Wilson’s disease protein (WNDP) is a copper-transporting ATPase essential for normal distribution of copper in human cells. Recent studies demonstrate that copper regulates WNDP through several mechanisms. Six metal-binding sites (MBS) at the N terminus of WNDP are predicted to be involved in copper-dependent regulation of WNDP; however, specific roles of MBS remain poorly understood. To address this issue, we generated WNDP variants with mutations or truncation in the N-terminal region and characterized their functional properties. We show that copper cooperatively stimulates catalytic activity of WNDP and that this effect requires the presence of both MBS5 and MBS6. Mutations of MBS6 or MBS1–5 result in non-cooperative activation of the enzyme by copper, whereas the deletion of MBS1–4 does not abolish cooperativity. Our data further suggest that MBS5 and MBS6 together regulate the affinity of the intramembrane-binding site(s) for copper. Analysis of the copper-dependent stimulation of catalytic phosphorylation demonstrate that the MBS6 and MBS1–5 mutants have a 7–8-fold lower EC₅₀ for copper activation, suggesting that their affinity for copper is increased. This conclusion is confirmed by a markedly decreased inhibition of these mutants by a copper chelator bathocuproine disulfonate. In contrast, deletion of MBS1–4 does not affect the affinity of sites important for catalytic phosphorylation. Rather, the MBS1–4 region appears to control access of copper to the functionally important metal-binding sites. The implications of these findings for intracellular regulation of WNDP are discussed.

Wilson’s disease protein (WNDP) is a copper-transporting P₁-type ATPase essential for regulation of copper concentration in human cells. The major functional activity of WNDP is to couple the energy of ATP hydrolysis with transport of copper from cytosol across cell membranes (1, 2). During ATP hydrolysis, WNDP becomes transiently phosphorylated at the invariant residue Asp-1027; catalytic phosphorylation from ATP is specifically stimulated by copper (3). Following copper translocation across the cell membrane, WNDP is dephosphorylated. In vitro, catalytic phosphorylation can also be achieved using inorganic phosphate (Pi) in the presence of Mg²⁺, this reaction reflects the reversibility of the dephosphorylation step (4). Similarly to other P-type ATPases, WNDP is thought to cycle between two major conformations: E₁, in which WNDP binds copper and ATP with high affinity and becomes phosphorylated, and E₂, in which the affinity for copper and ATP is low but WNDP can still be phosphorylated using inorganic phosphate.

WNDP has multiple copper-binding sites located at two different regions of the protein (Fig. 1A). In the N-terminal portion of WNDP, there are six metal-binding sites (MBS) formed by cysteine residues of the sequence motif GMXCXXC. Each motif belongs to a repetitive 70 amino acid sequence and binds one copper in the reduced Cu¹⁻ form (5, 6). In addition to six cystolic copper-binding sites, there is at least one site in the membrane portion of the protein. The structure of this site is unknown, but it is likely to be formed by a sequence motif CPC and other conserved intramembrane residues. By analogy with other P-type ATPases, it is believed that binding of copper to the intramembrane site is essential for copper-dependent catalytic phosphorylation of WNDP by ATP. In agreement with this assumption, mutations of the CPC motif abolish catalytic activity of copper-transporting ATPases (7–10).

Although the role of the CPC-based metal-binding sites seems clear, the function of multiple N-terminal MBS is poorly understood and is a subject of considerable interest. The bacterial and yeast orthologues of WNDP have one or two N-terminal MBS, suggesting that not all six MBS of WNDP are necessary for its function. This prediction was confirmed by experiments using a yeast complementation assay, which demonstrated that only MBS5 or MBS6 of WNDP are necessary for functional activity of WNDP (11, 12). Additional N-terminal MBS were proposed to be involved in WNDP regulation, which may include fine-tuning of WNDP activity in response to copper and/or regulation of such posttranslational events as a kinase-mediated phosphorylation and protein trafficking (13–17). The specific role of MBS5 and MBS6 for the WNDP function and the mechanism by which the N-terminal MBS regulate WNDP remain unknown.
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EXPERIMENTAL PROCEDURES

Generation of Recombinant Baculovirus and Protein Expression in Insect Cells—The generation of the plasmid encoding the full-length 4.4-kb WNDP cDNA (pFastBacDual-wild-type [WT] WNDP) and the catalytically inactive D1027A mutant of WNDP has been described previously (3). The WNDP variants with various changes in the N-terminal domain (coding region) were amplified by PCR using primers with a partly overlapping sequence (bold face). Fragment I was generated using forward primer 5′-CGCGGAATTCGGTGTCCGTGCGGG-3′ and reverse primer 5′-ACCAAAATCGATAAAACCGATTACAATC-3′. Fragment II was obtained with forward primers 5′-CGCCGATTACAATCCGCTGTGTCTAACATA-GGCCCTTCTTCTCAG-3′ and reverse primer 5′-CGTGGAAGAAGGGCAGCCCAGGCC-3′. Fragment II was exchanged with the ApaI and ClaI restriction endonucleases, and then exchanged with the ApaI-ClaI fragment of original pFastBacDual-WNDP plasmid. The presence of anticipated mutations as well as the absence of fortuitous mutations was verified by automated DNA sequencing. The generated constructs were utilized to transform the DH10Bac strain of Escherichia coli and produce bacmids using the commercially available Bac-to-Bac™ kit (Invitrogen). The bacmids were then transfected into Spodoptera frugiperda cells (S9F) to produce baculovirus expressing corresponding WNDP variants. Baculovirus was amplified as described in the Bac-to-Bac™ manual and in Ref. 18.

WNDP Expression in Insect Cells and Preparation of Membrane Fractions—Maintenance, infection, harvesting of S9F insect cells, and isolation of membrane fractions were carried out as described previously (3, 18). Protein concentration in membrane fraction was determined by the method of Lowry et al. (19). The expression of WT and mutant WNDP was analyzed by separation of 50 μg of total membrane protein on a 7.5% Laemmli gel (20) followed by Coomassie staining and Western blotting with polyclonal antibody a-ABD (1:20,000) as described previously (21).

Phosphorylation of WNDP Using [γ-32P]ATP—50 μg of total membrane protein was resuspended in 200 μl of the assay buffer: 20 mM bis-Tris propane, pH 6.0, 200 mM KCl, 5 mM MgCl₂. Radioactive [γ-32P]ATP (specific activity, 25 mCi/μmol) was added to a final concentration of 1 μM, and the reaction mixture was incubated on ice for 4 min or for various time periods when the kinetics of phosphorylation were measured. All additional treatments were performed as described in the figure legends. The reaction was stopped by addition of 50 μl of ice-cold 1 mM NaHPO₄, 50% trichloroacetic acid and then centrifuged for 10 min at 20,000 × g. The protein pellet was washed once with ice-cold water and resuspended in 40 μl of sample buffer (5 mM TCEP, pH 5.8, 6.7 mM urea, 0.4 M dithiothreitol, 5% SDS) and loaded on the acidic 7.5% polyacrylamide gel (22). After electrophoresis, the gels were fixed in 10% acetic acid for 10 min and dried on a blotting paper. The dried gels were exposed either overnight to the Molecular Imaging Screen CS (Bio-Rad) or for several hours at −80 °C to the Kodak BioMax MS film, and the intensity of the bands was quantified using a Bio-Rad Molecular Imaging System. The data were analyzed by a non-linear regression using SigmaPlot software.

WNDP Phosphorylation from [32P] Inorganic Phosphate—50 μg of membrane protein preparation was resuspended in 200 μl of the following buffer: 50 mM MES-Tris (pH 7.0), 10 mM MgCl₂, 20% Me₃SO (P buffer) containing 100 μM ascorbate and 100 μM tris-(2-carboxyethyl) phosphine hydrochloride (TCEP, Sigma). To initiate phosphorylation, [32P] phosphate (activity, 6000 Ci/mmol) was added to the mixture and incubated for 10 min at room temperature. The reaction was stopped by addition of 50 μl 50% (w/v) trichloroacetic acid in 1 mM NaHPO₄. Separation of precipitated protein and analysis of [32P] incorporation into WNDP was carried out as described above.

Treatment with Bathocuproine Disulfonate (BCS)—Membrane preparations from S9F cells expressing WT or mutant WNDPs were resuspended in the phosphorylation buffer, containing 100 μM TCEP, to a protein concentration of 0.25 mg/ml and then incubated on ice with the increasing concentrations of copper chelator BCS (ICN Biomedicals, Inc.) for 30 min. Analysis of phosphorylation from ATP was carried out as described above.

The Effect of Copper on Phosphorylation—The membrane-bound WT or mutant WNDPs were resuspended in the phosphorylation buffer to a 0.25 mg/ml protein concentration and incubated with 250 μg BCS on ice for 30 min. The chelator was then removed by centrifugation, and the pellets were washed with the phosphorylation buffer. The membrane pellets were resuspended in the phosphorylation buffer containing 100 μM ascorbate, 100 μM TCEP, and increasing concentrations of CuCl₂. Following 10 min of incubation on ice, radioactive ATP was added, and the phosphorylation reaction and analysis of [32P] incorporation were carried out as described above.
levels similar to WT WNDP. The functional activity of generated proteins was verified by measuring their ability to form a phosphorylated intermediate following incubation with \([\gamma^{32}\text{P}]\text{ATP}\). Phosphorylation levels of \(\Delta\text{MBS1–4}\) and \(\text{mMBS1–5}\) were indistinguishable from that of WT WNDP (Fig. 2B), whereas \(\text{mMBS6}\) had slightly lower level of phosphorylation (69.6 ± 9.1% of WT WNDP).

**Functional Consequences of the Cys → Ala Substitutions in the First Five MBS—**Studies on WNDP homologue (Menkes disease protein, MNKP) led to the suggestion that the N-terminal MBS of human copper-transporting ATPases were required for the ATPase function when concentration of copper was very low (23). This hypothesis predicts that in low copper, the \(\text{mMBS1–5}\) mutant is likely to be less active than WT WNDP because the sites necessary for efficient copper binding are missing in this mutant. To test this prediction, we compared catalytic phosphorylation of WT WNDP and \(\text{mMBS1–5}\) at different copper concentrations. For these experiments, WT WNDP and \(\text{mMBS1–5}\) were first inhibited by addition of the specific copper chelator BCS as described under “Experimental Procedures,” and then increasing concentrations of copper were added to reactivate the enzymes. As shown in Fig. 3A, the effect of copper on activity of WT WNDP and \(\text{mMBS1–5}\) is indeed strikingly different. However, contrary to the prediction, copper activated the \(\text{mMBS1–5}\) mutant with an EC\(_{50}\) that was ~7.6-fold lower (0.60 ± 0.16 \(\mu\)M) than the EC\(_{50}\) for the WT WNDP (4.6 ± 0.3 \(\mu\)M).

In the P-type ATPases, phosphorylation by ATP depends on binding of the exported ions to the intramembrane site(s). Therefore, in WNDP, the change in copper dependence of catalytic phosphorylation caused by mutations is likely to reflect changes in the intramembrane copper-binding site(s). The decrease in the EC\(_{50}\) for copper, observed for the \(\text{mMBS1–5}\) mutant, suggests that the affinity of the intramembrane site(s) for copper is increased as a result of the Cys → Ala substitutions in the N-terminal domain. Alternatively, the changes caused by the removal of the five functional MBS could have facilitated access of copper to the intramembrane sites without changing their affinity. To determine which of these explanations is correct, we compared the effect of the copper chelator BCS on the catalytic activity of WNDP and \(\text{mMBS1–5}\).

BCS inhibits WNDP, presumably by competing with WNDP for the metal; the inhibitory effect of BCS can be reversed by subsequent addition of copper to the protein (3). If \(\text{mMBS1–5}\) has a higher affinity for copper than WNDP, then higher concentrations of BCS would be necessary to inactivate the mutant. If the affinity of \(\text{mMBS1–5}\) for copper is unaltered, but sites became more exposed, the effect of BCS on \(\text{mMBS1–5}\) would be similar to its effect on WT WNDP or stronger. The measurements of catalytic phosphorylation in the presence of increasing concentrations of BCS demonstrate that BCS is a significantly less effective inhibitor of \(\text{mMBS1–5}\) as compared with the WT WNDP (Fig. 3B). To obtain 50% inhibition of \(\text{mMBS1–5}\), ~250 \(\mu\)M BCS was necessary as compared with 10 \(\mu\)M BCS required for similar inhibition of WT WNDP. This result is consistent with the increased affinity of the catalytically important copper-binding sites in the \(\text{mMBS1–5}\) mutant.

Experiments shown in Fig. 3 revealed another interesting difference between the \(\text{mMBS1–5}\) mutant and WT WNDP. The copper-dependent activation curve for WT WNDP is clearly cooperative, whereas for the mutant, it is not (Fig. 3A). The loss of cooperativity for \(\text{mMBS1–5}\) indicates that one or several MBS located in the \(\text{MBS1–5}\) region contributed to this process. To better understand the role of different MBS in cooperative activation of catalytic phosphorylation, we generated and characterized the \(\Delta\text{MBS1–4}\) mutant. We reasoned that \(\text{MBBS}\) is the part of the “core structure,” which is common for most copper-

![Fig. 2. Expression (A) and catalytic phosphorylation (B) of WT and mutant WNDPs.](image)
transporting ATPases and which is likely to be sufficient for their catalytic function. The metal-binding sites MBS1–4 are the "extra" sites in the mammalian ATPases, and their deletion should not have an effect on functional activity but may affect cooperativity.

The Functional Properties of the ΔMBS1–4 WNDP—The experiments on copper-dependent stimulation of catalytic phosphorylation demonstrate that the deletion of the first four metal-binding sites does not have a significant effect on the final level of catalytic activity (Fig. 4A). The effect of copper remains cooperative, and the slope of the curve becomes steeper. These results suggested that MBS1–4 did not play a key role in stimulation of WNDP activity and could even hinder effective interactions between remaining copper-binding sites, making the activation curve more shallow. At the same time, the EC50 value for copper-dependent activation of the ΔMBS1–4 mutant is 3-fold lower than that of WT WNDP (1.5 ± 0.1 μM versus 4.6 ± 0.3 μM). This observation was somewhat puzzling. If MBS1–4 are not important for stimulation of catalytic activity, one would expect to see no effect on affinity of the intramembrane site(s) from the deletion. However, the deletion may affect EC50 if MBS1–4 regulate the access of copper to other sites.

To clarify this issue, the experiments on BCS inactivation were carried out. These experiments demonstrate that the concentration of BCS necessary to obtain 50% inhibition of phosphorylation is essentially identical for WT WNDP and the ΔMBS1–4 mutant (both 0.1 μM, Fig. 4B), indicating that the affinity of sites important for catalytic phosphorylation is indeed unaffected by the deletion. Thus, in the case of ΔMBS1–4, the decrease in the EC50 value for copper activation is likely due to better access of copper to the remaining metal-binding sites and not due to change in the affinity of the intramembrane sites. If this hypothesis is correct, one may see the effect of the deletion on the kinetic of catalytic phosphorylation. To test this prediction, we compared the time dependence of the acyl-phosphate formation for WT WNDP and ΔMBS1–4. As shown in Fig. 5, the τ1/2 value for the ΔMBS1–4 mutant is lower than the τ1/2 value for WT WNDP (9.6 ± 3.1 s versus 24.2 ± 5.2 s). Assuming that copper delivery to the intramembrane sites is the rate-limiting step of the reaction, the decrease in the

Fig. 3. The effects of copper and BCS on catalytic phosphorylation of WT WNDP and the mMBS1–5 mutant. As shown in A, the stimulatory effect of copper on ATP-dependent phosphorylation of WNDP (●) or mMBS1–5 (▼) was determined following initial inhibition of their activity with BCS as described under "Experimental Procedures." The phosphorylation level of respective proteins prior to BCS treatment is set as 100%. B, inhibition of phosphorylation by BCS. The symbols for WT WNDP and mutant are the same as in panel A. The top portion of each panel shows the autoradiogram of a typical gel. The bottom portion is an average of five or three independent experiments, each normalized to protein.

Fig. 4. The effects of copper (A) and BCS (B) on catalytic phosphorylation of WT WNDP and the ΔMBS1–4 mutant. The experimental procedures are the same as in Fig. 3. The inset in panel A compares the effect of a lower concentration of copper (x axis, μM) on catalytic phosphorylation (y axis, %). For both panels and the inset: WT WNDP (●); ΔMBS1–4 (♦); and mMBS1–5 (▼). The average of five or three experiments is shown.
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The Functional Consequences of MBS6 Inactivation—As shown in Fig. 2, the MBS6 mutant had somewhat decreased catalytic phosphorylation (~70% of the WT level). This lower level of phosphorylation from ATP could be due to conformational shift toward the E2-like form, which is less favorable for ATP-dependent phosphorylation, or due to diminished ability of the mutant to undergo conformational transitions in response to copper binding. Such consequences of the mutation would complicate interpretation of the experiments on copper-dependent activation of the enzyme. Consequently, we examined the functional properties of mMBS6 in more detail. The ability of this mutant to undergo E1 → E2 conformational transitions was evaluated by comparing phosphorylation of WT WNDP and mMBS6 in the presence of P_i and Mg^{2+}. As shown in Fig. 6, the levels of the P_i-mediated phosphorylation of WNDP and the mutant are similar. Moreover, incubation of mMBS6 and WT WNDP with BCS results in an comparable increase in the P_i phosphorylation. Subsequent addition of copper prevents phosphorylation from P_i for both WT WNDP and the mutant. Thus, the MBS6 mutation does not seem to alter the ability of WNDP to undergo E1 → E2 transition. Additional experiments on limited proteolysis revealed that the tryptic patterns of the digested mMBS6 and WT WNDP were essentially identical, arguing against major effects of the mutation on protein folding (data not shown).

The effect of the MBS6 mutation on copper binding affinity and cooperativity was examined by measuring the copper dependence of catalytic phosphorylation. As shown in Fig. 7A, activation of mMBS6 by copper is non-cooperative despite the presence of five other functional N-terminal MBS. Furthermore, the EC_{50} value for mMBS6 (0.60 ± 0.17 μM) suggests that mMBS6 has an increased apparent affinity for copper, which is essentially identical to the apparent affinity of mMBS1–5 (0.60 ± 0.16 μM). For comparison, the EC_{50} value for WT WNDP is 4.6 ± 0.3 μM (Fig. 3). In addition to important similarities between mMBS1–5 and mMBS6, there are some differences in their functional properties. The maximum phosphorylation of mMBS6 after activation with copper is ~2-fold lower than that of mMBS1–5 and remained at this level even at 20 μM copper.

Further experiments on inactivation of mMBS6 by BCS confirmed the effect of the mutation on apparent affinity of the intramembrane sites for copper (Fig. 7B). The concentration of BCS required for 50% inhibition of mMBS6 is markedly increased as compared with WT WNDP (~250 μM versus 10 μM), indicating that in mMBS6, copper essential for catalytic phosphorylation is poorly exchangeable.

**DISCUSSION**

Since the discovery of the human copper-transporting ATPases, the role of their multiple metal-binding sites has been a subject of considerable interest. The work from several laboratories yielded information about copper binding properties of MBS (5, 24, 25) and their relative importance for overall function of MNKP and WNDP (11, 12). However, specific functions of the N-terminal MBS are yet to be understood. In this report, we provide experimental evidence that MBS5 and MBS6 play an important role in regulation of enzymatic properties of WNDP. Our data suggest that MBS5 and MBS6 work together to control affinity of the catalytically essential intramembrane sites for copper, whereas the more N-terminal MBS are not involved in this process. These results support conclusions of earlier yeast complementation experiments, which suggested the importance of MBS5 and MBS6 for WNDP function (11, 12). The biochemical assays recently developed by our group enabled us to further dissect the functional role of these MBS.

We demonstrate that mutations of either MBS1–5 or MBS6...
result in a 7–8-fold increase in sensitivity of WNDP for copper. Since catalytic phosphorylation of WNDP depends on binding of the transported ions within the transmembrane domain, the changes in MBS1–5 or MBS6 must be transmitted to the intramembrane domain either through direct contact of these sites with the membrane portion or indirectly through interactions with other WNDP domains. To better understand these events, it is useful to review the changes that occur in the individual MBS in response to copper binding and in response to Cys → Ala mutation.

The solution structures of individual MBS from human and yeast copper-transporting ATPases in their apo- and metal-bound states have been solved by nuclear magnetic resonance spectroscopy (26, 27). These structures yielded a very important conclusion, namely that upon copper binding the overall structure of individual MBS remains essentially unaltered. The only significant change occurs in the metal-binding loop where a Cys, exposed previously at the surface, binds copper and rotates toward the protein core (Fig. 8). Although this change is small, it has significant consequences at the level of the full-length WNDP, suggesting that the copper-induced rotation of cysteine alters interactions of the loop with other part(s) of the protein.

It seems likely that mutations of Cys residue for Ala may have similar consequences since they eliminate the exposed SH groups (Fig. 8). The mutations in the CXXC motifs of MBS6 and MBS1–5 increase an apparent affinity for copper, suggesting the possible role of free SH groups in maintaining a low affinity state of WNDP for the metal. The deletion of the first four MBS does not seem to have an effect on sites important for catalytic phosphorylation (Fig. 4B). Consequently, we propose that non-mutated MBS5 and MBS6 in their apo form stabilize WNDP in a state with low affinity for copper. This effect could be mediated through hydrogen bond interactions of cysteines in the CXXC motif with other domains of WNDP. Elimination of free SH groups by copper binding to MBS or by mutagenesis alters the intradomain interactions, resulting in increased affinity of the WNDP intramembrane sites for copper and subsequent stimulation of catalytic activity. Further experiments with substitution of individual Cys in various MBS will test this model.

It is particularly interesting that similar consequences of the mutations in the N-terminal MBS on copper binding affinity were recently reported for the bacterial copper-transporting ATPase CopA from E. coli (28). CopA has only two MBS; the Cys → Ala mutations in both MBS result in a 3-fold increase in the apparent affinity of CopA for copper. Thus, it is tempting to speculate that in the copper-transporting ATPases, the two MBS closest to the membrane have an important functional role regulating the affinity of the intramembrane sites. It could be that this regulatory role is common for all P-type ATPases involved in transport of transition metals (P1-type or CPx-ATPases). This conclusion is supported by recent studies using the cadmium-transporting ATPase CadA, which demonstrated that the deletion of the N-terminal MBS increased affinity of the intramembrane sites for cadmium (29).

Our model also suggests that specific consequences of the Cys → Ser substitutions, which preserve the ability to form hydrogen bonds, could be quite different from the effect of the Cys → Ala mutations. Recent work on functional analysis of MBS in MNKP, which is homologous to WNDP, illustrates this point. The Cys → Ser substitutions in all six N-terminal MBS of MNKP result in a mutant, which is stabilized in the low affinity state (18). This mutant is more sensitive to inhibition by copper chelator BCS than WT MNKP, in contrast to our mMBS6 and mMBS1–5 mutants, which are significantly more resistant to the chelator when compared with the WT WNDP (Figs. 3B and 7B). This difference could be due to the presence of at least one functional MBS in our mMBS1–5 mutant. However, a more likely explanation for the difference between our results and the data on MNKP is that the Cys → Ser mutant of MNKP is stabilized in the apo form, i.e. in the low affinity state.
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for copper, whereas our Cys → Ala mutants mimic the high affinity copper-bound state.

**Cooperative Effect of Copper**—The cooperative activation of WT WNDP by copper indicates that transition of WNDP from a low affinity state to a high affinity state involves binding of copper to more than one site. Our data suggest that both MBS5 and MBS6 are required for this process, whereas MBS1–4 are not important. The essentially identical change in apparent affinity for copper induced by the mMBS1–5 and mMBS6 mutations implies that MBS5 and MBS6 occupy very similar positions within WNDP and that binding of copper to one of these MBS allows metal binding to the other. The yeast complementation studies demonstrating that either MBS1 or MBS6, but not other N-terminal MBS, could support transport function of WNDP (11) are consistent with the idea that metal-binding loops of MBS5 and MBS6 are spatially very close.

Despite important similarities between mMBS1–5 and mMBS6, the properties of these mutants are not identical. The mMBS1–5 mutant has a WT catalytic activity in a wide range of copper concentrations, whereas mMBS6 phosphorylation does not reach the WT level even at copper concentration as high as 20 μM (Fig. 7A). Considering that the Cys → Ala mutation affects residues in a flexible loop, one has to conclude that the precise structure of this loop and/or interactions of this loop with the rest of the protein are important for the optimal catalytic activity of WNDP.

The involvement of MBS5 and MBS6 in cooperative stimulation of catalytic activity further clarifies the role of these two MBS in WNDP function. Cooperative binding permits a much more sensitive response to copper concentration. This property would enable WNDP to capture copper, which is scarcely available in the cytosol, and would provide efficient transport even when intracellular copper is low.

**The Functional Role of the N-terminal MBS1–4**—Our results demonstrate that independently of whether MBS1–4 are present in their normal form (in WT WNDP), mutated to Ala (in mMBS1–5), or deleted (in ΔMBS1–4), the level of catalytic phosphorylation of WNDP remains the same. Furthermore, the deletion of the first four MBS does not eliminate the sigmoidal character of the copper-dependent activation of WNDP. Therefore, we conclude that MBS1–4 are not important for copper-dependent stimulation of catalytic phosphorylation of WNDP and do not modulate the copper binding affinity of the transmembrane domain(s).

At the same time, the deletion of MBS1–4 has several interesting consequences. It shifts the sensitivity of WNDP for copper, makes the copper-dependent curve steeper, and increases the rate of phosphorylation from ATP. These results suggest that the presence of MBS1–4 may affect access of copper to the functionally important metal-binding site(s) and/or may regulate the WNDP turnover. As we have shown recently, the N-terminal domain of WNDP interacts with the ATP-binding domain in a copper-dependent manner, and this interaction alters the conformation of the ATP-binding domain (21). This result let us to hypothesize that the copper-dependent domain-domain dissociation may stimulate catalytic activity of WNDP.

Although more experimental evidence is needed, our current results are consistent with the role of the N-terminal MBS in regulating the enzyme turnover.

In a cell, the potential effect of the first four MBS on WNDP function is likely to be coupled to other regulatory events. Elevated copper induces phosphorylation of WNDP by a kinase (13) and redistribution of WNDP from the trans-Golgi network to the intracellular vesicles (7, 13, 14, 16). The MBS1–4 may play an important role in these events, regulating conformations of WNDP and its interactions with the regulatory proteins. The experiments testing this hypothesis are currently underway.

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