Cu(I) Binding and Transfer by the N Terminus of the Wilson Disease Protein

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Wilson and Menkes diseases are genetic disorders of copper metabolism caused by mutations in the Wilson (WND) and Menkes (MNK) copper-transporting P1B-type ATPases. The N termini of these ATPases consist of six metal binding domains (MBDs). The MBDs interact with the copper chaperone Atox1 and are believed to play roles in catalysis and in copper-mediated cellular relocalization of WND and MNK. Although all six MBDs have similar folds and bind one Cu(I) ion via a conserved CXXC motif, biochemical and genetic data suggest that they have distinct functions. Most studies aimed at characterizing the MBDs have employed smaller polypeptides consisting of one or two domains. The role of each MBD is probably defined by its environment within the six-domain N terminus, however. To study the properties of the individual domains within the context of the intact Wilson N terminus (N-WND), a series of variants in which five of the six metal binding CXXC motifs are mutated to SXSS was generated. For each variant, the Cu(I) binding affinity and the ability to exchange Cu(I) with Atox1 were investigated. The results indicate that Atox1 can deliver Cu(I) to and remove Cu(I) from each MBD, that each MBD has stronger Cu(I) retention properties than Atox1, and that all of the MBDs as well as Atox1 have similar $K_{Cu}$ values of $\left(2.2-6.3\right) \times 10^{10} \text{M}^{-1}$. Therefore, the specific role of each MBD is not conferred by its position within the intact N-WND but may be related to interactions with other domains and partner proteins.

The human Wilson (WND) and Menkes disease (MNK) proteins (1, 2) are P1B-type ATPases, integral membrane proteins that couple the energy of ATP hydrolysis with the translocation of Cu(I) ions across membranes (3, 4). Mutations in the genes encoding WND and MNK lead to genetic disorders of copper toxicity and copper starvation, respectively (5). The WND and MNK proteins are 69% similar and consist of a cytosolic N-terminal metal binding domain, eight transmembrane helices, an actuator domain (A-domain), and an ATP binding domain (ATPBD) (6). An intramembrane metal binding site is believed to involve a conserved CPC motif and several additional invariant residues. The N-terminal metal binding domain is composed of six homologous metal binding domains (MBDs). Each ~70-residue MBD exhibits a $\beta$αββαβ fold and contains a signature CXXC metal-binding motif (7–10). The two cysteine residues in the CXXC motif bind one Cu(I) in a distorted linear fashion (11–15). Prokaryotic (3) and yeast (16) homologs contain only one or two MBDs, and the Caenorhabditis elegans (17), Drosophila melanogaster (18), and rat (19) homologs have three, four, and five repeats, respectively.

An apparent function of the MBDs is to receive copper from the chaperone Atox1 (20, 21) via direct protein-protein interactions (7, 22–24). Atox1 and its homologs, both prokaryotic and eukaryotic, also contain a CXXC motif and exhibit a $\beta$αββαβ fold (25–29). According to yeast two-hybrid assays using both WND and MNK, MBD1–4 interact most strongly with Atox1, whereas no interaction is observed with domains 5 and 6 (20, 24). The interaction is abolished if the conserved cysteine residues in either the MBDs or Atox1 are mutated. In contrast to the two-hybrid data, an interaction between MNK domains 5 and 6 and Atox1 was detected by surface plasmon resonance studies (24). Cysteine labeling experiments further suggest that Atox1 preferentially delivers copper to WND domain 2 (30), and NMR chemical shift titrations indicate that Atox1 can interact with both MBD2 and MBD4 (7). Although it has been proposed that domain 4 transfers copper delivered by Atox1 to domains 5 and 6, which then shuttle it to the intramembrane CPC site (7), copper transfer from the MBDs into the transmembrane region has not been established. Copper binding to the WND MBDs also modulates an interdomain interaction with the ATPBD. In the absence of copper, the six MBDs interact with the ATPBD, reducing its affinity for ATP (31).

In addition to interacting with Atox1 and the ATPBD, the MBDs are believed to affect both catalysis and the cellular localization of WND and MNK. At elevated copper concentrations, WND and MNK relocalize from the trans-Golgi network to cytoplasmic vesicles (32) and the plasma membrane (33), respectively. For both MNK and WND, relocalization is coordinated with catalysis in that copper binding induces acyl phosphate formation as well as trafficking to the membrane (34, 35).
Mutagenesis studies on both WND and MNK suggest that either domain 5 or domain 6 is sufficient both for relocalization and for copper transport activity (36–39). Notably, recent studies of WND suggest that copper binding by neither the MBDs nor the intramembrane CPC motif is required for acyl phosphate formation and relocalization, raising the possibility of additional copper binding sites (35).

Collectively, the biochemical and genetic data suggest that WND MBD1–4 are important for interaction with Atox1 and that MBD5 and MBD6 are responsible for copper transport and copper-induced relocalization. The properties that confer distinct functions on the individual domains have not been elucidated; nor has the reason for the presence of six MBDs in the human proteins. The domains structurally characterized thus far are quite similar, although the electrostatic surfaces exhibit some variation (30, 40). Several attempts have been made to determine the copper binding affinities of the individual domains and to characterize their interactions with Atox1, but in all of these studies, smaller polypeptides consisting of one or two MBDs were employed (7, 41, 42). It is likely that the role of each MBD is dependent on and defined by its environment within the entire six-domain N terminus (N-WND). To probe the properties of the MBDs within the context of the intact N-WND, we generated a series of variants in which five of the six metal-binding CXXC motifs were mutated to SXXS as well as a variant lacking all CXXC sequences (Fig. 1). These proteins were then used to measure the copper binding affinities of each MBD and their ability to exchange Cu(I) with Atox1.

**MATERIALS AND METHODS**

**Construction of Template for Site-directed Mutagenesis**—The DNA sequence coding for the WND N-terminal MBDs was amplified from a plasmid containing the cDNA for the entire WND (supplied by Dr. Jonathan Gitlin, Washington University School of Medicine; GenBank™ accession number NM_000053) by PCR. Both forward and reverse primers, WND-f and WND-rev, were designed to include ligation-independent cloning (LIC) ends (Table S1). The pUC19 plasmid was partially cloned into the pET30Xa/LIC system using the WND-f and WND-rev primers (Table S1), sequenced, and transformed into Blue competent cells, and the DNA was isolated, sequenced, and used for the next round of mutations. The CXXC motifs in domains 1–4 were mutated to SXXS sequentially, yielding a construct with only domains 5 and 6 having intact metal-binding motifs. Subsequently, mutation of the CXXC motifs in domain 5, domain 6, or both domains 5 and 6 resulted in the constructs denoted MBD6, MBD5, and MBD0, respectively. The number in the mutant name refers to the domain with an intact CXXC motif. In all further steps, MBD0 was used as the template. By reversing the mutations (changing from SXXS to CXXC) in domains 1, 2, 3, and 4 individually using the primers shown in Table S1, the constructs MBD1, MBD2, MBD3, and MBD4 were generated.

**MBD Expression and Purification**—The mutants were initially cloned into the pET30Xa/LIC system using the WND-f and WND-rev primers (Table S1), sequenced, and transformed into Rosetta(DE3)pLysS cells. The proteins were overexpressed but accumulated in inclusion bodies under all experimental conditions. Switching to the pET32Xa/LIC system, which includes an additional thioredoxin tag, dramatically increased protein solubility. All variants were expressed at similar levels to the wild-type N-WND. Protein expression was induced at 18 °C with 0.9 mM isopropyl-β-D-thiogalactopyranoside at an optical density of 0.7–1.0 at 600 nm. Cells were harvested after an overnight growth, resuspended in 50 mM HEPES, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, and stored at −80 °C.

For purification, the cell suspension was thawed and stirred at ambient temperature for 30–60 min with EDTA-free protease inhibitor tablets (Roche Applied Science; 1 tablet for 40 ml of cell suspension) and a pinch of solid DNase I. The solution was centrifuged at 125,000 × g for 1 h, and the supernatant was applied to a 20-ml pre-equilibrated nickel-loaded chelating Sepharose column (Amersham Biosciences), rinsed with buffer A (50 mM HEPES, pH 7.5, 500 mM NaCl, 10% glycerol), and eluted with an eight-column volume gradient of 300 mM imidazole, pH 7.5, 500 mM NaCl. MBD-containing fractions were collected and dialyzed twice at 4 °C versus 2 liters of buffer H (50 mM phosphate, pH 7.5, 500 mM NaCl).
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mm HEPES, pH 7.5, 200 mm NaCl). The protein solution was then concentrated to 50 ml (~40 μM), and cleavage of affinity tags was initiated by the addition of 120 units of Factor Xa (Novagen) in buffer H supplemented with 5 mm CaCl₂. After 7–8 h, 1 mm phenylmethylsulfonyl fluoride was added to stop the proteolytic cleavage. Cleavage reactions were reloaded onto the nickel column, rinsed with buffer A, and eluted with ~24–36 mm imidazole. Fractions containing the cleaved protein were concentrated with a Centriprep 10 (Amicon) to 5 ml in the presence of 1 mm EDTA, 10 mm DTT, and 50 μM bathocuproine disulfonate (BCS), applied to a Superdex 200 gel filtration column (Amersham Biosciences), and eluted with 50 mm HEPES, pH 7.5, 300 mm NaCl, 1% glycerol. Rapid purification at 4 °C was critical to minimizing proteolysis. The typical yield was ~6–16 mg of pure protein (supplemental Fig. S1) per liter of Escherichia coli culture.

Atox1 Expression and Purification—BL21(DE3) cells containing pET21b with the gene encoding Atox1 (42) were grown at 37 °C to an optical density at 600 nm of 0.8–1.1, and then containing pET21b with the gene encoding Atox1 (42) were grown at 37 °C to an optical density at 600 nm of 0.8–1.1, and then protein expression was induced with 0.9 mm isopropyl-β-d-thiogalactopyranoside. After 3 h, the cells were harvested, resuspended in 20 mm MES, pH 6.0, 1 mm EDTA, 1 mm phenylmethylsulfonyl chloride, and stored at ~80 °C. For purification, the cell suspension was thawed with 5 mm DTT and protease inhibitor tablets (Amersham Biosciences; 1 tablet/50 ml), stirred for 30 min at ambient temperature, and centrifuged at 125,000 × g for 30 min. The supernatant was loaded onto a DEAE FF anion exchange column (HiLoad 26/10; Amersham Biosciences) equilibrated with 20 mm MES, pH 6.0, 5 mm DTT, 1 mm EDTA. Flow-through fractions containing Atox1 were concentrated and loaded onto an SP FF cation exchange column (HiLoad 26/10; Amersham Biosciences) preequilibrated with 20 mm MES, pH 6.0, 5 mM NaCl, 1 mm EDTA, 1 mm phenylmethylsulfonyl chloride, and stored at ~80 °C. For purification, the cell suspension was thawed with 5 mm DTT and protease inhibitor tablets (Amersham Biosciences; 1 tablet/50 ml), stirred for 30 min at ambient temperature, and centrifuged at 125,000 × g for 30 min. The supernatant was loaded onto a DEAE FF anion exchange column (HiLoad 26/10; Amersham Biosciences) equilibrated with 20 mm MES, pH 6.0, 5 mm DTT, 1 mm EDTA. Flow-through fractions containing Atox1 were concentrated and loaded onto an SP FF cation exchange column (HiLoad 26/10; Amersham Biosciences) preequilibrated with 20 mm MES, pH 6.0, 1 mm EDTA and eluted with a four-column volume gradient of 20 mm MES, pH 6.0, 1 m NaCl. Atox1-containing fractions eluted at ~200 mm NaCl were concentrated and purified further on a Superdex 75 column (HiLoad 16/60 prep grade; Amersham Biosciences) equilibrated with 50 mm HEPES, pH 7.5, 200 mm NaCl. The typical yield was ~9–15 mg of pure protein/liter of E. coli culture.

Cu(I) Loading of MBDS and Atox1—The copper-loaded forms of Atox1 (CuAtox1) and the MBD variants (CuMBD1–6) were prepared as follows. The MBD1–6 variants (CuMBD1–6) were incubated on ice for 30 min with 10 mM EDTA, 1 mM BCA, a Cu(I)-specific chelator. A separate set of experiments was conducted in the presence of 1 mM GSH, a physiological Cu(I) ligand (43, 44) or by inductively coupled plasma-atomic emission spectroscopy (Varian). Bovine serum albumin (BSA) and atomic absorption copper standard solution (990 μg/ml; Aldrich) were used as standards for protein and copper determinations, respectively. Protein concentrations of stock solutions were determined using extinction coefficients at 280 nm of 3,884 and 36,554 M⁻¹ cm⁻¹ for Atox1 and the MBD proteins, respectively (42). The starting apoMBDs had no detectable copper, and the typical Cu(I) stoichiometry per Atox1 monomer was 0.68 ± 0.12. Reverse copper transfer experiments from CuMBD1–6 to apoAtox1 were conducted in a similar manner. The starting CuMBD1–6 and apoAtox1 samples had copper/protein stoichiometries of 0.7–1.1 and 0.0002–0.0100, respectively. Both forward and reverse copper transfer data were used to estimate the equilibrium constants for copper redistribution between MBD1–6 and Atox1 (Reaction 1) as described previously (23).

$$\text{CuAtox1} + \text{apoMBD} \rightleftharpoons \text{apoAtox1} + \text{CuMBD}$$

REACTION 1

As a specificity control, similar experiments were conducted between CuAtox1 and apoMBD0 as well as between CuAtox1 and BSA, an unrelated copper-binding protein. Whereas MBDO has no metal binding cysteine residues and does not bind Cu(I), BSA binds up to 1.3 eq of Cu(I) when loaded according to the protocol described above.

To confirm that Cu(I) transfer occurs through direct protein-protein interactions (rather than by copper dissociation and subsequent binding), Cu(I) transfer experiments between CuAtox1 and apoMBD6 were repeated in the presence of 0.1–0.6 mm BCA, a Cu(I)-specific chelator. A separate set of experiments was conducted in the presence of 1 mm GSH, a physiologically relevant Cu(I) chelator. Both BCA and GSH bind Cu(I) to form Cu(II)(BCA)₂ (β₂ = 4.60 × 10¹⁴ M⁻²; see below) and Cu(II)(GSH)₂ (β₂ = 10⁻³⁸.² M⁻² (45)). APOMBD6 was first mixed with BCA or GSH and then incubated with increasing concentrations of CuAtox1 for >10 min, followed by separation of the mixture by gel filtration.

Chemical Modification of Cysteine Residues—Chemical modification was performed to test the surface accessibility of the metal binding cysteine residues. All manipulations except concentration steps were conducted anaerobically. The apo forms of MBDO and Atox1 were used as negative controls. Cu(I)-bound proteins and apoproteins were modified with the cysteine-directed nonfluorescent probe N-β-maleimidopropionic acid (BMPA) for 2 h. BMPA was then removed by two rounds of concentration, followed by dilution with buffer H, and all samples were treated with 0.5 mm BCS to remove bound...
copper. After this step, the apoproteins should have no free cysteines, whereas the samples that formerly had Cu(I) bound should have only the copper-binding cysteines available for modification. After BCS removal, all samples were treated with an ~50-fold excess of the cysteine-directed fluorescent probe, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; Molecular Probes, Inc., Eugene, OR) for 5 min and 2 h. Samples were nutated in the dark at room temperature. The reaction was quenched with β-mercaptoethanol, and the samples were analyzed by SDS-PAGE. AMS modification was detected by UV light, and the gel was then stained with Coomassie R-250.

Relative Copper Binding by MBD1–6 Using the BCA Competition Assay—The CuMBD1–6 proteins were diluted to 20 μM Cu(I) with buffer H containing 0.20 mM ascorbate. A 20 μM Cu(I) solution was prepared from a copper atomic absorption standard in the same buffer as a control. A series of 11 BCA samples with concentrations of 0.6, 0.8, 1.2, 1.6, 2, 3, 4, 5, 6, 7, and 8 mM were prepared. Copper control and protein solutions (90 μl) were mixed with 10-μl BCA solutions of increasing concentration and incubated for 1 h, 7 h, and overnight. The 1 h minimum time was chosen after optically monitoring copper binding by BCA as a function of time. The change in absorbance at 562 nm due to the formation of the Cu(I)(BCA)2 complex (extinction coefficient at 562 nm, 7,700 M⁻¹ cm⁻¹) plateaued after 45 min. Using an HP 8452A spectrophotometer, the absorbance at 562 nm was measured first for the buffer/ascorbate/BCA solution as a blank, followed by the 20 μM Cu(I)/ascorbate/BCA sample and finally by the CuMBD/ascorbate/BCA sample for each concentration of BCA. The estimated accuracy of this experiment is ±10%.

Determination of the Apparent Association Constant Kcb for the Cu(I)/(BCA)2 Complex by Isothermal Titration Calorimetry (ITC)—Binding of Cu(I) to BCA was measured by ITC using a MicroCal MCS-ITC instrument under an anaerobic atmosphere. Experiments were conducted at 23–60 °C in buffer H that was thoroughly degassed, purged with argon, and stored in the anaerobic chamber. Before use, all buffers and stock solutions were filtered (pore size 0.22 μm; Fisherbrand) to remove any particulate matter. The ITC cell was loaded with a 75–80 μM Cu(I) solution prepared from copper atomic absorption standard in buffer H with 0.2 mM ascorbate. A 2 mM BCA solution prepared fresh in buffer H was loaded into the titration syringe. After equilibration, the cell solution was titrated with 40–48 aliquots of 5–6.2-μl increments of BCA (up to a BCA/Cu(I) ratio of 4.5) at 180-s time intervals with constant stirring at 400 rpm. A preliminary 2-μl injection was included in each titration. The heat of dilution was determined from the prolonged titration of BCA into a saturated Cu(I) solution and used to correct the measured ΔH_{ITC} to determine the effective heat of binding.

The data were analyzed with Origin 5.0 from MicroCal using a one-site binding model. Best fit values are reported as an average of at least two titrations that gave an internally consistent heat under optimized experimental conditions. The known Cu(I)/BCA stoichiometry of 1:2 (43, 44) allows for calibration of the concentration scale. Thus, a fixed stoichiometry of 2 and variable concentrations of BCA were used in the regression analysis of the ITC data. The best fit model yields two equivalent binding constants, K_1 = K_2 = 2.145 ± 0.033 × 10^7 M⁻¹, with an overall binding constant β_2 = 4.60 × 10^{14} M⁻². Additional details of the Kcb determination are included in the supplemental materials.

Determination of the Apparent Association Constant Kcb for MBD1–6 and Atoxl—To determine the affinities of the MBDs and Atoxl for Cu(I), aliquots containing ~0.1 molar eq of freshly reduced apoproteins (1–4 μl, 0.3–0.6 mM) were titrated into 0.5–0.7 ml of 10 μM Cu(I) solution prepared from copper atomic absorption standard in the presence of 0.5–1 mM BCA. BCA/Cu ratios of 50–100 were used to ensure that all titrated Cu(I) was either chelated by BCA or incorporated into the protein. Excess BCA also serves as a competitor for nonspecific binding of Cu(I) to the MBDs. Both protein and Cu(I) solutions were prepared in thoroughly degassed buffer H containing 0.2 mM ascorbate. Absorption spectra were collected between 300 and 800 nm on an HP 8452A or a Cary 500 UV-visible spectrophotometer at ambient temperature using BCA/ascorbate as a blank. To ensure that equilibrium had been reached, the cuvette was incubated for at least 10 min after each addition of protein/ascorbate to the copper/BCA/ascorbate mixture (until there were no additional changes detected in the absorption spectrum). The additions were repeated until the intensity of the ligand-field absorption band at 562 nm had decreased to base-line levels. Individual absorption spectra collected following each addition of protein were corrected for dilution and base-line absorbance at 800 nm.

The spectra were analyzed with SPECFIT/32.3 SPECFIT/32 calculates binding constants based on a model that includes all colored species by the method of factor analysis. The model created to analyze the MBD/BCA competition for Cu(I) assumes that the Cu(I)(BCA) complex is initially present in solution and that CuMBD is formed during the titration, releasing free BCA. In the case of Atoxl, both CuAtoxl and Cu(Atoxl)2 species are formed during the titration. The Cu(I)(BCA) complex is the only colored species absorbing in the 450–800 nm region. The affinity of Cu(I) for BCA, K_1 = 2.145 ± 0.033 × 10^7 M⁻¹, and a known basis spectrum of the Cu(I)(BCA) complex were included in the binding model. There are precedents for the use of the K_1 (46) and β_2 (47) binding constants as the correction factors in such experiments. The use of K_1 avoids the risk of overstating the Cu(I) binding affinity. SPECFIT/32 fits all wavelengths simultaneously, resulting in high reliability of the binding constants obtained. Titration of the Cu(I)(BCA) solution with lysozyme was used as a negative control. No decrease in the absorbance of Cu(I)/BCA solution was detected upon the addition of increasing amounts of lysozyme, indicating that Cu(I) binding to MBD1–6 and Atoxl is specific (supplemental Fig. S4).

RESULTS

Copper Transfer between MBD1–6 and Atoxl—To test whether the metal-binding cysteine residues from each MBD are accessible for Atoxl docking and copper transfer within the context of all six MBDs, a chemical modification procedure was

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3 SPECFIT is a product of Spectrum Software Associates and is owned solely by the authors, Robert Binstead and Andreas Zuberbühler.
employed. Each variant contains the two metal-binding cysteines and seven additional cysteines throughout the sequence. MBD0–6 were reduced and modified with BMPA, a nonfluorescent probe, in the presence and absence of copper. For the six apoMBD variants (apoMBD1–6), BMPA modifies all nine cysteine residues present, whereas for the six copper-loaded MBD variants (CuMBD1–6), BMPA modifies the seven cysteine residues not involved in metal binding. Following removal of Cu(I) by the addition of BCS, the metal binding cysteines were labeled with the fluorescent probe AMS. All of the MBDs are labeled with AMS, indicating that the cysteines in the CXXC motif are surface-exposed, reduced, and able to receive copper from Atox1 (Fig. 2). On the basis of the fluorescence intensity, the efficiency of labeling was MBD2, MBD6 > MBD1 > MBD4 > MBD5 > MBD3 > MBD0.

To assess copper transfer from CuAtox1 to each apoMBD1–6 variant, the two proteins were incubated and separated by gel filtration. The purified apoMBD0–6 variants did not contain any measurable amounts of Cu(I) prior to mixing with CuAtox1. In the representative elution profile shown in Fig. 3A, CuAtox1 was mixed with apoMBD3 in a molar ratio of 10:1. Cu(I) was detected in the MBD3-containing fractions. No Atox1 was detected in the MBD3-containing fractions as analyzed by SDS–PAGE. Therefore, incubation of CuAtox1 with apoMBD3 leads to copper transfer from the chaperone to its target. The results are independent of incubation times, indicating that copper transfer is complete within a few minutes of mixing the components.

To test the efficiency and the level of copper transfer, the experiments were repeated for the same apoMBD0–6 samples incubated with increasing amounts of CuAtox1 up to a 60-fold excess. As shown in Fig. 3B for apoMBD3, this leads to a dose-dependent and saturable transfer of one Cu(I) ion from the chaperone, consistent with the presence of one CXXC motif in apoMBD3. Importantly, transfer is ≥70% complete even at an Atox1/MBD3 ratio of 1:1. Increasing the amount of CuAtox1 does not lead to binding of more than one Cu(I) to MBD3 (Fig. 3B), supporting the idea that Atox1 controls the delivery of copper to specific sites and confirming that the replacement of the CXXC motifs with SXXS prevents copper binding by the other MBDs. Similar results were obtained for Cu(I) transfer from CuAtox1 to MBD5 (Fig. 4A), MBD6 (Fig. 4B), MBD1, MBD2, and MBD4. These findings demonstrate that Atox1 can deliver Cu(I) to all of the MBDs in vitro.

To prove that Cu(I) transfer between Atox1 and the MBDs occurs through direct protein–protein interactions, and to ascertain whether physiological copper chelators can interfere with the transfer process, we repeated the copper transfer experiments in the presence of BCA and GSH. If Cu(I) transfer occurs by release of Cu(I) into solution with subsequent binding of aqueous Cu(I) by proteins, then chelators should abolish Cu(I) transfer. If Cu(I) transfer occurs through direct protein–protein interactions, the chelator should have no effect. As shown in Fig. 4B, neither competitor affects copper transfer.
To address the specificity of Cu(I) transfer, both MBD0 and BSA were used. As expected, no copper was transferred to MBD0 from CuAtox1, underscoring the importance of metal binding cysteines for copper acquisition by MBDs (Fig. 4C). Although we were able to load up to 1.3 eq of Cu(I) into BSA using aqueous Cu(I) (indicating that the metal ligands are available for binding), no copper was transferred from CuAtox1 even at a 20-fold excess of CuAtox1 over BSA (Fig. 4C).

To test for reverse copper transfer from CuMBD1–6 to apoAtox1, copper-loaded MBD1–6 were incubated with increasing amounts of freshly reduced apoAtox1 and separated by gel filtration. The analysis, as shown for MBD3 (Fig. 3C), indicates the presence of copper in Atox1-containing fractions. Therefore, apoAtox1 is able to receive copper from CuMBD3. Under conditions of a large apoAtox1 excess, only 0.2 coppers/protein remains in MBD3 (Fig. 3D). Similar results were obtained for MBD1, MBD2, MBD4, MBD5, and MBD6. In all cases, ~5 eq of apoAtox1 are required to remove most of the labile copper from the MBDs. The experimental results for forward and reverse copper transfer between the MBDs and Atox1 were used to estimate the equilibrium constant, $K_{ex}$, for copper redistribution between MBDs and the chaperone (23). The value of $K_{ex}$ was estimated to be 2.9 ± 0.1, 4.7 ± 0.3, 5.4 ± 0.3, 2.7 ± 0.2, and 3.9 ± 0.2 for MBD1 (Fig. 5), MBD2, MBD4, MBD5, and MBD6, respectively (Table 1).

Copper Retention by the MBD1–6 Variants—The copper retention properties of the MBDs were assessed by treatment with the Cu(I)-specific chelator BCA. The CuMBD1–6 variants were incubated under reducing conditions with increasing amounts of BCA, and the redistribution of copper between the MBDs and BCA was monitored spectrophotometrically at 562 nm after incubation for 1 h, 7 h, and overnight (Table 1). Representative data for MBD1 are shown in Fig. 6. For each MBD, no more than 15% of the Cu(I) was released after 1 h of incubation with a 45-fold excess of BCA. This finding is in agreement with the earlier observation that only 10% of copper redistributes from a single domain construct of MBD2 to BCA after 10 min of incubation (30). After 7 h of incubation at room temperature, 20, 10, 35, 10, 65, and 30% of the Cu(I) is acquired by BCA from MBD1, MBD2, MBD3, MBD4, MBD5, and MBD6, respectively (Table 1).
respectively. After overnight incubation at 4 °C, 50, 10, 50, 20, 80, and 80% of copper is redistributed to BCA by MBD1, MBD2, MBD3, MBD4, MBD5, and MBD6, respectively. The outcome of this experiment under prolonged incubation times depends somewhat on sample preparation and strongly on incubation temperature, however, suggesting that these latter results should not be overinterpreted. In contrast to the observation that the CuMBD1–6 variants retain most of the bound Cu(I) after 1 h of incubation with excess BCA, CuAtox1 releases all of its bound Cu(I) to BCA under identical conditions (Ref. 30 and data not shown), consistent with its chaperone function.

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**FIGURE 6. Copper retention by MBD1.** CuMBD1 was incubated with increasing concentrations of BCA for 1 h, 7 h, and overnight. The amount of copper redistributed from CuMBD1 to BCA was determined relative to the 20 μM Cu(I) standard, which was assigned as 100%.

**FIGURE 7. Determination of $K_{Cu}$.** A, absorption spectra collected during the titration of MBDS into a 10 μM Cu(I), 0.5 mM BCA solution in 50 mM HEPES, pH 7.5, 200 mM NaCl, 0.2 mM ascorbate. The absorbance decreases as increasing concentrations of MBDS are added. B, comparison of the calculated best fit (solid line) and observed data (squares) at 562 nm for the titration in A. C, comparison of the calculated best fit (solid line) and observed data (squares) at 562 nm for the titration of Atox1 into 10 μM Cu(I), 1 mM BCA. For Atox1, the model included both CuAtox1 and Cu(Atox1)$_2$ species.

**TABLE 1**

| Protein | Copper redistributed to BCA* | $K_{Cu}$ with Atox1 | $\Delta G_{298}$ | $K_{Cu}$ |
|---------|------------------------------|---------------------|-----------------|--------|
| MBD1    | 4.0%                         | 2.91 ± 0.14         | 0.63            | 2.6 ± 1.5 $\times 10^{10}$ |
| MBD2    | 0.0%                         | 4.70 ± 0.31         | 0.92            | 3.5 ± 0.9 $\times 10^{10}$ |
| MBD3    | 14%                          | 5.39 ± 0.27         | 1.00            | 2.5 ± 1.3 $\times 10^{10}$ |
| MBD4    | 3.0%                         | 5.39 ± 0.27         | 1.00            | 2.5 ± 1.3 $\times 10^{10}$ |
| MBD5    | 6.0%                         | 2.68 ± 0.21         | 0.58            | 2.2 ± 1.0 $\times 10^{10}$ |
| MBD6    | 7.0%                         | 3.92 ± 0.17         | 0.81            | 5.8 ± 2.5 $\times 10^{10}$ |
| Atox1   | 85%                          | NA                  |                 |       |

* Conditions were as follows: 1-h incubation time, 45-fold excess of BCA.

**Determinations for MBDS and Atox1** The Cu(I) binding affinities of various 2–6 domain constructs of N-WND and of Atox1 were determined previously by ITC (42). Our MBD1–6 variants aggregated upon direct Cu(I) titration and thus were not amenable to ITC studies. Therefore, a BCA competition assay was developed for $K_{Cu}$ determination. The addition of apoMBD1–6 to the Cu(I)/BCA solution resulted in a decrease in intensity of the optical feature at 562 nm (Fig. 7A), indicative of Cu(I) coordinating to the MBDs. The data for the MBD1–6/BCA competition experiments fit well to a model that includes a colored 1:1 Cu(I)(BCA) species ($K_1 = 2.145 ± 0.033 \times 10^7$ M$^{-1}$) and a noncolored CuMBD species. For Atox1, satisfactory fits were obtained only by including both

**FIGURE 6. Copper retention by MBD1.** CuMBD1 was incubated with increasing concentrations of BCA for 1 h, 7 h, and overnight. The amount of copper redistributed from CuMBD1 to BCA was determined relative to the 20 μM Cu(I) standard, which was assigned as 100%.

**FIGURE 7. Determination of $K_{Cu}$.** A, absorption spectra collected during the titration of MBDS into a 10 μM Cu(I), 0.5 mM BCA solution in 50 mM HEPES, pH 7.5, 200 mM NaCl, 0.2 mM ascorbate. The absorbance decreases as increasing concentrations of MBDS are added. B, comparison of the calculated best fit (solid line) and observed data (squares) at 562 nm for the titration in A. C, comparison of the calculated best fit (solid line) and observed data (squares) at 562 nm for the titration of Atox1 into 10 μM Cu(I), 1 mM BCA. For Atox1, the model included both CuAtox1 and Cu(Atox1)$_2$ species.

**TABLE 1**

| Protein | Copper redistributed to BCA* | $K_{Cu}$ with Atox1 | $\Delta G_{298}$ | $K_{Cu}$ |
|---------|------------------------------|---------------------|-----------------|--------|
| MBD1    | 4.0%                         | 2.91 ± 0.14         | 0.63            | 2.6 ± 1.5 $\times 10^{10}$ |
| MBD2    | 0.0%                         | 4.70 ± 0.31         | 0.92            | 3.5 ± 0.9 $\times 10^{10}$ |
| MBD3    | 14%                          | 5.39 ± 0.27         | 1.00            | 2.5 ± 1.3 $\times 10^{10}$ |
| MBD4    | 3.0%                         | 5.39 ± 0.27         | 1.00            | 2.5 ± 1.3 $\times 10^{10}$ |
| MBD5    | 6.0%                         | 2.68 ± 0.21         | 0.58            | 2.2 ± 1.0 $\times 10^{10}$ |
| MBD6    | 7.0%                         | 3.92 ± 0.17         | 0.81            | 5.8 ± 2.5 $\times 10^{10}$ |
| Atox1   | 85%                          | NA                  |                 |       |

* Conditions were as follows: 1-h incubation time, 45-fold excess of BCA.

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CuAtox1 and Cu(Atox1)$_2$ species in the binding model. This result agrees with the Cu(I) stoichiometry of 0.68 ± 0.12 measured per Atox1 monomer as well as with the copper-bridged dimer observed in the crystal structure (28). A comparison of the best fits to the observed data for a representative titration of MBD5 and Atox1 is shown in Fig. 7. Binding constants obtained from the best fits are shown in Table I. Binding of one Cu(I) ion to both Atox1 and the MBD1–6 variant proteins is characterized by similar binding constants of 2.2–6.3 × 10$^{10}$ M$^{-1}$. Binding of the second Atox1 molecule to CuAtox1 is much weaker, with a $K_{Cu}$ value of 3.3 × 10$^{6}$ M$^{-1}$.

**DISCUSSION**

We have systematically investigated the properties of the individual WND MBDS within the context of the entire N-WND (Fig. 1) unlike most prior studies, which focused on smaller constructs involving one or two MBDS. These previous studies did not account for the possibility that the presence of other MBDS might affect the behavior of the individual domains. Each MBD variant binds a single Cu(I) ion by direct copper loading as well as by transfer from CuAtox1 (Figs. 3 and 4). These findings are consistent with a previous report in which five or six copper ions were transferred from CuAtox1 to N-WND (21). In those experiments, a 30–40-fold molar excess of CuAtox1 over apo-N-WND was required for the transfer of six copper ions, however. According to NMR data, copper can be transferred from CuAtox1 to WND domains 2 and 4 (7), to MNK domains 2 and 5 (48), and to the three-domain construct comprising MNK MBD4–6 (48) but not to a two-domain WND protein consisting of domains 5 and 6 (7, 48). It is not clear why CuAtox1 can load domains 5 and 6 in our six-domain variants and in MNK domain 5 and MNK MBD4–6 but not in the two-domain constructs used for the NMR studies. One possibility is that copper transfer between CuAtox1 and MBDs–6 requires a higher CuAtox1/MBD ratio than the reported value of 2.5 (7). The current data show almost complete copper transfer at the same ratio, however (Fig. 4, A and B). Another potential explanation is that all or a certain combination of the MBDS may be required for efficient copper transfer from CuAtox1. In support of this notion, CuAtox1 delivers metal to each domain in the protein consisting of MNK domains 4–6 but cannot load the two-domain protein composed of just domains 5 and 6 (48). Whether CuAtox1 actually transfers Cu(I) to each domain in vivo remains unclear.

Copper transfer between Atox1 and the MBDS is highly specific, because no transfer was observed between CuAtox1 and BSA. In addition, the presence of metal-binding cysteine residues in the MBDS is essential for copper transfer, because CuAtox1 was not able to load copper into MBDS. Despite high physiological concentrations and tight binding ($K_{Cu}$ (Cu(I))(GSH)$_2$ = 10$^{30.8}$ M$^{-2}$), GSH does not interfere with the copper transfer process (Fig. 4B). Taken together, these results strongly support copper transfer via specific protein-protein interactions.

Atox1 not only delivers Cu(I) but can also remove Cu(I) from each MBD (Fig. 3, C and D). Reverse copper transfer was observed from N-WND to Atox1 (21) and from a single MBD of the yeast homolog Ccc2 to its chaperone, Atx1 (23). In the former case, a ~20-fold excess of Atox1 was required to remove most of the copper, whereas for the variants in this work, only ~5-fold excess is sufficient. This difference could arise from a cooperative effect in N-WND with all six metal binding sites intact, which would render copper removal more difficult and would not be observed for the MBD variants. ITC data for N-WND suggest that all six sites are equivalent, precluding cooperative binding, however (42). Lutsenko and co-workers (21) have demonstrated that excess apoAtox1 can extract up to five copper ions from fully loaded N-WND, leaving one copper ion bound. These data were interpreted to suggest that one domain may be inaccessible to Cu(I) removal by Atox1 (21). Since ~10–20% of the copper remains in each variant after treatment with excess apoAtox1 (Fig. 3D), the remaining copper ion in N-WND probably represents residual Cu(I) in all six domains rather than a particular domain loaded with a single Cu(I) and inaccessible to the chaperone. This interpretation is consistent with the surface accessibility of each variant observed by BMPA/AMS labeling.

Finally, the $K_{ex}$ values of 2.7–5.4 are consistent with the requirement for at least a 5-fold excess of Atox1 to remove Cu(I) from the MBDS (Table I). These values correspond to an estimated 0.58–1.00 kcal/mol thermodynamic gradient for copper transfer, which is shallow yet 3–5 times greater than that reported for the Atx1/Ccc2 system (23). As suggested for that system, this thermodynamic gradient must be coupled to ATP hydrolysis and/or conformational changes to enable directional copper transfer in vivo. Similar $K_{ex}$ values of 5–10 were obtained by NMR for Cu(I) exchange between Atox1 and MNK domains 2 and 5 (8). The $K_{ex}$ values of >1 suggest that the MBDS have a slightly higher affinity for Cu(I) than Atox1 under equilibrium conditions. The $K_{ex}$ value of ~0.8–0.9 measured recently for Cu(I) exchange between WND domains 2 and 4 (41) is consistent with our data as well (Table I).

The Cu(I) chelator BCA was used to compare the copper retention properties and copper binding affinities of the individual domains. Although a 45-fold molar excess of BCA results in complete redistribution of the Cu(I) from Atox1 to BCA (Ref. 30 and data not shown), <15% of the copper is removed from each of the MBD variants (Fig. 6). All six MBDS behave similarly to one another and to domain 2 (30), indicating that the retention properties alone do not confer upon domain 2 its proposed role as the initial site of Atox1 docking (30). Overall, these data suggest that either the Cu(I) affinity of the MBDS is higher than that of Atox1 or that the $k_{off}$ for Cu(I) is slower for the MBDS. The observation that only a small amount of Cu(I) is redistributed to BCA from our MBD variants but that Atox1 can remove ≥80% of the Cu(I) from the MBDS additionally demonstrates that metal transfer between chaperone and target occurs via direct protein–protein interactions.

The apparent copper binding affinities, $K_{Cu}$ for 1:1 Cu/MBD binding in the context of the intact N-WND determined by the BCA competition assay are 2.2–6.3 × 10$^{10}$ M$^{-1}$. The $K_{Cu}$ for 1:1 Cu/Atox1 binding is similar, 3.5 ± 1.0 × 10$^{10}$ M$^{-1}$ (Table I).

Thus, the slight difference in affinities of Atox1 and the MBDS for Cu(I) suggested by the measured $K_{ex}$ values is not detected by this competition method. The binding of a second Atox1 molecule to CuAtox1 is characterized by a $K_{Cu}$ of 3.3 ± 0.1 × 10$^{6}$ M$^{-1}$. Whereas fitting of the Atox1 data clearly requires the

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presence of a Cu(Atox1)₂ species, similar treatment of the MBD data resulted in a poor fit. In principle, copper-mediated dimerization of the MBD variants might be possible, but it is probably precluded by steric constraints.

By contrast, the K_Cu values for Atox1 and for constructs consisting of MBDA–2, MBDA–4, MBDB–5–6, MBDA–4, and MBDA–1–6 were determined to be 10⁴ to 10⁶ M⁻¹ by ITC (42). This discrepancy may reflect intrinsic differences between direct titrations and chelator competition methods as well as differences in buffer conditions. A discrepancy of similar magnitude was observed for Co(II) binding to nucleocapsid protein and Drs. S. Lutsenko and D. Winge for valuable discussions.

These findings are consistent with estimates of K_Cu by the two methods, the conclusion is the same; the six MBDs have similar affinities for Cu(I) to each other and to K₁ for Atox1. These findings are consistent with NMR data for MNK domains 4 and 6 (8) and with copper transfer data for WND domains 2 and 4 (41). Thus, the roles of the individual MBDs in WND function are not defined by different Cu(I) affinities.

In summary, the properties of each WND MBD have been analyzed within the context of the six-domain N-WND. Contrary to previous studies involving shorter polypeptides, all six MBDs in the variant proteins can receive Cu(I) from the chromophore Atox1, and Atox1 can remove Cu(I) from all six MBDs. When challenged with the Cu(I) chelator BCA, all six MBDs retain most of the bound Cu(I), whereas Cu(I) is readily removed from Atox1. These findings are consistent with estimated K_Cu values. Finally, the apparent K_Cu values for all six MBDs and Atox1 are 2.2–6.3 × 10¹⁰ M⁻¹. The seeming interchangeability of the MBDs in these assays is not compatible with recent proposals that Cu(I) is routed from Atox1 through the MBDs via specific pathways (7, 8, 30). Although the current approach of using N-WND variants is more physiologically relevant than using single domains, detailed understanding of the MBD functions will require studies in the presence of the other WND soluble domains. Given that the six MBDs interact with the ATPBD in the absence of copper (31), interactions with Atox1 and Cu(I) binding may be dependent on the specific involvement of each domain in this interaction. Furthermore, interactions of N-WND with other proteins, such as COMMD1 and Drs. S. Lutsenko and D. Winge for valuable discussions.

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