

The structure and function of heavy metal transport P_{1B}-ATPases

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Received: 12 July 2006 / Accepted: 28 November 2006 / Published online: 12 January 2007
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Abstract P_{1B}-type ATPases transport heavy metals (Cu⁺, Cu²⁺, Zn²⁺, Co²⁺, Cd²⁺, Pb²⁺) across membranes. Present in most organisms, they are key elements for metal homeostasis. P_{1B}-type ATPases contain 6–8 transmembrane fragments carrying signature sequences in segments flanking the large ATP binding cytoplasmic loop. These sequences made possible the differentiation of at least four P_{1B}-ATPase subgroups with distinct metal selectivity: P_{1B-1}: Cu⁺, P_{1B-2}: Zn²⁺, P_{1B-3}: Cu²⁺, P_{1B-4}: Co²⁺. Mutagenesis of the invariant transmembrane Cys in H6, Asn and Tyr in H7 and Met and Ser in H8 of the *Archaeoglobus fulgidus* Cu⁺-ATPase has revealed that their side chains likely coordinate the metals during transport and constitute a central unique component of these enzymes. The structure of various cytoplasmic domains has been solved. The overall structure of those involved in enzyme phosphorylation (P-domain), nucleotide binding (N-domain) and energy transduction (A-domain), appears similar to those described for the SERCA Ca²⁺-ATPase. However, they show different features likely associated with singular functions of these

proteins. Many P_{1B}-type ATPases, but not all of them, also contain a diverse arrangement of cytoplasmic metal binding domains (MBDs). In spite of their structural differences, all N- and C-terminal MBDs appear to control the enzyme turnover rate without affecting metal binding to transmembrane transport sites. In addition, eukaryotic Cu⁺-ATPases have multiple N-MBD regions that participate in the metal dependent targeting and localization of these proteins. The current knowledge of structure-function relationships among the different P_{1B}-ATPases allows for a description of selectivity, regulation and transport mechanisms. Moreover, it provides a framework to understand mutations in human Cu⁺-ATPases (ATP7A and ATP7B) that lead to Menkes and Wilson diseases.

Keywords P-type ATPases · Heavy metals · Membrane transport · Copper · Zinc · Cobalt · Cadmium

Defining features of P_{1B}-ATPases

P_{1B}-ATPases¹ are a subgroup of P-ATPases that transport heavy metals (Cu⁺, Cu²⁺, Zn²⁺, Co²⁺) across biological membranes (Lutsenko and

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¹ For simplicity P-type ATPases will be referred as P-ATPases, P_{1B}-ATPase, etc.

Kaplan 1995; Solioz and Vulpe 1996; Axelsen and Palmgren 1998; Rensing et al. 1999; Argüello 2003; Williams and Mills 2005). An immediate consequence of their metal specificity and the chemical similarities among transition metals is that these pumps transport alternative non-physiological substrates; for instance, Cu^+ -ATPases transport Ag^+ while Zn^{2+} -ATPases can transport Cd^{2+} and Pb^{2+} . $\text{P}_{1\text{B}}$ -ATPases share a common catalytic mechanism with the archetypical P-ATPases (Na^+ , K^+ -ATPase, Ca^{2+} -ATPase, H^+ -ATPase) (Fig. 1). This mechanism couples the catalytic phosphorylation of Asp in the invariant DKTGT sequence to the transmembrane translocation of the metals. $\text{P}_{1\text{B}}$ -ATPases have structural features such as metal-binding signature sequences in their transmembrane segments (TM), topological arrangements with eight or six TMs, and regulatory cytoplasmic metal binding domains (MDB), that distinguish them from their P-ATPase counterparts (Fig. 2). Consistent with their substrate specificities and direction of transport, i.e., metal efflux from the cytoplasm, they confer metal tolerance to archaea and bacteria, while in higher eukaryotes they are responsible for metal micronutrient absorption, distribution and clearance.

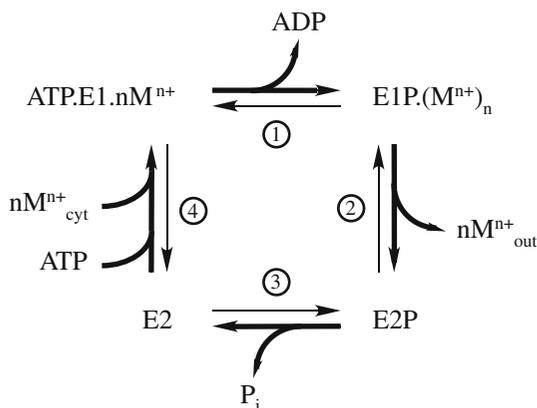


Fig. 1 $\text{P}_{1\text{B}}$ -ATPases catalytic cycle. E_1 , E_2 , E_1P and E_2P represent the basic conformations that the enzyme can assume. M^{n+} represents a metal transported by these enzymes. n indicates the uncertainty on the specific stoichiometry of transport. $\text{M}^{n+}_{\text{cyt}}$ and $\text{M}^{n+}_{\text{out}}$ represents the cytoplasmic or extracellular/luminal localization of the transported metal

Distribution and physiological roles

Different from other P-ATPases subfamilies, $\text{P}_{1\text{B}}$ -ATPases are found in all life kingdoms, from extremophilic archaea to man (Fig. 3). $\text{P}_{1\text{B}}$ -ATPases are present in most reported archaeal genomes (Argüello 2003; De Hertogh et al. 2004). Archaeal $\text{P}_{1\text{B}}$ -ATPases present a variety of substrate specificities and considerable structural stability (Mandal et al. 2002; Argüello 2003; Mana-Capelli et al. 2003; Baker-Austin et al. 2005) (Cattoni D, González-Flecha, FL, Argüello JM, unpublished results). These properties are linked to the extremophilic character of the hosting organisms. For instance, increased Cu^+ -ATPase transcript levels have been observed in *Ferroplasma acidarmanus*, an organism that tolerates copper at levels of 20 g/l (Baker-Austin et al. 2005). Alternatively, the chemolithoautotroph *Archaeoglobus fulgidus* has two $\text{P}_{1\text{B}}$ -ATPases, CopA and CopB, able to transport Cu^+ and Cu^{2+} respectively, suggesting the need to extrude alternative copper forms depending on the organism redox status (Mandal et al. 2002; Mana-Capelli et al. 2003). In view of their stability and diversity, these proteins appear as a relevant resource to explore structural-functional characteristics and biotechnological applications.

$\text{P}_{1\text{B}}$ -ATPases were initially identified in prokaryotic organisms, like *Staphylococcus aureus* plasmid pI258 (Nucifora et al. 1989), *Rhizobium meliloti* (Kahn et al. 1989) and *Enterococcus hirae* (Odermatt et al. 1993). In these organisms, their function is to maintain metal quotas, specially those of copper and zinc. This role has been demonstrated by gene knockout resulting in high sensitivity to the metal transported by the encoded protein. This experimental approach, together with functional complementation, has served to identify various $\text{P}_{1\text{B}}$ -ATPases and their multiple substrates (Odermatt et al. 1993; Phung et al. 1994; Rensing et al. 1997; Rutherford et al. 1999; Rensing et al. 2000; Tottey et al. 2001). It is interesting that some prokaryotes (*Mycobacterium tuberculosis*, *Lactococcus lactis*, *Pseudomonas aeruginosa*, etc.) have two or more $\text{P}_{1\text{B}}$ -ATPases of similar metal specificity (Argüello 2003). The reasons for this abundance,

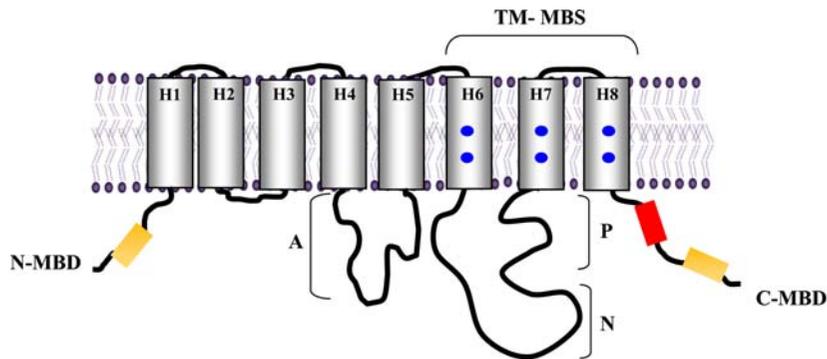


Fig. 2 Schematic illustration of the topology and main domains present in P_{1B} -ATPases. Transmembrane segments, H1 to H8, are indicated. The relative locations of the cytoplasmic actuator (A), phosphorylation (P) and nucleotide (N) domains are shown. The conserved amino

acids in H6, H7 and H8 forming the transmembrane metal binding sites (TM-MBS) are symbolized by blue dots. The presence of N- and C-terminal metal binding domains (MBDs) with diverse structures is represented by yellow and red rectangles

and apparent redundancy, have not been explored.

Genomes of fungi and animals contain two genes coding for Cu^+ -ATPases (Axelsen and Palmgren 1998; Argüello 2003). Among these, the better characterized are the two present in humans, ATP7A and ATP7B. Mutations in these genes lead to Menkes syndrome and Wilson disease respectively (Bull et al. 1993; Vulpe et al. 1993; Bull and Cox 1994; Lutsenko et al. 2003). Wilson disease is characterized by high copper levels in the liver, blood and brain, with consequent neurologic and hepatic disorders. Mutations in the Menkes ATPase (MNKP) lead to poor copper uptake from the intestine resulting in neurodegeneration and connective tissue abnormalities. These symptoms are associated with the particular sub-cellular distribution and specific organ expression of these enzymes. MNKP is expressed in the intestine, blood brain barrier and several other organs (Vulpe et al. 1993). In contrast, Wilson disease protein (WNDP) expression is predominant in the liver. Both enzymes localize to the trans-Golgi network and undergo Cu-dependent trafficking (Petris et al. 1996; Hung et al. 1997). However, while the MNKP is relocated to the cell plasma membrane in various tissues (Petris et al. 1996), the WNDP is targeted to vesicles proximal to the plasma membrane of liver canicular cells (Forbes et al. 1999; Schaefer et al. 1999).

Plants differ significantly from other organisms in the number and selectivity of their P_{1B} -ATPases

(Williams and Mills 2005). For instance, *Arabidopsis thaliana* contains four Cu^+ -ATPases (HMA5-8) and three Zn^{2+} -ATPases (HMA2-4). HMA1 has been reported to be a Cu^+ -ATPase although its transmembrane metal binding site (TM-MBS) appears to be different from that of Cu^+ -transporting ATPases (Seigneurin-Berny et al. 2006). Plant Cu^+ -ATPases are responsible for Cu^+ transport into a post-Golgi compartment (HMA7) (Woeste and Kieber 2000) and the chloroplast (HMA6, HMA8 and possibly HMA1) (Abdel-Ghany et al. 2005; Seigneurin-Berny et al. 2006). HMA5 appears to be involved in copper detoxification in roots (Andrés-Colas et al. 2006). The Zn^{2+} -ATPases HMA2 and HMA4 are located in the plasma membrane and expressed primarily in the vasculature of shoots and roots (Hussain et al. 2004). *hma2* knockout plants present high Zn^{2+} levels (Eren and Argüello 2004), while the *hma4* knockout plants show reduced Zn^{2+} levels (Hussain et al. 2004). Along this line, over-expression of HMA4 increases root-to-shoot Zn^{2+} translocation (Verret et al. 2004). Considering their distribution, these observations suggest that HMA2 and HMA4 participate in Zn^{2+} loading into the phloem and xylem respectively.

The catalytic mechanism

P_{1B} -ATPases transport metals across membranes following the classical E1/E2 Albers-Post

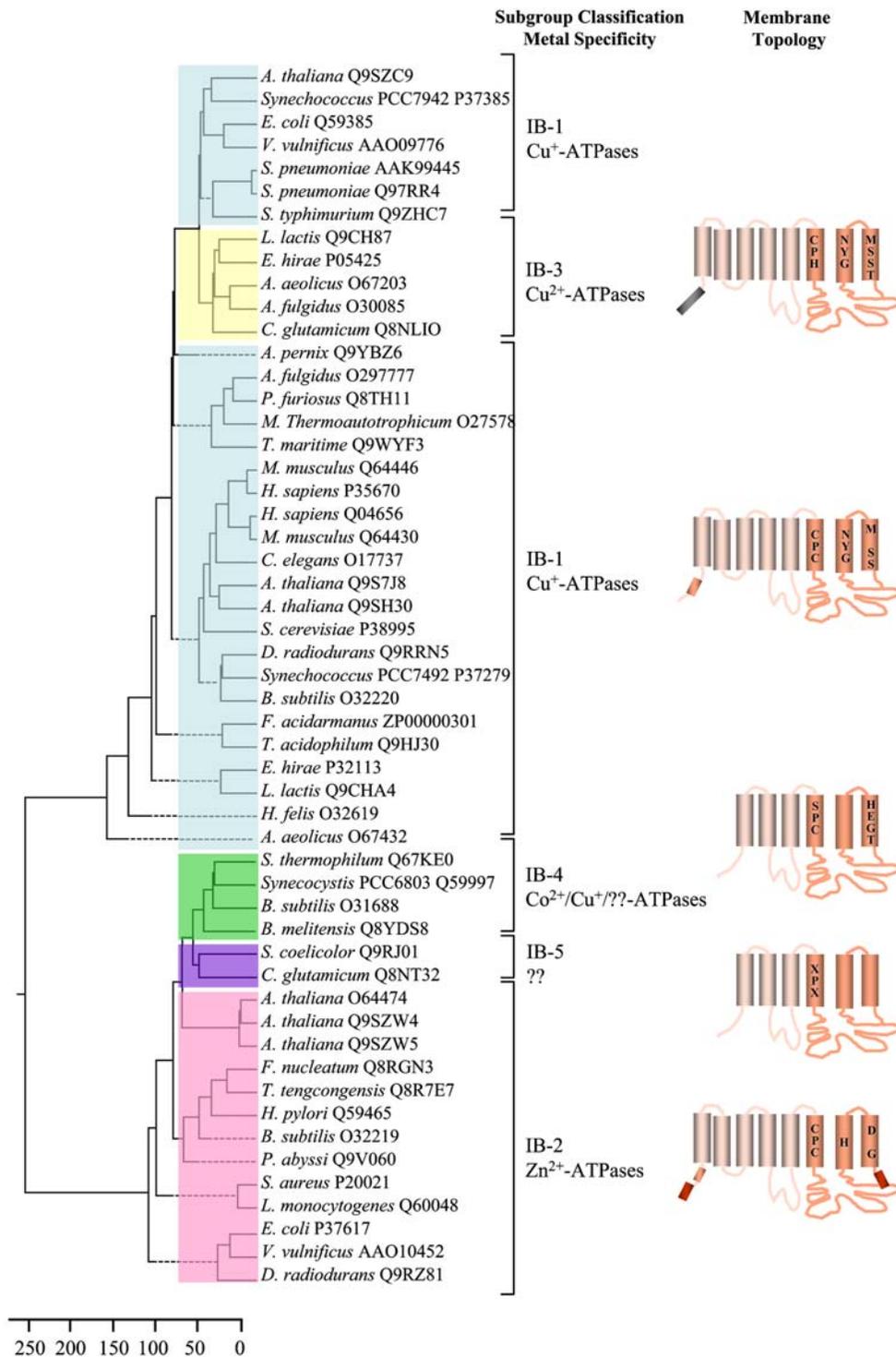


Fig. 3 Phylogenetic tree of the P_{1B}-type ATPases. The tree was prepared from a Clustal W alignment of representative sequences of P_{1B}-type ATPases. The relative abundance of sequences from each subgroup has been maintained. The metal specificity and the structural

characteristics are indicated next to the subgroup denomination. Amino acids in TMs proposed to participate in determining metal selectivity. Black blocks represent His-rich N-MBDs; orange blocks, CXXC N-MBDs; and red, His and Cys rich N- and C-MBDs

catalytical cycle (Fig. 1). This transport mechanism has been studied in detail in P_2 -ATPases (Na^+ , K^+ , Ca^{2+} , and H^+ , K^+ -ATPases) (MacLennan et al. 1997; Kaplan 2002). Catalytic enzyme phosphorylation occurs upon ATP binding with high (μM) affinity to the ATP binding domain (ATP-BD) and metal binding to the TM-MBS from the cytoplasmic side (Steps 4 and 1). Upon phosphorylation, the metal is likely occluded within the TM region and not accessible from the aqueous media. The subsequent conformational change allows for metal deocclusion, release to the extracellular (vesicular/luminal) compartment, and subsequent enzyme dephosphorylation (Step 3). The enzyme then returns to the E1 conformation upon ATP binding to the E2 form with low (mM) affinity (Step 4).

Biochemical studies have corroborated this model for various P_{1B} -ATPases while explaining aspects that are unique to heavy metal transport. Heterologously expressed eukaryote Cu^+ -ATPases have served to characterize transport into sealed vesicles, phosphorylation and dephosphorylation reactions in isolated proteins, and metal transfer among Cu-chaperones and N-terminal metal binding domains (MBDs) (Voskoboinik et al. 1998; Huffman and O'Halloran 2000; Wernimont et al. 2000; Tsivkovskii et al. 2002; Walker et al. 2002). ATPase activity, transport, direct metal binding to the TM-MBS, and phosphorylation reactions have been measured in eukaryote Zn^{2+} -ATPases and various prokaryote and archaeal enzymes, either in isolated membranes or solubilized pure enzyme preparations (Sharma et al. 2000; Fan and Rosen 2002; Mandal et al. 2002; Mana-Capelli et al. 2003; Mandal and Argüello 2003; Eren and Argüello 2004; Okkeri et al. 2004; Liu et al. 2006). These studies have allowed the characterization of mechanistic requirements, selectivity, and regulatory aspects independently of in vivo constraints associated with targeting, protein interactions, and inter-linked homeostasis mechanisms of non-metal substrates.

Direction of transport and stoichiometry

Transport experiments indicate that P_{1B} -ATPases drive metal efflux from the cytoplasmic

compartment (Rensing et al. 1997; Voskoboinik et al. 1998; Fan and Rosen 2002; Mana-Capelli et al. 2003; Eren and Argüello 2004). This is in agreement with a catalytic mechanism where the E1 form of the enzyme (TM-MBS open to the cytoplasmic side) binds the transported metal and ATP (Fig. 1). Early reports have suggested that some Cu^+ -ATPases, although structurally homologous to well-characterized enzymes, might drive metal influx into the cytoplasm (Odermatt et al. 1993; Tottey et al. 2001). However, the influx of metal would require binding of another transport substrate (proton?) to the E1 form to allow the coupled ATP hydrolysis and enzyme phosphorylation, with the inwardly transported metal driving enzyme dephosphorylation. Further experiments would be required to test this alternative mechanism.

The coupling of solute transport with ATP hydrolysis in a specific stoichiometry is a requirement of the E1/E2 mechanisms (MacLennan et al. 1997; Kaplan 2002). Because of experimental difficulties to obtain highly active preparations of sealed everted vesicles or, alternatively, conditions to stabilize the metal occluded (E1P(metal)) forms, these parameters have not been established for most P_{1B} -ATPases. However, Mitra and collaborators recently showed binding of 1 Zn^{2+} per ATPase to the TM-MBS of *E. coli* ZntA in the absence of other enzyme substrates (Liu et al. 2006). Although it could be argued whether Zn^{2+} TM-MBS were fully occupied, this relevant data is the first showing isolated metal binding to the TM-MBS.

The membrane topology of P_{1B} -ATPases

When compared to other P-ATPases, P_{1B} -ATPases have a distinct structure characterized by a reduced number of TMs, smaller ATP binding domains (ATP-BD), and the presence of N- or C-terminal MBDs in many of them (Fig. 2). The presence of eight TMs has been experimentally established in two bacterial enzymes (Melchers et al. 1996; Tsai et al. 2002). A similar topology emerges through computational analysis of most P_{1B} -ATPases (Fig. 3). However, a subgroup of these enzymes appears to have only six TMs. This

particular topology is observed in one of the major P_{1B} -ATPases subgroups, P_{1B-4} , and in a number of isolated proteins lacking signature sequences that define Cu^+ -, Zn^{2+} - or Cu^{2+} -ATPases (subgroup P_{1B-5}). The six TM topology is supported by single and multiple sequence analysis. Furthermore, while retaining the central cytoplasmic regions necessary for ATP binding and hydrolysis, these proteins are relatively small with short N- and C-terminal ends; thus, they contain no possible sequence to form the two additional segments observed in the P_{1B-1} , P_{1B-2} and P_{1B-3} subgroups (Fig. 3). The described topologies differentiate P_{1B} -ATPases from other subfamilies that contain ten (P_2 and P_3) or twelve (P_4 and P_5) TMs (Lutsenko and Kaplan 1995; Axelsen and Palmgren 1998). P_{1B} -ATPases also present a particular distribution of TMs with respect to the large cytoplasmic loop forming the ATP-BD. While P_{1B} -ATPases have two TMs on the C-terminal end of the ATP-BD, other P-ATPases have either four (P_{1A}) or six (P_2 , P_3 , P_4 and P_5).

In spite of the indicated differences, a common pattern is evident among these heavy metal ATPases. They all have a core structure formed by the five TMs flanking the two largest cytoplasmic loops (Fig. 3). These central components appear to confer their basic functionality to these enzymes, i.e., the capability to transport metals using the energy resulting from ATP hydrolysis.

The transmembrane metal binding site

The transmembrane metal binding sites (TM-MBS) of P_{1B} -ATPases are responsible for metal recognition and movement across the membrane permeability barrier. While there is a significant wealth of information on metal coordination in proteins where metals play structural and catalytic roles, little is known about the metal sites of transporters that bind and release the metals with high turnover (approximately 200 min^{-1} in the case of P_{1B} -ATPases). For instance, the requirement of conserved transmembrane His, Glu, Asp, Ser and Met residues for the metal translocation by various transporters has been established, but a

detailed description of the arrangement of these metal binding ligands is still lacking. Analysis of the first available sequences indicated that P_{1B} -ATPases have a CPC sequence in the center of their sixth TM (H6), with a few exceptions containing CPH (Bull and Cox 1994; Solioz and Vulpe 1996). While the Pro is conserved in all P-ATPases, the CPX motif appeared as a defining element of these enzymes that likely participate in metal coordination during transport. Mutations of these Cys impair enzyme function (Yoshimizu et al. 1998; Fan and Rosen 2002; Mandal and Argüello 2003; Lowe et al. 2004). These mutants seemed unable to bind Cu^+ but capable of interacting with nucleotides and undergoing basic conformational changes (Mandal and Argüello 2003). Although the CPC sequences are the most frequently observed, a current analysis of the available genomes reveals the presence of alternative sequences (SPC, CPS, CPT, CPA, CPG, CPD) in putative P_{1B} -ATPases. These proteins contain the DKTGT signature sequence and the characteristic topology of these enzymes. This observation highlights the diversity of these pumps and suggests the possibility of metal specificities yet to be identified.

Early studies of the Ca^{2+} - and Na^+, K^+ -ATPases (MacLennan et al. 1997; Lingrel et al. 1998) and more recently the evidence provided by the structure of the Ca^{2+} -ATPase SERCA1 (Toyoshima and Inesi 2004), indicate that their transmembrane cation binding sites are constituted by invariant polar side chains located in the three TMs flanking the ATP-BD (one in the N-terminal side and two on the C-terminal end). Following a similar pattern, conserved amino acids were identified in the equivalent TMs of P_{1B} -ATPases (Argüello 2003). The observed signature sequences pointed out the various TM-MBS present in these proteins and allowed for the classification of proteins into subgroups with distinct metal binding and transport specificities (Fig. 3).

Subgroup P_{1B-1} comprises all Cu^+ -ATPases, including the human WNDP and MNKP. In the center of their transmembrane region these proteins contain conserved residues Asn, Tyr in H7, Met and Ser in H8, and the CPC sequence in H6 (Argüello 2003). The functional roles of

these residues were tested by using as a model the hyperthermophilic CopA from *A. fulgidus* (Mandal et al. 2004). CopA carrying mutations in the mentioned residues were inactive. They were phosphorylated by inorganic phosphate (P_i) (Step 3, Fig. 1) showing that they retain their overall structure and could undergo major conformational transitions. However, mutant AfCopAs were not phosphorylated by ATP in the presence of Cu^+ (Step 1, Fig. 1) nor was Cu^+ able to prevent enzyme phosphorylation by P_i (Step 4, Fig. 1). Interestingly, a mutation of the conserved Ser in H8 of WNDP has been identified in some Wilson disease patients (Loudianos et al. 1999). Mutations in the immediate vicinity of these residues or in other TMs have no significant effects in enzyme activity or in Cu^+ dependent partial reactions (Lowe et al. 2004; Mandal et al. 2004). These observations suggest the participation of specific invariant residues (two Cys, Asn, Tyr, Met and Ser) in Cu^+ transport by P_{1B} -ATPases. The identification of these side chains provides a first view of the putative TM-MBS for Cu^+ -ATPases. However, further information is necessary to understand Cu^+ binding to the TM-MBS and subsequent transport. For instance, the stoichiometry of transport and the direct participation of these residues in initial ion binding, occlusion, or release remain to be established. In this direction, it seems likely that the second Cys in H6, proximal to the cytoplasmic side, would be required for metal binding, while the first Cys would be necessary for Cu^+ dissociation or enzyme dephosphorylation (Lowe et al. 2004).

The P_{1B-2} group includes the Zn^{2+} -ATPases. These enzymes contain the CPC in H6, a Lys in H7 and Asp and Gly in H8, as putative components of the corresponding TM-MBS (Fig. 3). Although the roles of the Lys and Gly have not been explored, mutations of Asp714 of *Escherichia coli* ZntA yields inactive enzymes (Dutta et al. 2006). Interestingly, Asp714His and Asp714Glu mutants were able to bind Zn^{2+} and undergo phosphorylation in the presence of P_i (in the absence of metal). However, they could not be phosphorylated by ATP in the presence of Zn^{2+} . These results further support the concept that TMs H6, H7 and H8 contribute amino acids to the TM-MBS.

Group P_{1B-3} corresponds to the Cu^{2+} -ATPases. In these proteins, the second Cys in H6 is replaced by His while amino acids in H7 and H8 are similar to those in the Cu^+ -ATPase subgroup (Fig. 3). Taking into account the presence of the imidazolium side chain (a hard Lewis base), the preferences of these enzymes to transport Cu^{2+} (an intermediate Lewis base) rather than Cu^+ , is not surprising (Argüello 2003; Mana-Capelli et al. 2003). Although the lack of activity in *E. hirae* CopB carrying a CPH \rightarrow SPH mutation has been reported (Bisig et al. 2001), no further studies have been performed on the TM-MBS of P_{1B-3} proteins. Similarly, the functional roles of amino acids conserved among proteins in subgroup P_{1B-4} have not been explored. Moreover, the metal specificity of these ATPases remains unclear. Studies of *Synechocystis* PCC6803 CoaT have shown that a knockout of the encoding gene leads to increased levels of intracellular Co^{2+} and reduced Co^{2+} tolerance (Rutherford et al. 1999). A recent study characterized *A. thaliana* HMA1, an enzyme that localizes in the chloroplast envelope and contains the signature amino acids and the topology that differentiate the P_{1B-4} -ATPases. The *hma1* knockout showed high copper content in the chloroplast and a phenotype compatible with this alteration. Enzymatic determinations using chloroplast membranes from plants over-expressing HMA1 showed higher levels of ATP hydrolysis in the presence of 3 mM copper. No activation was observed in the presence of 3 mM Co^{2+} , Zn^{2+} , Mn^{2+} or Fe^{2+} . Considering both studies, it is difficult to delineate the metal specificity of P_{1B-4} -ATPases and further characterization of other members of this subgroup seems necessary.

The central role of TM-MBDs for determining the specificity of P_{1B} -ATPases is also supported by experiments looking at the metal selectivity of enzymes lacking the N- and C-MBDs. The relative selectivity, both in terms of V_{max} and metal $K_{1/2}$ for activation, among the various transported metals (Cu^+/Ag^+ , $Cu^{2+}/Cu^+/Ag^+$ or Zn^{2+} , Cd^{2+}/Pb^{2+}) is maintained when the N-MBDs are removed or mutated (Mittra and Sharma 2001; Mana-Capelli et al. 2003; Mandal and Argüello 2003). Furthermore, *E. coli* ZntA chimeras containing N-MBDs

from the WNDP Cu^+ -ATPase retain Zn^{2+} -ATPase activity (Hou et al. 2001).

Metal binding to the TM-MBS

Enzyme phosphorylation by ATP, subsequent turnover and transport requires metal binding to the TM-MBS. This is independent of metal binding to the N- and C-MBDs, since truncated ATPases lacking these domains or proteins carrying mutations yielding N-MBDs unable to bind metals are phosphorylated and transport metals (Voskoboinik et al. 1999; Bal et al. 2001; Mitra and Sharma 2001; Fan and Rosen 2002; Mana-Capelli et al. 2003; Mandal and Argüello 2003). The case of PINA (Pineal Night-Specific ATPase), a splice variant of ATP7B gene lacking the WNDP six N-MBDs, is an example of truncated proteins in vivo functionality (Borjigin et al. 1999).

Considering the metal-protein interaction, it should be kept in mind the low in vivo free Cu^+ and Zn^{2+} concentrations associated with the presence of specific chaperone proteins and/or numerous ligands (Outten and O'Halloran 2001; Changela et al. 2003). Metals activate $\text{P}_{1\text{B}}$ -ATPases with apparent affinities ($K_{1/2}$) in the 0.1–3 μM range (Okkeri and Haltia 1999; Voskoboinik et al. 1999; Sharma et al. 2000; Fan and Rosen 2002; Mandal et al. 2002; Tsvikovskii et al. 2002; Mana-Capelli et al. 2003; Eren and Argüello 2004). Most of these assays have been performed in the presence of various metal ligands such as DTT, Cys, and of course, ATP (Sharma et al. 2000; Fan and Rosen 2002; Mandal et al. 2002; Eren and Argüello 2004). Moreover, WNDP phosphorylation by ATP is activated by the Cu-Atox1 complex with a 2 μM $K_{1/2}$ (Walker et al. 2002). Therefore, these $K_{1/2}$ values do not refer to free metal concentrations but to the total metal in the media. Considering the low dissociation constants for the soluble metal complexes (metal-thiolate, metal-chaperone, metal-ATP) (Martell and Smith 2004), it can be proposed that these complexes deliver the metal directly to the TM-MBS, perhaps by a kinetically controlled ligand exchange. This mechanism was proposed to explain the metal transfer between copper

chaperones and N-MBDs (Huffman and O'Halloran 2000). The question remains whether this is a plausible in vivo mechanism of metal delivery to the TM-MBS. This is particularly relevant in the case of Cu^+ -ATPases where cytoplasmic Cu^+ is bound to chaperones. Direct metal transfer from the Cu-chaperones to the TM-MBS (or from the N-MBDs to the TM-MBS) has not been shown. However, Lutsenko and her collaborators have shown that WNDP phosphorylation by ATP in the presence of Cu-Atox1, requires the second N-MBD (Walker et al. 2004). This finding suggests that at least in proteins with multiple N-MBDs, like WNDP or MNKP, Cu^+ might be delivered to the TM-MBS by the N-MBDs. Considering this result and the presence of enzymes lacking N-MBDs; for instance, the PINA variant and the members of subgroup $\text{P}_{1\text{B-4}}$, alternative mechanisms with direct cytoplasmic metal delivery to the TM-MBS might be expected to emerge. Independent of metal delivery to TM-MBS via a cytoplasmic MBD or directly to TM-MBDs by a chaperone, the feasibility of ligand exchange with a transmembrane binding site formed by residues from H6, H7, and H8 can be examined by comparison with the structurally related SERCA1. The structure of this Ca^{2+} -ATPase shows that Ca^{2+} access to the transmembrane binding sites is through a relatively wide (8–10 Å) funnel that contains one the Ca^{2+} ligands (equivalent to the proximal Cys in $\text{P}_{1\text{B}}$ -ATPases H6) at its bottom (Toyoshima and Nomura 2002). Then, among other possibilities, it could be hypothesized that if a similar funnel were present in $\text{P}_{1\text{B}}$ -ATPases, ligand-bound metals might be “delivered” directly into the TM-MBS.

The actuator and ATP binding domains

The large cytoplasmic loop between H6 and H7 of $\text{P}_{1\text{B}}$ -ATPases contains the DKTGT sequence conserved in all P-ATPases (Fig. 2). The loop, referred to as ATP-BD, encompasses the nucleotide binding (N) and the phosphorylation (P) domains. The smaller loop between H4 and H5 forms the actuator (A) domain. Recently the structures of the isolated ATP-BD and A-domain

of AfCopA and the N-domain of WNDP have been reported (Dmitriev et al. 2006; Sazinsky et al. 2006a, b) (Fig. 4). The A-domain of AfCopA shows a 10 β -strand core with two α -helices connecting with the TMs present in the full-length protein (Fig. 4A) (Sazinsky et al. 2006a). In spite of their small sequence homology (26%), the folding of AfCopA is highly similar to that of the A-domain of SERCA1, the P₂-type Ca²⁺-ATPase (Toyoshima et al. 2000; Toyoshima and Inesi 2004; Toyoshima et al. 2004). In particular, the presence of the highly conserved (S/T)GE(P/S) sequence at the tip of a solvent accessible loop on the side of the A-domain appears similar. This has relevant functional implications. In the Ca²⁺-ATPase, the interactions of this loop with the P-domain during enzyme phosphorylation/dephosphorylation appears critical since they drive the rotation of the A-domain with a subsequent rearrangement of TMs (Toyoshima and Nomura 2002; Olesen et al. 2004; Toyoshima and Inesi 2004; Toyoshima et al. 2004). This rearrangement, in turn, leads to metal deocclusion and release (Step 2, Fig. 1). Although the different disposition of TMs in the P_{1B}-ATPases might require different transmembrane movements, the structural similarities suggest an equivalent gating mechanism for metal release.

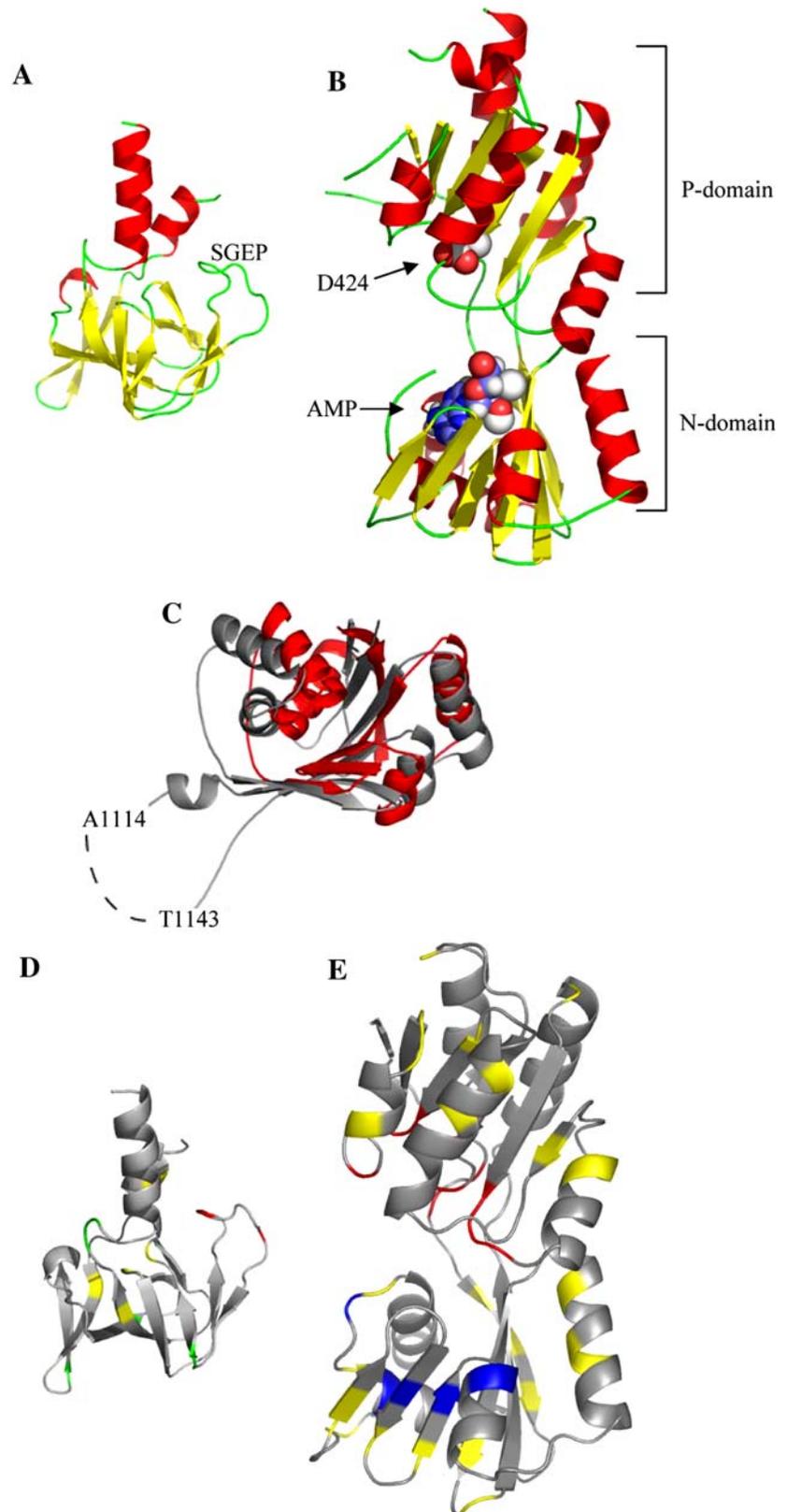
The ATP-BD structure shows the P- and N-domains joined by two short loops (the hinge region) (Sazinsky et al. 2006b). The P-domain consists of six-stranded parallel β -sheets sandwiched between three short α -helices (Fig. 4B). This domain contains the DKTGT sequence and a number of residues conserved in all P-ATPases that interact with the ATP γ -phosphate during binding and hydrolysis (Sørensen et al. 2004). Although the AfCopA P-domain is smaller and lacks some of the solvent exposed helices present in SERCA1 (Toyoshima et al. 2000) both proteins have a similar global fold.

The N-domain of both, WNDP and AfCopA, share a similar structure in accordance with their strong homology (Fig. 4C) (Dmitriev et al. 2006; Sazinsky et al. 2006b). The N-domains consist of six antiparallel β -sheets flanked by four α -helices. A significant difference is a 29 amino acid flexible loop present in the WNDP (Dmitriev et al. 2006) but not in AfCopA. This fragment might be

associated with roles peculiar to the eukaryote enzymes including perhaps alternative regulatory mechanisms and required targeting. Although these N-domains have little homology with that of SERCA1 (<25%), they reveal the same basic structure just lacking several 7–10 amino acid insertions located in the periphery of the SERCA1 structure (Toyoshima et al. 2000). More importantly, the amino acids forming the ATP binding site are distinct for the P_{1B}- and P₂-ATPases. P_{1B}-ATPases have a stronger similarity with the homologous region of KdpB, a P_{1A}-ATPase (Haupt et al. 2004; Dmitriev et al. 2006; Sazinsky et al. 2006a, b). The structures of KdpB and WNDP N-domains have been determined in the presence of nucleotides. Analysis of residues participating in nucleotide binding (His1069, Gly1099, Gly1101, Gly1149 and Asn1150 in the WNDP) shows that P_{1A}- and P_{1B}-ATPases have a unique ATP binding site distinct from that in P₂-ATPases. The involvement of some of these amino acids in ATP binding is supported by mutagenesis studies in the WNDP (Tsivkovskii et al. 2003; Morgan et al. 2004), *E. coli* ZntA (Okkeri and Haltia 1999; Okkeri et al. 2004), and *E. hirae* CopB (Bissig et al. 2001).

The structural description of the A, P, and N domains support the concept that, in spite of the differences among the sequences of the various P-ATPase subfamilies, they employ similar structures, not only to bind their ligands, but to implement the critical conformation changes required for energy transduction. Furthermore, these structures provide a framework to interpret the likely effect of many of the mutations leading to Wilson and Menkes diseases occurring in regions that are conserved in AfCopA. We have mapped the position of WNDP and MNKP mutations within the structures of the AfCopA ATP-BD and A-domain (Fig. 4D and E) (Cox and Moore 2002; Sazinsky et al. 2006a, b). This analysis allows differentiating likely effects on different enzyme functions. Mutations at positions highlighted in red in the ATP-BD likely affect enzyme phosphorylation, while the red positions in the A domain indicated those that might alter interactions among the A- and P-domains. Those in yellow might affect the overall enzyme stability rather than a specific ligand

Fig. 4 Structure of the A-, P- and N-domains of *Archaeoglobus fulgidus* CopA. Structures in *A*, *B*, *D* and *E* are presented with the segments that would be proximal to the TMs in the full-protein on the top of the models. *A*, AfCopA A-domain. Location of conserved SGEP sequence is shown. *B*, AfCopA ATP-BD. P-domain and N-domain are labeled. The location of conserved D424 is indicated. An AMP is modeled in the nucleotide binding site. Surfaces of conserved residues predicted to bind AMP are colored by atom type with oxygen *red*, carbon *grey*. *C*, Superimposed AfCopA (*red*) and WNDP (*grey*) N-domains. A flexible loop between A1114 and T1143 in the WNDP was removed from the model for clarity. *D*, Mapping of WNDP and MNKP mutations in the structure of AfCopA A-domain. *E*, Mapping of WNDP and MNKP mutations in the structure of AfCopA ATP-BD



interaction. Blue marks positions in the N-domain that when mutated might affect ATP binding, among these, His1069 in the WNDP. In the A-domain, mutations highlighted in red might affect the interactions of the A- and P-domain. Finally, in green are positions located in an accessible region of the A-domain that is opposite to the loop interacting with the P-domain. Considering their location these mutations might affect intra- or inter-molecular domain-domain interactions. Since rotation of the A-domain controls the rate limiting metal deocclusion (Step 3, Fig. 1), and a regulatory role controlling enzyme turnover has been suggested for the cytoplasmic MBDs (see below), it is tempting to hypothesize that these mutations might point out the sites of a functionally relevant N-MBD-A-domain interaction.

The cytoplasmic metal binding domains

Most P_{1B}-ATPases have various types of cytoplasmic metal binding domains (MBD) located either in the N-terminus (N-MBD) or C-terminus (C-MBD) (Figs. 2 and 3) (Tables 1 and 2). The N-MBDs observed in Cu⁺-ATPases and some bacterial Zn²⁺-ATPases are 60–70 amino acids domains with a highly conserved CXXC metal-binding sequence (Table 1) (Rensing et al. 1999; Arnesano et al. 2002; Lutsenko et al. 2003).

Eukaryote Cu⁺-ATPases contain multiple repeats of this domain. The human WNDP and MNKP have six N-MBDs that have been the focus of many studies (Arnesano et al. 2002; Lutsenko et al. 2003). Alternatively, archaea and prokaryote enzymes have usually one-two N-MBDs. The high-resolution structures of several of these domains in their apo and metal-bound forms have been reported (Gitschier et al. 1998; Banci et al. 2001; Banci et al. 2002). They present a β₂β₃β₁β₂ fold similar to the well-described Cu-chaperones like human Atox1, yeast Atx1, and prokaryote CopZ (Rosenzweig et al. 1999; Wimmer et al. 1999; Arnesano et al. 2002). In vitro, these N-MBDs can bind both monovalent and divalent cations including Cu⁺, Cu²⁺, Zn²⁺ and Cd²⁺ (DiDonato et al. 1997; Lutsenko et al. 1997; Jensen et al. 1999; Liu et al. 2005). In vivo, N-MBDs receive Cu⁺ from the corresponding Cu-chaperones (Hamza et al. 1999; Larin et al. 1999; Huffman and O’Halloran 2000; Wernimont et al. 2000). Their metal selectivity appears to be determined by the specific N-MBD-chaperone interaction via electrostatic and hydrophobic interactions that align the metal binding sites for rapid ligand exchange (Wernimont et al. 2000; Arnesano et al. 2002). This metal exchange has not been established for Zn²⁺-ATPases carrying CXXC containing N-MBDs since no Zn-chaperone (or equivalent molecule) has been identified.

Table 1 Cytoplasmic N-terminus metal binding domains of P_{1B}-ATPases

Type	Group ^a	Length ^b	Sequence	Protein
CXXC consensus	1B-1 1B-2	60–80	MVKDTYISSASKTPPMERTVVRVTGMT <u>CAMC</u> VKSJETAVGSLEGVEEVRVNLATETAFIRFD EKRIDFETIKRVIEDLGYGV	<i>A. fulgidus</i> CopA
CCXSE consensus	1B-2	90–100	MASKKMTKSYFDVLGIC <u>CTSE</u> VPLIENILNSM DGVKFEFSVIVPSRTVIVVHDTLILSQFQIVKA LNQAQLEANVRVTGETNFK	<i>A. thaliana</i> HMA2
(HX) _n (n = 2–6)	1B-2	100–150	MNQPVSE <u>HEHKHPHDHAH</u> GDDDDHGHAA <u>HGH</u> SCCGAKAAPPLVQLSETASQAQLSR FRIEAMDCPTEQTLIQDKLSKLAGIEQLEFN LINRVLGVRHTLDGTADIERAIDSLGMKA EPIAAQDDGSASVQPAKA	<i>P. putida</i> CadA-2
His rich	1B-3 1B-4	30–100	MNNGIDPENETNKKGAIGKNPEEKITVEQ TNTKNNLQEHGKMENMDQ <u>HH</u> THGHMER <u>HQ</u> QMDHGHMSGMDHSHMDHEDMSGMN <u>H</u> SHMGH <u>E</u> NMSGMDHSMHMGNFQK	<i>E. hirae</i> CopB

^a Refers to subgroup classification showed in Fig. 1

^b Number of amino acids

Table 2 Cytoplasmic C-terminus metal binding domains of P_{1B}-ATPases

Type	Group ^a	Length ^b	Sequence	Protein
Cys rich	1B-2	60–280	LNSM ^L TL ^L REEW ^K GGAKEDGAC ^R ATARS ^L VMRSQ ^L AADS QAPNAADAGAAGREQTNG ^C R ^C CPKPGMSPEHSVVID IRADGERQEERPAEAAVVA ^K CCGGGGGEGIRCGASK KPTATVVVA ^K CCGGGGGGEGTR ^C CGASKNPATAAVV AKCCSGGGGEGIG ^C CGASKKPTATAVVAKCCGGGGEG TRCAASKKPATAAVVA ^K CCGGDGGEGTG ^C CGASKRSP PAEGSCSGGEGGTNGVGR ^C CTSVKRPTCCDMGAA EVS ^D SSPETAKDCRNGRCCAKTMNSGEVKG	<i>O. sativa</i> HMA3
Cys/His rich	1B-2	260–480	MLLLSDK ^H K ^T GN ^K CYRE ^S SSSVLIAEKLEGDAAGDMEAG LLPKISDK ^H CKPG ^C CGTKTOEKAMKPAKASSD ^H SHSGC CETKQKDNVTVVKK ^S CCAEPVDLGHGHDSGCCGDKSQ QPH ^O HEVQVQOS ^C HN ^K PSGLDSGCCG ^G KSQOPH ^O HEL QOS ^C HDKPSGLDIGTGPK ^H EGSSTLVNLEGDAKEELKV LVNGFCSSPADLAITSLKVK ^S SDSHCKSNCSSRER ^C HHGS NCCRSYAKES ^C SHD ^H HHHTRAH ^H GVGTLKEIVIE	<i>A. thaliana</i> HMA2

^a Refers to subgroup classification showed in Fig. 1

^b Number of amino acids

Recent structural analysis of *E. coli* ZntA N-MBD, suggest that Asp in the DCXXC sequence might provide the electrostatic requirement to confer Zn²⁺ specificity to these domains (Banci et al. 2002).

Some prokaryote Zn²⁺-ATPases have His-rich N-MBDs [(HX)_n (n = 2–6)] alone or together with the CXXC N-MBDs (Table 1). Similar sequences have been observed in ZIP and Cation Diffusion Facilitator (CDF) families, located in cytoplasmic loops joining TMs (Paulsen and Saier 1997; Eng et al. 1998). Cu²⁺-ATPases (subgroup P_{1B-3}) and a few members of subgroup P_{1B-4} also have His rich N-MBDs. However, these are His stretches instead of HX repeats. Although it is clear that these have functional roles (Mana-Capelli et al. 2003), the metal binding specificity and properties of these domains have not been characterized.

All plant Zn²⁺-ATPases contain unique sequences in both the N-terminus and C-terminus. Plant N-MBDs appear homologous to those described for Cu⁺-ATPases and prokaryote Zn²⁺-ATPases. However, the CXXC sequences are replaced by CCXSE (x = S, T, P) (Table 1). These changes appear critical for selective Zn²⁺ binding since *A. thaliana* HMA2 N-MBD binds Zn²⁺ in vitro but not Cu⁺ (Eren and Argüello, unpublished results). Plant Zn²⁺-ATPases also have unusually long C-termini containing

numerous His and Cys (Table 2). These have various lengths and present two different patterns. The putative C-MBD from *A. thaliana* HMA3 and its homologs, have numerous Cys but no (or few) His residues. Alternatively, HMA2, HMA4 and homologous proteins have His- and Cys-rich C-MBD. We have characterized metal binding to the HMA2 C-MBD. This domain binds three Zn²⁺ (or Cd²⁺) with an apparent K_d in the nanomolar range. One of the Zn²⁺ binding sites is apparently formed by one Cys and three His, while the others are constituted by four His (Eren and Argüello, unpublished results).

While the various structures observed for N-MBDs and C-MBDs add to the complexity of P_{1B}-ATPases, these appear as different strategies to accomplish similar functions related to the regulation of these pumps.

Regulatory roles of cytoplasmic MBDs

MBDs are not essential for ATP hydrolysis and metal transport by P_{1B}-ATPases (Voskoboinik et al. 1999; Bal et al. 2001; Mitra and Sharma 2001; Fan and Rosen 2002; Mana-Capelli et al. 2003; Mandal and Argüello 2003). Numerous studies have pointed out alternative regulatory roles for these domains (Rensing et al. 1999; Fatemi and Sarkar 2002; Lutsenko et al. 2003).

They are required for the Cu-dependent targeting of MNKD and WNDP (Petris et al. 1996; Forbes et al. 1999; Schaefer et al. 1999). However, these critical physiological phenomena are beyond the scope of this discussion. Alternatively, it has also been observed that the N-MBDs control the rate of P_{1B} -ATPases turnover with little or no change in metal affinity (Voskoboinik et al. 1999; Mitra and Sharma 2001; Voskoboinik et al. 2001; Fan and Rosen 2002; Mana-Capelli et al. 2003; Mandal and Argüello 2003). These findings suggested that metal binding to N-MBDs affects the rate-limiting step of these enzymes catalytic cycle. Studies using *A. fulgidus* CopA and CopB as models confirmed that the rate limiting conformational change associated with metal release/dephosphorylation is slower when the N-MBDs are removed or made unable to bind metals by specific mutations (Mana-Capelli et al. 2003; Mandal and Argüello 2003). This effect is likely achieved by Cu-dependent changes in the interactions of N-MBDs with other protein domains. Supporting this idea, the Cu^+ dependent interaction of Wilson disease protein N-MBDs with the large ATP binding cytoplasmic loop has been shown (Tsivkovskii et al. 2001).

Summary and future directions

Significant advances have been produced in the understanding of the heavy metal transport mechanisms by P_{1B} -ATPases. The structures of domains responsible for ATP binding and hydrolysis, and energy transduction have been determined. The presence of the transmembrane metal binding site constituted by side chains from residues in TMs H6, H7 and H8, has been established. This has been an important step toward understanding the mechanisms of selectivity and metal translocation across the membrane. Understanding the catalytic cycle has facilitated probing the direction of transport and the regulatory role of cytoplasmic metal binding domains. Future molecular studies will likely address the detailed structure of the transmembrane region, the mechanisms of metal delivery to transmembrane metal binding site, the interactions among cytoplasmic domains and their functional consequences.

Acknowledgements We thank Matt Sazinsky, Danielle DeOssie, and Amy Rosenzweig for critical reading of this manuscript. This work was supported by NSF grant MCM-0235165 (J. M. A.).

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