The Distinct Functional Properties of the Nucleotide-binding Domain of ATP7B, the Human Copper-transporting ATPase

Analysis of the Wilson disease mutations E1064A, H1069Q, R1151H, and C1104F

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Running title: Nucleotide-binding domain of ATP7B

Key words: ATP7A, ATP7B, copper, P-type ATPase, nucleotide binding, isothermal titration calorimetry, Wilson disease

This work was funded by the National Health Institute Grants DK55719 and PPG 1-P01-GM067166-01 to S.L. R.T. is a recipient of the American Heart Association postdoctoral fellowship # 0325492Z.

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Summary

Copper transport by the P₁-ATPase ATP7B, or Wilson disease protein (WNDP), is essential for human metabolism. Perturbation of WNDP function causes intracellular copper accumulation and severe pathology known as Wilson disease (WD). Several WD mutations are clustered within the WNDP nucleotide-binding domain (N-domain), where they are predicted to disrupt ATP binding. The mechanism by which the N-domain coordinates ATP is presently unknown, since residues important for nucleotide binding in the better-characterized P₂-ATPases are not conserved within the P₁-ATPase subfamily. To gain insight into nucleotide binding under normal and disease conditions, we generated the recombinant WNDP N-domain and several WD mutants. Using isothermal titration calorimetry, we demonstrate that the N-domain binds ATP in a Mg²⁺-independent manner with a relatively high affinity of 75 µM, compared to millimolar affinities observed for the P₂-ATPase N-domains. The WNDP N-domain shows minimal discrimination between ATP, ADP, and AMP, yet discriminates well between ATP and GTP. Similar results were obtained for the N-domain of ATP7A, another P₁-ATPase. Mutations of the invariant WNDP residues E1064A and H1069Q drastically reduce nucleotide affinities, pointing to the likely role of these residues in nucleotide coordination. In contrast, the R1151H mutant exhibits only a 1.3-fold reduction in affinity for ATP. The C1104F mutation significantly alters protein folding, while C1104A does not affect the structure or function of the N-domain. Altogether, the results directly demonstrate the phenotypic diversity of WD mutations within the N-domain and indicate that the nucleotide-binding properties of the P₁-ATPases are distinct from those of the P₂-ATPases.
Introduction

The Wilson disease protein (WNDP) is a key regulator of copper homeostasis in a number of tissues, particularly in the liver, brain, and kidneys (1) (2). WNDP transports copper from cytosol across cell membranes, using the energy of ATP hydrolysis. Under basal conditions, WNDP delivers copper to enzymes within the secretory pathway; it is also essential for cellular copper excretion when copper concentrations are elevated (1-3). Mutations in WNDP result in marked accumulation of copper in the cytosol and a severe hepato-neurological disorder, Wilson disease (WD) (4,5). The clinical manifestations of WD are diverse (6), however the specific contributions of various WD mutations to phenotypic diversity remain poorly understood (7). Elucidating the consequences of mutations on WNDP structure and function is the first step toward a better understanding of molecular mechanisms underlying WD. In addition, an intriguing connection has recently been made between over-expression of WNDP and increased resistance of cells to the anti-cancer drug cisplatin (8-10). These findings point to the role of WNDP as a potential pharmacological target and further emphasize the need for a better understanding of the protein’s structure, function, and regulation. At present, such structural and biochemical information on WNDP is very limited.

WNDP belongs to the large family of the P-type ATPases and displays the key catalytic properties expected for the members of this family. Specifically, WNDP hydrolyzes ATP to form a transient phosphorylated intermediate at the invariant aspartate located in the DKTG sequence, a signature motif of the P-type ATPase (Figure 1). Copper, the transported ion, markedly stimulates the reaction (11). Recently, the first crystal structure of a P-type ATPase, Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum, was solved (12,13). The structure yielded important
information about the organization and conformational flexibility of this class of transporters. Ca\textsuperscript{2+}-ATPase was shown to be composed of several functional domains: the ATP-binding domain; the transmembrane domain, containing sites for transported ions; and the actuator domain (A-domain), which is critical for phosphatase activity and conformational transitions during the catalytic cycle (14,15). The ATP-binding domain of the P-type ATPases was shown to consist of two domains: the phosphorylation domain (the P-domain) containing the DKTG sequence and the nucleotide-binding domain (the N-domain), which contributes to ATP coordination.

The P-type ATPases are divided into five subfamilies based on their ion specificity and structural characteristics (16). WNDP is a member of the P\textsubscript{1B}-ATPase subfamily (16,17). The P\textsubscript{1}-ATPases share very limited sequence homology with Ca\textsuperscript{2+}-ATPase or other P\textsubscript{2}-ATPases. In fact, residues strictly conserved amongst all P-type ATPases are almost exclusively limited to the P-domain and the A-domain (Figure 1A). The P- and A-domains play essential roles in catalysis, thus it is not surprising that these two domains contain the sequence motifs found in all P-type ATPases. The N-domain is equally important, since it is involved in nucleotide binding. Therefore, it is intriguing that the amino acid residues known to participate in ATP coordination in the N-domain of Ca\textsuperscript{2+}-ATPase and other P\textsubscript{2}-ATPases are not conserved in the structure of the P\textsubscript{1}-ATPases. This observation suggests that the coordination environment of the nucleotide in these two P-type ATPase subfamilies could be quite different.

While there is little sequence similarity between the N-domains of the P\textsubscript{1} and P\textsubscript{2}-ATPases, the sequence of the N-domain is well conserved within the P\textsubscript{1}-ATPase subgroup. A number of the
residues are highly conserved or invariant (Figure 1B), suggesting their potentially important roles in the structure or function of the P$_1$-ATPase N-domains. In the WNDP sequence, these residues include E1064, S1067, H1069, P1070, Gly1101, and Gly1103. Importantly, invariant H1069 is the site of the most frequent disease-causing mutation, an observation that further emphasizes the functional significance of this residue.

Previous studies characterized the effect of mutations of H1069, or equivalent histidines, on the functional activity of WNDP and several homologous P$_1$-ATPases (18-22). The results confirmed the important role of this residue for the P-type ATPase function and suggested that H1069 may participate in the positioning of ATP within the catalytic site. However, direct evidence for the role of H1069 in ATP binding has been lacking. Similarly, a number of other WD-causing mutations have been identified in the N-domain of WNDP, but their effects on nucleotide-binding have not been characterized.

Experiments using site-directed mutagenesis of the full-length P$_1$-ATPases demonstrate that the dissection of the functional role of various residues could be impeded by additional effects of mutations on enzyme conformation (19). The specific role of the amino-acid residues in nucleotide coordination is particularly difficult to assess, since the nucleotide binding characteristics of the P$_1$-type ATPase mutants have been analyzed indirectly by monitoring their ability to utilize ATP to form a phosphorylated intermediate or to transport copper. Phosphorylation and transport are several steps removed from the initial nucleotide-binding event, making conclusions regarding nucleotide binding tenuous. Therefore, to directly investigate the role of several residues in nucleotide binding by WNDP, we generated the
recombinant WNDP N-domain and N-domain variants carrying known disease mutations, including H1069Q. In addition, we generated the recombinant N-domain of the Menkes disease protein (MNKP), the human copper-transporting ATPase ATP7A. MNKP is homologous to WNDP and is another member of the $P_1$-ATPase sub-family. Comparison of these two proteins helped us to dissect the commonalities in their nucleotide-binding properties. Using direct ligand binding measurements, we demonstrate that (i) the N-domains of WNDP and MNKP contain a single nucleotide-binding site, (ii) the properties of these sites are distinct from those of the $P_2$-ATPases, and (iii) the invariant residues E1064 and H1069 of WNDP are important for nucleotide coordination, while the less conserved C1104 and R1151 residues are not essential for nucleotide-binding.

**Materials and Methods**

*Expression constructs for the wild-type (wt) and mutant N-Domains of WNDP.* To generate the expression constructs for wt N-domain of WNDP (amino-acid residues V$^{1036}$-D$^{1196}$ of the full-length protein), the corresponding cDNA region was amplified via polymerase chain reaction (PCR) using pCDNA3.1(+)-WNDP plasmid as a template and the following primers: WT.ND-fwd, 5'-ACATATGGTCCCCAGGGTCATG-3' and WT.ND-rev, 5'-AGAATTC TTAGGTCTCGATTGCAGATC-3'. The PCR product, with 5' Nde1 and 3' EcoR1 endonuclease restriction sites flanking the coding sequence, was sub-cloned into the pCR1I-Blunt TOPO vector (Invitrogen). The resulting pCR1I-WT.ND plasmid was then used as a template for the generation of E1064A, H1069Q, C1104F, C1104A, and R1151H mutants by site-directed mutagenesis.
The site-directed mutagenesis has been carried out in two PCR steps. In the first step, two overlapping PCR products were generated using pCRII-WT.ND as a template. Product A was produced with WT.ND-fwd primer and mutagenesis-rev primer; product B was generated using mutagenesis-fwd primer and WT.ND-rev primer. In the second PCR step, the overlapping products A and B were used as templates and were amplified using the WT.ND-fwd and WT.ND-rev primers, producing mutant N-domain. Mutagenesis primers were: E1064A.ND-fwd: 5'-GTGGTGGGACTGCGGCCAGCAGT-3,’ E1064A.ND-rev: 5'-ACTGCTGGCCGCAGTCCCCACCAC-3,’ H1069Q.ND-fwd: 5'-AGCACAGTGGAACAACTGCTGGG-3,’ H1069Q.ND-rev: 5'-ACGCCCAAGGGTTTGTTCACCTGCTGG-3,’ C1104F.ND-fwd: 5'-TGTGGAATGGTTCAAAGTCAGCAAC-3,’ C1104F.ND-rev: 5'-GTTGCTGACTTTGAACCCAATTCCACA-3,’ C1104A.ND-fwd: 5'-TGTGGAATGGGGTGCTG-3,’ C1104A.ND-rev: 5'-GTTGCTGACTTTGGCCCCAATTCCACA-3,’ R1151H.ND-fwd: 5'-CTGATTGGAAACCAGTGCTG-3,’ and R1151H.ND-rev: 5’-CAGCCACTCGTGCTTGTTCCAATCAG-3.’ The mutant products were sub-cloned into the pCRII vector. Then, using the Nde1 and EcoR1 restriction sites, the coding regions of the wt and mutant N-domains were excised from the pCRII vector and ligated into the IMPACT-CN expression vector pTYB12 (New England BioLabs). Automated DNA sequencing was used to verify the desired nucleotide sequence of the entire coding region for all pTYB12-ND plasmid constructs.

The N-domain of MNKP. The expression construct for MNKP N-domain (residues T1048-D1230 of the full-length protein) was generated by PCR of the corresponding cDNA region of ATP7A using the following primers: MNKP-ND fwd. 5’-ATGAATGCTACCATTAG-3’ and MNKP-ND rev. 5’-CTGATTGGAAACCACGAGTGGCTG-3’.
CTCACCGAAC-3’ and MNKP-ND rev. 5’-ATGAATTCTTAGTCTGCAATGGCTATC-3.’

The PCR product, containing 5’ BsmI and 3’ EcoRI endonuclease restriction sites flanking the coding sequence, was then cloned into the pTYB12 expression vector. The nucleotide sequence of the pTYB12-MNKP.ND was verified by automated DNA sequencing.

*Expression and purification of the recombinant N-Domains.* All N-domains were expressed as fusion proteins with a chitin-binding domain and an intein protein. Briefly, *E. coli* BL21 (DE3) cells were grown in ampicillin supplemented (100 µg/ml) Luria-Bertani liquid media at 37°C until an OD$_{600}$ of ~0.6 was reached. The expression was then induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, with shaking at 250 rpm for ~12 hours at 23°C. After harvest by centrifugation, cell pellets were re-suspended in 50 mM Tris pH 8.2, 500 mM NaCl, with complete EDTA-free Protease inhibitor cocktail (Roche) and disrupted by passing the suspension through a French press twice at 15,000 p.s.i. The lysate was centrifuged for 45 min. at 15,000 rpm and the soluble fraction was passed over a chitin resin. Purified resin-bound fusion protein was obtained after washing the chitin resin (New England BioLabs) with approximately thirty column volumes of 50 mM Tris pH 8.2, 500 mM NaCl. Following the wash, the resin was equilibrated with the intein cleavage buffer containing 50 mM DTT, 50 mM Tris pH 8.2, and 150 mM NaCl. Cleavage buffer promotes intein-mediated excision of the N-domain from the fusion, allowing elution of non-tagged N-domain from the resin. The purified WNDP N-domains contained four non-native amino terminal residues (AGHM) derived from the pTYB12 vector, the MNKP N-domain did not contain any non-native amino acids. Expression and purification of the MNKP N-domain was carried out as described for the WNDP N-domain, except the induction with
IPTG was carried out at 16°C to improve solubility. Typical yields of wt and mutant N-domains ranged from 2-6 mg protein per liter of cell culture.

**CD-spectroscopy.** Purified wt and mutant N-domains were dialyzed extensively against 50 mM NaH$_2$PO$_4$, pH 7.0 buffer and concentrated to 0.4 mg/ml using Amicon Ultra PL-10 centrifugal filters (Millipore) with a 10 kDa molecular weight cutoff. Concentrated samples were then centrifuged at 90,000g for 45 minutes to remove insoluble particles. Protein concentrations were initially determined spectrophotometrically using a theoretical extinction coefficient of 8850 M$^{-1}$ cm$^{-1}$ at 280 nm; subsequent amino acid analysis confirmed the spectrophotometrically determined concentrations. CD spectra were measured using an AVIV CD model 215 spectrometer; deconvolution of the spectra was carried out with the program CDDN1 (23). The CD measurements were performed for three independent protein preparations of each mutant and the resulting secondary structure values were averaged.

Thermal denaturation was used to further compare folding of the wt and mutant N-domains. Temperature dependant structural transitions, measured as molar ellipticity changes at 222 nm, were monitored from 25°C to 80°C using CD spectroscopy. Spectra were collected with the AVIV CD 215 spectrometer using a 0.1 cm rectangular CD cell. Molar ellipticity was monitored in 0.5 nm increments with 30 s equilibrations between measurements.

**Nucleotide binding measurements.** Isothermal titration calorimetry (ITC) was used to analyze the nucleotide-binding properties of wt WNDP and MNKP N-domains, and the E1064A, H1069Q, C1104A, and R1151H N-domain mutants. Purified proteins were dialyzed
extensively against 50 mM NaH$_2$PO$_4$ pH 7.0 buffer and concentrated to 116 µM (~2 mg/ml). Nucleotide solutions of 4 mM adenosine 5'-triphosphate disodium salt (Sigma), adenosine 5'-diphosphate monosodium salt (Acros Organics), adenosine 5'-monophosphate (Acros Organics), or guanosine 5'-triphosphate dilithium salt (Alexis Biochemicals) were prepared in the dialysis buffer immediately prior to each titration. For titrations with ATP-Mg$^{2+}$ complex, 12 mM MgCl$_2$ (Sigma) was included in the 4 mM ATP disodium salt solution.

To ensure that the ITC data do not include enthalpy changes due to hydrolysis of nucleotides by the N-domain, 1 mM ATP in 50 mM MES, pH 6.0 buffer was incubated with or without 1 mM N-domain at room temperature for up to 6 hours. The amount of free P$_i$ resulting from ATP hydrolysis (10 µM Pi, 1% of total ATP) was determined by EnzChek® Phosphate Assay Kit (Molecular probes) and was found to be identical in the presence and absence of the N-domain.

ITC experiments were performed with the VP-ITC titration calorimeter (Microcal, Inc.). Protein and nucleotide solutions were degassed by vacuum aspiration for 5 min prior to loading the samples into the ITC cell and syringe, respectively. All titrations were carried out at 25°C, with a stirring speed of 300 r.p.m. and a 180 s duration between each 8 µl injection; thermal power was monitored every 8 s. Wt and mutant N-domains were titrated to saturation, where possible, with a 4 mM stock solution of nucleotide. Parallel experiments were performed, by injecting nucleotide into buffer or buffer into N-domain, to determine the heats of dilution. The heats of dilution were then subtracted from their respective N-domain nucleotide titrations prior to data analysis. Thermogram analysis was performed with the
Origin 5.0 data analysis software provided with the VP-ITC instrument. For each protein-nucleotide interaction, at least three titrations were performed on independently prepared N-domains. Titration thermograms were analyzed independently, and the obtained $K_d$ values were averaged.

To characterize binding of ATP using changes in the intrinsic tryptophan fluorescence, 11.5 µM WNDP N-domain in 50 mM NaH$_2$PO$_4$, pH 7.0 was titrated with increasing concentrations of ATP (0-3 mM) or the same volume of buffer. Using a model PTI-QM1 (Photon Technology International) fluorimeter, protein sample was excited at 295 nm (2 nm slit width) and fluorescence was monitored at an emission wavelength of 320 nm (1 nm slit width), which corresponds to the maximum emission wavelength for the N-domain. The final increase in protein sample volume due to ATP addition did not exceed 1% and was accounted for in final calculation. Data were plotted as ratio of $F/F_0$ vs. concentration of ATP.

*Molecular modeling and Molecular Dynamics simulations.* A spatial model of the ATP-binding domain of WNDP (residues M$^{996}$-R$^{1322}$) was built via homology modeling based on the X-ray structure of the nucleotide-binding domain (residues A$^{320}$-K$^{758}$) of Ca$^{2+}$-ATPase in the E1 (“open”) state (PDB entry 1EUL (13)). The details of the modeling experiments and validation criteria were described previously (24). In the current work, minor conformational changes were introduced into the loop 1062-1071 of the model using the following procedure. First, twenty models with different conformations of this loop were generated. Two models in which the side chains of E1064 and H1069 point towards the adenine binding cleft in the N-domain were selected for future studies. Both models were subjected to 5-ns molecular
dynamics (MD) simulations in explicit water. The conformers extracted from the equilibrium parts of MD trajectories were then employed in docking simulations with the ATP molecule as previously described (24). The molecular surfaces were mapped according to their hydrophobic properties using the molecular hydrophobicity potential (MHP) approach (25).

Results

The recombinant wt and mutant N-domains were expressed in *E.coli* and isolated following affinity chromatography on chitin beads and intein-mediated self-cleavage, as described in the experimental procedures. The expression levels, protein yields, and purities were similar for all N-domains.

*Folding and nucleotide-binding properties of the wt WNDP N-domain.* Previous NMR studies of the N-domain of Na,K-ATPase, a P$_2$-ATPase, revealed that the domain is folded and can bind ATP (26). However, the apparent affinity of the N-domain for the nucleotides was extremely low (5-20 mM), compared to the high affinity of the full-length protein (0.3-1 µM). Similarly, the analysis of ATP binding by the proteolytic fragment of Ca$^{2+}$-ATPase, containing the N-domain, yielded a high apparent $K_d$ value of ~ 0.7 mM (27), while characterization of the recombinant N-domain of Ca$^{2+}$-ATPase produced a $K_d$ value of approximately 2.4 mM (28). These results suggested that the formation of the high-affinity site for ATP in P$_2$-ATPases is a result of interaction between the N-domain and other regions of the proteins. Since the sequences of the P$_1$ and P$_2$-ATPases N-domains are dissimilar, we were interested in testing whether the isolated N-domain of WNDP, a P$_1$-ATPase, can alone form a high affinity site for the nucleotide.
Prior to nucleotide binding measurements, the folding of the recombinant wt N-domain of WNDP was examined. Analysis of the secondary structure of the N-domain using CD spectroscopy (Figure 2) revealed that the N-domain of WNDP has the following secondary structure composition: ~26 % α-helix, ~18.5 % β-turn, and ~22 % β-sheet (Table 1). Further, thermal denaturation experiments demonstrated a sharp transition curve typical of a well-folded soluble protein, with a $T_m$ of ~52°C (Figure 2, inset). Finally, the N-domain was found to be resistant to treatment with mild concentrations of trypsin (our data, not shown). All these results indicated that the recombinant N-domain of WNDP is well-folded.

The ability of the N-domain to bind nucleotides was examined using isothermal titration calorimetry (ITC). The N-domain does not contain the catalytic aspartate and is not expected to hydrolyze ATP. To verify this prediction and ensure that ITC measures only nucleotide-binding events and not hydrolysis of the nucleotides, we determined hydrolytic activity of the N-domain. The ATP hydrolysis in the presence of the N-domain did not exceed the spontaneous rate of ATP decay in the buffer even after prolonged (6 hours) incubation. Thus, the enthalpy changes observed in the ITC experiments are due to binding of the nucleotides to the N-domain.

A representative thermogram for the calorimetric titration of wt N-domain with ATP is presented in Figure 3A. The exothermic evolution of heat upon ATP injections, shown in the upper panel, illustrates saturable nucleotide binding by the N-domain. Calculation of the enthalpy changes at various molar ratios of ATP to the N-domain (Figure 3A, lower panel) revealed that the best fit for the data corresponds to the presence of one ligand-binding site in
the N-domain. In contrast, titration with GTP demonstrated no significant protein-nucleotide interactions. Enthalpy changes in this case were indistinguishable from the results of the nucleotide titration into buffer. Thus, the N-domain of ATP7B discriminates extremely well between adenosine and guanosine moieties, indicating selectivity for the nucleotides.

Calculations of the apparent affinity for ATP yielded a $K_d$ of $75.30 \pm 3.62 \, \mu M$, an unexpectedly low value, comparing to 0.7-5 mM $K_d$ values reported earlier for the N-domains of the $P_2$-ATPases (26-28). The ITC result was confirmed by measuring changes in the intrinsic tryptophan fluorescence of the N-domain upon addition of ATP (Figure 3B), which yielded similar $K_d$ value ($110 \pm 15 \, \mu M$). This relatively high affinity enabled us to elucidate the nucleotide binding characteristics of the N-domain in more detail. Based on known properties of the $P_2$-ATPases, we expected the following relative affinities of the N-domain for the nucleotides: ATP>ADP>>AMP (see for example, (29)). However, the experiments revealed that the nucleotide specificity of the WNDP N-domain did not follow this trend. As shown in Figure 4, there is very little difference in the affinity of the N-domain for ATP, ADP, and AMP ($K_d$ values summarized in the Table 2). In fact, the N-domain binds ATP with a slightly lower affinity than ADP or AMP.

To test whether the observed nucleotide-binding characteristics are unique for WNDP or representative of the $P_1$-ATPases, we expressed and purified the N-domain of MNKP, another copper-transporting ATPase (Figure 4B, inset). The MNKP N-domain is 50% identical to the WNDP domain, however it is larger (20 kDa versus 17 kDa) and contains a unique sequence insert (3). The ITC experiments demonstrated that the nucleotide-binding properties of the
MNKP N-domain are very similar to those of WNDP (Figure 4). Specifically, MNKP binds ATP and ADP with high affinity (Table 1) and discriminates poorly between these nucleotides. Interestingly, the $K_d$ value for ATP is higher than for ADP ($83.00 \pm 7.10 \mu$M and $44.50 \pm 0.70 \mu$M, respectively), similar to what was observed for the N-domain of WNDP. The N-domain of MNKP did not interact with GTP, confirming its selectivity towards the nucleotides containing the adenosine moiety.

Magnesium plays a critical role in the catalytic cycle of the P-type ATPase. The presence of Mg$^{2+}$ is required for the hydrolysis of ATP by the full-length WNDP (our data), suggesting that ATP-Mg$^{2+}$ is a substrate for this reaction. Although the N-domain lacks the catalytic aspartate required for the hydrolysis of ATP, it is unknown whether Mg$^{2+}$ plays a role in the docking of ATP to the N-domain of WNDP. To address this issue, we examined the effect of Mg$^{2+}$ on interaction between wt WNDP N-domain and ATP. The results shown in Figure 4A indicate that Mg$^{2+}$ increases the affinity of the N-domain for ATP, but only slightly (Table 1). In addition, no interactions were observed when the N-domain was titrated with magnesium in the absence of ATP. We conclude that the nucleotide binding by the N-domain is a magnesium independent event.

Residues E1064 and H1069 play important roles in nucleotide binding. Numerous disease-causing mutations have been identified in the N-domain of WNDP. The E1064A and H1069Q mutations affect amino acid residues that are highly conserved in P$_1$-ATPases, while C1104F and R1151H substitutions alter less conserved residues (Figure 1B). Consequently, we hypothesized that E1064 and H1069 could be essential for nucleotide coordination, while the
roles of C1104 and R1151 could be more structural.

To examine the involvement of H1069 and E1064 in ATP binding we introduced the above mutations into the N-domain and tested the effects of the mutations on folding and function. The secondary structure of the H1069Q and E1064A N-domains appeared unaltered by these mutations, as evidenced by essentially identical CD data for the mutants and wt N-domain (Figure 5A, Table 1). However, both mutations had a marked effect on nucleotide binding. As shown in Figure 5B and Table 2, E1064A lost the ability to bind ATP entirely, while the H1069Q mutant showed minimal binding, which did not reach saturation under the final conditions of our assay, i.e. at 600 µM ATP.

Efforts were made to evaluate the role of E1064 in nucleotide binding in more detail. We generated the N-domain mutant with a more conservative E1064D substitution, preserving the charge of the residue at this position. Surprisingly, the E1064D mutation had a significant negative effect on protein folding as evidenced by CD-spectroscopy and thermal stability experiments (data not shown). The nucleotide-binding properties of this mis-folded mutant were not investigated.

Cys1104 is not required for nucleotide binding. The residue corresponding to Cys1104 in WNDP is not conserved and can be replaced by alanine or glycine in other P1-ATPases (Figure 1B). At the same time, in WNDP the mutation of Cys1104 to Phe results in a WD phenotype. To better understand the role of C1104 in WNDP folding and function we generated the C1104F mutant of the N-domain and characterized its biochemical properties.
Unlike H1069Q and E1064A, the C1104F substitution had a marked effect on folding of the N-domain (Figure 6). Phenylalanine is a bulky residue and it is not surprising that the Cys>Phe mutation alters the N-domain structure. Therefore, to further elucidate the role of C1104 in protein folding and nucleotide binding, we substituted cysteine with the less bulky alanine.

In sharp contrast to the C1104F mutant, the secondary structure of the C1104A N-domain was very similar to that of the wt N-domain (Figure 6A). This result suggested that the amino-acid residues with a small side-chain are well tolerated at the 1104 position. Thermal denaturation experiments performed on the wt, C1104F, and C1104A N-domains confirmed this conclusion. As shown in Figure 6B, the C1104A mutant exhibits a temperature induced structural transition very similar to that of the wt protein. In contrast, the C1104F mutant did not undergo such a transition. Finally, the role of C1104 in nucleotide binding was tested by measuring the ability of the C1104A mutant to bind ATP. As shown in Table 2, C1104A binds both ATP and ADP similarly to the wt N-domain (the $K_d$ values are $59.97 \pm 0.39$ and $48.06 \pm 0.36$ µM, respectively). Thus, Cys1104 is not required for nucleotide binding.

**The effect of the R1151H substitution on nucleotide binding.** R1151 is another non-conserved residue, but substitution with histidine at this position results in WD phenotype (30). The CD spectrum of the R1151H mutant is indistinguishable from the wt N-domain (Table 1), suggesting that the effect of this mutation on the secondary structure of the protein is insignificant. However, the nucleotide-binding properties of the N-domain are altered by the R1151H substitution (Table 2). The $K_d$ value for ATP is increased by 25-30%, suggesting that
the affinity for the nucleotide was diminished, but not greatly affected. Similar effects were observed for ADP and AMP. Interestingly, binding of AMP was somewhat less affected, suggesting that the effect of the mutation could be in the vicinity of ATP’s $\beta$ and $\gamma$-phosphates.

*Molecular modeling.* To better understand the basis for the higher nucleotide-binding affinity of the WNDP N-domain and visualize the location of the mutant residues with respect to ATP we utilized a molecular modeling approach. This approach included fine-tuning of a recently generated 3D model of the WNDP ATP binding domain (24), molecular dynamics (MD) simulations, and ATP docking experiments. The modeling studies predict that all amino-acid residues characterized in our work are situated near the nucleotide-binding cleft (Figure 7). However, only E1064 and H1069 are in immediate proximity to the adenosine moiety of ATP. This prediction is consistent with our experimental data demonstrating a drastic effect of the E1064A and H1069Q substitutions on affinity of the N-domain for ATP. In the isolated N-domain, all mutated residues are also fairly exposed. This observation may explain why most of the mutations, with exception of bulky C1104F substitution, have little effect on folding of the N-domain.

Comparison of the WNDP N-domain model (Figure 7) with the high-resolution structure of Ca$^{2+}$-ATPase revealed that, upon ATP docking, the changes in accessible surface area are ~100-150 Å$^2$ for Ca$^{2+}$-ATPase and ~250 Å$^2$ for WNDP, although the spatial dimensions of the binding sites are quite similar. Thus, it appears that ATP is buried deeper within the WNDP’s nucleotide binding cleft, relative to Ca$^{2+}$-ATPase. Furthermore, the hydrophobic
surface of the WNDP N-domain exhibits greater complementarity with the nucleotide’s adenine ring, i.e. a larger fraction of the hydrophobic and hydrophilic surface of ATP is covered by the hydrophobic and hydrophilic atoms of the protein, respectively. Analysis of several protein-ATP complexes with known high-resolution structure suggests that the higher degree of complementarity correlates well with the higher affinity of binding (manuscript in preparation). Taken together, these results are consistent with WNDP having a higher nucleotide binding affinity than the P$_2$-ATPases.

**Discussion**

Within the large family of P-type ATPases, the P$_1$- and P$_2$-ATPases have distinct ion-specificities, characteristic trans-membrane topologies, and unique sequence motifs (17,31). The overall sequence similarity between the mammalian copper-transporting ATPases (P$_1$-ATPases) and Ca$^{2+}$-ATPase or Na,K-ATPase (P$_2$-ATPases) is less than 5%. Nevertheless, these proteins perform the same general function: coupling ATP binding and hydrolysis with the transport of ions across membranes. The marked sequence dissimilarity between the P$_1$- and P$_2$-ATPases may indicate that very few amino-acid residues are essential for ATP binding and catalysis. Alternatively, it could be that similar steps in the enzymatic cycle of the P$_1$- and P$_2$-ATPases are achieved via different structural means. Our characterization of the N-domains of two human copper-transporting P$_1$-ATPases, WNDP and MNKP, supports this latter hypothesis and identifies several key differences between the nucleotide-binding domains of the P$_1$- and P$_2$-ATPases.
Direct ligand binding measurements performed in this work indicate that the isolated N-domains of P₁-ATPases bind ATP with much higher affinity than the P₂-ATPase N-domains (70-80 µM versus 0.7–5 mM). In contrast, the full-length P₁- and P₂-ATPases have similar high apparent affinities for ATP (0.3-1 µM). These findings suggest that, compared to the P₂-ATPases, the formation of the nucleotide-binding site by WNDP and MNKP is significantly less dependent on interactions of the N-domain with other portions of the protein. While interactions with the P-domain and/or A-domain are still needed to obtain the low micromolar affinities for ATP observed during catalytic phosphorylation of WNDP, it seems clear that the nucleotide-binding site is largely pre-organized within the N-domain.

Our modeling studies suggest that favorable hydrophobic contacts and H-bonds between the adenosine moiety and the residues forming the binding cleft are likely to play a major role in higher affinity of the P₁-ATPase N-domain for the nucleotides. The significant hydrophobicity and the size of the binding cleft also help to explain the lack of interactions between the N-domain of WNDP and GTP, since in the WNDP N-domain the additional amino group of GTP would be placed in an unfavorable environment with sterical hindrances.

Another interesting difference between the P₁- and P₂- N-domains relates to their selectivity for adenosine-based nucleotides. The Na,K-ATPase N-domain binds ATP and ADP with K_d values of 5.1 ± 0.4 and 24.2 ± 5.3 mM, respectively (26). Neither the full-length Na,K-ATPase, nor the ATP-binding domain bind AMP with appreciable affinity (29). In contrast, the N-domains of WNDP and MNKP were found to bind ATP, ADP, and AMP, with very similar K_d values. Clearly, neither the ß nor γ-phosphates of ATP contribute significantly to
the binding of nucleotide to the P₁-ATPase N-domains. It seems likely that the phosphorylation domain, which contains D1027, the acceptor of γ-phosphate during catalytic cycle, could play a major role in the modulation of WNDP and MNKP nucleotide selectivity.

An unexpected, but reproducible finding was that the \( K_d \) values for ATP were slightly higher than those for ADP and AMP. Although the differences were not large, they were beyond error and observed repeatedly in both MNKP and WNDP N-domains. This result is consistent with the notion that in the isolated N-domain the γ-phosphate of ATP experiences weak repulsive interactions. In support of this conclusion, the addition of Mg\(^{2+}\) increases the binding affinity for ATP, bringing the \( K_d \) value close to those for ADP. It has been proposed that ATP binding facilitates movement of the N-domain towards the P-domain in the P-type ATPases (14). It is tempting to speculate that the observed repulsive interactions between the N-domain and ATP’s γ-phosphate may contribute to such a movement. The structural analysis of the N-domain in a complex with the nucleotide would directly test this hypothesis; these experiments are currently underway in our laboratory.

Previously, we expressed and characterized the ATP-binding domain of WNDP (ATP-BD), which included both the N-domain and the P-domain (32). These earlier studies utilized indirect measurements of nucleotide binding based on competition with the fluorescent ATP derivative, TNP-ATP. The experiments demonstrated that the isolated ATP-BD could bind the nucleotides at two sites (32). Our current studies illustrate that the N-domain has only one site. Therefore, the second set of the nucleotide-coordinating residues in ATP-BD is likely to be formed by the residues in the P-domain or by the P-domain and the linker region. This
conclusion is consistent with the above notion that the P-domain could be essential for providing selectivity to ATP in the full-length WNDP.

Our studies demonstrated that the recombinant N-domain of WNDP represents an excellent tool for dissecting specific consequences of various disease mutations and identification of residues directly involved in nucleotide binding. *A priori*, it is difficult to predict the effect of a given mutation on nucleotide coordination, protein folding, domain-domain interactions, or a combination thereof. Utilization of the purified N-domain enabled us to separate these various effects. We demonstrate that despite close proximity of all analyzed residues to the putative ATP-binding cleft (Figure 7) only E1064 and H1069 were important for nucleotide binding.

The strictly conserved nature of E1064 and a complete loss of nucleotide binding upon mutation of this residue suggest that E1064 is directly involved in ATP coordination in P1-ATPases. This conclusion is consistent with the earlier results of the experiments on zinc-transporting ATPase, ZntA. In these studies, the ZntA mutation E470A, which corresponds to the E1064A WD mutation, severely disrupted the enzyme’s function. Specifically, the ATPase activity of E460A ZntA was not detected in 0.45 mM ATP and was reduced by ~90% in the presence of 4 mM ATP (20). The important role of E1064 and its analogs in ATP coordination is also suggested by the predicted location of this residue in close proximity to the adenosine moiety of ATP (Figure 7).

Our data provide evidence that the invariant H1069 is also important for nucleotide binding by the WNDP N-domain. However, the role of this residue seems to go beyond direct
involvement in nucleotide coordination. Due to very weak ligand-substrate interactions, we were not able to precisely calculate the nucleotide affinity of the H1069Q disease mutant. However, based on the ratio of the ITC signals of wt and H1069Q titrations, we estimate the H1069Q mutant’s affinity for ATP to be reduced by at least 15-fold. The marked effect of equivalent H->Q substitution on apparent affinity for ATP was also reported for MNKP using measurements of the ATP-dependent transport of copper (21). The ZntA H475Q mutant, an equivalent of the WNDP H1069Q mutation, was shown to have the ATPase activity that was 4% and 43% of wt in 0.45 mM and 4 mM ATP, respectively (20). Altogether, these data indicate that the invariant histidine in the N-domain of the P$_1$-ATPase is important for ATP binding.

However, the mutation of H1069 in the full-length WNDP also affects reactions that are independent of nucleotide binding, such as phosphorylation from inorganic phosphate (22). Similar effects were observed for the equivalent mutation in ZntA (20), suggesting that in the full-length protein this histidine is located in close proximity to the phosphorylation domain and possibly the A-domain. Thus, it appears that H1069 and the equivalent residues in other P$_1$-type ATPases are strategically positioned to regulate the nucleotide-binding characteristics of the N-domain during catalytic cycle when several functional domains come together or move apart. The fairly exposed location of H1069, facing the P-domain (Figure 7), is consistent with such a regulatory role. Future high-resolution structural studies will determine whether this histidine residue is a direct ligand for ATP or whether it regulates the affinity of the nucleotide-binding site via interaction with other domains.
In contrast to the critical roles of the strictly conserved residues in nucleotide binding, mutations of the non-conserved C1104 and R1151 appear to affect the N-domain function through different mechanisms. Specifically, substitution of C1104 with Phe disrupts the N-domain folding. In ZntA, another P$_1$-ATPase, the position corresponding to C1104 is occupied by alanine (A508) indicating that the presence of Cys in this position is not essential for the ATPase function. Consistent with this conclusion, we found that substitution of C1104 with alanine in WNDP had no effect on either protein folding or nucleotide affinity. Thus, the C1104F WD phenotype likely results from folding defects, rather than loss of an important contact between the cysteine residue and ATP.

In yet another example of the variable effects of WD mutations, the R1151H mutation had very modest effects on either folding or affinity for ATP (Tables 1 and 2). These findings suggest that the residue is likely to play a role in domain-domain interactions or conformational transitions of the full-length protein. Such a role would help to explain why a mutation, which has negligible effects on protein folding or nucleotide binding, manifests as a disease mutation.

In summary, we have used direct nucleotide binding measurements to investigate the functional properties of the WNDP N-domain and several WD mutants. Analysis of the mutations unambiguously established their effects within the context of nucleotide binding. The observed spectrum of consequences on the N-domain structure and function contribute to our understanding of the phenotypic diversity of WD. In addition, the results demonstrate that the P$_1$-ATPase N-domains possess functional properties distinct from the P$_2$-ATPases.
Acknowledgments

The authors thank Ms. Tina Purnat for help with preparation of illustrations, the members of Lutsenko laboratory for critical reading of the manuscript and helpful discussions, and Dr. David Farrens for help with fluorescence measurements. The molecular modeling experiments were supported by the Russian Foundation for Basic Research (Grant 04-04-48875-a to R.G.E.).
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Table 1. The secondary structure composition (in %) of the wt WNDP N-domain and the WD mutants

|         | WT     | E1064A | H1069Q | C1104A | R1151H |
|---------|--------|--------|--------|--------|--------|
| α-helix | 26 ± 2 | 26 ± 3 | 26 ± 3 | 25 ± 3 | 24 ± 3 |
| β antiparallel | 11 ± 1 | 11 ± 1 | 12 ± 1 | 12 ± 1 | 12 ± 2 |
| β parallel | 11 ± 1 | 11 ± 1 | 11 ± 1 | 11 ± 1 | 12 ± 1 |
| β–turn | 18 ± 1 | 19 ± 1 | 19 ± 1 | 19 ± 1 | 19 ± 1 |
| Other   | 34 ± 2 | 33 ± 3 | 32 ± 3 | 33 ± 3 | 33 ± 3 |
Table 2. The $K_d$ values (in $\mu$M) for the experimental titrations of wt N-domains and the WNDP N-domain mutants with various nucleotides

|        | Wt WNDP | E1064A | H1069Q | C1104A | R1151H | MNKP    |
|--------|---------|--------|--------|--------|--------|---------|
| ATP    | 75.30 ± 3.62 | No binding | ~1200  | 59.97 ± 0.39 | 94.97 ± 0.39 | 83.00 ± 7.10 |
| ADP    | 61.51 ± 6.05 | No binding | ~1000  | 48.06 ± 0.36 | 84.57 ± 1.29 | 44.5 ± 0.70  |
| AMP    | 60.59 ±1.31 | ND    | ND     | 46.25 ± 3.68 | 68.60 ± 4.72 | ND      |
| ATP, Mg$^{2+}$ | 57.00 ± 3.00 | ND    | ND | ND | ND | ND |

ND: Not determined
Figure legends

**Figure 1. Schematic of WNDP structural organization and sequence alignment of the P$_1$-ATPase N-domains.** (A) TGEA, DKTG, TGDN, and GDGxND represent sequence motifs conserved in all P-type ATPases. The N-domain is independently folded and inserted into the P-domain; arrows indicate the domain’s boundaries. Letters CxxC indicate six copper-binding repeats in the N-terminal domain. E1064, H1069, C1104, and R1151 (in bold) show relative positions of the residues mutagenized in this work. (B) In the alignment, the asterisks indicate strictly conserved residues, dots indicate conserved residues, and divergent regions are in parenthesis. The alignment was generated using ClustalW ([www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/)). Protein data base accession numbers are given in parenthesis for the following: ATP7B_HUMAN, WNDP (P35670); ATP7A_HUMAN, MNKP (Q04656); ATZN_ECOLI, ZntA (P37617); ATU2_YEAST, CCC2 (P38995); and CADA_STAUU, CadA (P20021).

**Figure 2. Characterization of the recombinant WNDP N-domain.** Representative CD spectrum and thermal denaturation data (inset) for the wt N-domain. Data were recorded with 0.4 mg/ml N-domain in 50 mM NaH$_2$PO$_4$, pH 7.0.

**Figure 3. The N-domain of WNDP binds ATP specifically and with high affinity.** (A) Upper panel: Raw isothermal titration calorimetry (ITC) data demonstrating saturable exothermic evolution of heat upon sequential additions of ATP to wt N-domain. Lower panel: Normalized ITC data for ATP and GTP titrations plotted versus molar ratio of nucleotide/N-domain. Data analysis using Origin 5.0 software indicates that the ATP binding data fit well
to a single binding-site model (1.03 +/- 0.03 sites). (B) The effect of ATP (filled circles) on intrinsic tryptophan fluorescence of the wt N-domain was monitored following incremental additions of the concentrated solution of the nucleotide as described under "Experimental Procedures." The titrations with buffer (open circles) lacking nucleotides were carried out in parallel to account for changes in the fluorescence because of dilution; these changes were then subtracted from each data point.

Figure 4. WNDP and MNKP N-domains coordinate nucleotides primarily through contacts with the adenosine moiety. (A) The average normalized ITC data for titrations of wt WNDP N-domain with various nucleotides. (B) The average normalized ITC data for titrations of wt MNKP N-domain with ATP and ADP. Inset: Purified wt N-domain of MNKP (2 µg/lane) was separated on Laemmli gel and stained with Coomassie blue R250. The K_d values derived from these experiments are summarized in Table 2.

Figure 5. Mutations of highly conserved E1064A and H1069Q disrupt nucleotide binding without affecting protein folding. (A) Overlay of CD spectra for the wt N-domain and the H1069Q mutant. The spectrum for the E1064A mutant looks identical to the wt N-domain. The deconvolution data are summarized in Table 1. (B) The average normalized ITC data for titrations of wt WNDP N-domain and E1064, H1069 mutants with ATP.

Figure 6. The C1104F mutation causes defects in the N-domain folding while the folding properties of a C1104A mutant are unaltered. (A) The CD spectra of the wt, C1104F, and C1104A WNDP N-domains. The deconvolution data for the C1104A mutant are given in
Table 1. (B) Comparison of the thermal denaturation profiles of wt WNDP N-domain, and the C1104 mutants.

Figure 7. Structural model of the ATP binding site in the N-domain of WNDP obtained via molecular dynamics and docking simulations. The calculated orientation of ATP in the binding site and the molecular hydrophobicity potential on the accessible surface of the N-domain are shown. The phosphate tail of ATP may adopt different orientations; only one of the possible conformations is presented. The locations of the residues E1064, H1069, C1104, and R1151 characterized in this work are marked. Hydrophobic and hydrophilic regions are shown in light and dark gray colors, respectively.
Figure 2
Figure 3

A

Time (min)

Molar Ratio
(Nucleotide/N-domain)

\( \mu \text{cal/sec} \)

\( \text{kcal/mole nucleotide} \)

GTP

ATP

B

\( F/F_0 \% \)

ATP, mM
Figure 4

A

![Graph A](image)

B

![Graph B](image)
Figure 5

A

Molar Ellipticity, \( \xi \)

Wavelength (nm)

-40
-30
-20
-10
0
10

Wild-type
H1069Q

B

cal/mole ATP

Molar Ratio (ATP/N-domain)

0
1
2
3
4
5
6
7
8

-5000
-4000
-3000
-2000
-1000
0

Wild-type
E1064A
H1069Q
Figure 6

A

![Graph A](image)

- Molar Ellipticity, $\varepsilon$
- Wavelength (nm)
- Wild-type
- C1104A
- C1104F

B

![Graph B](image)

- $\Delta$ Molar Ellipticity, $\Delta\varepsilon$
- Temperature (C$^\circ$)
- Wild-type
- C1104A
- C1104F
Figure 7