Metallochaperone Atox1 Transfers Copper to the NH₂-terminal Domain of the Wilson’s Disease Protein and Regulates Its Catalytic Activity

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Copper is essential for the growth and development of mammalian cells. The key role in the intracellular distribution of copper belongs to the recently discovered family of metallochaperones and to copper-transporting P-type ATPases. The mutations in the ATPase ATP7B, the Wilson’s disease protein (WNDP), lead to intracellular accumulation of copper and severe hepatic and neurological abnormalities. Several of these mutations were shown to disrupt the protein-protein interactions between WNDP and the metallochaperone Atox1, suggesting that these interactions are important for normal copper homeostasis. To understand the functional consequences of the Atox1-WNDP interaction at the molecular level, we produced recombinant Atox1 and characterized its effects on WNDP. We demonstrate that Atox1 transfers copper to the purified amino-terminal domain of WNDP (N-WNDP) in a dose-dependent and saturable manner. A maximum of six copper atoms can be transferred to N-WNDP by the chaperone. Furthermore, the incubation of copper Atox1 with the full-length WNDP leads to the stimulation of the WNDP catalytic activity, providing strong evidence for the direct effect of Atox1 on the function of this transporter. Our data also suggest that Atox1 can regulate the copper occupancy of WNDP. The incubation with apo-Atox1 results in the removal of copper from the metalated N-WNDP and apparent down-regulation of WNDP activity. Interestingly, at least one copper atom remains tightly bound to N-WNDP even in the presence of excess apo-Atox1. We suggest that this incomplete reversibility reflects the functional non-equivalency of the metal-binding sites in WNDP and speculate about the intracellular consequences of the reversible Atox1-mediated copper transfer.

Copper is a trace element that has a dual role in human physiology. It is essential for normal cell metabolism serving as a cofactor to various enzymes involved in cellular processes as diverse as respiration, antioxidant defense, neurotransmitter biosynthesis, and iron homeostasis. At the same time, excess copper is toxic to the cell; therefore, the intracellular copper concentration is tightly controlled. Recent studies in yeast indicated that the amount of free copper in eucaryotic cells is negligible and that copper after being transported into the cell quickly becomes bound to various carriers (1). Among these carriers, the key role in further intracellular distribution of copper belongs to a recently discovered family of copper chaperones. These proteins temporarily sequester free copper while delivering it to specific target proteins throughout the cell (for reviews see Refs. 2–4).

Human copper chaperone Atox1 (also known as HAH1) is a small cytosolic protein that plays a key role in the distribution of copper to the cell secretory pathway. In yeast, the Atox1 ortholog, Atx1, was shown to facilitate copper delivery to the copper-transporting P-type ATPase Ccc2, which then transports copper into the late Golgi compartment (5, 6). In mammalian cells, Atox1 was proposed to bring copper to the copper-transporting ATPases, ATP7A and ATP7B, or the Menkes disease protein (MNKP) and the Wilson’s disease protein (WNDP), respectively. MNKP and WNDP then use the energy of ATP hydrolysis to either translocate the metal into the lumen of the trans-Golgi network for incorporation into copper-dependent enzymes or to export excess copper out of the cell. The deletion of the Atox1 gene in mice leads to the intracellular accumulation of copper and decreased activity of secreted copper-dependent enzymes such as tyrosinase, supporting the proposed role of Atox1 as a metal donor for the copper-transporting ATPases (7).

Although the important role of Atox1 for mammalian copper homeostasis was clearly demonstrated (7), the experimental data directly linking functions of the human chaperone Atox1 and the copper-transporting ATPases MNKP and WNDP are still lacking. Recent studies from several laboratories provided experimental evidence for physical interactions between Atx1 and the amino-terminal domains of MNKP and WNDP (8–10). In addition, several mutations found in Wilson’s disease patients were shown to disrupt the ability of WNDP to interact with Atox1, suggesting that this interaction was essential for normal copper homeostasis (9). Whether the protein-protein interactions lead to the transfer of copper from Atox1 to either WNDP or MNKP has not yet been demonstrated. Most importantly, it remains unknown whether the transfer of copper from the chaperone to the copper-transporting ATPases has a direct effect on activity of these transporters.

To address these important issues, we generated recombi...
nant Atox1 and examined whether Atox1 can transfer copper to the amino-terminal domain of WNDP (N-WNDP). We also determined the effect of Atox1 on the catalytic activity of WNDP using the full-length membrane-bound transporter. Finally, we characterized the reversibility of the Atox1-mediated transfer of copper and demonstrated that Atox1 can regulate the copper occupancy and activity of WNDP. In this work, we focused on the functional interactions between Atox1 and WNDP; however, our conclusions are probably applicable, at least in general, to the transfer of copper from Atox1 to MNKP, which is highly homologous to WNDP.

Experimental Procedures

Cloning, Expression, and Purification of Atox1—The human Atox1 cDNA was excised from the previously generated pET24b-Atox1 plasmid using NdeI and EcoRI endonucleases and cloned into the pTYB12 vector (New England Biolabs) to produce the pTYB12-Atox1 construct (see Fig. 1 and “Results”). This construct encoding the fusion protein composed of Atox1, intein, and a chitin-binding domain was then transformed into the Escherichia coli strain BL21 (DE3).

The expression of the Atox1-containing fusion was induced with 0.5 mm isopropyl-β-D-thiogalactopyranoside (Roche Molecular Biochemicals) for 19 h at room temperature. After centrifugation, the pellet was resuspended in lysis buffer (25 mm NaHPO4, 150 mm NaCl, pH 7.5) containing one Complete EDTA-free protease inhibitor mixture tablet (Roche Molecular Biochemicals). The cells were passed through a French Press (Sim-Amino) at 16,000 p.s.i., and the lysate was cleared by centrifugation at 30,000 g for 30 min. The soluble fraction of the lysate was passed through a chitin-bound column (New England Biolabs), allowing the Atox1 fusion to bind to the resin via its chitin-binding domain. The resin was then washed with 30-column volumes of lysis buffer. To induce the interein-mediated cleavage, the beads were incubated in 50 mm diethiothreitol (DTT), 25 mm NaHPO4, pH 8.15, 150 mm NaCl, for 40 h at room temperature. Atox1 was then collected in elution fractions and dialyzed against lysis buffer at 4 °C overnight to remove DTT. The protein concentration was measured using the Bradford assay (11), and protein purity was determined by a 15% Laemmli gel (12). The identity of Atox1 was confirmed by mass spectrometry and by immunohistochemistry using an Atox1-specific antibody. The yield of purified soluble Atox1 was ~2 mg of protein from a liter of cell culture.

Preparation of the Copper-bound Atox1—CuCl2 was mixed with glutathione at a 1:10 molar ratio, and the mixture was then added to Atox1 at a ratio of 1:1. Following a 10-min incubation at room temperature, Atox1 was extensively dialyzed against NaHPO4 buffer, and the stoichiometry of the copper-Atox1 complex was determined by a bischronic acid (BCA) assay in which the absorbance of a Cu(II)-BCA complex was monitored at 562 nm (13) or by atomic absorption (both methods produced very similar results). Electron paramagnetic resonance (EPR) measurements were carried out on one of the samples that had a typical copper-binding stoichiometry of 0.8 Cu/Atox1. EPR spectra were recorded on a Bruker E500 X-Band EPR spectrometer with a Super X microwave bridge and a Super High Q resonator. The instrument was equipped with a liquid nitrogen flow cryostat. No Cu2+ signal was detected, suggesting that copper bound to Atox1 was in the reduced Cu+ form.

Transfer of Copper from Atox1 to the N-terminal Domain of WNDP—N-WNDP used in this study was a fusion of 601 amino acid residues of human WNDP and the maltose-binding protein (14). N-WNDP was expressed and purified using affinity chromatography on amylase resin as described previously (14). For copper transfer experiments, N-WNDP bound to amylase resin (New England Biolabs) was washed with 30-column volumes of assay buffer (200 mm bis-Tris propane, pH 7.0, 200 mm KCl, 5 mm MgCl2) and then incubated with 100 μM DTT for 10 min at room temperature. After an additional wash with 10-column volumes of assay buffer, various amounts of soluble copper-bound Atox1 in the assay buffer were added to the resin and incubated with N-WNDP for 10 min at room temperature.2 Atox1 was then washed off the resin with assay buffer followed by the elution of N-WNDP with 10 mm maltose in the assay buffer. The protein concentration of each sample was measured using the Bradford assay, and the amount of copper bound to each protein was estimated using the BCA assay.

The presence of Atox1 in the N-WNDP elution fractions was examined by quantitative Western blot analysis. From the elution fractions, 2 μg of total protein were run on a Tris-Tricine gel (15) in parallel with the known amounts of purified Atox1 used to generate a calibration curve. The proteins were transferred to an Immobilon-P membrane (Millipore), then immunostained using an Atox1-specific antibody. The yield of purified soluble Atox1 was extensively dialyzed against Na2HPO4 at a 1:10 molar ratio, and the mixture was then added to Atox1 containing one Complete EDTA-free protease inhibitor mixture tablet (Roche Molecular Biochemicals) for 18 h at room temperature. The cells were then harvested by centrifugation at 20,000 g for 5 min, and BCS was removed. The membrane pellets were resuspended in 200 μl of assay buffer containing 100 μM TCEP, and the copper-Atox1 complex was added to the mixture at concentrations indicated in the legend to Fig. 3. After a 15-min incubation at room temperature, the samples were then placed on ice for 5 min, radioactively [γ-32P]ATP (5 μCi, specific activity 20 μCi/mmol) was added to 1 μl final concentration, and the reaction mixture was incubated at 37 °C for an additional 5 min.

The reaction was stopped by the addition of 50 μl of ice-cold 1 mm NaHPO4 in 50% trichloroacetic acid and then centrifuged for 10 min at 20,000 × g. The protein pellet was washed once with ice-cold water, resuspended in 40 μl of sample buffer (5 mm Tris-P04, pH 5.8, 6.7 μl urea, 0.4 μl DTT, 5% SDS), and loaded on the acid 7.5% polyacrylamide gel (17). After electrophoresis, the gels were fixed in 10% acetic acid for 10 min and dried on blotting paper. The dried gels were either overnight to the Molecular Imaging Screen-CS (Bio-Rad) or for several hours at ~80 °C to Kodak BioMax MS film, and the intensity of the bands was quantified using Bio-Rad molecular imager or Bio-Rad densitometer. The dried gels then were rehydrated, stained with Coomassie Blue, and the amount of protein in the WNDP-related bands was determined by a second round of densitometry. The 32P incorporation into WNDP was then normalized to the WNDP protein levels.

The results of the initial experiments with apo-Atox1 showed a significant variability. This was most probably attributed to rapid oxidation of the apo-chaperone, because the storage of apo-Atox1 decreased its effects on WNDP and N-WNDP, whereas subsequent treatment of apo-Atox1 with reducing TCEP restored the chaperone function. To make the results reproducible, additional treatment of apo-Atox1 with TCEP was carried out immediately before the reactions with either N-WNDP or the full-length WNDP.

Inactivation of the Catalytic Phosphorylation of WNDP by Apo-Atox1—50 μg of the membrane preparation containing WNDP was resuspended in 200 μl of the assay buffer containing 100 μM TCEP, and apo-Atox1 was added to final concentrations indicated in the legend for the N-WNDP protein levels.

The Effect of the Copper-Atox1 Complex on the Catalytic Activity of WNDP—The full-length WNDP was expressed in SF9 cells using the baculovirus-mediated infection, and the membrane fraction containing WNDP was isolated as described previously (16). Apo-Atox1 and copper-bound Atox1 were prepared as described above and were diazylated overnight against the assay buffer containing 100 μM of freshly prepared reducing reagent tris-2-carboxyethylphosphine hydrochloride (TCEP, Sigma) prior to incubation with WNDP.

For the Atox1-mediated reactivation of WNDP, 50 μg of total membrane protein was resuspended in 200 μl of ice-cold assay buffer containing 100 μM TCEP. The copper-chelator bathocuproine disulfonate (BCE, ICN Biomedicals) then was added to a final concentration of 100 μM. After a 15-min incubation on ice, the membrane protein was pelleted by centrifugation at 20,000 × g for 5 min, and BCE was removed. The membrane pellets were resuspended in 200 μl of assay buffer containing 100 μM TCEP, and the copper-Atox1 complex was added to the mixture at concentrations indicated in the legend to Fig. 3. After a 10-min incubation at room temperature, the samples were placed on ice for 5 min, radioactively [γ-32p]ATP (5 μCi, specific activity 20 μCi/mmol) was added to 1 μl final concentration, and the reaction mixture was incubated at 37 °C for an additional 5 min.

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Expression and Purification of Atox1—To simplify the purification of Atox1 and to obtain the purified protein without a large affinity tag, we generated an expression construct for Atox1 using a pTYB12 vector. In this construct, Atox1 was fused with an intein and a chitin-binding domain (CBD) (Fig. 1A). This enabled us to purify the expressed Atox1-CBD-intein fusion through the intein-mediated protein cleavage, leaving CBD and intein associated with the resin, the copper-glutathione complex to purified Atox1 (see “Experimental Procedures”) generates the metalated chaperone with a typical stoichiometry of 0.85 ± 0.1 copper atoms/Atox1.

Copper Transfer from Atox1 to N-WNDP—To determine whether Atox1 can transfer copper to WNDP, we utilized purified N-WNDP bound to amylose resin and a soluble copper-Atox1 complex (Cu-Atox1). Cu-Atox1 was passed through the resin containing bound N-WNDP, the resin was washed, and N-WNDP was then eluted from the resin using the maltose-containing buffer (Fig. 2A). The amount of protein and copper in each elution fraction was analyzed. This protocol allowed for a quick separation of Atox1 and N-WNDP and an easy determination of copper bound to both proteins after the transfer reaction.

In agreement with our earlier results (14), control N-WNDP eluted from the amylose resin did not contain measurable amounts of copper (data not shown). In contrast, when N-WNDP was first preincubated with Cu-Atox1 and then eluted, copper was detected in the N-WNDP-containing fractions (Fig. 2A, fractions 29 and 30), suggesting that copper was transferred from Atox1 to N-WNDP. It was previously shown that Atox1 interacted with N-WNDP and that these interactions could be detected using a
protocol similar to the procedure used for our transfer studies (8). Therefore, it was possible that in our experiments copper co-eluted with N-WNDP as a result of an association of Atox1 with N-WNDP and not because of copper transfer. To eliminate this possibility, we examined the presence of Atox1 in the N-WNDP-containing elution fractions. No Atox1 was detected in the N-WNDP-containing fractions analyzed by gel-electrophoresis and stained with Coomassie Blue. However, Atox1 was observed by immunostaining, indicating that small amounts of protein remained associated with N-WNDP after washes of the resin (data not shown). The amount of Atox1 that co-elutes with N-WNDP was determined using quantitative Western blot analysis and purified Atox1 as a standard. These calculations indicate that Atox1 is present in the N-WNDP elution fractions at a molar ratio of ~0.1 Atox1/N-WNDP and can account for 5–10% total copper bound to N-WNDP. Thus, we conclude that the incubation of Atox1 with N-WNDP leads to a transfer of copper from the chaperone to its target.

To characterize the transfer reaction in more detail, the experiments were repeated using a wide range of molar ratios of Atox1 with a 0.85 ± 0.1 copper to protein stoichiometry and

![Graph](image1)

**Fig. 3.** The effect of Atox1 on catalytic activity of WNDP. A, WNDP was treated with or without BCS as described under “Experimental Procedures.” BCS was then removed by centrifugation, WNDP was resuspended in the assay buffer, and the same amounts of apo-Atox1 or Cu-Atox1 were added to the BCS-pretreated sample. The catalytic activity of WNDP was monitored by measuring the amount of radioactive acylphosphate intermediate as described under “Experimental Procedures.” Autoradiogram of a typical gel is shown. B, the activation of the WNDP catalytic phosphorylation by Cu-Atox1 (●) and by free copper (○). The average of the densitometry data for five independent experiments is shown.

N-WNDP. As shown in Fig. 2B, the incubation of N-WNDP with increasing amounts of Cu:Atox1 leads to a dose-dependent and saturable transfer of copper to N-WNDP. Up to six copper atoms per N-WNDP can be transferred when a 30–40-fold molar excess of Cu:Atox1 over N-WNDP is present. N-WNDP is known to contain six metal-binding sites; therefore, it appears that Atox1 can deliver copper to all metal-binding sites in N-WNDP. A further increase in the amount of added Cu:Atox1 does not lead to an additional binding of copper to N-WNDP.

![Graph](image2)

**Fig. 4.** Apo-Atox1 removes copper from N-WNDP. A, metalated N-WNDP was bound to amylose resin and extensively washed. Apo-Atox1 was then passed through the resin, the resin was further washed, and N-WNDP was eluted with the maltose-containing buffer. The amount of copper (●) and protein (○) in each elution fraction was measured. The additions of the chaperone and the maltose-containing elution buffer are indicated by the arrows. B, dose-dependent effect of apo-Atox1 on the amount of copper that remains bound to N-WNDP. The average of five independent experiments is shown. The solid line is the theoretical hyperbolic curve with a $R^2$-factor of 0.99. C, copper was removed from N-WNDP as in B using increasing amounts of apo-Atox1. N-WNDP eluted from the resin was labeled with fluorescent reagent CFP and electrophoresed on a 10% Laemmli gel.
consistent with the idea that the chaperone controls the delivery of copper to specific sites.

In N-WNDP, copper is coordinated by Cys residues in the highly conserved GMTCXXC sequence motifs. The binding of copper to N-WNDP in a cell protects these Cys residues against labeling with the fluorescent coumarine maleimide CPM (14). A similar decrease in fluorescent labeling of N-WNDP was observed following copper transfer from Cu-Atox1 to N-WNDP (data not shown), suggesting that copper was transferred to Cys residues.

The Effect of Atox1 on WNDP Activity—Recently, we demonstrated that the full-length WNDP expressed in insect cells was catalytically active and was able to form a phosphorylated acylphosphate intermediate when incubated with ATP (16). This catalytic reaction is inhibited by the copper chelator BCS. An addition of copper to the inhibited enzyme restores its activity. We utilized this assay to test whether Cu-Atox1 could reactivate the BCS-treated WNDP by transferring copper to the transporter. The membrane-bound WNDP was incubated with either apo-Atox1 or Cu-Atox1, and [γ-32P]ATP was then added and the ability of WNDP to form an acylphosphate intermediate was analyzed by measuring the amount of radioactivity associated with the WNDP band on an acidic polyacrylamide gel. As shown in Fig. 3A, the addition of Cu-Atox1 to the BCS-treated WNDP leads to the reactivation of the enzyme, indicating that Cu-Atox1 transfers copper to the WNDP metal-binding sites, which are essential for the stimulation of its catalytic phosphorylation. The lack of WNDP reactivation by apo-Atox1 confirms that copper transfer and not a mere interaction with Atox1 is necessary for the stimulation of the WNDP activity (Fig. 3A).

In the copper transfer experiments shown in Fig. 2, purified N-WNDP was the only protein that could accept copper from Atox1. In contrast, in the membrane preparations used for the reactivation experiments, WNDP represents 2% or less of the total protein (as shown in this study). Thus, it was interesting to compare the ability of Atox1 to transfer copper to purified N-WNDP with its ability to activate WNDP in the presence of a large excess of other proteins. To do that, the amount of WNDP in the membrane fraction was estimated using quantitative Western blot analysis. Atox1 then was added to the BCS-treated WNDP at the same molar ratios that were used previously for the copper transfer experiments with purified N-WNDP.

As shown in Fig. 3B, the effect of copper-Atox1 on catalytic activity was dose-dependent and saturable with a maximum reactivation reached in the presence of a 20–30-fold molar excess of Atox1 over WNDP. Thus, Atox1 was at least as efficient in activating WNDP in the presence of a large excess of unrelated proteins as it was in transferring copper to purified N-WNDP. Also, we compared the efficiency of Cu-Atox1 in the stimulation of the WNDP activity with respect to free copper. As shown in Fig. 3B, Cu-Atox1 activated WNDP with EC_{50} equal to 0.18 ± 0.07 μM. Free copper added in the presence of 100 μM ascorbate stimulated the WNDP phosphorylation with comparable EC_{50} = 0.17 ± 0.04 μM.

The Reverse Transfer of Copper from N-WNDP to Atox1—It has been proposed that copper transfer from the chaperone to N-WNDP could be reversible (8). In fact, a reversible copper exchange was demonstrated using the yeast copper-chaperone Atox1 and a purified single metal-binding repeat of the copper-transporting ATPase Ccc2 (18). However, it remains unknown whether apo-Atox1 can remove copper from WNDP and how the presence of the multiple copper-binding sites in this protein (a situation typical for the eucaryotic copper-ATPases) affects this process. To determine whether copper could be transferred from N-WNDP back to Atox1, N-WNDP loaded with 5–6 copper atoms in cell culture (14) was bound to amylose resin and then incubated with 30–40-fold molar excess of purified apo-Atox1. As shown in Fig. 4A, the addition of apo-Atox1 to the N-WNDP-containing resin leads to a co-elution of the chaperone and copper, indicating that apo-Atox1 was able to strip copper from N-WNDP. However, some copper remained bound to N-WNDP, suggesting that the reverse transfer was partial. To verify these conclusions, the experiments were repeated using a wide range of molar ratios of apo-Atox1 and copper-bound N-WNDP. As shown in Fig. 4B, the addition of increasing amounts of apo-Atox1 to N-WNDP resulted in a saturable decrease in the amount of copper bound to N-WNDP. Interestingly, 3–4 copper atoms can be stripped from N-WNDP using a fairly small excess of apo-Atox1 over N-WNDP, whereas the remaining copper seems to be much less exchangeable. In fact, 1.11 ± 0.11 copper atoms remained associated with N-WNDP even after incubation with a large excess of apo-Atox1, suggesting that one metal-binding site had a much lower affinity for Atox1 or was much less exposed.

It was interesting to determine whether removal of copper by apo-Atox1 leaves the Cys residues in N-WNDP in the reduced...
state and thus available for subsequent loading with copper. To examine the accessibility of cysteines, we carried out the fluorescent labeling of N-WNDP before and after the incubation of N-WNDP with increasing amounts of apo-Atox1. Copper bound to N-WNDP protects cysteine residues in the metal-binding sites against labeling with the fluorescent coumarine maleimide CPM (Fig. 4C) (14). After incubation with apo-Atox1, copper is removed from N-WNDP; the decrease in copper binding is associated with the increase in the fluorescent labeling of Cys residues in N-WNDP (Fig. 4C). The availability of the Cys residues for labeling with the fluorescent probe indicates that after copper is removed by the chaperone, these residues remain reduced and available for a new round of copper binding.

Atox1 Down-regulates WNDP Activity—We have previously proposed that copper binding to N-WNDP regulates the functional activity of WNDP (16). The ability of apo-Atox1 to remove copper from N-WNDP suggested that treatment of the fully active transporter with apo-Atox1 may decrease the copper occupancy of WNDP and consequently reduce its catalytic activity. To test this hypothesis, the fully active membrane-bound WNDP was incubated with increasing concentrations of apo-Atox1, and subsequently, the chaperone was separated from WNDP by centrifugation. WNDP was resuspended in assay buffer, and its ability to undergo catalytic phosphorylation was measured using [γ-32P]ATP. As shown in Fig. 5, pre-incubation with apo-Atox1 leads to a concentration-dependent and saturable reduction of the WNDP activity, presumably because of a reverse transfer of copper from the amino-terminal domain of WNDP to Atox1. Interestingly, even after treatment with a large excess of apo-Atox1, WNDP retains a significant portion of its activity (~50%), suggesting that the apo-Atox1 removes some but not all coppers from WNDP, leading to the down-regulation of the enzyme.

**DISCUSSION**

With this work, we began characterizing the biochemical processes important for the distribution of copper from the cytosol to the secretory compartment of human cells. We demonstrate that the previously reported interaction between Atox1 and N-WNDP leads to a transfer of copper from the chaperone to the copper-transporting ATPase and that a maximum of six copper atoms can be transferred from Atox1 to N-WNDP.

Furthermore, we found that the addition of increasing amounts of Atox1 to the full-length WNDP led to a concurrent stimulation of the catalytic activity of WNDP measured through the formation of the phosphorylated intermediate. Significantly, this effect was observed using fairly low concentrations of Atox1 (EC50 ~200 nM). The presence of a large excess of various proteins in the membrane preparation did not interfere with the transfer reaction, suggesting that Cu-Atox1 specifically targeted N-WNDP and activated the transporter.

Altogether, our results provide the first experimental demonstration of the direct effect of Atox1 on WNDP function and illustrate that Atox1 can indeed act as a physiological copper donor for this human copper-transporting ATPase. Although it is still technically difficult to make a precise quantitation of how many copper atoms need to be transferred to the full-length WNDP to induce phosphorylation, it is clear that the Atox1-mediated copper transfer correlates with the activation of WNDP. In a cell, such activation would most probably lead to copper transport from the cytosol into the secretory pathway of the cell.

It seems particularly interesting that Atox1 cannot only deliver copper to N-WNDP but can also regulate its metal occupancy through the removal of copper. It is possible that in a cell, the ratio of copper-bound and apo-chaperones would fluctuate depending on how much copper is taken up by the cell and how much of it is exported or utilized. Our results suggest that the apo-chaperone is not simply an inert carrier waiting to be occupied by copper. Instead, apo-Atox1 can remove copper from N-WNDP and decrease the activity of the full-length transporter, suggesting that both metalated and apo forms of Atox1 may contribute to the regulation of WNDP.

It has been previously shown that the metal-binding sites in N-WNDP are functionally non-equivalent (19). Only one of them is necessary to sustain the copper transport activity of WNDP (19, 20), whereas the other metal-binding sites in N-WNDP are probably involved in the regulation of the transporter. Our results provide further evidence of distinct properties of the metal-binding sites in N-WNDP. In experiments with apo-Atox1, one copper atom remained bound to N-WNDP even when the chaperone was present in a large excess, suggesting that a certain metal-binding site in N-WNDP was unavailable for interactions with Atox1. This conclusion is in apparent contradiction with the results of the forward transfer experiments in which all of the binding sites in N-WNDP were filled by copper using the copper-Atox1 complex.

There are two possible explanations for these results. First, it is possible that in the forward reaction, one of the metal-binding sites in N-WNDP is filled with copper indirectly, i.e. copper is transferred to this site not from Atox1 but from another metal-binding site in N-WNDP. Alternatively, in apo-N-WNDP, all of the metal-binding sites could be available for interactions with the chaperone, whereas in the copper-bound N-WNDP, some sites could be less exposed. This interpretation would be consistent with the results of recent studies from DiDonato et al. (21) who demonstrated that the binding of copper to N-WNDP induced the conformational transitions in this protein (21). Experiments are currently underway to understand the sequence of the events during copper transfer to N-WNDP and to identify the site(s) that can retain copper in the presence of excess apo-chaperone.

It is also interesting that the incubation of WNDP with apo-Atox1 lowers the WNDP activity to a certain level but does not lead to a full inactivation of the transporter. This finding is in contrast to our earlier results demonstrating that the copper chelator BCS can eliminate WNDP activity (16). Apo-Atox1 interacts quite efficiently with WNDP. A dose-dependent curve for the chaperone shows its half-maximum effect at a concentration of 1.0 ± 0.24 μM (Fig. 5), whereas the half-maximum effect of BCS on the WNDP activity was observed only in the presence of 50 μM chelator (16). It appears that apo-Atox1, unlike BCS, strips copper only from some presumably "regulatory" metal-binding sites in WNDP, thus down-regulating the enzyme, whereas the site(s) essential for the WNDP activity remains inaccessible to apo-chaperone probably because of steric hindrances.

The ability of Atox1 to alter the copper occupancy of N-WNDP and the activity of WNDP in an asymmetric fashion may have important physiological consequences. In a cell, the changes in copper occupancy of WNDP were proposed to affect the intracellular localization (19, 21), posttranslational modification (22), and activity of WNDP (16). Thus, Atox1 may play a key role in these events by controlling the amount of copper bound to the transporter and hence contribute to the regulation of the intracellular localization or posttranslational modification of WNDP while keeping the transporter active at a wide range of copper concentrations.

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REFERENCES

1. Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C. & O’Halloran, T. V. (1999) Science 284, 805–808
2. O’Halloran, T. V. & Culotta, V. C. (2000) J. Biol. Chem. 275, 25057–25060
3. Harrison, M. D., Jones, C. E., Soliz, M. & Dameron, C. T. (2000) Trends Biochem. Sci. 25, 29–32
4. Culotta, V. C., Lin, S. J., Schmidt, P., Klomp, L. W., Casareno, R. L. & Gitlin, J. (1999) Adv. Exp. Med. Biol. 448, 247–254
5. Pufahl, R. A., Singer, C. P., Pearies, K. L., Lin, S. J., Schmidt, P. J., Fahrni, C. J., Culotta, V. C., Penner-Hahn, J. E. & O’Halloran, T. V. (1997) Science 276, 853–856
6. Lin, S. J., Pufahl, R. A., Dancis, A., O’Halloran, T. V. & Culotta, V. C. (1997) J. Biol. Chem. 272, 9215–9220
7. Hamza, I., Faisst, A., Prohaska, J., Chen, J., Gruss, P. & Gitlin, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6848–6852
8. Larin, D., Mekios, C., Das, K., Ross, B., Yang, A. S. & Gilliam, T. C. (1999) J. Biol. Chem. 274, 28497–28504
9. Hamza, I., Schaefer, M., Klomp, L. W. & Gitlin, J. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13363–13368
10. Lockhart, P. J. & Mercer, J. F. (2000) Biochim. Biophys. Acta 1490, 11–20
11. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
12. Laemmli, U. K. (1970) Nature 227, 680–685
13. Brenner, A. J. & Harris, E. D. (1995) Anal. Biochem. 226, 80–84
14. Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T. & Kaplan, J. H. (1997) J. Biol. Chem. 272, 18939–18944
15. Schagger, H. & Von Jagow, G. (1987) Anal. Biochem. 166, 368–379
16. Tsekovskii, R., Eisses, J. F., Kaplan, J. H. & Lutsenko, S. (2002) J. Biol. Chem. 277, 976–983
17. Sarkadi, B., Enyedi, A., Foldes-Papp, Z. & Gardos, G. (1986) J. Biol. Chem. 261, 9552–9557
18. Huffman, D. L. & O’Halloran, T. V. (2000) J. Biol. Chem. 275, 18611–18614
19. Forbes, J. R., Hsi, G. & Cox D. W. (1999) J. Biol. Chem. 274, 12408–12413
20. Izda, M., Terada, K., Sambongi, Y., Wakabayashi, T., Miuna, N., Koyama, K., Futai, M. & Sugiyama, T. (1998) FEBS Lett. 428, 281–285
21. DiDonato, M., Hsu, H. F., Narindrasorasak, S., Que, L., Jr. & Sarkar, B. (2000) Biochemistry 39, 1890–1896
22. Vanderwerf, S. M., Cooper, M. J., Stetsenko, I. V. & Lutsenko S. (2001) J. Biol. Chem. 276, 36289–36294