

Biochemistry of the Wilson's Disease Protein

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1. INTRODUCTION

Copper is an essential metal, utilized as a cofactor by numerous enzymes regulating vital cellular functions, including oxidative phosphorylation, neurotransmitter biosynthesis, radical detoxification, iron uptake, and many others (for review, *see* refs. 1 and 2). The importance of copper for normal cell metabolism is best illustrated by the existence of severe genetic disorders, in which the normal distribution of copper is disrupted (3–5). Menkes disease (MNK) is an inborn copper deficiency associated with severe developmental delays, mental retardation, poor temperature control, and connective tissue abnormalities. All of these symptoms can be ascribed to the malfunction of various enzymes, which require copper as a cofactor. Such enzymes include cytochrome-*c* oxidase, tyrosinase, lysyl oxidase, peptidyl- β -amidase, and many others. Recent identification of the Menkes disease gene (*ATP7A*) revealed that it encoded a copper-transporting ATPase or the Menkes disease protein (6–8), which has a dual function: to transport copper from the cytosol to copper-dependent enzymes located within the secretory pathway and to export excess copper out of the cell.

Although the Menkes disease protein is indispensable for copper distribution from the intestine to various tissues, by itself it is insufficient for normal copper homeostasis. The product of another gene, *ATP7B*, plays a key role in removing excess copper from human body by transporting copper from the liver to the bile (9–11). Mutations in *ATP7B* lead to vast accumulation of copper in the liver, brain, and kidneys, causing a set of pathological symptoms, known as Wilson's disease (WND). Severe liver lesions, neurological problems, and a wide spectrum of psychiatric abnormalities are common symptoms of WND (12). The Wilson's disease gene, *ATP7B*, was isolated and fully characterized in 1993–1994; these studies revealed that it encodes a copper-transporting ATPase with over 50% sequence homology to the Menkes disease protein (9–11, 13, 14).

Although the Menkes disease protein (MNKP) and Wilson's disease protein (WNDP) have significant structural identity and also function similarly in the *in vitro* systems (15–17), their distinct tissue distribution and alterations in their expression during development (18) suggest that the relative abundance and activity of these proteins are controlled by a specific set of environmental cues. Understanding the regulatory mechanisms acting on these proteins represents one of the most unexplored and exciting area of copper homeostasis.

The detailed biochemical characterization of the Menkes and Wilson's disease proteins is the first step toward elucidation of their specific physiological roles in a cell. Such studies can be aided

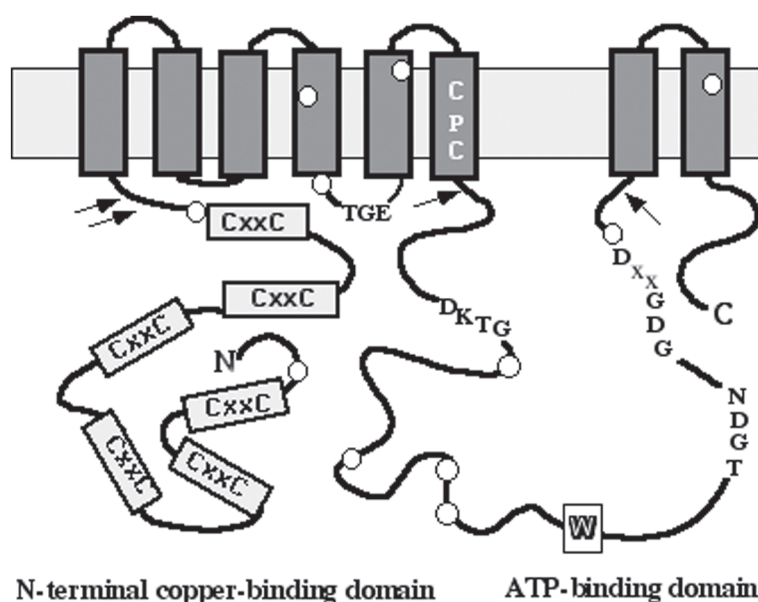


Fig. 1. Schematic representation of the transmembrane organization of WNDP. The blocks in the N-terminal portion indicate the position of copper-binding sites with a conserved sequence motif GMTCxxC. The single arrows indicate the beginning and the end of the ATP-binding domain; the double arrow indicates the end of the N-terminal domain. Open circles mark the location of some of the Wilson's disease mutations and TGE, DKTG, TGDN, and GDGxxD are the sequence motifs conserved in all P-type ATPases; W in a block shows the position of a single tryptophan residue in the ATP-binding domain. CPC is a sequence motif that is specific for copper-transporting ATPases.

considerably by comparative analysis of WNDP or MNKP with much better characterized cation-transporting ATPases, such as Ca^{2+} -ATPase or Na^+ , K^+ -ATPase, which belong to the same protein family as WNDP and MNKP.

2. HUMAN COPPER-TRANSPORTING ATPASES AS MEMBERS OF THE P-TYPE ATPASE FAMILY

Analysis of the primary sequence of the *ATP7A* and *ATP7B* gene products revealed that the corresponding proteins, MNKP and WNDP, belong to a family of cation-transporting P-type ATPases. The P-type ATPases are a large group of membrane proteins that utilize energy of ATP hydrolysis to transport various ions across cell membranes. During the catalytic cycle the γ -phosphate of ATP is transferred to the invariant Asp residue within the nucleotide-binding site of ATPase with the formation of acylphosphate intermediate; this property distinguishes the P-type ATPases from other cation-transporting pumps. Because both Wilson's disease and Menkes disease are associated with defects in copper distribution, it was proposed that WNDP and MNKP function in a cell as copper-transporting P-type ATPases. In agreement with this proposal, recent studies by Voskoboinik et al. have shown that MNKP and WNDP transport copper across cell membranes and ATP stimulates the MNKP- and WNDP-dependent transport (19,20).

Today, over 100 members of the P-type ATPase family have been described and a wealth of information has been accumulated regarding the structure and function of some of these proteins (for review, see refs. 21 and 22). The first crystal structure of a P-type ATPase, the Ca^{2+} -ATPase from sarcoplasmic reticulum, has been recently solved, providing an important framework for studies on

molecular mechanisms of the ATP-driven ion transport (23). To understand how this information can be utilized for analysis of WNDP and MNKP, it could be beneficial to dissect features that are common for the human copper-transporting ATPases and other P-type ATPases, as well as to identify the unique structural and functional characteristics of MNKP and WNDP.

Like all P-type ATPases, WNDP and MNKP have several highly conserved sequence motifs, such as DKTG, TGDN, GDGxxD, and TGEA/S (Fig. 1). The invariant residues in these motifs are known to play key roles in catalysis and accompanying conformational transitions, indicating that the basic mechanisms of ATP hydrolysis and coupling between the hydrolytic and ion-transport steps are likely to be the same for human copper-transporting ATPases and other P-type pumps.

At the same time, human copper-transporting ATPases have several unique structural and functional characteristics (*see* Sections 2.2. and 2.4.), indicating that specific details of their molecular mechanism and their intracellular behavior differ from those of well-characterized P-type ATPases. For this reason, in our early attempt to classify the P-type ATPases, we placed the copper-transporting and other structurally similar transient metal-transporting ATPases into a separate subgroup (P₁-type ATPases), in contrast to P₂-ATPases, such as Ca²⁺-ATPase and Na⁺,K⁺-ATPase, which transport alkali and alkali-earth ions (24). Solioz and Vulpe later suggested an alternative name for the P₁-ATPases, CPx-ATPases, based on the presence of characteristic motif CPx in the transmembrane portion of these proteins (25). In a recent and more complete classification scheme, Axelson and Palmgren also placed the P₁-ATPases in a separate group (type IB), distinct from four other P-type ATPase subfamilies (26).

The comparison of structural and functional properties of mammalian copper-transporting ATPases (P₁-ATPases) and P₂-type ATPases reveals the following interesting differences between these two groups of pumps.

2.1. Physiological Role

The major function of all well-characterized P₂-type ATPases is to maintain the concentration gradient of the transported cations across cell membranes; the generated gradients then serve as a driving force for such physiological processes as muscle contraction, nutrients uptake, or electrical activity of neurons. Whether human copper-ATPases have a similar role and maintain a concentration gradient of copper across cell membranes remains to be elucidated. In the cytosol, essentially all copper apparently exists in a protein-bound form (27), but its status in the intracellular compartments is less clear. If, in organelles, copper is present in a free form and therefore the transmembrane copper gradient is generated, it remains unknown whether such gradient drives any secondary process. However, it is now well established that the eucaryotic copper-transporting ATPases represent key components of a biosynthetic, cofactor-delivery pathway, transporting copper to copper-dependent enzymes. Incorporation of copper into proteins in the secretory pathway is essential for numerous physiological functions, including respiration, neurotransmitter biosynthesis, and high-affinity iron uptake; however, the role of copper in these processes is indirect.

In addition to their important role in delivering copper to the copper-dependent enzymes, human copper-transporting ATPases regulate the intracellular concentration of copper by removing excess copper from the cell. This "detoxification" function of copper-transporting ATPases is very similar to the functional role of bacterial Cd²⁺-ATPase and Pb²⁺-ATPases and likely appeared first during evolution. It was later extended to accommodate eucaryotic cell needs in having copper inside various cell organelles. To carry out this dual function, WNDP and MNKP have to be located, at least temporarily, in different cell compartments.

In agreement with this prediction, MNKP was shown to cycle between the trans-Golgi network (TGN) and the plasma membrane: Under basal conditions, MNKP was detected predominantly in TGN, whereas increase in copper concentration led to the redistribution of MNKP from TGN to the plasma membrane (28,29). Similarly, the intracellular localization of WNDP depends on copper

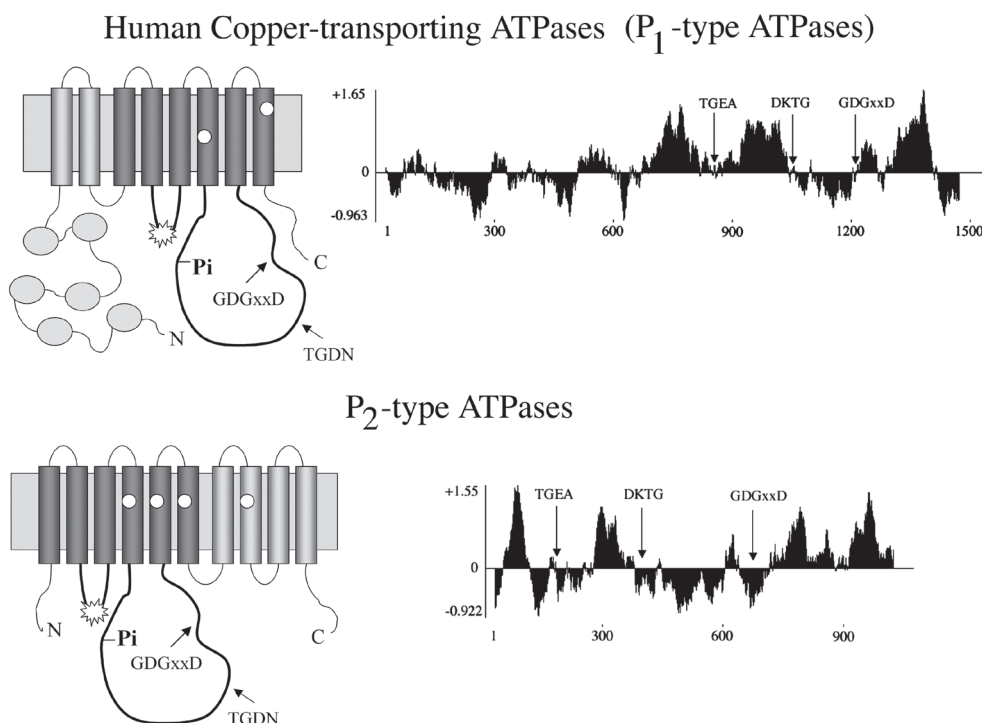


Fig. 2. Comparison of the transmembrane topology and organization of the cation-translocation pathway in human copper-transporting ATPases (P₁-type ATPases) and P₂-type ATPases. The letters and the arrows/vertical lines mark the positions of the conserved sequence motifs; Pi indicates the Asp residue in the DKTG motif that is phosphorylated during the catalytic cycle and the asterisk shows the position of the TGEA/S sequence in the transmembrane model of ATPases. The transmembrane segments that are common for the P₁- and P₂-ATPases are dark colored, the membrane segments unique for each group of ATPases are light gray; the open circles indicate the transmembrane segments known to be important for cation coordination and transport activity in both groups of pumps.

concentration (30,31). In response to increased copper, WNDP redistributes from its primary localization site, TGN, to a vesicular compartment (probably endosomes). Thus, changes in copper concentration seem to regulate copper transport across various cell membranes by altering the number of copper transporters present at these membranes. So far, the dependence of intracellular localization on concentration of the transported ion seems to be a unique property of human copper-transporting ATPases. Whether this mode of regulation is the only way of altering copper transport across the membranes or whether the changes in copper concentrations also control the activity of MNKP and WNDP remains to be elucidated.

2.2. Transport Characteristics

Copper can bind to proteins in either reduced, Cu¹⁺, or in the oxidized, Cu²⁺, form. The ability of copper to exist in different oxidation states raises an interesting possibility that copper oxidation may occur in the secretory pathway or in another intracellular organelle as the last step of the copper-transport process. Copper binds to WNDP and MNKP in the reduced form (*see* Section 3.) and is likely to be transported in the same form (32). Copper is then released from the transporters, possibly with a change in oxidation state, and becomes incorporated into copper-dependent enzymes. [Inter-

estingly, chloride ions seems to play an important role in this process, at least in yeast (33)]. The alternative possibility is that copper is picked up from the ATPases through direct intermolecular interactions either by target proteins or by low-molecular-weight copper carriers. If this last scenario is correct, then the intracellular transport of copper is unique, because the entire ion-transport process would be mediated through a chain of specific protein-protein interactions.

2.3. Cation Recognition

The extremely low concentrations of free copper in a cell (27) results in another interesting property of WNDP and MNKP. Unlike many P-type ATPases, which recognize free cations present in the cytosol, eucaryotic copper-transporting ATPases receive copper from so-called copper chaperones, small cytosolic proteins that presumably work as shuttles between the copper uptake system and other components of the copper distribution pathway (34–36). Thus, specificity of MNKP and WNDP for the transported ion is defined not only by the stereochemistry of copper binding sites, but also by specific recognition of the copper-carrier protein, HAH1.

2.4. The Structural Differences Between the P_1 - and P_2 -ATPases

It is probably not a coincidence that in addition to the functional differences described earlier, copper-transporting ATPases have structural features that make them quite distinct from the P_2 -ATPases. The most obvious difference is the organization of the cation-translocation pathway (Fig. 2). In their membrane portion, P_2 -ATPases have 10 transmembrane segments: 4 segments before the ATP-binding domain and 6 segments in the C-terminal portion after the ATP-binding domain. The transmembrane segments involved in cation coordination and transport in the P_2 -ATPases contain a large number of hydrophilic and helix-breaking amino acid residues, which are essential for binding of the positively charged ions in the membrane (37–39).

In contrast, the P_1 -ATPases have a total of eight membrane-spanning regions: six before the ATP-binding domain, and only one pair after the ATP-binding domain. The membrane portion of the P_1 -ATPases has fewer hydrophilic and helix-breaking residues, and the segments, corresponding to the last four C-terminal transmembrane helices of P_2 -ATPases, are absent in the structure of copper pumps (Fig. 2). Interestingly, these last four transmembrane segments play an important role in the insertion and maintenance of the ion-binding segments in P_2 -ATPases and may even contribute to cation coordination (40,41). The absence of these fragments in the copper-ATPases suggests that the P_1 - and P_2 -ATPases likely to have different mechanisms for insertion of some transmembrane hairpins and for overall assembly of the cation-translocation pathway.

It is also interesting that the transmembrane segments immediately after the ATP-binding domain, which play a central role in ion coordination in the P_2 -type ATPases, do not contain any obvious ligands for copper binding in WNDP and MNKP. Currently, the only candidate for copper binding in the membrane is the CPC motif in the sixth transmembrane helix (*see* Fig. 1). This suggests that additional coordination of copper could be provided either by side chains of Ser/Thr and Tyr residues or by the backbone carbonyls of other transmembrane segments. Alternatively, it is possible that two additional transmembrane segments present at the N-terminal part of the protein are required to form the copper-translocation pathway. If the first membrane hairpin, which is absent in the structure of the P_2 -ATPases, is directly involved in copper transport, then the mechanism of coupling between the ATP hydrolysis and cation transport could be quite different for the P_1 -type and P_2 -type pumps.

Another obvious difference between the P_1 - and P_2 -type ATPases is the role of the N-terminal domain in cation binding and selectivity. Mutations in the N-terminal domain do not have a dramatic effect on the cation affinity of the characterized P_2 -ATPases, which is defined mainly by the residues located in the transmembrane portion of these proteins ([22,42] and Fig. 2). What specific role the N-terminus of the P_2 -ATPases plays in the transport process, if any, is still not clear. In contrast, the N-terminal domain of the copper-transporting ATPases contains multiple copper-binding sites (*see* Fig.

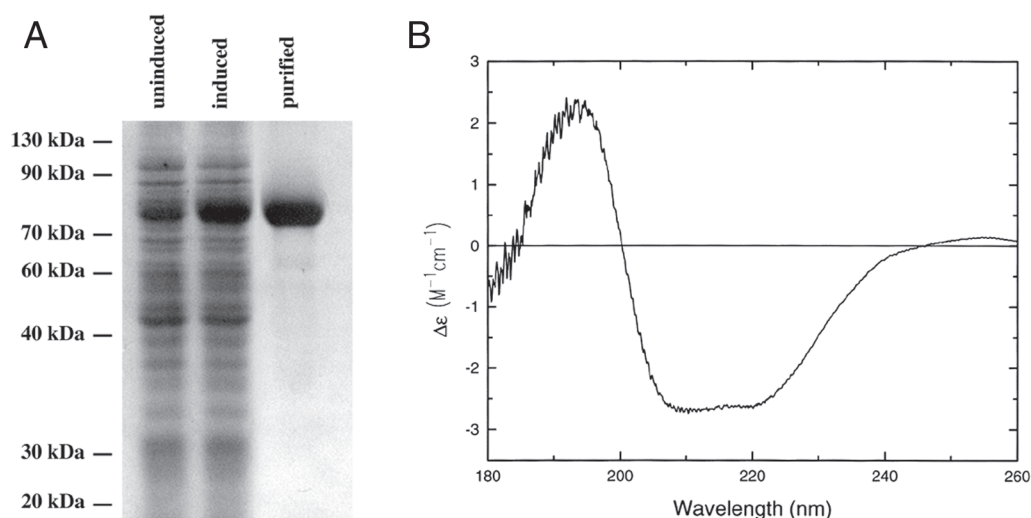


Fig. 3. (A) Expression of N-WNDP-HT in *Escherichia coli* and purified soluble protein used for further analysis of the secondary structure. (B) Circular dichroism spectroscopy of N-WNDP-HT reveals the following secondary structure elements for this domain: 19.5% α -helix, 26% β -sheet, 21% β -turn, and 33.5% random coil.

1 and text below), and the presence of at least one or more of these repeats is essential for copper transport by MNKP and WNDP (34,43–45). This interesting difference between the P_1 - and P_2 -AT-Pases seemed critical for dissecting the molecular mechanism of copper transport and prompted us and other investigators to focus our attention on biochemical characterization of the N-terminal domain of WNDP and MNKP.

3. N-TERMINAL DOMAIN AND COPPER-BINDING PROPERTIES OF WNDP

The N-terminal domain of WNDP and MNKP is an unique and intriguing feature in the structure of these proteins. It is composed of 6 repetitive sequences, each of which is about 70 amino acid residues long (Fig. 1). Each repeat contains a highly conserved motif GMTCxxCxxxIE, which is also present in the bacterial mercury-binding protein MerP and in bacterial Cd^{2+} -transporting ATPase. Based on this sequence homology with bacterial metal-binding proteins, it was proposed that the sequence repeats in the N-terminus of WNDP and MNKP play a role in copper binding (6–11). Later studies from Gitschier et al. (46) and Steele and Opella (47) revealed that the overall fold of the single sequence repeat from Menkes disease protein and MerP are the same, highlighting the remarkable conservation of this particular metal-binding motif through evolution.

The involvement of the N-terminal domain in copper binding was directly demonstrated by us and other groups following heterologous expression of this domain and biochemical characterization of purified proteins (48–51). We found that the N-terminal domains of WNDP and MNKP (N-WNDP and N-MNKP, respectively) bind copper with stoichiometry close to six coppers per domain or one copper per metal-binding repeat (48). Copper binds to these proteins in vivo and in vitro, however, efficient in vitro binding is only achieved in the presence of reducing agents (48), suggesting that copper binds to the protein in the reduced copper(I) form. These studies also demonstrated that Cys residues play an important role in copper coordination. Recent X-ray absorption spectroscopy analysis directly demonstrate that copper is bound to N-MNKP and N-WNDP in the reduced Cu(I) form and is coordinated primarily by two Cys residues (52,53). Different copper stoichiometry (eight or

four coppers bound per domain) with alternative stereochemistry was reported by the Dameron group (50,54). These studies utilized the refolded protein, and discrepancies between these results and our data are likely to reflect the difference between the *in vitro* and *in vivo* loading of the N-terminal domains with copper.

Although it is clear that in the isolated metal-binding repeat, copper is coordinated by Cys residues in a flexible and fairly exposed loop (46), the arrangement of the multiple copper sites in the fully loaded N-terminal domain remain unknown. We expressed N-WNDP with a short histidine tag (N-WNDP-HT) in a soluble form and characterized the secondary structure of the purified N-WNDP-HT using circular dichroism spectroscopy (Fig. 3). These experiments revealed that the composition of the secondary-structure elements of N-WNDP (*see* Fig. 3 legend) closely resembles those of the single metal-binding repeat, suggesting that when all six domains come together there is no marked alterations in the overall fold of these repeats.

Recent studies by Sarkar and colleagues demonstrated that copper binding to N-WNDP is accompanied by conformational changes and by changes in the tertiary structure of the protein (53). These results are very interesting, but it remains uncertain how closely the reported structural rearrangements resemble the changes taking place under more physiological conditions. Our studies using limited proteolytic digestion of soluble copper-free and copper-bound N-WNDP maltose-binding fusion (N-WNDP-MBP) demonstrate that, although some protein regions slightly change their exposure to protease as a result of copper binding, the overall proteolytic pattern of copper-free and copper-bound N-WNDP remain the same. This result suggests that there is no dramatic alterations in the overall fold of these proteins upon *in vivo* copper binding (unpublished observation).

It seems most likely that the copper-induced conformational changes affect the configuration of loops connecting the metal-binding repeats and/or the distance between various metal-binding repeats. This is not to say that the small changes in the conformation of the N-terminal domain do not have a major impact on the WNDP function. In fact, as we have shown recently, copper binding to the N-terminal domain has a marked effect on domain–domain interactions within WNDP, which, in turn, may lead to changes in the enzyme activity and intracellular trafficking (*see* Section 4.).

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

The energy-driven translocation of copper across cell membranes is likely to require coordination and interaction among three major domains of copper-transporting P-type ATPases; the copper-binding domain, the ATP-hydrolyzing domain, and the membrane portion of the protein, containing the ion-translocation pathway. In our earlier work, we hypothesized that the N-terminal domain can play a regulatory role modulating the catalytic properties of WNDP and MNKP in response to copper binding (13). We also suggested that the regulatory function can be carried out via specific protein–protein interactions of the N-terminal domain with the second large cytosolic loop, containing the ATP-binding domain (13). To test this hypothesis, we have recently expressed, purified, and characterized the ATP-binding domain of WNDP (ATP-BD) and analyzed the domain–domain interactions within WNDP (55). The results of these studies are discussed next.

Current expression systems do not permit direct measurements of the nucleotide-binding properties of human copper-transporting ATPases. However, one can get important and reliable information about the nucleotide specificity and relative nucleotide-binding affinities of proteins by using their isolated nucleotide binding domains (56–58). In determining the borders of the putative ATP-binding domain of WNDP, we were aided by studies on other members of the P-type ATPase family, such as Ca^{2+} -ATPase and Na^+, K^+ -ATPase. In these proteins, the major cytosolic loop that contains the highly conserved motifs DKTG, TDGN, and GDGxxD (*see* Fig. 1) was shown to be sufficient for selective binding of nucleotides (56–58). Although the overall homology between Ca^{2+} -ATPase and copper-ATPases is just 5%, the similarity between regions corresponding to the ATP-binding domain

Table 1
Apparent Affinities of the Purified ATP-BD for Various Nucleotides in the Absence or Presence of the Copper-Free and Copper-Bound N-Terminal Domain (N-WNDP-Cu and N-WNDP+Cu, Respectively)

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

is higher (18–23%), suggesting that the overall fold and some nucleotide-binding properties could be well preserved among all members of the P-type ATPase family.

We expressed the fragment K¹⁰¹⁰–K¹³²⁵ of WNDP (Fig. 1) as a histidine tag (HT) fusion in *E. coli*, purified it from the soluble fraction, and demonstrated that it formed an independently folded domain (ATP-binding domain, or ATP-BD) (55). ATP-BD has both the nucleotide-binding and ATP-hydrolyzing activities (55); the affinities of the purified ATP-BD for nucleotides are summarized in Table 1.

Analysis of the nucleotide-binding properties of ATP-BD yielded several interesting results. First, the ATP-binding domain of WNDP was found to bind ADP and AMP equally well and with significant affinity (*see* Table 1), in contrast to previously characterized domains of P₂-type ATPases, which show fairly low affinity for these nucleotides and a large difference in the affinity for ADP and AMP (56,57). The lower selectivity of ATP-BD toward nucleotides resembles the property of the P-type ATPase from *Methanococcus jannaschii* (59), a soluble protein structurally equivalent to the isolated ATP-binding domain, and probably reflects the early evolutionary origin of copper-transporting ATPases.

Interestingly, both ATP-BD and the *Methanococcus* P-type ATPase have a low but measurable ATPase activity (55,59), a property that has not been observed in the isolated ATP-binding domains of the P₂-ATPases. This interesting difference likely reflects a more compact folding of the ATP-binding domain of WNDP and the bacterial P-type ATPases; ATP-BD is 70–80 amino acid residues shorter than the corresponding domain of the P₂-type ATPases and lacks several loops, which could be important for precise nucleotide selection in Ca²⁺- or Na⁺,K⁺-ATPase. The molecular modeling of the WNDP ATP-binding domain using the published crystal structure of Ca²⁺-ATPase further illustrates these points (Fig. 4).

As shown in Fig. 4, the ATP-binding domain of P-type ATPases consists of two distinct parts, the phosphorylation domain (P-domain), which includes the highly conserved residues DKTG, TGDN, and GDGxxD, and the N domain, which contains residues important for binding of the adenosine moiety of nucleotides (23). The P domains of WNDP and Ca²⁺-ATPase are structurally very similar (*i.e.*, consistent with the common role these regions play in catalytic cycle of ATPases). In contrast, the N domains involved in the nucleotide binding are quite different. The differences in the number, length, and position of several loops in the N domain of P₁-type and P₂-type ATPases (*see* Fig. 4) are likely to be responsible for the differences in their nucleotide selectivity (*see* above).

Another novel and interesting property of the WNDP ATP-binding domain is its ability to bind ATP (the substrate of ATP hydrolysis) and ADP (the product of the reaction) simultaneously (55). Given the distinct subdomain organization of ATP-BD (Figs. 4 and 5), it is tempting to speculate that ATP binds in close proximity to the Asp residue in the DKTG motif (the residue, which in P-type ATPases accepts γ -phosphate from ATP, forming phosphorylated intermediate) while ADP is bound in the adenosine-binding pocket of the N domain. During the catalytic cycle, two subdomains would

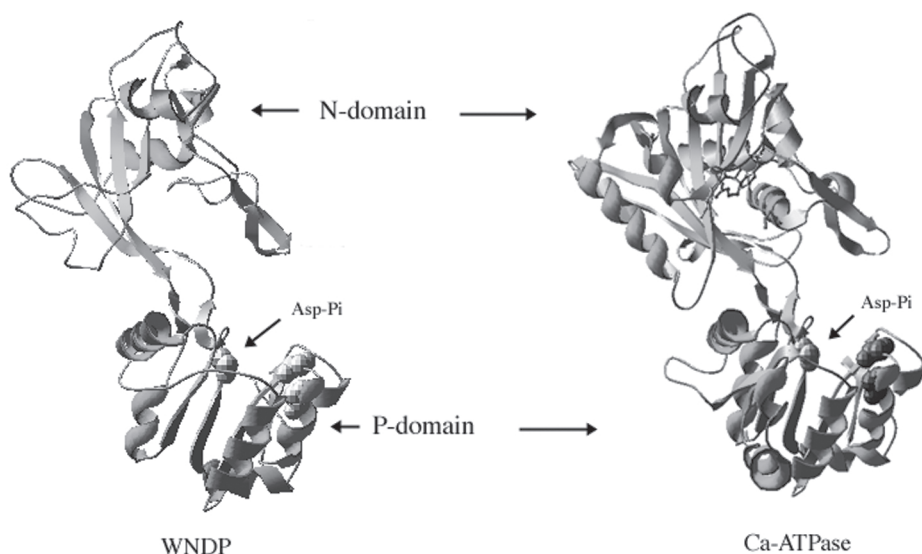


Fig. 4. Comparison of three-dimensional fold of the ATP-binding domains of WNDP and Ca-ATPase. The homology modeling was carried out using published coordinates for Ca^{2+} -ATPase (accession N. 1EUL) and SwissPdbViewer software. The balls in the lower P-domain indicate the positions for invariant Asp in the DKTG motif, two Asp residues in the GDGxxD sequence, and the location of the TGDN motif. The chain of balls in the upper N domain marks the site of the TNP-AMP (the AMP analog) binding in the crystal structure of Ca^{2+} -ATPase and the equivalent region in the structure of ATP-BD.

come together [as described for Ca^{2+} -ATPase in (23)], forming a “closed state.” The hydrolysis of ATP would then be accompanied by transfer of the adenosine moiety from the P domain to the N domain as shown in Fig. 5 with formation of an “open state” in which both ATP- and ADP-binding sites are accessible.

It is significant that the nucleotide-binding properties of ATP-BD are modified in the presence of the N-terminal domain (*see* Table 1 and Section 5.). This change reflects the interaction between two functional domains of WNDP and suggests that domain–domain interactions play an important role in the functional activity of WNDP and homologous MNKP. The ability of ATP-BD to fold independently, to bind and hydrolyze ATP, and to interact with the N-WNDP specifically makes this isolated nucleotide-binding domain a convenient tool for analysis of numerous disease-causing mutations located in this region of WNDP (60,61) (*see* Fig. 1).

5. COPPER-DEPENDENT DOMAIN–DOMAIN INTERACTIONS AND THE REGULATORY ROLE OF THE N-TERMINAL DOMAIN

The N-terminal domain of human copper-transporting ATPases is essential for the copper-dependent functions of these proteins. Mutations of Cys residues in the metal-binding motifs inactivate the copper-transporting activity of WNDP and prevent copper-dependent trafficking of WNDP in a cell (34,45). At the same time, recent studies conducted in several laboratories convincingly demonstrated that the entire N-terminal domain was not essential for the transport function of copper-ATPases: the large portion of this domain could be deleted or mutagenized without significant loss of the copper-transport activity (34,43,44). These results are consistent with the fact that the bacterial, yeast, and plant copper-ATPase can carry out their functions with fewer than six (one to three) metal-binding repeats (e.g., *see* ref. 62). Because extra metal-binding repeats are not important for function, it seems likely that they play a role in regulation of WNDP and MNKP.

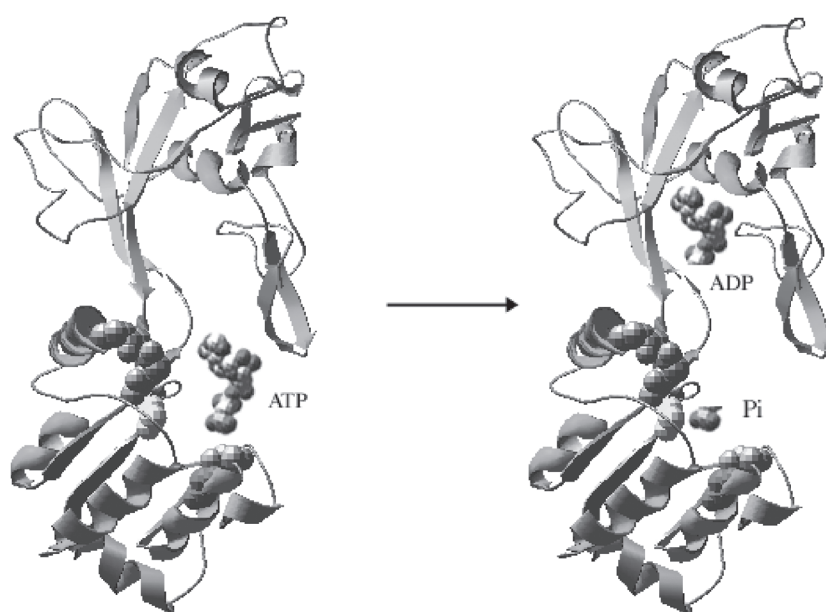


Fig. 5. Hypothetical model showing how ATP and ADP could bind to ATP-BD simultaneously (55) in the isolated ATP-BD (*see* text for details).

The regulatory role for the N-terminal domain has been suggested by several authors (20,44,48,53), and recent studies indicate that copper binding to the N-terminal domain triggers the intracellular relocalization of MNKP and WNDP (44,45). However, only one or two metal-binding repeats, which are important for transport function, seem to be necessary and sufficient for copper-induced trafficking (44,63,64). Therefore, the first four metal-binding repeats were proposed to function by preventing protein trafficking before they are filled up with copper (*see* the model in ref. 53).

Our recent studies shed some light on how the N-terminal domain may regulate the copper-dependent functions of WNDP. We found that N-WNDP interacts specifically with the ATP-binding domain of WNDP and that the interactions between these two domains are copper dependent: In the absence of copper, two domains interact tightly, whereas copper-bound N-WNDP does not bind to ATP-BD very well (55). How many bound copper atoms are sufficient to induce the change in intradomain interactions is a subject of future investigations.

Importantly, the domain–domain interactions have a clear effect on the conformational state of the ATP-binding domain: when N-WNDP is bound to ATP-BD, the affinity of the latter to nucleotide is decreased several-fold (*see* Table 1). Therefore it is tempting to speculate that binding of copper to the N-terminal domain is accompanied by conformational changes that alter the interaction between N-WNDP and the ATP-binding domain (Fig. 6). This change, in turn, may modify the nucleotide-binding properties of WNDP and possibly the rate of ATP-hydrolysis.

It is also quite possible that copper-induced changes in domain–domain interactions and the subsequent conformational transitions lead to exposure of sites for the intracellular trafficking machinery, resulting in copper-dependent relocalization of WNDP in a cell. The mutagenesis studies and analysis of the naturally occurring mutants (63,65) revealed that the copper-dependent trafficking of MNKP and WNDP can be disrupted by amino acid substitutions in various regions of these proteins. These results suggest that a certain conformation of a functionally active protein rather than its mere ability to bind copper is important for trafficking of copper-ATPase. Although the precise molecular mecha-

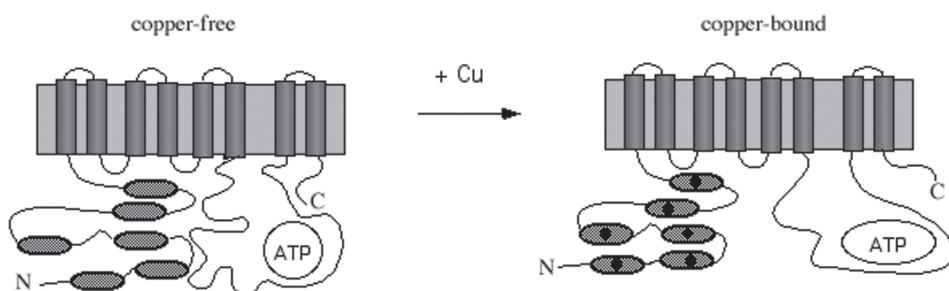


Fig. 6. Copper binding to N-WNDP decreases the interactions of N-terminal domain with ATP-BD, which in turn, changes the ATP-BD conformation.

nism of the WNDP trafficking and targeting is still unclear, it is likely to involve series of copper-dependent posttranslational events, based on changes in domains conformation induced by copper.

6. PRACTICAL ASPECTS OF EXPRESSION, PURIFICATION, AND BIOCHEMICAL ANALYSIS OF THE WNDP FUNCTIONAL DOMAINS

6.1. Expression of the N-Terminal Domain

Most of the biochemical studies described earlier utilized the heterologous expression, purification, and analysis of isolated functional domains of WNDP. Although, in general, this approach has certain limitations, it proved to be very fruitful and informative for WNDP. The key to successful biochemical characterization of isolated domains is the ability to obtain them in a soluble and well-folded form. The N-terminal domains of WNDP and MNKP contain over 600 amino acid residues and a large number of cysteines, which makes their expression in a soluble form a challenging task. In fact, in our first experiments the expression of N-MNKP and N-WNDP as fusions with maltose-binding protein (N-MNKP-MBP and N-WNDP-MBP, respectively) led to largely insoluble proteins deposited in inclusion bodies. Using the fluorescent Cys-directed probe (*see* Section 6.2. for details), we determined that most of the Cys residues in these proteins were unavailable for modification without prior reduction with dithiotreitol or β -mercaptoethanol, suggesting that Cys residues were involved in the formation of disulfide bridges (unpublished observation). It also suggested that incorrect S–S bridge formation could have led to protein misfolding and insolubility.

To overcome this problem, we utilized the innovative approach proposed by Yasukawa et al. (66). In this work, the solubility of eucaryotic proteins expressed in *E. coli* was shown to be markedly enhanced by presence of thioredoxin expressed from a separate plasmid. Indeed, in our experiments, coexpression of N-WNDP-MBP and N-MNKP-MBP with thioredoxin led to a dramatic increase in protein solubility (up to 60% of expressed protein was found in the soluble portion). Analysis of soluble N-WNDP-MBP and N-MNKP-MBP using Cys-directed probe revealed that the soluble domains have their Cys in the reduced form, not in S–S bridges (Fig. 7).

6.2. Copper Loading and Analysis of Copper Binding

The ability to keep Cys residues in the reduced form in a cell was extremely important, because it allowed us to develop a procedure for the *in vivo* loading of N-WNDP and N-MNKP with copper (48) (*see* Fig. 7). N-WBDP and N-MNKP in a copper-bound form can then be purified from cells using affinity chromatography and the amount of bound copper can be determined using a spectrophotometric assay (67) based on complexation of Cu(I) with bicinchoninic acid (BCA), as shown in

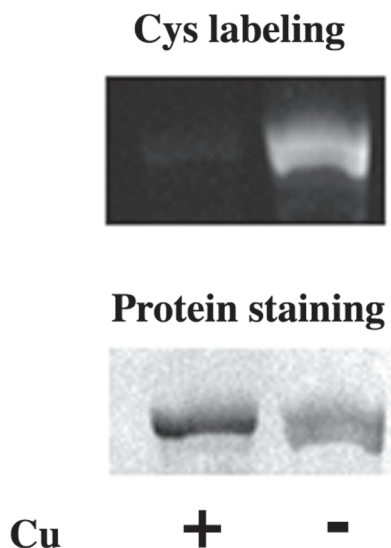


Fig. 7. Expression of N-MNKP-MBP in the presence of thioredoxin and in vivo loading with copper. *E. coli* cells transformed with N-MNKP-MBP and thioredoxin-containing plasmids were grown under standard conditions. Copper was added up to 500 μ M (+) to one of the cultures and the protein expression in control and copper-treated sample was induced with IPTG as described (48). Following affinity purification, the availability of Cys residues for labeling with fluorescent coumarin maleimide (CPM) and the amount of copper bound to the protein were determined. In this experiment, the (+) copper sample contained 5.19 mol Cu/mol protein. Similar results were obtained with N-WNDP-MBP fusion.

Fig. 8, or by atom absorption spectroscopy. The two procedures yield similar results, although care should be taken using the BCA assay, because the accuracy of this procedure may be affected by buffer composition, (e.g., by presence of imidazole (67; and our unpublished data).

Binding of copper to N-WNDP, N-MNKP, or to copper chaperones, such as HAH1, all of which contain the same CxxC motif for copper coordination, can be monitored by decrease of the Cys residues reactivity toward fluorescent reagents following copper binding to the protein, as shown in Fig. 7. Although this assay is indirect and has to be confirmed by the BCA-based analysis or by atom absorption spectroscopy, it could be very valuable when comparing multiple samples. The labeling assay is fairly independent on buffer composition and requires significantly less protein than the BCA-based procedure and atom absorption spectroscopy. We observed good agreement in copper stoichiometry values comparing the BCA procedure with the fluorescent probe-based assay, and we routinely used both protocols for the copper-binding measurements.

6.3. Choosing a Tag for Affinity Purification of the Copper-Binding Domain

For the characterization of the copper-binding domain of WNDP, we utilized the MBP fusion and a histidine tag. The advantage of the MBP fusion is that MBP does not bind copper and therefore does not appear to interfere with copper-binding properties of WNDP, in contrast to HisTag, which binds copper (Cu^{2+}). The disadvantage of MBP fusion is its large size (42 kDa), which complicates structural analysis of the fusion protein. For the characterization of MerP, expressed as a MBP fusion, MBP was cleaved prior to spectroscopic analysis (47); however, trombin cleavage of N-WNDP-MBP, which is significantly larger than MerP-MBP, was fairly inefficient (our data). Therefore, for characterization of the N-WNDP secondary structure, we utilized a HisTagged version of this protein (Fig. 3).

Interestingly, N-WNDP-HT can be expressed in a soluble form in *E. coli* in the absence of thioredoxin, but it has to be reduced in vitro in order to get copper bound (Fig. 9).

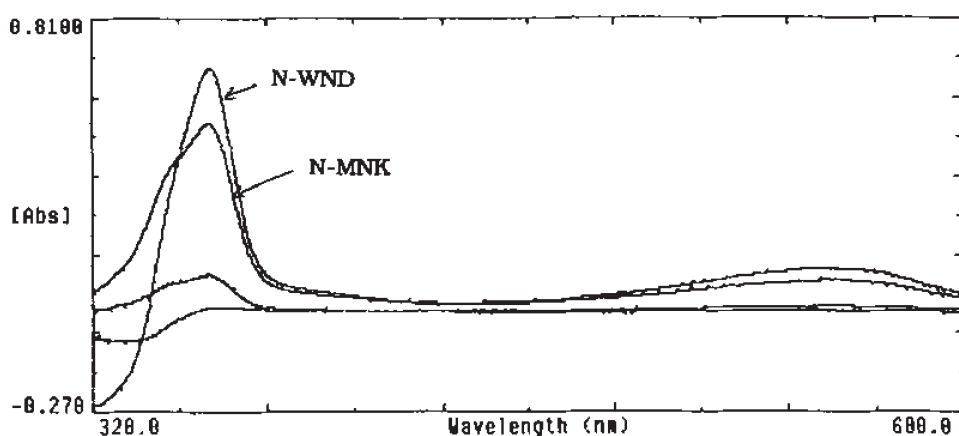


Fig. 8. Measurements of copper-binding to N-WNDP (N-WND) and N-MNKP (N-MNK) using bicinchoninic acid. Proteins loaded with copper *in vivo* as in Fig. 7 were used to estimate the amount of bound copper.

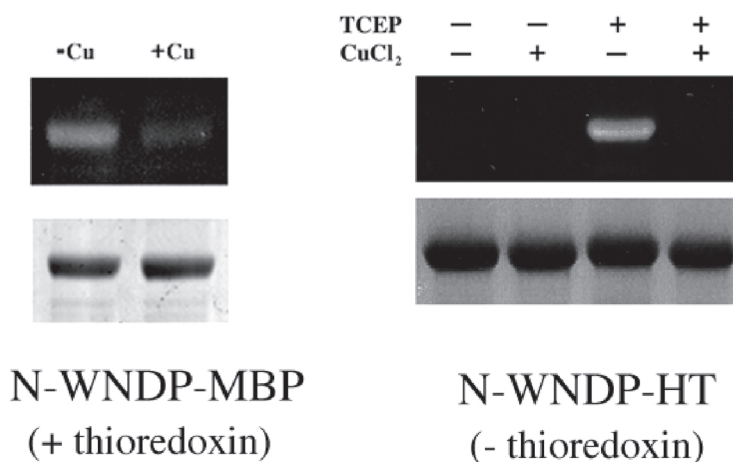


Fig. 9. Comparative analysis of the *in vitro* copper-binding properties of N-WNDP-MBP and N-WNDP-HT. Left panel: N-WNDP-MBP was expressed in the presence of thioredoxin, purified using amylose resin, and loaded with copper *in vitro* in the presence of ascorbate as described in ref. 48. Copper-binding was monitored by labeling of Cys residues with fluorescent coumarin maleimide and confirmed with BCA assay. Right panel: N-WNDP-HT was expressed without thioredoxin, purified on NTA resin and was either eluted with Imidazole (first two lanes) or was incubated with or without copper in the presence of reducing reagent [tris-(2-carboxyethyl)phosphine hydrochloride, TCEP] while it was bound to the resin. Note that without reduction, Cys residues are unavailable for labeling with the fluorescent probe. Following washes and elution with imidazole, the amount of copper bound to N-WNDP-HT was determined as above.

Although the N-WNDP-HT is useful for analysis of copper-independent properties of this domain, such as overall folding, and structure, its usefulness for analysis of copper binding is somewhat ambiguous because of the ability of HisTag to bind copper. In fact, our initial attempts to load N-WNDP with copper either *in vivo* or *in vitro* led to protein precipitation. The precipitation problem can be avoided if copper is added to N-WNDP-HT during purification while protein is still bound to the NTA resin and the His tails are sequestered by interactions with Ni. This protocol allowed copper to bind to Cys residues, as shown in Fig. 9.

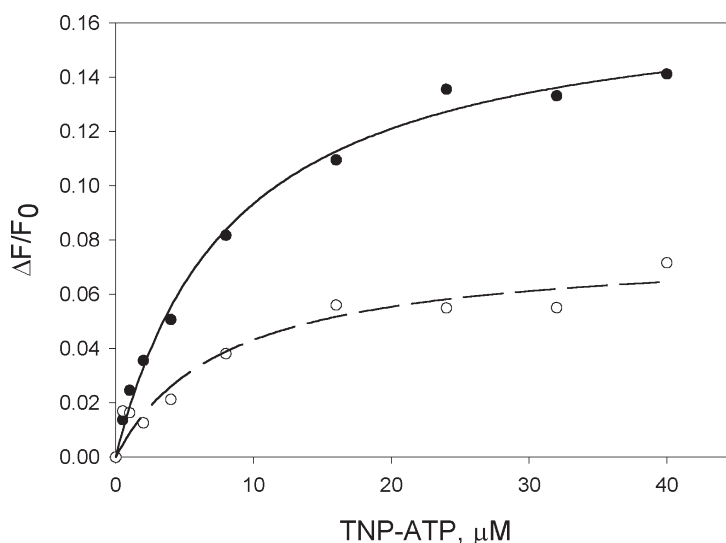


Fig. 10. The comparison of the nucleotide-binding properties for the ATP-binding domains of WNDP (filled circles) and Na⁺,K⁺-ATPase (the typical representative of the P₂-type ATPases, empty circles). The identical amounts of purified nucleotide-binding domains were mixed with increasing concentrations of TNP-ATP and the nucleotide-binding was monitored by the increase in TNP-ATP fluorescence.

The column-based procedure yields a copper-bound N-WNDP with stoichiometry close to what was found for in vitro-loaded N-WNDP-MBP; however, there is a marked difference between N-WNDP-HT and N-WNDP-MBP in the stability of the copper–protein complex. Although copper is bound tightly to N-WNDP-MBP, such that copper-bound protein can be dialyzed or concentrated significantly without losing copper, the HisTag fusion of WNDP quickly loses its copper upon concentration and then begins to aggregate when protein concentration exceeds 1–2 mg/mL. We conclude that the MBP fusion expressed in the presence of thioredoxin and loaded with copper *in vivo* currently represents a much better system for characterization of N-WNDP and N-MNKP properties.

6.4. ATP-Binding Domain

The problems associated with the HisTag, which we discussed earlier, could be the result of the fact that both the HisTag and the N-terminal domain can bind copper, and the presence of two copper-binding motifs generates protein with completely new properties. Using the HisTag, however, works fairly well for expression and purification of the ATP-binding domain of WNDP (ATP-BD). Although solubility of this domain is rather limited, and is not improved by coexpression with thioredoxin, it is possible to obtain up to 500 μg of purified protein from 2 L of cell culture following induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at room temperature (55).

The ability of the ATP-binding domain to bind the nucleotides can be quickly assessed using the fluorescent analog ATP (thrinetrophenyl-ATP, TNP-ATP). In solution, this reagent has low fluorescence; binding of TNP-ATP to proteins is accompanied by an increase in fluorescence, as shown in Fig. 10.

The specificity of TNP-ATP binding and relative affinities toward various nucleotides can then be determined by competition studies as in refs. 55–58. The disadvantage of TNP-ATP as a probe for the nucleotide-binding site is a relatively high nonspecific binding because of protein interaction with TNP moiety. However, there are also certain advantages. The TNP-ATP-based assay not only estimates the ability of the isolated domain to binds nucleotides, it also can be used to monitor the

changes in the microenvironment of the probe. As shown in Fig. 10, whereas the affinities of ATP-BD and the ATP-binding domain of the Na pump for TNP-ATP are comparable, the increase in the TNP-ATP fluorescence is larger when it binds to ATP-BD, indicating that the microenvironment of TNP-ATP differs in ATP-BD and Na⁺,K⁺-ATPase ATP-binding domain. Therefore, one can utilize this protocol to estimate whether mutations in ATP-BD alter the surrounding environment of the nucleotide-binding site.

7. CONCLUSION

WNPD and MNKP represent a novel group of ion transporters with fascinating structural and functional properties. The first important steps in biochemical analysis of these proteins have been made and further studies will undoubtedly uncover new and exciting information about molecular mechanisms of copper distribution in human cells.

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