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Copper uptake across rainbow trout gills: mechanisms of apical entry

Martin Grosell* and Chris M. Wood

McMaster University, Department of Biology, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1

*Present address: Zoophysiological Laboratory, August Krogh Institute, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark

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Summary

Several components of branchial copper uptake were identified in juvenile freshwater rainbow trout (*Oncorhynchus mykiss*) using ^{64}Cu. On the basis of competitive interactions between sodium and copper uptake, inhibition of copper uptake by a proton pump inhibitor (bafilomycin A1, 2 μmol l^{-1}) and a Na^+ channel blocker (phenamil, 100 μmol l^{-1}), it appears that a proportion of the branchial copper uptake occurs *via* an apical Na^+ channel. This sodium-sensitive copper uptake demonstrates saturation kinetics, with a K_m of 7.1 nmol l^{-1} and a J_{max} of 21.2 pmol g^{-1} h^{-1}, and is characterized by an IC_{50} of 104 μmol l^{-1} sodium. On the basis of residual copper uptake in the presence of high sodium concentrations (20 mmol l^{-1}) and differential inhibition of sodium and copper uptake by phenamil (100 μmol l^{-1}), a sodium-insensitive component of copper uptake is also present in trout gills. It demonstrates saturation kinetics with a comparably low K_m (9.6 nmol l^{-1}) but a lower maximum transport capacity (J_{max}=3.5 pmol g^{-1} h^{-1}) than the sodium-insensitive system. Sodium uptake exhibits saturation kinetics with a K_m of 69 μmol l^{-1}. Copper reduced branchial sodium transport affinity but increased the maximal sodium transport capacity.

Key words: copper, homeostasis, sodium uptake, copper/sodium interactions, gill, rainbow trout, *Oncorhynchus mykiss*.

Introduction

The redox nature of copper is utilized in a large number of enzymatic processes, including that catalysed by mitochondrial cytochrome *c* oxidase, which makes copper an essential element for all aerobic organisms (Soloman and Lowery, 1993). However, the redox properties of copper can cause rapid generation of reactive oxygen species when cellular copper levels are elevated (Harris and Gitlin, 1996). In addition, copper binds to histidine-, cysteine- and methionine-containing proteins with high affinity, which can result in their dysfunction. Maintaining optimal levels of copper is therefore critical, and the mechanisms of copper homeostasis in mammals have received considerable attention, particularly because of two genetically linked fatal disorders in copper metabolism, Menke's disease and Wilson's disease (Camakaris et al., 1999). Several transport proteins involved in cellular copper uptake, vectorial intracellular copper transport, copper sequestration and the regulated efflux of copper have been identified in mammals (for a review, see Camakaris et al., 1999). In contrast, very little is known about the mechanisms of copper metabolism in lower vertebrates, including fish.

At the organ level, fish resemble higher vertebrates with respect to copper homeostasis. As in mammals, the liver is the major homeostatic organ, and biliary copper excretion is elevated in situations of elevated copper uptake (Grosell et al., 1998a,b, 2001b; Kamunde et al., 2001). Circulating levels of copper in the plasma are under tight regulation (Grosell et al., 1997, 2001b), and copper derived from recent uptake from the water is associated predominantly with a 70 kDa protein (presumably albumin) (Grosell, 1996), as in mammals (Cousins, 1985; Frieden, 1980; Weiner and Cousins, 1983). Renal loss of copper from fish and mammals is low (Grosell et al., 1998b) and does not appear to be stimulated under conditions of copper excess.

While part of the copper requirements in fish is clearly met by dietary intake, the role of the gills in copper homeostasis has been overlooked until recently. However, it has long been known from toxicological studies that elevated copper concentrations in water can lead to increased copper levels, particularly in the gills and the liver (Buckley et al., 1982; Grosell et al., 1996, 1997, 1998a,b; Laurén and McDonald, 1987a,b; McCarter and Roch, 1984), suggesting that the gills can serve as a route of copper uptake. In the light of the high volume of water passing through the gills (18 l kg^{-1} h^{-1}) (Wood and Jackson, 1980), even the low ambient copper concentrations normally present in fresh water (8–80 nmol l^{-1}=0.5–5.0 μg l^{-1}) (Spry et al., 1981) offer a potential source for normal copper assimilation by the gills. A recent study revealed that significant copper uptake occurs across the gills under normal conditions and that as much as 60 % of the whole-body copper requirement in fast-growing

juvenile rainbow trout fed a copper-deficient diet can be obtained directly from the water across the gills (Kamunde et al., 2002).

Recent findings of regulated copper transport across the branchial epithelium suggest the involvement of more specific copper transport proteins. For example, in rainbow trout *Oncorhynchus mykiss*, copper uptake across the gills was reduced by exposure to elevated ambient copper concentrations (Grosell et al., 1997). Furthermore, Kamunde et al. (2001, 2002) reported reduced branchial copper uptake in rainbow trout fed a copper-rich diet and increased branchial copper uptake in trout fed a copper-deficient diet. In addition, copper deficiency in juvenile rainbow trout could be induced only when copper levels in both the diet and the water were reduced. Fish held in water with normal copper levels and fed a copper-deficient diet maintained normal growth through the increased branchial copper uptake rate (Kamunde et al., 2002). Branchial copper uptake thus appears to be modulated depending on the copper status of the fish, strongly suggesting the involvement of specific, regulated transport pathways.

The main objective of the present study was to characterize the branchial copper uptake pathways in rainbow trout. Our initial hypothesis was that copper shares transport pathways with sodium since copper, at higher concentrations, impairs branchial sodium uptake, a vital part of freshwater osmoregulation (Laurén and McDonald, 1985, 1987a,b). Furthermore, recent evidence indicates that silver, which is known to serve as an analogue of copper in certain specific transport pathways (e.g. Solioz and Odermatt, 1995), is taken up *via* the apical sodium pathway in rainbow trout (Bury and Wood, 1999). This sodium pathway is *via* an apical, H^+-ATPase-coupled, Na^+ channel (Fenwick et al., 1999) and a basolateral Na^+/K^+-ATPase extruding sodium from the gill epithelial cells to the blood plasma (for a review, see Wood, 2001). We tested this hypothesis by cation competition studies and pharmacological manipulation of the apical sodium entry step. A detailed analysis of copper and sodium uptake kinetics and their interactions at environmentally realistic copper levels was performed to test whether multiple copper uptake pathways exist. Because of the essential nature of copper, relatively high background levels occur in most organs, making an isotopic approach necessary for detailed studies of copper uptake at environmentally realistic copper concentrations. In the present study, we employed the radioactive ^{64}Cu isotope to trace copper uptake rates of the order of pmol g^{-1} h^{-1} accurately.

Materials and methods

Experimental fish

Juvenile rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were obtained from Humber Springs Trout Hatchery, Ontario, Canada, and acclimated to laboratory conditions for a minimum of 2 weeks prior to experimentation. During this initial acclimation period, the fish were held in 264 l fibreglass tanks supplied with flow-through dechlorinated aerated Hamilton City tap water ($[Na^+]$, 0.6 mmol l^{-1}; $[Cl^-]$, 0.7 mmol l^{-1}; $[Ca^{2+}]$, 1.0 mmol l^{-1}; $[HCO_3^-]$, 1.9 mmol l^{-1}; pH 7.9–8.2; background [copper], 50 nmol l^{-1}=3.2 μg l^{-1}). Subsequently, fish were slowly acclimated to artificial 'soft water' over a period of 2 weeks by mixing hard water with increasing amounts of ion-reduced water (produced by reverse osmosis; Anderson Water Systems, Dundas, Ontario, Canada) until the desired water chemistry was reached ($[Na^+]$, 0.05 mmol l^{-1}; $[Cl^-]$, 0.04 mmol l^{-1}; $[Ca^{2+}]$, 0.02 mmol l^{-1}; pH 5.6–6.0; background [copper], 18 nmol l^{-1}=1.2 μg l^{-1}). The fish were held in this soft water for an additional 2 weeks prior to experimentation. The goal here was to work with fish acclimated to a medium containing low copper and sodium concentrations. The temperature was kept at 12±1 °C throughout acclimation and all subsequent procedures. The fish were fed trout pellets (Martin's Feed Mills, Ontario, Canada) at a rate of 1 % of their body mass (wet mass) three times a week. The copper content of the food was 46 nmol g^{-1} (2.9 μg g^{-1} dry mass).

Time course of copper accumulation

To establish the appropriate incubation time and sampling protocol for copper influx studies in softwater-acclimated juvenile rainbow trout (mean mass 4.44 g; range 2.12–10.40 g), copper accumulation as a function ambient copper concentration was measured during 0.5, 1, 2, 4 and 8 h of ^{64}Cu incubation at a range of copper concentrations (29±3, 30±2, 44±2, 88±2 and 145±5 nmol l^{-1}, means ± S.E.M., N=6 in all cases). The ^{64}Cu was obtained by radiation of $CuNO_3$ (solid form) in the Nuclear Research Reactor at McMaster University. After radiation, ^{64}Cu was dissolved in 0.5 % HNO_3. For all the above concentrations, groups of eight fish were placed in Pyrex beakers (1 l) and allowed to settle, after which each of the beakers was spiked with ^{64}Cu (specific activity at the time of addition; 3.26 MBq μmol l^{-1}) to yield each of the above copper concentrations. The addition of small amounts of the highly concentrated ^{64}Cu stock solution had negligible effects on water pH. After the isotope incubation period (see above), fish were netted out of the exposure containers and placed in a rinsing solution containing 200 μmol l^{-1} non-radioactive $CuSO_4$ and 0.1 g l^{-1} MS-222, an anaesthetic that completely anaesthetized the fish. This cold displacement rinse was performed to remove any non-specifically surface-bound isotope from the gills and skin of the fish. After 1 min, the fish were removed and the gills were obtained by dissection. The gills and the rest of the body were then blotted dry, placed in pre-weighed plastic vials and assayed for gamma radioactivity. On the basis of the results of this experiment, all the following experiments employed 2 h incubation periods, and copper uptake was measured by assaying the appearance of ^{64}Cu in the whole fish.

Cation competition studies

To test interactions between the major freshwater cations and copper uptake, copper uptake rates were measured in separate experiments in the presence of 0.05, 1, 2.5, 10 and

20 mmol l^{-1} Na^+, K^+ and Ca^{2+} (all as chloride salts). Juvenile rainbow trout (mean mass 0.52 g; range 0.19–1.46 g) were placed in 15 individual 100 ml Nalgene containers (eight fish per chamber) containing 50 ml of standard soft water and one of the above concentrations of either Na^+, K^+ or Ca^{2+}. After a 1 h pre-incubation period, ^{64}Cu was added to each container to reach a final concentration of 200 nmol l^{-1}. After a 2 h isotope incubation period, fish were netted out of the exposure containers and placed in a rinsing solution containing 200 μmol l^{-1} non-radioactive $CuSO_4$ and 0.1 g l^{-1} MS-222, after which each fish was blotted dry, placed in a pre-weighed plastic vial and assayed for gamma radioactivity.

Pharmacological studies

To test the involvement of an H^+-ATPase in branchial copper uptake, juvenile rainbow trout (mean mass 0.18 g; range 0.09–0.29 g) were placed in four 50 ml Nalgene containers (eight fish per chamber) each containing 20 ml of aerated soft water. Bafilomycin A1 (2 μmol l^{-1}; Biomol, PA, USA), a specific H^+-ATPase inhibitor, dissolved in dimethylsulphoxide (DMSO) (final concentration 0.2 %) was added to two of the four containers. The other two chambers contained only 0.2 % DMSO and acted as controls. Very small fish were employed because of the cost of the drug.

After a 1 h pre-incubation period, ^{64}Cu was added to one bafilomycin-treated container and to the corresponding control container to reach a final concentration of 200 nmol l^{-1}. The two remaining containers received 74 kBq l^{-1} ^{22}Na (Amersham, specific activity 11.21 MBq g^{-1} Na^+). After a 2 h isotope incubation period, fish were netted out of the exposure containers and placed in a rinsing solution containing 200 μmol l^{-1} non-radioactive $CuSO_4$ or 100 mmol l^{-1} non-radioactive NaCl for the ^{64}Cu and ^{22}Na experiments, respectively. Both rinsing solutions contained 0.1 g l^{-1} MS-222. Each fish was blotted dry, placed in a pre-weighed plastic vial and assayed for gamma radioactivity.

To test the involvement of the apical Na^+ channel in branchial copper uptake, a similar experiment was conducted using phenamil (RBI, Sigma-Aldrich, Canada), an amiloride analogue that is an irreversible Na^+ channel inhibitor (Garvin et al., 1985; Kleyman and Cragoe, 1988). Studies with amiloride analogues are complicated by the fact that these compounds bind metals avidly (cf. Bury and Wood, 1999). However, the irreversible nature of phenamil, unlike many other analogues, means that, after a pre-incubation period, the compound can be removed from the bathing solution and still be pharmacologically active. This approach avoids the problem of the drug complexing copper in solution and inadvertently affecting copper influx by this non-specific mechanism.

Juvenile rainbow trout (mean mass 0.80 g; range 0.26–1.49 g) were placed in four 100 ml Nalgene containers (eight fish per chamber) each containing 50 ml of aerated soft water. To two of these four containers, 100 μmol l^{-1} phenamil (RBI, Sigma-Aldrich, Canada), dissolved in DMSO (final concentration 0.1 %), was added. Again, the remaining two tanks contained only 0.1 % DMSO and acted as controls. Exploiting the fact that phenamil acts irreversibly on Na^+ channels, fish were removed from the pre-incubation containers, quickly rinsed and placed in identical containers holding pure aerated soft water. Subsequently, ^{64}Cu and ^{22}Na incubations were performed. Performing the ^{64}Cu exposure after removal of phenamil eliminated any potential problem with phenamil–^{64}Cu complex formation possibly rendering copper unavailable for uptake.

Interactions between copper and sodium uptake: dual-labelling experiments

To investigate the interactions between copper uptake and the ambient sodium concentration further, a detailed analysis of simultaneous copper and sodium uptake was performed. For these experiments, the short half-life of the ^{64}Cu isotope (12.7 h) was a clear advantage because it allowed for a dual radio-labelling experimental approach (see below). Juvenile rainbow trout (mean mass 4.23 g; range 1.66–9.91 g) were exposed to combinations of copper concentrations (background, no added ^{64}Cu; 18±1 and 29±3, 30±2, 44±2, 88±2 and 145±5 nmol l^{-1}) and sodium concentrations (10±1, 51±3, 96±2, 142±2, 217±2, 583±14 and 1059±12 μmol l^{-1}; means ± S.E.M., N=6–7); the 42 combinations each contained eight fish per experimental group. For each of the above sodium concentrations, six Pyrex beakers (1 l) containing aerated soft water (prepared with a background sodium concentration of only 10 μmol l^{-1}) supplemented with the appropriate sodium concentration were prepared. Of the six beakers with identical sodium concentrations, each was spiked with ^{22}Na (37 kBq) and a different concentration of ^{64}Cu (specific activity at the time of addition 3.26 MBq μmol^{-1}) to yield each of the six target copper concentrations. After a 2 h isotope incubation period, fish were netted out of the exposure containers and placed in a rinsing solution containing 200 μmol l^{-1} non-radioactive $CuSO_4$, 1 mol l^{-1} NaCl and 0.1 g l^{-1} MS-222, after which each fish was blotted dry, placed in a pre-weighed plastic vial and assayed for gamma radioactivity.

Analytical techniques and calculations

For all isotope flux experiments, an initial water sample (5 ml) was obtained 10 min after isotope addition, and a final water sample (5 ml) was obtained just before termination of the flux experiment. The water samples were acidified with 1 % HNO_3 (trace metal analysis grade; BHD Chemicals). From the metal uptake kinetic studies, an additional set of water samples was passed through a 45 μm filter (Acrodisc polyethersulphone inline filters; Gelman) prior to acidification to reveal the amount of metal truly in solution. Water copper concentrations were measured by graphite atomizer, atomic absorption spectroscopy (Varian, SpectrAA220 with a SpectrAA GTA110), while concentrations of other cations were measured by atomic absorption spectroscopy (Varian Spectra AA220).

The geochemical speciation model MINEQL revealed that

more than 97 % of the free copper in solution in the experimental water at all concentrations was present as Cu^{2+}.

Radioactivity from ^{64}Cu and ^{22}Na in the water, gills and fish samples was determined using a Canberra Packard Minaxi auto gamma 5000 series gamma-counter. The ^{64}Cu isotope was counted immediately after the experiments, because of its short half-life, with a window of 433–2000 keV, and decay correction was performed automatically by an onboard program.

For the dual-labelling experiments (^{64}Cu and ^{22}Na), the sum of the two isotopes was counted immediately using the appropriate ^{22}Na window, after which the ^{64}Cu isotope was allowed to decay. After a minimum of 12 half-lives, equivalent to 99.95 % loss of the ^{64}Cu isotope, the samples were recounted to reveal the activity of only the ^{22}Na in each sample. By subtracting the final activity measured (^{22}Na counts only) from the activity measured initially (^{64}Cu+^{22}Na), the ^{64}Cu radioactivity at the time the samples were taken could be calculated. All ^{64}Cu activities obtained in this manner were decay-corrected mathematically, to a common reference time, prior to uptake calculations (see below).

The average of the measured specific activity (cts min^{-1} $nmol^{-1}$) of the appropriate isotope in the initial and final water sample served as the basis for the uptake rate calculations. In practice, these values usually differed by no more than 10 %. In these calculations, the activity recorded in each gill or fish, the mass of the gill or fish and the isotope incubation time elapsed were related to the average specific activity to yield uptake rates (nmol g^{-1} h^{-1}) of copper and sodium.

Statistical analyses

Non-linear regression analyses of both copper and sodium uptake kinetics were performed with Sigmaplot for Windows version 4.00. Statistically significant differences between control groups and treated groups from the pharmacological studies were evaluated by a two-tailed Student's *t*-test. General cation competition studies and affinity constants for sodium uptake at different copper concentrations were evaluated by a two-tailed Student's *t*-test with a Bonferroni multisample comparison correction. A significance level of $P<0.05$ was employed throughout.

Results

Time course and pattern of copper accumulation

Copper uptake into the whole body of juvenile rainbow trout increased with increasing ambient copper concentration and, under the conditions applied in the present study, copper accumulation increased during the first 1–2 h of isotope incubation, after which it levelled off at a more-or-less constant concentration (Fig. 1A). A similar pattern was observed for the gills, except in fish incubated at the highest copper concentration, in which a clear biphasic branchial pattern was observed (Fig. 1B). At this copper concentration (145 nmol l^{-1}), the concentration of radiolabelled copper in the gills increased with time initially; after 2 h, branchial ^{64}Cu levels decreased considerably. On the basis of this data set, a 2 h incubation period was chosen for the remaining experiments, with all measurements being made on a whole-body basis. This choice represents a compromise between, on the one hand, having constant incubation conditions (i.e. as short an incubation period as possible) and thus constant accumulation rates throughout the isotope incubation and, on the other hand, ensuring sufficient isotope accumulation by the fish for accurate detection of gamma radiation. The relative contribution of the gills to whole-body copper accumulation was high, ranging from 30 to 70 %. Furthermore, the branchial contribution to whole-body copper accumulation (measured at 2 h) increased with increasing copper concentration (Fig. 1, inset).

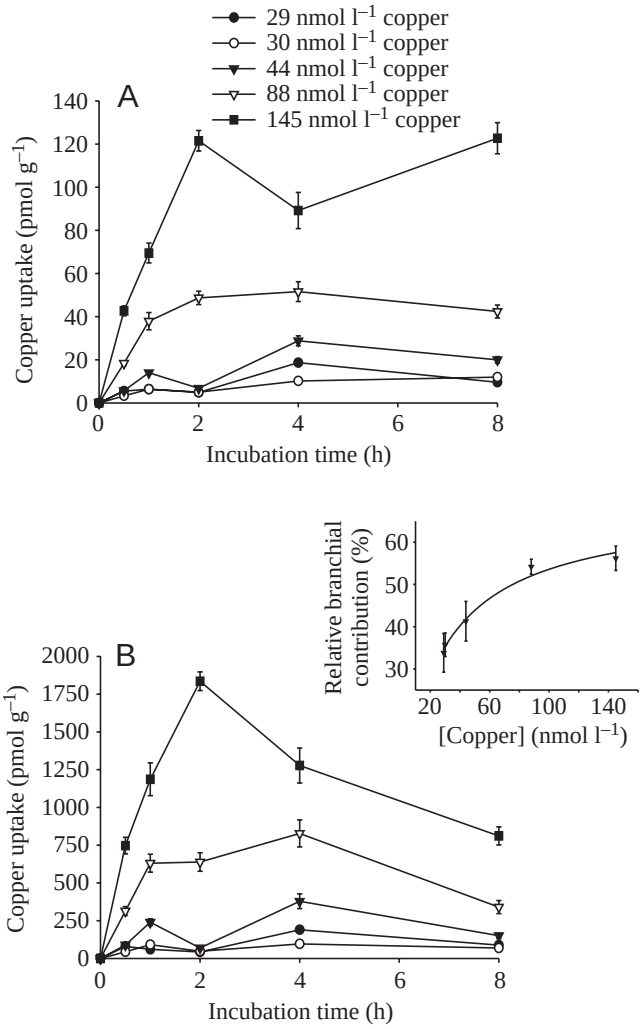

Fig. 1. Copper (^{64}Cu) uptake (pmol g^{-1}) as a function of incubation time (h) in juvenile rainbow trout, whole body (including gills) (pmol g^{-1}) (A) and gills (pmol g^{-1} gill) (B) assessed at different copper concentrations. Inset: relative proportion (%) of whole-body ^{64}Cu in the gills as a function of ambient copper concentration after 2 h of isotope incubation. Values are means ± S.E.M., N=8 in all cases.

Cation competition

Only sodium suppressed copper uptake across the gills of juvenile rainbow trout in a statistically significant manner (Fig. 2). Increasing the ambient sodium concentration from 50 μmol l^{-1} to 1 mmol l^{-1} reduced branchial copper uptake (at a concentration of 200 nmol l^{-1}) by more than 50 %. Increasing the sodium concentration further did not result in additional inhibition of copper uptake. Increasing potassium (Fig. 2B) and calcium (Fig. 2C) concentrations over the same range had no significant effect on branchial copper uptake, although there was a trend for inhibition by high potassium levels.

Pharmacology of copper and sodium uptake

Both bafilomycin A1 (2 μmol l^{-1}) and phenamil (100 μmol l^{-1}) treatments were successful in inhibiting branchial sodium uptake by 80 % and 70 %, respectively (Fig. 3). In addition, bafilomycin A1 and phenamil significantly reduced branchial copper uptake by 68 % and 39 %, respectively (Fig. 3). The different absolute rates of copper and sodium uptake in the bafilomycin A1 *versus* the phenamil experiments reflects the very different sizes of the fish used in the different trials. Smaller fish generally have higher sodium uptake rates than larger fish. This is seen also in the present study, where the controls in the bafilomycin A1 experiment with a mean mass of 0.18 g exhibited sodium uptake rates of 950 nmol g^{-1} h^{-1} (Fig. 3A), which is substantially higher than that of the controls in the phenamil experiment with a mean mass of 0.80 g, which exhibited sodium uptake rates of 450 nmol g^{-1} h^{-1} (Fig. 3B).

Interactions between copper and sodium uptake

Increasing the ambient sodium concentration (from 10 to 1059 μmol l^{-1}) reduced branchial copper uptake considerably (Fig. 4A–C). Copper uptake rates at all copper concentrations exhibited a negative hyperbolic relationship with ambient sodium concentration (illustrated for 145 nmol l^{-1} copper in the

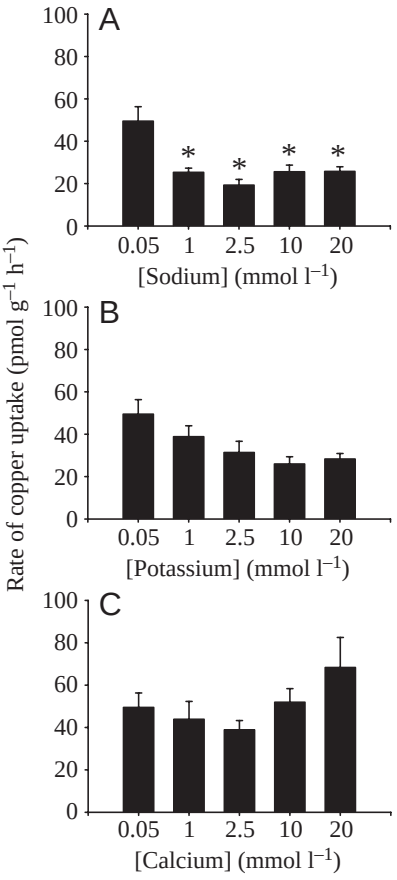

Fig. 2. Rates of copper (^{64}Cu) uptake (pmol g^{-1} h^{-1}) as a function of ambient (A) sodium, (B) potassium and (C) calcium concentration (nmol l^{-1}) in juvenile rainbow trout assessed during a 2 h incubation in the presence of 200 nmol l^{-1} copper. Values are means + S.E.M., *N*=8 in all cases. An asterisk indicates a statistically significant difference from the corresponding control value (0.05 mmol l^{-1}); two-tailed Student's unpaired *t*-test with multisample comparison correction (P<0.05).

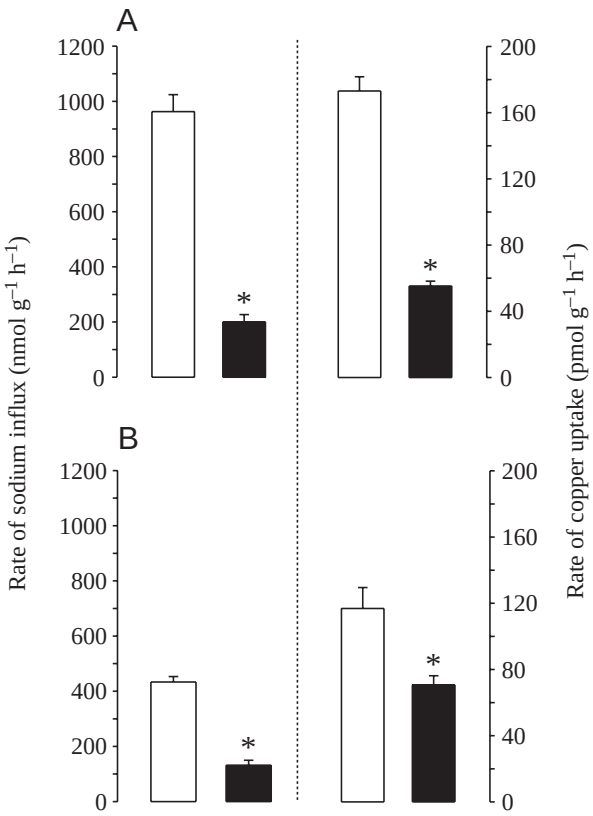

Fig. 3. Rates of sodium (nmol g^{-1} h^{-1}; left of dotted vertical line) and copper (pmol g^{-1} h^{-1}; right of vertical dotted line) uptake in juvenile rainbow trout (A) during treatment with 2 μmol l^{-1} bafilomycin A1 and (B) after treatment with 100 μmol l^{-1} phenamil assessed during a 2 h incubation in the presence of 200 nmol l^{-1} copper. Filled columns indicate results from pharmacologically treated fish, and open columns indicate results from corresponding vehicle (DMSO) control fish. Values are means + S.E.M., *N*=8 in all cases. An asterisk indicates a statistically significant difference from the corresponding control value; two-tailed Student's unpaired *t*-test (P<0.05).

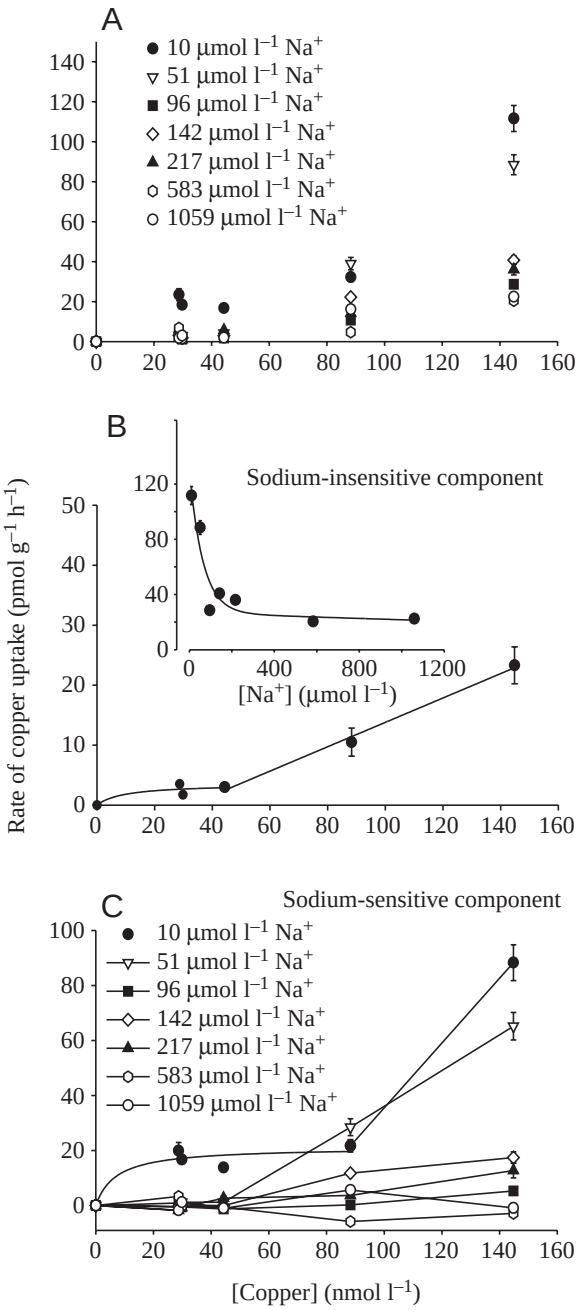

Fig. 4. (A) Overall copper (^{64}Cu) uptake rates (pmol g^{-1} h^{-1}) in the presence of different ambient sodium concentrations (indicated by different symbols), (B) calculated 'sodium-insensitive' copper uptake rates (pmol g^{-1} h^{-1}) and (C) 'sodium-sensitive' copper uptake rates (pmol g^{-1} h^{-1}) as a function of ambient copper concentration in juvenile rainbow trout during a 2 h incubation period. The 'sodium-insensitive' copper uptake rates were determined mathematically as the J_{min} (see text) in a hyperbolic curve fit (SigmaPlot 4.0 for Windows) to copper uptake rates as a function of ambient sodium concentration. 'Sodium-sensitive' copper uptake rates were determined as the difference between the overall copper uptake and the 'sodium-insensitive' copper uptake. Inset: example of a hyperbolic curve fit: copper uptake during a 2 h incubation at 145 nmol l^{-1} copper as a function of ambient sodium concentration. Values are means ± S.E.M., N=8 in all cases.

inset of Fig. 4B, r^2=0.90, P<0.05). The sodium concentration required for a 50 % reduction in the rate of branchial copper uptake (IC_{50}) was 103.8±9.9 μmol l^{-1} (mean ± S.E.M., N=7) The copper uptake remaining at high sodium concentrations was determined as the constant b in the hyperbolic relationship, $y=ax/(b+x)$, where y is the copper uptake rate and x is the ambient sodium concentration, and is referred to as J_{min} in the following. Plotting the J_{min} kinetic constant from these hyperbolic relationships as a function of ambient copper concentrations revealed the 'sodium-insensitive' component to branchial copper uptake. This sodium-insensitive copper uptake exhibits a high-affinity, low-capacity saturation kinetic component (J_{max}=3.5 pmol g^{-1} h^{-1}; K_m=9.6 nmol l^{-1}; r^2=0.92, P<0.05) at lower copper concentrations; at copper concentrations above 44 nmol l^{-1}, there is a linear relationship between copper uptake rates and ambient copper concentration (r^2=0.91, P<0.05) (Fig. 4B).

Subtracting the sodium-insensitive copper uptake (Fig. 4B) from the total copper uptake (Fig. 4A) revealed the sodium-sensitive copper uptake component (Fig. 4C). At the lowest sodium concentration (10 μmol l^{-1}), this sodium-sensitive component exhibits saturation kinetics, with a comparable high affinity to that of the sodium-insensitive uptake, but a sixfold higher capacity at copper concentrations below 88 nmol l^{-1} (J_{max}=21.2 pmol g^{-1} h^{-1}; K_m=7.1 nmol l^{-1}; r^2=0.89, P<0.05). At the higher sodium concentrations, copper uptake *via* this sodium-sensitive pathway is essentially totally inhibited at concentrations below 88–145 nmol l^{-1} copper.

Sodium uptake in control fish exhibited saturation kinetics characterized by a maximum transport capacity (J_{max}) of 683 nmol g^{-1} h^{-1} and an affinity (K_m) of 69 μmol l^{-1} and was clearly affected by copper exposure (Fig. 5). Ambient copper concentrations at and above 29 nmol l^{-1} tended to increase apparent sodium transport capacity (J_{max}), a trend that was significant at 30, 44, 88 and 145 nmol l^{-1} copper, and to reduce sodium affinity (i.e. increase K_m) significantly at 30 and 88 nmol l^{-1} copper.

Discussion

The present study demonstrates two different pathways for branchial copper uptake in rainbow trout. On the basis of the competition of copper uptake by low ambient sodium concentrations (IC_{50}=104 μmol l^{-1}) and the sensitivity of a proportion of the copper uptake to phenamil and bafilomycin A1, one of these uptake pathways appears to be through the apical Na^+ channel present in rainbow trout gills. A second pathway for copper uptake is suggested by continued copper uptake even in the presence of very high (1–20 mmol l^{-1}) ambient sodium concentrations. Further evidence for this component to copper uptake is the discrepancy between the inhibitory effect of phenamil (a Na^+ channel blocker) on sodium and copper uptake, phenamil inhibiting sodium uptake more effectively than copper uptake (Fig. 3). In the following, we refer to these two mechanisms of copper uptake as 'sodium-sensitive' and 'sodium-insensitive' copper uptake, respectively.

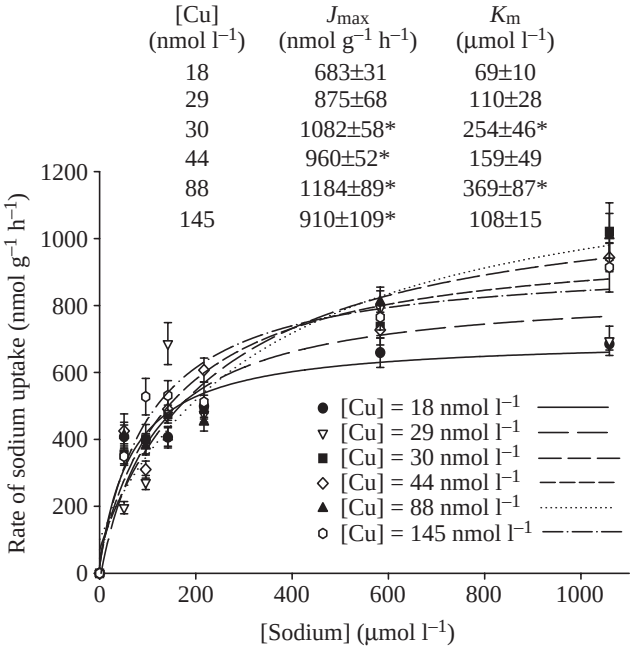

Fig. 5. Rates of sodium uptake (nmol g^{-1} h^{-1}) as a function of ambient sodium concentration in the presence of different ambient copper concentrations in juvenile rainbow trout during a 2 h incubation period. The lines are Michaelis–Menten curves (SigmaPlot 4.0 for Windows), and the inset summarizes the derived sodium uptake kinetic parameters J_{max} (nmol g^{-1} h^{-1}) and K_m (μmol l^{-1}). Values are means ± S.E.M., N=8 in all cases. In the inset, an asterisk indicates a significant difference from the control condition (18 nmol l^{-1} copper).

Both the sodium-sensitive and the sodium-insensitive components of branchial copper uptake exhibit saturation kinetics at low, environmentally realistic concentrations (see below), but also a linear component at concentrations above 88 and 44 nmol l^{-1} copper, respectively. These linear components could reflect non-specific uptake or binding to sodium-sensitive and sodium-insensitive sites directly on the gill surface. Notably, they extend into the concentration range that can be acutely toxic to rainbow trout (for a review, see Wood, 2001). Copper binding to rainbow trout gills exceeding an apparent saturable component has previously been reported for rainbow trout (Taylor et al., 2000).

Time course of copper uptake

The tendency for whole-body ^{64}Cu levels to stabilize after 2–4 h observed in the present experiment and the decrease in gill copper accumulation over time, especially at higher ambient copper concentrations, reinforces the view that branchial copper uptake is subject to homeostatic regulation.

Stabilization of the gill ^{64}Cu concentration over time must reflect the establishment of an equilibrium between uptake and elimination from the gill tissue. At the highest copper concentration, a biphasic accumulation pattern was observed, with an initial increase followed by a marked drop in branchial ^{64}Cu levels even during continuous exposure. This is in close agreement with previous reports of an initial peak in branchial copper levels in rainbow trout during continuous exposure (Grosell et al., 1997). One possible interpretation is that the basolateral transport mechanism is limiting and that, as copper builds up in the cytosol of the gill transport cells, this accumulation may initiate a stimulation of extrusion mechanisms such as the copper-specific P-type ATPases (see below). Alternatively, there may be a reduction in the apical entry of copper. Regardless of its origin, the mechanism would appear to serve as a protective response against elevated copper levels in the gill tissue because it occurs only at higher copper concentrations.

Sodium-sensitive copper uptake

Metal transport through the apical Na^+ channel of rainbow trout gills has been reported previously. Bury and Wood (1999) demonstrated that silver uptake across rainbow trout gills was sensitive to both phenamil and bafilomycin A1 at the concentrations used here. Furthermore, silver uptake was reduced in the presence of 500 μmol l^{-1} sodium, but was not influenced by 200 μmol l^{-1} sodium. This indicates that the IC_{50} of sodium against silver uptake is higher than the 104 μmol l^{-1} sodium reported for sodium against copper uptake in the present study (Fig. 4). In turn, this suggests that the affinity of the Na^+ channel for silver is higher than for copper. In parallel, it is known that the affinity of the whole gill surface for silver is more than two orders of magnitude higher than for copper (Wood, 2001). It appears from the copper uptake kinetics of the sodium-insensitive pathway that it comprises a high-affinity, low-capacity transport system. The sodium-sensitive copper uptake has a similar high affinity but a much higher capacity. Depending on the ambient copper concentration, the copper uptake rates *via* this mechanism are 2–5 times greater than *via* the sodium-insensitive copper uptake pathway (Fig. 4B *versus* Fig. 4C). Clearly, in low-sodium fresh water, typical of ion-poor softwater areas, this will be the dominant mechanism of branchial copper uptake. Particularly noteworthy for both mechanisms are their extremely high affinities (i.e. low K_m values of 7.1–9.6 nmol l^{-1}), which means that they will be functional at environmental copper concentrations in non-contaminated environments (8–80 nmol l^{-1}) (Spry et al., 1981). This again points to an important role for the gill in normal copper homeostasis.

Additional support for a link between sodium and copper transport is provided by the recent work of Pyle and co-workers (G. G. Pyle, C. Kamunde, C. M. Wood and D. G. McDonald, unpublished observations), who found that rainbow trout fed a high-sodium diet for 1 week exhibited reduced unidirectional uptake of both sodium and copper at the gills. This suggests that the sodium-sensitive pathway may be modulated by internal sodium status, in addition to external water sodium levels. A link between sodium and copper transport has been largely overlooked in higher vertebrates, although it has previously been suggested in rat intestine on the basis of amiloride-sensitivity and sodium-sensitive tissue copper retention (Wapnier, 1991). These observations are in

agreement with the present study suggesting a role for the apical Na^+ channel in copper uptake not only across fish gills but possibly also across mammalian intestinal epithelia.

Effects of copper on branchial sodium transport

Sodium clearly inhibited copper uptake, and the reverse was also true (Fig. 5). This is in parallel to findings of an immediate partial inhibition of sodium uptake in rainbow trout during exposure to toxic levels of silver (Morgan et al., 1997) followed by a greater inhibition at 8 h. It is also in agreement with findings of a progressively developing inhibition of sodium uptake over 24 h in rainbow trout exposed to elevated copper levels (Laurén and McDonald, 1985). The effect of both silver and copper on sodium uptake is associated with inhibition of the basolateral Na^+/K^+-ATPase (for a review, see Wood, 2001). Detailed measurements of the parallel time course of inhibition of sodium uptake and maximal Na^+/K^+-ATPase activity during copper or silver exposure in rainbow trout gills have not, to our knowledge, been reported. Clearly, these would be useful in determining whether all the inhibition can be explained by a slowly developing blockade of the Na^+/K^+-ATPase or whether there is an additional more rapid effect on apical or intracellular mechanisms.

In the present study, which employed only 2 h of exposure, the inhibition observed might reflect actions of copper on these latter components, rather than on basolateral Na^+/K^+-ATPase activity. In support of this suggestion are the different effects of copper on sodium transport kinetics observed in the present study and in the study by Laurén and McDonald (1987a). Both studies found a reduced apparent affinity of branchial sodium transport (increased K_m); however, where the present study documents a somewhat increased maximal transport capacity (J_{max}) after only 2 h of exposure, the study by Laurén and McDonald (1987b) reported a decreased J_{max} after 24 h, when Na^+/K^+-ATPase activity was inhibited. This difference may mean that different components of the branchial sodium transport pathway are affected by copper, depending on the duration of the exposure. The increased J_{max} observed in the present study is in agreement with findings of stimulated transepithelial short-circuit current and conductance in copper-exposed isolated ventral frog skin, as reported by Flonta et al. (1998). A similar response was reported for zinc uptake across the gills of rainbow trout as ambient calcium concentration was increased (Spry and Wood, 1989); these two metals are also thought to share a common apical uptake mechanism (for a review, see Wood, 2001).

The present study shows that copper may be transported by the apical Na^+ channel, and inhibitory interactions between sodium and copper could thus occur at this channel. Another possible site of inhibition of sodium transport is the branchial carbonic anhydrase. This enzyme provides the substrate for the apical proton pump (from carbon dioxide and water), and inhibition of carbonic anhydrase could thus reduce the activity of the proton pump simply by depletion of substrate. Carbonic anhydrase readily binds copper (Ditusa et al., 2001), and copper-induced inhibition of branchial carbonic anhydrase has been reported in the estuarine crab *Chasmagnathus granulata* (Vitale et al., 1999). It is not known whether this also occurs in the freshwater fish gill, although silver is effective in this regard (Morgan et al., 1997).

Sodium-insensitive copper transport

The saturable component of sodium-insensitive copper uptake suggests the involvement of a specific carrier in addition to the apical Na^+ channel. The recent identification of a group of high-affinity copper uptake carriers, Ctr-type copper transporters, offer an appealing potential mechanism for sodium-insensitive copper uptake across trout gills. This type of copper transporter has been documented in phylogenetically distinct species such as yeast (Dancis et al., 1994; Kamphenkel et al., 1995; Knight et al., 1996), mouse (Lee et al., 2000) and human (Zhou and Gitschier, 1997) and is essential for normal development (Lee et al., 2001). Thus, it may well be present in teleost fish.

The mouse Ctr1 copper transporter exhibits an affinity constant of 1000–2000 nmol l^{-1} copper (J. Lee and D. Thiele, personal communication). The affinity constant of the sodium-insensitive copper uptake across trout gills in the present study was only 9.6 nmol l^{-1} (similar to that of the sodium-sensitive pathway) and is therefore very relevant to normal environmental copper levels in natural fresh water. However, this value is at least two orders of magnitude lower than that found for the murine Ctr1 copper transporter. Assuming that sodium-insensitive copper uptake across trout gills is mediated by a Ctr1-type transporter, this difference in apparent affinity could be explained by the different incubation media employed in the mouse Ctr1 transport study and the present study. Copper uptake kinetic measurements for the mouse Ctr1 transporter were performed in cell culture media that offer a large number of complexing agents, possibly rendering much of the copper unavailable for copper-specific transporters. In contrast, the present study was performed in ion-poor fresh water at slightly acidic pH, at which more than 90 % of the copper occurs in ionic form. The difference in apparent affinity between the present study and the Ctr transporter study could simply reflect different chemical forms of copper present in the different media.

While the sodium-sensitive copper uptake pathway clearly dominates at low ambient sodium concentrations, the sodium-insensitive copper uptake pathway dominates at sodium concentrations above 200 μmol l^{-1}. It therefore probably plays the dominant role in most natural fresh waters, all except those endemic to very ion-poor watersheds. Both the sodium-sensitive and the sodium-insensitive copper uptakes exhibited saturation kinetics within the range of concentrations employed in the present study. This observation is in agreement with a previous report of copper uptake kinetics in juvenile rainbow trout, employing similarly low copper concentrations, in which saturation occurred at copper concentrations below 95 nmol l^{-1} (Kamunde et al., 2002). The concentrations employed in the present study range from low environmentally realistic concentrations (Spry et al., 1981) to

levels that will probably cause toxic effects in ion-poor water (for a review, see Wood, 2001). At higher copper concentrations, which cause severe acute toxicity, saturation kinetics for branchial copper uptake and binding by rainbow trout has been observed (Campbell et al., 1999; Laurén and McDonald, 1987a,b; Taylor et al., 2000). The interpretation of the saturation transport kinetics at concentrations of copper that may cause acute toxicity is, however, complicated by potential lamellar damage and gill surface mucification (Wilson and Taylor, 1993), which may create an artificial condition of 'apparent saturation'.

Possible site of regulation of branchial copper uptake

Branchial copper uptake across rainbow trout gills is regulated depending on the copper status of the fish. This regulated copper uptake is likely to be mediated by one or more specific copper carriers rather than by the apical Na^+ channel primarily involved in maintaining sodium homeostasis. Sodium-insensitive copper uptake, possibly through an apical Ctr-type transporter, is one potential regulated copper uptake pathway in rainbow trout gills. As noted above, the time-dependent pattern of branchial copper accumulation and the increasing relative branchial contribution to whole-body copper accumulation with increasing ambient copper concentration during isotope incubation (Fig. 1B, inset) strongly suggest that the basolateral membrane is the rate-limiting site in branchial copper uptake in rainbow trout. This makes the basolateral membrane another likely candidate for a site of regulation of branchial copper uptake. The extrusion of copper across the basolateral membrane in the copper-assimilating intestinal epithelium in mammals is mediated by a copper-specific P-type ATPase, the Menke's (MNK) Cu-ATPase (Camakaris et al., 1995). Regulation of this step of intestinal copper uptake occurs *via* copper-sensitive MNK protein trafficking between the *trans*-Golgi network and the basolateral membrane (Petris et al., 1996).

A similar mechanism of copper transport across the basolateral membrane could be involved in regulated copper uptake by the rainbow trout gill, an organ clearly involved in copper assimilation. Copper uptake across the gills of rainbow trout is sensitive to vanadate, a P-type ATPase inhibitor, which could indicate the involvement of a Cu^{2+}-ATPase (Campbell et al., 1999). Furthermore, a putative Cu^{2+}-ATPase from teleost fish gills has been identified on the basis of 80 % amino acid homology with mammalian MNK proteins (Grosell et al., 2001a), supporting the possible involvement of a Cu^{2+}-ATPase in branchial copper assimilation. In further support of this hypothesis are reports of ATP-dependent, vanadate-sensitive silver transport across basolateral membrane vesicles from trout gill cells (Bury et al., 1999). These observations support the presence of a Cu^{2+}-ATPase in the basolateral membrane because silver has been shown to act as a substrate for bacterial Cu^{2+}-ATPases and also because vanadate is a specific inhibitor of P-type ATPases (Solioz and Odermatt, 1995).

Copper uptake through Na^+ channels may make an important contribution to copper homeostasis not only in fish but also in mammals, in which some evidence for an interaction between sodium and copper uptake has been demonstrated (Wapnier, 1991). Copper uptake across the gills offers an exciting area for further studies of the mechanisms of regulation of copper homeostasis. Studies of the potential involvement of a Ctr-type transporter and Cu^{2+}-ATPases in branchial copper uptake may provide information about evolutionary aspects of copper homeostasis. While copper transport by Ctr-type transporters in yeast is regulated at the transcriptional level on the basis of copper levels (Labbé et al., 1997), mammalian Ctr1 mRNA is not sensitive to cellular copper availability (Lee et al., 2000). Similarly, transport by Cu^{2+}-ATPase is regulated by trafficking between the *trans*-Golgi network and the plasma membrane in mammals rather than by changes in gene expression. The Cu^{2+}-ATPase resides in the *trans*-Golgi network under conditions of normal copper concentration, but it relocates to the plasma membrane in the presence of excess copper (Camakaris et al., 1999). In contrast, Cu^{2+}-ATPase mRNA levels in bacteria are sensitive to copper levels (Odermatt and Solioz, 1995). The possible involvement of these copper transporters in the regulation of branchial copper transport in fish offers a fruitful area for further understanding of the mechanisms of regulated copper transport not only in fish but also in higher vertebrates.

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