Toxicology and Applied Pharmacology **159,** 1–8 (1999)
Article ID taap.1999.8706, available online at http://www.idealibrary.com on IDEAL®

ATP-Dependent Silver Transport across the Basolateral Membrane of Rainbow Trout Gills

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Received February 2, 1999; accepted May 19, 1999

ATP-Dependent Silver Transport across the Basolateral Membrane of Rainbow Trout Gills. Bury, N. R., Grosell, M., Grover, A. K., and Wood, C. M. (1999). *Toxicol. Appl. Pharmacol.* **159, 1–8.**

Silver has been shown to be extremely toxic to freshwater teleosts, acting to inhibit Na^+ uptake at the gills, due to the inactivation of branchial Na^+/K^+-ATPase activity. However, the gills are also a route by which silver may enter the fish. Therefore, this study focuses on the mechanism of transport of this nonessential metal across the basolateral membrane of the gill cell, using basolateral membrane vesicles (BLMV) prepared from the gills of freshwater rainbow trout. Uptake of silver by BLMV was via a carrier-mediated process, which was ATP-dependent, reached equilibium over time, and followed Michaelis–Menten kinetics, with maximal transport capacity (V_{max}) of 14.3 ± 5.5 (SE) nmol mg membrane protein^{-1} min^{-1} and an affinity (K_m) of 62.6 ± 43.7 μM, and was inhibited by 100 μM sodium orthovanadate (Na_3VO_4). The ionophore monensin (10 μM) released transported silver from the BLMV. Acylphosphate intermediates, of a 104 kDa size, were formed from the BLMV preparations in the presence of ATP plus Ag. These results demonstrate that there is a P-type ATPase present in the basolateral membrane of the gills of rainbow trout that can actively transport silver, a process which will remove this heavy metal from its site of toxic action, the gill. © 1999 Academic Press

Key Words: **Silver; fish; gills; heavy metal transport.**

Silver is considered to be relatively nontoxic to humans and other mammals (Hollinger, 1996), but in the aquatic environment, the silver ion (Ag^+) can be extremely toxic. Silver, when present as $AgNO_3$, has an LC50 value for freshwater fish of between 5 and 70 μg Ag L^{-1} (Hogstrand and Wood, 1998). However, speciation governs toxicity (Hogstrand and Wood, 1998), and in the case of rainbow trout, toxicity is associated only with the fraction which is present as the free silver ion (Ag^+) and not with silver bound to chloride, dissolved organic matter, or other naturally occurring anions (Hogstrand *et al.*, 1996a; Galvez and Wood, 1997; McGeer and Wood, 1998; Bury *et al.* 1999a,b).

Freshwater fish live in a dilute medium; consequently, they lose ions to the environment via their integument (principally the gills) and urine. To maintain ionic balance, they actively take up ions from the environment via the gills. It is the gills that are the primary site for acute heavy metal toxicity (McDonald and Wood, 1993). The toxic action of silver to freshwater rainbow trout is via inhibition of branchial Na^+ and Cl^- influx (Morgan *et al.*, 1997; Webb and Wood, 1998; Bury *et al.*, 1999a,b). More specifically, silver inhibits the Na^+/K^+-ATPase enzyme situated on the basolateral membrane of the gill (Morgan *et al.*, 1997; Bury *et al.*, 1999b). The disturbance to ionoregulation results in perturbed fluid-volume regulation and hemoconcentration, with death occurring due to cardiovascular collapse (Wood *et al.*, 1996).

Recently, Bury and Wood (1999) showed that silver, probably in the ionic form (Ag^+), can enter freshwater rainbow trout via a Na^+ channel situated on the branchial apical membrane. *In vivo*, the silver uptake rate across the gills and into the body follows Michaelis–Menten kinetics, suggesting that part of the uptake process is carrier-mediated. Furthermore, the temporal pattern of uptake shows an initial accumulation of silver in the gills prior to its appearance in the body, which suggests that the rate-limiting step is extrusion across the basolateral membrane.

A number of uptake processes have been described for heavy metals in vertebrate systems. Specific P-type ATPases have been characterized for the transport of copper (Bull and Cox, 1994; Dijkstra *et al.*, 1996a, b), and recently a divalent-cation transporter (DCT1) has been identified which transports a variety of divalent ions including Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and Pb^{2+} (Gunshin *et al.*, 1997). In addition, heavy metals may mimic other ions in various carrier-mediated processes (Clarkson, 1993). For example, intestinal Ni^{2+} uptake can occur via an iron transport process (Tallkvist and Tjälve, 1998); inorganic heavy metals–bicarbonate complexes may be transported via the Cl^-/HCO^- exchanger (Lou *et al.*, 1991; Alda and Garay, 1989, 1990) and heavy metal– organic complexes may be transported via an organic anion transporter

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0041-008X/99 $30.00

(Ballatori and Clarkson, 1985; Zaulps and Barfuss, 1995; Zaulps, 1998). In the case of silver, Havelaar *et al.* (1998) recently identified a general heavy metal P-type ATPase in the lysosomal membrane that transported silver. This transporter differed from the DCT1 because both the divalent ions cadmium and copper competed with the monovalent silver ion for silver transport. Silver has also been shown to be transported by the copper ATPase (CopB ATPase) of the bacterium *Enterococcus hirae*, an enzyme which confers bacterial resistance to this metal. The evidence from our previous study concerning silver uptake in rainbow trout suggests that silver may be transported across the basolateral membrane of gills by a carrier-mediated process (Bury and Wood, 1999). Consequently, the main aim of this study was to characterize silver uptake using basolateral membrane vesicles prepared from the gills of rainbow trout.

MATERIALS AND METHODS

Fish husbandry. Rainbow trout (*Oncorhynchus mykiss*) were obtained from Spring Valley Hatchery (Petersburg, ON, Canada). Fish were kept in dechlorinated Hamilton tap water (in mM: [Na^+], 0.5; [Cl^-], 0.7; [Ca^{2+}], 1; [Mg^{2+}], 0.2; [K^+], 0.05; pH 7.8–8.0, 13°C) and fed commercial trout food (Martin Mills Inc., Tavistock, ON, Canada) at 1.5% of their body weight daily.

Basolateral membrane vesicle preparation. Basolateral membrane preparations followed the methods of Perry and Flik (1989). Briefly, rainbow trout (approx. 250 g) were stunned with a blow to the head and killed by severance of the spine. The gills were then perfused with 60 ml of ice cold saline (0.9% NaCl; 0.5 mM Na_2-EDTA; 20 IU L^{-1} heparin; pH 7.8 adjusted with Tris). All subsequent procedures were performed at 4°C or on ice. Gill epithelium, devoid of blood, was scraped from the cartilage of the filaments with a glass slide and placed into 30 ml of a hypotonic solution (25 mM NaCl, 1 mM Hepes adjusted to a pH of 8 with Tris). Protease inhibitors were omitted from the preparation media because of the possibility of these chemicals binding to silver and interfering with the uptake studies. The scrapings were either homogenized using a polytron homogenizer set at 30% of maximum speed for 2 min or dispersed with 30 strokes of a loose fitting dounce homogenizer. This solution was adjusted to 45 ml with hypotonic solution and centrifuged at 550*g* for 15 min to remove cellular debris. The supernatant was centrifuged at 50000*g* (JA21 Beckman Sorval centrifuge) for 30 min. The resulting pellet consisted of a top fluffy white layer and a firm brownish bottom. The white fluffy layer was resuspended in an isotonic solution (250 mM sucrose; 5 mM $MgSO_4$; 5 mM Hepes adjusted to 7.4 with Tris) by gentle agitation. The resuspended pellet was adjusted to a volume of 20 ml and received 100 strokes from a tight-fitting dounce homogenizer. The supernatant was centrifuged at 1000*g* for 10 min and then at 10000*g* for 10 min, and the final supernatant was further centrifuged at 50000*g* for 20 min. Vesicles were formed from the resulting pellet by resuspending via 10 passages through a 23-gauge needle in the appropriate media (see below). Typically, such a procedure yields the BLM preparation as 20% inside-out vesicles, 30% right-side out vesicles, and the remaining 50% of membranes as unsealed (Perry and Flik, 1989; Hogstrand *et al.,* 1996). The protein concentration was determined by commercial kit (Bio-Rad) using bovine serum albumin standards and the concentration adjusted to between 1 and 1.5 mg membrane protein ml^{-1}. To confirm the purity of the BLMV preparation, the Na^+/K^+-ATPase activity (see below for methods) was measured in the initial homogenate and the final BLMV, and calcium transport was measured using the protocol of Perry and Flik (1989) and Hogstrand *et al.* (1996b).

Silver uptake studies. The standard resuspension solution, used for the kinetic analysis, time course, and monensin treatment studies, consisted of: 250 mM sucrose; 10 mM KNO_3; 0.8 mM $MgSO_4$, and 0.5 mM Na_2-EDTA. The standard assay solution contained 250 mM sucrose, 10 mM KNO_3, 11 mM $MgSO_4$, 6 mM Na_2-EDTA, and either 5 mM Na_2-ATP or 5 mM Na_2-ADP. All resuspension and assay media contained 20 mM Hepes and were adjusted to pH 7.4 with Tris. $^{110m}AgNO_3$ (Amersham International Ltd.) was added to a concentration of 80 μM (0.02 μCi/ml), except in the kinetic analysis where the concentration was varied from 5 to 80 μM and the time course analysis where the concentration was 20 μM. When the concentration of salts was adjusted (as described below), the osmolarities of the resuspension and assay medium were maintained by the addition of an appropriate concentration of sucrose.

BLMV silver uptake was assessed by prewarming 125 μl of assay medium at 36°C, after which 30 μl of BLMV preparation total was added and the solution was mixed on a vortex mixer. Silver uptake was assessed over a 3-min incubation period, except in the time course experiment. After the appropriate incubation period, the BLMV were collected on Schleicher and Schüell nitrocellulose filters, pore size 0.45 μm, by rapid filtration. The filters had received a 1-min incubation in 250 mM sucrose, 10 mM $MgSO_4$, 10 mM KNO_3, 2 mM $AgNO_3$, and 20 mM Hepes adjusted to pH 7.4 with Tris to ensure that the majority of the silver binding sites on the membrane were occupied with cold Ag. The filtered membranes were washed twice with the aforementioned buffer to displace surface-bound ^{110m}Ag. Radioactivity present on the filters was measured on a gamma counter (Packard Instruments, Downers Grove, IL), and ATP-dependent silver transport was determined by the equation

$$\text{nmol/mg membrane protein/min} = (\text{FATP} - \text{FADP})/\text{SA} \times [P] \times T$$

where FATP and FADP are the cpm on the filters in the presence of ATP or ADP, respectively, SA is the specific activity of the assay media (nmol/ml), [*P*] represents the concentration of BLMV protein filtered (mg), and *T* is time of incubation. The values for FATP and FADP were corrected for nonspecific binding of silver to the nitrocellulose filters. Nonspecific binding was derived from performing the same experiment in the absence of BLMV and measuring the radioactivity present on the filter. When expressed as a percentage of the total counts, nonspecific binding in the presence of ATP was 4.6 ± 0.4% (mean ± STD, $N = 18$) and in the presence of ADP was 11 ± 1.4% (mean ± STD, $N = 18$). Calculations in the time course study were essentially the same as above, with the time component being omitted.

Monensin treatment. The leaking of Ca^{2+} from Ca^{2+}-loaded BLMV after the addition of the ionophore A23187 has routinely been used to confirm that BLMV accumulate Ca^{2+} (for example, Perry and Flik, 1989). There is no specific ionophore for Ag^+. However, the Na^+ ionophore monensin (Choy *et al.,* 1974) can act as an ionophore for other cations including Ag^+ (Tsukube and Sohmiya, 1989). To verify that Ag^+ had been transported into the vesicular space, BLMV were loaded with silver using standard resuspension and assay conditions (see above) over a 10-min period, after which either 10 μM monensin (dissolved in ethanol) or 0.01% ethanol (vehicle control) was added. The concentration of radiolabeled silver present in the vesicles was measured at 3 and then 10 min after this point.

Competition of BLMV silver uptake by Na^+ or K^+. For the sodium competition studies, all vesicles were resuspended in standard resuspension medium and silver uptake was assessed in either standard assay medium or standard assay medium with either 20 or 80 mM of Na_2SO_4 added. For the potassium competition studies, the vesicles from five fish were combined and split into three aliquots. One aliquot was resuspended in potassium-free resuspension medium and silver uptake studies were then performed in potassium-free assay medium. The other two pellets were resuspended in standard resuspension medium and silver uptake was assessed in standard assay or assay medium containing 80 mM KNO_3.

BLMV silver uptake in the absence of Mg^{2+}. Magnesium acts as a cofactor for dephosphoryation of ATP by P-type ATPases (Pedersen and Carafoli, 1987). Its importance for BLMV silver uptake was assessed by performing transport experiments in the absence of $MgSO_4$. For the magnesium-free experiment, vesicles were prepared as described above. Each vesicle preparation was divided and the vesicles were disrupted by an additional 30

strokes of a tight dounce homogenizer. These membranes then received an additional centrifugation at 50,000*g* for 20 min, and vesicles were formed from one of the pellets in resuspension buffer from which $MgSO_4$ had been omitted by 10 passages through a 23-gauge needle. Silver uptake was assessed in assay media with no $MgSO_4$. The other pellet was resuspended in standard resuspension medium and silver uptake was assessed in standard assay medium (see above).

BLMV silver uptake in the presence of P-type ATPase inhibitor sodium orthovanadate. Sodium orthovanadate (Na_3VO_4) was prepared as described by Gordon (1991). BLMV were resuspended and silver transport was assessed in standard medium containing 100 μM sodium orthovanadate. Vesicles were then incubated for 4 h on ice in the presence of sodium orthovanadate prior to assessment of silver uptake.

BLMV Na^+/K^+-ATPase activity. Rainbow trout basolateral membrane vesicles were prepared as described above. Membranes were resuspended to a concentration of 1 mg protein ml^{-1} in a solution of 250 mM sucrose, 0.8 mM $MgSO_4$, 20 mM HEPES and adjusted to pH 7.4 with Tris. BLMV were treated with saponin (0.2 mg/mg membrane protein) prior to assessing Na^+/K^+-ATPase activity. Na^+/K^+-ATPase activity was determined by the method of Bonting *et al.* (1961). Briefly, the phosphate liberated by the enzyme in two media was assessed. The first medium contained 100 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 5 mM Na_2ATP, 5 mM EDTA, 30 mM imidazole and was adjusted to pH 7.4 with Hepes. The second medium was similar, except KCl was omitted and 1.6 mM ouabain was added. $AgNO_3$ was added to both media at various concentrations ranging from 0.1 pM to 1 mM. A 10-μl aliquot of BLMV was added to 400 μl of assay medium on ice and then incubated at 37°C for 15 min. The reaction was stopped by the addition of 1 ml of ice cold 8.6% (w/v) TCA and 1 ml of ice-cold Bonting's color reagent (9.6% $FeSO_4.6H_2O$ (w/v); 1.15% ammonium heptamolybdate (w/v) in 0.66 M H_2SO_4). The inorganic phosphate liberated (P_i) was determined colorimetrically at a wavelength of 700 nm. Ouabain sensitive Na^+/K^+-ATPase activity was calculated from the difference in P_i between the two media.

Acylphosphates intermediate formation. Trout gills were perfused and scraped from the cartilage as described above, and all procedures performed at 4°C or on ice. The initial homogenization procedure was performed as described above. However, the hypotonic buffer was supplemented with 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulphonyl fluoride (PMSF). The homogenate was centrifuged at 550*g* to remove cellular debris and the resulting supernatant centrifuged at 10,000*g* for 10 min. The supernatant was further centrifuged at 50,000*g* for 45 min. The resulting pellet was then washed with 300 mM sucrose, 20 mM Hepes adjusted to pH 7.4 with Tris and centrifuged at 10,500*g* for 15 min. The pellet received a second wash in resuspension buffer (50 μM EGTA, 20 mM Hepes adjusted to pH 6.8 with Tris) to remove the trace amounts of Na^+, K^+, DTT, and PMSF that may still have been present. The final pellet was resuspended in 1 ml of resuspension buffer with a final protein concentration of 4.7 mg BSA equivalents ml^{-1}.

All subsequent procedures were performed at 0°C unless otherwise stated. A 40-μl aliquot of membrane solution was incubated for 10 min in a solution of 20 mM Hepes/Tris pH6.8 containing either 100 or 200 μM $AgNO_3$, or 10 mM $NaNO_3$, with or without 100 mM KNO_3, after which, a nominal concentration of [γ-^{32}P]ATP was added. After 1 min, the reaction was stopped by the addition of 750 μl of TCAP (10% trichloroacetic acid, 50 mM phosphoric acid, and 1 mM ATP). The samples were mixed using a vortex mixer, left for 10 min on ice, and then centrifuged at 13,000*g* at 4°C for 10 min. The supernatant was discarded and the pellet washed with 1.25 ml of TCAP and then centrifuged as above, and the pellet was resuspended in 50 μl of 10 mM Mops, 40 mM DTT, 1 mM EDTA, 3% sodium dodecyl sulfonic acid, 10% sucrose, adjusted to pH 5.5 and containing 0.1% of the tracking dye methyl green. The samples were vortexed vigorously for 10 min and electrophoresed in 7.5% polyacrylamide acid gels at pH 4 until the tracking dye reached the gel front and then electrophoresed for a further hour. Teleost acylphosphate formations have not been demonstrated before, consequently to verify the assay conditions an additional experiment was performed in parallel using Ca^{2+} (added as $CaCl_2$) and the rabbit cardiac Ca^{2+}-ATPase (for details of preparation see Grover and Samson, 1988). The formation of cardiac Ca^{2+} ATPase acylphosphate intermediates is routinely performed in our laboratory and gives a characteristic band at a molecular weight of 104 $\pm$ 3 kDa (for example Grover and Samson, 1988).

Statistical methods. The concentration of silver present in the BLMV after monensin treatment, sodium orthovanadate treatment, or in magnesium-free conditions was compared to the appropriate controls by a Student's *t* test. The statistical significance of varying the concentrations of K^+ or Na^+ in the assay media on BLMV silver uptake was determined by ANOVA, followed by a least significant difference (LSD) test. Michaelis–Menten kinetic constants were derived from nonlinear regression analysis and best fit curves were generated using SPSS 6 computer package.

RESULTS

Rainbow trout basolateral membrane vesicle preparations were enriched in Na^+/K^+-ATPase activity by 9.2 times compared to the initial homogenate, a value similar to that reported by Perry and Flik (1989). Ca^{2+} transport rate was 0.5 nmol mg membrane protein^{-1} min^{-1}. This value is lower than the rate reported by Perry and Flik (1989), as well as by Hogstrand *et al.* (1996b) (1.2 and 4.5 nmol mg protein^{-1} min^{-1}, respectively), and may reflect the fact that our vesicles were prepared without the protease inhibitor aprotonin and thiol-reducing agent DTT.

Rainbow trout basolateral membrane vesicles exhibited an ATP-dependent silver uptake mechanism (Fig. 1). The difference in the accumulation rate of silver in the presence of either 5 mmol L^{-1} ATP or 5 mmol L^{-1} ADP reached equilibrium over time (Fig. 2) and had a maximal transport capacity (V_{max}) of 14.3 $\pm$ 5.5 (SE) nmol mg membrane protein^{-1} min^{-1} and an affinity (K_m) of 62.6 $\pm$ 43.7 μM (Fig. 1).

The Na^+ ionophore monensin has also been shown to act as an ionophore for Ag^+ (Tsukube and Sohmiya, 1989). BLMV were preloaded with silver; after 3 min of adding 10 μM monensin to the preparation, the concentration of silver in the vesicles was significantly reduced, and this decrease was more pronounced at 10 min after the addition of monensin (Fig. 3). There was no significant change in the controls.

Increasing Na^+ or K^+ concentrations in the assay medium significantly reduced BLMV silver uptake in a dose-dependent manner (Fig. 4). The maximal BLMV silver uptake rate was observed in the absence of K^+ (Fig. 4). BLMV silver uptake was significantly inhibited by 100 μM sodium orthovanadate and was reduced, but was not significantly different from controls ($p = 0.09$), in the absence of Mg^{2+} (Fig. 5).

BLMV Na^+/K^+-ATPase activity was inhibited by $AgNO_3$ in a dose-dependent manner, the IC50 value being 5.3 μM $AgNO_3$ (Fig. 6).

Na^+, Ag^+ and K^+-dependent acylphosphate intermediates were formed and corresponded to a size of 104 kDa, as confirmed by the well characterized acylphosphate formed by the cardiac muscle in the presence of 0.5 mM $CaCl_2$ (Grover and Samson, 1988; Fig. 7). The degree of acylphosphate intermediate formation when potassium was administered in conjunction with Na^+ or Ag^+, was reduced when compared to the

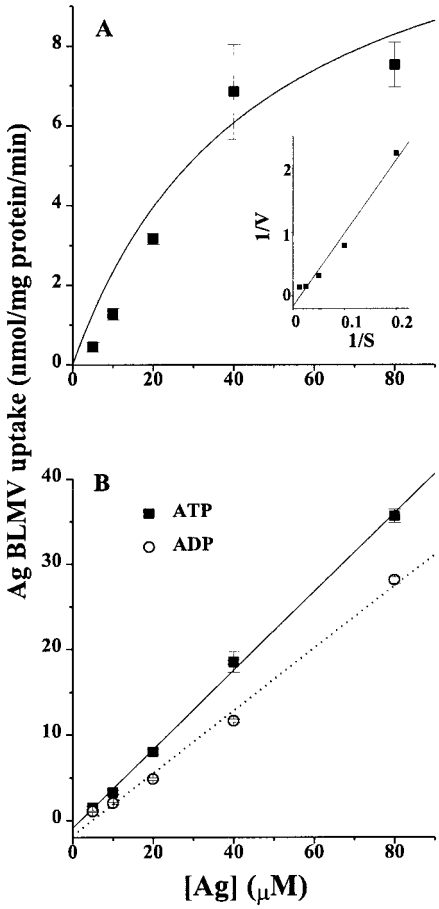

FIG. 1. Rainbow trout gill basolateral membrane vesicle ATP-dependent silver uptake over a range of silver concentrations (A), calculated from the difference between the BLMV silver uptake rate in the presence of ATP or ADP (B). The curve in (A) is generated from the Michaelis–Menten kinetics equation $y = V_{max}/(K_m + [Ag])$, where $V_{max} = 14.3 \pm 5.5$ (SE) nmol mg membrane protein^{-1} min^{-1} and $K_m = 62.6 \pm 43.7$ μM, and the lines in (B) are based on linear regression lines with equation $y = 0.5x - 0.9$, $R = 0.99$, $p < 0.0001$, and $y = 0.4x - 1.8$, $R = 0.98$, $p = 0.002$ for the ATP and ADP data, respectively. Inset of A represents the Eadie/Hofstee plot from the values from graph (A), and the line is based on the linear regression line with an equation of $y = 11.4x - 0.16$, $R = 0.98$, $p = 0.002$. Values are means ± SEM; $N = 5$.

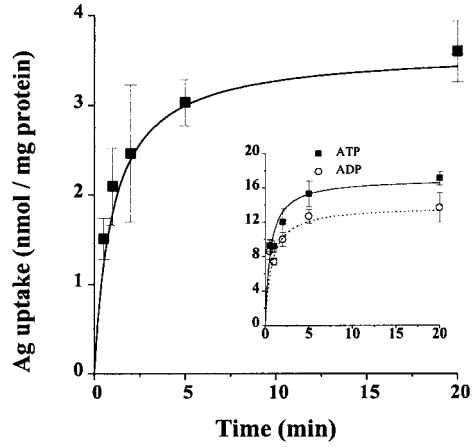

FIG. 2. Time course of silver uptake by rainbow trout gill basolateral membrane vesicle in the presence of 20 μM $^{110m}AgNO_3$. The line is generated from the best fit curve to a rectangular hyperbola with an equation of $y = a/(b + x)$, where $a = 3.5 \pm 0.3$ (STD) and $b = 0.7 \pm 0.2$ (STD). BLMV silver uptake rate is calculated from the uptake rate in the presence of ATP or ADP, represented by the inset graph. The best fit lines also follow the equation for a rectangular hyperbola, where $a = 17 \pm 0.8$ (STD) and $b = 0.6 \pm 0.1$ (STD) for the ATP data and $a = 13.8 \pm 1$ (STD) and $b = 0.7 \pm 0.2$ (STD) for the ADP data. Values are means ± SEM; $N = 4$–5.

acylphosphate intermediate formation in the presence of only either Ag^+ or Na^+ (Fig. 7). Acylphosphate formation was greater in BLMV incubated with 200 μM $AgNO_3$, compared to the formation at 100 μM $AgNO_3$ (Fig. 7).

DISCUSSION

The identification of an ATP-dependent silver uptake by basolateral membrane vesicles (BLMV) of the gills of rainbow trout confirms our hypothesis based on earlier *in vivo* observations that there is a carrier-mediated silver uptake process involved in branchial silver uptake (Bury and Wood, 1999). The transport of silver by BLMV saturated over a range of silver concentrations and reached equilibrium over time. In addition, BLMV silver transport was inhibited by micromolar quantities of sodium orthovanadate and acylphosphate intermediates were formed in the presence of ATP and silver, demonstrating that this transporter is probably a P-type ATPases (Pedersen and Carafoli, 1987).

The accumulation of Ag into the BLMV was confirmed by the observation that Ag leaked from vesicles preloaded with

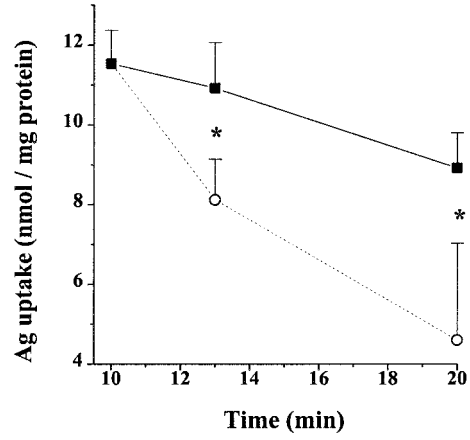

FIG. 3. The accumulation of silver in rainbow trout gill basolateral membrane vesicles after a 10-min incubation with $^{110m}AgNO_3$, and after the addition of 10 μM monensin (○----○) or the vehicle control (0.01% ethanol; ■——■). Values are calculated as the difference between the concentration of silver accumulated in the presence of ATP or ADP. Values are means ± SEM; $N = 3$–4. *Significant difference from the value at the start of treatment ($t = 10$ min, $p < 0.05$, t test)

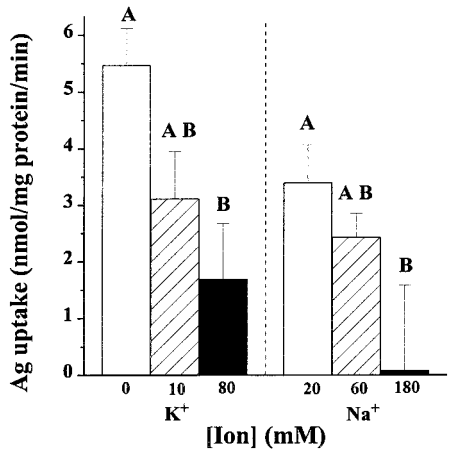

FIG. 4. Rainbow trout gill basolateral membrane vesicle silver uptake rate in the presence of 80 μM $^{110m}AgNO_3$ and differing concentrations of KNO_3 (0, 10, 80 mM) or $NaNO_3$ (20, 60, 180 mM). The values are calculated from the difference between the silver uptake rate in the presence of ATP or ADP. Values are means ± SEM; $N = 4$–5. Columns containing different letters are significantly different from each other (ANOVA followed by a least significant differences test (LSD), $p < 0.05$).

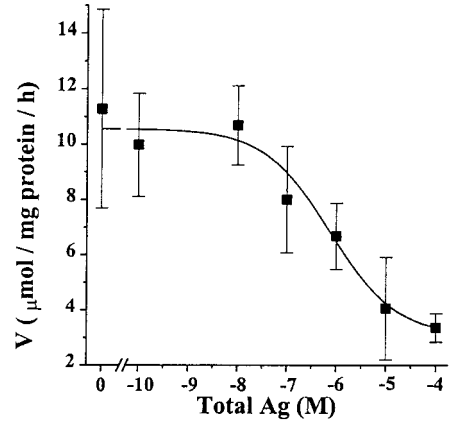

FIG. 6. Na^+/K^+-ATPase activity of rainbow trout gill basolateral membrane preparations in the presence of increasing concentrations of $AgNO_3$. Values are means ± SEM; $N = 5$. IC50 = 5.3 μM $AgNO_3$.

Ag after the addition of monensin, a chemical which can act as a Ag^+ ionophore (Tsukube and Sohmiya, 1989). A similar validation technique was performed by Perry and Flik (1989) using the ionophore A23187 to verify that rainbow trout BLMV accumulated Ca^{2+}. The maximum transport velocity (V_{max}) for the silver uptake in rainbow trout gill BLMV preparations was approximately 100-fold greater than that for the bacterium *E. hirae* membrane vesicle (14 nmol mg^{-1} min^{-1} compared to 0.07 nmol mg^{-1} min^{-1}, Solioz and Odermatt, 1995) and approximately 70-fold greater compared to lysosomal Ag transport (approximately 0.2 nmol mg^{-1} min^{-1}, Havelaar *et al.*, 1998). The affinity of trout gill BLMV Ag transport is 60× lower (i.e., K_m is higher) than that of *E. hirae* membrane vesicles (62 μM compared to 1 μM, Solioz and Odermatt, 1995). These discrepancies may be explained simply by species difference, or by the presence of more silver-binding proteins in our preparations. Both Solioz and Odermatt (1995) and Havelaar *et al.* (1998) highlighted the problems of nonspecific binding of the free metal to proteins, and in their studies an excess of glutathione was required for silver transport to be observed. In our studies glutathione was not needed for Ag to accumulate in the BLMV. This suggests that the Ag transport process across the gills differs from that in lysosomes and *E. hirae* membranes.

No acylphosphate intermediates have previously been identified in fish gill preparations. The acylphosphates formed in

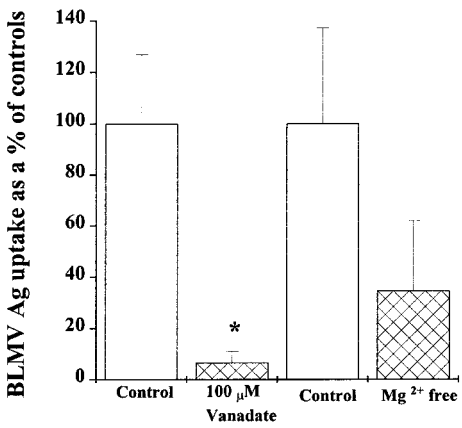

FIG. 5. Rainbow trout gill basolateral membrane vesicle silver uptake rate in the presence of 80 μM $^{110m}AgNO_3$ expressed as a percentage of control values (□) in the presence of 100 μM sodium orthovanadate or in magnesium free conditions (▨). Values are means ± SEM; $N = 4$–5. *Significant difference from controls ($p < 0.05$, *t* test).

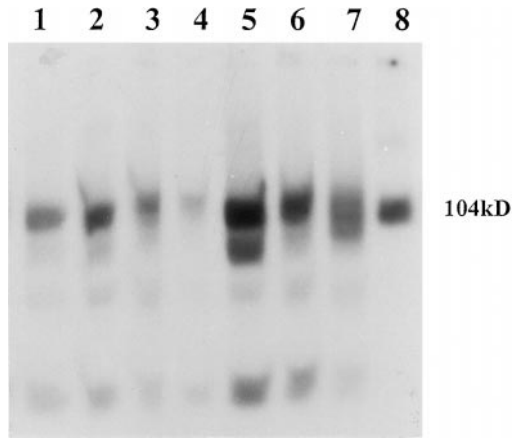

FIG. 7. Acylphophate formation in the presence of 100 μM $AgNO_3$ (lane 1), 200 μM $AgNO_3$ (lane 2), 100 μM $AgNO_3$ and 100 mM KNO_3 (lane 3), 200 μM $AgNO_3$ and 100 mM KNO_3 (lane 4), 10 mM $NaNO_3$ (lane 5), 10 mM $NaNO_3$ and 100 mM KNO_3 (lane 6), and 100 mM KNO_3 (lane 7). Lane 8 contains the acylphosphate formed by the cardiac heart Ca^{2+}-ATPase in the presence of 0.5 mM $CaCl_2$ (Grover and Samson, 1988); this acted as an internal control and molecular marker, the band corresponding to 104 kDa.

the presence of ATP and Na^+, Ag^+, or K^+ correspond to a size, 104 kDa, similar to the acylphosphates formed by the α-subunit of the Na^+/K^+-ATPase (Yoda and Yoda, 1986) and Ca^{2+}-ATPase (Grover and Samson, 1988). It is unclear what the other fainter bands of smaller size represent, but they may be a consequence of proteolysis. There are three possibilities for the decrease in the intensity of the band formed in the presence of Na^+ plus K^+, as well as Ag^+ plus K^+. First, the reduction may be due to the availability of the substrates required for the complete cycle of the sodium pump (Yoda and Yoda, 1986). Second, K^+ may compete with either Na^+ or Ag^+ at their binding sites and therefore this K^+-enzyme complex is unable to undergo a conformational change. Finally, the converse may also occur. Thus, K^+, on its own, would induce acylphosphate formation, and the weaker band observed in the presence of Na^+ plus K^+, as well as Ag^+ plus K^+, may also be a consequence of competition between the ions.

At present, there are no specific transport processes described for silver, but there are a number of possible candidate P-type ATPases including the general heavy metal transporter described in lysosomal preparations (Havelaar *et al.*, 1998) and the CopB ATPase of *E. hirae* (Solioz and Odermatt, 1995). The merits and drawbacks of these enzymes as candidates for silver transport in fish, as well as other P-type ATPases (Na^+/K^+-, Ca^{2+}- and Na^+-ATPase) which have been identified in fish gills (Pfeiler and Kirschener, 1972; Pfeiler, 1978; Karnaky *et al.*, 1976; Flik *et al.*, 1993) are discussed below in the context of our present results.

Copper Transport Processes

Evidence that silver may replace copper in a transport process comes from the bacteria *E. hirae* (Solioz and Odermatt, 1995), where Ag is transported by the CopB ATPase. Cu also competitively inhibits Ag uptake into lysosomes, but this Ag transport is also inhibited by Cd, indicating that enzyme is probably a general heavy metal P-type ATPase (Havelaar *et al.*, 1998). No Cu ATPase has been identified in teleosts. However, fish possess enzymes that require copper, and freshwater rainbow trout accumulate copper from the water via the gills (Grosell *et al.*, 1997); thus, there is circumstantial evidence for a branchial copper uptake mechanism. It is not clear whether Cu crosses the gills as an ion (Cu^+ or Cu^{2+}) or as an inorganic or organic complex. However, the need for ATP for BLMV Ag uptake indicates that if Ag were transported by a Cu transport system, it would probably be via a Cu-ATPase.

Acylphosphate intermediate formation in the presence of silver and ATP demonstrates the presence of a functional enzyme. Recently, Solioz and Camakaris (1997) showed acylphosphate formation by Menke's copper ATPase, but to visualize the phosphorylation of the Menke's ATPase, an immunoprecipitation step had to be included prior to electrophoresis. If this step was omitted, then the Menke's ATPase formed irreversible aggregations. The procedure for visualizing acylphosphate intermediates in the present study used more classical methods where the proteins are precipitated using trichloroacetic acid. Consequently, if the silver transport protein is in fact a copper ATPase, then this enzyme, based on the conditions for acylphosphate formation, would differ from the Menke's ATPase. This does not rule out the possibility that silver is transported via a Cu-ATPase which has different characteristics from those of the Menke's ATPase, but for verification more evidence is required for the presence of this enzyme in the gills of fish.

Ca^{2+}-ATPase

Silver specifically inhibits Na^+ influx (Morgan *et al.*, 1997; Webb and Wood, 1998; Bury *et al.*, 1999a,b) and does not perturb calcium influx rates in fish (Wood *et al.*, 1996), illustrating that silver discriminates between the proteins involved in Na^+ and Ca^{2+} transport. Increasing water Ca^{2+} does not reduce silver uptake rates (Bury and Wood, 1999). In addition, ionic silver is monovalent (Ag^+) and is unlikely to replace the divalent calcium ion (Ca^{2+}) in the branchial Ca^{2+}-ATPase, and is thus probably not transported by such a system.

Na^+- and Na^+/K^+-ATPase

Silver has been shown to enter the fish via the apical Na^+-channel (Bury and Wood, 1999), and thus silver could also mimic Na^+ at the extrusion step across the basolateral membrane. Na^+/K^+-ATPase is the major enzyme identified in teleost gill involved in basolateral extrusion of Na^+ (see reviews by Perry, 1997 and Flik *et al.*, 1997). However, there are also reports of ouabain-insensitive Na^+-activated ATPase activity in gill homogenates (Pfeiler and Kirschener, 1972; Pfeiler, 1978; Borgatti *et al.*, 1985). An increase in $[K^+]$ and $[Na^+]$ reduced BLMV silver uptake, but both ions are present in large excess (1000$\times$ greater than the concentrations of silver), which may indicate that the reduction in silver uptake may be solely due to nonspecific binding of K^+ and Na^+ at the transport site. Unfortunately, it was not possible to use ouabain (a specific inhibitor of Na^+/K^+-ATPase), due to the uncertainty as to whether this chemical binds silver. However, the maximal BLMV silver uptake was observed in the absence of K^+, suggesting that uptake is unlikely to be via the Na^+/K^+-ATPase. Furthermore, it is unlikely, but not impossible, that silver would be transported by an enzyme (Na^+/K^+-ATPase) which it actually poisons. Silver inhibits the activity of the Na^+/K^+-ATPase in these BLMV preparations in a dose-dependent manner, with an IC50 of 5.3 μM $AgNO_3$. A similar IC50 value was obtained for silver inhibition of Na^+/K^+-ATPase activity in crude gill homogenates (Morgan *et al.*, 1997), but is a lot higher than the IC50 for a purified dog kidney Na^+/K^+-ATPase preparation (IC50 = 9 nM; Hussain *et al.*, 1994). Consequently, if Ag is mimicking Na^+ in extrusion across the basolateral membrane, then it is probably via a different ATP-dependent Na^+ uptake pathway than Na^+/K^+-ATPase.

A General Heavy Metal Transport Protein

Recently two heavy metal transport proteins have been shown to be able to utilize a number of different metals as substrates, for example, the DCT1 transports a number of divalent ions (Gunshin *et al.*, 1997), while the general heavy metal ion transporter of the lysosomal membrane does not discriminate between divalent and monovalent ions (Havelaar *et al.*, 1998). The characteristics of BLMV silver transport are different from those of the silver transport of lysosomal membranes (Havelaar *et al.*, 1998). BLMV silver transport did not require glutathione, was inhibited by sodium orthovanadate, and occurred across a plasma membrane. It remains to be seen if the BLMV silver transport system is in fact via a general heavy metal transport protein or another ATP-dependent transport process in the gills of fish.

In conclusion, we have demonstrated that there is an ATP-dependent silver transport mechanism in the gills of freshwater adapted rainbow trout. The inhibition by sodium orthovanadate, and acylphosphate formation in the presence of ATP and Ag, suggest that this protein is a P-type ATPase. However, further studies are required to identify the enzyme responsible for silver transport. The prospect of either a fortuitous or specific heavy metal transport process in the gills of fish raises a number of important toxicological questions. Heavy metals are known to exert their acute toxic action by inhibiting certain ion transport processes in the gills of fish (McDonald and Wood, 1993). Branchial heavy metal transport would remove the incriminating metal and thus may be a defense mechanism against toxicity.

ACKNOWLEDGMENTS

We thank Joe Gorsuch and Ken Robillard for valuable support and critical reading of the manuscript. This work was supported by a Natural Sciences and Engineering Research Council of Canada Industrially Oriented Research grant in conjunction with grants from Kodak Canada Inc.

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