

## Copper and Silver Transport by CopB-ATPase in Membrane Vesicles of *Enterococcus hirae*\*

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Marc Solioz‡ and Alex Odermatt

From the Department of Clinical Pharmacology, University of Berne, 3010 Berne, Switzerland

The P-type ATPase, CopB, of *Enterococcus hirae* is required for the copper resistance displayed by this organism and thus was postulated to be a copper pump. Using  $^{64}\text{Cu}^+$  and  $^{110m}\text{Ag}^+$ , we here show ATP-driven copper and silver accumulation catalyzed by CopB in native inside-out membrane vesicles of *E. hirae*. CopB ATPase exhibited an apparent  $K_m$  for  $\text{Cu}^+$  and  $\text{Ag}^+$  of 1  $\mu\text{M}$  and for ATP of 10  $\mu\text{M}$ . Transport was maximal at pH 6 and had an apparent  $V_{\text{max}}$  of 0.07  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  for both copper and silver transport. Vanadate displayed a biphasic effect on transport: maximal inhibition was observed at 40  $\mu\text{M}$  vanadate for copper transport and 60  $\mu\text{M}$  for silver transport, respectively. At higher vanadate concentrations, these inhibitions were reversed. The CopB ATPase of *E. hirae* is thus a pump for the extrusion of monovalent copper and silver ions, with copper probably being the natural substrate.

Copper is an essential heavy metal ion by its function as a cofactor of many enzymes. Since copper can be very toxic to both eukaryotic and prokaryotic cells, homeostatic mechanisms have evolved to balance the intracellular copper concentration. We have recently described one such mechanism in *Enterococcus hirae* that is dependent on the activity of two ATPases, CopA and CopB, that are regulated in their expression by the copper concentration (1, 2). Studies with whole cells had suggested that CopA serves in the uptake and CopB in the extrusion of copper (3). Based on their primary structure, CopA and CopB were considered to be novel members of the family of P-type ATPases.

Typical features of P-type ATPases, for which  $\text{Ca}^{2+}$ -ATPases are paradigms, are a protein subunit of around 100 kDa, sensitivity to vanadate, and the formation of an acylphosphate intermediate in the transport reaction. The relatedness of P-type ATPases is also apparent by sequence features that are conserved in these enzymes from bacteria to man, such as the "phosphatase" domain, TGES, or the "phosphorylation" domain, DKTGT (4). Besides the presence of such conserved sequence elements in the CopB-ATPase, this protein also exhibits two features that are known from other heavy metal-binding proteins. These are (i) cysteine flanking the intramembranous conserved proline that forms the ion channel in P-type ATPases, and (ii) the N-terminal motif MXHXXMSGMXHS that is also found in a copper-binding protein of *Pseudomonas*

*syringae* (5). Based on these observations, we had suggested that CopB is a copper ATPase (6).

Several putative copper ATPases similar to CopA and CopB of *E. hirae* have since been described from other organisms. Most notably, in humans, two ATPases with over 30% amino acid sequence identity to CopB were identified in connection with the copper metabolic diseases Menkes (7–9) and Wilson (10–12). Two related ATPases, PacS and CtaA, were described for the cyanobacteria *Synechococcus* 7942 (13, 14). Also, hp-CopA of *Helicobacter pylori* (15) and *ccc2* of *Saccharomyces cerevisiae* (GenBank™/EMBL Data Bank accession number L36317) were postulated to be copper-pumping ATPases. Similar genes that have as yet not been associated with any function are *fixI* of *Rhizobium meliloti* (16), open reading frame YBR295w of *S. cerevisiae*, and open reading frame o732 of *Escherichia coli* (GenBank™/EMBL Data Bank accession numbers Z36164, L33259, and U00039, respectively).

Of particular interest in this context are the cadmium resistance ATPases that have been cloned from three different microorganisms (17–19). These proteins have metal binding motifs similar to those of the putative copper ATPases and thus appear to form, together with these, a family of heavy metal ATPases. The cadmium ATPases are the only members of this family for which heavy metal ion transport has actually been demonstrated. Native inside-out membrane vesicles of *Bacillus subtilis* harboring the *Staphylococcus aureus* *cadA* ATPase gene were shown to catalyze ATP-driven  $^{109}\text{Cd}^{2+}$  accumulation (20). In contrast, the evidence that any of the postulated copper ATPases is indeed a copper pump has so far been indirect and rested on the behavior of whole cells in response to copper and on the special structural features of these pumps (1, 21). Here, we show ATP-driven  $^{64}\text{Cu}^+$  and  $^{110m}\text{Ag}^+$  accumulation by the CopB ATPase in native membrane vesicles, thus providing direct evidence that CopB of *E. hirae* is a heavy metal ATPase.

### EXPERIMENTAL PROCEDURES

**Strains and Culture Conditions**—*E. hirae* wild-type (ATCC 9790, formerly called *Streptococcus faecalis* or *faecium*) was obtained from the American Type Culture Collection. The generation of the gene-disrupted strains *copA*, *copB*, *copAB*, and *copY* have been described previously (2, 22). Cells were grown semi-anaerobically (the cultures were sealed but not made anaerobic) in 1%  $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$ , 1% trypticase peptone (BBL), 0.5% yeast extract (BBL), 1% glucose. Growth was monitored by measuring the absorption of a 1:10 dilution in water at 546 nm.

**Vesicle Preparation**—5-Liter cultures were inoculated from frozen stocks of logarithmically growing cells and grown in a fermentor at 37 °C. The cultures were induced with 500  $\mu\text{M}$   $\text{CuSO}_4$ , added at an OD of 1.5–2, and grown for an additional 30 min. Cells were harvested by centrifugation for 10 min at 2000  $\times g$  and washed once with 1.5 liters and once with 0.5 liter of 2 mM  $\text{MgSO}_4$ . Protoplasts were obtained by suspending 10 g of cells in 60 ml of 250 mM  $\text{K}_2\text{SO}_4$ , 50 mM glycylglycine, 2 mM  $\text{MgSO}_4$ , pH 7.2, and treating with 40 mg of lysozyme (Sigma) in the presence of 1 mM phenylmethylsulfonyl fluoride (Merck, Darmstadt, Germany) for 1 h at 37 °C in an orbital shaking water bath. The protoplasts were centrifuged for 25 min at 23,000  $\times g$  and the pellet resuspended in 20 ml of CT buffer (100 mM Tris-Cl, 150 mM KCl, 50 mM

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‡ To whom correspondence should be addressed: Dept. of Clinical Pharmacology, University of Berne, Murtenstrasse 35, 3010 Berne, Switzerland. Tel.: 031-632-32-68; Fax: 031-381-47-13; E-mail: solioz@ikp.unibe.ch.

NaCl, 5 mM MgSO<sub>4</sub>, pH 6.5) for copper transport experiments or ST buffer (100 mM Tris-SO<sub>4</sub>, 25 mM K<sub>2</sub>SO<sub>4</sub>, 25 mM Na<sub>2</sub>SO<sub>4</sub>, 5 mM MgSO<sub>4</sub>, pH 7) for silver transport experiments. The protoplasts were supplemented with 1 mg of DNase I (Sigma) and homogenized in a Potter-Elvehjem-type homogenizer before passing them through a French press at 70 Megapascal. Unlysed cells were collected by centrifugation for 12 min at 23,000 × *g* and the membrane fraction collected by ultracentrifugation for 1 h at 90,000 × *g*. The membrane pellets were washed by resuspension in the same buffer and centrifuging as before. The final pellet was suspended in 2.5 ml of the same buffer and frozen in aliquots at -70 °C. The protein concentration of these preparations was 3–4 mg/ml as determined with the Bradford protein assay (23). For all experiments, frozen membranes were thawed just before use. No loss in activity of frozen membranes was observed over 2 months.

**Copper Transport Measurements**—0.1 mg of membrane protein was suspended in 1 ml of CT buffer, supplemented with 5 mM dithiothreitol, 0.5–5 μCi of <sup>64</sup>Cu<sup>+</sup> (University of Missouri Research Reactor, Columbia, MO; specific activity: 1–300 Ci/mg, depending on the time of use) and 0.05–10 μM cold copper, added as CuSO<sub>4</sub>, as specified under "Results." After 30 min preincubation at 37 °C with rotary shaking, transport was initiated by adding 0.5 mM Na-ATP, pH 6.5. 100-μl samples were taken at intervals and filtered through 25-mm filter disks of 0.22-μm nitrocellulose membranes (Millipore) that had been stored for at least 1 h in CS buffer containing 1 mM CuSO<sub>4</sub>. The vesicle deposits on the filters were immediately washed twice with 1 ml of CT buffer containing 1 mM CuSO<sub>4</sub>, the moist filters dissolved in scintillation mixture, and the radioactivity determined in a β-counter. For the measurements of the pH dependence of transport, CT buffer was adjusted to the corresponding pH values with Tris base. Incubations at low pH were performed in phosphate buffer of the following composition: 50 mM NaP<sub>i</sub>, 50 mM Tris-Cl, 75 mM KCl, 25 mM NaCl, 5 mM MgSO<sub>4</sub>, adjusted to the desired pH with NaOH. The wash steps were performed with CT buffer of the corresponding pH and containing 1 mM CuSO<sub>4</sub>. For the measurement of vanadate inhibition, VO<sub>4</sub><sup>3-</sup> was added to the vesicles at the onset of the preincubation.

**Silver Transport Measurements**—Silver transport experiments were conducted in the absence of dithiothreitol. 0.1 mg of membrane protein was preincubated for 10 min at 37 °C with 1 μM tetrachlorosalicylanilide and 0.5 μM valinomycin in ST buffer. 0.2 μCi of <sup>110m</sup>Ag<sup>+</sup> (Amersham Corp., 2 mCi/mg) and 0.2–10 μM AgNO<sub>3</sub>, as required by the experiment, were added and preincubation continued for 10 min. Other details of the procedure were as described for copper transport, except that the filters were preincubated and washed with ST buffer containing 1 mM AgNO<sub>3</sub>.

## RESULTS

To demonstrate copper transport by the CopB ATPase of *E. hirae*, native membrane vesicles proved to be a suitable preparation. Such vesicles are a mixed population of inside-out and right-side-out vesicles. However, since the bacterial membrane is impermeable to ATP, only vesicles with the ATP binding site of the ATPase facing outside (inside-out vesicles) will respond to added ATP. Fig. 1 shows ATP-stimulated copper accumulation into vesicles. The most rapid copper accumulation was observed in the complete reaction, which contained 5 mM dithiothreitol. Since dithiothreitol reduces the added copper(II) to copper(I), the transported species under these conditions is copper(I). Very little copper was taken up without ATP. If dithiothreitol was omitted from the reaction, a basal level of copper uptake and high background binding were observed. 40 μM vanadate, a specific inhibitor of P-type ATPases, inhibited the dithiothreitol-stimulated copper uptake (see also below). *E. hirae* membrane vesicles thus display ATP-stimulated, vanadate-sensitive accumulation of Cu<sup>+</sup>. Approximately the same basal level of transport under nonreducing conditions or in the presence of 40 μM vanadate was observed in all experiments. It was not sensitive to uncouplers, and its origin is not clear. Conceivably, it is due to nonspecific secondary transport or binding. For calculation, we subtracted the basal dithiothreitol-independent rate of transport.

The experiments of Fig. 1 were conducted with a strain that overexpresses both the CopA and the CopB ATPase due to disruption of the regulatory gene *copY* (22). To ascertain whether CopA or CopB was responsible for the observed copper

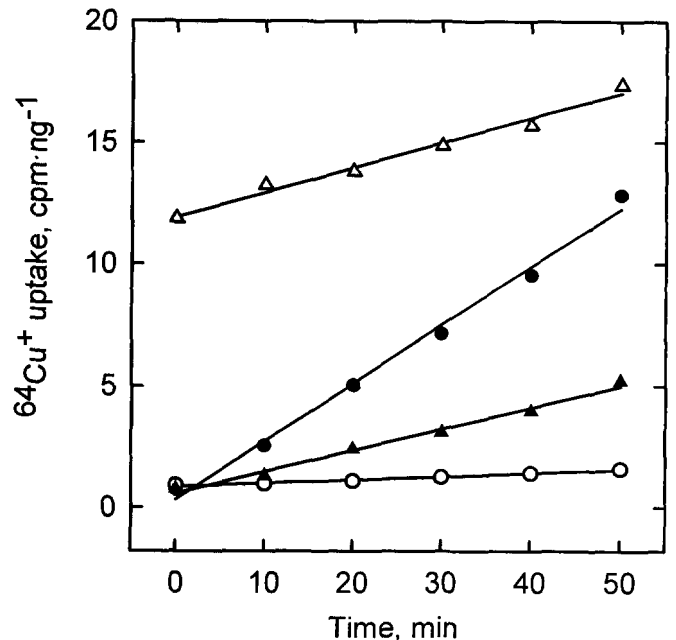


FIG. 1. Copper accumulation by native membrane vesicles. At time 0, transport was initiated by the addition of ATP. The copper concentration was 50 nM. Other details were as described under "Experimental Procedures." ●, complete reaction; ○, without ATP; ▲, without dithiothreitol; △, in presence of 40 μM VO<sub>4</sub><sup>3-</sup>.

accumulation, we employed strains that were null mutants in either CopA ( $\Delta copA$ ), CopB ( $\Delta copB$ ), or both ( $\Delta copAB$ ). Fig. 2 shows that wild-type and a strain only possessing CopB exhibited high copper transport activity, whereas strains deficient in either CopB, or both CopA and CopB, only displayed the basal, dithiothreitol-independent level of transport activity. We thus conclude that copper(I) is accumulated in vesicles by the action of the CopB ATPase. This is in line with the proposed *in vivo* function of CopB in copper resistance by exporting copper from the cytoplasm. Further investigation of CopB was conducted in the  $\Delta copA$  strain only expressing CopB.

We had previously shown that whole cells can extrude silver and suggested that the enzyme involved in the process is the CopB ATPase (3). To corroborate these *in vivo* observations, we investigated silver transport by native membrane vesicles. Fig. 3 shows that vesicles containing only the CopB ATPase showed ATP-stimulated accumulation of <sup>110m</sup>Ag<sup>+</sup>. In the absence of ATP or in membranes devoid of CopB, there were basal levels of silver accumulation similar to those observed in copper transport experiments. These basal rates were somewhat, but not completely, suppressed by the K<sup>+</sup> and H<sup>+</sup> ionophores valinomycin and tetrachlorosalicylanilide. Dithiothreitol, which proved essential to measure Cu<sup>+</sup> transport, was not required for silver transport; in fact, it interfered with the Ag<sup>+</sup> measurements, probably by interacting with Ag<sup>+</sup>. These results show that silver(I) is a substrate for the CopB ATPase and that dithiothreitol employed in the copper transport experiments is not required by the vesicles, but strictly serves to reduce copper(II) to copper(I). That Ag<sup>+</sup> can replace Cu<sup>+</sup> had also been observed in other instances, such as for the copper-dependent yeast transcription factor ACE1 (24).

Fig. 4 shows the analysis of kinetic parameters of copper transport by CopB. The reaction had an apparent V<sub>max</sub> of 0.07 nmol·min<sup>-1</sup>·mg<sup>-1</sup> and a K<sub>m</sub> for Cu<sup>+</sup> of 1 μM. However, these values must be interpreted cautiously; the reaction contains the copper binding entities Tris, dithiothreitol, and the membrane vesicles. We thus cannot know the exact concentration of free Cu<sup>+</sup> ions, and the value we derived for the K<sub>m</sub> is most

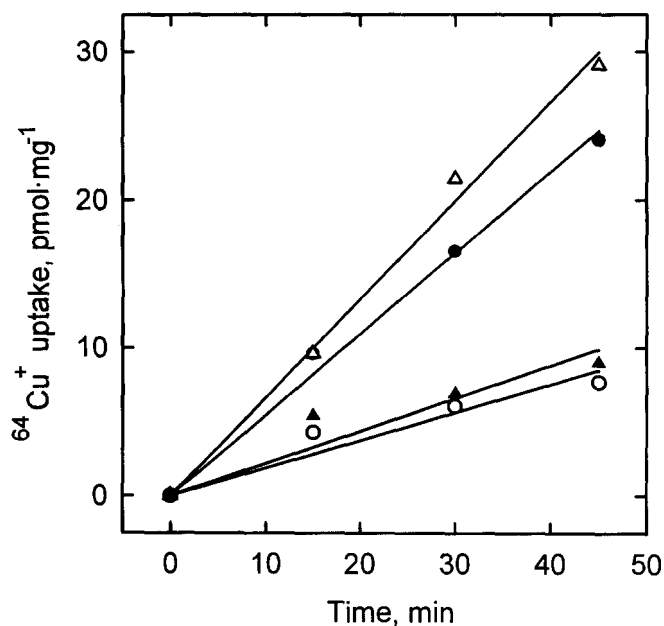


FIG. 2. Copper accumulation by membrane vesicles of wild-type and mutant strains. The copper concentration was 50 nM. Other details were as described under "Experimental Procedures." ●, wild type; ○, strain only expressing CopA ( $\Delta copB$ ); △, strain only expressing CopB ( $\Delta copA$ ); ▲, double-disrupted strain ( $\Delta copAB$ ).

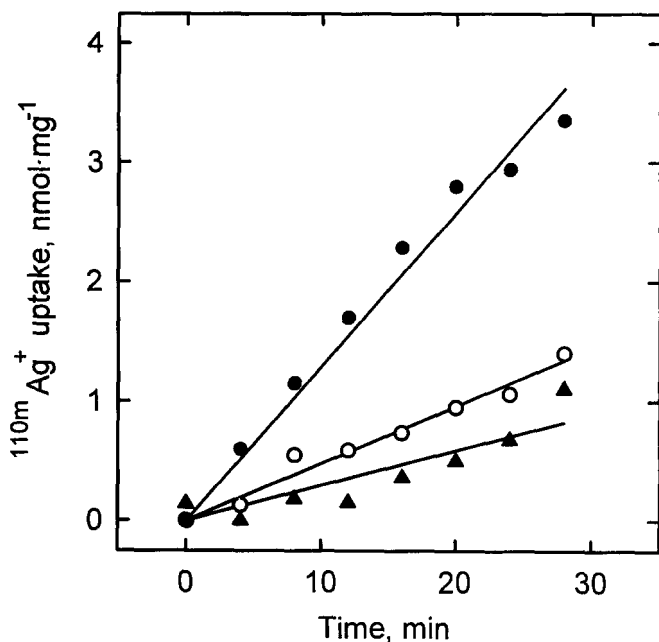


FIG. 3. Silver transport by membrane vesicles. The silver concentration was 4  $\mu$ M; other details of the procedure were as described under "Experimental Procedures." ●,  $\Delta copA$  membrane vesicles in the presence of ATP; ○,  $\Delta copA$  membrane vesicles in the absence of ATP; ▲,  $\Delta copAB$  membrane vesicles in the presence of ATP.

likely an overestimate. Nevertheless, the enumeration of kinetic parameters is useful in connection with the analysis of mutant forms of CopB. The  $K_m$  for ATP was 10  $\mu$ M, and the kinetic parameters for silver transport by CopB were essentially the same as those for copper, although the same uncertainty regarding the free silver concentration applies (data not shown).

Fig. 5 shows the pH dependence of the transport by the CopB ATPase. Maximal activity was observed at pH 6. At more acidic pH values, the ATPase activity dropped rapidly, whereas at

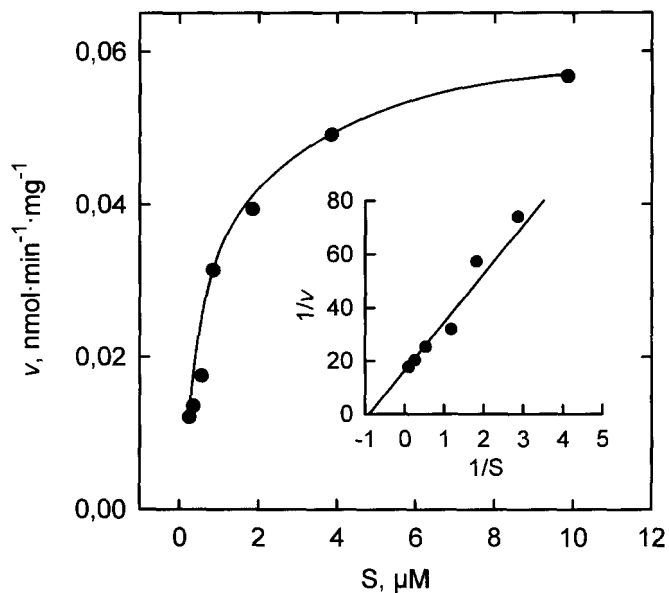


FIG. 4. Plot of the copper transport rate,  $v$ , versus the copper concentration,  $S$ . Transport rates at varying copper concentrations were measured with  $\Delta copA$  membrane vesicles as outlined in the legend to Fig. 2. Inset, Lineweaver-Burk plot of the same data.

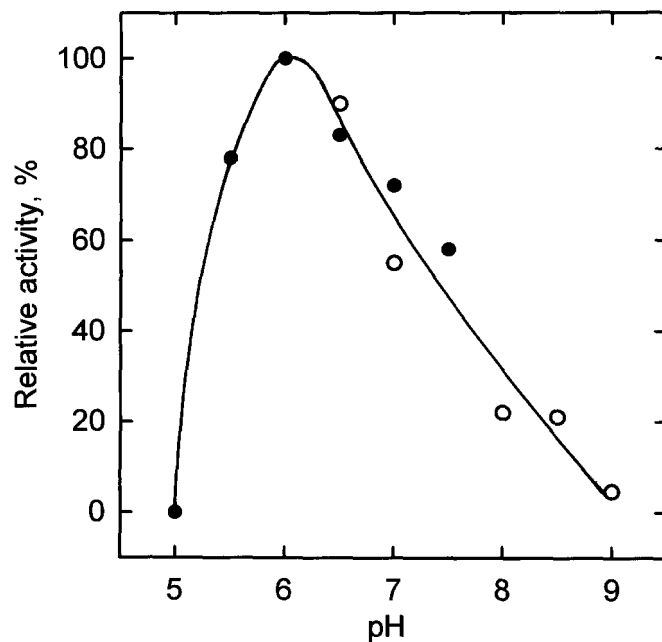


FIG. 5. pH dependence of CopB activity. The rate of copper transport as a function of pH was determined with  $\Delta copA$  membrane vesicles. The copper concentration was 1  $\mu$ M; other details of the experiment were as described under "Experimental Procedures." ●, measurements conducted in phosphate buffer; ○, measurements conducted in CT buffer.

increasing pH, only a gradual decline in activity was observed. We previously showed that the Na<sup>+</sup>-ATPase that is present in *E. hirae* is also active up to pH 9. At high pH, *E. hirae* can have a very small or no proton motive force and can no longer rely on symporters and antiporters for the ionic control of the cytoplasm and becomes dependent on the operation of primary transport systems (25). If the regulation of cytoplasmic copper is vital at high pH, the cell needs a copper pump that can operate under these conditions.

Vanadate is a specific inhibitor of P-type ATPases and therefore is a diagnostic tool for these enzymes (26). The vanadate

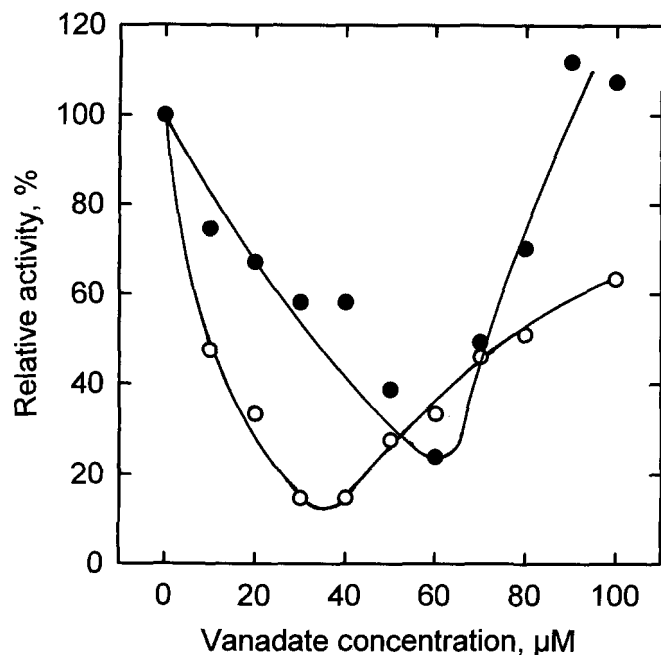


FIG. 6. Vanadate inhibition of transport. Transport rates were determined with  $\Delta copA$  membrane vesicle at a copper concentration of  $1 \mu\text{M}$  and a silver concentration of  $4 \mu\text{M}$ , respectively. Other details of the experiment were as described under "Experimental Procedures." ●, inhibition of silver transport; ○, inhibition of copper transport.

inhibition of copper and silver transport was analyzed in more detail in Fig. 6. An unusual, biphasic inhibition curve was observed. Copper transport was progressively inhibited by up to  $40 \mu\text{M}$   $\text{VO}_4^{3-}$  and silver transport by up to  $60 \mu\text{M}$   $\text{VO}_4^{3-}$ . A maximal level of 85% inhibition was observed for copper and 76% for silver transport. These inhibitions were reversed at higher vanadate concentrations. We have no obvious explanation for this behavior. The different minima in the inhibition curves for copper and silver transport may be due to the presence of dithiothreitol in only the copper experiments. Vanadium has a complex chemistry with many different oxidation states and polymeric forms (27). Conceivably, under the reducing conditions of the copper transport reaction, species of vanadium other than  $\text{VO}_4^{3-}$  act on CopB. Indeed, it has been observed that the combination  $\text{VO}_4^{3-}$ /glutathione leads to the formation of a vanadium species that activates adiposite cyclic nucleotide phosphodiesterase (28). Overall, both silver and copper transport show the same unusual, biphasic vanadate inhibition curve. The meaning of this inhibition/activation pattern remains to be elucidated.

#### DISCUSSION

We here show that CopB of *E. hirae* is an ATP-driven pump that can expel  $\text{Cu}^+$  and  $\text{Ag}^+$  from the cytoplasm. It is the first example of a P-type ATPase for which copper (and silver) transport has been shown. Wild-type *E. hirae* can tolerate up to 8 mM copper in the media, and we have shown by genetic analysis that CopB is required for this resistance (2). The physiological role of the CopB ATPase is thus the control of cytoplasmic copper by extruding it from the cell. The growth requirement of microorganisms for copper is satisfied by 1–10  $\mu\text{M}$  copper (29); these concentrations are not toxic to *E. hirae* and do not induce the *cop*-operon. Cytoplasmic copper concentrations may therefore be in this micromolar range and a  $K_m$  of  $1 \mu\text{M}$  for copper transport by CopB appears reasonable. Silver, in contrast to copper, is not essential for cell growth and is extremely toxic to *E. hirae*:  $5 \mu\text{M}$   $\text{Ag}^+$  inhibits cell growth. Silver transport catalyzed by CopB is probably fortuitous and

does not serve a physiological role.

That copper(I) and not copper(II) is the substrate of CopB suggests that in the cytoplasm, copper not sequestered by copper enzymes occurs in the copper(I) form, either due to the reducing environment of the cytoplasm or through the action of reductases. The  $V_{\text{max}}$  of  $0.07 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  for copper transport by vesicles is very low. The only comparable system, the CadA ATPase of *S. aureus*, displayed transport rates for  $^{109}\text{Cd}^{2+}$  that were roughly 100-fold higher (20). However, CadA of *S. aureus* is a plasmid-encoded resistance mechanism only present in some strains, whereas the CopB ATPase of *E. hirae* is a chromosomally coded function of copper homeostasis. The observed transport rates may be sufficient to deal with excess cytoplasmic copper, although we cannot entirely rule out that our experimental conditions do not result in full activity.

P-type ATPases, which evidently have evolved from a common precursor, have developed into a versatile group of ion pumps capable of transporting  $\text{H}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cd}^{2+}$  and, as shown here,  $\text{Cu}^+$  and  $\text{Ag}^+$ . Of this collection of ions,  $\text{Cd}^{2+}$ ,  $\text{Cu}^+$ , and  $\text{Ag}^+$  are distinct in that they are heavy metal ions and thus have special properties. In particular, they strongly interact with the side chains of cysteine and histidine residues. This must be reflected by the primary structures of heavy metal ion ATPases. When inspecting the amino acid sequences of the *S. aureus*  $\text{Cd}^{2+}$ -ATPase and the *E. hirae*  $\text{Cu}^+$ ( $\text{Ag}^+$ )-ATPase, the only two heavy metal ATPases of known ion specificity, two features are particularly apparent: one or two cysteines flanking the proline residue that is believed to be part of the ion channel through the membrane, and the presence of heavy metal binding motifs in the N terminus.

The proline residue of the purported ion channel is located in a putative transmembranous helix and is invariant in all known P-type ATPases. Site-directed mutagenesis of this amino acid in the  $\text{Ca}^{2+}$ -ATPase of the sarcoplasmic reticulum showed that this proline is essential for transport (30). It is embedded in the sequence IPE-309 in the sarcoplasmic  $\text{Ca}^{2+}$ -ATPase. Although no cysteines are found adjacent to this proline in any of the non-heavy metal ATPases, it is flanked by two cysteines in the  $\text{Cd}^{2+}$ -ATPase (CPC-373) and a cysteine and a histidine (CPH-398) in the CopB ATPase. All the postulated copper ATPases display the motif CPC in corresponding positions (CPC in the putative *H. pylori* ATPase). This clearly suggests that the presence of the CPC motif is indicative of heavy metal ion transport.

The other structural element that is being considered typical for heavy metal ATPases is the heavy metal ion binding domain in the polar N-terminal region of these enzymes. The consensus sequence GXXCXXC, present in the N terminus of CadA, occurs in all conjectured copper ATPases, but is absent from CopB of *E. hirae*. In the proposed copper ATPases encoded by the human Wilson and Menkes disease genes, the motif GXXCXXC is even repeated five and six times, respectively, and forms part of a reiterated building block of 60–70 amino acids (31). The notion that this motif forms a general heavy metal ion binding site originates from the observation that the same structural element also occurs in mercury reductases and periplasmic mercury binding proteins (32).

Rather than the consensus sequence GXXCXXC, the N terminus of CopB features three repeats of the type MXHXMS-GMXHS. Similar motifs are also found in a periplasmic copper binding protein of *P. syringae* and has been postulated to form copper binding domains (5). Also, CopB is the only ATPase known to date that possesses the sequence CPH at the site of the proposed ion channel. Why CopB differs from the other heavy metal ATPases in these regards is at present not clear. A possibility that should not be discarded at this point is that the

substrates for at least some of the putative "copper" ATPases is in fact not copper, but another heavy metal ion.

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