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Structure and Function of Cu(I)- and Zn(II)-ATPases

Oleg Sitsel,[†] Christina Grønberg,[‡] Henriette Elisabeth Autzen,[‡] Kaituo Wang,[‡] Gabriele Meloni,[§] Poul Nissen,[†] and Pontus Gourdon*[,‡,∥]

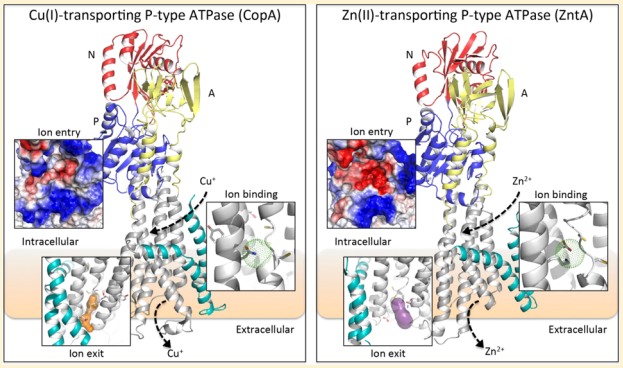
[†]Centre for Membrane Pumps in Cells and Disease (PUMPkin), Danish National Research Foundation, Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10C, DK-8000 Aarhus C, Denmark

[‡]Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3B, DK-2200 Copenhagen, Denmark

[§]Division of Chemistry and Chemical Engineering and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California 91125, United States

[∥]Department of Experimental Medical Science, Lund University, Sölvegatan 19, SE-221 84 Lund, Sweden

ABSTRACT: Copper and zinc are micronutrients essential for the function of many enzymes while also being toxic at elevated concentrations. Cu(I)- and Zn(II)-transporting P-type ATPases of subclass 1B are of key importance for the homeostasis of these transition metals, allowing ion transport across cellular membranes at the expense of ATP. Recent biochemical studies and crystal structures have significantly improved our understanding of the transport mechanisms of these proteins, but many details about their structure and function remain elusive. Here we compare the Cu(I)- and Zn(II)-ATPases, scrutinizing the molecular differences that allow transport of these two distinct metal types, and discuss possible future directions of research in the field.



All cells are separated from their extracellular environment by lipid membranes to maintain a controlled internal chemical composition. Transmembrane protein channels and transporters perform the crucial task of controlling the flux of ions and molecules across the membrane, thereby maintaining cell homeostasis in a responsive manner. Primary transporters utilize, for example, light or metabolic energy to allow transfer against electrochemical gradients, whereas secondary transporters couple pre-established gradients to transfer.

P-type ATPases make up a large family of primary active transmembrane transporters found in virtually all life forms. They exploit energy from ATP hydrolysis to catalyze ion or lipid transport across cellular membranes. Their cellular function varies from generation of electrochemical gradients and membrane potentials to ion clearance, transition metal detoxification, and establishment of lipid bilayer asymmetry. Since the later Nobel prize winning discovery of the Na^+,K^+-ATPase in 1957,[1] the number of known P-type ATPase proteins exceeds 100000 entries (UniProtKB,[2] as of March 2015). P-type ATPases fall into five classes (P_1−P_5) with up to four subclasses (A−D) based on their transport specificity,[3] which ranges from protons to phospholipids (Table 1). However, despite substantial diversity, P-type ATPases share a common topology and are likely to exploit the same general reaction cycle. In this review, we focus on how Cu(I)- and Zn(II)-ATPases have adapted the P-type topology and reaction cycle for specific transport of transition metals.

CATALYTIC CYCLE OF P-TYPE ATPASES

The mechanism of P-type ATPases is summarized by the so-called Post−Albers cycle,[4,5] with structurally distinct E1 and E2 states, which were originally recognized by changes in e.g. proteolytic patterns (Figure 1a). The cycle describes how conformational changes orchestrated by ATP-driven phosphorylation and dephosphorylation as well as transport site binding events trigger an alternating access mechanism for transport and countertransport to opposite sides of the membrane through four cornerstone intermediates: E1, E1P, E2P, and E2.[6] The inward-open E1 state has a high affinity for the extruded ion(s) from the cytoplasmic side. Ion binding triggers phosphorylation in formation of the occluded E1P phosphoenzyme state. Similarly, the outward-open E2P state is in some P-type ATPases associated with counterion binding from the extracellular/luminal side that triggers occlusion and dephosphorylation in formation of the occluded E2 state. The E1P to E2P and E2 to E1 transitions are associated with large conformational changes and release of the occluded ions.[7]

P-TYPE ATPASE TOPOLOGY

Information about the topology and three-dimensional structure of P-type ATPases has arisen from decades of biochemical characterization of representative members,[8] and

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Table 1. Subclasses of P-type ATPases[a]

subclass	structures	transport (out)	countertransport	electrogenic?	TMs[40]
P_{1A}	none	likely none[97]	2 K^+, in complex with a KcsA-like K^+ channel subunit[97]	yes	7[98]
P_{1B}	CopA, ZntA	see Table 2	likely none[39,96]	yes	8[31]
P_{2A}	SERCA1a	2 Ca^{2+} per cycle[33] or Mn^{2+}[99]	2−3 H^+ per cycle[34]	yes	10 or 11
P_{2B}	none	1 Ca^{2+} per cycle[100]	0−1 H^+ per cycle[100,101]	yes	10
P_{2C}	Na^+,K^+-ATPase	3 Na^+ per cycle[36] or 1−2 H^+ per cycle[102−104]	2 K^+ per cycle (Na^+,K^+),[36] 1−2 K^+ per cycle (H^+,K^+)[102−104]	yes, no	10 + 1 + 1
P_{2D}	none	1 Na^+ per cycle[105,106]	possibly 1 H^+ per cycle[107]	unknown	10[107]
P_{3A}	H^+-ATPase	1 H^+ per cycle[108]	none[109]	yes	10
P_{3B}	none	unknown	unknown	unknown	10
P_4	none	unknown	phospholipids[110]	unknown	10 + 2
P_5	none	unknown	N/A	unknown	11 or 12

[a]Subclasses, structurally determined representatives, transported ions and counterions, electrogenic properties, and numbers of transmembrane helices.

from more than 70 structures deposited in the PDB within the past 15 years.[9−32] A majority of this structural information originates from rabbit fast-twitch muscle SERCA1a,[7] a Ca^{2+}-transporting P_{2A}-ATPase responsible for resequestration of Ca^{2+} from the cytoplasm to the sarco/endoplasmic reticulum internal store. SERCA1a transports two Ca^{2+} in exchange for two or three H^+ per hydrolyzed ATP molecule.[33,34] The successful crystallization of SERCA1a in different conformational states has allowed visualization of a range of intermediate states, sampling the entire transport cycle, and has provided a wealth of information about how a P-type ATPase functions as a molecular pump. Similarly, significant insight has been obtained from crystal structures of the Na^+,K^+-ATPase, which has also been determined in multiple conformations.[16,26,28,35] This protein maintains the electrochemical gradients of sodium and potassium ions and is of fundamental importance in animal cells. Thus, for SERCA1a and the Na^+,K^+-ATPase, both archetypical P_2-ATPases, countertransport is integral to the functional cycle.[36] Another important and well-characterized P-type ATPase that is structurally determined is the P_{3A}-type plasma membrane H^+-ATPase,[18] which generates pH gradients and membrane potential in e.g. plants and fungi. In contrast to subclass P_{2A}, this enzyme exports a single H^+ per cycle against a steep concentration gradient and with no apparent countertransport.[37,38] Finally, the most recently determined P-type ATPase structures belong to the P_{1B}-type Cu(I)- and Zn(II)-ATPases, which are the main topic of this review.[23,31,39]

The crystal structures and sequence analysis of P-type ATPases reveal four core components: a membrane-spanning domain (M-domain), which encompasses the ion-binding site(s), and the cytoplasmic actuator-, phosphorylation-, and nucleotide-binding domains (denoted A, P, and N, respectively) responsible for autophosphorylation (N and P) and autodephosphorylation (A and P) (Figure 1b). Tight coupling of these domains, most notably via linkers connecting the M-domain to the A- and P-domains and tertiary interactions through extended helices of the M-domain, lies at the heart of P-type ATPase function (for recent reviews of the conformational changes in SERCA1a, see refs 40−42). The M-domain consists of 7−12 transmembrane helices (8 in P_{1B}-ATPases) and is responsible for ion uptake, translocation, and release, as well as countertransport in certain cases, and is thus evolutionarily adapted for specific functions. Six of these helices (M1−M6) are conserved throughout the P-type ATPase superfamily, with an important feature being an omnipresent proline in M4 that unwinds the helix, creating space for the bound ion(s) and allowing E1−E2 transitions. Subclass-specific N-terminal and C-terminal transmembrane helices expand P-type ATPase diversity by providing, for example, additional ion/counterion-binding sites and pathways.[26,28,41,43] In addition, P_{1B}-ATPases and also some other P-type ATPases have additional domains at their termini that are likely to have regulatory functions,[44] but unlike P_{1A}-ATPases, they seemingly have no associated subunits.

P_{1B}-TYPE ATPASES

Cu(I)- and Zn(II)-ATPases (commonly denoted CopA and ZntA) belong to the transition metal-transporting subclass P_{1B}, which is found in all three domains of life, but most abundantly in prokaryotes.[40] They are traditionally divided into five groups (P_{1B-1}−P_{1B-5}), based on conserved residues in M4−M6 predicted to confer metal selectivity (Table 2), but the existence of at least two other groups and additional orphan sequences has recently highlighted an even higher degree of diversity.[45] P_{1B-1}-ATPases transport Cu(I) in addition to the nonphysiological Ag(I) as a result of the similar chemical properties of these ions. P_{1B-2}-ATPases transport Zn(II) but can also translocate, for example, Cd(II) and Pb(II). The remaining groups P_{1B-3}−P_{1B-7} occur less frequently and are not as well-studied as the first two; P_{1B-3}-ATPases are proposed to transport Cu(II) and P_{1B-4}-ATPases Co(II), Ni(II), and/or Zn(II). P_{1B-5}-ATPases may export Fe(II) or Ni(II), while the transport specificity of group P_{1B-6}- and P_{1B-7}-ATPases is still unknown (Table 2).[45−48] The possibility that the apparent diversities of P_{1B}-ATPase groups may also relate to different transport kinetics is noteworthy, although many such groups are poorly characterized.

The primary physiological function of P_{1B}-ATPases is to protect organisms against excess transition metals and to supply essential transition metals for transmembrane or periplasmic assembly and maturation of essential metalloenzymes, such as cytochrome *c* oxidase.[49−52] In higher organisms, P_{1B}-type ATPases perform these functions through transfer of cytoplasmic metals to internal organelles, such as the trans-Golgi network, and the extracellular milieu.[53,54]

Except for isolated, cytoplasmic domains, the first and so far only crystal structures of the P_{1B} subclass are those of a Cu(I)-ATPase from *Legionella pneumophila* (LpCopA) and a Zn(II)-ATPase from *Shigella sonnei* (SsZntA). They revealed a number of subclass-specific structural features and provided novel insight into the transport mechanism of P_{1B}-ATPases.[23,39] Notably, the crystals of both LpCopA and SsZntA were

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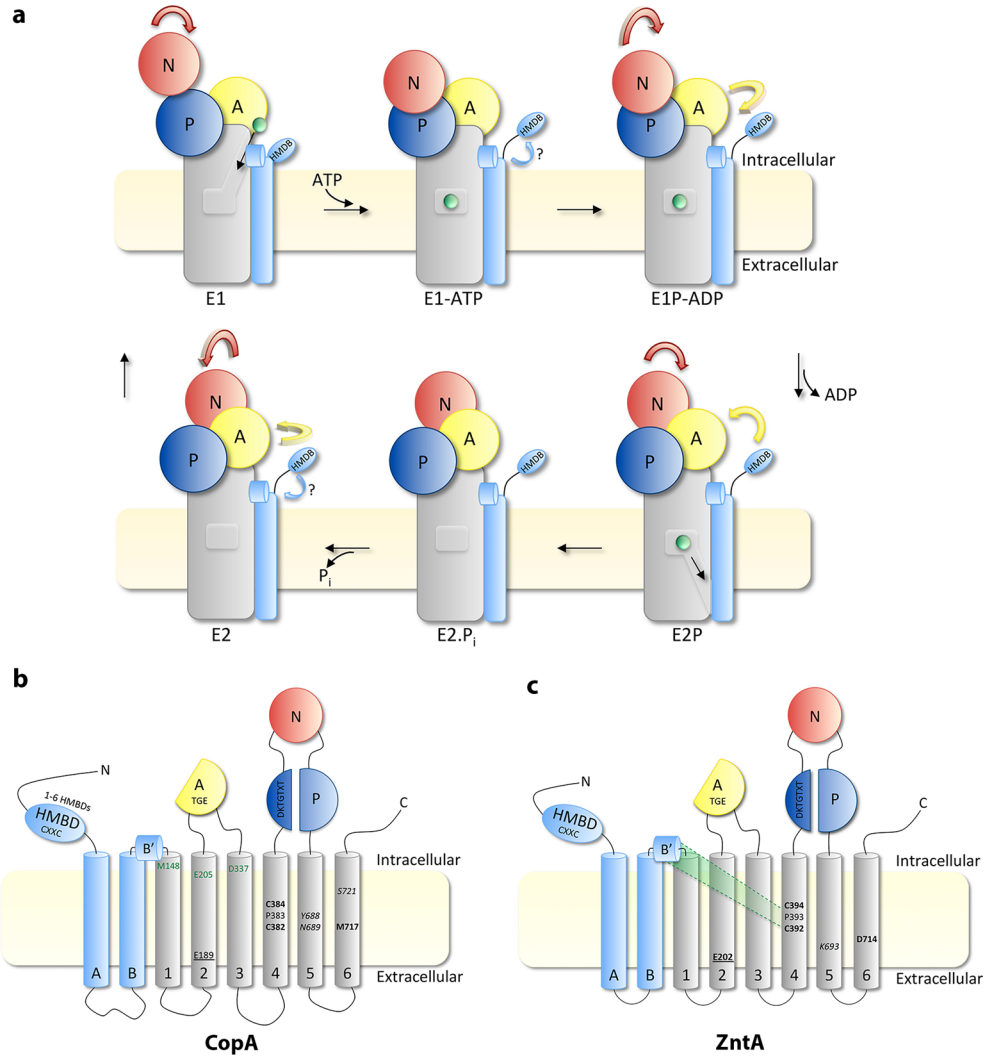

Figure 1. Schematic reaction cycle and topology of P_{1B}-type ATPases. These proteins form M (transmembrane, wheat and cyan), A (actuator, yellow), P (phosphorylation, blue), and N (nucleotide-binding, red) domains as well as one or several heavy metal-binding domains (HMBDs, cyan). (a) Post−Albers reaction cycle of P-type ATPases, in which the proteins cycle among four main conformations, E1, E1P, E2P, and E2. In the E1 state, the metal ion (green) binds to the membranous high-affinity binding site and triggers conformational changes, which result in occlusion and phosphorylation of an omnipresent aspartic acid residue in the P-domain, thus forming the E1P-ADP state. The protein then releases ADP and reorganizes to the outward-open E2P state, which allows for metal release (note that countertransport has not been demonstrated for any P_{1B}-ATPase). Dephosphorylation is associated with occlusion and release of the inorganic phosphate (via the E2.P_i transition conformation), yielding the fully dephosphorylated E2 state. Finally, restoration of the E1 state initiates a new reaction cycle. (b) Topology with key residues in the domains highlighted. Structural features implicated in metal uptake are colored green: ligands involved in entry for CopA, and a negatively charged entry funnel in ZntA. Highly conserved residues proposed to be involved in membranous high-affinity metal ion binding, release, and other transport processes are shown in bold, underscored, and/or in italics.

obtained by the high lipid-detergent technique (HiLiDe) yielding crystals formed by stacked bilayers.[55] The structures capture the E2P outward-open phosphoenzyme state through co-crystallization with beryllium fluoride, and the E2.P_i intermediate, a dephosphorylation transition state mimicked by aluminum fluoride. Thus, structural information about the metal-bound E1 and E1P states is still missing for the P_{1B}-ATPases. The crystal structures display a unique, P_{1B}-specific topology with two extra transmembrane helices, MA and MB, positioned N-terminal of the M1−M6 core. This extension and lack of the N-terminal part of the A-domain that is present in P_2- and P_3-ATPases result in the absence of the A-domain−M1 linker present in all other structurally determined P-type ATPases, and a significantly smaller A-domain.[9,16,18,44]

Furthermore, so-called heavy metal-binding domains (HMBDs), rich in metal-attracting cysteine and/or histidine residues, are frequently fused to the MA N-terminus or occasionally also to the C-terminus.[56] HMBDs typically possess compact ferredoxin-like $\beta\alpha\beta\beta\alpha\beta$ folds, but in multiple cases, the structure is either different[57] or hitherto unknown. These domains are not essential for transport and likely act as regulatory and stimulatory sensors of cellular metal levels.[58] They can thus be compared to the so-called R-domains[40] of calmodulin-binding Ca^{2+}-ATPases[59] and plasma membrane H^+-ATPases.[40,60]

CU(I)- AND ZN(II)-ATPASES

Cu(I)-ATPases are the only P_{1B}-type ATPases present in animals and are the most numerous and perhaps the best

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Table 2. P_{1B}-type ATPase Groups[a]

group	transported metal	M4 motif[45,46,111,112]	M5 motif[45,46,111,112]	M6 motif[45,46,111,112]	M-domain binding stoichiometry	coordination geometry	PDB entry
P_{1B-1}	Cu(I), Ag(I)	**C**P**C**	**Y**N(X)$_4$P	**M**XXS**S**	1 or 2[90]	trigonal[90]	3RFU 4BBJ 4BEV
P_{1B-2}	Zn(II), Cd(II), Pb(II)	**C**P**C**	T(X)$_5$QN(X)$_7$**K**	**D**XG(X)$_7$N	1[86,91]	tetrahedral[91]	3UMV 3UMW
P_{1B-3}	Cu(II)	**C**P**H**	G**Y**N(X)$_4$P	**MS**XS**T**	1[113]	square pyramidal[113]	–
P_{1B-4}	Co(II), Ni(II) (?), Zn(II) (?)	**S**P**C**		**HE**GX**T**	1[111]	tetrahedral[111]	–
P_{1B-5}	Fe(II) (?), Ni(II) (?)	PCP		QEXXD	unknown	unknown	–
P_{1B-6}	Fe(II) (?)	SCA[b]		TXXNHN	unknown	unknown	–
P_{1B-7}	unknown	CSC[b]			unknown	unknown	–

[a]Groups, group-specific motifs in transmembrane helices M4–M6 with putative metal ion-binding residues highlighted in bold, metal binding stoichiometries, and coordination geometries. PDB entries of the available structures derived from full length protein are indicated. [b]Note that the invariant proline of M4, present in all classes of P-type ATPases, is missing.

studied proteins of the subclass. Malfunction of the human Cu(I)-ATPases ATP7A and ATP7B causes the severe Menkes' and Wilson's diseases, respectively. Classical Menkes' syndrome is lethal at an early age and is characterized by a general copper deficiency, as the ubiquitously distributed ATP7A is unable to deliver copper required for vital metalloproteins.[61] Because of the primarily hepatic distribution of ATP7B (where it is responsible for efflux of copper into the bile), Wilson's disease predominantly affects the liver through copper overload and can usually be managed by a combination of copper dietary restriction and copper chelating drugs.[62] Zn(II)-ATPases make up the second largest subgroup of P_{1B}-ATPases and are prevalent in bacteria and plants. As with Cu(I)-ATPases,[63] knockouts of ZntA genes in plants result in severely stunted growth that can be rescued by increasing the zinc content of the growth medium.[64]

Cu(I)- and Zn(II)-ATPases share a similar architecture (Figure 1b). In contrast to P_{1B-4}-type ATPases, the intracellular N-terminus forms either a single HMBD or multiple [six in the human Cu(I)-ATPases] HMBDs connected to MA. The following helix, MB, is kinked almost perpendicularly at one or two conserved glycine residue(s) located in the cytoplasmic–membrane interface and forms a "platform" dubbed MB′. This structural feature is anchored to the membrane as an amphipathic helix with positively charged side chains exposed to the cytoplasm, possibly allowing interactions with the HMBD and/or metal-delivering chaperones, at least in the case of the Cu(I)-ATPases.[23,65] Helix M4 contains the CPC motif, which is characteristic of both Cu(I)- and Zn(II)-ATPases as the central part of the transition metal-binding site together with group-specific motifs in M5 and M6 that are likely to impose Cu(I) versus Zn(II) specificity (Table 2).

TRANSPORT CYCLE OF CU(I)- AND ZN(II)-ATPASES

Details of the transport mechanisms and conduits of Cu(I)- and Zn(II)-ATPases diverge. This discrepancy is likely due to the different chemical nature of copper and zinc ions with significantly different coordination chemistries (see below). Furthermore, the Cu(I)/Cu(II) redox potential is close to that of the cytoplasm, implying that copper can redox-cycle between the I and II oxidation states if it is unbound. This can result in free oxygen radical production via Fenton-type and Haber–Weiss chemistry that, together with ectopic metalation of iron–sulfur clusters, may cause severe damage to the cell when excess levels are present.[66,67] Therefore, copper levels are always kept under tight control by an array of copper-binding protein chaperones and low-molecular weight compounds such as glutathione. As a result, hydrated copper ions have been found to be practically nonexistent in *Escherichia coli* cells under normal conditions.[68] On the other hand, Zn(II) has only one accessible oxidation state at biological redox potentials and is therefore not redox active. Consequently, Zn(II) is less toxic, and a pool of free ions in the picomolar or low nanomolar ranges[69] is constantly present in cells; however, at higher concentrations, free Zn(II) may also cause damage.[70,71] The fact that both Cu(I) and Zn(II) are much less abundant in cells than the transported ions of other classes of P-type ATPases, together with the distinct biological roles played by the different members of the superfamily [e.g. transition metal homeostasis for P_{1B} pumps compared to fast Ca(II) reuptake in the sarco/endoplasmic reticulum to induce muscle relaxation for SERCA1a], provides a likely physiological explanation for the significantly higher affinity for the transported compound, and lower maximal transport rates, that evolved in P_{1B}-ATPases compared to other prominent P-type ATPases.[72]

PRETRANSPORT REGULATION OF CU(I)- AND ZN(II)-ATPASES

The unique properties of copper and zinc ions are likely also to have a profound effect on the delivery of metal to the ATPases. Prokaryotic CopA proteins often come in three-component operons encoding the ATPase, a <100-amino acid ferredoxin-like metallochaperone dubbed CopZ, and a Cu(I)-sensing transcriptional regulator termed CopY that controls the expression of the former two.[73] Upon metal-induced stress, CopZ binds Cu(I) via a CXXC motif and likely delivers the ion to the HMBD and the transmembrane domain of the Cu(I)-ATPase, thereby allowing further transport to occur.[44,58] Supplying CopA with copper in the presence of DTT provides a lower level of ATPase turnover,[58] reaffirming the notion that chaperones stimulate CopA-facilitated Cu(I) transport.

ZntA expression is regulated by the Zn(II)-sensing transcriptional regulator ZntR,[74] but no zinc chaperone protein has been identified. The set of genes induced at high Zn(II) concentrations rather include elements of the cysteine synthesis pathway,[75] indicating that cytoplasmic zinc buffering and transfer to the P-type ATPase occur via a set of actors different from those in the case of Cu(I) and involves metal-binding, low-molecular weight thiolates (e.g. cysteine and glutathione).

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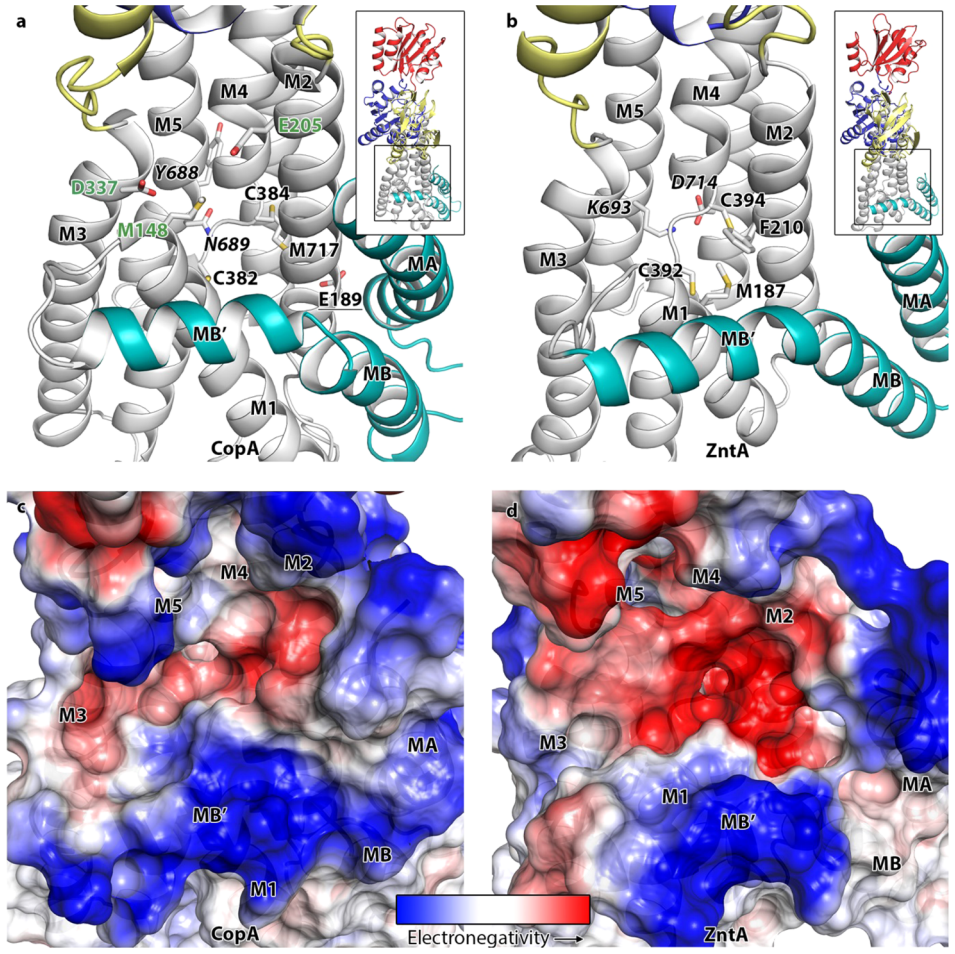

Figure 2. Metal ion entry. The E2P states (linked to metal release) are displayed with overviews (insets) with the domains colored as in Figure 1b. (a and c) Close-up of the entry pathway of CopA. In contrast to ZntA, the surface of CopA is less electronegative, and uptake to the high-affinity binding sites (at C382, C384, and M717) has been suggested to be assisted by M148, E205, and D337. (b and d) Close-up of the entry pathway of ZntA. Uptake is likely facilitated by a highly electronegative funnel (colored red in panel d) rather than specific residues. M187 and F210 may operate as gatekeepers, preventing back-flow of metal from the high-affinity site (at C392 and C394).

■ ION UPTAKE AND THE ROLE OF THE HMBD

On the basis of structural analysis and analogy to P_2-ATPases, ion uptake is expected to occur adjacent to the MB′ platform in P_{1B}-ATPases (Figure 2).[23,29,30] Reflecting the different nature of the copper and zinc complexes as metal donors, this region is marked by distinct structural features in CopA and ZntA. In Cu(I)-ATPases, the highly positively charged platform likely facilitates binding of CopZ-type chaperones that possess complementing patches on their surface, potentially in an orientation stimulating transfer to the Cu(I)-ATPase.[23,44,76] In LpCopA, M148, possibly assisted by E205 and D337, is believed to be responsible for transfer of Cu(I) from the CopZ CXXC motif to the membranous high-affinity metal-binding site(s), and these sites are presumably coupled through ligand exchange as suggested by indications of a M148-CPC-Cu(I) complex.[77,23] In the case of SsZntA, this metal uptake mechanism is replaced by a highly electronegative funnel-like structure that extends to the CPC motif,[39] a mechanism well suited for uptake of weakly complexed or noncomplexed ions. Upon high-affinity binding, Zn(II) is likely occluded by the M187-F210 residue pair positioned at the CPC motif end of the funnel,[39] a gating mechanism reminiscent of that proposed in H^+-ATPases via an asparagine.[18,78]

What then is the role of the HMBD, which has been proposed to interact with the MB′ platform through electrostatic interfaces?[23,39,76,79] While an understanding of whether the HMBDs have one common function in P_{1B}-ATPases remains elusive, a study of the interactions among the *Archaeoglobus fulgidus* CopZ, HMBD, and Cu(I)-ATPase (AfCopA) core has provided some insight.[58] The CopZ chaperone is capable of donating Cu(I) to both the HMBD and the ATPase core in both the wild type and the HMBD-truncated form. However, a separate Cu(I)-loaded HMBD cannot transfer the metal to the core, and metal binding to this domain also appears to be unnecessary for the *in vivo* Cu(I) transport activity;[57] removing the HMBD generally reduces the activity of both Cu(I)- and Zn(II)-ATPases,[23,39,80] although the contrary has also been observed.[58] The HMBD was not visible in the crystal structures of the E2P or E2.P_i states of LpCopA or SsZntA,[23,31,39] indicating a high degree of flexibility and a lack of stable interaction with the core in these states. Therefore, available data lead to the conclusion that rather than transferring metal from chaperones to the ATPase core,[44] the HMBD may play an indirect, autoregulatory function: autoinhibitory when transported metal ions are absent through pathway blockade and stimulatory when the ion is bound. The mechanism of this putative function is not well understood, but

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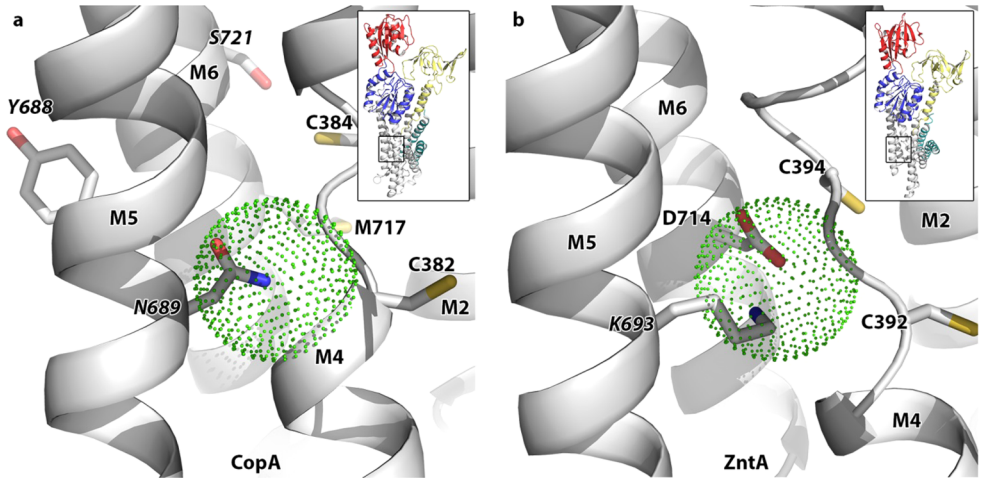

Figure 3. Membranous high-affinity metal ion binding. The binding region of CopA (a) and ZntA (b) with invariant key residues in the nonbound E2.P_i states (PDB entries 3RFU and 4UMW) and overviews (insets) with the domains colored as in Figure 1b. The number of metal-binding sites in CopA is debated (but C382, C384, and M717 are ligands), while the high-affinity site in ZntA is formed by C392, C394, and D714. The role of Y688, N689, and S721 in CopA remains unclear, but K693 has been proposed to operate as a built-in counterion in ZntA. The center of the green sphere is located between the two calcium ions in SERCA1a (using PDB entries 3B9R and 3N8G).

it may be in cooperativity with the A-domain as proposed previously,[23,39,81] in line with the N-terminal region of other P-type ATPases being integral to the A-domain.[9,16,18] The HMBD might therefore increase the phosphorylation (or less likely dephosphorylation) rates by facilitating A-domain movements. This is in line with data demonstrating slower dephosphorylation kinetics of ZntA when the HMBD is removed.[82] In the case of Cu(I)-ATPases in higher organisms, which have up to six N-terminal HMBDs, the complexity of regulation seems to be taken to even more sophisticated levels.[83] Notably, it has been proposed that the HMBD of the Zn(II)-ATPase AtHMA4 of *Arabidopsis thaliana* serves as not only a regulator of export but also a sensor.[44,84]

■ INTRAMEMBRANOUS METAL BINDING

Once the metal has entered the M-domain, it binds to the region with the CPC motif. The affinity for the metal ions at this location has been reported to be very high: in the femtomolar range for Cu(I) in CopA[58,85] and in the nanomolar range for Zn(II), Cd(II), and Pb(II) in ZntA.[86] The selectivity of transport may be determined by the differences in the coordination chemistry of Cu(I) and Zn(II) in terms of coordination number, geometry, and ligands as imposed by unique sets of invariant residues in CopA and ZntA. According to the Pearson acid base theory (also known as HSAB theory), transition metal binding depends on the hardness as a Lewis acid. As a soft Lewis acid, Cu(I) will preferably be coordinated by soft Lewis bases such as cysteines and methionines.[87] On the other hand, Zn(II) is a harder Lewis acid because of its higher charge density, allowing coordination also by harder Lewis bases such as aspartates and glutamates.[88] Furthermore, lower coordination numbers are expected for Cu(I), which often has three ligands, compared to Zn(II), which prefers tetrahedral coordination.

Indeed, for LpCopA, a single Cu(I) is coordinated in a trigonal planar geometry by three sulfur ligands (Figure 3), most likely C382 and C384 of the M4 CPC motif and M717 in M6.[89] However, a different copper binding model containing two independent trigonal planar sites having mixed S and N/O coordination was previously suggested for AfCopA,[90] with one site formed by the two cysteine residues on M4 along with a tyrosine on M5, and a second site with similar affinity centered on an asparagine on M5 together with a methionine and a serine on M6, corresponding to C382, C384, and Y688 and N689, M717, and S721 in LpCopA, respectively.[90] Notably, all six residues are fully conserved in P_{1B-1}-ATPases, but the latter three may also have functional roles other than copper binding e.g. thiol/thiolate stabilization and proton shuttling.[44] Considering the high degree of sequence conservation among CopA proteins, a uniform mechanism should be anticipated, and additional experiments are thus required to resolve this issue.

In Zn(II)-ATPases, Zn(II) is bound with a tetrahedral coordination geometry involving both sulfur and oxygen/nitrogen atoms.[39,91] The current model is that the CPC motif (residues 392−394 in SsZntA) again provides two metal-binding sulfurs and forms the high-affinity binding site (Figure 3) using oxygen coordination from likely one (D714 in SsZntA) or possibly two (the adjacent E202) carboxylate side chains.[39,92,93]

Similar to the residues of the putative second site in CopA, a universally conserved lysine residue (K693 in SsZntA) is also located in the direct vicinity of the CPC motif in Zn(II)-ATPases.[39] However, this residue does not appear to participate in coordination of the bound cation but rather forms a salt bridge with the coordinating D714 once Zn(II) is released, thereby possibly acting as a built-in counterion. A similar mechanism was also proposed for P_{3A}-ATPases, in which a central aspartate is charge-compensated by an arginine at the equivalent position.[18] Interestingly, these two subclasses therefore appear to act as uniporters unlike the P_2-ATPases, showing how different transport mechanisms have adapted to the common overall topology of the P-type ATPase superfamily (Table 1).

■ ION RELEASE

How then is the metal released from the high-affinity binding site in the M-domain? The available E2P and E2.P_i structures provide insights. According to the classical Post−Albers cycle, E2P represents the state in which the ATPase is opened toward the extracytoplasmic side for exchange of ions, and E2.P_i

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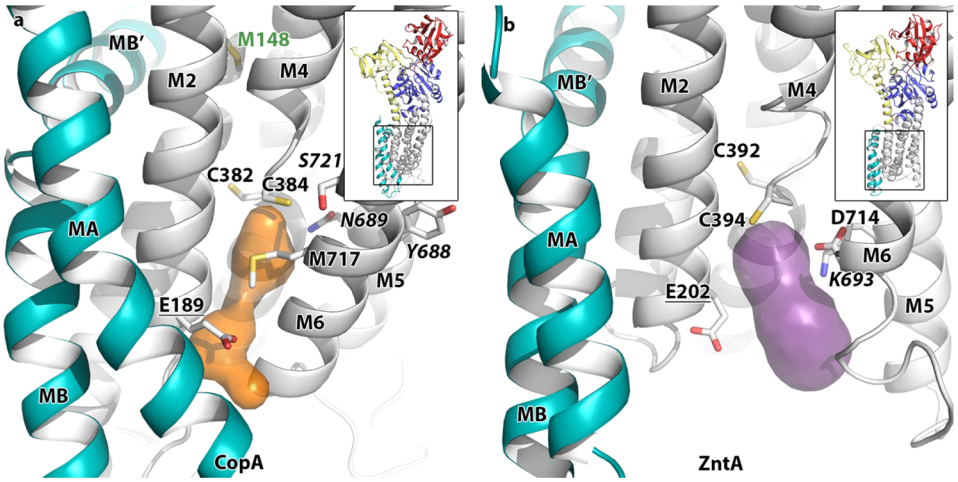

Figure 4. Metal release. Overviews (insets) are colored as in Figure 1b. (a) Close-up of the proposed release pathway of CopA in the E2P and E2.P_i states. Release of Cu(I) from the coordinating residues C382, C384, and M717 likely occurs through a narrow passage lined by MA, M2, and M6 (as calculated with CAVER[114]). (b) Close-up of the release pathway of ZntA in the E2P state associated with release. In contrast to CopA, a wide opening from metal-coordinating residues C392, C394, and D714 is obtained by substantial movements of helices M5 and M6 (as obtained with CAVER).

represents the occluded state with bound counterions (if present). A most striking feature of the SsZntA structure in the E2P state is a wide opening extending from the CPC motif to the periplasmic side, formed by M5 and M6 moving away from the rest of the M-domain (Figure 4), similar to e.g. SERCA1a, from which the ions are released in free form.[17] A highly conserved glutamate, E202 in SsZntA, is located in the metal discharge conduit within reach of the high-affinity metal-binding residues. Considering that mutation of this residue severely affects both protein activity and metal-binding stoichiometry,[39] it may be suggested that E202 serves as a transient ligand for the metal ion during release from the CPC motif as also proposed for the equivalent residue in SERCA1a.[17] With the M5−M6 cavity closed again in the E2.P_i state of SsZntA, and the aforementioned salt bridge between the Zn(II)-binding aspartate and the nearby lysine, there is no apparent need for a proton or other types of counterions. This conclusion is supported by functional studies of SsZntA in proteoliposomes.[39]

The LpCopA crystal structure in the E2P state differs notably from the corresponding SsZntA structure by having no opening via M5 and M6, as confirmed by two crystal forms of LpCopA of this state.[31] Instead, molecular dynamics simulations suggest that the membranous high-affinity metal-binding site can be accessed by a narrow, Cu(I)-passable conduit lined by MA, M2, and M6 (Figure 4).[31] A unique release mechanism for Zn(II) seems likely as Cu(I) is strictly bound to protein metallochaperones or low-molecular weight chelators and not allowed to be in free form.[94] It has recently been shown that in *E. coli*, the soluble periplasmic chaperone CusF is able to specifically interact with CopA and unidirectionally accept extruded Cu(I).[31,95] An *in silico* analysis of this interaction shows that the metal-binding residues of CusF may dock specifically to the proposed exit pathway of CopA.[31,95] The conserved exit pathway glutamate present in Zn(II)-ATPases may also be important in Cu(I)-ATPases. Molecular dynamics simulations and *in vitro* assays have indicated that E189, the corresponding residue in LpCopA, plays an important role in the release of the metal ion from the CPC motif.[31] The E2.P_i structure of LpCopA (represented by a E2-AlF_4^- complex) shows that the cytoplasmic domains reorient for dephosphorylation as expected upon transition from the E2P state, yet the M-domain remains almost identical and depicts a narrow but open release pathway. This may suggest that Cu(I)-ATPases stay outward-open for a longer time with a different coupling of dephosphorylation to outward occlusion, a missing factor involved in a CopA release complex, or a crystal packing artifact that alters the M-domain structure in the E2-AlF_4^- structure. Assuming a different coupling of release, it could be related to a specific need in CopA proteins for protein-to-protein transfer of the extruded metal, as opposed to the direct release into the extracellular environment observed for other P-type ATPases.

With regard to countertransport, no proteoliposome studies of CopA proteins, similar to those of SsZntA, have yet been performed. However, currents produced by both LpCopA and the human Cu(I)-ATPase ATP7B have been shown to be insensitive to pH, as observed by charge transfer measurements on solid-supported membranes,[89,96] implying that Cu(I)-ATPases like Zn(II)-ATPases may operate without net countertransport. Still, transient current such as in thiolate deprotonation/reprotonation reactions may be important and could then exchange only on the cytoplasmic side. Nonetheless, the apparent lack of net countertransport may represent a common unifying theme for CopA and ZntA proteins, perhaps even for the entire P_{1B} subclass.

■ FUTURE DIRECTIONS

Several major questions about the transition metal-transporting P_{1B}-type ATPases remain to be answered. For instance, how do Cu(I)-ATPases receive metal ions in organisms that lack classical CopZ chaperones as is the case for many bacteria? Do they use a different set of metalloproteins for metal delivery [as was shown to be the case for the *Streptococcus pneumoniae* Cu(I)-ATPase[57]], or do small compounds such as cysteine and glutathione perform this role? Other questions concern the role of the HMBDs. Why does deletion of HMBDs significantly reduce the transport activity, even though they do not seem to participate in metal transfer? When do these domains interact with the ATPase core during the catalytic cycle? Why do vertebrate Cu(I)-ATPases have up to six HMBDs while some

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bacterial ATPases have none? What functions do the C-terminal and N-domain insertions in the human transporters serve? Several questions regarding ion binding also remain to be answered. How are selectivity, specificity, and transport established? What is the transport stoichiometry of Cu(I)-ATPases? Is it one or two metal ions that bind at their high-affinity binding region in the M-domain? How are protons shuttled in and out to thiolates when Cu(I)- and Zn(II)-ATPases seemingly do not execute any countertransport? Which chaperones accept Cu(I) from the ATPase on the extracytoplasmic side, and how is transfer established? These and many other questions stimulate continued research and promise to make metal P-type ATPases a very important and exciting field in the years to come.

AUTHOR INFORMATION

Corresponding Author

*E-mail: pontus@sund.ku.dk. Phone: +45 50339990.

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ABBREVIATIONS

PDB, Protein Data Bank; SERCA1a, sarco/endoplasmic reticulum Ca^{2+}-ATPase isoform 1a; CopA, Cu(I)-ATPase; ZntA, Zn(II)-ATPase; ATP7A, human copper-transporting ATPase 1; ATP7B, human copper-transporting ATPase 2; HMBD, heavy metal-binding domain; LpCopA, CopA from *L. pneumophila*; SsZntA, ZntA from *S. sonnei*; HiLiDe, high lipid-detergent method of membrane protein crystallization; CopZ, soluble cytoplasmic Cu(I) chaperone; ZntR, Zn(II)-sensing transcriptional regulator.

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