The Lys\textsuperscript{1010}–Lys\textsuperscript{1325} Fragment of the Wilson’s Disease Protein Binds Nucleotides and Interacts with the N-terminal Domain of This Protein in a Copper-dependent Manner*

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Wilson’s disease, an autosomal disorder associated with vast accumulation of copper in tissues, is caused by mutations in a gene encoding a copper-transporting ATPase (Wilson’s disease protein, WNDP). Numerous mutations have been identified throughout the WNDP sequence, particularly in the Lys\textsuperscript{1010}–Lys\textsuperscript{1325} segment; however, the biochemical properties and molecular mechanism of WNDP remain poorly characterized. Here, the Lys\textsuperscript{1010}–Lys\textsuperscript{1325} fragment of WNDP was overexpressed, purified, and shown to form an independently folded ATP-binding domain (ATP-BD). ATP-BD binds the fluorescent ATP analogue trinitrophenyl-ATP with high affinity, and ATP competes with trinitrophenyl-ATP for the binding site; ADP and AMP appear to bind to ATP-BD at the site separate from ATP. Purified ATP-BD hydrolyzes ATP and interacts specifically with the N-terminal copper-binding domain of WNDP (N-WNDP). Strikingly, copper binding to N-WNDP diminishes these interactions, suggesting that the copper-dependent change in domain-domain contact may represent the mechanism of WNDP regulation. In agreement with this hypothesis, N-WNDP induces conformational changes in ATP-BD as evidenced by the altered nucleotide binding properties of ATP-BD in the presence of N-WNDP. Significantly, the effects of copper-free and copper-bound N-WNDP on ATP-BD are not identical. The implications of these results for the WNDP function are discussed.

Copper is an essential trace element that serves as a cofactor for a variety of key metabolic enzymes, such as tyrosinase, cytochrome c oxidase, superoxide dismutase and many others. Inborn copper deficiency, known as Menkes disease, leads to a dramatic decrease in the activity of these enzymes, causing severe developmental delays, poor temperature control, and defects of connective and vascular tissues. Recent genetic studies linked this disease to various mutations in the ATP7A gene located on the X chromosome (1, 2). The ATP7A gene product, or Menkes disease protein, is a copper-transporting P-type ATPase, the primary function of which is to export dietary copper from intestinal cells for further delivery to various tissues.

Copper overload is as deleterious to cells as copper deficiency, presumably because of the ability of copper to participate in reactions that generate highly reactive oxygen species (3). Wilson’s disease (WD)\textsuperscript{1} is an autosomal disorder associated with vast accumulation of copper in the liver, brain, and kidneys (4). The disease is caused by mutations in the ATP7B gene that encodes another copper-transporting P-type ATPase (5–7). The Wilson’s disease protein (WNDP) and Menkes disease protein (MNKP) share over 50% sequence similarity and are likely to have similar mechanistic properties (8).

All P-type ATPases can be divided into five major branches based on their substrate specificity (9). WNDP and MNKP belong to the P\textsubscript{2}-(P2x) subfamily of the P-type ATPases (10, 11). All members of this subfamily utilize energy of ATP hydrolysis to transport either transition or heavy metals across cell membranes, and all have characteristic metal-binding motifs at the N-terminal portion of the molecule (Fig. 1). In contrast, the P\textsubscript{2}-ATPases are involved in transport of alkali or alkali earth metals and, as a rule, lack the characteristic metal-binding sites at their N termini. N\textsuperscript{2},K\textsuperscript{2}-ATPase is a typical representative of the P\textsubscript{2}-ATPase subfamily with known nucleotide binding properties, and it has been utilized in this study for comparative purposes.

Although mechanistic properties of the P\textsubscript{2}-ATPases are well studied, very few members of the P\textsubscript{2}-ATPase subfamily have been functionally characterized (12–15). ATP-dependent transport has been recently demonstrated for MNKP (16), but the detailed molecular mechanism for either MNKP or WNDP remains unknown. Location of the copper-binding sites in the N-terminal domain, separately from the ATP-binding region and the transmembrane cation-translocation pathway (Fig. 1), suggests that the ATP-driven copper transport by WNDP and MNKP is likely to involve cooperation between several protein domains. The results obtained in this work substantiate this hypothesis.

At present, purification and reconstitution procedures for either MNKP or WNDP are unavailable, severely limiting the detailed analysis of their structure and function. However, the use of recombinant proteins corresponding to the putative functional domains of WNDP and MNKP proved to be very informative. Recently, we and others produced and biochemically characterized the recombinant N-terminal copper-binding domain of both WNDP and MNKP (17–20). The studies revealed

\textsuperscript{1} The abbreviations used are: WD, Wilson’s disease; WNDP or WND, Wilson’s disease protein; MNKP, Menkes disease protein; N-WNDP or N-WND, N-terminal domain of the Wilson’s disease protein; ATP-BD, ATP-binding domain of the Wilson’s disease protein; MAM5 loop, ATP-binding domain of Na\textsuperscript{+},K\textsuperscript{+}-ATPase; MBP, maltose-binding protein; N-WNDP-MBP, maltose-binding protein fusion of N-WNDP; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)-ATP; Ab, antibody; IPTG, isopropyl-\textbeta-D-thiogalactopyranoside; Ni-NTA, nickel-nitritoltriacetic acid; MOPS, 4-morpholinepropanesulfonic acid; GuHCl, guanidine hydrochloride.

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that this 70-kDa domain binds copper specifically in vivo and in vitro with stoichiometry of 5.5–6 copper/domain, suggesting that each of the six GMMTXCX motifs present in this domain participate in copper coordination (17, 18). Furthermore, it has been shown that copper binds to Cys residues in the GMMTXCX motif as Cu\(^{2+}\) via novel linear coordination (20, 21).

Present studies demonstrate that in addition to its copper binding sites, WNDP participates in copper coordination (17, 18). Furthermore, it has been shown that copper binds to Cys residues in the GMMTXCX motif as Cu\(^{2+}\) via novel linear coordination (20, 21). Present studies demonstrate that in addition to its copper binding sites, WNDP participates in copper coordination (17, 18). Furthermore, it has been shown that copper binds to Cys residues in the GMMTXCX motif as Cu\(^{2+}\) via novel linear coordination (20, 21).

In the case of WNDP and MNKP, analysis of recombinant domains offers another important advantage: it allows us to assess individually their overall folding and functional characteristics. This information is particularly important if one wants to understand how mutations in certain regions of WNDP and MNKP alter their specific properties. A number of the WD-causing mutations have been identified in the Lys\(^{1010}\)–Lys\(^{1325}\) region of WNDP (Fig. 1) (22, 23). For only one of them, His\(^{1070}\) to Gln, there is evidence suggesting that the substitution of His for Gln is associated with the WNDP misfolding in a cell (24). The specific molecular consequences for most of the other mutations are still unknown. Consequently, the ability to analyze folding and functional properties of normal and mutant domains of WNDP would be very valuable. In this paper we describe several biochemical procedures that can be utilized for these purposes.

The WNDP segment including the amino acid residues Lys\(^{1010}\)–Lys\(^{1325}\) and the homologous region of MNKP were predicted to act as the ATP-binding domains (ATP-BDs) of these proteins. The suggestion was based on the presence in this region of several highly conserved motifs (Fig. 1) previously shown to be involved in ATP binding and hydrolysis in such P\(_2\)-type ATPases as Na\(^{+}\)-K\(^{+}\)-ATPase and Ca\(^{2+}\)-ATPase. At the same time, the overall homology between the corresponding segments of WNDP (P\(_2\)-ATPase) and Na\(^{+}\)-K\(^{+}\)-ATPase or Ca\(^{2+}\)-ATPase (P\(_2\)-ATPases) is only 18–23%, suggesting that the specific nucleotide binding properties and the regulatory mechanisms modulating binding of ATP may differ for the P\(_2\) and P\(_2\)-ATPases. Our analysis of the ATP-binding domain of WNDP supports this hypothesis.

In this paper, we provide experimental evidence that the recombinant Lys\(^{1010}\)–Lys\(^{1325}\) fragment of WNDP forms an independently folded ATP-BD, which has distinct nucleotide binding properties and measurable ATPase activity, that ATP-BD interacts with the N-terminal copper-binding domain (N-WNDP) in a copper-dependent manner, and that the copper-dependent domain-domain interactions induce conformational changes in ATP-BD. These results shed a light on some steps in molecular mechanism of WNDP and highly homologous MNKP.

**EXPERIMENTAL PROCEDURES**

**Materials**—The α-n-WND is a polyclonal antibody (Ab) developed against the peptide GMMTCCCCVHNIE, which corresponds to the sixth metal-binding repeat Gly\(^{722}\)–Glu\(^{796}\) in the N-WNDP. The polyclonal Ab a-AB was raised against the recombinant ATP-BD (for cloning and expression of ATP-BD, please see below). The Ab against the His tag was purchased from Qiagen, anti-maltose-binding protein antibodies were obtained from NeoMarkers. The construct encoding the amino acid residues Lys\(^{1010}\)–Lys\(^{1325}\) was amplified by polymerase chain reaction using the following primers: WD-ATP-Fwd, 5′-GAATCTCATATGAGGGAGGACAG-3′, and WD-ATP-Rev, 5′-GAATTCATATGCTGTTATCTTGAGGAC-3′. The polymerase chain reaction product was used as template to incorporate NdeI sites that were used to clone the polymerase chain reaction product into the PET-28b vector (Novagen). The (lack of) unwanted mutations in the obtained construct was verified by DNA sequencing. The construct was used to transform Escherichia coli BL21 (DE3) cells; the protein expression was induced by treatment of cells with either 0.1 mM IPTG (for purification from soluble cell fraction) or 1 mM IPTG (for purification from inclusion bodies) for 1 h at room temperature.

E. coli cells were resuspended in lysis buffer prepared by dissolving 1 Complete protease inhibitor tablet (Roche Molecular Biochemicals) in 50 ml of 500 mM NaCl, pH 7.5, and then lysed by passing twice through the French press (American Instrument Co.) at 20,000 p.s.i. To decrease viscosity, lysate was additionally passed two times by syringe through a medium gauge needle. Soluble proteins were separated from the insoluble fraction following centrifugation of the lysate at 30,000 g for 20 min. To analyze the distribution of ATP-BD between soluble and insoluble fractions, insoluble material was re-suspended in the lysis buffer to the same volume as soluble protein. Identical aliquots were taken from both fractions and separated by 10% Laemmli gel, and then proteins were transferred to Immobilon-P membrane as described (25). The amount of ATP-BD in each fraction was determined by staining of the blot with the a-AB Ab (dilution 1:20,000) followed by densitometry of immunostained bands. The solubility of ATP-BD depended on the level of expression. Following induction with 1 mM IPTG, almost all ATP-BD was quickly deposed into the inclusion bodies. When protein expression was significantly lowered by changing the IPTG concentration from 1.0 to 0.1 mM, the overall amount of produced ATP-BD decreased greatly, but proportionally more protein was found in the soluble fraction. Analysis of the distribution of ATP-BD revealed that at 0.1 mM IPTG ~20% of the produced ATP-BD was soluble, and only this protein was used for characterization of its functional properties. The expression of the M4M5 loop and the ABC domain and preparation of soluble fractions containing these proteins were carried out as described below for ATP-BD.

**Purification of the His\(_6\)-tagged Proteins**—Purification of the His\(_6\)-tagged proteins (ATP-BD, M4M5 loop, and ABC domain) by Ni\(^{2+}\) affinity chromatography was carried out at room temperature except where indicated. The soluble fraction of lysate was loaded on Ni-NTA-agarose resin (Qiagen); the amount of Ni-NTA-agarose was used to determine the yield of purified protein (200 μl slurry/mg for M4M5 loop, 1 ml/mg for ATP-BD). Imidazole was added to the lysate to 50 mM prior to binding. Following a 1-h incubation of the lysate with constant agitation, the resin was applied on a column and washed with at least 30 volumes of wash buffer (50 mM imidazole, 50 mM Tris, 500
mM NaCl, pH 7.5). The target proteins were then eluted in several fractions of elution buffer (200 mM imidazole, 50 mM Tris, 500 mM NaCl, pH 7.5). Protein concentration was determined by the method of Bradford (26). 0.5–2.0 µg of protein were loaded onto a 10% SDS-polyacrylamide gel, and protein purity was ascertained by Coomassie staining. For analysis of folding and nucleotide binding properties, copper-free and copper-bound proteins (M4M5 loop and N-WNDP) were purified using the same protocol (17).

The Recombinant N-terminal Domain of WND—The recombinant N-terminal domain of WND tagged to the maltose-binding protein (N-WND-MBP) in its copper-bound and copper-free form was overexpressed as described previously (17). The recombinant 42-kDa maltose-binding protein (MBP) was generated using the pMal-c vector (New England Biolabs) and purified using the same protocol (17).

The ATP-BD Folding—The ATP-BD folding was analyzed using the intrinsic tryptophan fluorescence. The measurements were performed on a model PTI-QM1 (Photon Technology International) fluorimeter. Emission spectra were recorded from 305 to 405 nm (bandwidth, 2 nm) with an excitation wavelength of 295 nm (bandwidth, 3 nm) for 1.25 µM ATP-BD solution in 50 mM MOPS, pH 7.5, 500 mM NaCl in the absence or presence of 6 M guanidine hydrochloride (GuHCl). The solution of tryptophan in the above buffer at concentration similar to concentration of ATP-BD was used in these experiments as a standard. For determination of which the effect of nucleotides on the Trp fluorescence was tested, the excitation was set at 295 nm, emission was set at 345 nm, and 800 µM of the same protein solution was titrated with 2 µl aliquots of ATP or AMP to obtain final concentration ranging from 0 to 800 µM. Identical aliquots of buffer were added to control sample to account for a change in the fluorescence because of dilution; the final increase in volume was less than 2%.

The ATP-BD Folding—Fluorescence changes upon binding of TNP-ATP to ATP-BD or the M4M5 loop were measured at room temperature in transparent polystyrene 96-well plates (Falcon, Becton Dickinson) using a PLUOstar microplate reader (BMG Labtechologies Inc.). The excitation was set at 390 nm, and the emission was recorded at 538 nm. The reaction was carried out in 50 mM MOPS pH 7.5 buffer containing various NaCl concentrations (20–500 mM). The 1 µl aliquots of the TNP-ATP stock solution were added to 100 µl of a 5 µM protein solution for final concentrations ranging from 0 to 40 µM. (The increase in the reaction volume did not exceed 10% and was taken into account.) To determine the background fluorescence, identical TNP-ATP titrations were made in 50 mM MOPS pH 7.5 buffer containing 20–500 mM NaCl. The apparent binding affinities of ATP-BD and M4M5 loop for TNP-ATP were determined after subtracting the background TNP-ATP fluorescence using SigmaPlot software package. For most experiments, NaCl concentration was kept at 20 mM, because both the TNP-ATP affinity and the fluorescence increase upon TNP-ATP binding to ATP-BD were higher at lower salt concentrations. (For example, the $K_a$ for TNP-ATP at 20 mM NaCl was 1.89 ± 0.72 µM, whereas at 500 mM the $K_a$ was 6.3 µM ± 1.5.) These differences in the TNP-ATP binding parameters are likely to reflect small changes in the nucleotide-binding pocket, because changes in NaCl concentrations do not have noticeable effect on overall protein folding as evidenced by the Trp fluorescence spectra.

The effect of N-WND on TNP-ATP binding was analyzed as described above using equimolar amounts of ATP-BD and either copper-free or copper-bound N-WNDP in 100 µl of MOPS, pH 7.5, 500 mM NaCl buffer. The background TNP-ATP binding to N-WNDP was similar for copper-free and copper-bound proteins and was subtracted.

The Displacement of TNP-ATP by Nucleotides—The displacement of TNP-ATP by nucleotides was analyzed using PTI-QM1 fluorimeter with excitation set at 410 nm and emission set at 545 nm. For most experiments, NaCl concentration was kept at 20 mM, because both the TNP-ATP affinity and the fluorescence increase upon TNP-ATP binding to ATP-BD were higher at lower salt concentrations. (For example, the $K_a$ for TNP-ATP at 20 mM NaCl was 1.89 ± 0.72 µM, whereas at 500 mM the $K_a$ was 6.3 µM ± 1.5.) These differences in the TNP-ATP binding parameters are likely to reflect small changes in the nucleotide-binding pocket, because changes in NaCl concentrations do not have noticeable effect on overall protein folding as evidenced by the Trp fluorescence spectra.

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The ATPase Activity of ATP-BD—The ATPase Activity of ATP-BD was measured using the EnzChek® Phosphate Assay (Molecular Probes). Inorganic phosphate released during ATP hydrolysis was utilized by purine nucleoside phosphorylase to convert the substrate 2-amino-6-mercaptopurine ribose to riboside 1-phosphate and 2-amino-6-mercaptopurine. This leads to a shift in maximum absorbance from 330 nm for the substrate to 360 nm for the product that can be monitored spectrophotometrically. For the ATPase measurements, the purified ATP-BD was dialyzed against the reaction buffer (20 mM Tris-HCl, 1 mM MgCl$_2$, 200 mM NaCl, pH 7.5) and then incubated in 10–20 µl of volume with 1 µg ATP at protein concentration of 0.1 mg/ml for 15 min at 37°C. The reaction was stopped by transferring samples on ice, and the amount of released phosphate was determined using the Molecular Probes protocol. The His tag protein corresponding to the fragment of the WNDP copper-binding domain was expressed in the same strain of cells as ATP-BD and then purified under identical conditions. The elution fractions containing this protein or just buffer were used as negative controls.

The Domain-Domain Interactions—The domain-domain interactions were evaluated by an optimized quantitative copurification procedure. In preparation for the interaction experiment, a preliminary purification of ATP-BD and the control His tag proteins was carried out to determine the relative molar concentrations of the target proteins in their respective lysates. The amount of each His-tagged protein was quantified by densitometry of the Coomassie-stained gel using bovine serum albumin as a standard. The amount of ATP-BD in the soluble portion of the lysate was lower than that of the M4M5 loop and the ABC protein, consequently the soluble lysate from the nontransformed BL21 cells was used to dilute the M4M5 loop and ABC lysates appropriately and bring the concentration of all His tag proteins to the same molar concentration.

Miniature affinity chromatography columns were constructed using glass wool, standard 1-ml pipette tips, and 5 µl of Qiagen Ni-NTA agarose resin. The equal amounts of the primary His tag proteins (ATP-BD, M4M5 loop, and ABC) or lysate from nontransformed BL21 were incubated with the Ni-NTA resin. The resin was washed as described in the purification procedure above, and then the secondary proteins (NWN-MBP, Cu, NWND-MBP + Cu, or MBP) were run over the columns. After two additional wash steps with 200 bed volumes of buffer, the bound proteins were eluted in 200 mM imidazole. The presence of proteins in the elution fractions was detected by Western blotting using the appropriate Ab. The anti-MBP Ab was used at a 1:40,000 to 1:100,000 dilution, the a-ABD and a-N-WND were used at 1:20,000 dilution, and protein bands were visualized using Pierce SuperSignal chemiluminescence procedure.

Domain-Domain Interaction Assay on Amylose Resin—For domain-domain interaction assay on amylose resin, cells expressing N-WNDP with or without copper (for expression conditions see Ref. 17) from a 1-liter culture were lysed in 40 ml of PB buffer (50 mM sodium phosphate, pH 7.5, 250 mM NaCl) and then incubated in 10–20 ml of lysis buffer, the mixture was incubated on ice for 20 min with occasional stirring. Then resin was washed three times with 500 µl of PB buffer and once with 500 µl of TB buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl). Next, 1 µg of ATP-BD in 100 µl of TB buffer was added to the resin and incubated at room temperature for 15 min. The unbound ATP-BD was removed, and the resin was washed twice with 500 µl of TB buffer. Finally, all proteins were eluted from the resin with 60 µl of PB buffer containing 10 mM maltose. The elution fractions were analyzed for the presence of N-WND or ATP-BD by immunoblotting using appropriate antibodies.

The Effect of N-WND on the Nucleotide Binding Properties of ATP-BD—To test the effect of N-WND on the nucleotide binding properties of ATP-BD, TNP-ATP at 0.5 µM was mixed with 2.5 µM ATP-BD solution in 50 mM MOPS, 200 mM NaCl, pH 7.5 buffer and then 2.5 µM of either copper-free or copper-bound N-WND-MBP was added to the mixture. The mixture was then titrated with ATP, AMP (0–800 µM) or with the same volumes of buffer as a control using PTI-QM1 fluorimeter with excitation set at 410 nm and emission set at 545 nm. The protein fluorescence before TNP-ATP addition served as a background and was subtracted.

**RESULTS**

**Expression and Purification of ATP-BD**—The recombinant Lys$^{1010}$–Lys$^{1325}$ fragment, corresponding to the putative ATP-BD of WNDP, was produced in E. coli as a His tag fusion protein.
protein following induction of protein expression with IPTG (Fig. 2A). The calculated molecular mass of the ATP-BD His tag fusion, 38 kDa, agreed well with the molecular weight of the expressed protein (Fig. 2). Soluble ATP-BD was purified to about 95% of homogeneity (Fig. 2B), and the yield of purified protein (200–500 μg from 1 liter of the cell culture), although limited, was sufficient to determine the major functional characteristics of this putative ATP-binding fragment.

Intrinsic Trp Fluorescence as a Measure of the ATP-BD Folding—ATP-BD has a single Trp residue at the position corresponding to Trp1154 of the full-length WNDP (Fig. 1). In general, Trp fluorescence is very sensitive to local environment, a property that can be utilized to monitor protein folding or protein interactions with ligands. To determine whether the purified ATP-BD is folded, the local environment around the Trp1154 residue was examined by comparing the steady-state fluorescence emission spectrum of ATP-BD with the spectrum of free 1-tryptophan present at the same molar concentration.

The ability of ATP-BD to bind the nucleotides was further verified in competition experiments in which TNP-ATP fluorescence was monitored in the presence of increasing concentrations of either ATP or AMP (Fig. 5). ATP is structurally similar to TNP-ATP and is expected to induce fluorescence decrease by displacing TNP-ATP from the nucleotide-binding site. Fig. 5 demonstrates that the additions of ATP lead to a saturable decrease in TNP-ATP fluorescence (the apparent $K_a$ for ATP is 268 ± 23 μM), in agreement with the above prediction.

AMP, in contrast, was expected to have little or no effect on TNP-ATP fluorescence, because AMP was earlier shown not to bind or bind with very low affinity to the recombinant ATP-
Fig. 5. The effects of ATP, ADP, and AMP on TNP-ATP fluorescence. TNP-ATP was first bound to ATP-BD in 50 mM MOPS, 20 mM NaCl, pH 7.5 buffer, and the increase in fluorescence at 545 nm caused by TNP-ATP-binding ($F_0$) was taken as 100%. $A$, the effect of ATP (filled symbols) or AMP (open symbols) was monitored following incremental additions of the concentrated solution of corresponding nucleotides as described under “Experimental Procedures.” The titrations with buffer 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 to get $F$ values. The changes in the TNP-ATP fluorescence caused by nucleotide binding is expressed as a percentage of initial fluorescence ($F/F_0 \times 100\%$). The $arrow$ indicates the position at which ATP (filled triangles) was added to the sample preincubated with AMP (open circles) and AMP (open triangles) was added to ATP-pretreated sample (filled circles). $B$, the effects of ATP (filled symbols) and ADP (open symbols) on TNP-ATP fluorescence were compared under the same conditions as above.

binding domains of the P$_2$-type ATPases (29, 31). Contrary to this expectation, AMP decreased fluorescence of bound TNP-ATP, although differently than ATP. The AMP effect on fluorescence intensity was significantly smaller than the ATP effect even at saturating concentrations of the nucleotide (Fig. 5A); however, the apparent affinity for AMP ($K_\text{d} = 79 \pm 18 \mu M$) was higher than that for ATP.

The nucleotides can decrease the TNP-ATP fluorescence intensity either directly by displacing TNP-ATP from its binding site or indirectly by binding at a separate site and inducing changes in the TNP-ATP environment. It seems unlikely that AMP would directly compete with TNP-ATP with higher affinity than ATP, as our data seem to suggest. Rather, AMP is likely to bind at a separate site and cause modest fluorescence decrease by inducing changes in protein structure. The apparent affinity for AMP in this case could be higher than that for ATP, because it would reflect direct binding of the nucleotide, whereas the $K_\text{d}$ for ATP would measure the ability of ATP to displace TNP-ATP.

To test the possibility that ATP-BD binds ATP and AMP at two separate sites, the TNP-ATP displacement experiments were repeated with the following modification. When the nucleotide-induced decrease in TNP-ATP fluorescence reached a saturation level, the increasing concentrations of the other nucleotide were added to the sample, i.e. AMP was added to the sample pretreated with ATP and vice versa (Fig. 5A, arrow). Addition of ATP to the AMP-bound protein led to further decrease in TNP-ATP fluorescence, indicating that ATP binds to ATP-BD and affects TNP-ATP even in the presence of saturating AMP. In contrast, additions of AMP to ATP-pretreated sample had little effect on fluorescence, which is precisely what one would expect if ATP already displaced TNP-ATP from its nucleotide-binding site. (In fact, AMP consistently produced a small increase in the fluorescence of the ATP-pretreated sample, further illustrating that AMP was bound to ATP-BD, but did not compete with TNP-ATP.)

Neither ATP nor AMP decreases TNP-ATP fluorescence to the background level. This effect was observed with many ATP-binding proteins (29, 31, 32), and it is likely to reflect an additional, nonspecific, binding of TNP-ATP to proteins through the TNP-moiety.

Binding of AMP at the site separate from ATP suggested two possibilities: (i) either ATP-BD contains an additional, noncatalytic, nucleotide-binding site or (ii) in isolated ATP-BD the catalytic site is split into two distinct nucleotide-binding regions, which accommodate separately the substrate and product of the reaction. This latter possibility seems plausible given a well defined two-subdomain structure of the ATP-binding domain of Ca$^{2+}$-ATPase, the P-type ATPase for which crystal structure has been solved (33). To gain more insight into the nature of the second nucleotide-binding site in ATP-BD, we repeated the TNP-ATP displacement experiments using ATP (the substrate) and ADP (the product) (Fig. 5B). Our reasoning was as follows.

If the AMP-binding site represents a noncatalytic site, then AMP would likely to interact with the ATP-binding region (where it normally binds during catalysis) and in the nucleotide displacement experiments ADP would behave similarly to ATP. In contrast, if AMP was interacting with the product-binding site, then AMP would bind to this site, and its effect on TNP-ATP fluorescence would resemble the AMP effect. As shown in Fig. 5B, the behavior of ADP in the nucleotide displacement experiments was strikingly similar to AMP, as evidenced by the $K_\text{d}$ value (85.2 ± 4.7 $\mu M$), the overall effect on TNP-ATP fluorescence, and the ability of ADP and ATP to interact with ATP-BD simultaneously (see experiments marked on Fig. 5 by an arrow). These results are consistent with the hypothesis that ATP-BD has separate binding regions for the substrate and product of the ATPase reaction.

The ATP Hydrolysis Measurements—We further tested whether the purified ATP-BD can hydrolyze ATP. As shown in Fig. 6, incubation of ATP-BD with ATP led to release of inorganic phosphate, detected by increase of absorbance at 360 nm using EnzChek phosphate assay (see “Experimental Procedures”). The specific activity (67.6 ± 15.7 nmol P$_i$/mg/min) was reproducible for protein batches with different ATP-BD concentrations, and essentially no ATPase activity was detected in control eluates from the Ni$^{2+}$ affinity resin, containing a similar amount of unrelated His tag protein or just a buffer (Fig. 6). Interestingly, the ATPase activity of isolated ATP-BD, although fairly low compared with known values for the full-
length P-type ATPases, is similar to the activity of soluble protein from *Methanococcus jannaschii*, which structurally corresponds to the ATP-binding domain of P-type ATPases (34).

**ATP-BD Interacts Specifically with N-WNDP**—The above experiments confirmed that the recombinant fragment Lys<sup>1012</sup>-Lys<sup>1326</sup> folds independently and is sufficient for the nucleotide binding by WNDP. It seemed very likely that the ATP-driven copper transport by WNDP would require a crosstalk between two major functional domains participating in this process: the copper-binding domain and the ATP-binding domain. Consequently, we tested whether ATP-BD is involved in specific protein-protein interactions with N-WNDP, the copper-binding domain of WNDP. To do that, a copurification strategy has been employed.

ATP-BD was produced as a His tag fusion, whereas N-WNDP was expressed as a maltose-binding protein fusion (N-WNDP-MBP) in either copper-free or copper-bound form (17). By itself, N-WNDP-MBP does not bind to the Ni-NTA resin (Fig. 7A), in agreement with our earlier data on the N-WNDP-MBP metal-binding specificity (17). In contrast, when ATP-BD was bound first to Ni-NTA, significant retention of N-WNDP-MBP-MBP was observed (Fig. 7A), indicating interaction between these two domains.

The observed interaction is specific, because neither ABC (unrelated His tag protein) nor the M4M5 loop (ATP-binding domain of Na<sup>+</sup>-K<sup>+</sup>-ATPase with the size and the nucleotide binding properties similar to ATP-BD) were able to retain N-WNDP-MBP-MBP with the efficiency comparable with ATP-BD (Fig. 7B). Also, no significant interactions were detected between ATP-BD and MBP, further confirming that the retention of N-WNDP-MBP-MBP on a ATP-BD-Ni-resin was due to interactions between the ATP-BD and N-WNDP domains.

Strikingly, the interactions between N-WNDP and ATP-BD were found to be copper-dependent. When identical amounts of copper-free and copper-bound N-WNDP-MBP were loaded on the ATP-BD-containing resin, significantly less copper-bound N-WNDP was coeluted with ATP-BD (Fig. 7, A and B), suggesting that the interaction site on the N-WNDP-MBP was altered by copper binding.

A similar difference in binding of ATP-BD by copper-free and copper-bound N-WNDP was observed when the experiments were repeated in the reverse orientation, i.e. when equal amounts of N-WNDP-MBP with or without copper were bound to amylose resin, and purified ATP-BD was let to interact with these proteins (Fig. 7C). As in the first series of experiments, more ATP-BD was associated with copper-free N-WNDP-MBP than with copper-bound N-terminal domain, and no significant binding was detected with empty resin or with the resin containing bound MBP, further confirming the specificity and copper dependence of domain-domain interactions.

**Interdomain Interactions Lead to Distinct Conformational Changes in ATP-BD**—To determine whether the interdomain interactions had some functional consequences, we analyzed the effect of the N-terminal domain on TNP-ATP binding by ATP-BD and on nucleotide competition. As shown in Fig. 8, the apparent affinity of ATP-BD for TNP-ATP decreased in the presence of either copper-free or copper-bound N-WNDP, suggesting that the domain-domain interactions led to a conformational change in ATP-BD. The copper-free N-WNDP induced a larger increase in the *K<sub>i</sub>* for TNP-ATP (from 1.89 ± 0.24 to 10.36 ± 0.46 μM) than the copper-bound N-WNDP (from 1.89 ± 0.72 to 6.72 ± 1.45 μM) in agreement with our data showing that the copper-free N-WNDP interacted more tightly with ATP-BD. (The background binding of TNP-ATP to N-WNDP
DISCUSSION

In this paper we provide experimental evidence that the Lys^{1010-1325} segment of the Wilson’s disease protein represents an independently folded ATP-BD of this protein and that ATP-BD binds the nucleotides, hydrolyzes ATP, and interacts with the N-terminal copper-binding domain of WNDP in a copper-dependent manner. We further demonstrate that the domain-domain interactions lead to conformational changes in ATP-BD.

A number of biochemical tests have been utilized here to describe the characteristic properties of ATP-BD. The obtained information and developed procedures form an experimental framework for analysis of various WD-causing mutations that have been mapped to the Lys^{1010-1325} region (22, 23). The precise molecular consequences of these mutations for the WNDP structure and function can now be assessed by (i) analyzing the ATP-BD folding using intrinsic fluorescence of Trp^{1454}, (ii) measuring ATPase activity of ATP-BD, (iii) characterizing the ATP binding properties using TNP-ATP binding and nucleotide competition studies, and (iv) testing the effect of mutations on the copper-dependent interactions between ATP-BD and N-WNDP. The experimental approaches described here are also applicable for analysis of the Menkes disease protein, which is highly homologous to WNDP.

Nucleotide Binding Properties of ATP-BD—ATP-BD binds TNP-ATP with affinity in a low micromolar range, and ATP competes with TNP-ATP for binding to ATP-BD. These properties and observed nucleotide affinities are similar to characteristics previously reported for the recombinant ATP-binding domains of P2-ATPases (29, 31). Further analysis of nucleotide binding properties of ATP-BD yielded several unexpected and interesting results. First, we found that ATP-BD has a fairly high affinity for both ADP and AMP, indicating that ATP-BD and possibly the full-length WNDP do not discriminate between these nucleotides as well as P2-ATPases. This difference in nucleotide selectivity between the ATP-binding domains of P2-ATPases and ATP-BD is unlikely due to a less precise folding of ATP-BD, because ATP-BD not only has higher affinity for ADP and AMP but it also can hydrolyze ATP. This latter property was not reported for the isolated domains of the P2-type ATPases.

Secondly, AMP and ADP appear to bind at the site that is different from the ATP-binding site, suggesting that in ATP-BD the substrate and product-binding regions are separated. Recent crystal structure of Ca^{2+}-ATPase offers possible explanation for this unexpected result. The cytosolic loop of Ca^{2+}-ATPase corresponding to ATP-BD consists of two subdomains, the adenosine domain, which in crystals binds TNP-AMP molecule, and the phosphorylation domain (33). It is tempting to speculate that in ATP-BD ATP binds in close proximity to phosphorylation domain, whereas ADP and AMP interact with the adenosine domain. Binding of ATP (the substrate) and ADP (the product) at different sites observed in our experiments also suggests an interesting possibility that following ATP hydrolysis, the nucleotide migrates from the ATP site to the ADP site when two subdomains come together.

We carried out molecular modeling studies using the crystallographic information and sequence alignments between ATP-BD and the ATP-binding domain of Ca^{2+}-ATPase. These studies demonstrate that the amino acid sequence and three-dimensional structure of the regions corresponding to the phosphorylation subdomains of these two proteins are very similar, whereas the adenosine domains differ considerably in the position, length, and number of various loops (not shown). The difference in the structure of the N domains may explain why the nucleotide selectivity of ATP-BD and the nucleotide-binding domains of P2-ATPases are different.

Domain-Domain Interactions—The observation that ATP-BD interacts with N-WNDP and that binding of copper to N-WNDP alters this interaction seems particularly interesting. Although the involvement of N-WNDP in copper binding is firmly established, the precise role of the N-terminal domain (Fig. 1) for the WNDP function is still unclear. Recent studies revealed that the large portion of the N-terminal domain including most of the metal-binding sites is not critical for the overall transport activity of human copper-transporting...
the TNP-ATP fluorescence of this initial complex ($F_0$) was taken as 100%. The change in TNP-ATP fluorescence induced by ATP (A) or AMP (B) was monitored following incremental additions of the concentrated solution of corresponding nucleotide as described in Fig. 5, and the results were compared with the data obtained in the absence of N-WNDP (filled circles).

Fig. 9. The copper-free and copper-bound N-WNDP have different effects on nucleotide binding by ATP-BD. TNP-ATP was bound to ATP-BD as in Fig. 5, and then either copper-free N-WNDP (open circles) or copper-bound N-WNDP (triangles) was added to the mixture, and the TNP-ATP fluorescence of this initial complex ($F_0$) was taken as 100%. The change in TNP-ATP fluorescence induced by ATP (A) or AMP (B) was monitored following incremental additions of the concentrated solution of corresponding nucleotide as described in Fig. 5, and the results were compared with the data obtained in the absence of N-WNDP (filled circles).

Fig. 10. Sequence alignment of the ATP-binding region of various copper-transporting ATPases (fragment corresponding to residues Ser<sup>1065</sup>-Asn<sup>1223</sup> of WNDP). CCC2-yeast copper-transporting ATPase (NP_010556), PacA-putative copper-transporting ATPase from Synechococcus sp. PCC7942 (ATCS_SYNP7), CopA-copper-transporting ATPase from Enterococcus hirae (COPA_ENTHR), WNDP (AT7B_HUMAN), and MNKP (AT7A_HUMAN). Identical residues are boxed, and similarities are shaded.

ATPases (35–38). At the same time, copper binding to the N terminus is required for the intracellular redistribution of WNDP and homologous Menkes disease protein, a process that is probably triggered by a copper-dependent conformational change in the protein (39, 40).

Earlier, we and others have proposed that the N-terminal copper-binding domain may act as a regulator of WNDP (5, 38). The results obtained in these studies agree with this hypothesis and give us a hint as to how N-WNDP may carry out its regulatory function. We demonstrated that two functional domains of WNDP interact specifically and that binding of copper to the N terminus alters the domain-domain interactions (Fig. 7). Furthermore, the copper-bound and copper-free N-WNDPs induce distinct conformational changes in ATP-BD (Figs. 8 and 9). We hypothesize that in WNDP, the copper-free N-terminal domain binds tightly to ATP-BD and holds ATP-BD in certain conformation. Binding of copper to N-WNDP decreases domain-domain interactions, which allows ATP-BD to adopt a new conformation with higher affinity for ATP. This hypothesis is consistent with our observations that copper-free N-WNDP binds tighter to ATP-BD and that the affinity of ATP-BD for nucleotide triphosphates is the lowest in the presence of copper-free N-WNDP. Interestingly, the results suggesting the interdomain interactions between the N terminus and the ATP-binding domain have been recently obtained for another P-type ATPase, Pmr1 (41).

It has to be stressed that our data and the proposed hypothesis do not contradict the studies on copper-induced WNDP trafficking. In fact, it is very likely that the observed change in domain-domain interactions in response to copper leads to the exposure of previously hidden sites on either domain for interactions with the components of cell trafficking machinery.

Analysis of domain-domain interaction data shown in Fig. 7 and the results of the nucleotide-displacement experiments in Fig. 9 give us some hints as to how N-WNDP and ATP-BD may interact. The TNP-ATP displacements demonstrate that both nucleotide-binding sites in ATP-BD (for ATP and AMP) are affected by N-WNDP, suggesting that several regions of ATP-BD could be involved in recognition of N-WNDP. It is interesting that although we did not see any interactions of N-WNDP with unrelated protein ABC, weak interaction was detected with the ATP-binding domain of Na<sup>+</sup>,K<sup>+</sup>-ATPase (Fig. 7B), which has a sequence and folding homology to the phosphorylation region of ATP-BD. Therefore, it seems plausible that phosphorylation region may somehow contribute to domain-domain interactions, although most of the interaction occurs through other, unique regions of ATP-BD.

Recently, a number of bacterial ATPases homologous to WNDP and MNKP have been described (12–15). Unlike their mammalian counterparts, bacterial ATPases have only one or two metal-binding repeats at the N-terminal portion of their molecule. At the same time, the bacterial copper-ATPases are missing 45–50 amino acid residues in the region corresponding to ATP-BD of WNDP (Fig. 10). A similar segment is absent in the sequence of CCC2, a yeast homologue of MNKP and WNDP, and RAN1, a putative copper-ATPase from Arabidopsis thaliana, which also have only two metal-binding repeats. It is tempting to speculate that the extra protein segment in ATP-BD of WNDP and MNKP could be important for regulatory interactions with the extended part of the N-terminal domain of these proteins. Consistent with this hypothesis, the identified region has no homology with the corresponding segments of the P<sub>2</sub>-ATPases. In summary, this paper characterizes the nucleotide-binding domain of WNDP, provides some insight into the possible mechanism by which N-terminal copper-binding domain may regulate WNDP, and supplies convenient tools for detailed analysis of mutations identified in the Wilson's disease patients.
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The Lys$^{1010}$–Lys$^{1325}$ Fragment of the Wilson's Disease Protein Binds Nucleotides and Interacts with the N-terminal Domain of This Protein in a Copper-dependent Manner

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