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The transport mechanism of bacterial Cu⁺-ATPases: distinct efflux rates adapted to different function

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Abstract

Cu^+-ATPases play a key role in bacterial Cu^+ homeostasis by participating in Cu^+ detoxification and cuproprotein assembly. Characterization of *Archaeoglobus fulgidus* CopA, a model protein within the subfamily of P_{1B-1} type ATPases, has provided structural and mechanistic details on this group of transporters. Atomic resolution structures of cytoplasmic regulatory metal binding domains (MBDs) and catalytic actuator, phosphorylation, and nucleotide binding domains are available. These, in combination with whole protein structures resulting from cryo-electron microscopy analyses, have enabled the initial modeling of these transporters. Invariant residues in helixes 6, 7 and 8 form two transmembrane metal binding sites (TM-MBSs). These bind Cu^+ with high affinity in a trigonal planar geometry. The cytoplasmic Cu^+ chaperone CopZ transfers the metal directly to the TM-MBSs; however, loading both of the TM-MBSs requires binding of nucleotides to the enzyme. In agreement with the classical transport mechanism of P-type ATPases, occupancy of both transmembrane sites by cytoplasmic Cu^+ is a requirement for enzyme phosphorylation and subsequent transport into the periplasmic or extracellular milieus. Recent transport studies have shown that all Cu^+-ATPases drive cytoplasmic Cu^+ efflux, albeit with quite different transport rates in tune with their various physiological roles. Archetypical Cu^+-efflux pumps responsible for Cu^+ tolerance, like the *Escherichia coli* CopA, have turnover rates ten times higher than those involved in cuproprotein assembly (or alternative functions). This explains the incapability of the latter group to significantly contribute to the metal efflux required for survival in high copper environments.

Keywords

Cu^+-ATPases; Cu^+ transport; Cu^+ chaperone; Cu^+ coordination; Homeostasis

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Roles of Cu^+-ATPases in bacteria

Copper is an essential micronutrient for all organisms. It is involved in numerous biochemical processes, such as oxidative phosphorylation, photosynthesis, and free radical control (Fraústro da Silva and Williams 2001). In bacteria, Cu is mostly present in periplasmic and plasma membrane cuproproteins such as superoxide dismutase C and cytochrome c oxidase (Desideri and Falconi 2003; Brunori et al. 2005; Tottey et al. 2005; Osman and Cavet 2008). However, in spite of its importance, a build up in Cu cellular levels has several adventitious effects due to the participation of this metal in Fenton-type reactions, or the damage of key iron-sulfur clusters (Fraústro da Silva and Williams 2001; Macomber and Imlay 2009). A complex machinery involving Cu-dependent transcription factors, metallothioneins, Cu-chaperones and transporters keeps Cu levels within narrow physiological margins (O'Halloran and Culotta 2000; Tottey et al. 2005; Argüello et al. 2007; Espariz et al. 2007; Osman and Cavet 2008; Robinson 2008; Boal and Rosenzweig 2009; Ma et al. 2009; Solioz et al. 2010).

Cu^+-ATPases are responsible for cytoplasmic Cu^+ efflux across the plasma membrane (Argüello et al. 2007; Osman and Cavet 2008; Solioz et al. 2010). Therefore, they are typically associated with Cu^+ detoxification. In line with this function, Cu^+ stimulates their transcriptional up regulation, and mutation of the coding genes leads to decreased metal resistance (Kanamaru et al. 1994; Francis and Thomas 1997; Rensing et al. 2000). Due to this essential role in Cu^+ tolerance, most bacterial genomes possess genes encoding for these proteins, suggesting that this function appeared early in the evolution of life. However, many bacterial genomes contain multiple genes coding for Cu^+-ATPases pointing out their likely participation in other processes (Fig. 1) (Francis and Thomas 1997; Tottey et al. 2001; Argüello 2003; Agranoff and Krishna 2004; Pontel et al. 2007; González-Guerrero et al. 2010).

Cu^+-ATPases required for cytochrome c oxidase assembly represent ATPases with alternative cellular functions (Preisig et al. 1996; Koch et al. 2000; González-Guerrero et al. 2010; Hassani et al. 2010). These ATPases, referred to as FixI/CopA2-like ATPases (Fig. 1), constitute a significant portion of the accessible sequences (17%), and always alongside the ubiquitous ATPases conferring Cu^+ tolerance, are present in 30% of available genomes (González-Guerrero et al. 2010). A diverse range of organisms contain FixI/CopA2-like proteins, suggesting that coding genes appeared early in evolution via gene duplication or horizontal transfer but were kept only in some organisms for a specific biological function. In any case, it is noticeable that most of the FixI/CopA2-like encoding bacteria are endosymbiotic (*Rhizobium leguminosarum*) or pathogenic (*Pseudomonas syringae*) organisms. These ATPases are usually part of a ferredoxin encoding operon immediately downstream of a cytochrome-cbb_3 oxidase assembly polycistron. Characterization of *fixI/copA2* mutant strains has shown significant deficiencies in oxidase activity (Preisig et al. 1996; Koch et al. 2000; González-Guerrero et al. 2010; Hassani et al. 2010) and increased sensitivity to oxidative stress (González-Guerrero et al. 2010). Nevertheless, *fixI/copA2* mutants show Cu^+ tolerance similar to wild type strains. This is not surprising considering that this capacity is provided by the always-present classical Cu^+-ATPases. Moreover, simultaneous mutation of both homologous genes does not lead to higher Cu^+ sensitivity. These phenotypes led to propose that FixI/CopA2-like ATPases might be Cu^+ importers (Preisig et al. 1996; Koch et al. 2000; Hassani et al. 2010). However, recent biochemical characterization has shown that FixI/CopA2-like proteins perform the same basic molecular work as the ATPases conferring Cu^+ tolerance; i.e., to drive cytoplasmic Cu^+ efflux, albeit with different kinetic characteristics that explain their inability to contribute to Cu^+ resistance (see below) (González-Guerrero et al. 2010).

Cu^+-ATPases present in photosynthetic cyanobacteria (*Synechocystis* PCC 6803 and *Synechococcus* PCC 7942) also illustrate additional cellular roles of these transporters. Mutation of either of these ATPases, CtaA or PacS, leads to reduced cytochrome *caa3* oxidase activity and plastocyanin mediated electron transport (Tottey et al. 2001). On the other hand, PacS appears to be required for Cu^+-tolerance while CtaA does not (Kanamaru et al. 1994; Phung et al. 1994; Tottey et al. 2001). These clear phenotypes along with the proposed location of CtaA in the plasma membrane (Kanamaru et al. 1994) and PacS in the thylakoid membrane supports a model where CtaA imports periplasmic Cu^+ into the cytoplasm and PacS transports cytoplasmic Cu^+ into the thylakoid lumen (Tottey et al. 2001). However, since the common structure of Cu^+-ATPases determines an essentially similar transport mechanism; both proteins should drive cytoplasmic Cu^+ efflux across the corresponding membranes. The apparent conflict between the observed phenotype and the mechanistic constrains is explained, as it was the case of FixI/CopA-like ATPases, by a slow rate of transport by CtaA. The direction of transport and kinetics of these Cu^+-ATPases are described in detail later in this review.

It is also relevant that Cu^+-ATPases appear to be required for bacterial infection. As part of their bactericidal mechanisms, macrophages secrete Cu^+ into phagosomes (White et al. 2009). Consequently, ATPases conferring Cu^+ tolerance are essential for virulence (Francis and Thomas 1997; Schwan et al. 2005; Zhang and Rainey 2007; White et al. 2009; González-Guerrero et al. 2010). Considering this, it is tempting to hypothesize that the evolutionary pressure that has preserved the Cu^+ detoxification machinery has been the protection against phagocytosis (either by host immune responses or predatory amoeba) rather than high environmental Cu since this metal is not abundant in most ecological niches. On the other hand, pathogens and endosymbionts also endure hostile environments rich in free radicals (Levine et al. 1994; Jabs et al. 1997). Accordingly, FixI/CopA2–like ATPases are also required for virulence; in this case, it is probably because of their participation in free radical protection mechanisms (González-Guerrero et al. 2010).

Structure of Cu^+-ATPases

Cu^+-ATPases constitute the P_{1B-1} subgroup of P-type ATPases[1] (Axelsen and Palmgren 1998;Argüello 2003). All Cu^+-ATPases present eight transmembrane segments where the transport metal binding sites are located (see below), and two major intracellular loops containing the actuator (A-domain) and the ATP-binding domains (ATP-BD) (Fig. 2a) (Axelsen and Palmgren 1998;Argüello 2003;Argüello et al. 2007). In addition, bacterial Cu^+-ATPases contain one or two cytoplasmic metal binding domains in their N-terminus (N-MBDs). The atomic resolution structures of all soluble domains have been established (Sazinsky et al. 2006a;Sazinsky et al. 2006b;Lubben et al. 2007;Boal and Rosenzweig 2009;Tsuda and Toyoshima 2009). Subsequently, a model of the overall structure of these proteins emerged from mapping these cytoplasmic domains into structures resulting from cryo-electron microscopy studies (Fig. 2a) (Chintalapati et al. 2008;Wu et al. 2008). A relevant aspect of these structures is the great structural homology of the catalytic A-domain and ATP-BD with those of P_2-ATPases for which full structures are available (Morth et al. 2007;Olesen et al. 2007;Pedersen et al. 2007;Toyoshima 2008). On the other hand, the model is compatible with the described interaction between N-MBD and the ATP-BD (González-Guerrero et al. 2009). This Cu^+ and nucleotide sensitive interaction, which has also been described in eukaryotic Cu^+-ATPases (Tsivkovskii et al. 2001), is probably determinant for the well-described regulatory role of N-MBDs (Fan and Rosen 2002;Mandal and Argüello 2003;Argüello et al. 2007). That is, at low Cu^+ levels when the N-MBD sites

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

are not occupied, the N-MBD would restrict A-domain and ATP-BD movements required for catalysis (Hatori et al. 2007;Wu et al. 2008;González-Guerrero et al. 2009).

The defining characteristic of Cu^+-ATPases is their capability to bind Cu^+ in their transmembrane region and translocate the ion across the membrane. These binding and translocation events determine the enzyme specificity, transport stoichiometry, and energetics. Significant progress has been made in recent years to establish the number and nature of Cu^+ transmembrane binding sites (TM-MBSs). Analysis of the sequences of founding members of the subfamily pointed out the likely participation of two Cys in H6 in metal coordination. Subsequent systematic analysis of a larger set of Cu^+-ATPases showed the presence of four additional invariant residues (a Tyr, an Asn, a Thr and a Ser) in the neighboring transmembrane segments H7 and H8 (Argüello 2003). Interestingly, these six residues are located in transmembrane segments flanking the ATP-BD in positions equivalent to the ion coordinating amino acids of P_2-ATPases (Morth et al. 2007; Olesen et al. 2007; Pedersen et al. 2007; Toyoshima 2008). Recent studies combining metal binding, mutagenesis and X-ray spectroscopy have shown that these amino acids constitute two Cu^+ binding sites (Site I and II) (Fig. 2b) (Mandal et al. 2004; González-Guerrero et al. 2008). In both cases, the ions are coordinated in a trigonal planar geometry. It is notable that similar coordination (trigonal planar) is likely present in the Ctr Cu^+ transporters responsible for Cu^+ influx in eukaryotes (Eisses and Kaplan 2002; Puig et al. 2002), CusA and CusB components of the CusABC transporter present in Gram-negative bacteria (Su et al. 2009; Long et al. 2010). Thus, it could be proposed that this geometry probably facilitates the vectorial metal binding and release with high turnover.

Transport and catalytic mechanisms of Cu^+-ATPases

Cu^+-ATPases transport their substrate following the E1/E2 Albers-Post mechanism (Fig. 3) (Argüello et al. 2007). The basic features of this cycle have been described in detail (Fan and Rosen 2002;Mandal et al. 2002;Mandal and Argüello 2003;Mandal et al. 2004;Hatori et al. 2007;Hatori et al. 2008). Thus, it is now accepted that: (1) metal transport is coupled to ATP hydrolysis, (2) the enzyme assumes two basic conformations (E1/E2), (3) it requires metal binding for catalytic phosphorylation by ATP, (4) nucleotides interact with the protein with two apparent affinities, (5) dephosphorylation appears to be a rate limiting step, and (6) the cycle is regulated by Cu^+ binding to cytoplasmic MBDs. In recent years, distinctive aspects with significant mechanistic and physiological implications have emerged. Unsurprisingly, these appear to be associated with the cell metal homeostasis requirements and shaped by the specific chemistry of Cu^+.

The identification of two TM-MBSs suggests a transport stoichiometry of two metal atoms transported per hydrolyzed ATP molecule (González-Guerrero et al. 2008). In agreement with the participation of both sites in transport, mutation of either binding site prevents enzyme phosphorylation (Mandal et al. 2004). A significant characteristic of the TM-MBSs is their ability to bind the metal with affinities in the femtomolar range, which are much higher than affinities observed for the corresponding Cu^+-chaperones and the regulatory MBDs when these are measured under similar conditions (Sazinsky et al. 2007; González-Guerrero and Argüello 2008; González-Guerrero et al. 2008). A consequence of these high affinities is that the enzyme does not need to occlude the ions to prevent the backward release of metal into the cytoplasmic media. That is, even in the absence of nucleotide, Cu^+ bound to TM-MBSs have a very slow k_{off}.

Perhaps the most remarkable feature of Cu^+-ATPases is their capability to acquire their substrate from the Cu^+-chaperones that shuttle the metal within the cytoplasm. Cu^+ exchange between CopZ (bacterial Cu^+-chaperones) and Cu^+-ATPase N-MBDs has been

extensively characterized (O'Halloran and Culotta 2000; Tottey et al. 2005; Osman and Cavet 2008; Boal and Rosenzweig 2009; Ma et al. 2009; Solioz et al. 2010). This well-described Cu^+ exchange led to the early assumption that Cu^+ reached the TM-MBSs from N-MBDs. However, removal of Cu^+ binding to N-MBDs (by mutagenesis of Cu^+ binding residues or domain truncation) does not prevent either Cu^+-dependent ATPase activity (Fan and Rosen 2002; Mandal and Argüello 2003) or the capability of the enzyme to be activated by CopZ-Cu^+ (González-Guerrero and Argüello 2008). Moreover, soluble Cu^+ loaded MBDs cannot stimulate Cu^+-ATPase activity. Alternatively, Cu^+ loaded CopZ transfers the metal to TM-MBSs stimulating Cu^+-ATPase activity, even in enzymes lacking cytoplasmic MBDs (González-Guerrero and Argüello 2008). Confirming these findings, the transfer of Cu^+ from CopZ to TM-MBS was also observed under non-turnover conditions. Thus, experimental evidence supports a model where Cu^+ is directly transferred from CopZ to TM-MBSs independently of MBDs. Within the framework of this model (Fig. 3), the interaction of CopZ-Cu^+ with the enzyme and the metal binding to TM-MBSs have mechanistic properties essential for an appropriate Cu^+ homeostasis. For instance, only CopZ-Cu^+ binds the enzyme while apo-CopZ has no observable interaction with Cu^+ loaded TM-MBSs (González-Guerrero and Argüello 2008). Therefore, there is a vectorial transfer of Cu^+ from the chaperone to the transport sites; i.e., there is no metal exchange among them. The unidirectional transfer is likely determined by protein–protein interactions where the relative high Cu^+ affinities prevent backward release rather than determine the transfer. The critical role of specific protein–protein interactions in the Cu^+ transfer is also pointed out by the requirement of nucleotide binding for CopZ-Cu^+ loading of the second TM-MBS (Fig. 3) (González-Guerrero et al. 2009). In this case, the simpler assumption is that the binding of the nucleotide does not change the affinity of TM-MBS for the metal but more likely stabilizes the ATPase conformation required for binding of CopZ-Cu^+. In spite of this requirement, occupancy of TM-MBSs does not appear to be sequential; e.g., Cu^+ does not need to pass first through a "cytosolic" TM-MBS to access the "external" TM-MBS. CopZ mediated Cu^+ transfer to ATPase mutants separately lacking one or other TM-MBSs has shown a "parallel" arrangement of the TM-MBSs; i.e., CopZ delivers one Cu^+ per ATPase molecule indistinctly to Site I or II (González-Guerrero et al. 2009).

Direction of metal transport by Cu^+-ATPases

The conformational coupling of cytoplasmic substrate binding to transmembrane sites with the phosphorylation of the ATP-BD, as well as the structural movements required for substrate translocation, have been clearly shown in the case of the Ca^{2+}-ATPase for which various structures are available (Olesen et al. 2007; Toyoshima 2008). The structural similarities among P-ATPases allow the prediction that Cu^+-ATPases will undergo similar catalytic and transport steps with the consequent outward transport of cytoplasmic Cu^+. However, as pointed out earlier, various Cu^+-ATPases (FixI/ CopA2-like proteins, *Synechocystis* CtaA, and *E. hirae* CopA) seem not to follow these mechanistic determinants in spite of their close structural homology. In fact, the phenotypes observed upon mutation of these enzymes strongly suggest that they might well be Cu^+ influx ATPases (Odermatt et al. 1993; Kanamaru et al. 1994; Phung et al. 1994; Preisig et al. 1996; Koch et al. 2000; Tottey et al. 2001; González-Guerrero et al. 2010; Hassani et al. 2010).

This apparent discrepancy was explained recently by experiments in which the direction of transport of *Pseudomonas aeruginosa* FixI/CopA2-like Cu^+-ATPase was determined (González-Guerrero et al. 2010). It was observed that the enzyme was indeed only able to drive cytoplasmic Cu^+ efflux, although at a very slow rate (compared to that of its paralog CopA1) (Table 1). This slow rate of transport is clearly incompatible with a role in Cu^+-detoxification but appears to be adequate for the function of these proteins, which is to contribute to the assembly of Cu-containing cytochrome c oxidases (Preisig et al. 1996;

Koch et al. 2000; González-Guerrero et al. 2010; Hassani et al. 2010). Similar experiments performed with other proposed "influx" Cu^+-ATPases yield analogous results. Figure 4 shows the initial rates of ATP-driven Cu^+ uptake into *E. coli* everted vesicles heterologously expressing *Synechocystis* PCC 6803 CtaA, *E. hirae* CopA, and, for comparison, *E. coli* CopA and *Synechocystis* PCC 6803 PacS. These experiments were performed in mixed vesicle populations; that is, "inside-out" and "right side-out" vesicles pools. However, only "inside-out" vesicles, where the ATP-BD is exposed to the media participate in the experiment. The data showed that, as expected, these enzymes transported cytoplasmic Cu^+ into the vesicles. Moreover, *E. hirae* CopA and *Synechocystis* PCC 6803 PacS transported Cu^+ at rates similar to *E. coli* CopA. This is particularly interesting in the case of *E. hirae* CopA since it was originally proposed to be a Cu^+ importer (Odermatt et al. 1993). On the other hand, *Synechocystis* PCC 6803 CtaA showed a V_{max} ten-fold smaller than that of E. coli CopA but similar to that of *P. aeruginosa* CopA2 (Table 1). Consequently, this characteristic would be compatible with a role of *Synechocystis* PCC 6803 CtaA in cuproprotein assembly. Furthermore, in vivo, the slow-transporting ATPase CtaA would not significantly contribute to Cu^+-detoxification. This characteristic is supported by the lack of reversion of the Cu^+-sensitive phenotype observed in assays testing *E. coli* ΔCopA complementation by CtaA (not shown). Interestingly, distinct from *P. aeruginosa* Cu^+-ATPases, PacS and CtaA showed small differences in substrate apparent affinities; however, it should be kept in mind that, in contrast to physiological conditions, in these assays Cu^+ was not bound to the specific chaperones. While these data provide a coherent structural–functional framework to analyze the roles of these proteins, it is more complex to explain the described decrease in cytoplasmic Cu^+ content after mutation of CtaA in *Synechocystis* PCC 6803 (and in some cases of FixI/ CopA2-like proteins). It could be speculated that, since these ATPases are required for assembly of proteins involved in the energy metabolism, the observed changes in Cu^+ content might well be pleiotropic effects. However, it is beyond the scope of the described transport experiments to explain the interplay among PacS and CtaA in photosynthetic bacteria toward the assembly of relevant cuproproteins.

Future directions

The described findings have given us significant insight into the structure and transport mechanisms of the Cu^+-ATPases; as well as, the manner in which Cu^+ binds these proteins in the cell. However, little is known on how the Cu^+ is released from the TM-MBSs in the E2 state. This is of special interest in Cu^+-ATPases from Gram negative bacteria, as well as for those Cu^+-ATPases responsible for providing Cu^+ for plasma membrane cuproproteins, since the presence of Cu^+ in the periplasm could have damaging effects similar to free Cu^+ in the cytosol. In fact, the periplasm contains a large number of Cu-binding proteins that would ensure that no Cu^+ is "free" in a CopZ-like manner (Huffman et al. 2002; Bagai et al. 2008; Petit-Haertlein et al. 2010). This raises the question of whether some periplasmic proteins or equivalent plasma membrane bound ones, could interact with the Cu^+-ATPase accepting the released Cu^+ and affecting the kinetics of transport in a manner similar to CopZ in the cytosol. Supporting this, it has been shown that mutation of the *scoI*-like gene *senC* in *P. aeruginosa*, has similar effects on cytochrome c oxidase activity as the mutation of the *copA2* or cytochrome oxidase itself (Frangipani and Haas 2009; González-Guerrero et al. 2010). ScoI proteins in the mitochondria are responsible for shuttling Cu^+ for the assembly of active cytochrome oxidases (Robinson and Winge 2010). It could be speculated that SenC mediates the transfer of Cu^+ from ATPase to cytochrome oxidase, being a candidate for direct interaction with the transporter. Alternatively, given that some periplasmic/plasma membrane cuproproteins are secreted unfolded through the Sec pathway (Saier 2006), it would be interesting to explore their interaction with Cu^+-ATPases. As a

corollary of this hypothetical scenario, it is likely that different Cu^+ accepting proteins would interact with alternative Cu^+-ATPases regulating their differential function.

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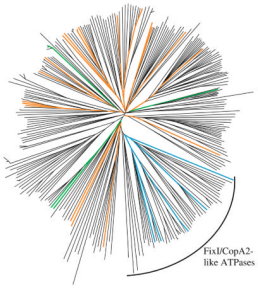

Fig. 1.

Unrooted tree of Cu^{+}-ATPases from all sequenced bacteria (González-Guerrero et al. 2010). Characterized Cu^{+}- ATPases in this subgroup are indicated in blue (*R. capsulatus* CcoI, *B. japonicum* FixI, *S. meliloti* FixI, *R. gelatinosus* CtpA, *P. aeruginosa* CopA2). Characterized Cu^{+}-ATPases involved in Cu^{+} detoxification are *indicated in orange. E. hirae* CopA, *L. monocytogenes* CtpA, *Synechocystis* PCC 6803 CtaA and *Synechococcus elongatus* PCC 7942 CtaA *are indicated in green*

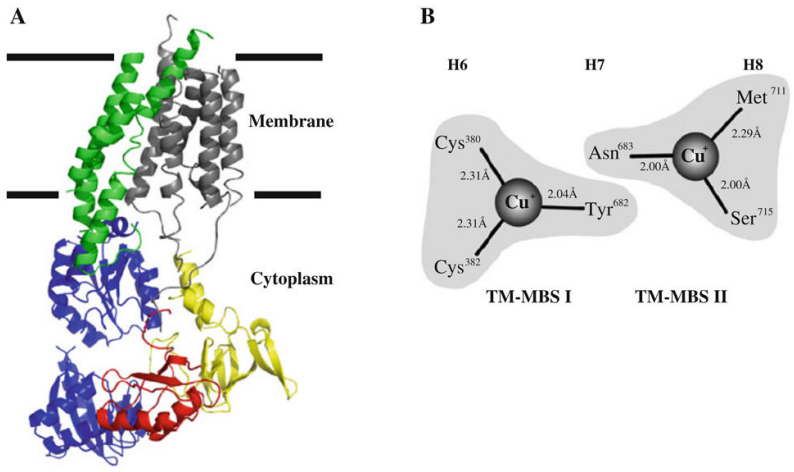

Fig. 2.

a Model for *A. fulgidus* ΔN,C-CopA obtained using PDB 2VOY (Wu et al. 2008). Domains were colored as follows: transmembrane helices (*green and grey*), P/N domain (*blue*), A domain (*yellow*). **b** Scheme showing amino acids forming the trans-membrane Cu^{+} binding sites I and II

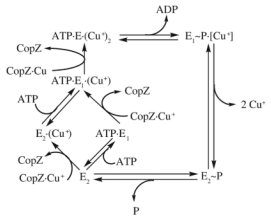

Fig. 3.

Proposed catalytic and transport cycle of Cu^{+}-ATPases (González-Guerrero et al. 2009)

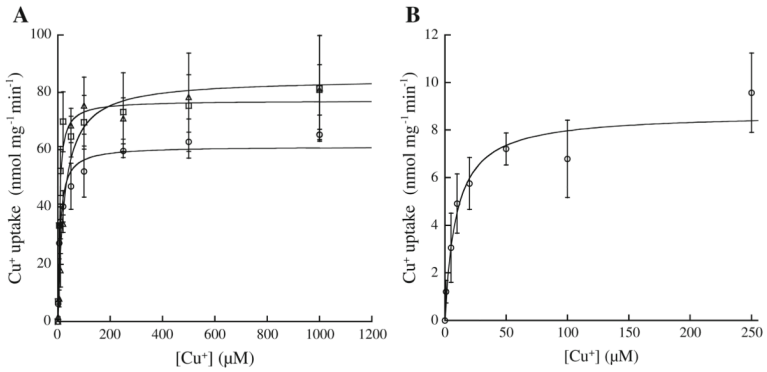

Fig. 4.

Cu^+ transport kinetics in E. coli DC194 everted vesicles expressing **a** *E. coli* CopA (*triangle*), *Synechosystis* PCC 6803 PacS (*square*), *E. hirae* CopA (*circle*) and **b** *Synechosystis* PCC 6803 CtaA (*circle*). Fitted parameters are shown in Table 1. In all cases, Cu^+ uptake by vesicles from empty vector transformed *E. coli* DC194 was subtracted and values were normalized by relative protein expression levels. Methodological details associated with these experiments have been previously described (González-Guerrero et al. 2010). Data are the mean ± SE of three independent experiments

Table 1

Kinetic parameters of Cu^+ uptake into *E. coli* DC194 everted membrane vesicles expressing the indicated bacterial Cu^+-ATPases

Organism	Protein	V_{max} (nmol mg^{-1} min^{-1})	$K_{1/2}$ (μM)	Reference
E. coli	CopA	86.3 ± 8.4	27.2 ± 8.5	González-Guerrero et al. (2010)
P. aeruginosa	CopA1	63.1 ± 1.5	152.6 ± 7.9	González-Guerrero et al. (2010)
P. aeruginosa	CopA2	6.7 ± 0.4	20.7 ± 3.7	González-Guerrero et al. (2010)
Synechocystis PCC 6803	CtaA	8.7 ± 0.6	9.1 ± 2.5	This publication
Synechocystis PCC 6803	PacS	77.1 ± 2.5	5.5 ± 1.0	This publication
E. hirae	CopA	61.1 ± 1.4	8.4 ± 1.9	This publication