Interactions between Copper-binding Sites Determine the Redox Status and Conformation of the Regulatory N-terminal Domain of ATP7B

Copper-transporting ATPase ATP7B is essential for human copper homeostasis and normal liver function. ATP7B has six N-terminal metal-binding domains (MBDs) that sense cytosolic copper levels and regulate ATP7B. The mechanism of copper sensing and signal integration from multiple MBDs is poorly understood. We show that MBDs communicate and that this communication determines the oxidation state and conformation of the entire N-terminal domain of ATP7B (N-ATP7B). Mutations of copper-coordinating Cys to Ala in any MBD (2, 3, 4, or 6) change the N-ATP7B conformation and have distinct functional consequences. Mutating MBD2 or MBD3 causes Cys oxidation in other MBDs and loss of copper binding. In contrast, mutation of MBD4 and MBD6 does not alter the redox status and function of other sites. Our results suggest that MBD2 and MBD3 work together to regulate access to other metal-binding sites, whereas MBD4 and MBD6 receive copper independently, downstream of MBD2 and MBD3. Unlike Ala substitutions, the Cys-to-Ser mutation in MBD2 preserves the conformation and reduced state of N-ATP7B, suggesting that hydrogen bonds contribute to interdomain communications. Tight coupling between MBDs suggests a mechanism by which small changes in individual sites (induced by copper binding or mutation) result in stabilization of distinct conformations of the entire N-ATP7B and altered exposure of sites for interactions with regulatory proteins.

Copper is an essential trace element used as a cofactor by numerous enzymes, which take advantage of its low redox potential to catalyze electron transfer reactions. Perhaps because of this high reactivity, copper is kept under strict homeostatic control. The copper-transporting P-type ATPases (Cu-ATPases), ATP7A and ATP7B, are essential for maintaining cellular copper levels. These transporters deliver copper into the secretory pathway for the biosynthesis of cuproenzymes and facilitate export of excess copper from the cells. In humans, mutations in either ATP7A or ATP7B disrupt copper homeostasis and lead to the severe disorders Menkes disease and Wilson disease, respectively (1). Copper is not only transported by Cu-ATPases; it also acts as a regulator of their activity, post-translational modification, and intracellular localization (2). The mechanism of copper-dependent regulation of Cu-ATPases is poorly understood.

ATP7A and ATP7B are highly homologous proteins and share many aspects of function and regulation. In this study, we focus on ATP7B. Cu-ATPase ATP7B is a 165-kDa protein with eight transmembrane segments and most of the soluble parts exposed to the cytosol. The large cytosolic N-terminal domain of ATP7B (N-ATP7B) binds copper and plays a key role in regulation of ATP7B. The intracellular copper donor metallochaperone Atox1 docks to N-ATP7B, transfers copper, and stimulates the activity of ATP7B. N-ATP7B is also phosphorylated by a kinase in response to copper binding (3), houses the sequence determinants for the copper-dependent apical targeting of ATP7B (4), and recognizes dynactin, a component of the trafficking machinery, in a copper-dependent manner (5). It remains unknown whether all of these copper-induced events are directly coupled. It is also unclear how many copper-binding sites in N-ATP7B participate in each regulatory event or how signals from multiple copper-binding events are integrated, if at all.

N-ATP7B contains six metal-binding subdomains (MBDs), each coordinating one copper between 2 Cys residues in an invariant CxxC motif (6, 7). The six MBDs are connected by flexible loops of varying lengths (Fig. 1A). The three-dimensional structure of N-ATP7B is unknown; however, the structures of individual subdomains and two pairs (MBD3/4 and MBD5/6) have been solved (8–15). These studies revealed that all MBDs have a ferridoxin-like \( \beta/\alpha \) sandwich structure (6, 7). In individual recombinant MBDs, copper binding to this loop does not induce significant changes in the structure of MBDs: the loop takes on a more fixed, rigid state (9), whereas the rest of the protein shows minor changes. However, when copper binds to N-ATP7B, significant changes in secondary structure are detected by circular
Cross-talk between Metal-binding Sites in ATP7B

**FIGURE 1. Organization of N-ATP7B.** A, schematic illustrating the relative length of the loops connecting the N-terminal MBDS; copper-binding sites, tryptophan residues, and trypsin recognition sites are indicated. B, structure of a representative MB (MBDS; adapted from Ref. 8) (Protein Data Bank code 2ew9) The GMxCxC metal-binding loop is shown in light gray; Cys residues are indicated.

...for small perturbations in structure (because of mutation or copper binding) to alter the tertiary structure of the entire N-ATP7B.

**MATERIALS AND METHODS**

Constructs/Mutants—Mutations in N-ATP7B were generated using the pMal-N-ATP7B plasmid as a template and the Stratagene QuikChange XLII kit with the following mutagenic primers (or combinations thereof in the case of m2/3A): M2A, GCAATGACGCC-CAGTCCGCTGTCAGTCCAT-TGAAGG (forward) and CTGAC-AGCGGACTGGGCGGTCATGC-CCTCCACC (reverse); M2S, AGG-GCATGACCCAGCCATCCAGTG-TCAGCTCC (forward) and GGA-TGACACTGGACTGGTCTAGCCCT (reverse); M3A, GGAATGACCTGCTAGTCTCGGCTTGAAT-ATTG (forward) and CAAGACGCGACTAGTCA-TCCATCTATTTC (reverse); M4A, CATTGCGCGGATGACCCGTCA-TCCGCTGCCATCCGCCTGCTCCATAATGG (forward) and CTTCATGGAATGACGCTAGCAGCGGTC- ATGGCCGCAATTG (reverse); and M6A, GGAATGACCCGCCTCCCTGCTACCACATAGGTGTC (forward) and GGACAGGAGCAGCCGCTGCATCCGCTGTCCATTCCAT (reverse). The correctness of the coding sequences in all constructs was verified by automatic DNA sequencing.

**Expression and Purification of Recombinant Proteins**—N-ATP7B (and mutants thereof) was expressed and purified as a fusion with maltose-binding protein (MBP) as described previously (6). In brief, the fusion protein was coexpressed with thioredoxin in *Escherichia coli* to maintain solubility and reducing state, purified by affinity chromatography over amylose resin (New England Biolabs, Ipswich, MA), and eluted into buffer containing 10 mM maltose, 25 mM NaH2PO4, and 150 mM NaCl (pH 7.5). The levels of protein expression for mutants and wild-type N-ATP7B were comparable. For copper binding in *vivo*, 250 μM CuCl2 was added to the growth medium prior to inducing protein expression for 3 h at 25 °C. Atox1 was expressed in *E. coli* as a fusion with the chitin-binding protein in the pTYB12-intein vector as described previously (18) and purified by affinity chromatography over chitin resin (New England Biolabs). Intein-mediated cleavage of chitin-binding protein and elution of Atox1 were accomplished by incubating resin-bound protein with buffer containing 50 mM dithiothreitol, 25 mM NaH2PO4, and 150 mM NaCl (pH 7.5) for 36 h at room temperature. Atox1 was concentrated using Amicon ultrafiltration devices (Millipore, Billerica, MA) and then dialyzed against buffer containing 25 mM NaH2PO4 and 150 mM NaCl (pH 7.5) overnight at 4 °C to remove dithiothreitol.

**Atox1-mediated Copper Transfer**—Prior to copper transfer experiments, 1 mg of purified N-ATP7B (at 9 mM) or 1 mg of Atox1 (at 130 μM) was reduced with 1 mM tris(carboxymethyl)phosphine (TCEP) and then dialedyzed into 1 liter of buffer A...
(25 mM NaH₂PO₄ and 150 mM NaCl (pH 7.5)) for 1 h. A 10:1 GSH/CuCl₂ solution made in buffer A was added to the reduced Atox1 at a 1:1 molar ratio for 15 min at room temperature, followed by overnight dialysis to remove excess glutathione. For transfer reactions, increasing amounts of Cu-Atox1 were added to 100 ng of apo-N-ATP7B for 15 min at room temperature. Atox1 was then removed from the mixture by centrifugation through a Microcon YM-50 filter (Millipore); the concentrate containing N-ATP7B was diluted with buffer A, and centrifugations were repeated three times to completely remove Atox1. N-ATP7B was eluted with 100 μl of 25 mM NaH₂PO₄, pH 7.5 containing 25 mM NaCl.

Copper-binding Stoichiometry—The concentration of purified proteins was determined with the Lowry assay (19). Copper concentration in samples was measured using atomic absorption spectroscopy (AA-6650G, Shimadzu, Columbia, MO), and concentration in samples was measured using atomic absorption spectroscopy (AA-6650G, Shimadzu, Columbia, MO), and a copper/protein molar ratio was averaged for each mutant in four independent experiments.

X-ray Absorption Spectroscopy—N-ATP7B mutants were loaded with copper in cells as described above, purified, and concentrated to concentrations yielding >100 μM bound copper. CuK edge (8.9 keV) extended x-ray absorption fine structures (EXAFS) were collected at the Stanford Synchrotron Radiation Laboratory operating at 3 GeV with currents between 100 and 50 mA. All samples were measured and analyzed as described previously (20). For edge comparisons, all edges were normalized to have an intensity of 0 at 8970 eV and an intensity of 1 at 9000 eV.

Fluorescent Labeling of Cys Residues—10 μg (91 pmol) of purified apo-N-ATP7B (wild-type and mutant) in elution buffer was used for labeling of cysteines. 7-Diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin (CPM; Invitrogen, Carlsbad, CA) was added in the dark for 5 min at a concentration of 10 μM and then quenched with 100 μM glutathione. Samples were run on a 12% Laemmli gel and rinsed 2×10 min in double-distilled H₂O, and fluorescent images were taken using a FluorChem 5500 (Alpha Innotech Corp., San Leandro, CA). Gels were then fixed in 50% ethanol and 8% phosphoric acid for a minimum of 3 h, stained with colloidal Coomassie Blue G-250, and imaged again to normalize fluorescence intensity per protein present in the corresponding band. The fluorescence of fully copper-loaded N-ATP7B was defined as the background and was subtracted. Where indicated, samples were incubated in solutions containing either 100 μM TCEP for 10 min (to reduce disulfide bonds) or 6 M urea for 30 min (to expose buried Cys residues) prior to labeling with 10 μM CPM as described above.

Quantitation of Reduced Cys Residues—Further quantitation of available cysteine residues was carried out using Ellman’s reagent (5,5’-dithiobis(2-nitrobenzoic acid)) as described (21). Briefly, 10 μg (91 pmol) of purified apo-N-ATP7B (wild-type ± copper and m2A) in elution buffer was used for Ellman’s reaction after adjusting the protein concentration for A₂₈₀ measurements. Where indicated, samples were incubated in solutions containing either 100 μM TCEP for 10 min (to reduce oxidized cysteines) or 6 M urea for 30 min (to expose buried Cys residues), with all samples suspended in final volumes of 50 μl. Samples were added to 750 μl of 1 M Tris-HCl (pH 8.0), followed by the addition of 50 μl of 2 mM 5,5’-dithiobis(2-nitrobenzoic acid) (in 50 mM sodium acetate) for 15 min and reading the absorbance at 412 nm. A calibration curve of acetylcysteine ranging from 31.25 μM to 1 mM was assayed in duplicate along with each set of test samples. The assay was repeated three times using the calibration curve to estimate total cysteines available per protein in the N-ATP7B samples.

Limited Proteolysis—15 μg of eluted apo-N-ATP7B (wild-type and mutant) at 10 μM was digested with tosylphenylalanyl chloromethyl ketone-treated bovine pancreatic trypsin (Sigma) for 3 h at room temperature at a 1:3000 (w/w) protease/protein ratio in 83 mM ammonium bicarbonate (pH 8.0) containing 10 mM CaCl₂. The reaction was stopped by the addition of 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride. The protein fragments were separated on a 15% Laemmli gel and then fixed and stained with colloidal Coomassie Blue G-250. In some instances, CPM labeling and quenching were performed as described above prior to proteolysis.

Native Gel Electrophoresis—20 μg of wild-type N-ATP7B was subject to limited proteolysis (see above) and then separated on a 10% Tris/glycine gel that did not contain urea, SDS, or reducing agents. The gel was fixed in 50% ethanol and 8% phosphoric acid for a minimum of 3 h and stained overnight with colloidal Coomassie Blue G-250. The lane containing the protein fragments was destained in 7% acetic acid, equilibrated in Laemmli running buffer containing 2 M urea for 1 h to redissolve proteins, layered onto a 12.5% Laemmli gel, and separated in the presence of 2 M urea and 0.1% SDS. This gel was then fixed and silver-stained as described (22).

In-gel Digests/Extractions—For protein identification by tandem mass spectrometry, Coomassie Blue-stained gels were washed in double-distilled H₂O, and relevant spots were excised. Spots were incubated twice in 500 μl of 50 mM NH₄HCO₃ in 50% acetonitrile for 30 min and then dried by removing the wash buffer and incubating in 100% acetonitrile for 2 min. If the samples had not been previously reduced and alkylated, 100 μl of buffer containing 10 mM dithiothreitol and 100 mM NH₄HCO₃ was added for 45 min at 56 °C and removed, and 100 μl of buffer containing 55 mM iodoacetamide and 100 mM NH₄HCO₃ for 30 min at room temperature. This solution was removed, and 500 μl of buffer containing 50 mM NH₄HCO₃ and 50% acetonitrile was added for 15 min at room temperature. This solution was replaced with 200 μl of 100% acetonitrile for 2 min at room temperature. 20 μl of digestion solution (0.01 μg of trypsin, 44 mM NH₄HCO₃, and 4.4 M CaCl₂) was added for 15 min at 4 °C. (For large spots, this was repeated until liquid was no longer absorbed.) Excess solution was removed, and 60 μl of digestion solution (without trypsin) was added at 37 °C for 16 h. To extract peptides, 3 μl of 88% formic acid was added for 15 min at 37 °C, and all of the supernatant was then collected for tandem mass spectrometry analysis.

Fluorescence Spectroscopy—Purified N-ATP7B-MBP was dialyzed into buffer containing 25 mM NaH₂PO₄ and 150 mM NaCl (pH 7.5) for 1 h and diluted to 0.3 mg/ml in the same buffer using A₂₈₀ to evaluate and equate final concentrations. MBP was prepared in the same manner as N-ATP7B-MBP, dialyzed, and diluted to a final concentration of 0.12 mg/ml.
400-μl samples were taken for fluorescence emission scans using a SpectraMax M2 fluorescence cuvette reader (Molecular Devices, Sunnyvale, CA). Excitation wavelengths were set at either 280 or 295 nm, and emission readings were taken between 250 and 450 nm at 1-nm intervals using 10 scans/emission wavelength. Following scans, $A_{280}$ measurements were taken to normalize the data for protein concentration. The experiment was performed three times, with a representative scan presented herein.

**Modeling and Molecular Dynamics Simulations**—Structures for MBD1 and MBD2 were generated through homology modeling (Modeler version 9v6) using human ATP7B (NCBI Protein Database accession number P35670) and then used to generate distance constraints for *ab initio* modeling. *Ab initio* structure prediction was carried out using locally installed Rosetta *ab initio* software (version 2.0, licensed through the University of Washington). The fragment libraries were generated using the Web version of the Rosetta fragment server (Robetta). Briefly, fragments for residues 56–214 of ATP7B were generated using the Robetta fragment server, and 10,000 independent structures were predicted using the above-generated distance constraints for MBD1 and MBD2. The connecting loop along with 5-residue anchors from MBD1 and MBD2 (residues 121–148) were extracted using the MMTSB tool set and subjected to a clustering analysis. Loop extraction was performed to avoid biasing the clustering algorithm through constrained MBDs (which are significantly longer than the connecting loop). The centers of the three largest clusters were chosen as the best models. The MBDs were then “ligated” back to the models for the connecting loops using the 5-residue anchors. The final models were minimized using CHARMM27 force fields and validated as described previously (23).

A 10.0-ns molecular dynamics simulation of MBD2 from ATP7A in explicit solvent was conducted using the NAMD version 2.6 molecular dynamics simulation package and the CHARMM27 force field. Because the structure of MBD2 from ATP7B was not available and because one can argue that the *ab initio* models generated from Rosetta may not be accurate enough for molecular dynamics simulations, we used the structure of MBD2 from ATP7A (Protein Data Bank code 1s6o) (13). This structure of MBD2 (code 1s6o) was introduced in a box of TIP3P explicit water molecules that extended at least 10 Å away from the protein surface, and counterions were introduced to obtain a neutral system. The protonation status of the titratable residues corresponds to pH 7.0. The system was minimized in two steps: water minimization and entire system minimization. First, the system was subjected to 1000 steps of conjugate gradient energy minimization with the coordinates of the protein frozen to allow the solvent molecules to relax. A second 1000 steps of conjugate gradient energy minimization were then performed after unfreezing the protein to remove steric clashes found in the protein structure. The root mean square deviation (r.m.s.d.) as a function of time for the simulated structures was stable for 200 ps. The final equilibrated system that uses periodic boundary conditions has dimensions of 59.18, 64.22, and 64.92 Å. The resulting system was used in a 10.0-ns simulation that was performed in the NVT ensemble using a 2-fs time step and a Langevin thermostat with a 5 ps$^{-1}$ damping parameter. The system temperature was coupled using the Berendsen algorithm at 300 K. Electrostatic interactions were calculated using the particle mesh Ewald with a real space cutoff of 0.9 nm. Cutoff for van der Waals interactions was set at 0.9 nm. A SHAKE algorithm was applied using a tolerance of 10$^{-8}$ Å to maintain all bonds containing hydrogen at their equilibrium length. The time step for integration was 2 fs, and coordinates and velocities were saved every 2 ps. Simulations were analyzed using NAMD routines, and the data were plotted using GraphPad version 4.0.

**RESULTS**

**MBD2 Influences the Copper-binding Capability of Other N-terminal MBDs**—To understand the relationships between different MBDs, we first generated and characterized the m2A mutant of N-ATP7B, in which the copper-binding CxxC motif of MBD2 was replaced with AxxA (Fig. 2A). Earlier experiments suggested that MBD2 may regulate access to other MBs (see above). We hypothesized that MBD2 performs this gating role by interacting with other MBDs and changing the protein conformation of N-ATP7B (thus allowing access to other sites) in response to copper binding. Mutating MBD2 would disrupt this communication and cause insufficient exposure of other MBDs to Atox1 and inadequate copper transfer. To test this hypothesis, we compared the copper loading of wild-type N-ATP7B and the m2A mutant by Cu-Atox1. For wild-type N-ATP7B, as shown previously (18), all six MBDs could be loaded with copper (Fig. 2B). However, for the m2A mutant, instead of the expected stoichiometry of 5 copper atoms/protein, <3 copper atoms were transferred (maximum of 2.8 ± 0.5) (Fig. 2B) even using up to 60-fold excess of Cu-Atox1 over N-ATP7B.

To test whether the decreased copper-binding stoichiometry of the m2A mutant was due to structural changes, we performed limited proteolysis on wild-type and m2A N-ATP7B and compared proteolytic patterns by separating fragments on an SDS-polyacrylamide gel (Fig. 3). At the concentration used for limited proteolysis, trypsin cuts N-ATP7B at a limited number of specific sites in the loops connecting the MBDs, whereas individual 7–8-kDa MBDs and the 16-kDa MBD5/6 pair remain intact (see Fig. 1A) (3, 17). The fragmentation patterns of wild-type and mutant N-ATP7B were markedly different (Fig. 3), demonstrating that the m2A mutation caused a conformational change in N-ATP7B. The mobility of several bands was increased (*i.e.* the fragments became shorter), suggesting that additional trypsin recognition sites in the loops were exposed. We investigated the kinetics of the proteolysis and found that the difference in pattern remained stable for several hours before patterns converged (supplemental Fig. 1). Thus, the change in the MBS led to a new configuration of the loops connecting MBDs (see more under “Discussion”).

If the structural changes in the m2A mutant preclude appropriate exposure of the remaining MBDs to Atox1, then incubating m2A with free copper (rather than with a relatively bulky Atox1) is likely to yield higher copper-binding stoichiometry. To test this prediction, wild-type N-ATP7B and the m2A mutant were loaded with copper in cells by growing *E. coli* in medium supplemented with CuCl$_2$. Although *E. coli* lacks...
Atox1 orthologs, wild-type N-ATP7B can be loaded with ~5.5 copper atoms/protein by metal taken up by cells (6). Contrary to our expectations, the m2A mutant still showed significantly reduced copper binding (2.6 ± 0.3 copper atoms/protein compared with the control at 5.3 ± 0.1 copper atoms/protein) (Fig. 4). This result suggested that the m2A mutation impeded more than one MBD from binding (or retaining) copper and that the copper-binding status of the N-terminal MBDs depends on a functional MBD2.

**Mutations of Different MBDs Have Distinct Effects on Copper Binding by N-ATP7B**—To determine whether the CxxC-to-AxxA mutations in other MBDs would have a similar effect on copper binding by N-ATP7B, we generated a series of mutants (Fig. 2A). The mutant proteins were expressed in *E. coli*, loaded with copper in cells, and purified, and their copper-binding stoichiometry was measured (Fig. 4). Mutating MBD3 (m3A) decreased copper binding (2.2 ± 0.4 copper atoms/molecule) to a level similar to the m2A mutation. In contrast, mutants of MBD4 (m4A) and MBD6 (m6A) bound copper with stoichiometry close to what was expected for domains containing five intact MBSs (4.6 ± 0.2 and 4.9 ± 0.1, respectively). Interestingly, when both MBD2 and MBD3 were mutated (in the m2/3A variant), the remaining sites bound copper with a stoichiometry close to the expected 4 copper atoms (3.6 ± 0.3) as if a negative effect of each individual mutation was counteracted. Altogether, these data suggest that MBD2 and MBD3 may work together to regulate access to other MBSs, whereas MBD4 and MBD6 receive copper independently, following transfer of copper to MBD2 and MBD3.

**Decrease in Copper-binding Stoichiometry Is Due to Cys Oxidation**—To better understand how mutation in MBD2 decreases the number of available copper-binding sites, we first examined whether the mutation altered the arrangement of the

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**FIGURE 2.** Atox1-mediated copper transfer is impaired in the MBD2 mutant. A, diagram of the N-ATP7B mutants used in this work. B, copper transfer from Atox1 to N-ATP7B. Points represent the amount of copper that remains bound to N-ATP7B after incubation with the indicated amounts of Cu-Atox1 and removal of Cu-Atox1 from the mixture. Error bars indicate S.D. across three independent experiments. wt, wild-type N-ATP7B.

**FIGURE 3.** Mutation m2A induces structural changes in N-ATP7B. The Coomassie Blue-stained gel compares limited proteolytic patterns of wild-type (WT) and m2A N-ATP7B.

**FIGURE 4.** Mutations in MBDs have distinct effects on the copper-binding stoichiometry of N-ATP7B mutants. Wild-type (wt) and mutant N-ATP7B were loaded with copper in *E. coli* prior to purification. Light gray bars indicate the number of CxxC motifs left intact. Dark gray bars represent experimental values as determined by atomic absorption (copper) and Lowry assay (protein). Error bars indicate S.D. across three experimental repeats.
remaining intact sites, possibly causing more than one MBD to coordinate the same copper molecule. In wild-type N-ATP7B, each copper is coordinated by two sulfur groups of the CxxC motif in a linear fashion with bond lengths of 2.2 Å (24). EXAFS characterization of copper bound to m2A showed no significant deviation from this wild-type coordination (supplemental Fig. 2). Similar copper coordination was also observed for the m2/3A mutant. These results suggested that the decrease in copper-binding stoichiometry was unlikely due to changes in the copper-coordinating environment but rather was caused by the loss of copper-binding sites.

To estimate the number of available sites, Cys residues in both wild-type and mutant apo-N-ATP7B were labeled with the Cys-directed fluorescent maleimide CPM (Fig. 5A). The m2A mutant showed a marked decrease in CPM labeling (∼33% compared with wild-type N-ATP7B). To further test for specificity of the m2A mutation with respect to Cys reactivity, we performed CPM labeling of other mutants described in Fig. 2A. The only mutant besides m2A to have a significant reduction in CPM labeling was m3A (Fig. 5B), which was also the only mutant to have a sharp decrease in copper binding. To ensure that this observation was not the artifact of the purification procedure, we labeled crude E. coli lysates with CPM prior to protein purification (supplemental Fig. 3A). Lower fluorescence of m2A was again detected (supplemental Fig. 3A), demonstrating that the loss of available cysteines happened during the expression of the protein, consistent with the loss of in-cell copper binding by these mutants (Fig. 4).

To examine whether the decrease in CPM labeling was due to blocked access to the sites or oxidation of Cys residues, we repeated labeling experiments after treatment of N-ATP7B with the disulfide-reducing agent TCEP (Fig. 5A). With TCEP treatment, the m2A mutant regained about half of the lost fluorescence (to 67% of the wild-type protein). (The wild-type protein also gained ∼10% CPM labeling after TCEP treatment, which we ascribe to the recovery from normal oxidation during protein purification.) The lower free thiol reactivity of the m2A mutant was further confirmed using Ellman’s reaction (supplemental Fig. 3B). Altogether, these data suggest that the m2A and m3A mutations render N-ATP7B susceptible to oxidation.

**Tight Packing of MBDs Allows for Cross-communication**—The marked effect of the m2A and m3A mutations on other MBDs suggested that the MBDs closely interact with each other despite being connected by long loops. To examine how tightly the MBDs interact, we subjected wild-type apo-N-ATP7B to limited proteolysis and separated the resulting fragments on a native Tris/glycine gel. Under these conditions, most of the fragments remained associated and migrated together. The 8–12-kDa bands were also detected in the second dimension, indicating that these fragments interact with the rest of the bundle, but perhaps less tightly.

The interdependence of MBDs in N-ATP7B was further confirmed by performing limited proteolysis of other mutants described in Fig. 2A. Distinct proteolytic patterns for these mutants (supplemental Fig. 5) indicated that MBD packing controls the exposure/orientation of connecting loops in a very precise manner.
Flexibility of the Copper-binding Loop Allows for Changing Exposure of Sulphhydryl Groups—Having established interaction between MBDs, we investigated the mechanism of interdomain communication. The copper-binding CxxC site is situated at the surface of MBD2 and must be sufficiently exposed for Atox1 to dock and transfer copper (Fig. 1B). Therefore, it was not apparent how mutating Cys residues to Ala in the exposed loop would affect the conformation of the entire N-ATP7B. We considered that the sulphhydryl group of Cys, unlike the side chain of Ala, is capable of forming a hydrogen bond. Others have demonstrated differential dynamics and flexibility of the metal-binding loops of various MBDs using molecular dynamics simulations (25). We hypothesized that, in the apo form, the metal-binding loop of MBD2 may be flexible enough to reach and interact with other MBDs and that the ability of sulphhydryl groups to hydrogen bond would be important for the interdomain contacts.

To test this hypothesis, we first examined the flexibility of MBD2 using molecular dynamics simulations. The structure of MBD2 of ATP7B is not available; rather than using a model, we characterized the dynamics of MBD2 of ATP7A, for which structures have been solved (13) and which has 60% identity and 77% similarity to MBS2 of ATP7B. Supplemental Fig. 8 compares the structure of MBD2 (Protein Data Bank code 1s60) with the TIP3P explicit solvent-equilibrated structure used for the molecular dynamics simulation. The results show that the initial structure and the equilibrated structure used for this simulation display a r.m.s.d. of <1.0 Å. Following a 10,000-ps simulation of dynamic movement in solution, we calculated r.m.s.d. values for the backbone carbons, as well as distances between the α-carbons of metal-binding cysteines. Blue dots (S–S) indicate the distance between sulfur atoms of metal-binding cysteines. Black dots (Cα–Cα) indicate the distance between α-carbons of metal-binding cysteines. Green dots (Cα–Cα) indicate the distance between α-carbons of metal-binding cysteines. Blue dots (S–S) indicate the distance between sulfur atoms of metal-binding cysteines. Black dots (Cα–Cα) indicate the distance between α-carbons of metal-binding cysteines. Green dots (Cα–Cα) indicate the distance between α-carbons of metal-binding cysteines.

In addition, when subjected to limited proteolysis, the m2S mutant displayed a fragmentation pattern that was more similar to the wild-type than to the m2A pattern (Fig. 8C). Similarly to m4A and m6A, which did not show any significant decrease in CPM labeling (Fig. 5B). In addition, when subjected to limited proteolysis, the m2S mutant displayed a fragmentation pattern that was more similar to the wild-type than to the m2A pattern (Fig. 8B).

These results strongly suggest that the hydrogen-bonding ability of the Cys residues is critical for the overall folding of N-ATP7B. To further test this conclusion, we compared intrinsic Trp fluorescence in wild-type N-ATP7B and the m2 mutants (Fig. 8C). N-ATP7B contains 2 Trp residues: at the very N terminus before MBD1 (residue 29) and in the loop between MBD1 and MBD2 (Fig. 1A). Compared with apo-N-ATP7B, the m2A mutant displayed a fragmentation pattern that was more similar to the wild-type than to the m2A pattern (Fig. 8B).
ATP7B, the m2A mutant had a higher fluorescence (110 ± 4% of the wild-type protein), suggesting a greater exposure of Trp to solution. Unlike m2A, the m2S mutant did not show an increase in fluorescence (96 ± 2% compared with the wild-type protein), a result consistent with its behavior in other tests. Similar results were obtained when fluorescence was measured using an excitation wavelength of 295 nm (data not shown).

Finally, if the metal-binding Cys residues in MBD2 play a role in interdomain communication, then the transfer of copper from Atox1 to N-ATP7B is expected to have an effect on N-ATP7B conformation. We have demonstrated that the regulatory N-terminal domain of ATP7B is arranged with the MBDs in close contact and communication with each other. The tight packing of MBDs fixes the connecting loops in distinct conformations and allows for small structural changes in individual MBDs to be transmitted to other MBDs and modify the exposure of the connecting loops. The interdomain communication appears to require hydrogen bonding, which also contributes to the maintenance of the redox status of metal-binding Cys residues. MBD2, the site of primary copper transfer from Atox1 (when the fully folded N-ATP7B is used), along with MBD3 plays a central role in maintaining the conformation and reduced state of the entire N-terminal domain.

NMR and crystal structures of individual MBDs show only minor differences between the copper-bound and apo forms (8–15). However, the conformation and secondary structure of apo-N-ATP7B and copper-loaded N-ATP7B differ significantly (3, 7). Our data show that, even in the apo form, MBDs are packed tightly, albeit not rigidly, together. Copper binding to the CxxC site, which causes the inward movement of the loop, making it rigid and less exposed (13), appears to alter the contacts between MBDs (as do the Cys-to-Ala mutations) and induces changes in the structure of loops connecting MBDs. The change in loop structures/orientations is likely to be an important event in regulating ATP7B because it may expose sites for kinase-mediated phosphorylation located within the loops (3, 26).

**DISCUSSION**

We have demonstrated that the regulatory N-terminal domain of ATP7B is arranged with the MBDs in close contact and communication with each other. The tight packing of MBDs fixes the connecting loops in distinct conformations and allows for small structural changes in individual MBDs to be transmitted to other MBDs and modify the exposure of the connecting loops. The interdomain communication appears to require hydrogen bonding, which also contributes to the maintenance of the redox status of metal-binding Cys residues. MBD2, the site of primary copper transfer from Atox1 (when the fully folded N-ATP7B is used), along with MBD3 plays a central role in maintaining the conformation and reduced state of the entire N-terminal domain.
Conformational changes in the loops may also provide docking sites for trafficking machinery (5) while burying residues that interact with the ATP-binding domain and disrupting interdomain interactions (27). It is interesting that the conformational effects of mutating different MBDS (or sequential copper binding) are not identical as evidenced by distinct and stable proteolytic patterns. This diversity of consequences may be necessary for a fine-tuned response to various concentrations of copper and may explain the poorly understood need for multiple copper-binding sites in N-ATP7B.

The effect of m2A and m3A mutations on the oxidation state and copper-binding ability of other MBDS was unexpected. The drastic difference between m2A and m2S was particularly striking. These results suggest that hydrogen bonding may be required for both the precise packing and maintenance of the reduced state of N-ATP7B. The flexibility of the metal-binding loop in MBDB could be critical for allowing communications between MBDSs (and between MBD and Atox1) (see Ref. 25). It is interesting to consider that the stabilization of a Cys residue in a more extended conformation through hydrogen bonding is likely to decrease the probability of forming disulfide bonds and thus preserve the reduced state of the MBDSs. This view is supported by our observation that, in wild-type N-ATP7B, MBDB is reduced and available for Cys labeling, whereas following proteolysis (when precise contacts are disrupted), MBDB rapidly oxidizes and cannot be labeled with CPM (supplemental Fig. 7). Our data also suggest that some Cys residues could be oxidized irreversibly (supplemental Fig. 3B), possibly to sulfenic or sulfonic acid, as has been observed for other Cys-containing metalloproteins (29). Although this may be a by-product of our expression system, these in vitro data raise an interesting question as to how the reduced state of N-ATP7B is maintained in a cell. A previous report on a copper-dependent interaction between N-ATP7B and glutaredoxin suggests one possible reduction mechanism (30).

It is also intriguing that the m2A and m3A mutants have a similar increase in oxidation and loss of copper binding, whereas mutating both MBDSs in tandem relieves this effect (Figs. 4 and 5B). This observation suggests that, although both MBDSs play an important role in regulating the status of other MBDSs, they each do not have individual downstream partners. The proteolytic pattern of the m2/3A mutant is distinct from that of either m2A or m3A (supplemental Fig. 5B). It is possible that this distinct conformation favors the “proper” orientation of the remaining MBDSs, although we did not explore this further in this study. It is clear that not every change in conformation is associated with increased susceptibility to oxidation. The m6A mutant had a similar proteolysis pattern to the m2A mutant (supplemental Fig. 5A), even though it bound copper and labeled with CPM at predicted levels. This suggests that the conformational changes in loops and redox sensitivity of MBBS are related but not dependent on one another and further demonstrates the unique role MBDB plays in regulating access to other MBBSs in N-ATP7B.

We attribute the changes in the proteolytic pattern of the m2A mutant to be predominantly due to the dissociation of MBDB and MBDB1 from MBDB2 and exposure of additional trypsin sites that are inaccessible when the domains are packed tightly together (Fig. 1A). This is supported by the increase in tryptophan fluorescence in the mutant, which we think is likely due to the exposure of the Trp residue in the loop connecting MBDB1 and MBDB2. We also observed some dissociation of MBDB1 and MBDB2 when the digested domain was separated on a native gel (Fig. 6A). Our ab initio structures (Fig. 9) provide a clue to the potential interaction between MBDB1 and MBDB2. The loop connecting the two domains seems to have some inherent α-helical tendencies, which could bring the two MBDSs close together, but can also unfold and increase the distance between the MBDSs. Molecular dynamics simulations of apo- and Cu-Atox1, which is a structural homolog of MBDSs, suggest that copper binding may increase the flexibility of the portion of the MBDB opposite from the metal-binding loop, thus influencing pairing of other MBDSs (28). This change could be transmitted through the loop region, imparting more structure to the loop in the copper-bound state and bringing the MBDSs closer together.

**FIGURE 9. Structures of pairs of neighboring MBDSs.** A, structure of MBDB5 and MBDB6 of ATP7B (adapted from Achila et al. (8); Protein Data Bank code 2ew9). B–D, ab initio structures for MBDB1 and MBDB2. MBDB1 is shown in green, the inter-MBD loop in cyan, and MBDB2 in blue. The GMxCxxC loop is shown in red, and copper-binding Cys residues are shown in yellow.
together. Previous EXAFS analysis of copper-loaded N-ATP7B consistently showed a Cu–Cu distance of 2.6 Å (24), indicative of close proximity between pairs of MBSs.

In summary, we have presented a working model in which close packing of N-ATP7B allows cross-talk among MBSs. This interdomain communication is necessary for maintenance of reduced cysteines and proper copper acquisition. The MBD interactions may involve a hydrogen-bonding network that allows copper binding to be translated into larger structural rearrangements via the loops between the MBDs. These rearrangements are likely to play a major role in regulating ATPase activity as well as mediating interactions with cellular trafficking machinery. Maintaining the precise interdomain contacts could also be essential for success of high resolution structural studies of N-ATP7B, which, so far, have represented a significant challenge.

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