Expression and Mutagenesis of ZntA, a Zinc-Transporting P-Type ATPase from *Escherichia coli*†

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ABSTRACT: Cation-transporting P-type ATPases comprise a major membrane protein family, the members of which are found in eukaryotes, eubacteria, and archaea. A phylogenetically old branch of the P-type ATPase family is involved in the transport of heavy-metal ions such as copper, silver, cadmium, and zinc. In humans, two homologous P-type ATPases transport copper. Mutations in the human proteins cause disorders of copper metabolism known as Wilson and Menkes diseases. *E. coli* possesses two genes for heavy-metal translocating P-type ATPases. We have constructed an expression system for one of them, *ZntA*, which encodes a 732 amino acid residue protein capable of transporting Zn^{2+}. A vanadate-sensitive, Zn^{2+}-dependent ATPase activity is present in the membrane fraction of our expression strain. In addition to Zn^{2+}, the heavy-metal ions Cd^{2+}, Pb^{2+}, and Ag^{+} activate the ATPase. Incubation of membranes from the expression strain with [γ-^{33}P]ATP in the presence of Zn^{2+}, Cd^{2+}, or Pb^{2+} brings about phosphorylation of two membrane proteins with molecular masses of approximately 90 and 190 kDa, most likely representing the ZntA monomer and dimer, respectively. Although Cu^{2+} can stimulate phosphorylation by [γ-^{33}P]ATP, it does not activate the ATPase. Cu^{2+} also prevents the Zn^{2+} activation of the ATPase when present in 2-fold excess over Zn^{2+}. Ag^{+} and Cu^{+} appear not to promote phosphorylation of the enzyme. To study the effects of Wilson disease mutations, we have constructed two site-directed mutants of ZntA, His475Gln and Glu470Ala, the human counterparts of which cause Wilson disease. Both mutants show a reduced metal ion stimulated ATPase activity (about 30−40% of the wild-type activity) and are phosphorylated much less efficiently by [γ-^{33}P]ATP than the wild type. In comparison to the wild type, the Glu470Ala mutant is phosphorylated more strongly by [^{33}P]P_i, whereas the His475Gln mutant is phosphorylated more weakly. These results suggest that the mutation His475Gln affects the reaction with ATP and P_i and stabilizes the enzyme in a dephosphorylated state. The Glu470Ala mutant seems to favor the E2 state. We conclude that His475 and Glu470 play important roles in the transport cycles of both the Wilson disease ATPase and ZntA.

P-type ATPases[1] are polytopic membrane proteins, the majority of which are involved in the uphill transport of cations (*1*−*5*). The best known members of the P-type ATPase family, characterized by extensive mutagenesis (*6*−*8*) and electrophysiology (*9*) studies, include the eukaryotic plasma membrane Na,K-ATPase and Ca-ATPase from the sarcoplasmic reticulum. Recently, two novel types of P-type ATPases have been found: lipid flippases (*10*) and P-type ATPases involved in the transport of heavy-metal ions (*11, 12*).

A hallmark of all P-type ATPases is a phosphorylated aspartate (hence the term P-type) which is formed transiently when the energy derived from the hydrolysis of ATP is used to translocate a substrate ion against a concentration gradient. The transport cycle of P-type ATPases is thought to consist of two enzyme states designated E1 and E2 (*1, 13*−*15*). The E1 state has a high affinity for the cytoplasmic substrate cation, whereas in the E2 state the affinity is low. Cation binding activates the ATPase which catalyzes the transfer of the γ-phosphate of ATP to a conserved aspartyl residue, turning the ATPase into the E1−P state. This high-energy state spontaneously converts into the low-energy state E2−P. The E1−P to E2−P conversion is among the key events in the transport cycle: it is linked to the transmembrane movement of a substrate cation. After hydrolysis of the aspartyl phosphate intermediate, the enzyme returns back to the E1 state.

All P-type ATPases have a similar core structure (*16*) which comprises two cytoplasmic and six transmembrane domains (Figure 1A). The phosphorylation motif Asp-Lys-Thr-Gly (in which the aspartate becomes phoshorylated during the transport cycle), along with 2 other well-conserved

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[1] Abbreviations: ATP, adenosine triphosphate; ATPase, adenosine-triphosphatase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; P_i, inorganic phosphate; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactoside; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; A, Ala, alanine; D, Asp, aspartate; C, Cys, cysteine; Q, Gln, glutamine; E, Glu, glutamate; G, Gly, glycine; H, His, histidine; P, Pro, proline; S, Ser, serine; T, Thr, threonine.

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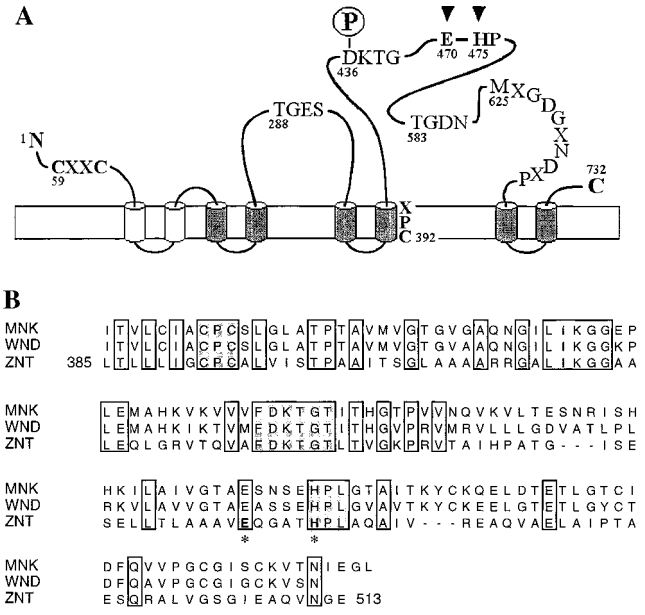

FIGURE 1: (A) Schematic model of the membrane topology of ZntA based on hydropathy analysis. The sequence motifs indicated with the one-letter amino acid code are discussed in the text. The phosphorylated aspartate is labeled with circled P. The two residues mutated in this study are marked with arrowheads. Conserved motifs specific for heavy-metal ATPases are in boldface. The six transmembrane domains which are thought to constitute the common core of P-type ATPases are shaded. (B) Sequence alignment showing the region of the second cytoplasmic domain of ZntA (ZNT), Menkes disease protein (MNK), and Wilson disease protein (WND). The residues identical in all three sequences are boxed. Sequence motifs discussed in the text have been highlighted, and the mutated residues, marked with asterisks, are in boldface.

sequence motifs, resides in a large cytoplasmic domain composed of 280 amino acid residues in ZntA. Sequence analysis suggests that this catalytically central domain adopts the haloacid dehalogenase fold (*17*). Another smaller peripheral domain (about 145 residues) contains the motif Thr-Gly-Glu-Ser/Thr, thought to be involved in the hydrolysis of the phosphointermediate.

Na,K-ATPases and Ca-ATPases are thought to have 10 transmembrane domains with a cytoplasmic N-terminus, whereas the heavy-metal ATPases may have up to 8 transmembrane helices (*11, 12*). Heavy-metal transporting P-type ATPases have a proline flanked by one or two cysteine residues (Cys-Pro-Xaa, Xaa = Cys, His, or Ser) in the transmembrane domain that precedes the large cytoplasmic phosphorylation domain (Figure 1A). This site is likely to participate in binding of the translocated metal ion on its way across the membrane (*11, 18*). Two additional sequence features are specific to heavy-metal ATPases: at least one N-terminal Cys-Xaa-Xaa-Cys [or, in CopB and SilP, histidine-rich motifs (*19, 20*)] and a His-Pro sequence some 30−40 residues C-terminal from the phosphorylated aspartate. Both sites have been shown to be functionally important (*21, 22*). The Cys-Xaa-Xaa-Cys motif binds a metal ion (*23, 24*).

Two human genes, ATP7A and ATP7B, code for copper-transporting P-type ATPases (*25*−*30*). Mutations in either of these genes lead to characteristic disease phenotypes: Menkes disease in the case of ATP7A and Wilson disease[2] in the case of ATP7B mutations. The human proteins are expressed in different tissues. The Menkes protein transports copper in the gut and other tissues but not in the liver, while the ATPase mutated in Wilson disease is part of the copper export pathway from the liver. Consequently, in Menkes disease, there is a systemic lack of copper, resulting in lack of important enzyme activities, while in Wilson disease there is excess of copper in the liver and in the brain. Most of the Wilson disease mutations are amino acid substitutions, which probably perturb the transport function of the ATPase (*31*). For this reason, studying the molecular consequences of the disease mutations can give insight into the mechanism of heavy-metal ATPases. Moreover, the detailed mechanism of any P-type ATPase remains to be elucidated. We believe that studying an ATPase which has a heavy-metal substrate, normally present in the micromolar concentration range, can provide certain advantages over the classical P-type ATPases.

E. coli has two P-type ATPases, named ZntA and YbaR (ATCU_Ecoli), which on the basis of their amino acid sequence belong to the same class of P-type ATPases as the two human gene products (*32, 33*). The ZntA protein consists of 732 amino acid residues with about 30% identity with the product of the Wilson disease gene (see the sequence alignment in Figure 1B). ZntA has been reported to transport Zn^{2+} and Cd^{2+} (*34, 35*) and possibly Pb^{2+} (*36*). The function of the protein encoded by *YbaR* is not known.

In the present study, we report expression, characterization, and site-directed mutagenesis of recombinant His-tagged ZntA (designated ATP732 in this study). We show that the membrane fraction of the expression strain contains a vanadate-sensitive, Zn^{2+}-induced ATPase activity. Moreover, we demonstrate that [γ-^{33}P]ATP phosphorylates a membrane protein with characteristics expected for ZntA. We have also constructed site-directed mutants of two highly conserved residues which mimic pathogenic mutations in Wilson disease. In particular, the human equivalent of His475Gln is the most common Wilson disease mutation in northern European populations (*37, 38*). Analyses of these mutants suggest that the mutated residues play important functional roles in the heavy-metal ATPases.

MATERIALS AND METHODS

Cloning of the ZntA Gene. ZntA was cloned by PCR with Vent-polymerase (NEB) using *E. coli JM 109* genomic DNA as a template and the following oligonucleotide primers: forward, CTC GGA TCC ATG TCG ACT CCT GAC AAT CAC G; reverse, CTC GGT ACC CCT TAT CTC CTG CGC AAC AAT C. The primers were designed on the basis of GenBank entry U00039, ATZN_ECOLI (*32*), with the following added unique restriction sites (underlined): forward, *Bam*HI; reverse, *Kpn*I. The amplified gene (2.2 kb) was ligated into pTrcHisA (Invitrogen) and sequenced. The expression plasmid was named pATP732.

Mutagenesis. Site-directed mutagenesis to introduce the His475Gln mutation was carried out using the following primers: forward, TAAAACCGGTAC**C**CTGACCGTCG-GTAAACCGCGC; reverse, TCGCGTACGATGGCTTG-CGCCAGTGG**T**TGCGTCGC. In addition to the desired

[2] Wilson disease, or hepatolenticular degeneration, is named after Kinnier Wilson, who in 1912 published an extensive article on the disease (*40*). However, the first clinicopathological description of the disease was published in 1890 by E. A. Homen (*41*). Homen's work was known to Wilson, who discussed Homen's results at length as well as republished a patient photograph taken by Homen (*42*).

mutation (T in boldface in the reverse primer), the forward primer contains a silent mutation (boldface C) which creates a *Kpn*I site. This was used to rapidly verify the presence of the desired mutation after cloning of the PCR fragment into the expression construct. The PCR fragment also contains *Age*I and *Bsi*WI sites, which were used for cloning of the fragment. The Glu470Ala mutation was constructed using the overlapping primers method (*39*). Fragment 1 was generated with the same forward primer as His475Gln together with the following reverse primer: TCGCGCCT-TGCGCGACCGCCGC. For fragment 2, the forward and reverse primers GCGGCGGTCGCGCAAGGCGCG and GCACTACCGTTTGCCCGGCGCTTTCCA were used. The final amplified fragment was cloned using unique *Age*I and *Pac*I sites. All mutations were verified by sequencing the relevant region of the expression construct.

Expression of His-Tagged ZntA (ATP732). In this work, the ZntA protein expressed from pATP732 is called ATP732. For production of ATP732, bacterial cultures harboring the expression plasmid were inoculated with frozen glycerol stocks. All cultures were grown at 37 °C with shaking at 300 rpm in a medium containing 25% (v/v) LB and 75% (v/v) JM109-minimal medium (1 × M9 salt solution, 20 mM glucose, 0.5 mg/L thiamin, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$). Five milliliters of an overnight culture was used to inoculate 100 mL of the above medium, and the culture was grown for 1 h, 45 min. The expression of ATP732 was then induced with 100 μM isopropyl-β-thiogalactoside (IPTG). Cells were harvested 5 h post-induction and stored at −20 °C.

Isolation of the Membrane Fraction. All the following steps were carried out on ice or at 4 °C. Wet cells were suspended into the membrane buffer (40 mM Tris-HCl, pH 8.0, 5 mM EDTA, 500 mM sucrose) (10 mL/g of wet cells) followed by the addition of 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.2 mg/mL lysozyme (Sigma). The mixture was incubated for 15 min with occasional mixing. The resulting spheroplasts were centrifuged at 12 000 rpm for 20 min (Sorvall SS34 rotor). The spheroplasts were suspended at 15 mL/g original wet weight into the lysis buffer (10 mM KP_i, pH 8.0, 2 mM EDTA, 1 mM PMSF). The suspension was homogenized using a rotating-blade homogenizer and incubated for 15 min with mixing every 5 min. After the addition of 10 mM $MgCl_2$ and 0.01 mg/mL pancreatic DNase I (Sigma), the incubation was continued for 30 min. The membranes were centrifuged (18 000 rpm, 20 min, Sorvall SS34) and washed with the storage buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 20% glycerol, 2 mM β-mercaptoethanol, 0.5 mM PMSF). Finally, the membranes were pelleted at 40 000 rpm for 1 h (Beckman Ti50 rotor), suspended into the storage buffer to a final concentration of 10 mg of protein/mL, and stored at −20 °C. Protein concentration was measured with the BCA protein assay kit (Pierce). For SDS−PAGE, 400 μg of membrane protein was solubilized in 4% Triton X-100 in a total volume of 50 μL. The solubilized membranes were incubated 30 min on ice, followed by centrifugation in a microfuge (14 000 rpm, 15 min, 4 °C). Three microliters of the supernatant was analyzed on a 12% SDS−PAGE gel.

ATPase Activity Measurements. ATPase activity was determined using the phosphate analysis method (*43*) with a few modifications. Two parallel samples contained 0.5 mL of ice-cold reaction buffer (100 mM MES, pH 6.0, 50 mM NaCl, 5 mM $MgCl_2$), to which 50 μg of a freshly made membrane protein preparation (10 mg of protein/mL) and 10 μL of a metal ion solution [1 mM solution of $ZnSO_4$, $Cd(NO_3)_2$, $PbCl_2$, $CuSO_4$, $AgNO_3$, or $NiCl_2$] or water were added. To form Cu^+, 5 mM DTT was included in the mixture containing Cu^{2+}. The tubes were incubated on ice for 10 min and then transferred to a water bath at 37 °C for 2 min. ATP hydrolysis was initiated by the addition of 20 μL of 100 mM ATP in 0.5 M Tris, pH 8.0, giving a final ATP concentration of 4 mM. After 10 min, 1 mL of stop solution (0.5% ammonium molybdate in 0.35 M H_2SO_4) and 2 mL of 0.5% SDS were added to each tube. The colorimetric reaction was started by addition of 50 μL of 0.5 M ascorbate−KOH, pH 7.0. After 30 min, the absorbance at 750 nm was measured.

Phosphorylation Assays. To 140 μL of ice-cold reaction buffer (20 mM Bis-tris-propane, pH 6.0, 200 mM KCl, 10 μM EDTA) 5 μL of membranes (50 μg of protein) was added, and the mixture was incubated on ice for 5 min. Metal ion solution (5 μL of a 1 mM stock solution) or water was added, and the samples were incubated again for 10 min. Cu^+ was generated as described in the ATPase assay above. When measuring the sensivity of phosphorylation to vanadate, a total vanadate concentration of 50 μM in the presence of 2 mM $MgCl_2$ was added together with Zn^{2+}. (The 100 mM vanadate stock solution was prepared by titrating a solution prepared from Na_3VO_4 with HCl to pH 7.0. This stock was stored at −20 °C. The color of the vanadate solution was orange, most likely owing to the presence of some polymeric decavanadate. For this reason, the inhibitory species can be either monovanadate or decavanadate or both.) The reaction was started by adding 2.5 μCi of [γ-^{33}P]ATP (Amersham Pharmacia) and $MgCl_2$ to final concentrations of 25 nM and 8 mM, respectively. The reaction was stopped after 30 s by the addition of 40 μL of ice-cold 50% TCA. After a 10 min incubation on ice, the membranes were collected by centrifugation (14 000 rpm, 5 min). The pellets were washed with water and then with 50 mM H_3PO_4−NaOH, pH 2.4, and dissolved in 20 μL of sample buffer (1 volume of 2 × standard SDS−PAGE sample buffer diluted with 1 volume of 50 mM H_3PO_4−NaOH, pH 2.4, 2% SDS). A 10 μL sample was analyzed on acidic SDS−PAGE (see below).

The phosphorylation assay with [^{33}P]P_i (Amersham Pharmacia) was carried out at room temperature. The reaction mixture was as above, except that the reaction buffer was 100 mM MES, pH 6.0, containing 25% DMSO when metal ions were included. When the heavy-metal ions were not present, the reaction mixture also contained 0.5 mM EDTA. The 10 min reaction was started by the addition of 40 μCi of [^{33}P]P_i and 8 mM $MgCl_2$, giving a final P_i concentration of 100 nM. All subsequent steps were the same as in the assays with [γ-^{33}P]ATP.

Analysis of Phosphointermediates by Acidic SDS−PAGE. Gels were made essentially as described by Fairbanks and Avruch (*44*). Continuous 8% acrylamide gels in 50 mM H_3PO_4−NaOH, pH 2.4, 0.5% SDS (which was also used for running the electrophoresis) were polymerized with 20 μM $FeSO_4$, 0.625 mM ascorbate−KOH, pH 7.0, and 0.06% H_2O_2. Gels were run at 30 V (3−4 h) at room temperature. After electrophoresis, the gels were dried and analyzed with a BAS-1800 Bio-imaging analyzer (Fuji). The exposure times

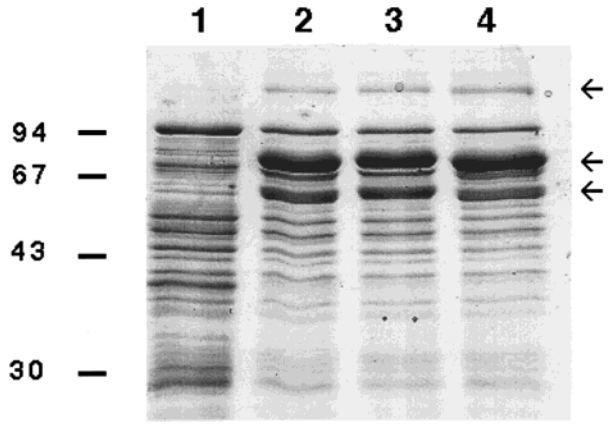

FIGURE 2: SDS−PAGE analysis of solubilized membrane fractions of strains expressing the wild-type and mutant ATP732 proteins. Lane 1, the host strain *E. coli Top10* harboring the expression plasmid pTrcHisA without cloned insert; lane 2, membranes isolated from the strain carrying the expression construct pATP732; lane 3, the mutant His475Gln; lane 4, the mutant Glu470Ala. The positions of molecular mass markers are shown on the left. The arrows on the right point at the bands of ATP732 dimer, monomer, and the 60 kDa fragment. The gel was stained with Coomassie blue.

for gels containing samples labeled with [γ-^{33}P]ATP and [^{33}P]-P_i were 10 min and 1 h, respectively. The cpm number of each lane, measured using a rectangular area limited by and including the 190 and 60 kDa bands, is shown under each lane in Figures 4, 5, 6B, and 7B.

RESULTS AND DISCUSSION

Expression of the ATPase. Despite extensive biochemical studies, there is no detailed understanding of the transport mechanism(s) of P-type ATPases (*4*, *5*). In part, this is due to the lack of a high-resolution structural model. This work had two major aims: first, construction of an expression system for ZntA, which would enable the production of the ZntA protein on a milligram scale; and, second, development of a model system for studying Wilson disease mutations and their molecular consequences.

Figure 2 shows an SDS−PAGE analysis of membrane fractions isolated from the pATP732 expression strain producing ATP732 (wild-type recombinant ZntA carrying an N-terminal polyhistidine tag; lane 2) and two mutant proteins (Glu470Ala, lane 3; His475Gln, lane 4). A comparison with the vector-only control (lane 1) indicates that a major protein band with an apparent molecular mass of about 80 kDa represents the ATPase (for which the calculated molecular mass is 80 kDa). In all strains overexpressing variants of ZntA, an additional 60 kDa band is seen. It is likely to be a proteolytic fragment that can undergo phosphorylation by ATP (cf. Figure 4 and below). The mutant proteins are expressed as well as the wild type (cf. lanes 2, 3, and 4). One liter of culture yields about 1 mg of pure ATPase (unpublished data).

Effect of Wilson Disease Mutations on the ATPase Activity of ATP732. The results of an ATPase assay (Figure 3A) show that the membrane fraction of the strain expressing ATP732 has a marked Zn^{2+}-stimulated ATPase activity[3] [about 400−500 nmol of ATP (mg of protein)$^{-1}$ min^{-1}]. The activity is sensitive to vanadate, a common inhibitor of P-type ATPases (Figure 3B). Notably, membranes from the vector-only

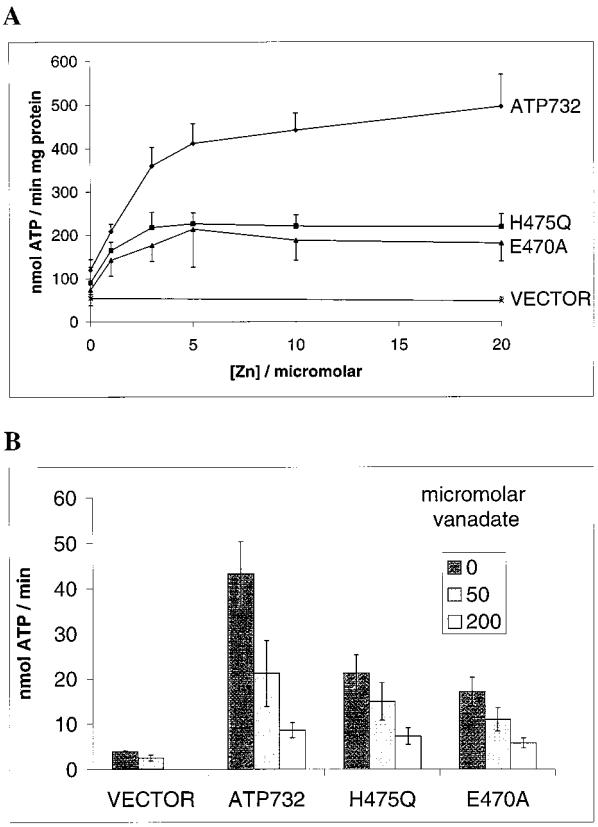

FIGURE 3: (A) Effect of [Zn^{2+}] on the ATPase activity of membrane fractions isolated from the strains expressing wild-type ATP732 and the His475Gln and Glu470Ala mutants of ATP732. (B) Vanadate inhibition of the Zn^{2+}-dependent ATPase activity. All reactions contain 20 μM $ZnSO_4$ and 0, 50, or 200 μM vanadate.

control strain show less than 10% of the vanadate-sensitive ATPase activity of the membranes from the expression strain, and the control activity is not Zn^{2+}-dependent. For membranes from the strain expressing ATP732, 50 μM vanadate causes 50% inhibition of the ATPase activity measured in the presence of 20 μM Zn^{2+} (Figure 3B). Under the same conditions, the His475Gln and Glu470Ala mutants are 60% and 70% less active than the wild type; this activity is vanadate-sensitive. Moreover, the dependence of the ATPase activity on Zn^{2+} concentration may differ between the wild type and the mutants. The wild-type enzyme reaches its maximal turnover rate at 20 μM Zn^{2+}, whereas the mutants are already maximally active at 5 μM Zn^{2+}. It appears as if the mutants had lost one metal binding site required for maximal activity of the ATPase (see Conclusions).

While the activity of both Wilson disease mutants is significantly lower than that of the wild-type protein, the mutants show significant activity. This finding is consistent with the results obtained with the Wilson disease protein using other types of activity measurements (*21*, *22*). Although we assume that the ATPase activity is coupled to ion translocation in the wild type as well as in the mutants, a direct transport assay remains to be carried out.

Phosphorylation by [^{33}P]ATP. A hallmark of P-type ATPases is the absolutely conserved sequence Asp-Lys-Thr-

[3] While it is possible that the N-terminal His tag of ATP732, included to aid purification, also binds a heavy-metal ion, it is unlikely that this binding would activate the ATPase or stimulate phosphorylation. Moreover, it is likely that the substrate heavy metals prefer the soft cysteine side chain ligands over the imidazoles in the His tag.

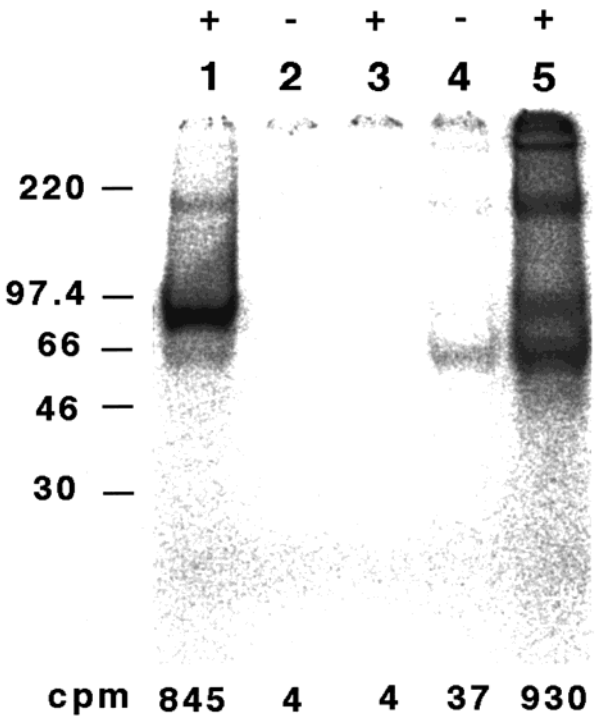

FIGURE 4: Phosphorylation of membrane proteins by [^{33}P]ATP in the absence (−) or presence (+) of 31 μM Zn^{2+}, analyzed using acidic SDS−PAGE as described under Materials and Methods. Lane 1, membranes from wild-type *E. coli Top10* grown in the presence of 1 mM $ZnSO_4$; lane 2, membranes from the *E. coli Top10* harboring only the expression vector pTrcHisA; lane 3, same as lane 2 but in the presence of Zn^{2+}; lane 4, ATP732 membranes without Zn^{2+} ; lane 5, ATP732 membranes with Zn^{2+} present during the phosphorylation assay. The positions of molecular mass markers are shown on the left. The numbers on the bottom of each lane indicate the cpm's of the region between the 60 and 190 kDa bands (see Materials and Methods).

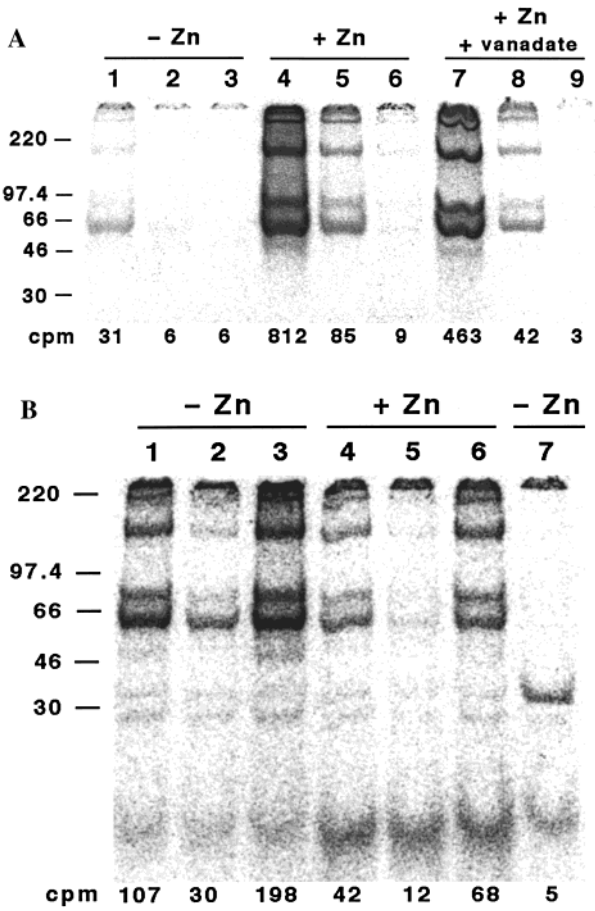

FIGURE 5: (A) An acidic SDS−PAGE analysis of phosphorylation of ATP732 and the two mutant proteins by [^{33}P]ATP in the presence of Zn^{2+} (31 μM) and vanadate (50 μM). Lane 1, ATP732; lane 2, His475Gln; lane 3, Glu470Ala; lane 4, ATP732 + Zn^{2+}; lane 5, His475Gln + Zn^{2+}; lane 6, Glu470Ala + Zn^{2+}; lane 7, ATP732 + Zn^{2+} + vanadate; lane 8, His475Gln + Zn^{2+} + vanadate; lane 9, Glu470Ala + Zn^{2+} + vanadate. The positions of molecular mass markers are shown on the left. (B) Phosphorylation by [^{33}P]P_i. Lane 1, ATP732; lane 2, His475Gln; lane 3, Glu470Ala; lane 4, ATP732 + Zn^{2+}; lane 5, His475Gln + Zn^{2+}; lane 6, Glu470Ala + Zn^{2+}; lane 7, membranes from the vector-only control. The concentration of Zn^{2+}, when used, was 31 μM.

Gly in which the aspartate residue is transiently phosphorylated by ATP during the transport cycle. Figures 4 and 5A show the results of an assay of the membrane preparations with [γ-^{33}P]ATP as the labeling agent, analyzed under conditions in which the aspartyl phosphate is stable. Figure 4 (lane 5) shows that in the presence of Zn^{2+} two major membrane protein bands with molecular masses of about 92 and 190 kDa are phosphorylated. As dimerization appears to be typical for P-type ATPases (*1, 16*), these two bands most likely represent phosphorylated monomers and dimers of ZntA. Both radioactive bands are also visible in the membranes of the parent strain, *E. coli Top 10*, when grown in the presence of Zn^{2+} (Figure 4, lane 1), which is known to induce the expression of the chromosomal *ZntA* (*35*). However, these bands are almost absent from the membranes of bacteria grown without added zinc (lane 2). In lane 5, the highest molecular weight bands near the top of the gel are likely to be aggregated forms of ATP732, as these are not found in the Zn^{2+}-induced parent strain (lane 1) nor in the vector-only control sample (lane 2). The band with the lowest molecular mass of about 60 kDa in lane 5 is probably a proteolytic fragment of the overexpessed recombinant protein, because it is not present in lane 1 (ZntA produced from the chromosomal gene). The labeling of the 60 kDa species by [^{33}P]ATP is also sensitive to 50 μM vanadate (Figure 5A, lane 7), as is the labeling of the monomer and dimer bands regardless of whether they are mutated or not (compare lanes 7, 8, and 9 in Figure 5A).

Based on the data discussed above, the following conclusions can be drawn. It is possible to study the recombinant ATP732 in the wild-type background because the expression of the chromosomal *ZntA* is negligible in the absence of Zn^{2+}. ZntA produced from the chromosomal gene appears mostly monomeric, but some dimers can be detected. ATP732 is present as monomers, as dimers, and as a 60 kDa form as well as higher oligomers, all of which can be phosphorylated by ATP.

Both mutant proteins are clearly less phosphorylated than ATP732 (Figure 5A, lanes 4, 5, and 6). In particular, Glu470Ala is phosphorylated very weakly. The presence of Zn^{2+} causes 25-fold stimulation of phoshorylation of wild-type ATP732 (cf. the cpm numbers beneath lanes 4 and 5 in Figure 4 and lanes 1 and 4 in Figure 5A). The phosphorylation of the His475Q mutant is stimulated by Zn^{2+}, whereas that of the Glu470A mutant is not.

Phosphorylation by [^{33}P]P_i. In the E2 state, P-type ATPases can be phosphorylated by P_i (*5, 7*). This phosphorylation is sensitive to the cytoplasmic substrate cations because these tend to turn the enzyme into the E1 state. With

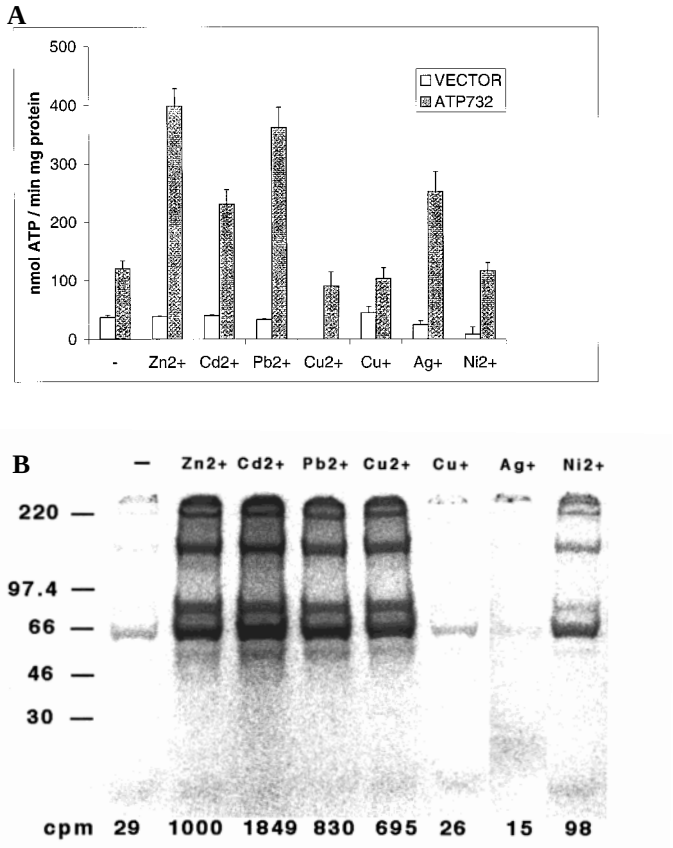

FIGURE 6: (A) Metal ion specificity of the ATPase activity. The final concentration of each metal ion was 20 μM. The metal ions used are indicated beneath each pair of columns. White bars, membranes from the vector-only control strain; gray bars, ATP732-containing membranes. (B) Metal ion dependence of phosphorylation of ATP732, analyzed with acidic SDS−PAGE. Lane 1, ATP732; lane 2, ATP732 + Zn^{2+}; lane 3, ATP732 + Cd^{2+}; lane 4, ATP732 + Pb^{2+}; lane 5, ATP732 + Cu^{2+}; lane 6, ATP732 + Cu^{+}; lane 7, ATP732 + Ag^{+}; lane 8, ATP732 + Ni^{2+}. The cpm numbers are indicated on the bottom of each lane. The positions of molecular mass markers are shown on the left.

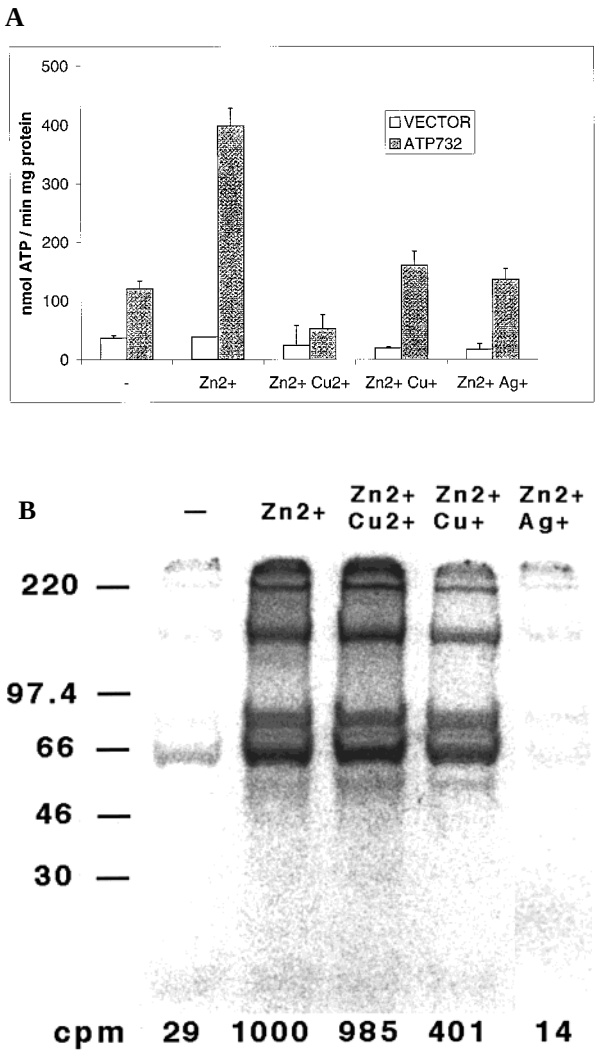

FIGURE 7: (A) Inhibition of Zn^{2+}-stimulated ATPase activity of ATP732 by heavy-metal ions. The metal ions used are indicated beneath each pair of columns. White bars, membranes from the vector-only control strain; gray bars, ATP732-containing membranes. The concentrations of Zn^{2+} and the other metal ion were 20 and 40 μM, respectively. (B) Inhibition of Zn^{2+}-stimulated phosphorylation by [^{33}P]ATP with other metal ions. Lane 1, no metal ion added; lane 2, Zn^{2+}; lane 3, Zn^{2+} + Cu^{2+}; lane 4, Zn^{2+} + Cu^{+}; lane 5, Zn^{2+} + Ag^{+}. Concentrations of the metal ions: Zn^{2+}, 31 μM; the other metal ion, 62 μM. The cpm numbers are indicated on the bottom of each lane. The positions of molecular mass markers are shown on the left.

this in mind, we have studied the phosphorylation of the wild-type ZntA and the two mutants by [^{33}P]P_i. From this experiment (Figure 5B), the following points emerge: The wild-type ATP732 protein is phosphorylated by P_i in the absence of Zn^{2+}. The labeling becomes weaker in the presence of Zn^{2+} (Figure 5B, cf. lanes 1 and 4). The mutants show a similar trend: Zn^{2+} weakens phosphorylation by P_i to about 40% of the level obtained without the metal ion present (cf. lanes 2 and 5 and lanes 3 and 6 in Figure 5B). However, concerning the extent of labeling by [^{33}P]P_i, the two mutants differ not only from the wild type but also from each other: Glu470Ala is more strongly phosphorylated than the wild type, whereas His475Gln is phosphorylated significantly less than the wild type. This behavior suggests that the Glu470Ala mutation causes the ATPase to favor the E2 state.

Metal Specificity. Both phosphorylation by ATP and hydrolysis of ATP are stimulated by Zn^{2+}. In the experiment described below, we set out to study the effect of other heavy-metal ions on these reactions. Besides Zn^{2+}, the ATPase activity is stimulated by Pb^{2+} and, to a lesser extent, by Cd^{2+} and Ag^{+} (Figure 6A). None of the other metal ions tested (Cu^{2+}, Cu^{+}, Ni^{2+}, Hg^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}) were able to enhance the hydrolysis of ATP. We then studied the effect of these metals on the phosphorylation of ATP732 by [^{33}P]-ATP (Figure 6B). Zn^{2+}, Cd^{2+}, and Pb^{2+}, all of which activate the ATPase, also markedly increase the phosphorylation of the protein. Ag^{+}, despite its ability to stimulate the ATPase, seems not to enhance phosphorylation. In addition, Cd^{2+} appears to stimulate phosphorylation more strongly than Zn^{2+}, although the latter is more potent in stimulating the ATPase activity. Likewise, Cu^{2+} clearly enhances phosphorylation, although it cannot activate the ATPase at all.

Ni^{2+} fails to prevent the stimulatory effect of Zn^{2+} in the ATPase assay (not shown), suggesting that Ni^{2+} does not bind tightly to the sites required to be occupied for the turnover of the ATPase. The addition of Cu^{2+}, Cu^{+}, or Ag^{+} in the presence of Zn^{2+} at a concentration twice that of Zn^{2+} strongly inhibits the ATPase activity (Figure 7A). In this experiment, Cu^{+} or Ag^{+} also inhibit phosphorylation by

ATP, while the presence of 60 μM Cu^{2+} and 30 μM Zn^{2+} together results in strong phosphorylation of the enzyme (Figure 7B).

CONCLUSIONS

Heavy-metal transporting ATPases are of special interest because they offer novel ways to study the mechanism of P-type ATPases in general, and because members of the family are mutated in two human diseases. Understanding the effects of the disease mutations can contribute toward the elucidation of the mechanism of P-type ATPases.

The aims of this work were (i) to create an (over)-expression system for ZntA, and (ii) to test whether ZntA could be used as a model for human proteins involved in Wilson and Menkes diseases. We have expressed and characterized a P-type ATPase encoded by the ZntA gene of *E. coli*. Our results show that an active, Zn^{2+}-dependent, and vanadate-sensitive ATPase is produced and inserted into the cell membrane upon expression of *ZntA* from pATP732. The wild-type protein is phosphorylated in a metal-dependent fashion by the terminal phosphate of ATP and also by P_i under a separate set of conditions. The two mutants which mimic common mutations found in Wilson disease patients show significantly reduced catalytic activities as well as specific changes in their phosphorylation properties. The His475Gln mutant is inefficiently phosphorylated by both ATP and P_i, whereas the Glu470Ala mutation weakens phosphorylation by ATP but enhances phosphorylation by P_i. This suggests that the latter mutation causes a shift toward the E2 state. We conclude that both mutated residues play important roles in the function of the heavy-metal transporting P-type ATPases.

The metal specificity studies show that a heavy-metal ion can give rise to one of the following effects: First, a substrate ion such as Zn^{2+} stimulates both ATP hydrolysis and phosphorylation. The substrate ions Zn^{2+} and Cd^{2+} differ, however, in their relative capability to stimulate the ATPase activity and phosphorylation. In comparison with Zn^{2+}, Cd^{2+} is more effective in promoting phosphorylation and less effective in stimulating ATP hydrolysis. Second, Cu^{2+} clearly enhances the phosphorylation of the protein, but it is unable to activate the ATPase. Third, Ag^{+} stimulates the ATPase activity, although under our experimental conditions it seems not to cause (stable enough) phosphorylation of the protein. Finally, although Cu^{+} neither stimulates the ATPase nor affects its phosphorylation, it can still prevent the effects of Zn^{2+} on these reactions.

Together these results may suggest that the enzyme has at least two functionally different metal binding sites with slightly different metal specificities: one that activates the phosphoryl transfer from ATP and another to which the ion to be translocated ion binds. It may be the latter binding event that is necessary for turnover of the ATPase. Incidentally, the changes in the shape of the ATPase activity vs $[Zn^{2+}]$ plots in Figure 3A could be interpreted as evidence for a loss of a high-affinity metal binding site in the mutants. An alternative explanation could be that there is only one metal binding site which has to be occupied before the phosphointermediate can be formed. Hydrolysis of the intermediate can only occur once the bound metal has been released or moved forward along the translocation pathway. Thus, too tight binding, as in the case of Cu^{2+}, would result in a phosphorylated protein which does not catalyze ATP hydrolysis. Clearly, further studies on the metal binding properties of ZntA are needed.

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