Minireview

Escherichia coli Soft Metal Ion-translocating ATPases*

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Life may have first arisen in deep oceanic hydrothermal vents that were rich in metals such as arsenic, lead, copper, and zinc (1). Maintaining suitable intracellular concentrations of essential metals such as copper and zinc while excluding toxic metals such as arsenic, lead, and cadmium was one of the earliest challenges of the first cells. This ancient environmental challenge was a driving force for the evolution of mechanisms for metal ion homeostasis and detoxification. Even today toxic metals enter the ecosphere from geochemical sources. For example, parts of the Midwestern and Northeastern United States have arsenic concentrations that exceed 10 μg/liter, the provisional guideline of the World Health Organization for arsenic in drinking water, or 50 μg/liter, the present United States Environmental Protection Agency recommended maximum. Arsenic in the water supply in southern and western Bangladesh and the adjacent regions of India has triggered a health catastrophe. It is little wonder that in every organism examined there are transport systems that detoxify metal ions by catalyzing extrusion from the cytosol.

In this review three families of Escherichia coli transport ATPases that catalyze uptake or confer resistance to ions of the transition metals copper and zinc, the heavy metals cadmium and lead, and the metalloids arsenic and antimony will be described. As a group these pumps will be designated soft metal ion-translocating ATPases or, for convenience, soft metal ATPases, because many ion species of these elements are chemically soft Lewis acids, as opposed to the hard Lewis acids of Groups I and II elements such as Na⁺ and Ca²⁺. Hard Lewis acids bind to proteins through relatively weak ionic interactions with hard Lewis bases such as the carboxyl oxygens of glutamate or aspartate residues. In contrast, soft Lewis acids (or simply soft metal ions) form strong bonds with soft Lewis bases such as the thiolates of cysteine residues and the imidazolium nitrogens of histidine residues. These nearly covalent interactions with cysteines and histidines in proteins account for much of the biological properties and toxicity of soft metal ions.

ATPases That Catalyze Uptake of Soft Metal Ions in E. coli

A thorough description of soft metal metabolism requires knowledge of the pathways of entry and efflux of these metals into and out of cells. Fig. 1 shows the soft metal ion-translocating ATPases of E. coli, and similar systems have been identified in many prokaryotes. There are two known high affinity uptake ATPases, one for zinc and the other for arsenic. Both are members of the ABC superfamily of solute-translocating ATPases. Zinc is accumulated by the ZnuABC ATPase, a member of the ABC superfamily (2). The 36-kDa ZnuA protein is a periplasmic Zn(II)-binding protein in E. coli that donates Zn(II) to the 28-kDa ZnuB membrane protein, which translocates Zn(II) into the cytosol. ZnuC is the 28-kDa ATPase subunit that couples energy into the complex.

Sb(III) (and probably As(III)) is accumulated by the GlpF polyol transporter, suggesting that metalloids exist in solution as the inorganic polyols Sb(OH)₃, As(OH)₃, or their polymers (3). In an oxidizing atmosphere, arsenic is usually present as arsenate (As(V)). Uptake of arsenate occurs via the phosphate-translocating ABC-type ATPase complex formed by PstA, PstB, PstC, and PstS (4). PstS is a 34-kDa periplasmic binding protein; the 32-kDa PstA and the 34-kDa PstC form the membrane complex; the 29-kDa PstB is the ATPase subunit of the transport system. The substrate of the ArsAB efflux pump is As(III), so arsenate is first reduced to arsenite by the ArsC arsenate reductase (5).

No copper uptake systems have been identified in E. coli. In the present day oxidizing atmosphere, copper is present as Cu(II), whereas it is usually Cu(I) inside of cells. NDH-2, an alternate NADH dehydrogenase of E. coli, has Cu(II) reductase activity (6), but it is not yet clear if copper reduction is its physiological function. Another candidate Cu(II) reductase is YacK (CueO), a putative multicopper oxidase (7). Alternatively, CueO may serve to oxidize Cu(I) exported from the cytosol. In Saccharomyces cerevisiae the proteins involved in copper metabolism have been extensively characterized, and the pathways are similar in most eukaryotes including humans (8). Yeast and other eukaryotes have evolved metal ion chaperones that ferry Cu(I) from the cytoplasmic membrane permease to their intracellular targets, but no E. coli Cu(I) chaperone has yet been identified.

Efflux of soft metal ions is catalyzed by specific transport ATPases. Cu(I) is extruded by CopA, a P-type ATPase encoded by the chromosomal copA gene of E. coli (9). Zn(II), Cd(II), and Pb(II) are transported out of cells by ZntA, also a P-type ATPase, that is encoded by the chromosomal zntA gene of E. coli (10, 11). As(III) and Sb(III) are extruded by the ArsAB ATPase, a novel pump with similarities to ABC transporters (12). The best characterized system is the one encoded by the arsKDBC operon of E. coli plasmid R773 (13). Properties of these soft metal ion-translocating ATPases are described below.

P-type ATPases for Monovalent Soft Metals: Cu(I) and Ag(I) Pumps

Members of the P-type ATPase superfamily transport a variety of cations, including H⁺, Na⁺, K⁺, Ca²⁺, Mg²⁺, Cu(I), Ag(I), Pb(II), Zn(II), and Cd(II), and aminophospholipids. They share common features including a conserved aspartate that is phosphorylated to form the phosphoenzyme intermediate, a conserved proline in a transmembrane segment that is probably part of the translocation pathway, and consensus domains for ATP binding and hydrolysis. A core structure in all P-type

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1 Review article.
As(V) to As(III), which is then extruded from the cells by the ArsAB ATPase, an ABC transporter. The ArsC arsenate reductase reduces arsenate to arsenite cycle through the cell by uptake of arsenate by a phosphate-transporting ATPase, an ABC transporter. The ArsC arsenate reductase reduces As(V) to As(III), which is then extruded from the cells by the ArsAB arsenite-translocating ATPase.

P-type ATPases consists of two extramembrane loops and three pairs of transmembrane segments. The larger loop contains the ATP-binding and phosphorylation sites, and the smaller loop contains residues involved in dephosphorylation.

P-type ATPases fall into five phylogenetic groups (14). One, referred to as soft metal, heavy metal, transition metal, or monovalent soft metal P-type ATPases, can be further divided into two distinct subgroups, one for the monovalent cations Cu(I) and Ag(I), and the other for the divalent cations Co(II), Zn(II), Cd(II), and Pb(II) (12, 15). Soft metal P-type ATPases have several features different from hard metal P-type ATPases. There are 1–6 metal binding motifs in the hydrophilic N-terminal domain, most commonly GXXCXXC or (M/H)XXM-DH/S/O/XXM. Soft metal ATPases have eight transmembrane segments, whereas hard metal pumps have 10 (16, 17). An invariant proline in transmembrane segment 6 is flanked by two cysteines in most of the soft metal pumps; in a few cases, this is CPH, CPS, or SPC.

The most widely recognized monovalent soft metal P-type ATPases are the human copper pumps ATP7A (or MNK) and ATP7B (or WND) (18). Mutations in these pumps produce Menkes’ disease, respectively. Both have six N-terminal metal binding motifs. Many bacterial Cu(I) pumps have been identified; the physiological functions of these pumps are to mediate energy-dependent copper uptake or efflux. The Cu(I)-ATPases from Enterococcus hirae (CopA and CopB) participate in copper homeostasis (15). CopB has a histidine-rich N terminus instead of the more common CXXC metal binding motif. Everted vesicles containing CopB accumulate both $^{64}$Cu(I) and $^{110}$Ag(I) in an ATP-dependent manner. CopB has been purified and reconstituted into proteoliposomes (19). Purified and reconstituted CopB exhibits low ATPase activity that is not stimulated by Cu(I). Other bacterial monovalent soft metal P-type ATPases include SiIP from Salmonella typhimurium (20). SiIP confers resistance to Ag(I) but not Cu(I). SiIP has a histidine-rich N terminus unrelated to the N terminus of E. hirae CopB. The Helicobacter pylori CopA has eight transmembrane segments with cytosolic N and C termini (16). It is likely that other soft metal P-type ATPases have a similar topology.

CopA from E. coli is an 834-residue protein highly similar to the human Menkes and Wilson’s disease copper pumps (9). It has two metal binding motifs, $G_{11}^{11}LSCGHC$ and $G_{107}^{107}MSCASC$. CopA is inducible by either copper or silver, but disruption of copA resulted in sensitivity to copper salts but not Ag(I). Everted membrane vesicles from cells expressing copA accumulate $^{64}$Cu in an ATP-dependent vanadate-inhibited manner. No transport of copper ion is observed in the absence of dithiothreitol (9). The requirement for a strong reductant indicates that Cu(I), and not Cu(II), is the substrate of the pump.

The phylogenetically distinct second branch of the soft metal P-type ATPases includes ZntA, CadA, and CadT. Disruption of the E. coli zntA resulted in sensitivity to Zn(II), Cd(II), and Pb(II) but not Cu(I) (10, 11, 21). Disruption of both soft metal pumps, copA and zntA, results in the same degree of sensitivity to Cu(I), Zn(II), Cd(II), and Pb(II) as the single disruptions, indicating that ZntA and CopA have non-overlapping specificities. Everted membrane vesicles containing ZntA catalyze ATP-coupled accumulation of $^{65}$Zn(II) or $^{109}$Cd(II) in a vanadate-sensitive reaction (10). The 727-residue CadA, which confers cadmium resistance in Staphylococcus aureus (22), forms a

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**FIG. 1.** Soft metal ATPases of E. coli. Right, zinc ions are accumulated by the ZnuABC ATPase, an ABC transporter. Excess Zn(II) is extruded by the ZntA P-type ATPase. The opposing activities of these two pumps provide for zinc homeostasis. The proteins responsible for uptake and intracellular transport of copper have not been identified in E. coli. Left, arsenic oxyanions cycle through the cell by uptake of arsenate by a phosphate-translocating ATPase, an ABC transporter. The ArsC arsenate reductase reduces As(V) to As(III), which is then extruded from the cells by the ArsAB arsenite-translocating ATPase.

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**FIG. 2.** Structural features of the ArsA ATPase. A, the molecular dyad. A view of ArsA looking down the pseudo 2-fold axis of molecular symmetry. Secondary structure elements are drawn as ribbons, with strands in red and helices and loops in ivory (A1) or green (A2), Sb(III) and Mg$^{2+}$ are represented as CPK (blue and magenta, respectively). ADP in the NBSs is represented as transparent CPK and also as sticks colored by atom types (P, yellow; O, red; N, blue). Inserts, molecular surface of the two NBSs (A1, ivory; A2, green), B, the signal transduction pathway. D$^{142}$/447/TAFTPGH$^{146}$/453 connects the A1 and A2 NBS to the metal site. Strands (red) and helices and the P-loops (ivory, A1; green, A2) are drawn as ribbons. Nucleotides are shown as transparent CPKs and also as stick models colored according to atom type. The DTAPTH sequence is shown as stick models (cyan). Sb (blue) and Mg$^{2+}$ (magenta) are shown as CPK. Generated with MOLSCRIPT and RASTER3D and modified with permission from Ref. 34.

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Cd(II)-dependent phosphoenzyme intermediate during the catalytic cycle and catalyzes ATP-dependent accumulation of $^{109}$Cd(II) (23). A *Synechocystis* ZntA homologue, CoaT, confers Co(II) resistance (24). CoaT lacks an N-terminal metal binding motif and has an intramembrane SPC sequence. CadA from *H. pylori* has 8 transmembrane segments, similar to CopA (17). Thus, both monovalent and divalent soft metal P-type ATPases have similar topologies that are distinct from the hard metal P-type ATPases.

Purified ZntA catalyzes soft metal ion-dependent ATPase activity at rates equal to those for the hard metal P-type ATPases, the first clear demonstration of metal ion-dependent ATPase activity by a soft metal P-type ATPase (25). Activity is stimulated by Pb(II) > Cd(II) > Zn(II) > Hg(II). The rates of ZntA-catalyzed ATP hydrolysis are 4-fold higher when thiolates of cysteine or glutathione were present, though the apparent $K_m$ values for Pb(II) and Zn(II) increase ~10-fold. It is tempting to speculate that in vivo the N-terminal metal binding domain of ZntA receives its substrate from the metal-glutathione conjugates.

**The ArsAB ATPase: As(III)/Sb(III) Efflux Pump**

The *E. coli* ArsAB complex is an As(III)/Sb(III)-translocating ATPase. ArsB is a 429-residue inner membrane protein with 12 membrane-spanning segments (26). Although bound to ArsB in *vivo*, ArsA can be purified as a soluble protein (27). ArsA has two homologous halves, A1 and A2, connected by a short linker. Each has a consensus nucleotide-binding site (NBS), and both NBSs are required for activity (28, 29). As(III) or Sb(III) stimulates ATP hydrolysis (27). Cys-113 and Cys-172 in A1 and Cys-422 in A2 are required for metalloid activation, indicating that Sb(III) or As(III) interacts with ArsA as soft metals (30). ArsA homologues have a signature sequence DTAPTGH (31) that may correspond to the Switch II region of other nucleotide-binding proteins (32). In ArsA there are two signature sequences, $^{142}$TAPTGH$^{146}$/$^{142}$TAPTGH$^{146}$, one in A1 and the other in A2, that transmit information on occupancy of the metalloid site to the NBSs. Using two single tryptophan ArsAs (Trp-141 and Trp-159), conformational changes in the A1 domain were monitored during the individual steps of the catalytic cycle (33). In the unactivated state a conformational change is rate-limiting for the overall reaction. Following ATP hydrolysis and product release, the enzyme slowly isomerizes to the original conformation. Binding of metalloid bypasses this rate-limiting step, allowing the enzyme to return to its active conformation following ATP hydrolysis. This is reminiscent of the E1–E2 transition in P-type ATPases, where conformational change is a key feature of the mechanism.

The crystal structure of ArsA has been determined (34). A1 and A2 halves display nearly identical folds and together form almost a perfect dyad (Fig. 2). Two NBSs, each filled with MgADP, are at the interface between A1 and A2, and residues from both halves contribute to nucleotide binding (Fig. 2, insets). In each NBS, Mg$^{2+}$ is octahedrally coordinated by the β-phosphate of ADP, several water molecules, and a threonine hydroxyl. In the A1 NBS Asp-45 is directly coordinated to Mg$^{2+}$. In the A2 NBS Asp-364 is indirectly coordinated to Mg$^{2+}$ via a water molecule. Despite extensive topological similarities, the two NBSs appear to be in different conformations, with A1 NBS almost completely closed and A2 NBS fully open (Fig. 2, insets). The observed asymmetry of the two NBSs may reflect a multisite catalysis mechanism in which the two sites are alternatively open and closed (35). On the other hand, the two sites might be intrinsically different, for example, one regulatory and the other catalytic.

Also at the interface between A1 and A2, but over 20 Å apart from the NBSs, is the single allosteric site of the enzyme, where three Sb(III) bind to form a novel metal cluster (Fig. 2A). One Sb(III) is coordinated to His-148 (A1) and Ser-420 (A2), a second to Cys-113 (A1) and Cys-422 (A2), and the third to Cys-172 (A1) and His-453 (A2). Thus, each Sb(III) is coordinated by one A1 residue and one A2 residue. Binding of the three metalloids...
is expected to stabilize the interaction between A1 and A2, generating functional NBSs. Consistent with this belief, mutation of the cysteine ligands results in a substantial decrease in allosteric activation (30). Mutation of the ligands His-148 and His-453 also produces a modest decrease in allosteric activation (36). This effect may be because of the fact that His-148 and His-453 are the C-terminal ends of the two DTAPTGH sequences (Fig. 2B) whose N-terminal ends, Asp-142 and Asp-447, coordinate Mg\(^{2+}\) indirectly at the NBSs via water molecules. Thus, the crystal structure of ArsA supports the hypothesis that D\(^{142/447}\)TAPTGH\(^{146/453}\) are intramolecular signal transduction pathways.

ArsA is a distant relative of NiFH, the iron protein of nitrogenase that couples ATP hydrolysis to electron transfer. The structure of the iron protein from Azotobacter vinelandii can be taken as a paradigm for the similar component of the nitrogenase. NiFH is a symmetric homodimer held together by a [4Fe-4S] metal center (34), with two NBSs at the dimer interface. When a monomer of NiFH is superimposed with either ArsA A1 or A2, the iron-sulfur cluster of NiFH and the metalloid cluster of ArsA are almost coincident (Fig. 3A). The two cysteines at position 97 (one from each NiFH subunit), which are ligands of the iron-sulfur center, correspond to Cys-113 (A1) and Cys-422 (A2) of the antimony-binding site. The other two cysteine ligands of NiFH [4Fe-4S] cluster correspond to His-148 (A1) and His-453 of ArsA (Fig. 3B). The structural similarities between ArsA and NiFH suggest common mechanistic features. Coincident with ATP hydrolysis NiFH assumes a more compact conformation that projects the tetranuclear center toward the P-cluster of the Mo-Fe protein (38). This conformational change is tightly linked with transfer of electrons from a ferredoxin to the Mo-Fe subunit of nitrogenase. Thus, NiFH can be described as an "electron pump," in which an ATP-driven decrease in the redox potential of the metal center is coupled with vectorial movement of the ions into ArsB. Thus ArsA might expand to pull antimonite ions from the cytosol and contract to expel them into the membrane channel of ArsB.

It is tempting to speculate that ArsA evolved from an ancestor of the nitrogenase NiFH by conversion of an iron-sulfur center into an Sb(III)-binding site. We hypothesize that ArsB arose first as a secondary carrier, and the ArsAB pump evolved subsequently by association of the common ancestor of ArsA and NiFH with ArsB. The same principle of association of soluble ATPases with secondary membrane transporters may have been operational during the evolution of other transport ATPases such as the F- or ABC-type pumps.

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