

Absorption of copper and copper–histidine complexes across the apical surface of freshwater rainbow trout intestine

Chris N. Glover · Chris M. Wood

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Abstract Bioavailability is integral in mediating the delicate balance between nutritive and potentially toxic levels of copper in fish diets. Brush-border membrane vesicles isolated from freshwater rainbow trout intestine were used to characterise apical copper absorption, and to examine the influence of the amino acid histidine on this process. In the absence of histidine, a low affinity, high capacity copper uptake mechanism was described. However, when expressed as a function of ionic copper (Cu^{2+}), absorption was linear, rather than saturable, suggesting that the saturable curve was an artifact of copper speciation. Conversely, in the presence of L-histidine (780 μM) saturable uptake was characterised. The uptake capacity discerned (J_{max} of $354 \pm 81 \text{ nmol mg protein}^{-1} \text{ min}^{-1}$) in the presence of histidine indicated a significantly reduced capacity for copper transport than that in the absence of histidine. To determine if copper uptake was achievable through putative histidine uptake pathways, copper and histidine were incubated in the presence of tenfold greater concentrations of amino acids proposed to block histidine transporters. Accounting for changes in copper speciation, significant inhibition of uptake by glycine and lysine were noted at copper levels of

699 and 1,028 μM . These results suggest that copper–histidine complexes may be transportable via specific amino acid-transporters in the brush-border membrane.

Keywords Copper · Amino acid · Uptake · Metal chelates · Bioavailability

Abbreviation

BBMV Brush-border membrane vesicles
 J_{max} maximal rate of uptake
 K_{m} Michaelis–Menten uptake affinity constant

Introduction

Assessing the bioavailability of copper, and determining the mechanism by which bioavailable forms are absorbed, is of critical importance for understanding the roles of copper in both nutrition and toxicology. Copper performs vital functions as a co-factor in proteins involved in a wide range of key biological processes including antioxidant defence and cellular respiration (Bury et al. 2003). However, copper in excess can cause toxicity, by either impairing sodium uptake at the gills (Wood 2001), or by inhibiting digestive enzymes and disturbing gastrointestinal function (Clearwater et al. 2002). The biological impact of copper is largely governed by bioaccumulation, itself dependent upon copper bioavailability.

As alluded to above, copper uptake is achieved by two epithelia in fish: the branchial and the gastrointestinal (Bury et al. 2003). The mechanism of copper transport across the gill is relatively well understood. Apical transport appears to occur via a sodium-sensitive pathway, possibly an epithelial sodium channel, and a sodium-insensitive mechanism, hypothesised to be a high affinity carrier of the Ctr

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C. N. Glover
National Institute of Nutrition and Seafood Research (NIFES),
Bergen, Norway

C. N. Glover (✉)
Scion Research, Te Papa Tipu Innovation Park,
49 Sala St, Private Bag 3020, Rotorua, New Zealand
e-mail: chris.glover@scionresearch.com

C. M. Wood
Department of Biology, McMaster University,
Hamilton, Canada

family (Grosell and Wood 2002). Basolateral transfer is likely to be carrier-mediated, with physiological evidence suggesting the involvement of a P-type ATPase (Campbell et al. 1999). Gastrointestinal copper transport in fish has been the subject of several recent papers that have contributed significantly to our understanding of this process. Apical copper uptake appears to be passive, with basolateral transport suggested to be active and rate limiting (Clearwater et al. 1998; Handy et al. 2000). Some reports indicate that copper uptake is strongly dependent on luminal sodium, with increased sodium promoting uptake (Nadella et al. 2007). Other studies, however, have failed to show this (Burke and Handy 2005), possibly indicating species or technique-related differences.

Previous studies of copper absorption in fish have focussed primarily upon the uptake of free, ionic copper (as Cu^+ or Cu^{2+}). However, recent evidence suggests that the amino acid histidine may also exert an important influence on copper bioavailability in fish (Nadella et al. 2006b). This finding was not surprising in the light of similar conclusions drawn in studies of dietary copper bioavailability in other organisms (e.g. Chen and Mayer 1998). In fact the ability of histidine to enhance copper bioavailability is the basis of a treatment for human copper deficiency (Deschamps et al. 2005).

The positive effect of histidine on health parameters in fish nutrition has been previously demonstrated. For example, the addition of histidine to fish diet has been implicated with an improvement in cataract status (Breck et al. 2003; Bjerkaas and Sveier 2004), an effect that could be attributed to enhanced micronutrient uptake. Physiological studies have shown that histidine can facilitate the uptake of the divalent metal zinc across rainbow trout intestine (Glover et al. 2003), and can act to redistribute the metal to tissues where it may enhance fish health (Glover and Hogstrand 2002).

Two hypotheses explain the effect of histidine on metal bioavailability. Either the addition of histidine creates a transportable chelate, which opens up a new transport pathway (e.g. Wapnir et al. 1983), or histidine acts as a donor, facilitating the passage of the metal to its transporter (e.g. Bobilya et al. 1993). Previous attempts to elucidate which mechanism is responsible for the effect of histidine on metal bioavailability have been inconclusive, partly owing to a lack of information regarding intestinal histidine uptake (Glover et al. 2003). Recent research characterised histidine transport in the fish intestine for the first time (Glover and Wood 2007). This study also examined the effect of luminal copper on histidine uptake. The characteristics of histidine absorption in the presence of copper were kinetically distinct from those of histidine alone, demonstrating a unique transport mechanism for a potential chelated species (Glover and Wood 2007). The present investigation examined the reciprocal relationship, the

effect of histidine on copper uptake across the apical surface of rainbow trout intestine, using brush-border membrane vesicles (BBMV's).

Materials and methods

Animals

Rainbow trout (*Oncorhynchus mykiss* ~200–300 g) from Humber Springs trout farm (Orangeville, ON, Canada), were acclimated to conditions in the laboratory at McMaster University: water temperature 12–13°C; water chemistry Na^+ , 0.5 mM; Ca^{2+} , 1 mM, Cl^- , 0.7 mM; hardness, 140 ppm as CaCO_3 ; pH 8. Fish were fed three times a week, ad libitum, on standard commercial feed (Martin Mills) with a minimum-estimated histidine content of 7 g kg^{-1} (NRC 1993) and a copper content of 27 mg kg^{-1} , based on measurements of previous batches of this feed (Nadella et al. 2006b).

Brush-border membrane vesicle preparation

Vesicle preparation and transport assay protocol were closely based on that of Glover et al. (2003). Intestinal scrapings from two fish were combined, dounce homogenised (buffer 50 mM mannitol, 2 mM EGTA, 0.5 mM MgSO_4 , 0.1 mM phenylmethanesulfonylfluoride, pH 7.4), centrifuged ($8,500 \times g$, Sigma 4K15 refrigerated centrifuge, 4°C, 15 min) and the resulting fluffy layer collected. This was then further homogenised (buffer 320 mM sucrose, 10 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), pH 7.4), before a membrane aggregation step (10 mM MgCl_2) was performed. Two consecutive centrifugations (1: $20,000 \times g$, 10 min, 4°C, Sigma 4K15; 2: $45,000 \times g$, 25 min, 4°C, Beckman L8M ultracentrifuge, SW28 rotor) resulted in a final pellet. Activities of the enzymes alkaline phosphatase (brush-border), and Na^+ , K^+ -ATPase (basolateral) were monitored as markers of membrane purity (Glover et al. 2003). Membranes were resuspended in vesicle buffer [149 mM KCl, 1 mM NaCl, 10 mM *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES)] with 30 passages through a 23-gauge needle. For all transport assays the membrane fraction was diluted to a final concentration of $1 \text{ mg protein ml}^{-1}$, as determined by the Bradford (1976) assay.

Measurement of unidirectional copper uptake

Kinetic characterisation of unidirectional copper transport across the apical intestinal membrane of rainbow trout was assessed by a rapid filtration method. Membrane vesicle aliquots of $35 \mu\text{l}$ ($35 \mu\text{g protein}$) were added to $125 \mu\text{l}$ of

assay buffer (149 mM NaCl, 1 mM KCl, 10 mM HEPES, pH 7.4) containing copper (as $\text{Cu}(\text{NO}_3)_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$) levels of 7.4, 14, 63, 155, 232, 699, or 1,028 μM . These concentrations were derived from measurement of $2\times$ copper stock solutions in assay buffer, determined by graphite furnace atomic absorption spectroscopy (Varian Spectra AA-220). Radiolabelled ^{64}Cu (half-life 12.9 h) was prepared by irradiating dried $\text{Cu}(\text{NO}_3)_2$ (McMaster Nuclear Reactor) and redissolving the salt in equal volumes of 0.1 M HNO_3 and 0.01 M NaHCO_3 . At the start of the experiment radiolabel was added to each copper stock solution at a level of 0.2-mCi ml^{-1} . Once the assay was initiated by the addition of the vesicles to the buffer, the reaction was permitted to proceed for 30 s in a water bath set at 10°C . This reaction time was chosen after initial investigation of the time-dependence of copper uptake (Fig. 1), which showed this interval to be close to the linear range of uptake. Triplicate 45 μl aliquots were then added to membrane filters (0.45 μm , Schleicher and Schuell) placed in a rapid filtration sampling manifold (Millipore), and filters were then washed with 5 mls of stop buffer (ice cold assay buffer, with 1 mM EDTA). EDTA is an effective chelator of copper, and should act to remove any non-specific surface-bound metal, that may obscure true absorption. The ^{64}Cu activity of filters was determined by gamma counting (Perkin–Elmer, 1480 Wallac Wizard 3). Values were corrected for background activity and radioactive decay, and converted using specific activity calculations. Uptake was expressed as $\text{nmol mg protein}^{-1} \text{min}^{-1}$.

The effect of two concentrations of L-histidine (0.78 and 780 μM) on the kinetics of apical copper uptake was tested. These reactions were performed identically to those with copper alone, with the exception of a 1-min equilibration between the amino acid and the copper before the addition of vesicles. To identify potential transport systems, tenfold

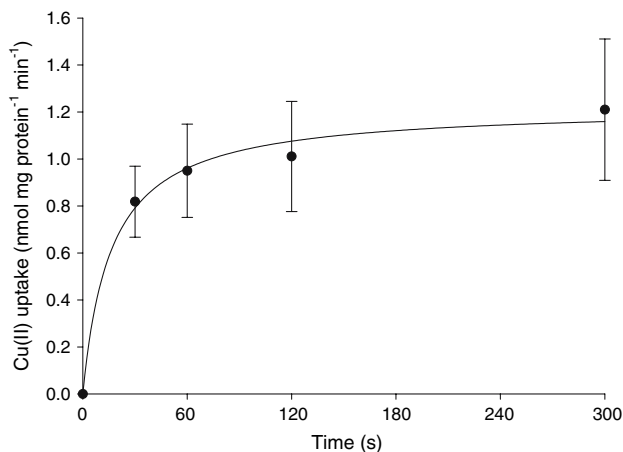


Fig. 1 Copper uptake ($\text{nmol mg protein}^{-1} \text{min}^{-1}$) in rainbow trout intestinal BBMV's as a function of time, at an extravesicular copper concentration of 6.0 μM , at 10°C . Plotted points represent the means (\pm SEM) of four replicates, measured in triplicate

excess (i.e. 7.8 μM at 0.78 μM L-histidine or 7.8 mM at 780 μM L-histidine) of potential competing amino acid/dipeptide substrates (glycine, glutamine, lysine, phenylalanine, and glycylglycine) were added to solutions containing copper and L-histidine. These were also incubated for 1 min before transport initiation. The copper levels tested were 7.4, 699 and 1,028 μM , chosen owing to their different speciation at the histidine levels tested.

Speciation modelling

Copper and amino acid speciation was determined using the geochemical modelling software MINEQL+ (ver. 4.5; Environmental Research Systems; Schecher and McAvoy 1992). All stability constants used were those provided with the program. Where constants were not pre-existing, as for glutamine and glycylglycine, these were obtained from the literature (Berthon 1995; Martin et al. 1971, respectively).

Calculations and statistical analysis

Michaelis–Menten uptake parameters were calculated directly from curves using Sigmaplot 8.02 (SPSS Inc.). Statistical differences were assessed by one-way analysis of variance, at the $\alpha = 0.05$ level, using an LSD post-hoc test (Statistica ver. 5; StatSoft).

Results

The membrane vesicle isolation resulted in a highly enriched brush-border membrane component, with activity of the marker enzyme alkaline phosphatase 6.7-fold higher in the final pellet than the initial homogenate. However, a significant enrichment of basolateral membranes was also observed, with Na^+, K^+ -ATPase activity 2.6-fold higher in the final pellet.

Uptake of copper into membrane vesicles showed saturation with time (Fig. 1). A rapid initial rate of uptake was noted over the initial 30 s of incubation in extravesicular copper, with increased incubation time inducing only minor increases in membrane vesicle copper accumulation. On the basis of this relationship, 30 s was chosen as the incubation time for all subsequent manipulations.

When calculated on the basis of total copper, the passage of this metal across the apical membrane of rainbow trout intestine proceeded via a low affinity, saturable mechanism (Fig. 2, filled data points). The Michaelis–Menten characteristics of uptake derived from this curve show a maximal uptake rate (J_{max}) of $1,272 \pm 287 \text{ nmol mg protein}^{-1} \text{min}^{-1}$, and a K_m (copper concentration required to reach 50% J_{max}) of $1,447 \pm 502 \mu\text{M}$ ($r^2 = 0.9951$, $P < 0.0001$). However, as demonstrated in Table 1, the proportion of free, ionic copper

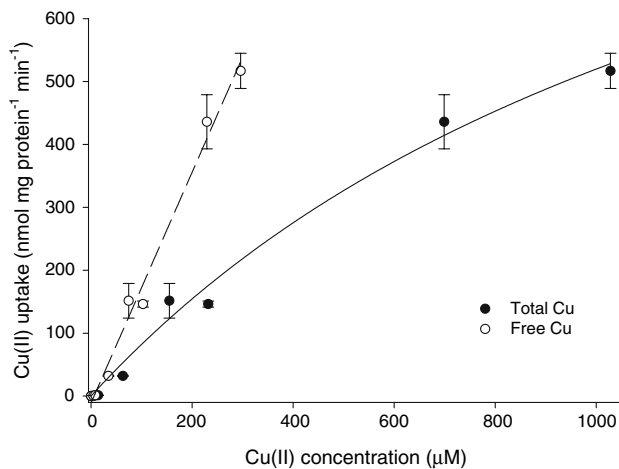


Fig. 2 Copper uptake ($\text{nmol mg protein}^{-1} \text{min}^{-1}$) in rainbow trout intestinal BBMVs as a function of total copper (closed circles) or ionic Cu^{2+} (open circles) levels following incubation for 30 s at 10°C . Copper speciation determined using MINEQL⁺ (Table 1). Curve fitting and calculation of Michaelis–Menten parameters performed using SigmaPlot (ver. 5; StatSoft). Plotted points represent the means ($\pm\text{SEM}$) of 4 (63, 232, 699, and 1,028 μM concentrations) or 8 (7.4, 14 and 154 μM concentrations) replicates, measured in triplicate

(Cu^{2+}) decreased as copper concentration increased. Recalculating the uptake curve on the basis of Cu^{2+} the uptake curve became linear (with a y-intercept of $-11 \text{ nmol mg protein}^{-1} \text{min}^{-1}$, and a slope of $1.8 \text{ nmol mg protein}^{-1} \text{min}^{-1} \mu\text{mol l}^{-1}$; $r^2 = 0.9947$, $P < 0.0001$).

The addition of a low histidine concentration ($0.78 \mu\text{M}$), had very little influence on the copper uptake curve characteristics (data not shown). On the basis of total copper, a K_m of $1,613 \pm 592$ and a J_{max} of $1,435 \pm 353 \text{ nmol mg protein}^{-1} \text{min}^{-1}$ were described ($r^2 = 0.9953$; $P < 0.0001$). Accounting for only free Cu^{2+} and copper–histidine complexes (bioavailable copper species; Niyogi and Wood 2004; Nadella et al. 2006b), uptake was again best described by a linear function (y-intercept $-15 \text{ nmol mg protein}^{-1} \text{min}^{-1}$, slope $1.9 \text{ nmol mg protein}^{-1} \text{min}^{-1} \mu\text{mol l}^{-1}$; $r^2 = 0.9949$; $P < 0.0001$).

In the presence of higher histidine levels ($780 \mu\text{M}$) copper was present almost exclusively in a bis-chelated form ($\text{Cu}(\text{His})_2$; Table 1). The exception was at the highest copper level ($1,028 \mu\text{M}$), where the monochelate ($\text{Cu}(\text{His})^+$) dominated speciation. The resulting uptake curve exhibited saturation, with a J_{max} of $354 \pm 81 \text{ nmol mg protein}^{-1} \text{min}^{-1}$ and a K_m of $1,103 \pm 421 \mu\text{M}$ ($r^2 = 0.9916$; $P < 0.0001$; Fig. 3). The K_m value was statistically equivalent to that obtained in the presence of low histidine and copper alone, while the J_{max} value was significantly lower than that derived from both other kinetic curves.

No significant effect of a tenfold excess ($7.8 \mu\text{M}$) of any of the tested potential inhibitors of the transport system could be shown on copper uptake at $7.4 \mu\text{M}$ and in the pres-

Table 1 Copper speciation (% of each species present) under experimental conditions

Total Cu (μM)	Cu^{2+}	$\text{Cu}(\text{His})^+$	$\text{Cu}(\text{His})_2$
Cu(II) alone			
7.4	61		
14	60		
63	55		
155	48		
232	44		
699	33		
1,028	29		
Cu(II) + $0.78 \mu\text{M}$ His			
7.4	55	11	
14	57	5.6	
63	54	1.2	
155	48	0.8	
232	44	0.6	
699	33	0.3	
1,028	29	0.2	
Cu(II) + $780 \mu\text{M}$ His			
7.4			100
14			100
63			100
155			100
232			100
699		13	86
1,028	11	74	0.8

Speciation determined using MINEQL⁺ (Schecher and McAvoy 1992). Where percentage values do not add to 100, the remainder of copper was as present as hydroxide ($\text{Cu}_2\text{OH}^{2+}$, CuOH^+ , $\text{Cu}(\text{OH})_2$) and/or chloride (CuCl^+) species

ence of $0.78 \mu\text{M}$ L-histidine (data not shown). At a similar copper level, but in the presence of higher extravesicular histidine ($780 \mu\text{M}$), tenfold excess of transport inhibitors also had no effect (Fig. 4a). Conversely, at this same high histidine level ($780 \mu\text{M}$), but elevated copper levels of $699 \mu\text{M}$ (Fig. 4b) and $1,028 \mu\text{M}$ (Fig. 4c), the amino acids glycine, glutamine and lysine all significantly inhibited copper transport across the intestinal brush-border. The magnitude of this effect was greatest for glycine with a 65% inhibition at $699 \mu\text{M}$, and a 78% inhibition at $1,028 \mu\text{M}$ copper. Glutamine reduced uptake by 35 and 36%, and lysine by 57, and 46% at 699 and $1,028 \mu\text{M}$ copper, respectively.

However, the potential inhibitors also have some binding affinity for copper. As such they will compete with histidine for copper binding, thus altering copper speciation. Inhibitors may therefore act to inhibit transport specifically (by blocking potential uptake pathways traversed by copper–histidine chelates), or non-specifically (by binding copper, reducing the level of copper–histidine chelates and thus

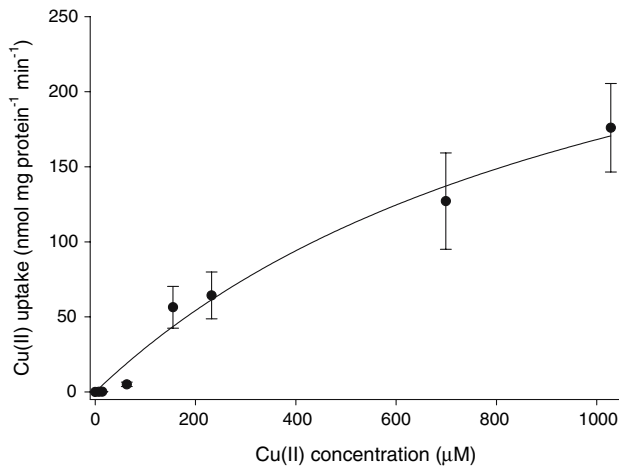


Fig. 3 Copper uptake (nmol mg protein⁻¹ min⁻¹) in the presence of 780 μM L-histidine in rainbow trout intestinal BBMVs as a function of total copper (=Cu–His chelate) levels following incubation for 30 s at 10°C. Curve fitting and calculation of Michaelis–Menten parameters performed using SigmaPlot (ver. 5; StatSoft). Plotted points represent the means (±SEM) of 4 (63, 232, 699, and 1,028 μM concentrations) or 8 (7.4, 14 and 154 μM concentrations) replicates, measured in triplicate

reducing uptake). The correct interpretation of the results of the inhibitor studies depends on accounting for this non-specific effect. Table 2 shows that the amino acids glycine, glutamine, phenylalanine, and to a lesser extent the dipeptide glycylglycine, have strong effects on copper speciation. The inhibitory effects of glycine and glutamine (Fig. 4b, c) may therefore represent the effect of a lowered transport substrate (copper–histidine chelate) concentration, rather than a competitive effect on a transport pathway. Predicted levels of copper–histidine substrates (Table 3) were substituted into the respective concentration-dependent uptake equations (see above), and the uptake at the predicted copper–histidine levels was calculated (Table 3). This was then compared with the experimentally measured uptake. The results of this analysis (Table 3) showed that the effect of glycine persisted when accounting for speciation changes, whereas the effect of glutamine was abolished. This suggests that the impact of glutamine on copper transport in the presence of 780 μM of histidine is likely to be an artifact of altered speciation. It is also interesting to note that although phenylalanine has a strong affinity for copper, and therefore reduced the substrate concentration (Table 2), copper transport was maintained at control levels.

Discussion

Apical copper uptake

Previous investigations of copper transport across the intestinal epithelium of fish, have speculated that the rate-limit-

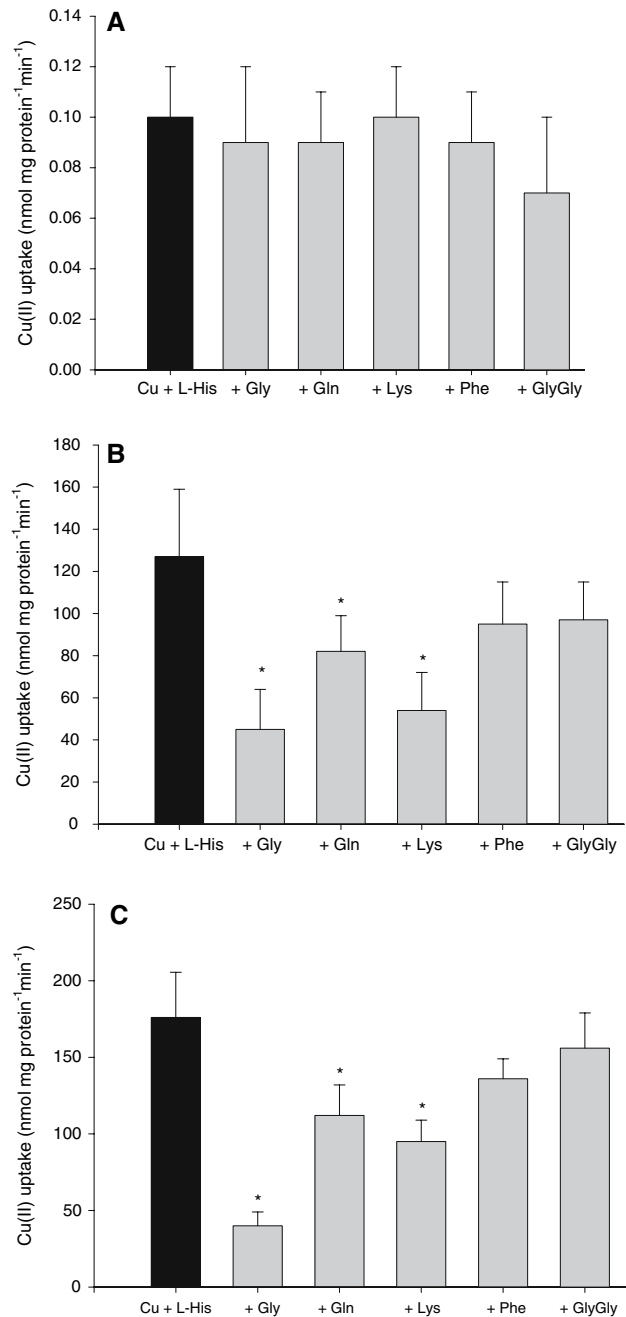


Fig. 4 Intestinal BBMVs uptake of **a** 7.4 μM, **b** 699 μM, and **c** 1,028 μM copper (nmol mg protein⁻¹ min⁻¹) in the presence of 780 μM L-histidine and tenfold excess of potential transport-system inhibitors. Plotted points represent the means (±SEM) of 4 (699 and 1,028 μM) or 8 (7.4 μM) replicates, measured in triplicate. Significant differences (**P* < 0.05) were determined by one-way ANOVA, followed by LSD post-hoc analysis. Gly glycine, Gln L-glutamine, Lys L-lysine, Phe L-phenylalanine, GlyGly glycylglycine

ing step of copper absorption is movement across the basolateral membrane, with passive entry across the apical membrane (e.g. Clearwater et al. 1998; Handy et al. 2000; Nadella et al. 2006b). Using a technique that isolates the

Table 2 Copper speciation (%) in the presence of high total copper (699 or 1,028 μM), high L-histidine (780 μM), and 7.8 mM of putative histidine transport competitors (X)

	Cu ²⁺	Cu(His) ⁺	Cu(His) ₂	Cu(X) ⁺	Cu(X) ₂
699 μM Cu; 780 μM His	13		86		
+Gly			54		46
+Gln			53		47
+Lys	14		84		
+Phe			50		49
+GlyGly	40		36	24	
1,028 μM Cu; 780 μM His	11	74	0.8		
+Gly			37		62
+Gln			36		63
+Lys	8.3	74	1.0		7.4
+Phe			35		64
+GlyGly	39		19	42	

Speciation determined using MINEQL+ (Schecher and McAvoy 1992). Where percentage values do not add to 100, the remainder of copper was as present as hydroxide (Cu₂OH²⁺, CuOH⁺, Cu(OH)₂) and/or chloride (CuCl⁺) species

Gly glycine, Gln L-glutamine, Lys L-lysine, PheL-phenylalanine, GlyGly glycylglycine

apical intestinal membrane, the current study showed that apical copper uptake proceeds via a low affinity process. Based on total copper in the extravesicular solution, this process was saturable. However, speciation analysis showed that as copper increased, the proportion of free copper decreased, meaning that the appearance of uptake saturation was likely an artifact of speciation. Calculation on the basis of free copper (Cu²⁺) resulted in a linear curve, demonstrating that apical copper uptake is speciation-dependent, and copper absorption is likely diffusive, though linear kinetics in themselves do not eliminate mediation by channels or carriers. Nevertheless, a passive entry mechanism would be consistent with some previous data regarding copper transport across this membrane in fish (Clearwater et al. 1998; Handy et al. 2000; Nadella et al. 2006b).

Table 3 Predicted versus measured copper uptake in the presence of high total copper (699 or 1,028 μM), high L-histidine (780 μM), and 7.8 mM of putative histidine transport competitors glycine and glutamine

	Histidine-liganded Cu (μM)	Predicted Cu uptake (nmol mg protein ⁻¹ min ⁻¹)	Measured Cu uptake (nmol mg protein ⁻¹ min ⁻¹)
699 μM + 780 μM histidine + 7.8 mM glycine	368	88	45 \pm 19
1,028 μM + 780 μM histidine + 7.8 mM glycine	374	90	40 \pm 9
699 μM + 780 μM histidine + 7.8 mM glutamine	374	90	82 \pm 17
1,028 μM + 780 μM histidine + 7.8 mM glutamine	377	90	112 \pm 20

Predicted uptake was determined using the equation of the curve shown in Fig. 3. See text for details. Speciation determined using MINEQL+ (Schecher and McAvoy 1992)

Pharmacological and physiological evidence currently points to apical intestinal copper uptake in fish via either DMT1 (Nadella et al. 2007) or Ctr1 (Burke and Handy 2005). However, there remains some evidence that copper may be taken up via a phenamil-sensitive sodium channel (Burke and Handy 2005; Nadella et al. 2007). A channel mechanism may more easily explain the linear pattern of uptake observed herein. The high extravesicular sodium levels used in the present study may have outcompeted copper for transport via such a mechanism thus accounting for the relatively low affinity of apical copper uptake.

It remains possible that the methodological approach may have obscured uptake via a low capacity, high affinity transporter. In the present study, copper uptake was examined over a wide range of concentrations. This approach is favoured in that it generally permits the delineation of multiple transport pathways with different affinities and transport characteristics, should they exist (e.g. Glover et al. 2003). In this study, however, it was not possible to mathematically isolate high affinity copper transport. Saturable copper transport pathways with affinity constants of 32 μM (Nadella et al. 2006b) and 216 μM (Burke and Handy 2005) have previously been described in rainbow trout intestine, although these studies were conducted on more complex preparations incorporating additional uptake barriers. In rat intestinal BBMVs a high affinity, low capacity transporter has been described (Knöpfel et al. 2005). The affinity constant of 0.3 μM elucidated by these authors would be completely saturated by even the lowest copper level tested in the present study. It would also be saturated at chyme copper levels in the intestine (Nadella et al. 2006a), so the biological relevance of such a transporter, should it exist in fish gut, would be questionable.

Apical copper uptake in presence of histidine

The presence of 780 μM histidine significantly altered the uptake of copper (Fig. 3). At this histidine level, in the absence of potential competitors, almost all copper at all concentrations examined was present chelated to histidine (Table 1). This indicates that this form of copper is

bioavailable for uptake by the apical brush-border of rainbow trout intestine, via a pathway likely to be distinct from that facilitating transport in the presence of copper alone. The presence of copper was recently described as diverting histidine uptake to a transport system different from that used to achieve uptake in the presence of histidine alone (Glover and Wood 2007). This suggests that a distinct transport system exists for copper–histidine chelates. This is further supported by the data obtained for copper uptake in the presence of histidine (Fig. 4) in the present study. Under these conditions, copper uptake at high concentrations was significantly inhibited by putative histidine transport blockers, glycine and lysine (also glutamine, although see discussion below). In contrast, histidine uptake across rainbow trout intestinal brush-border could not be inhibited by these substrates (Glover and Wood 2007). These results indicate that copper uptake in the presence of histidine occurs via a transport system that is distinct from both the system responsible for copper uptake alone, and that mediating histidine alone.

The use of putative uptake competitors is a useful experimental approach to determining the nature of the transport system facilitating absorption (Christensen 1989). In accordance with proposed mammalian transport systems for histidine (Bröer 2002), potential inhibitors of histidine uptake were tested for their effect on copper absorption in the presence of this amino acid. There is an additional complication, however, given the speciation-dependence of copper absorption across the brush-border. Copper binds histidine with high affinity, but will also bind other amino acids. The chelate formed between copper and the inhibiting amino acid may simply act to reduce free copper and/or copper–histidine levels and may thus reduce copper uptake by lowering transporter substrate levels, rather than by competitively blocking uptake. It is important to account for this effect, a process, which can be aided by geochemical speciation analysis.

In this study glycine, glutamine, and lysine were all shown to inhibit histidine-facilitated copper uptake (Fig. 4b,c). A geochemical modelling approach was performed (MINEQL+; Schecher and McAvoy 1992) to determine the nature of this effect. The levels of copper–histidine chelates were calculated in the presence of tenfold excess of these potential competitors (Table 2). Clearly, lysine had limited impact on copper–histidine speciation, and therefore the inhibitory effect should be considered a genuine competitive interaction. The negative effect of lysine is curious given its reported lack of effect on copper bioavailability in dietary feeding trials in fish (Kjoss et al. 2006). Glycine and glutamine, in contrast, did significantly lower copper–histidine concentration. For these amino acids the predicted copper–histidine concentration from speciation analysis was substituted into the kinetic uptake curve

(Fig. 3), and a predicted uptake was calculated. As shown in Table 3, the uptake of copper–histidine chelate in the presence of glutamine was likely explained simply by the change in speciation, whereas for glycine the measured uptake remained significantly reduced compared to the predicted uptake, indicating a competitive effect. Interestingly, although the amino acid phenylalanine has a relatively high affinity for copper, and consequently reduced the copper–histidine substrate levels (see Table 2), transport was not impaired. This suggests that copper–phenylalanine may be bioavailable, and uptake of this complex may proceed by a pathway distinct from that of copper–histidine chelates.

The conclusion of the speciation analysis was that glycine and lysine significantly impaired copper–histidine uptake. According to the transport scheme described by Bröer (2002), these two amino acids do not share a common uptake pathway with histidine. This scheme was derived for mammals, and is quite possibly inappropriate for use in fish. It has been suggested that amino acid uptake in fish is more promiscuous than that in mammals (Huang and Chen 1975), although definitive evidence supporting this view is lacking. It is therefore possible that these substrates could share a single common transport pathway in fish gut. A more likely proposition is the presence of two transporters, one blocked by lysine, the other by glycine. Studies combining these inhibitors in the presence of copper and histidine would be required to confirm this.

The nature of these two transporters can be hypothesised, based on the scheme for amino acid transport specificities in mammals (Bröer 2002). Lysine shares transport systems $b^{0,+}$ and $B^{0,+}$ with histidine, suggesting that competitive interactions at these loci may explain the negative effect of lysine on histidine-mediated copper uptake. However phenylalanine is also a $b^{0,+}$ and $B^{0,+}$ substrate, yet did not inhibit uptake likely eliminating this as a potential transport route. Systems $y+$ and $y+L$ also facilitate transport of lysine and histidine, although the latter system is also shared with glutamine (Bröer 2002). Intriguingly the $y+$ system has previously been proposed as a candidate for histidine-facilitated zinc uptake in human erythrocytes (Horn et al. 1995) and is known to have a gastrointestinal distribution in mammals (Ganapathy et al. 2001). Glycine shares transport systems A and N with histidine (Bröer 2002). Although transcripts of these systems have been located to mammalian intestine (Hatakana et al. 2000; Wang et al. 2000; Nakanishi et al. 2001), transport is also shared with glutamine, which after speciation calculations, was not found to inhibit copper–histidine uptake. It is clear that further analysis, including molecular characterisation, is required to ascertain the mechanisms by which copper–histidine chelates are transported across the apical surface. In particular the dichotomy between the apparent lack of

glutamine effect, as predicted by speciation, and its shared specificity with amino acids shown to be inhibitors in the present study, requires investigation. It is possible that copper–glutamine chelates, like those of phenylalanine, may in fact be transportable substrates. This would account for the observed effects, with possible stimulation of copper uptake by transport of a copper–glutamine species, offsetting the competitive effects of glutamine on the copper–histidine moiety.

Are copper-histidine chelates transported?

Amino acid supplementation of diets has positive effects on whole body metal accumulation (e.g. Apines et al. 2003), and may be responsible for amelioration of important aquacultural diseases such as cataracts (e.g. Breck et al. 2003). The positive effects of amino acid chelation on metal bioaccumulation are clearly a function of enhanced bioavailability. Key questions remain regarding the mechanism of this effect. The enhanced bioavailability could be the result of histidine acting as a donor–ligand shuttling copper from inhibitory ligands in the diet to a dedicated copper transporter, or due to the creation of an alternative transport pathway, which accepts the copper–histidine chelate as a substrate.

These competing theories have been tested in rat hepatocytes (Darwish et al. 1984). On the basis of shared transport kinetic characteristics of copper alone and in the presence of histidine, the donor ligand hypothesis was favoured (Darwish et al. 1984). Conversely, in the light of new information regarding histidine uptake (Glover and Wood 2007), the transported chelate theory can now be considered most likely to explain histidine-mediated copper and zinc transport in rainbow trout intestine (Glover et al. 2003; Nadella et al. 2006b). Similarly, a transported substrate, hypothesised to traverse the epithelium via a peptide transporter, was proposed for histidine–zinc chelates in lobster intestine (Conrad and Ahearn 2005). The transport characteristics of copper in the present study clearly differ in the presence of histidine. Accounting for speciation, the linear copper uptake in the absence of histidine, is transformed to a saturable mechanism in its presence. Furthermore, copper–histidine transport was specifically inhibited by amino acid transport system blockers, suggesting that an amino acid transporter was involved.

Considerations and conclusions

Mechanistic examination of histidine-mediated metal uptake is technically challenging. Factors such as metal concentration; oxidation state; the nature, concentration, and binding affinities of potential ligands; the bioavailability of these chelates of different stoichiometries; the pres-

ence and concentration of moieties that may compete, or be cofactors, for potential transporters; the chemical state of the gut environment and epithelial microenvironment (e.g. pH) may all exert significant influence over uptake. Additionally biological factors such as the transporters themselves (their kinetic characteristics, substrate specificities, exposure history, and systemic regulation) can also have important impacts. These factors will clearly vary considerably between studies and are likely to contribute to the disparities noted between laboratories and techniques used to investigate ligand effects on metal uptake.

For metals, uptake may also be complicated by potential toxic effects that impair absorption. Bioavailability, and consequently bioaccumulation, play important roles in determining the nutritional value and toxicological threat of dietary metals (Bury et al. 2003; Borgmann et al. 2006). This knowledge is therefore critical to both aquaculture and environmental protection. As demonstrated herein, considerable basic knowledge of uptake mechanisms is required to enhance our general understanding, before the principles may be applied to prediction of metal bioavailability and uptake in complex mixtures such as the diet (Borgmann et al. 2006).

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