Published in final edited form as:

Curr Top Membr. 2012 ; 69: 113–136. doi:10.1016/B978-0-12-394390-3.00005-7.

Toward a Molecular Understanding of Metal Transport by P_{1B}-Type ATPases

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Abstract

The P_{1B} family of P-type ATPases couples the transport of cytoplasmic transition metals across biological membranes to the hydrolysis of ATP. These ubiquitous transporters function in maintaining cytoplasmic metal quotas and in the assembly of metalloproteins, and have been classified into subfamilies (P_{1B-1}–P_{1B-5}) on the basis of their transported substrates (Cu⁺, Zn²⁺, Cu²⁺, and Co²⁺) and signature sequences in their transmembrane segments. In addition, each subgroup presents a characteristic membrane topology and specific regulatory cytoplasmic metal-binding domains. In recent years, significant major aspects of their transport mechanism have been described, including the stoichiometry of transport and the delivery of substrates to transport sites by metallochaperones. Toward understanding their structure, the metal coordination by transport sites has been characterized for Cu⁺ and Zn²⁺-ATPases. In addition, atomic resolution structures have been determined, providing key insight into the elements that enable transition metal transport. Because the Cu⁺-transporting ATPases are found in humans and are linked to disease, this subfamily has been the focus of intense study. As a result, significant progress has been made toward understanding Cu⁺-ATPase function on the molecular level, using both the human proteins and the bacterial homologs, most notably the CopA proteins from *Archaeoglobus fulgidus*, *Bacillus subtilis*, and *Thermotoga maritima*. This chapter thus focuses on the mechanistic and structural information obtained by studying these latter Cu⁺-ATPases, with some consideration of how these aspects might differ for the other subfamilies of P_{1B}-ATPases.

1. INTRODUCTION

1.1. Overall Architecture

The P-type ATPases are a large family of integral membrane proteins that use the energy of ATP hydrolysis to transport cations and lipids across membranes (Bublitz, Morth, & Nissen, 2011; Palmgren & Nissen, 2011). In these enzymes, the hydrolysis of ATP and formation of a covalent phosphoenzyme intermediate are coupled to conformational changes in the transmembrane segments (TM) that allow ion translocation across the membrane (Palmgren & Nissen, 2011). The P-type ATPases are divided into subclasses on the basis of substrate specificity. These subfamilies include the P_{1A}-ATPases (K⁺), the P_{1B}-ATPases (heavy metals), the P₂-ATPases (Ca²⁺, Na⁺/K⁺, and H⁺/K⁺), the P₃-ATPases (H⁺), the P₄-ATPases (phospholipids), and the P₅-ATPases (substrate unknown) (Lutsenko & Kaplan, 1995; Palmgren & Nissen, 2011). The P₂-ATPases include the well-characterized sarcoplasmic

reticulum Ca^{2+} -ATPase (SERCA) and the Na^+/K^+ -ATPase, both of which have been structurally characterized (Morth et al., 2007; Toyoshima, 2008). The P_3 -ATPases are plasma membrane H^+ pumps and structurally resemble the P_2 -ATPases (Pedersen, Buch-Pedersen, Morth, Palmgren, & Nissen, 2007).

The $\text{P}_{1\text{B}}$ -ATPases, which transport heavy metal ions, are found in all kingdoms of life and are the prevalent P-type ATPases in bacteria and archaea (Palmgren & Nissen, 2011). While they share structural elements with P-type ATPases, there are several unique aspects to the architecture of the $\text{P}_{1\text{B}}$ -ATPases. All P-type ATPases include two soluble cytoplasmic domains, the ATP-binding and actuator domains (ATPBD and A domain). The ATPBD comprises two distinct domains, the nucleotide-binding or N domain and the phosphorylation or P domain. All P-type ATPases also have a central core of six TM helices (denoted H1–H6) (Fig. 1). In most, but not all, $\text{P}_{1\text{B}}$ -ATPases (Section 1.2), two additional TM helices are inserted at the N-terminal side of the core domain (denoted MA and MB) (Argüello, Eren, & González-Guerrero, 2007; Palmgren & Nissen, 2011). A second defining feature of $\text{P}_{1\text{B}}$ -ATPases is the presence of various soluble MBDs at the N- and C-termini (Argüello et al., 2007; Argüello, González-Guerrero, & Raimunda, 2011).

1.2. Substrate Diversity

The $\text{P}_{1\text{B}}$ -ATPases transport a wide array of substrates, including $\text{Zn}^{2+}/\text{Cd}^{2+}/\text{Pb}^{2+}$ (Rensing, Sun, Mitra, & Rosen, 1998; Sharma, Rensing, Rosen, & Mitra, 2000), Cu^{2+} (Mana-Capelli, Mandal, & Argüello, 2003), Cu^+/Ag^+ (Mandal, Cheung, & Argüello, 2002), and Co^{2+} (Rutherford, Cavet, & Robinson, 1999; Scherer & Nies, 2009). Residues in the last three TM helices (H4–H6) are proposed to form the metal-binding site(s) (MBS) and specificity is believed to derive from the identities and positions of residues within these helices (Argüello, 2003) (Fig. 1). In particular, mutagenesis data indicate that a three-residue, cysteine-containing sequence motif in H4 [CPC, CPH, SPC, (T/S) PCP, YPC, and APC] is important for metal transport activity (Argüello et al., 2007; Dutta, Liu, Hou, & Mitra, 2006; Fan & Rosen, 2002; Forbes & Cox, 1998; Lowe et al., 2004). These sequence motifs and limited experimental data linking various $\text{P}_{1\text{B}}$ -ATPases to specific metal ions have been used to develop a subclassification scheme in which five families are designated $\text{P}_{1\text{B-1}}$ through $\text{P}_{1\text{B-5}}$ (Fig. 1) (Argüello, 2003; Argüello et al., 2007). The $\text{P}_{1\text{B-1}}$, $\text{P}_{1\text{B-2}}$, and $\text{P}_{1\text{B-3}}$ families include the additional TM helices MA and MB at the N-terminus. The $\text{P}_{1\text{B-1}}$ -ATPases are specific for Cu^+ and Ag^+ , $\text{P}_{1\text{B-2}}$ -ATPases are $\text{Zn}^{2+}/\text{Cd}^{2+}/\text{Pb}^{2+}$ transporters, $\text{P}_{1\text{B-3}}$ -ATPases are Cu^{2+} -ATPases, and $\text{P}_{1\text{B-4}}$ -ATPases are believed to transport primarily Co^{2+} and perhaps Zn^{2+} and Cd^{2+} . The $\text{P}_{1\text{B-5}}$ -ATPase subgroup contains the (T/S)PCP sequence in H4 and QEXXD in H6, but their substrates have not been determined (Argüello, 2003; Traverso et al., 2010). Lastly, a small number of $\text{P}_{1\text{B}}$ -ATPases with unknown substrates contain different signature sequences in their fourth TM (YPC, APC, etc.) and do not fit this classification (Argüello, 2003; Völlmecke, Lorenz Drees, Reimann, Albers, & Lübben, 2012).

The subfamilies are further differentiated on the basis of the type of MBDs present. Most bacterial $\text{P}_{1\text{B-1}}$ -ATPases and $\text{P}_{1\text{B-2}}$ -ATPases contain one or more MBDs (Fig. 1) (Argüello, 2003; Solioz & Vulpe, 1996). These resemble the Atx1-like family of Cu^+ chaperones, small soluble proteins that bind Cu^+ and can transfer it directly to partner $\text{P}_{1\text{B-1}}$ -ATPases (Boal & Rosenzweig, 2009; Robinson & Winge, 2010; Rosenzweig, 2001) (Section 2.4). Eukaryote $\text{P}_{1\text{B-1}}$ -ATPases contain up to six repeating N-terminal MBDs (Argüello, 2003; Solioz & Vulpe, 1996). A number of these MBDs have been characterized in vitro and bind a single Cu^+ ion via a conserved CXXC motif (Boal & Rosenzweig, 2009; Singleton & LeBrun, 2007) (Section 3.1). The Atx1-like MBDs in the $\text{P}_{1\text{B-2}}$ -ATPases contain, in addition to the two cysteines in the CXXC motif, conserved carboxylate residues that provide additional ligands for Zn^{2+} and Cd^{2+} (Banci et al., 2002; Eren, González-Guerrero, Kaufman, &

Argüello, 2007). Some P_{1B-2}-ATPases, notably those in plants, contain other types of histidine- and cysteine-rich MBDs in their C-termini (Argüello, 2003; Eren et al., 2007; Eren, Kennedy, Maroney, & Argüello, 2006). The Cu²⁺-specific P_{1B-3}-ATPases have a histidine-rich N-terminal MBD, which is necessary for maximal Cu²⁺ transport activity (Mana-Capelli et al., 2003). The two subfamilies that lack helices MA and MB also lack N-terminal MBDs. The P_{1B-4}-ATPases have no MBDs, but a number of P_{1B-5}-ATPases contain a novel C-terminal MBD, classified as a hemerythrin (Hr)-like domain and demonstrated to bind a diiron center (Traverso et al., 2010).

1.3. Biological Functions

The P_{1B}-ATPases have two important roles in both prokaryotes and eukaryotes (Lutsenko, Gupta, Burkhead, & Zuzel, 2008; Raimunda, González-Guerrero, Leeber, & Argüello, 2011). First, they efflux cytoplasmic metal ions, protecting cells from the deleterious effects of elevated heavy metal concentrations. Second, they provide metal cofactors for biosynthesis of metalloproteins. In prokaryotes, there are many examples of systems in which genetic disruption of P_{1B}-ATPases leads to toxic metal accumulation, including *Escherichia coli* ZntA (Rensing, Mitra, & Rosen, 1997), *E. coli* CopA (Rensing, Fan, Sharma, Mitra, & Rosen, 2000), *Synechocystis* sp. PCC6803 PacS (Tottey, Rich, Rondet, & Robinson, 2001), *Enterococcus hirae* CopB (Odermatt, Suter, Krapf, & Solioz, 1993), and *Synechocystis* sp. PCC6803 CoaT (Rutherford et al., 1999). Prokaryotic P_{1B-1}-ATPases also play an important role in protein maturation, extruding Cu⁺ ions to the periplasm for insertion into metalloenzymes. This dual functionality is consistent with the presence in many genomes of multiple genes encoding P_{1B-1}-ATPases (Raimunda et al., 2011). In *Pseudomonas aeruginosa*, the CopA1 Cu⁺-ATPase provides copper tolerance, whereas disruption of a second Cu⁺-ATPase, CopA2, results in reduced cytochrome oxidase activity and sensitivity to oxidative stress, suggesting a role in copper delivery to periplasmic chaperone proteins (González-Guerrero, Raimunda, Cheng, & Argüello, 2010). In the photosynthetic cyanobacteria *Synechocystis* sp. PCC6803, two P_{1B-1}-ATPases are present, CtaA in the plasma membrane and PacS in the thylakoid membrane, and are suggested to be involved in copper tolerance and copper delivery to *caa*₃ oxidase/plastocyanin, respectively (Tottey, Harvie, & Robinson, 2005).

The metal transport activity of P_{1B}-ATPases is also required for bacterial infection. Phagosomal copper and zinc overload has emerged as a potential bactericidal mechanism (Botella et al., 2011; Wagner et al., 2005; White, Lee, Kambe, Fritsche, & Petris, 2009) and bacterial Cu⁺-ATPases responsible for maintaining low cytoplasmic Cu⁺ levels appear essential for survival in the host (González-Guerrero et al., 2010; Schwan, Warrener, Keunz, Stover, & Folger, 2005; White et al., 2009; Zhang & Rainey, 2007). The Cu⁺-ATPases associated with cytochrome *c* oxidase are also necessary for infection, apparently to cope with the phagosomal oxidative burst (González-Guerrero et al., 2010). It is likely that the observed induction of various P_{1B}-ATPases during infection might be linked to survival in the phagosomal noxious environment (Botella et al., 2011; Sasseti & Rubin, 2003).

The eukaryotic P_{1B}-ATPases are found in the trans-Golgi network, plasma membrane, vacuole, and chloroplast. In *Arabidopsis thaliana*, there are eight P_{1B}-ATPases (Williams & Mills, 2005). These include P_{1B-1}-, P_{1B-2}- and P_{1B-4}-ATPases involved in both organismal metal distribution and metalloenzyme assembly (Williams & Mills, 2005). For instance, the participation of the Cu⁺-ATPases HMA6, HMA7 in the assembly of plastocyanin and chloroplast Cu/Zn-SOD has been shown, as well as the role of HMA8 in the assembly of ethylene receptor (Abdel-Ghany, Muller-Moule, Niyogi, Pilon, & Shikanai, 2005; Hirayama et al., 1999). The function of the Zn²⁺-ATPases HMA2 and HMA4 in controlling Zn²⁺ organismal distribution has also been described (Eren & Argüello, 2004; Mills, Krijger, Baccarini, Hall, & Williams, 2003). Fungi and animals contain primarily P_{1B-1}-ATPases.

Among the earliest characterized P_{1B-1} -ATPases was the *Saccharomyces cerevisiae* Ccc2 ATPase that delivers Cu^+ to the multicopper oxidase Fet3 (Yuan et al., 1995). In humans, there are two Cu^+ -transporting P_{1B-1} -ATPases, ATP7A and ATP7B (Bull & Cox, 1994). ATP7A is primarily expressed in the intestine and the choroid plexus; ATP7B is primarily expressed in the liver and the brain (Lutsenko et al., 2008). These proteins cycle between the trans-Golgi network, endocytic vesicles, and the plasma membrane as a function of copper concentrations, and function both in Cu^+ export and in biosynthesis of metalloenzymes, including tyrosinase, lysyl oxidase, peptidylglycine α -monooxygenase, and ceruloplasmin (Lutsenko, Barnes, Bartee, & Dmitriev, 2007; Lutsenko et al., 2008). In addition, the trafficking of ATP7A to the phagosome membrane during infection contributes to phagosomal copper accumulation and resultant bacterial killing (White et al., 2009).

Mutations in ATP7A and ATP7B lead to Menkes syndrome and Wilson disease, respectively (Bull & Cox, 1994). Menkes syndrome is caused by decreased Cu^+ uptake across the small intestine, compounded by the lost ability to transfer copper from the cell cytosol into the lumen of the secretory pathway, as well as impaired copper distribution to a variety of tissues, including the brain (Kaler, 1998). The resultant deficiencies in copper-containing enzymes are manifested as severe impairment of neurological and connective tissue function (Mercer, 2001). By contrast, Wilson disease is characterized primarily by toxic copper overload in the liver due to reduced biliary excretion. The presence of yellow “Kayser–Fleischer” rings around the cornea of the eye, which signifies copper buildup in the brain, is sometimes the first symptom of Wilson disease (Das & Ray, 2006; Gitlin, 2003). Notably, increased expression of the Menkes and Wilson ATPases is also associated with resistance to the anticancer drug cisplatin (Katano et al., 2002; Safaei, Holzer, Katano, Samimi, & Howell, 2004).

2. MECHANISM

2.1. Catalytic Cycle

The Cu^+ -ATPases follow the general steps of the classical Post–Albers cycle that describes the transport and catalysis of P-type ATPases with some differences associated with the chemistry and biological availability of their substrates (Argüello et al., 2011; Post, Hegyvary, & Kume, 1972) (Fig. 2). In this mechanism, enzymes assume two major conformational states, E1 and E2, presenting differential affinities for the transported substrates (Hatori, Majima, Tsuda, & Toyoshima, 2007; Mandal, Yang, Kertesz, & Argüello, 2004). In its E1 form, the enzyme binds nucleotides at the ATPBD and the metal substrate at the transmembrane transport sites from the cytoplasmic side with femtomolar affinity (González-Guerrero & Argüello, 2008). Although both nucleotide and metal can independently bind to the ATPase, the essential coupling of solute transport with ATP hydrolysis requires simultaneous ATP and metal binding to drive catalytic phosphorylation of the aspartic acid residue in the invariant DKTGT sequence and the subsequent enzyme turnover. Moreover, in the case of Cu^+ -ATPases, which transport two Cu^+ ions per ATP, full occupancy of the transport sites is required for catalysis (González-Guerrero, Eren, Rawat, Stemmler, & Argüello, 2008; Mandal et al., 2004). Phosphorylation causes conformational changes leading to metal coordination rearrangements that force the release of metal into a luminal/periplasmic compartment as the enzyme transitions to a phosphorylated E2 state. The metal release appears to be a rate-limiting step in the catalytic cycle. This phenomenon explains the various transport rates observed with alternative substrates when the activity is measured in vitro (Mandal et al., 2002). It is significant that P_{1B} -ATPases turnover at relatively slow rates $<10/s$ (Hatori et al., 2007; Mandal et al., 2002; Sharma et al., 2000). These rates are much lower than those observed for P_2 - and P_3 -ATPases and highlight the differences between transition metal and alkali metal transport. Unlike Na^+ , K^+ , or Ca^{2+} , transition metal ions are not free/hydrated in the cell. Thus,

although the free metal is transported, it binds the transport sites via a mechanism of metal exchange involving chaperone proteins and other sequestering molecules (see Section 2.4).

2.2. Transmembrane Metal-Binding Sites

The presence of distinct transmembrane metal-binding sites has been established for Cu^+ - and Zn^{2+} -ATPases. A combination of metal-binding and mutagenesis experiments on *Archaeoglobus fulgidus* CopA clearly showed the presence of two TM sites with femtomolar affinity for Cu^+ (González-Guerrero & Argüello, 2008). This high affinity prevents release of the Cu^+ ions back into the cytoplasm prior to transfer. According to extended X-ray absorption fine structure (EXAFS) data, both sites exhibit trigonal planar coordination geometry with a mixture of sulfur and oxygen/nitrogen ligation. The first site involves Cys 380 and Cys 382 from the conserved CPC motif in H6 and Tyr 682 from H7 (González-Guerrero et al., 2008; Mandal et al., 2004). The second site comprises Asn 683 from H7 and residues Met 711 and Ser 715 from H8 (Fig. 3C). All these residues are required for Cu^+ binding and translocation. The high degree of conservation of these amino acids suggests that the binding of two Cu^+ ions and consequent transport stoichiometry is a general phenomenon in all Cu^+ -ATPases. It should be noted, however, that the metal-binding stoichiometry may vary across the $\text{P}_{1\text{B}}$ -ATPase subfamilies. For example, the *E. coli* Zn^{2+} $\text{P}_{1\text{B-2}}$ -ATPase ZntA is reported to have a single TM Zn^{2+} -binding site (Liu, Dutta, Stemmler, & Mitra, 2006) in which the ion is coordinated in a tetragonal geometry by Cys 392 and Cys 394 in H4, an Asp 714 in H8 and likely Lys 693 in H5 (Dutta et al., 2006; Dutta, Liu, Stemmler, & Mitra, 2007; Okkeri & Haltia, 2006; Raimunda, Subramanian, Stemmler, & Argüello, 2012).

2.3. Direction of Transport

The structural coupling of cytoplasmic substrate efflux to ATP hydrolysis and catalytic phosphorylation has been demonstrated in the P_2 -ATPases by studies characterizing ion transport, ATP hydrolysis and various partial reactions (Palmgren & Nissen, 2011; Toyoshima & Inesi, 2004). Crystal structures of the SERCA Ca^{2+} -ATPase in various conformations have provided further support for this paradigm (Palmgren & Nissen, 2011; Toyoshima, 2008). As counterpart to this, when a second substrate is counter-transported (Na^+ , K^+ -ATPase, H^+ , and K^+ -ATPase), the inward transported ions bind the enzyme in an E2 form and consequently cannot drive catalytic phosphorylation. For the $\text{P}_{1\text{B}}$ -ATPases, the efflux of cytoplasmic metals has been shown for Cu^+ -, Zn^{2+} -, Cu^{2+} -, and Co^{2+} -ATPases (Fan & Rosen, 2002; Mana-Capelli et al., 2003; Rensing et al., 1998; Scherer & Nies, 2009; Voskoboinik et al., 1999). In these cases, the direct measurement of transport was achieved by monitoring radioisotope uptake into everted vesicles obtained from systems heterologously expressing the target ATPases. However, phenotypes resulting from mutation of specific Cu^+ -ATPases are not always consistent with the mechanism of transport described above. Whereas mutation of most bacterial Cu^+ -ATPases leads to cytoplasmic copper overload, no changes or even a small decrease in the cytoplasmic copper content has been observed in some cases (González-Guerrero et al., 2010; Odermatt et al., 1993; Tottey et al., 2001). However, kinetic characterization combined with functional analysis indicates that Cu^+ -ATPases involved in cuproprotein assembly do transport cytoplasmic Cu^+ to extracellular milieus, but at a low rate (González-Guerrero et al., 2010; Raimunda et al., 2011). This property, while compatible with their functional role, is not suitable to maintain cytoplasmic copper quotas, a task performed by homologous ATPases present in the genome.

2.4. Role of Metallochaperones

A unique feature of the Cu^+ -ATPases is that, in the majority of the currently characterized systems, the Cu^+ substrate is delivered to the TM sites by soluble Cu^+ chaperone proteins.

These ~70 residue proteins exhibit a conserved ferredoxin-like $\beta\alpha\beta\beta\alpha\beta$ fold and bind Cu^+ ions via a conserved CXXC motif in a linear two-coordinate fashion (Boal & Rosenzweig, 2009; Robinson & Winge, 2010). The prototypical Cu^+ chaperone is *S. cerevisiae* Atx1. Disruption of Atx1 interferes with Cu^+ delivery from the Cu^+ -ATPase Ccc2 to the multicopper oxidase Fet3, which is involved in iron transport (Lin, Pufahl, Dancis, O'Halloran, & Culotta, 1997). Atx1 and numerous homologs from humans (called Atox1), bacteria, and archaea (called CopZ) have been biochemically and structurally characterized (Robinson and Winge, 2010).

Observations that these chaperones are able to exchange Cu^+ with the N-terminal MBDs of their partner Cu^+ -ATPases (Hamza, Schaefer, Klomp, & Gitlin, 1999; Walker et al., 2004; Walker, Tsivkovskii, & Lutsenko, 2002) initially led to a proposed mechanism in which substrate is routed from chaperone to MBDs and then from MBDs to the TM sites (Achila et al., 2006; Huffman & O'Halloran, 2000; O'Halloran & Culotta, 2000; Pufahl et al., 1997). In support of this pathway, the second MBD of ATP7B appears necessary for copper-dependent phosphorylation with Atox1 providing Cu^+ (Walker et al., 2004). However, in bacterial systems, removal of the MBDs or mutation of their metal-binding motifs does not affect copper-dependent ATPase activity (Fan & Rosen, 2002; Mandal & Argüello, 2003) and in the case of *A. fulgidus* CopA does not prevent activation by the copper-loaded chaperone CopZ (González-Guerrero & Argüello, 2008). Instead, the TM sites can be directly loaded by the chaperone and the MBDs cannot substitute for the chaperone in this role.

Metal transfer from CopZ to the TM-MBS in the ATPase is key to Cu^+ homeostasis because it allows the efflux of cytoplasmic Cu^+ pools while preventing the release of free ion into the cytoplasmic milieu. Similarly, this represents a departure from the well-characterized reversible interaction of alkali and alkali earth metals with their transporters (Argüello, Raimunda, & González-Guerrero, 2012). While the apo form of the chaperone apparently does not interact with the protein, the Cu^+ -loaded chaperone probably docks with the "platform" region close to the TM site (Argüello et al., 2011; Gourdon et al., 2011) (Fig. 3A) (Section 3.2). The chaperone sequentially delivers Cu^+ to either TM site, and mutation of one site does not affect delivery to the other (González-Guerrero & Argüello, 2008). Once the Cu^+ is bound to a TM site, it cannot be exchanged with the apo chaperone. This, together with the high affinity of TM sites, leads to a practically irreversible Cu^+ delivery to TM sites (Fig. 2). Interestingly, once the first TM site is occupied with Cu^+ , nucleotide binding is required for loading of the second TM site (González-Guerrero & Argüello, 2008).

2.5. Regulation

Although the MBDs are not required for activity of the bacterial $\text{P}_{1\text{B}}$ -ATPases, they do affect the turnover rates (Fan & Rosen, 2002; Mana-Capelli et al., 2003; Mandal & Argüello, 2003; Mitra & Sharma, 2001). It is apparent that metal binding to the MBDs precludes their interaction with the ATPBD, allowing faster enzyme turnover (González-Guerrero, Hong, & Argüello, 2009; Hatori et al., 2007). The similar affinities of the MBDs and their cognate chaperones for Cu^+ are also consistent with a regulatory role (Huffman & O'Halloran, 2000; Walker et al., 2002; Wernimont, Yatsunyk, & Rosenzweig, 2004). Through these mechanisms, the MBDs can provide a direct readout of Cu^+ concentrations in the cell to the transporter.

In addition, the overall $\text{P}_{1\text{B}}$ -ATPase cellular activity is controlled by a number of metal-sensing transcriptional regulators. Members of the MerR family of Cu^+ sensors (CueR like) are activators that respond to cellular Cu^+ excess and elicit Cu^+ -ATPase expression (Ma, Jacobsen, & Giedroc, 2009; Osman & Cavet, 2008). CopY is a copper-responsive repressor

present in *Lactobacillus lactis*, *Enterococcus hirae* and other gram-positive bacteria (Solioz, Abicht, Mermod, & Mancini, 2010). At low Cu^+ , CopY remains bound to the promoter regions preventing transcription of Cu^+ -ATPases. Finally, the recently described *Mycobacterium tuberculosis* CsoR is the founding member of a large family of Cu^+ -sensing transcriptional repressors (Ma et al., 2009; Osman & Cavet, 2008).

The human Cu^+ -ATPases are also regulated by Cu^+ binding to MBDs. Removal of the first five MBDs of ATP7B affects the rates of phosphorylation, and binding of Cu^+ to the MBDs prevents interaction with the ATPBD (Huster & Lutsenko, 2003; Tsivkovskii, MacArthur, & Lutsenko, 2001). Similar control of ATP7A Cu^+ transport has been described (Voskoboinik, Mar, Strausak, & Camakaris, 2001). In mammals, an additional layer of regulation derives from copper-dependent trafficking of the Cu^+ -ATPases (Lutsenko et al., 2007). At basal copper concentrations, ATP7A and ATP7B are localized to the trans-Golgi network. At increased copper concentrations, both relocate to the membrane for Cu^+ export, ATP7A to the basolateral membrane and ATP7B to the canalicular membrane to export Cu^+ into the bile.

3. STRUCTURE

3.1. Soluble Domains

Initial efforts to structurally characterize Cu^+ -ATPases focused on the soluble domains, which are readily cloned, expressed, and purified in quantities sufficient for structure determination by X-ray crystallography or nuclear magnetic resonance (NMR). Numerous NMR structures of the N-MBDs have been reported, including single MBDs of yeast Ccc2, human ATP7A (all six), *Bacillus subtilis* CopA, and *Synechocystis* sp. PCC6803 PacS (Boal & Rosenzweig, 2009) (Fig. 3B). These structures have been determined both with Cu^+ bound and in the apo forms. The crystal structure of the apo C-terminal MBD from *A. fulgidus* CopA has also been reported (Agarwal et al., 2010). All MBDs exhibit the classic ferredoxin-like $\beta\alpha\beta\beta\alpha\beta$ fold and bind Cu^+ with the conserved CXXC motif in a two-coordinate linear or approximately linear fashion (Fig. 3B). Structural alterations upon metal binding are typically confined to coordinating residues or residues adjacent to the metal-binding site. Other common features include stabilization of the metal-binding loop by hydrogen bonding interactions and hydrophobic interactions with residues in the protein core (Boal & Rosenzweig, 2009). Finally, analysis of the electrostatic surfaces of these domains has provided some insight into protein–protein interactions with Cu^+ chaperones (Banci, Bertini, McGreevy, et al., 2010; Huffman & O'Halloran, 2001).

Given that yeast and many bacterial Cu^+ -ATPases have two sequential N-terminal MBDs and ATP7A and ATP7B have six N-terminal MBDs, it is also important to determine how these MBDs interact with one another on the molecular level and whether Cu^+ affects this interaction. Circular dichroism and EXAFS spectroscopic data suggest that Cu^+ binding affects the structure of the entire N-terminal soluble domain of ATP7B (DiDonato, Hsu, Narindrasorasak, Que, & Sarkar, 2000; Ralle, Lutsenko, & Blackburn, 2004). Structural studies of proteins containing multiple MBDs have been hampered by the presence of flexible linker sequences, but several NMR structures have been reported. In the structure of MBDs 5 and 6 from ATP7B, the two domains are oriented with the CXXC motifs at opposite sides of the molecule and the eight-residue linker in between the two domains (Achila et al., 2006). MBDs 3 and 4 of ATP7B have also been characterized. In this case, the two domains are connected by a 31-residue linker, which is disordered (Banci, Bertini, Cantini, Rosenzweig, & Yatsunyk, 2008). Interestingly, in all these structures of multiple MBDs, including a partial characterization of ATP7A domains 4–6, minimal perturbations are observed upon Cu^+ binding. Similar results were obtained from an NMR study of the

two MBDs from *B. subtilis* CopA (Banci, Bertini, Ciofi-Baffoni, Gonnelli, & Su, 2003; Singleton et al., 2008).

Protein–protein interactions between the MBDs and the Cu⁺ chaperone proteins have also been probed by NMR. These studies have focused on whether all the MBDs or just selected MBDs can interact with the chaperones and which residues specifically facilitate docking and recognition (Banci, Bertini, McGreevy, et al., 2010; Boal & Rosenzweig, 2009). Most of these studies involve chemical shift mapping, but NMR structures of complexes between *S. cerevisiae* Atx1 and MBD 1 of Ccc2 (Banci et al., 2006) and between Atox1 and domain 1 of ATP7A (Banci, Bertini, Calderone, et al., 2009) have also been determined. In these structures, a Cu⁺ ion is primarily coordinated by two cysteine residues from the CXXC motif in the MBD and one cysteine from the chaperone CXXC motif. A non-physiological Cd²⁺-bridged complex between Atox1 and MBD 1 of ATP7A is also available. The complexation surfaces in these complexes include complementary electrostatic as well as hydrophobic interactions (Banci, Bertini, McGreevy, et al., 2010). Although these structures have provided insight into metal coordination and protein–protein recognition, limited conclusions can be drawn in the absence of the additional Cu⁺-ATPase domains.

The other cytoplasmic domains of the Cu⁺-ATPases, the ATPBD and the A domain, have also been characterized separately. Located between H4 and H5, the ATPBD comprises two subdomains, the N and P domains. The P domain contains an invariant DKTGT motif, of which the aspartic acid residue is phosphorylated during the catalytic cycle. The crystal structure of the ATPBD from *A. fulgidus* CopA reveals that the two domains are linked by a hinge region with the nucleotide-binding site in a cleft between domains (Sazinsky, Mandal, Argüello, & Rosenzweig, 2006). The overall fold of the P domain is a six-stranded β sheet flanked by α helices, and it structurally and sequentially resembles P domains from the other branches of the P-type ATPase family. The N domain also forms a mixed $\alpha\beta$ structure, similar to those of other P-type ATPases, but its sequence is only conserved among members of the P_{1B}-ATPase family. Although bound nucleotide is not present in the *A. fulgidus* ATPBD structure, the two domains exhibit a closed conformation. However, the open (apo) and closed (nucleotide bound) forms of the ATPBD from *Thermotoga maritima* have also been crystallographically characterized, as well as the His462Gln variant (Tsuda & Toyoshima, 2009), which serves as a model for a frequent disease-causing mutation in ATP7B. These structures reveal that the N domain recognizes the adenine ring of ATP in a different fashion from that in other classes of P-type ATPases. The structure of the ATPBD from *Sulfolobus solfataricus* is also available and houses a sulfate ion at the phosphorylation site (Lübbers et al., 2007). Finally, NMR structures of the N domains of both ATP7B and ATP7A have been reported (Banci, Bertini, Cantini, et al., 2010; Dmitriev et al., 2006). The overall structures of the ATPBDs from prokaryotes and eukaryotes are quite similar, with the exception of some additional loops and structural elements present in the ATP7A and ATP7B N domain structures.

The A domain, located between H2 and H3, has also been structurally characterized. A conserved GE sequence in this domain required for dephosphorylation (Petrus et al., 2002) likely interacts with the DKTGT motif in the P domain. An initial crystal structure of the *A. fulgidus* CopA A domain revealed 10 β strands with helices at the N- and C-termini leading into the membrane domains (Sazinsky, Agarwal, Argüello, & Rosenzweig, 2006). With the exception of several loop regions, the fold is very similar to that of the SERCA1 A domain. In SERCA1, the A domain rotates extensively during catalysis (Toyoshima & Inesi, 2004), and proteolytic mapping studies suggest similar conformational changes that occur in *T. maritima* CopA (Hatori et al., 2007). The solution structures of the A domains from ATP7A and ATP7B have also been reported and exhibit a similar overall fold to that of *A. fulgidus* CopA with an insertion between the first two β strands that are suggested to interact with the

N-terminal MBDs (Banci, Bertini, Cantini, et al., 2009). The NMR data indicate that the GE loop is relatively rigid, suggesting that major rearrangements do not occur during the catalytic step.

3.2. Intact CopA

Although soluble domain structures have provided much insight into P_{1B}-ATPase function, a complete understanding requires structural characterization of a whole enzyme, including the TM regions. In the absence of suitable three-dimensional crystals of any P_{1B}-ATPase, the structures of intact Cu⁺-ATPases were pursued by cryoelectron microscopy. Structures of *A. fulgidus* CopA lacking the C-terminal MBD and lacking both the N- and C-terminal MBDs were determined to ~17 Å resolution, and domains were assigned by docking the soluble domain crystal structures into the electron density (Wu, Rice, & Stokes, 2008). Comparison of the two resultant structures suggested several areas of conformational flexibility and, most importantly, identified a region between the ATPBD N domain and the A domain that might correspond to the N-terminal MBD. This location is consistent with cross-linking and proteolytic digestion studies (Hatori et al., 2007; Lübber et al., 2009). A 7 Å projection map obtained using two-dimensional crystals of CtrA3 Cu²⁺-ATPase from *Aquifex aeolicus* has also been reported and shows the arrangement of the eight TM helices (Chintalapati, Al Kurdi, van Scheltinga, & Kuhlbrandt, 2008). Both of these studies indicate a dimeric oligomerization state.

The first crystal structure of P_{1B}-ATPase, that of the Cu⁺-ATPase CopA from *Legionella pneumophila*, was recently determined to 3.2 Å resolution (Gourdon et al., 2011). This structure has provided a unique scaffold to model other P_{1B}-ATPases and onto which Wilson and Menkes disease mutations can be mapped. In the structure, the six core TM helices are organized similarly to other P-type ATPases, but the N-terminal MA and MB helices have an unusual arrangement (Fig. 3A). The MA helix interacts with H2 and H6 and the MB helix interacts with H1 and H2. MB is divided into two segments, a short TM helix and an amphipathic helix at the membrane interface, linked by a kinked region. The structure was determined in the presence of AlF₄⁻ and is thus locked in a metal-free E2 conformation. Importantly, despite the absence of bound Cu⁺, the structure reveals the locations of the ligands proposed on the basis of mutagenesis and spectroscopic studies of *A. fulgidus* CopA (González-Guerrero et al., 2008; Mandal et al., 2004). These residues, Cys 382 and Cys 384 from the M4 CPC motif, Tyr 688 and Asn 689 from M5, and Met 717 and Ser 721 of M6, occupy the same positions as the calcium-binding residues of SERCA1 in the calcium-free form (Olesen et al., 2007). Some rearrangement of these residues is necessary to form an appropriate Cu⁺-binding site, and it remains to be answered how the atypical metal-binding residues Tyr 688, Asn 689, and Ser 721 might actually coordinate the metal ion. Although the entire CopA protein was crystallized, very little electron density for the N-MBD was observed, allowing only its tentative placement adjacent to the linker regions of the A domain. The proposed location is consistent with cryoelectron microscopy (Wu et al., 2008) and chemical cross-linking data (Hatori et al., 2007), however.

A key finding of the CopA structure is the identification of a “platform” formed by helix MB (Fig. 3A). Importantly, this “platform” contains positively charged residues, including Lys 135, Arg 136, and Lys 142, which may provide a complementary electrostatic surface patch for docking of a Cu⁺ chaperone and subsequent Cu⁺ delivery (Argüello et al., 2012; Gourdon et al., 2011). Given that the apo and Cu⁺-loaded chaperones have similar surface properties, it is puzzling that Cu⁺ transfer experiments and calculations of the docking energetics suggest that the apo chaperone does not interact with the ATPase platform (Argüello et al., 2012; González-Guerrero & Argüello, 2008). It may be that the positive Cu⁺ charge is sufficient to transiently stabilize the interaction by interacting with negatively

charged amino acids located proximal to the end of “platform”. Three potential metal-binding residues, Met 148, Glu 205, and Asp 337, are proximal to the end of the “platform” and may represent the initial site of Cu^+ coordination (Fig. 3A and C). These putative ligands are fully conserved in all Cu^+ -ATPase sequences. Although this site is unable to stably bind Cu^+ , a transport pathway might involve delivery of Cu^+ to this transient-binding site on the platform followed by translocation to the TM sites. Conformational changes in helices H4, H5 and H6 might then facilitate release of Cu^+ to the luminal/periplasmic compartment. It has been proposed that residues close to the external side of the membrane may form an exit site, which in the structure contains electron density modeled as a K^+ ion (Gourdon et al., 2011). Several partially conserved residues, including Glu 189, Met 100, and Met 711, are located in this region. They may interact with sequestering molecules handling the metal upon release from the transporter.

4. FUTURE DIRECTIONS AND CHALLENGES

Although much is now known about the mechanism and structure of the Cu^+ -ATPases, major questions remain to be addressed. From a mechanistic point of view, details of the metal release step need to be elucidated. For gram-negative bacteria, it is not clear whether specific periplasmic proteins receive the Cu^+ and facilitate efflux or distribution to periplasmic or plasma membrane proteins. From a structural point of view, the location of the MBDs and the details of Cu^+ coordination need to be addressed. Although specific amino acid ligands have been identified in Cu^+ - and Zn^{2+} -ATPases, the changes in metal coordination and metrical/geometric parameters during transport will be of great interest. Finally, an overall structural and functional understanding requires structures of a Cu^+ -ATPase in the different conformational states, with and without Cu^+ substrate, and ideally, in complex with the Cu^+ chaperone. Such studies will also provide new insight into the molecular basis for Wilson and Menkes diseases.

The study of the other $\text{P}_{1\text{B}}$ -ATPase subfamilies has not reached the level of understanding that has been achieved for the Cu^+ -ATPases. For example, transport of Co^{2+} (and alternative substrates) by the $\text{P}_{1\text{B-4}}$ -ATPases has not been firmly established (Rutherford et al., 1999) and the substrate for the $\text{P}_{1\text{B-5}}$ -ATPases remains unknown (Traverso et al., 2010). The functional diversity of these families is also relatively unexplored, and it is not known whether they play roles beyond efflux, perhaps in metalloprotein assembly. In addition, the details of metal binding by the TM regions have not been elucidated. Conserved residues have been identified, including the essential cysteine-containing motif in H4 (Fig. 1), but the stoichiometry of metal binding and the identities of specific ligands have barely been investigated. Similarly, the roles of the soluble MBDs have not been explored for these subfamilies. The histidine-rich N-terminal MBDs in the $\text{P}_{1\text{B-3}}$ -ATPases are necessary for maximal Cu^{2+} transport activity (Mana-Capelli et al., 2003), but have not been characterized, and the function of the C-terminal Hr domain in the $\text{P}_{1\text{B-5}}$ -ATPases (Traverso et al., 2010) is not known. Unraveling the coordination chemistry, mechanism, and structure of these ubiquitous metal ion transporters is critical to the field of metal ion homeostasis and has long-term implications for understanding the role of these enzymes in disease and virulence.

Acknowledgments

Work in the Rosenzweig laboratory on $\text{P}_{1\text{B}}$ -ATPases is supported by National Institutes of Health (NIH) grant GM058518. Work in the Argüello laboratory on $\text{P}_{1\text{B}}$ -ATPases is supported by NIH grant 1R21AI082484-01, National Science Foundation (NSF) grant MCB-0743901, and United States Department of Agriculture-National Institute of Food and Agriculture (USDA-NIFA) grant 2010-65108-20606.

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Figure 1. Structural features of P_{1B}-ATPase subgroups. Boxes represent TM and cytoplasmic metal-binding domains (MBDs). The aspartic acid residue phosphorylated during catalysis and the identified signature sequences involved in metal coordination are indicated.

Figure 2. Catalytic and transport cycle of Cu^+ -ATPases. E_1 and E_2 represent the two main enzyme conformations. CopZ is the bacterial cytoplasmic Cu^+ chaperone. PCh indicates a hypothetical periplasmic Cu^+ chaperone/acceptor. The thicker arrows represent largely irreversible partial reactions.

Figure 3.

Structures of Cu^+ -ATPases. (A) Crystal structure of *L. pneumophila* CopA (PDB accession code 3RFU). The TM regions are shown in white, the A domain is shown in gray, and the ATPBD is shown in black with the N and P domains labeled. The N-terminal MA and MB helices are labeled and platform and metal-binding residues, shown in more detail in panel C, are represented as ball and sticks. The model begins with residue 74; the N-terminal MBD is not visible in the electron density. (B) Solution structure of the N-terminal MBD of *B. subtilis* CopA with bound Cu^+ (PDB accession code 1KQK). (C) Close-up view of the platform and proposed metal-binding residues in *L. pneumophila* CopA (PDB accession code 3RFU).