Human Copper-Transporting ATPase ATP7B (The Wilson's Disease Protein): Biochemical Properties and Regulation

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Wilson's disease protein (WNDP) is a product of a gene *ATP7B* that is mutated in patients with Wilson's disease, a severe genetic disorder with hepatic and neurological manifestations caused by accumulation of copper in the liver and brain. In a cell, WNDP transports copper across various cell membranes using energy of ATP-hydrolysis. Copper regulates WNDP at several levels, modulating its catalytic activity, posttranslational modification, and intracellular localization. This review summarizes recent studies on enzymatic function and copper-dependent regulation of WNDP. Specifically, we describe the molecular architecture and major biochemical properties of WNDP, discuss advantages of the recently developed functional expression of WNDP in insect cells, and summarize the results of the ligand-binding studies and molecular modeling experiments for the ATP-binding domain of WNDP. In addition, we speculate on how copper binding may regulate the activity and intracellular distribution of WNDP, and what role the human copper chaperone Atox1 may play in these processes.

KEY WORDS: Copper; ATP7B; P-type ATPase; Wilson's disease; ATP-binding; molecular modeling; regulation.

THE PHYSIOLOGICAL ROLE OF THE WILSON'S DISEASE PROTEIN (WNDP)

WNDP is a copper-transporting ATPase with a very important role in cell physiology. Mutations in the gene encoding WNDP are associated with changes in human copper metabolism leading to a severe hepato-neurological disorder, Wilson's disease. In Wilson's disease, copper accumulates in a number of tissues, particularly in the liver, brain, and kidneys, causing DNA damage, inactivation of certain enzymes, and lipid peroxidation (Carmichael *et al.*, 1995; Gu *et al.*, 2000; Nair *et al.*, 1998). At the organism level, patients with Wilson's disease have neurological and psychiatric problems and/or various degree of liver pathology (Scheinberg and Sternlieb, 1984). The precise molecular mechanisms of copper toxicity in hu-

mans, and particularly the effect of accumulated copper on human behavior, are still poorly understood.

The Wilson's disease gene, *ATP7B*, encodes a 165 kDa membrane protein, which belongs to a large family of ion-transporting ATPases (Petrukhin *et al.*, 1994). Members of this family couple the enzymatic reaction of ATP-hydrolysis with transport of various ions across cell membranes. The physiological consequences of Wilson's disease, such as accumulation of copper in tissues, decreased export of copper from the liver into the bile, and decreased incorporation of copper into the secreted copper-dependent ferroxidase, ceruloplasmin (Scheinberg and Sternlieb, 1984), suggest that normal WNDP has two important functions: to facilitate export of copper from the cell and to deliver copper to the secretory pathway for incorporation into copper-dependent enzymes. Which of these functions is carried out at any given moment appears to depend on intracellular concentration of copper.

Normal copper intake into the cell is fairly low (Linder and Hazegh-Azam, 1996). Thus, under regular conditions the need for removal of copper from the cell is likely to be insignificant, while the demand for incorporation of copper into copper-dependent enzymes in the

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secretary pathway could be relatively high. Consistent with these likely copper requirements, under normal conditions WNDP is located primarily in the trans-Golgi network (TGN), where it transports copper to such copper-dependent enzymes as ceruloplasmin (Terada *et al.*, 1998). However, when the copper concentration is increased above a certain threshold, WNDP is found primarily in a vesicular compartment, distinct from TGN (Schaefer *et al.*, 1999). It is believed that in this compartment WNDP sequesters copper into the lumen of the vesicles, thus protecting cells against potentially toxic effects of copper. How copper is then transported from the vesicles across the plasma membrane is still unclear. It is possible that WNDP itself traffics from the vesicular compartment to the plasma membrane, albeit at a very low level (Roelofsen *et al.*, 2000). Alternatively, it was proposed that copper trapped in the vesicles is exocytosed, while WNDP returns back to the TGN (Payne *et al.*, 1998; Suzuki and Gitlin, 1999). Further studies are needed to elucidate the precise route of copper excretion from the WNDP-expressing cell and to understand the molecular mechanisms of the WNDP relocalization and the protein machinery involved in this process.

MOLECULAR ARCHITECTURE OF WNDP

The predicted topological organization of WNDP is shown in Fig. 1(A). The protein is composed of four major domains and a long C-terminal tail. The transmembrane portion of the protein that forms a copper-translocation pathway consists of eight transmembrane segments (TM). One of these segments, TM6, contains CPC sequence motif. This motif is highly conserved in all ATPases involved in transport of transition metals (so called P_1-type AT-Pases or CPX-ATPases), and is likely to be a part of the intramembrane metal-binding site (Bissig *et al.*, 2001). A high degree of sequence conservation is also seen in the last two transmembrane segments, pointing to their potential role in metal translocation (Fig. 1(B)). Interestingly, the TM 7 of WNDP contains a two-residues motif YN, which is seen in all ATPases shown or predicted to be involved in transport of copper (Fig. 1(B)). The ATPases transporting zinc or cadmium lack this motif (Fig. 1(B)), suggesting that YN could be important in defining the metal specificity of the membrane portion of the copper-transporting ATPases.

The extracellular loops of WNDP are predicted to be very short, and all functional domains of WNDP, other than its transmembrane portion, are cytosolic. The N-terminal copper-binding domain of WNDP (N-WNDP) is about 650 amino acid residues long and contains six repetitive sequences, each bearing the conserved sequence
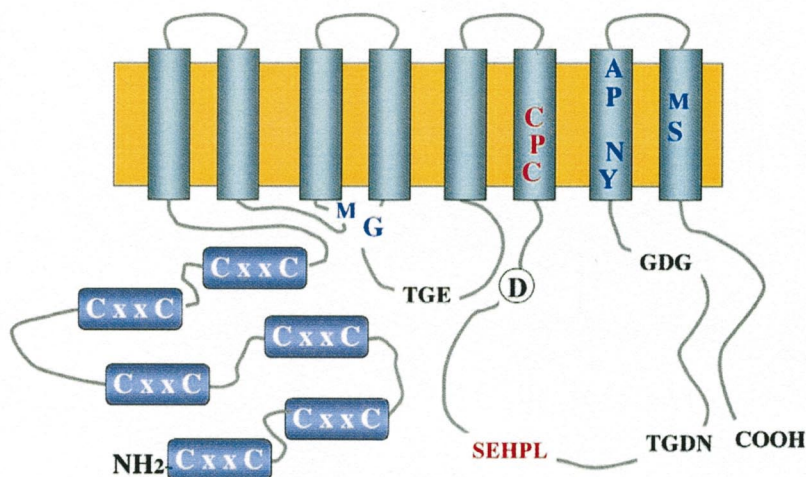
motif GMT/HCxxCxxxIE. The structure of the N-terminal domain of WNDP has yet to be determined; however, the NMR structure of a single metal-binding repeat (MBR) from the highly homologous human copper-transporting ATPase ATP7A has been solved (Gitschier *et al.*, 1998). Using this structure, homology models were generated for all the MBRs of WNDP, demonstrating that these 70 amino acid residues protein segments of N-WNDP are likely to be folded very similarly into the ferredoxin-like units (Arnesano *et al.*, 2002). In these units, copper binds to cysteines in the GMT/HCxxC sequence, which is located in the exposed loop at the "top" of the otherwise compactly folded MBR.

We and others have shown that N-WNDP binds six copper atoms, suggesting that each of the MBRs in N-WNDP is involved in copper coordination (DiDonato *et al.*, 1997; Lutsenko *et al.*, 1997). The MBRs are connected by linkers of different length. The longest linker is between MBR4 and MBR5, and it subdivides N-WNDP into two parts: MBRs1-4 and MBR5,6. This is interesting, because the bacterial and yeast copper-transporting AT-Pases have only one or two MBRs, suggesting that most of the MBRs in WNDP are not essential for the WNDP function. This conclusion is supported by recent studies, demonstrating that only one MBR is necessary to support the transport activity of WNDP expressed in yeast cells (Forbes *et al.*, 1999; Iida *et al.*, 1998). As discussed below, the other MBRs and the linkers connecting these repeats are likely to be important for regulation of WNDP in response to copper binding.

While the N-terminal domain is unique for WNDP and its mammalian homologues, the other cytosolic domains can be found in a structure of all cation-transporting P-type ATPases. The ATP-binding domain (ATP-BD) is about 350 amino acid residues long and includes the motifs DKTG, TGDN, and GDGxxD (Fig. 1(A)) that were shown to be essential for ATP-hydrolysis in the P-type ATPases. In addition, the ATP-BD is the site of the most frequent mutation H1069Q found in Wilson's disease patients (Tanzi *et al.*, 1993; Thomas *et al.*, 1995). This mutation occurs in the sequence motif SEHPL (see Fig. 1(A)), which is highly conserved in the P_1-type ATPases. The functional role of this sequence motif remains unknown, although the presence of the HP dyad in all P_1-ATPases points to a critical involvement of these two residues in the ATP-dependent transport of transition metals.

The cytosolic loop located between N-WNDP and ATP-BD is shorter and contains about 140 residues. The key functional residues in this region form the highly conserved TGE motif (Fig. 1). It is very likely that this loop is folded into a separate domain, as was shown for Ca^{2+}-ATPase, another member of the P-type ATPase family

Fig. 1. Molecular architecture of WNDP. (A) The predicted transmembrane organization of WNDP. The cylinders in the N-terminal portion with the CxxC motif represent the cytosolic copper-binding sites. TGE, TGDN, and GDG (GDGxxD in the text) are the sequence motifs conserved in all P-type ATPases. D in a circle is an invariant Asp residue in the DKTG sequence motif that is phosphorylated during ATP hydrolysis. CPC and SEHPL are the sequence motifs characteristic of the P_1-type ATPases. Other letters mark location of the amino-acid residues highly conserved in the copper-transporting ATPases. (B) Sequence alignment of the putative transmembrane segments TM7 and TM8 for several P_1-type ATPases. Red letters indicate the amino acid residues that are highly conserved in the copper-transporting ATPases. In blue are the counterparts of these residues in the P_1-type ATPases with different ion specificity.

(Toyoshima *et al.*, 2000). In Ca^{2+}-ATPase this region is called A-domain (from actuator), because it interacts with the ATP-BD and plays an important role in conformational transitions associated with the catalytic activity of the transporter (Toyoshima *et al.*, 2000).

Lastly, the 80 amino acid residues C-terminal tail of WNDP could be important for trafficking and/or regulation of this copper-transporting ATPase. The C-terminus contains three Leu residues, two of which are present in homologous copper-transporting ATPase ATP7A. In ATP7A this dileucine motif was required for the trafficking of ATP7A from the plasma membrane to the TGN (Petris *et al.*, 1998). Whether the three leucine residues in the C-terminal tail of WNDP have the same function remains to be determined. However, truncation of the C-terminal region due to a frame-shift mutation at position

4973 of the *ATP7B* gene results in the Wilson's disease phenotype in patients (Majumdar *et al.*, 2000). This finding suggests that alteration of the WNDP C-terminus may cause abnormal intracellular localization of WNDP, and consequently disrupts its ability to transport copper into appropriate compartments (Majumdar *et al.*, 2000). The lack of the C-terminus may also have an indirect negative effect on the WNDP folding and activity.

FUNCTIONAL EXPRESSION AND CATALYTIC PROPERTIES OF WNDP

The general mechanism of copper transport by WNDP is likely to resemble the mechanism of other P-type ATPases. In other words, the overall sequence of events is the following. WNDP binds ATP and copper from the cytosolic side; ATP is then hydrolyzed, and during this process the γ-phosphate of ATP is transferred to the invariant Asp residue in the DTKG sequence motif. Copper is then released at the opposite side of the membrane, Asp becomes dephosphorylated, and WNDP returns to the initial state so that the cycle can be repeated. Although there is little doubt that in general WNDP follows this basic sequence of events, the specific details of the WNDP-mediated copper transport are much less clear. One of the most intriguing questions is how copper is delivered from the cytosol to the plasma membrane and how it is then released?

In a cell, copper binds to proteins as Cu^+ and Cu^{2+}. It is assumed that WNDP transports Cu^+, maintaining its reducing state all the way through the transport cycle. This is a plausible assumption, since the environment of the cell cytosol is reducing and N-WNDP was shown to bind Cu^{+1} (DiDonato *et al.*, 2000). The concentration of free copper ion in the cytosol is extremely low and most of the copper is believed to be present in a protein-bound form (Rae *et al.*, 1999). This suggests that there has to be a mechanism that enables WNDP to retrieve this bound copper from its carriers. Recent studies revealed that the intracellular copper is delivered to the cytosolic copper-binding sites of WNDP by a metallochaperone Atox1 (see Copper-Dependent Regulation of WNDP), but specific details of this process are still unclear. Similarly, it remains unknown how copper relocates from these cytosolic sites to the membrane portion of WNDP. Another issue that remains to be understood is the role of protons in the WNDP-mediated copper transport. Unlike most of the plasma membrane P-type ATPases, WNDP simultaneously faces environments with quite different pH: the cytosolic portion of WNDP is at neutral pH, while the protein surface at the opposite side of the membrane is exposed to the lumen of the TGN and vesicles, where the environment is more acidic. It is likely that the exposure of the intramembrane metal-binding site(s) to lower pH facilitates release of copper from WNDP into the TGN lumen. It is also possible that specific luminal proteins or small molecular weight molecules contribute to copper retrieval from WNDP. Whether a proton facilitates copper release and is subsequently transported as a counterion remains to be tested. To address these and other important questions related to the transmembrane transport of copper it is crucial to have functional assay that permits direct characterization of the biochemical properties of WNDP.

Functional characterization of WNDP endogenously expressed in human cells is difficult because of its very low expression level ($\sim$0.005% of total membrane protein, Tsivkovskii *et al.*, 2002) and the predominant localization of this protein in the intracellular membranes. Initial studies of WNDP and its various mutants were carried out following heterologous expression of these proteins in the $\Delta ccc2$ yeast strain, where their ability to complement the function of the deleted yeast copper-transporting ATPase Ccc2 was monitored (Forbes *et al.*, 1999; Iida *et al.*, 1998). Although relatively fast and convenient for screening of the WNDP mutants, this assay by its nature cannot address the questions related to the molecular mechanism of WNDP. Recently, expression of WNDP in mammalian cells was reported, permitting measurements of the ATP-dependent copper transport (Voskoboinik *et al.*, 2001). However, the level of WNDP expression in these cells is insufficient for analysis of the enzymatic steps that accompany copper transport. The baculovirus-mediated functional expression of WNDP in insect cells which was recently developed by our group (Tsivkovskii *et al.*, 2002), overcame the limitation of earlier assays.

The baculovirus-based system permitted robust expression of WNDP at levels that can be detected on a standard Coomassie-stained gel (Tsivkovskii *et al.*, 2002). The yield of WNDP per milligram of membrane protein in *Sf9* cells is about 20-fold higher than the amount of WNDP expressed in COS cells and about 400-fold higher than the amount of endogenous WNDP in HepG2 cells. Interestingly, the presence of copper in the cell growth media increases the amounts of the expressed WNDP (our unpublished observation). Since polH baculovirus promoter is not known to be regulated by copper, the higher yields of WNDP in the presence of the metal could be due to the stabilizing effect of copper binding on WNDP. Alternatively, copper may induce some cellular factors protecting WNDP from proteases or may simply inactivate the proteases. In *Sf9* cells, the large portion of expressed WNDP is present in the Golgi fraction in agreement with its known primary localization in mammalian cells. Thus, it appears that the TGN-retention signal of WNDP is functional in the insect cells. A significant portion of WNDP is also accumulated in ER possibly due to high levels of expression.

WNDP expressed in insect system displays properties characteristic of a P-type ATPase (Tsivkovskii *et al.*, 2002). Incubation of WNDP with 1–2 μM radioactive γ-ATP leads to formation of a phosphorylated intermediate, a process that can be monitored following separation of the protein on a specially formulated acidic gel (Fig. 2). The aspartyl–phosphate intermediate that is formed following the ATP-hydrolysis step is sensitive to treatment with hydroxylamine. As shown in Fig. 2, incubation of phosphorylated WNDP with 250 mM hydroxylamine at pH 6.0 leads to cleavage of the acyl–phosphate bond, dramatically decreasing the amount of radioactive phosphate associated with WNDP. This property helps to distinguish the product of catalytic phosphorylation from the products of phosphorylation by kinases such as phosphoserine, phosphothreonine, and phosphotyrosine. The transient nature of the intermediate can also be demonstrated by chasing phosphorylated WNDP with nonradioactive ATP or by reversing the reaction with ADP (Tsivkovskii *et al.*, 2002). As discussed above, WNDP contains the DKTG sequence motif, in which the invariant aspartate residue, D1027, was predicted to serve as an acceptor of the γ-phosphate during ATP hydrolysis. The D1027A substitution does not have a negative effect on the WNDP expression in insect cells but prevents catalytic phosphorylation from ATP, providing experimental verification of this prediction (Fig. 2(A)). The hydroxylamine-insensitive phosphorylation by a kinase (Copper-Dependent Regulation of WNDP) is unaltered by the D1027A mutation.

For many P-type ATPases, formation of the acyl–phosphate intermediate is known to be facilitated by the ions extruded from the cytosol by these proteins. Interestingly, catalytic phosphorylation of WNDP occurs in the absence of added copper, suggesting that trace amounts of copper present in the cell growth medium and in buffers (about 1–1.5 μM, our data) are sufficient to fully activate WNDP. (The ability of trace copper to sustain catalytic activity was also demonstrated for the bacterial copper-transporting ATPase CopB, Bissig *et al.*, 2001.) In agreement with this assumption, addition of the copper chelator bathocuproine disulfonate (BCS) to WNDP markedly decreases the level of catalytic phosphorylation. Importantly, the effect of BCS on WNDP is reversible. As shown in Fig. 2(B), additions of copper to the BCS-treated WNDP lead to restoration of its ability to form a phosphorylated intermediate. It is interesting that the reactivation of WNDP strongly depends on the presence of a highly efficient cistine-reducing reagent TCEP, but not DTT or glutathione. We speculated that the removal of copper by BCS results in rapid oxidation of cysteines, presumably in the intramembrane metal-binding site(s) of WNDP. This apparent rapid oxidation of cysteines (during 15-min treatment on ice) into stable S—S bond is very intriguing and

raises the question as how such rapid oxidation is prevented in a cell.

Reactivation of the BCS-treated WNDP with copper is metal specific and pH- and temperature-dependent (Tsivkovskii *et al.*, 2002; Walker *et al.*, 2002). At pH 6.0, the effect of copper on the WNDP activity appears to be cooperative, suggesting that more than one copper-binding site has to be occupied to activate phosphorylation of WNDP (Fig. 2(B)). The EC_{50} for copper in these experiments was 1.5 ± 0.6 μM (Tsivkovskii *et al.*, 2002). Interestingly, shifting the pH to 7.0 led to a simple hyperbolic dependence of phosphorylation from added copper, and to a 10-fold decrease in the EC_{50} value for copper (Fig. 2(B)). It seems that protonation and deprotonation of certain group(s) in WNDP has a significant effect on the WNDP conformation and its affinity for the transported metal.

It is worth noting that a fairly high concentration of BCS (about 100 μM) is needed to inactivate WNDP (Tsivkovskii *et al.*, 2002). This is interesting, because copper concentration in the buffers is only 1–1.5 μM and affinity of BCS for copper is very high (10^{-20} M, Rae *et al.*, 1999). Therefore, it seems that BCS inactivates WNDP not only because it sequesters copper in the buffers, but also because it competes with WNDP for copper, which is tightly bound to the metal-binding sites of the protein. Significantly, removal of copper from these sites not only inhibits WNDP, but also leads to a decrease in the apparent affinity of WNDP for copper. In other words, although catalytic phosphorylation of the BCS-treated WNDP can be restored by addition of the metal, more copper has to be added to obtain a comparable level of activity than before the BCS treatment (Tsivkovskii *et al.*, 2002). We speculate that prior to BCS treatment copper-bound N-WNDP confers a high affinity state of WNDP. In this state, copper binds efficiently to the intramembrane site(s) inducing catalytic phosphorylation. Removal of copper from N-WNDP alters WNDP conformation, leading to decreased affinity for copper and down-regulation of the enzyme. Although direct evidence for this hypothesis has yet to be obtained, several results discussed under Copper-Dependent Regulation of WNDP and Copper Delivery and Regulation of WNDP by the Copper Chaperone Atox1 support the important role of N-WNDP in regulation of the WNDP activity.

THE ATP-BINDING DOMAIN AND THE NUCLEOTIDE-BINDING PROPERTIES OF WNDP

Molecular Modeling Experiments.

Recently, the first high-resolution structure of a P-type ATPase, the Ca^{2+}-ATPase of sarcoplasmic reticulum (SR), was determined (Toyoshima *et al.*, 2000). This
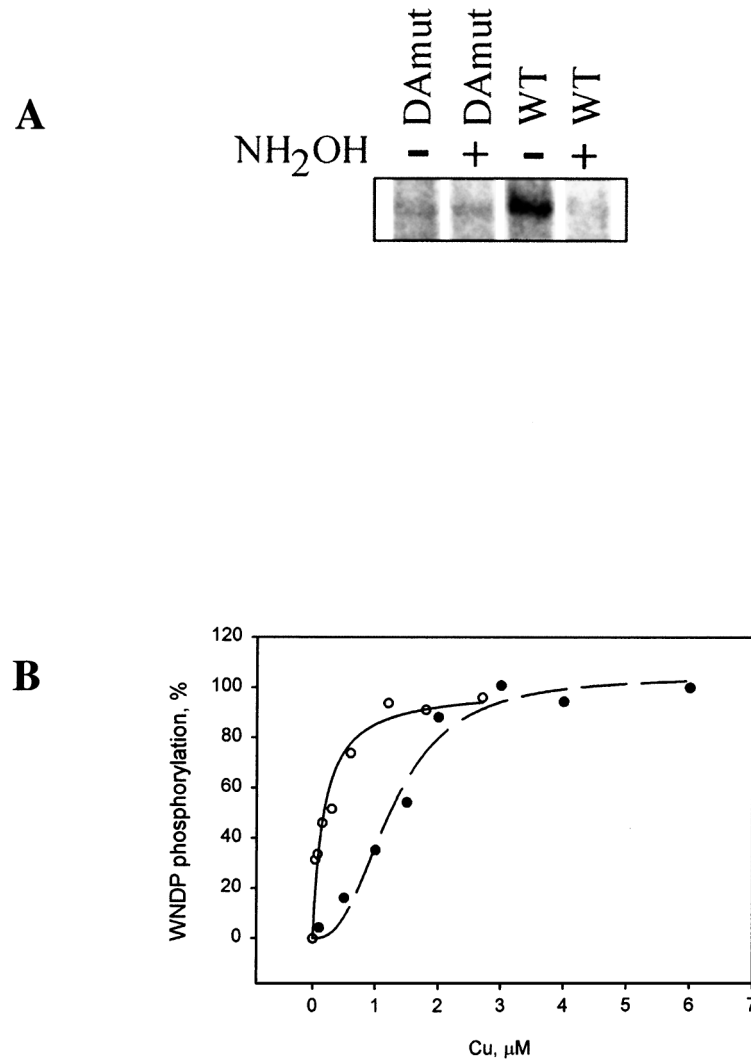
A

B

Fig. 2. WNDP forms phosphorylated intermediate in a copper-dependent manner. (A) The membrane preparations of *Sf9* cells containing either WNDP (WT) or the D1027A mutant (DA) in the 20 mM Bis-tris-propane pH 6.0, 200 mM KCl, 5 mM $MgCl_2$ buffer were incubated for 4 minutes on ice with 1 μM [^{32}P] γ-ATP, precipitated by adding trichloroacetic acid up to 10%, and resuspended in 50 mM MES pH 6.0 buffer with or without 250 mM hydroxylamine. Following 0.5 h incubation at room temperature the samples were analyzed on acidic gel as described (Tsivkovskii *et al.*, 2002). Note that hydroxylamine does not affect noncatalytic kinase-mediated phosphorylation, which can be seen in the D1027A mutant. (B) WNDP was treated with copper chelator BCS, BCS was then removed by centrifugation, and the protein resuspended in 20 mM Bis-tris-propane, 200 mM KCl, 5 mM $MgCl_2$ buffer was incubated with increasing copper concentration at pH 6.0 (black circles) or pH 7.0 (open circles). Phosphorylation reaction and analysis of acylphosphate intermediate were carried out as described in (Tsivkovskii *et al.*, 2002). The results of the ^{32}P densitometry are shown.

structure not only confirmed the results of many earlier biochemical experiments, it also provided a greater degree of understanding of the molecular basis of the ATP-driven ion transport. The crystallization work has been rapidly followed by molecular modeling studies for other P-type ATPases in the attempt of dissecting what is common and what is unique for the ion pumps with different ion specificity (Sweadner and Donnet, 2001).

WNDP is only 5% homologous to SR Ca^{2+}-ATPase. This degree of similarity is too low to carry out conclusive modeling studies for the full-length WNDP. However, the ATP-BDs of WNDP (residues V^{997}-R^{1322}) and SR Ca^{2+}-ATPase (residues L^{321}-K^{758}) share a much larger degree of similarity. The identical amino acid residues are predominantly clustered in the following regions of WNDP: V^{997}-A^{1065}, D^{1185}-I^{1236}, and F^{1240}-I^{1311}, with respectively 21, 33, and 47% of identity to the corresponding fragments of Ca^{2+}-ATPase. The ATP-BD of Ca^{2+}-ATPase was previously shown to consist of two subdomains: the P-domain, which contains the site of catalytic phosphorylation, and the N-domain, which is believed to form a binding site for the adenine moiety of ATP (Toyoshima *et al.*, 2000). Interestingly, the majority of the residues homologous between ATP-BDs of WNDP and Ca^{2+}-ATPase belong to the P-domain. Only two short segments of WNDP P^{1037}-A^{1065} and D^{1185}-A^{1195} do not map to the P-domain, but correspond to the Ca^{2+}-ATPase segments M^{360}-A^{390} and D^{590}-A^{600} that form a three-strand β-sheet layer, which connects the P-domain and the N-domain. Unexpectedly, the predicted N-domain of WNDP (residues S^{1066}-I^{1184} did not show even distant homology either with the corresponding region in SR Ca^{2+}-ATPase (P^{391}-T^{589}) or with any other proteins of known 3D structure.

In the absence of sequence homology, we used a number of threading algorithms to estimate the putative spatial fold of the WNDP N-domain (Etremov *et al.*, manuscript in preparation). These experiments revealed some similarity of the predicted fold for the N-domain to the experimental fold of the A chain of 5-carboxymethyl-2-hydroxymuconate isomerase (CHI, PDB accession code 1OTG). Interestingly, the 3D structure of CHI includes a four-strand β-sheet layer–exactly as observed for the corresponding part of SR Ca^{2+}-ATPase (P^{391}-T^{589}). Consequently, we hypothesized that the N-domain of WNDP has the topology of β-strands similar to that of SR Ca^{2+}-ATPase. Based on this assumption, the nonhomologous parts of the N-domains were aligned via superposition of the four β-strands. Obtained alignment served as a basis for the 3D model of both the P- and N-domains of WNDP that was built using the Modeller software (Sali and Overington, 1994). The generated 3D model is shown in Fig. 3.

Functional Properties of ATP-BD

The above analysis predicts that the overall catalytic reaction, which takes place in the conserved P-domain, is likely to be very similar for WNDP and Ca^{2+}-ATPase, while the nucleotide binding properties of the structurally

dissimilar N-domains, such as their selectivity for nucleotides and relative nucleotide affinities, could be different. Our experimental data support this prediction. To elucidate the nucleotide binding properties of the WNDP ATP-binding domain, we have expressed this domain in bacterial cells and carried out a series of biochemical studies (Tsivkovskii *et al.*, 2001). The purified ATP-BD could fold independently and was able to hydrolyze ATP, although with low efficiency, suggesting that the major structural features were preserved in the isolated ATP-BD. The nucleotide-binding properties of the isolated ATP were characterized by ligand competition assays.

In these experiments ATP-BD was first incubated with the fluorescent ATP analogue trinitrophenyl-ATP (TNP-ATP). TNP-ATP in solution has low fluorescence; however, upon binding to ATP-BD the reagent's fluorescence is increased. Subsequent addition of ATP leads to a competition between the ligands and displacement of TNP-ATP from the binding site, resulting in a decrease in fluorescence (Tsivkovskii *et al.*, 2001). In contrast, ADP and AMP appear to decrease the TNP-fluorescence by inducing conformational change in ATP-BD and altering the environment of the reagent rather than by direct competition (Tsivkovskii *et al.*, 2001). The nucleotide-binding characteristics of ATP-BD are summarized in Table I.

Interestingly, the isolated ATP-BD of WNDP binds ADP and AMP with higher affinity than the ATP-BDs of Ca^{2+}-ATPase or Na$^+$,K$^+$-ATPase, but does not discriminate between ADP and AMP (Tsivkovskii *et al.*, 2001) unlike the latter domains (Capieaux *et al.*, 1993; Gatto *et al.*, 1998; Moutin *et al.*, 1994). Thus, it appears that sequence dissimilarities between the N-domains of WNDP and Ca-ATPase (see above) do translate into their somewhat different nucleotide-binding properties. Our experiments also indicate that ATP and ADP bind to ATP-BD independently and at different sites (Tsivkovskii *et al.*, 2001). We hypothesize that ATP-BD has two nucleotide-binding pockets, which are located in close proximity to each other and may serve to accommodate the adenine moiety of the substrate, ATP, before and after the hydrolysis. In order to understand where in the ATP-BD the two nucleotide-binding sites could be located we carried out nucleotide-docking experiments using the generated 3D model for ATP-BD and ADP molecule. ADP was used in this series of experiments to identify the various regions in which adenine moiety can bind, rather than to distinguish between the ADP- and ATP-binding sites.

The Nucleotide Docking Experiments

The docking simulations were performed without any preliminary constraints of the binding site region—in
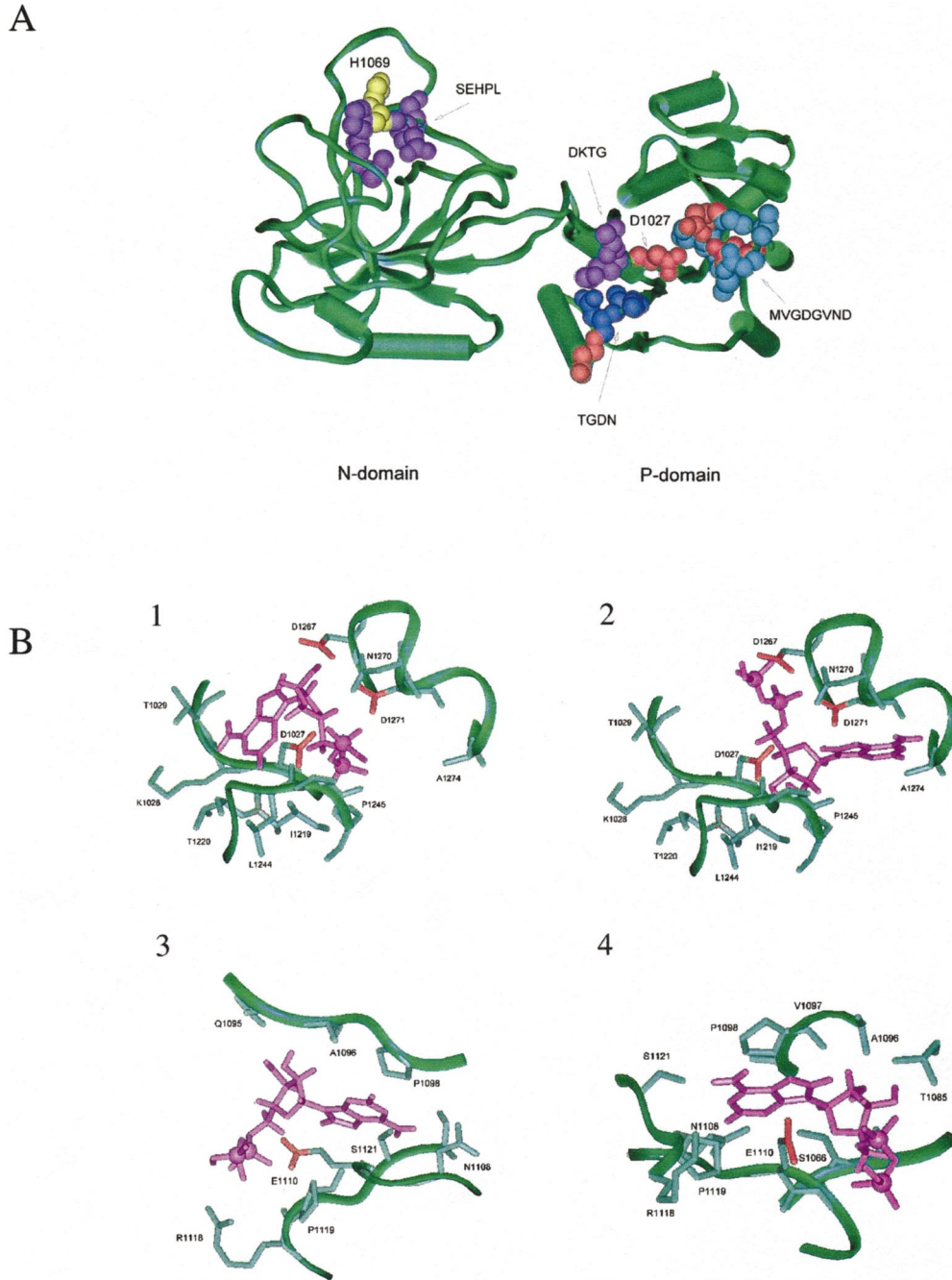
Fig. 3. Molecular model of ATP-BD (A) and the nucleotide-binding sites predicted by the ADP-docking experiments (B). (A) The model was built using the Modeller software. Quality of 10 generated models was verified using the Profiles_3D program (Luthy *et al.*, 1992). Several short fragments which showed poor compatibility score *S* (i.e. the segments of the model, which did not satisfy the general principles of folding observed in the high resolution structures of globular proteins) were iteratively refined to achieve the best score for the entire model. The final model with the highest *S* value was used for the nucleotide docking experiments. The conserved sequence motifs are indicated by space-filling models for corresponding residues. SEHPL is located in the putative N-domain; DKTG , TGDN, and MVGDGVND (GDG in Fig. 1) are the sequence motifs present in all P-type ATPases. These motifs are located in the predicted P-domain. Location of the H1069 and D1027 are marked by the arrows. (B) Orientation of ADP in the predicted nucleotide-binding sites. (1) and (2) show two different orientation of ADP in the first site, (3) site #2, and (4) site #3.

Table I. Nucleotide-Binding Characteristics of ATP-BD

	K_a (MM)			
	TNP-ATP	ATP	ADP	AMP
ATP-BD	1.89 ± 0.72	268 ± 23	85 ± 5	79 ± 18
ATP-BD + N-WND(−Cu)	10.36 ± 0.46	1137 ± 238	n.d.	52 ± 31
ATP-BD + N-WND(+Cu)	6.72 ± 1.45	339 ± 80	n.d.	168 ± 37

Note. The apparent Ka for TNP-ATP, ADP, and AMP reflect their direct binding to ATP-BD, while the Ka for ATP is a measure of competition between TNP-ATP and ATP (Tsivkovskii *et al.*, 2001).

other words, all the surface of the ATP-BD model was accessible for the ADP binding. Using the DOCK program (Kuntz, 1992), independent calculations were carried out, for several randomly chosen mutual orientations of the protein and ADP. As a result, four groups of the best-scoring conformations of ADP were found. They fall into three different sites (Fig. 3(B)). The first site is located in the P-domain and includes the conserved sequence motifs DKTG (residues 1027-1030), TGDN (1220-1223), LPSH (1244-1247), and GDGxxD (1266-1271). This result is consistent with a known role of these residues in catalysis in the P-type ATPases. Interestingly, there are two distinct orientations of ADP molecule in the binding site. In one orientation, the carboxyl groups of D1027, D1267, and D1271 are close to the adenine and ribose moiety of ADP (Fig. 3(B1)), while in another orientation the carboxyl groups are in contact with the ribose and phosphate groups (Fig. 3(B2)). This latter orientation is more likely to reflect the position of the nucleotide during catalysis.

The second and third predicted sites overlap, and two orientations of ADP are observed in these two regions (Fig. 3(B3,4)). The sites are formed by residues A^{1065}-S^{1066}, T^{1085}, V^{1097}-P^{1098}, N^{1108}-I^{1112}, and P^{1119}-S^{1121} of the N-domain. Interestingly, these sites are adjacent to the sequence motif SEHPL, which is highly conserved in all P_1-type ATPases (see above). Thus, it is tempting to speculate that mutations in the SEHPL motif may have a considerable effect on functions of WNDP associated with binding of the nucleotides. As seen in Fig. 3(B), in sites 2 and 3 the ADP molecule binds mainly via its adenine ring, in agreement with the prediction that the N-domain of ATP-BD is responsible for recognition of the nucleotide moiety rather than the phosphates. Future experiments will test our modeling results and determine whether the identified sites represent the portions of the same nucleotide-binding pocket or two different sites, only one of which is involved in catalysis.

COPPER-DEPENDENT REGULATION OF WNDP

Our recent results suggest that copper binding to the N-terminal domain of WNDP (N-WNDP) is likely to be associated with several regulatory events. First, we found that N-WNDP interacts with ATP-BD and that this interaction is metal-dependent. The copper-bound N-WNDP associates with ATP-BD less tightly than apo-N-WNDP, suggesting that copper binding to N-WNDP disrupts domain–domain interactions within WNDP (Tsivkovskii *et al.*, 2001). The decrease in interdomain interactions leads to the change in conformation of ATP-BD, as evidenced by increased affinity of ATP-BD for ATP (Table I). The functional consequences of these events could be two-fold. First, if apo-N-WNDP plays an autoinhibitory role, the catalytic or transport activity of WNDP could be increased as a result of copper binding and domain–domain dissociation. Second, it is possible that copper-dependent change in conformation or mutual orientation of the major functional domains would lead to exposure of certain regions of WNDP for interactions with other intracellular proteins.

As described in the Introduction, the intracellular localization of WNDP is copper-dependent, i.e. WNDP responds to the change in copper concentration by interacting with the cell trafficking machinery and subsequent relocalization. It seems likely that under low copper conditions only some, but not all metal-binding sites in WNDP are occupied by copper, and this could be sufficient for the basal transport activity of WNDP and for delivery of copper to the TGN. When copper concentration is increased and all metal-binding sites in N-WNDP become occupied with the metal, the conformation of N-WNDP changes (DiDonato *et al.*, 2000), weakening interactions between N-WNDP and ATP-BD. This in turn may stimulate the activity of WNDP and allow proteins involved in protein trafficking to interact with WNDP. MBR 1-4 and MBR5,6 in N-WNDP are connected by a fairly long linker (see Molecular Architecture of WNDP). It seems likely that MBR5 and/or MBR6 are required to maintain the basal copper-transport activity of WNDP, while MBR1-4 can be involved in the copper-dependent regulation. The flexible linker connecting two portions of N-WNDP could be necessary to accommodate the conformational changes that occur when N-WNDP becomes saturated with copper and when interdomain interactions in WNDP begin to change. Experiments are now underway to thoroughly test this hypothesis.

The domain–domain dissociation may also expose certain regions in WNDP to intracellular regulators, such as kinases. Our recent data suggest that the kinase-dependent phosphorylation of WNDP is another way by

which copper may control the intracellular distribution of WNDP or its function (Vanderwerf *et al.*, 2001). In a cell grown in the standard medium WNDP is phosphorylated by a kinase at a basal level, which is increased two- to three fold if cells are treated with 2–50 μM copper. The basal and copper-induced phosphorylation occur at two different sites and coincide with different intracellular localization of WNDP (Vanderwerf *et al.*, 2001). At low copper, the basally phosphorylated WNDP is located primarily at the TGN. When the concentration of copper is elevated the hyperphosphorylated protein is seen in the vesicular compartment; subsequent decrease in copper concentration leads to dephosphorylation of WNDP and its return to the TGN (Vanderwerf *et al.*, 2001). The kinase that phosphorylated WNDP appears to be ubiquitous, since kinase-mediated phosphorylation of WNDP was observed not only in mammalian cells but also in insect cells where WNDP is heterologously expressed. In this latter case, it was shown that the Ser residue(s) represent the targets of modification (Vanderwerf, 2002).

COPPER DELIVERY AND REGULATION OF WNDP BY THE COPPER CHAPERONE ATOX1

All intracellular copper is believed to be present in a bound form, and WNDP must interact with specific copper donor(s) to receive the metal for further transport. The role of copper carriers belongs to metallochaperones, a class of molecules that function in sequestering copper ions and delivering them to specific protein targets throughout the cell (Harrison *et al.*, 1999; O'Halloran and Culotta, 2000). Genetic studies by Hamza *et al.* suggested that the copper chaperone Atox1 (formerly known as HAH1) acts as a copper-donor for human copper-transporting ATPases (Hamza *et al.*, 2001). Atox1 is a 8 kDa cytosolic protein that has a copper-binding motif MxCxxC. The crystal structure of Atox1 has been recently solved (Wernimont *et al.*, 2000). It revealed that copper is coordinated between the cysteine residues in the MTCxxC motif, and that the overall fold of Atox1 was very similar to the fold of the metal-binding repeat 4 of the human copper-transporting ATPase ATP7A, a close homologue of WNDP (Gitschier *et al.*, 1998). Molecular modeling experiments suggest that Atox1 could dock with the MBRs and deliver copper to their metal-binding sites (Arnesano *et al.*, 2002; Wernimont *et al.*, 2000). Our recent biochemical data supplied direct evidence that copper can be transferred from Atox1 to WNDP and that the transfer of copper has a regulatory effect on WNDP activity.

For these experiments, human recombinant copper chaperone Atox1 was expressed in bacterial cell and then purified using the intein-based expression system (Walker *et al.*, 2002). Metallation of Atox1 with a copper–glutathione complex produces the Cu^{+}-Atox1, which has approximately one (0.85 ± 0.1) copper bound per protein (Walker *et al.*, 2002). Cu^{+}-Atox1 can transfer copper to either isolated N-terminal domain of WNDP (N-WNDP) or to the full-length transporter. The copper transfer by Cu^{+}-Atox1 is a saturable process leading to loading of N-WNDP with approximately six copper atoms (Walker *et al.*, 2002). This value correlates well with the amount of copper bound to N-WNDP in cell culture (Lutsenko *et al.*, 1997). Significantly, transfer of copper is associated with the increase in the activity of the full-length WNDP expressed in insect cells. This is indicated by the ability of Cu^{+}-Atox1, but not apo-Atox1 to restore the catalytic phosphorylation of the BCS-treated WNDP in a concentration-dependent and saturable manner (Walker *et al.*, 2002).

While these results provide a convincing demonstration of the functional connection between Atox1 and WNDP, several important questions remain. Our in vitro experiments demonstrated that a 5–30–fold excess of Cu^{+}-Atox1 is necessary for transfer of one to six copper atoms to N-WNDP or for activation of the full-length WNDP. How closely the in vitro copper transfer reflects the intracellular reaction remains to be determined. It is currently unknown whether Atox1 is present in a cell in excess to WNDP and what is the intracellular stoichiometry of the copper Atox1 complex. It is also important to determine which specific metal-binding site(s) of WNDP accept copper from Atox1 and in which order these sites are filled with copper. Experiments are currently underway to address these important issues.

We also found that Atox1 not only brings copper to WNDP and stimulates its activity, but can function as a regulator by removing copper from N-WNDP and decreasing the functional activity of the full-length WNDP (Walker *et al.*, 2002). The experiments performed using apo-Atox1 and copper-loaded N-WNDP showed that apo-Atox1 can strip copper from N-WNDP down to about one copper per protein. Interestingly, this "reverse" transfer proceeds fairly easy for the first three to four copper atoms and only a small excess of apo-Atox1 is needed to remove copper from N-WNDP. The removal of the remaining one to two copper atoms is more difficult and cannot be fully completed even using 40–60–fold excess of apo-Atox1 over N-WNDP (Walker *et al.*, 2002). This result points to a functional or spatial nonequivalency of the metal-binding sites in WNDP. It is tempting to speculate that copper, which cannot be easily removed by apo-Atox1, is bound to sites essential for enzymatic activity of WNDP, while

easily exchangeable copper is bound to the metal sites involved in regulation of WNDP.

This hypothesis is supported by the experiments on inactivation of WNDP by apo-Atox1 (Walker *et al.*, 2002). Apo-Atox1 inhibits catalytic phosphorylation of WNDP with EC_{50} equal to 1.0 ± 0.24 μM, which is significantly lower than the EC_{50} value for BCS (50 μM). However, BCS inactivates WNDP completely, while apo-Atox1 decreases the WNDP activity by no more than 50% (Walker *et al.*, 2002). This suggests that although apo-Atox1 is more specific and efficient in removing copper from certain, presumably regulatory, copper-binding sites on WNDP, it cannot remove copper from all its sites. The remaining copper is sufficient to sustain the basal activity of WNDP. Which copper-binding site(s) is involved in maintaining this basal WNDP activity has yet to be determined.

Our work suggests that Atox1 may play a complex role in a cell by regulating the copper occupancy of WNDP and modulating the WNDP activity. This additional regulatory function of Atox1 may have important physiological consequences for the WNDP in vivo. As we described in earlier sections, copper controls trafficking of WNDP from the trans-Golgi network to a vesicular compartment and modulates the posttranslational modification of WNDP. It seems likely that Atox1 could be essential for these processes acting as an intracellular copper sensor, which determines how much copper has to be delivered to or removed from WNDP.

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