeats in the N-terminal portion of human copper-transporting ATPases and their unique spatial arrangement are required to provide high selectivity toward copper.

Our data indicate that there are 5–6 copper molecules bound per N-terminal domain molecule, suggesting that each of the sequence repeats is likely to be involved in copper binding. The stoichiometry of copper incorporation was reproduced in three independent experiments; however, binding of 6 copper ions per domain should be considered as an approximate stoichiometry until confirmed by other methods. The precise number of copper atoms bound per domain will be determined in future crystallization studies.

The obtained stoichiometries reflect the amount of tightly bound copper because our experimental conditions include multiple washes or long dialysis. The binding we observed must be of a rather high affinity because the presence of 1 mM EDTA in the buffer during purification of the copper-containing N-WND and N-MNK did not decrease the stoichiometry of copper binding. These data also indicate that in the native enzyme, additional sites may exist for exchangeable copper (conserved CPC motif in the membrane portion is the obvious candidate for such a site), or else interactions of the N-terminal

**Fig. 7.** Copper protects Cys residues in the N-WND domain against labeling with fluorescent coumarin. Purified N-WND protein at 20 μg was labeled with CPM in the presence of CuCl₂ or CdCl₂ at indicated micromolar concentrations. Labeled proteins were separated on a 10% Laemmli polyacrylamide gel, and fluorescence was detected under an UV lamp. The results of the experiment with the N-MNK domain were identical and not shown.

**Fig. 8.** Binding of copper in vivo to the N-WND and N-MNK proteins protects Cys residues against labeling with CPM. The N-WND and N-MNK proteins were isolated from the cells grown in the presence of copper (N-WND+Cu and N-MNK+Cu) or in the absence of copper (N-WND and N-MNK) and then labeled with CPM as described under “Experimental Procedures.” A, Coomassie staining; B, fluorescence.
domain with other domains in the copper-ATPase during the cycle allow copper to be released from the N-terminal domain and then be transported. In any case, direct involvement of the N-terminal domains in copper binding suggests that they play an important role in copper-dependent functions of the Wilson’s and Menkes disease proteins. Future experiments with the full-length copper-ATPase will show whether the cations that are bound to the N-terminal domains are then transported by human copper-ATPases.

We have also demonstrated that the presence of reducing agents facilitates copper binding to the domains and that copper is likely to be bound to the N-MNK and N-WND domains in the Cu(I) form. The N-MNK domain incorporates a similar amount of copper in vitro and in vivo, whereas differences between in vivo and in vitro stoichiometry were observed for N-WND. The lower copper binding by purified copper-free N-WND can be explained by partial nonspecific formation of the S-S bridges in N-WND during overexpression and purification.

This explanation also agrees with lesser labeling of cysteines in S-S bridges in N-WND during overexpression and purification. The lower copper binding by purified copper-free N-WND can be explained by partial nonspecific formation of the S-S bridges in N-WND during overexpression and purification. Further experiments, including EPR studies, are under way to confirm our conclusion about the oxidation state of copper in the N-WND and N-MNK complexes.

We also found that N-MNK and in particular N-WND have a “hot spot” for proteolytic digestion. As shown in Fig. 8, the 65-kDa fragment is a major, and often the only, contaminant in our preparation. Western blot analysis with the antibodies directed either against MBP or against the N terminus and copurification of the 65-kDa protein and full-length domain on amylose column and on metal-chelate column indicate that the 65-kDa fragment contains both parts (MBP and two or three repeats of N-WND). We speculate that folded N-WND and N-MNK are composed of two subdomains connected by a flexible and proteolytically sensitive linker.

Finally, we have demonstrated that Cys residues in metal-binding motifs seem to be directly involved in copper coordination. Most probably, these cysteines are not the only amino acid residues that interact with copper. Experiments are currently under way to identify other participating residues.

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