The Wilson’s disease protein (WNDP) is a copper-transporting ATPase regulating distribution of copper in the liver. Mutations in WNDP lead to a severe metabolic disorder, Wilson’s disease. The function of WNDP depends on Atox1, a cytosolic metallochaperone that delivers copper to WNDP. We demonstrate that the metal-binding site 2 (MBS2) in the N-terminal domain of WNDP (N-WNDP) plays an important role in this process. The transfer of one copper from Atox1 to N-WNDP results in selective protection of the metal-coordinating cysteines in MBS2 against labeling with a cysteine-directed probe. Such selectivity is not observed when free copper is added to N-WNDP. Similarly, site-directed mutagenesis of MBS2 eliminates stimulation of the catalytic activity of WNDP by the copper-Atox1 complex but not by free copper. The Atox1 preference toward MBS2 is likely due to specific protein-protein interactions and is not due to unique surface exposure of the metal-coordinating residues or higher copper binding affinity of MBS2 compared with other sites. Competition experiments using a copper chelator revealed that MBS2 retained copper much better than Atox1, and this may facilitate the metal transfer process. X-ray absorption spectroscopy of the isolated recombinant MBS2 demonstrated that this sub-domain coordinates copper with a linear biscysteinate geometry, very similar to that of Atox1. Therefore, non-coordinating residues in the vicinity of the metal-binding sites are responsible for the difference in the copper binding properties of MBS2 and Atox1. The intramolecular changes that accompany transfer of a single copper to N-WNDP are discussed.

Copper is essential for cell growth and development as a cofactor of cytochrome c oxidase, copper, zinc-dependent superoxide dismutase, ceruloplasmin, lysyl oxidase, and other important metabolic enzymes. However, abnormally elevated copper disrupts cell metabolism, presumably by stimulating the production of reactive oxygen species. Toxic effects of copper are evident in patients with Wilson’s disease, a genetic disorder characterized by accumulation of copper in tissues and severe hepatic and neurological problems (1). To regulate the intracellular copper concentration, cells have developed a sophisticated network of copper-transporting proteins that includes the copper-transporting ATPases and metallochaperones (2–4).

The Wilson’s disease protein (WNDP) is a copper-transporting P-type ATPase that plays a key role in copper distribution in the liver, kidney, and, the brain. WNDP utilizes the energy of ATP hydrolysis to transport the metal into the secretory pathway for incorporation into such copper-dependent enzymes as ceruloplasmin and to export excess copper from the cell (5). WNDP and other eucaryotic copper-ATPases are unique among the P-type ATPases because they do not bind copper directly from the cytosol (where the amounts of free copper are extremely low (6)) but receive the metal ion from a small cystolic protein called a metallochaperone through direct protein-protein interactions (7–10).

Atox1 (previously known as HAH1) serves as a metallochaperone for WNDP. Several mutations in WNDP originally found in Wilson’s disease patients were shown to disrupt the Atox1-WNDP interaction (11), suggesting that Atox1 is required for WNDP function. In agreement with this prediction, we demonstrated that Atox1 directly transfers copper to WNDP and that copper delivery results in stimulation of the WNDP catalytic activity (9). Conversely, apoAtox1 can strip copper from WNDP, leading to inhibition of WNDP (9). Therefore, Atox1 can regulate the functional activity of WNDP by modulating the amount of copper bound to the protein.

Although the role of Atox1 in copper delivery to WNDP seems clear, the molecular details of this intriguing process remain uncertain. WNDP contains a large N-terminal domain (N-WNDP) with six metal binding subdomains (MBS) that have homologous sequences (Fig. 1) and a very similar $\beta_6\beta_6\beta_6$ fold (12, 13). Each MBS includes a conserved GMXGXC sequence, which is situated in the exposed loop. It has been shown that N-WNDP binds up to six Cu$^+$ ions and that copper is coordinated by the two cysteines of the GMXGXC sequence (14–16). Atox1 contains a similar copper-binding motif, MXCXC (Fig. 1), and binds Cu$^+$ per subunit (17, 18). Interestingly, Atox1 has the same overall fold as the individual copper binding subdomains of N-WNDP (19). This similarity in structure and the presence of complimentary charges at the surface of Atox1 and some of the N-terminal MBSs of the copper-transporting ATPases led to the suggestion that Atox1 docks to MBS and transfers copper via ligand exchange (13, 19, 20). The crystallographic structure of Atox1, in which one copper...
bonds two Atox1 monomers, provides an attractive model for such a copper-transfer intermediate (19).

Despite considerable progress in the structural characterization of Atox1 and individual MBSs, it is still unknown how copper migrates within N-WNDP after transfer from Atox1. In fact, even the first step of this process is poorly understood. For example, we do not know whether Atox1 docks randomly to any MBS and transfers copper with equal efficiency or if there is a specific and unique entry pathway in the N-WNDP for copper. It is also unclear what happens after copper is transferred to N-WNDP. Previous studies demonstrated that although six MBSs of N-WNDP are structurally similar, their functions are distinct. MBS5 and MBS6 are important for copper delivery to the intramembrane copper-binding site(s) (21) and appear to control the affinity of these sites for the metal (22). In contrast, MBS 1–4 do not affect the affinity of the intramembrane sites but may regulate the access of copper to these sites and modulate the enzyme turnover (22).

Interestingly, the fragment of N-WNDP including MBS1–4, but not the MBS5- and MBS6-containing fragment, was shown to interact with the copper-bound Atox1 (8). Thus, taken together the experimental data suggest that copper translocation through N-WNDP toward the intramembrane portion is likely to involve several MBSs and represent a multistep process. To better understand the molecular mechanism of this process, here we characterized the consequences of the Atox1-mediated transfer of a single copper ion to N-WNDP. We demonstrate that Atox1 selectively delivers copper to MBS2 and that this step is essential for further migration of copper to the intramembrane copper-binding sites of N-WNDP.

Experimental Procedures

Recombinant Proteins—Expression and purification of Atox1 were carried out using a published protocol (9). Briefly, the recombinant Atox1 was expressed in Escherichia coli as an intein fusion and purified from a soluble fraction of cell lysate using affinity chromatography on chitin beads (New England Biolabs). After washes with 25 mM Na2HPO4, 150 mM NaCl, pH 7.5 (Buffer A), Atox1 was cleaved from the fusion protein and eluted from the resin with 50 mM dithiothreitol in 25 mM Na2HPO4, 150 mM NaCl, pH 8.15. The protein was then dialyzed into Buffer A and used for copper binding and transfer experiments. The copper-bound form of Atox1 (8) was incubated with N-WNDP for 10 min. To remove the chaperone, the resin was washed extensively with 10 volumes of Buffer A, and N-WNDP was eluted with Buffer A containing 10 mM maltose. The protein concentration and the copper stoichiometry were measured in the eluted samples of N-WNDP as described above. For copper binding in the absence of Atox1, N-WNDP was incubated with increasing concentrations of CuCl2 (Sigma) dissolved in buffer A containing freshly prepared 200 μM ascorbate (Fisher). Under these conditions all copper is present in the reduced form (our data). The time of incubation and subsequent washes and elution steps were the same as for experiments with Cu*-Atox1.

Chemical Labeling and Proteolysis of N-WNDP—To produce recombinant metal binding subdomain 2 (MBS2, residues 141–212 of N-WNDP), the corresponding segment of the ATP7B cDNA was amplified using the following primers: forward, 5'-CATAT-GGAGGAGCCTGCTGTC3'; reverse, 5'-GTCGACCTAGCTTGTAGTGCAACG-3'. The fragments were designed such that 5' Ndel and 3' SalI restriction sites were incorporated into the MBS2 PCR product. The PCR fragment was cloned into the pTYB12 IMPACT expression vector (New England Biolabs) to produce the pTYB12-MBS2 expression plasmid, and the sequence fidelity was verified by automated DNA sequencing. E. coli ER2566 cells were transformed with the pTYB12-MBS2 plasmid, and the expression of the MBS2 fusion was induced by isopropyl-β-D-thiogalactopyranoside added to a final concentration of 100 μM at 25°C for 20 h. The MBS2 was purified from the soluble fraction using the protocol described above for Atox1 and dialyzed into Buffer A. The copper-bound form of MBS2 was generated by incubation of apo-MBS2 with equimolar amounts of a copper-glutathione complex and dialyzed into 20 mM Na2HPO4, pH 7.5, 80 mM NaCl, and 15% Tris-Tricine gel (25), and the separation of the CPM-labeled peptides was monitored under UV light using a Gel-Doc system (Bio-Rad).

Copper Transfer Experiments—Before copper transfer experiments, the purified N-WNDP bound to amylase resin was reduced by incubating with 100 μM Tris(2-carboxyethyl)phosphine hydrochloride (Sigma) for 10 min. After a wash with 3 resin volumes of buffer A, apoAtox1 or Cu*-Atox1 was added and incubated with N-WNDP for 10 min. To remove the chaperone, the resin was washed extensively with 10 volumes of buffer A, and N-WNDP was eluted with buffer A containing 10 mM maltose. The protein concentration and the copper stoichiometry were measured in the eluted samples of N-WNDP as described above. For copper binding in the absence of Atox1, N-WNDP was incubated with increasing concentrations of CuCl2 (Sigma) dissolved in buffer A containing freshly prepared 200 μM ascorbate (Fisher). Under these conditions all copper is present in the reduced form (our data). The time of incubation and subsequent washes and elution steps were the same as for experiments with Cu*-Atox1.
Surface labeling of N-WNDP was performed by incubating 373 pmol of apo-N-WNDP with 0, 1, and 2 mol equivalent of CPM in buffer A. The reaction was quenched with β-mercaptoethanol. The labeled samples were then digested with trypsin and analyzed by Tris-Tricine gel electrophoresis as described above.

Comparison of the Copper Binding Characteristics of MBS2 and Atox1

Using Competition with the Copper Chelator BCA—Copper-bound Cu-‘Atox1 and Cu-‘MBS2 were diluted with 20 mM NaH₂PO₄, 150 mM NaCl, pH 7.5, buffer containing freshly prepared 40 μM ascorbate to obtain a 7.5 μM concentration of the copper-containing complex. The proteins were then incubated with increasing concentrations of BCA for 10 min. The formation of the BCA-Cu(I) complex was monitored spectrophotometrically at 562 nm with a Beckman DU 640B spectrophotometer. A solution containing 7.5 μM BCA, 10 mM CuCl₂, 40 μM ascorbate, 20 mM NaH₂PO₄, 150 mM NaCl, pH 7.5, was used as a control.

X-ray Absorption Spectroscopy (XAS) Data Collection and Analysis

For the XAS experiments the reconstitution of MBS2 with copper was performed in an inert atmosphere using an anaerobic chamber to prevent the oxidation of cysteine residues. Before metal reconstitution, a 10-fold molar excess of dithiothreitol was added to MBS2 on ice, and the mixture was incubated for 10 min. The protein was then dialyzed overnight under argon into a buffer containing 50 mM HEPES and 10% acetonitrile at pH 7.5. Copper was added as a tetra-acetonitrile complex, [Cu(C₄H₈NO₂)]PF₆, dissolved in the same buffer to an equimolar ratio to the protein. The Cu-‘MBS2 was then dialyzed in successive steps of 12 h each against 50 mM HEPES, 10% acetonitrile, pH 7.5; 50 mM HEPES, 5% acetonitrile, pH 7.5; 50 mM HEPES, pH 7.5. The copper content was monitored by atomic flame absorption spectroscopy (Varian AA-5). The protein was then concentrated using a modified ultrafilter centrifugation system (Millipore, molecular weight cut-off 3500 Da). Final concentrations were 140 μM in copper and 220 μM in MBS2 with a copper stoichiometry of 0.7. The sample was sealed and stored at −80°C.

XAS data were collected at the Stanford Synchrotron Radiation Laboratory (beamline 9–3, 3.0GeV, 50–100 mA). A bend rhodium-coated mirror positioned upstream of the fully tuned Si(111) monochromator was used to cut off all energy above 12 keV. Cu-‘MBS2 was analyzed in fluorescence mode using a Canberra 30 element array detector and was cooled down to 10 K using a liquid He flow cryostat (Oxford Instruments). The energy was calibrated by simultaneously measuring a copper metal foil and assigning the first inflection (26) point of the copper edge to 8890.3 eV. For Cu-‘MBS2, 10 scans were collected to 12.8 Å⁻¹.

Data reduction and analysis were performed using the EXAFSPAK computer suite (27). Theoretical phase and amplitude functions were calculated as described in FFEF 8.2 (26). The inspected raw data were averaged, and the background was subtracted and normalized. The EXAFS data were simulated by curve fitting in the OPT module of EXAFSPAK using a non-linear Marquadt algorithm, where the difference between the experimental and the calculated model is minimized. The following parameters were refined: ΔE₀ (a small energy correction at k = 0, ranging from −5 to −20 eV), R₀ (the distance between the central absorber and atom i), and σ² (the Debye-Waller factor, defining the mean square deviation of R₀). A goodness-of-fit (Fᵦ) parameter displayed at the end of each cycle was used to evaluate the merit of the fit. Fᵦ is defined as

\[ Fᵦ = \sum \frac{k^2(\text{data} - \text{ modelo})^2}{k^2(\text{data})^2} \]  (Eq. 1)

Preparation and Expression of mMBS2 WNDP in Insect Cells—The generation of the plasmid encoding the full-length 4.4-kilobase WNDP/H9262 spectrophotometer. A solution containing 7.5 μM total membrane protein was resuspended in 200 μl of 100 μM Tris(2-carboxyethyl)phosphine hydrochloride, 20 mM bis-Tris propane, pH 7.0, 200 mM KCl, 5 mM MgCl₂ and then incubated on ice for 5 min with 500 μl copper chelator batho-
prine disulfonate (BSC, ICN Biomedicals) for 30 min to inhibit catalytic phosphorylation. The chelator was then removed by centrifugation, and the pellets were washed with 20 mM bis-Tris propane, pH 7.0, 200 mM KCl, 5 mM MgCl₂ (phosphorylation buffer). The membranes were resuspended in phosphorylation buffer containing 100 μM ascorbate, 100 μM Tris(2-carboxyethyl)phosphine hydrochloride followed by the addition of increasing concentrations of CuCl₂ or Cu-‘Atox1. After a 10-min incubation at room temperature, [γ-³²P]ATP (specific activity 25 mCi/μmol) was added to a final concentration of 1 μM, and the reaction was incubated on ice for 4 min. The reaction was stopped by the addition of 50 μl of ice-cold 1 mM NaH₂PO₄ in 50% trichloroacetic acid and then centrifuged for 10 min at 20,000 × g. The protein pellet was washed, resuspended in 40 μl of 5 mM Tris-PO₄, pH 5.8, 6.7 mM urea, 0.4 mM diethiothreitol, 5% SDS and loaded on an acidic 8.0% polyacrylamide gel (31). After electrophoresis, the gels were fixed in 10% acetic acid for 10 min and dried on blotting paper. The dried gels were exposed either to the Molecular Imaging screen C9 (Bio-Rad) or at −80°C to Kodak BioMax MS film. The intensity of the bands was quantified using a Bio-Rad Molecular Imager GS-525. The dried gels were rehydrated and stained with Coomasie Blue R250, and the amount of protein in the WNDP-related bands was determined by densitometry. The incorporation of [³²P] into WNDP was normalized to the WNDP protein levels.

RESULTS

The Atox1-mediated Transfer of Copper to N-WNDP Selectively Protects Cysteines in MBS2 against Labeling with CPM—Previously, we demonstrated that Atox1 transferred copper to N-WNDP, stimulating the catalytic activity of WNDP (9). To better understand the molecular details of this process, we sought to determine whether Cu-‘Atox1 delivered copper to a specific site on N-WNDP. Our approach is outlined in Fig. 2A. Apo-N-WNDP was incubated either with Cu-‘Atox1 to transfer one copper (9) or with apoAtox1 as a control. After copper transfer and subsequent removal of Atox1, the cysteine residues in N-WNDP were labeled with the fluorescent reagent CPM. Copper binding to N-WNDP protects the metal-coordinating cysteines in N-WNDP from labeling with CPM (14); therefore, the difference in the intensity of fluorescent labeling can be utilized for identification of the copper-bound MBS.

To facilitate identification of the copper-bound MBS we also developed a protocol for limited proteolytic digestion of N-WNDP. MBSs in N-WNDP are thought to be compactly folded subdomains (12, 13) connected by fairly loosely connected loops with the exception of MBS5 and MBS6, which are linked by a very short sequence). Therefore, there was expected that under mild condition, the proteolytic digestion would occur within the linkers, leaving the MBSs intact. Indeed, treatment of labeled N-WNDP with low amounts of trypsin (1:2000 w/w) produced a series of the 8–16-kDa fragments that were stable to proteolysis for a period of about 3 h (the predicted mass for individual MBS without the linker sequence is ~8 kDa). The tryptic fragments were separated by gel electrophoresis, and the fluorescent intensities of the peptide bands from the apo- and copper-bound N-WNDP were compared (Fig. 2).

If Atox1 transfers copper to more than one site in N-WNDP, one would expect to see a partial decrease in fluorescence of several fragments. However, if Atox1 delivers copper to a preferential site, only one band should be protected against the
Copper Transfer from Atox1 to Wilson's Disease Protein

To evaluate the surface exposure of various metal-binding sites, we examined the reactivity of the cysteine residues in N-WNDP using brief labeling with limited amounts of CPM. The apparent molecular mass of 8 kDa indicates that this fragment is ~79–80 amino acid residues long and, therefore, contains only one metal binding motif, GGMTCXCC, corresponding to MBS2. The next GXXCXXC site is 133 residues away and, if included, would generate a 15-kDa fragment. Consistent with these conclusions, we identified a cleavage site Arg^232^Ala^238^ between MBS2 and MBS3 (our data). Thus, transfer of copper to N-WNDP causes a selective loss of fluorescence in MBS2, suggesting that MBS2 could be a site that preferentially accepts copper from Atox1.

Cysteines in MBS2 Are Not Unique with Respect to Their Surface Exposure or Affinity for Copper—The selectivity toward MBS2 during copper transfer reaction suggested that the properties of this MBS were unique. For example, the cysteine residues of MBS2 could be the most exposed and, hence, the most likely residues to receive copper from Cu^+^-Atox1, or MBS2 could have the highest affinity for copper among the metal-binding sites in N-WNDP. In this latter case, copper can be delivered by Atox1 to other sites and then migrate to MBS2.

To evaluate the surface exposure of various metal-binding sites we examined the reactivity of the cysteine residues in N-WNDP using brief labeling with limited amounts of CPM, a fairly bulky reagent. As shown in Fig. 3, even when CPM is sub-stoichiometric with respect to cysteines, multiple bands were fluorescently labeled. Therefore, judging by their chemical reactivity, several MBS in N-WNDP appeared to be similarly exposed (Fig. 3). This conclusion was further confirmed using a cysteine-directed reagent with different chemistry, 2-((biotinoyl)amino)ethyl-methanethiosulfonate-biotin (our data, not shown).

To test whether MBS2 is the site with the highest affinity for copper, transfer experiments were repeated using free copper added in the presence of the reducing reagents such as ascorbate or glutathione. In this case, binding of one copper to N-WNDP results in the decreased fluorescence of several fragments, including MBS2 (Fig. 4). This result suggests that not only are several sites available for copper binding, but also, the apparent affinities of these MBSs for the metal are not significantly different. Because neither the exposure nor affinity of MBS2 for copper appears to be unique, it seems likely that specific protein-protein interactions with Atox1 are essential for delivery of copper to the preferential site.

Binding of a Single Copper Induces Conformational Change in N-WNDP—Interestingly, the transfer of one copper leads to a small but reproducible change in the proteolytic pattern of N-WNDP. As shown in Figs. 2C and 4, although a single 16-kDa band is observed in the apo-N-WNDP sample, the digestion of the copper-bound N-WNDP results in a 16-kDa doublet (Fig. 2C) and is often accompanied by an increase in the intensity of the 14-kDa band (Fig. 4). The N-terminal amino acid sequencing revealed that the 14-kDa fragment began with the sequence NH$_2$-NQVQGTC. This sequence corresponds to a segment Asn^{352}Cys^{358} located in the loop before MBS4 (Fig. 2D).

The selectivity to various sites was directed to the specific site (Ordered) or to any MBS (Random). The right panel shows the protein pattern (Coomassie), which remains unaltered in both cases. B, the actual result of the experiment. The fluorescence pattern is shown on the left panel; the Coomassie R250 staining is on the right. A, a separate gel showing a typical change in the tryptic cleavage of the 16-kDa band after transfer of one copper.

A, a schematic representation of two possible outcomes of the experiment on copper transfer and subsequent fluorescent labeling. The two left panels show the change in the pattern of the CPM-labeled peptides if transfer is directed to the specific site (Ordered) or to any MBS (Random). The right panel shows the protein pattern (Coomassie), which remains unaltered in both cases. B, the actual result of the experiment. The fluorescence pattern is shown on the left panel; the Coomassie R250 staining is on the right. The arrow indicates the differentially labeled 8-kDa band corresponding to MBS2 (see "Results" for details). C, a separate gel showing a typical change in the tryptic cleavage of the 16-kDa band after transfer of one copper.

Fig. 2. Identification of MBS that accepts copper from Cu^+^-Atox1. A, a schematic representation of two possible outcomes of the experiment on copper transfer and subsequent fluorescent labeling. The two left panels show the change in the pattern of the CPM-labeled peptides if transfer is directed to the specific site (Ordered) or to any MBS (Random). The right panel shows the protein pattern (Coomassie), which remains unaltered in both cases. B, the actual result of the experiment. The fluorescence pattern is shown on the left panel; the Coomassie R250 staining is on the right. The arrow indicates the differentially labeled 8-kDa band corresponding to MBS2 (see "Results" for details). C, a separate gel showing a typical change in the tryptic cleavage of the 16-kDa band after transfer of one copper.

Fig. 3. Surface labeling of N-WNDP with CPM. N-WNDP was incubated in the absence or presence of 1 or 2 mol equivalent of CPM. The protein was proteolyzed, and the labeling of various fragments was analyzed (Fluorescence). The same gel was then stained with Coomassie R250.
predicted molecular mass of the Asn^{392}-Arg^{483} fragment is 14 kDa. Therefore, the 14-kDa fragment includes the entire MBS4 and the linker sequences between MBS4 and MBS5 but does not include MBS5. The two bands of the 16-kDa doublet have the same N-terminal sequence, NH_2-AVAPQKC, corresponding to the segment Alw^{484}-Cys^{490}. Thus, the 16-kDa doublet encompasses the MBS5 and MBS6 region, and the difference in mobility of the two bands within the doublet is a result of different cleavage at their C termini. Overall, it appears that the binding of one copper to N-WNDP leads to a change in the conformation of N-WNDP, exposing new sites for cleavage with trypsin.

The Difference in Copper Binding Characteristics of MBS2 and Atox1—Why is copper transferred from Atox1 to MBS2? To address this question we compared the ability of Atox1 and MBS2 to retain copper in the presence of the specific copper chelator BCA. Atox1 and MBS2 were expressed using the same expression system, purified, and loaded with copper under identical conditions (see “Experimental Procedures” for details). The copper-bound Atox1 or MBS2 were then incubated under reducing conditions with increasing concentrations of BCA and redistribution of copper between each protein, and BCA was monitored spectrophotometrically (copper-BCA complex has maximum absorbance at 562 nm).

There was a marked difference between Atox1 and MBS2 in these experiments (Fig. 5). A 45-fold molar excess of BCA over copper-bound Atox1 caused complete redistribution of copper from Atox1 to the chelator. In contrast, only 10% of copper was removed from MBS2 under the same conditions, suggesting that either the affinity of MBS2 for copper was significantly higher than that of Atox1 or the dissociation rate of copper from MBS2 was much slower. This difference in copper retention could either be due to a difference in copper coordination by these two proteins (two-coordinate versus three-coordinate, for example) or due to a difference in the local environment of metal-binding sites. To examine these possibilities we used XAS.

MBS2 Coordinates Copper with a Linear Bis cysteinate Geometry, Very Similar to That of Atox1—We have shown recently that Atox1 binds copper with a linear biscysteinate coordination geometry (18). The extended x-ray absorption spectroscopy fine structure studies of MBS2 yielded a similar result. EXAFS of MBS2 was dominated by strong Cu-S backscattering out to \( k = 12.8 \text{ Å}^{-1} \) (Fig. 6A, inset a), in agreement with the key role of the cysteine residues of the GMTCCXC motif in Cu\(^{+}\) coordination. The Fourier transform revealed a strong feature at \( R \sim 2 \text{ Å} \) and several less intense features centered around \( R + \Delta = 3.5 \) and 4.3 Å, respectively (Fig. 6A).

The 2-Å feature was fitted with 2× Cu-S at 2.16 Å (1). When the outer shells were included in the refinement, the \( P \) value improved slightly (Table I). The best fit was obtained with 2× Cu-S at 2.16 Å, 2× Cu-C at 3.38 Å and multiple scattering paths for Cu-S1-Cu-S2-Cu at 4.32 Å (Table I). As suggested by Penner-Hahn and co-workers (32), the Cu-S-Cu-S-Cu multiple scattering is very sensitive to the S-Cu-S angle and can only be observed if the angle is >175° (32). Thus, observing this scattering path is an indication of an almost linear S-Cu\(^{+}\)-S coordination. The linear Cu\(^{+}\) coordination in MBS2 was confirmed by comparing the pre-edge features at 8983 eV for MBS2 to that of a synthetic linear S-Cu\(^{+}\)-S complex, which were found very similar in their shape and intensity (Fig. 6A, inset b).

Overall, the data and the refinement results for Atox1 and MBS2 are very similar (Table 1, Fig. 6B). The differences in intensity of the Fourier transform and EXAFS can be traced to a small variation in the Debye-Waller factor for the first shell intensity of the Fourier transform and EXAFS can be traced to a small variation in the Debye-Waller factor for the first shell. However, the overall shape and intensity of the Fourier transform and EXAFS can be traced to a small variation in the Debye-Waller factor for the first shell. Therefore, all the data and the refinement results for Atox1 and MBS2 are in agreement with the linear Cu\(^{+}\) coordination in MBS2.

Mutation of MBS2 Disrupts Atox1-mediated Stimulation of Catalytic Activity of WNPD—The experiments with differential labeling of N-WNDP (Fig. 2) suggested that MBS2 was the site receiving copper from Atox1. However, these experiments did not exclude the possibility that copper binding protects MBS2 against fluorescent labeling indirectly. In addition, previous experiments using a yeast complementation assay demonstrated that mutation of the cysteines in MBS2 to serines did not disrupt the ability WNPD to transport copper across the membrane (15). Therefore, to independently test whether MBS2 is required for copper transfer from Atox1 to the full-length WNPD, both copper-coordinating cysteines in MBS2 of the full-length WNPD were mutated to alanines, a mutation that is expected to inactivate metal binding more efficiently than cysteine to serine substitution. The generated mMBS2-WNPD was expressed in Sf9 cells, and the catalytic activity of the mutant in the presence of copper or copper-Atox1 was characterized using the protocol developed for the wild-type WNPD (28). In this procedure, the ability of WNPD to form a catalytic phosphorylated intermediate is first inhibited by removing copper with the copper chelator BCS. The reactivation is then monitored upon the addition of free copper or a copper-

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**Fig. 4.** Comparison of selectivity of protection against CPM labeling after transfer of one copper to N-WNDP using various copper donors. Copper was added to N-WNDP in the presence of ascorbate or as a complex with glutathione or Atox1 to obtain binding of approximately one copper per N-WNDP in each case. The N-WNDP was then CPM-labeled and proteolyzed, and fluorescent patterns were compared. Each lane contains the same amount of protein, as evidenced by Coomassie staining (not shown).

**Fig. 5.** Copper retention by MBS2 and Atox1 in the presence of the high affinity copper chelator BCA. The purified copper complexes with MBS2 (○) and Atox1 (▲) were incubated with increasing concentrations of the copper chelator BCA. The amount of copper redistributed from the protein to BCA was determined by comparing the absorbance to the standard. The initial amount of copper-bound protein was taken as 100%, and the amount of copper redistributed to BCS was subtracted.
Atox1 complex. Because the formation of a catalytic intermediate critically depends on binding of copper to the intramembrane-binding site(s) of WNDP, the disruption of copper delivery from the cytosol to the membrane site(s) would result in the inactivation of catalytic phosphorylation.

The membrane fraction containing the full-length WNDP and mMBS2-WNDP mutant was isolated from Sf9 cells and treated with BCS to inhibit their activity. The subsequent addition of free copper in the presence of ascorbate reactivated the wild-type WNDP and the mMBS2-WNDP mutant in a very similar manner and to the same level (Fig. 7A). This result indicated that (a) copper was able to reach the intramembrane copper-binding site despite the mutation at MBS2, and (b) under these conditions the mutant had activity similar to wild-type WNDP. In contrast, when Cu²⁺-Atox1 was used as a metal donor, there was a drastic difference between reactivation of wild-type WNDP and mMBS2-WNDP (Fig. 7B). Even the large excess of Cu⁺-Atox1 could not stimulate the catalytic activity of mMBS2-WNDP, suggesting that MBS2 was essential for the initial steps of copper delivery from Atox1 to the catalytically essential intramembrane sites.

**DISCUSSION**

The current work was undertaken to understand the molecular mechanisms underlying copper transport by WNDP. Specifically, the described experiments focus on transfer of copper from Atox1 to the N-terminal domain of WNDP (N-WNDP), the first step of a poorly understood journey of copper from the cytosol to the intramembrane copper-binding site(s) of WNDP. Our results suggest that the chaperone delivers copper specifically to MBS2 of N-WNDP. The involvement of MBS2 in the Atox1-mediated copper transfer is consistent with the earlier yeast two-hybrid data showing interactions of Atox1 with the MBS1–4 fragment and the lack of interactions with the MBS5/6-containing fragment (8).

The importance of MBS2 for copper transfer led us to examine the properties of this site in more detail. The labeling with CPM suggests that with respect to solvent exposure, MBS2 is not unique and that other MBS can potentially accept copper from Atox1. Indeed, when present in excess, Atox1 metallates all six MBSs in N-WNDP (9). This earlier observation seems at odds with our current results showing that mutation of a single MBS2 in the full-length WNDP abolishes the Atox1-mediated
delivery of copper (Fig. 7). There are two possible explanations of this apparent contradiction. First, it could be that Atox1 can only dock to MBS2 via specific protein-protein interactions, and copper then migrates from MBS2 to other MBSs. In this case, all N-terminal metal-binding sites can be loaded via MBS2, and the mutation of MBS2 would disrupt this process. This explanation seems unlikely since we observed no redistribution of copper from MBS2 to other copper-binding sites in N-WNDP. In fact, unless interactions of MBS2 with other domains in the full-length WNDP facilitate copper dissociation from MBS2, this site appears to retain copper remarkably well (Fig. 5).

The following alternative explanation better accommodates the experimental data. It seems likely that the folding of N-WNDP and its interaction with other domains of WNDP, such as the ATP-binding domain (39), limit accessibility of the N-terminal MBSs to Atox1 (which is bulkier than the chemical probe) and make the delivery of copper to these MBSs strongly dependent on initial binding of copper to MBS2. In this scenario, copper transfer to MBS2 may trigger a change in the conformation of N-WNDP, allowing Atox1 sufficient access to other MBSs. This hypothesis is consistent with our observation that the binding of a single copper to N-WNDP is accompanied by the appearance of new tryptic sites during proteolysis (Figs. 2C and 4). Also, circular dichroism experiments of N-WNDP showed subtle changes in the overall fold upon copper binding (16).

Our experiments further suggest that the affinity of several metal binding sites in N-WNDP for copper are comparable and higher that that of Atox1. In the competition experiments, MBS2 displayed a much better retention of copper than Atox1, suggesting that under equilibrium copper is likely to redistribute to this site. It is clear that the difference in properties of MBS2 and Atox1 is not due to copper coordination. The EXAFS experiments demonstrate that MBS2 binds Cu⁺ with linear coordination and the distance between the sulfurs in the cysteine residues to the copper was 2.16 Å. This distance is the same as the distance between Cu⁺ and the cysteines of Atox1 (18). Therefore, the local protein environment plays an important role in stability of the copper-bound form of Atox1 and MBS2.

The high resolution structure of MBS2 of WNDP is not yet available, but this sub-domain is homologous to MBS2 and MBS4 of the Menkes disease protein (58% identity to MBS2), the structures of which were determined by NMR (12, 13). The NMR experiments revealed some structural features that could account for the difference in the retention of copper by MBS2 and Atox1. In MBS2 of either Menkes disease protein or WNDP there is a conserved phenylalanine that lies within the loop adjacent to the cysteines at the MBS (18). Therefore, the local protein environment plays an important role in attracting and positioning Atox1 with respect to MBS2. The interactions between MBS2 and Atox1 are most likely not tight, since in our experiments we were unable to detect stoichiometric amounts of bound Atox1 after incubation with N-WNDP (our data, not shown). It is also clear that the charge distribution at the surface of MBS2 is not unique for this subdomain (Fig. 8B) and, consequently, the key role of MBS2 in the Atox1-mediated copper transfer is likely due to specific location of this site in N-WNDP.

If all the metal-binding sites in N-WNDP have a comparable affinity for copper, why doesn’t copper migrate from MBS2 to other metal-binding sites? Although available data do not allow us to unambiguously answer this question, it seems that extremely poor dissociation of copper from MBS2 is a likely reason for this result. The strong retention of copper by MBS2 also reinforces our conclusion that binding of copper to MBS2 is likely to work as a switch, allowing subsequent loading of other sites, rather than a specific entrance for copper. The experiments testing this model are currently under way in our laboratory.

In summary, we demonstrated the specific role of the N-terminal metal-binding site 2 of WNDP in the first step of the
Atox1-mediated delivery of copper to N-WNDP. This first step appears to involve specific protein-protein interactions between the donor and acceptor and is likely to be facilitated by the difference in the copper binding affinity of the Atox1/MBS2 pair. We speculate that binding of copper to MBS2 works as a switch, which opens the access of the chaperone to other metal-binding sites in WNDP.

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Fig. 8. Comparison of the local environment of the metal-coordinating cysteines in MBS2 and Atox1 (A) and surface charge distribution on all MBSs and Atox1 (B). A, the copper-coordinating cysteines in the GMXGXXC motif are shown in yellow, and the residues in close proximity to this motif (Phe in MBS2 and Lys in Atox1) are shown in green. B, the negative charges on the surface of MBSs and Atox1 are indicated in red, and the positive charges are in blue. The figure was generated with the program MOLMOL (34).

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