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Biochimica et Biophysica Acta 1614 (2003) 211–219
BIOCHIMICA ET BIOPHYSICA ACTA
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Zinc uptake across the apical membrane of freshwater rainbow trout intestine is mediated by high affinity, low affinity, and histidine-facilitated pathways

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Received 5 February 2003; received in revised form 28 May 2003; accepted 4 June 2003

Abstract

Zinc is both a vital nutrient and an important toxicant to aquatic biota. In order to understand the interplay between nutrition and toxicity, it will be important to determine the mechanisms and the factors that regulate zinc uptake. The mechanism of apical intestinal Zn(II) uptake in freshwater rainbow trout and its potential modification by the complexing amino acid histidine was investigated using brush-border membrane vesicles (BBMVs). Following characterisation of the BBMV preparation, zinc uptake in the absence of histidine was both time- and concentration-dependent and consisted of two components. A saturable phase of uptake was described by an affinity constant of 57 ± 17 μM and a transport capacity of 1867 ± 296 nmol mg membrane protein^{-1} min^{-1}. At higher zinc levels (>500 μM) a linear, diffusive component of uptake was evident. Zinc transport was also temperature-dependent, with Q_{10} values suggesting zinc uptake was a carrier-mediated process. Zinc uptake by vesicles in the presence of histidine was correlated to a mono-histidine species ($Zn(His)^{+}$) at all Zn(II) concentrations examined.

Keywords: Dietary metal; Intestine; Amino acid chelate; Fish; Zinc; Absorption

1. Introduction

The absorption of zinc is a physiological necessity. By virtue of vital structural and/or catalytic roles in more than 300 proteins, the failure to maintain adequate zinc levels impedes a wide range of physiological processes. For example, zinc deficiency impairs growth and development, and compromises immune and reproductive status in mammals (see Ref. [1]). Similar disturbances in piscine health and nutrition are also associated with inadequate zinc intake [2]. Conversely, zinc toxicity may prevail when fish are exposed to elevated environmental zinc levels [3]. The dual uptake pathways associated with immersion in a polluted medium, and the subsequent reliance on a contaminated diet, is a toxicological dilemma unique to aquatic biota. This is further complicated by the need to maintain adequate nutrition, while limiting toxic actions. Consequently, zinc absorption and assimilation is under homeostatic regulation.

For fish, two major routes of absorption exist—the gut and the gills [3]. In freshwater fish waterborne zinc is primarily absorbed via the gills, whereas uptake via the gastrointestinal tract is associated with dietary, particulate and sedimentary zinc. Quantitatively these latter sources are likely to be more important, as reflected in the greater relative contribution of the gut towards total zinc body burden [4]. Branchial zinc uptake is relatively well characterised, with the passage of zinc through an apical calcium channel in the ion transporting cells believed to be a major route of absorption (see Ref. [3] and references therein). In contrast, the mechanism of zinc uptake in the alimentary canal of fish is less well studied. In marine teleosts, two components of zinc absorption have been described [5,6]. A saturable component exists at physiological luminal concentrations, while an additional, linear component is apparent with increasing luminal zinc levels. This was similar to the reported pattern in mammalian systems [7]. In freshwater fish, however, only saturable uptake has been described [8]. In this in vivo study it was proposed that a significant mucus

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doi:10.1016/S0005-2736(03)00178-0

secretion response obscured the linear, diffusive, uptake component reported in the gut of marine teleosts and mammals [8]. The mechanism of intestinal zinc transport in freshwater teleosts clearly requires further characterisation to determine if a diffusive component of uptake exists in a system free from the confounding effects of mucus.

Using this same in vivo perfusion technique, important influences of luminal amino acids on zinc uptake were described for freshwater trout intestine [9]. Histidine and cysteine chelation of zinc was not found to greatly influence the amount of zinc absorbed. However, these amino acids significantly altered the distribution of zinc within the body, in a stereoisomer-independent but speciation-dependent manner [9]. These findings could explain apparent discrepancies between aquacultural studies that showed no increase in zinc uptake (e.g. Ref. [10]), and those exhibiting beneficial health effects (e.g. Ref. [11]), upon the addition of amino acid-chelated zinc to piscine diets. In fact, by analogy with studies in other organisms (e.g. Ref. [12,13]), including mammalian intestine [14], it was suggested that a specific transport pathway involving zinc–histidine chelates may exist in trout intestine [9].

Considerable advances in knowledge of the molecular nature of zinc transporters have been made in recent years (see Ref. [15]). Despite this, there is still little information regarding the physiological aspects of zinc absorption pathways across the intestinal epithelium. In the present study brush-border membrane vesicles (BBMVs) were used to examine intestinal zinc transport across the apical membrane of freshwater rainbow trout. This technique circumvents the confounding influence of mucus secretion, which considerably obscured mechanistic analysis of zinc absorption in vivo. The BBMV preparation was analysed for purity and quality, before characterisation of zinc uptake, in the presence or absence of histidine, across the apical membrane was performed.

2. Materials and methods

2.1. Animals

Adult rainbow trout (*Oncorhynchus mykiss*, ~ 200–600 g) were obtained from Houghton Springs fish farm in Dorset, England. Fish were maintained in 300–400 l fibreglass tanks supplied with flowing and aerated City of London tap water that was passed through activated carbon, mechanical and biological filters. Water temperature was maintained between 11 and 15 °C, varying with season. Fish were fed to satiation three times a week on Mazuri Aquatic trout pellets containing 83 mg kg^{-1} Zn(II).

2.2. Membrane collection and purification

Collection and isolation of brush-border membranes was based on the protocol described by Pelletier et al. [16]. Following euthanasia, the entire length of the intestine (from pyloric sphincter to anus) was removed and flushed with ice-cold saline (0.9% NaCl, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol; Sigma). All subsequent steps were performed on ice or at 4 °C. The intestine was split longitudinally, blotted to remove mucus, and scraped with a glass microscope slide. Scrapings were immediately transferred to 35 ml of buffer 'A' (50 mM mannitol, 2 mM EGTA, 0.5 mM $MgSO_4$, 0.1 mM phenylmethanesulfonylflouride (PMSF; Sigma); pH 7.4) and homogenised with 20 strokes of a Dounce homogeniser ('loose' pestle). This homogenate was then centrifuged at 12 000 × *g* for 15 min (Beckman J2-21 refrigerated centrifuge, 4 °C). The resulting white 'fluffy' layer was removed and made up to 30 ml with buffer 'B' (0.32 M sucrose, 10 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris; Sigma), pH 7.4), and homogenised with 25 strokes by Dounce homogenisation ('tight' pestle). Solid $MgCl_2 \cdot 6H_2O$ was then added to a final concentration of 10 mM and the homogenate left for 15 min on ice with occasional agitation. The supernatant obtained by a 10-min centrifugation at 20 200 × *g* was further spun for 20 min at 43 500 × *g* (Beckman L8-80M Ultracentrifuge). The resulting pellet contained the brush-border membrane fraction. This pellet was resuspended in standard assay buffer (149 mM KCl, 1 mM NaCl, 10 mM *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; Sigma), pH 7.4) by 30 passages through a 23-gauge needle [17]. Protein content of the brush-border fraction was determined by measuring the absorbance at 595 nm resulting from protein interaction with Coomassie brilliant blue dye [19]. Vesicle solutions were subsequently made up to 1 mg protein ml^{-1} with assay buffer.

The major deviation from the protocol described by Pelletier et al. [16] was the substitution of magnesium for calcium at the vesicle aggregation step. Calcium is known to activate membrane phospholipases potentially causing membrane degradation [20], while magnesium is reported to result in piscine (i.e. Tilapia) intestinal vesicle populations that are completely oriented with the brush-border exposed to the external medium [17].

2.3. Analysis of membrane purity

Marker enzymes were analysed to determine the recovery and purity of the isolated membranes. Alkaline phosphatase (EC 3.2.1.48; Sigma Diagnostics kit 104) was used as a marker of brush-border membrane recovery [16,21]. The contamination of the brush-border membrane preparation by basolateral membranes and mitochondrial membranes was determined by analysis of the activities of the enzyme markers, Na^+, K^+-ATPase (EC 3.6.1.3) and succinate dehydrogenase (EC 1.3.99.1), respectively [16,22]. The Na^+, K^+-ATPase assay procedure followed that described by Bury et al. [23].

For all enzymes assays activities in initial homogenates were compared to activities in the membrane vesicle solu-

tion to determine the enrichment and purification of each membrane fraction. These results are illustrated in Table 1.

2.4. Analysis of vesicle quality

To determine the membrane orientation of vesicles, alkaline phosphatase activity in untreated preparations was compared to activity in detergent-treated preparations. Vesicles were incubated in Triton X-100 (0.2% w/v) at 37 °C for 1 min, to permeabilise the vesicles before alkaline phosphatase activity was measured (Sigma diagnostics kit 104). The ratio of permeabilised to untreated alkaline phosphatase activity was used as a measure of the proportion of correctly oriented (i.e. right-side out) vesicles [24].

BBMV integrity was also characterised experimentally. Vesicles were added to 100 μM Zn(II) in the presence of 200, 400, 600 or 800 mM D-mannitol. Membrane preparations were incubated for 10 min and assayed as described below. The differing osmolarity between the inside of the vesicles (assay buffer only) and the mannitol solutions on the exterior should alter the intravesicular space, and consequently the vesicular Zn(II) accumulation [25].

2.5. BBMV fluxes

The time- and concentration-dependent uptake of Zn(II) by BBMVs was analysed by a rapid filtration method. Aliquots of membrane vesicles (35 μl; i.e. 35 μg of protein) were preincubated at 37 °C for 1 min. This incubation temperature has been used successfully for membrane vesicle experiments in rainbow trout [23]. Vesicles were then added to 125 μl of pre-warmed reaction buffer (149 mM KCl, 1 mM NaCl, 10 mM HEPES, pH 7.4, ~62.5 kBq ml^{-1} $^{65}Zn(II)$ ($ZnCl_2$; Perkin Elmer)). Solutions were made up to the appropriate Zn(II) concentration by the addition of unlabelled $ZnSO_4 \cdot 7H_2O$ (see below).

BBMVs were incubated for 0.5, 1, 5 or 10 min before 45-μl aliquots were added in triplicate to 0.45-μm pore size methylcellulose membrane filters (ME25; Schleicher and Schüell) placed in a rapid filtration unit (Millipore). Filters were presoaked in ice-cold stop/wash buffer (149 mM KCl, 1 mM NaCl, 0.5 mM EGTA, pH adjusted to 7.4 with Tris). Following the vacuum filtration of membrane vesicles, the filters were washed twice with 2 ml of ice-cold stop/wash buffer. Membrane filters were then counted for $^{65}Zn(II)$ activity (LKB Wallac, 1282 compugamma).

Kinetic analysis of Zn(II) uptake was performed at Zn(II) concentrations of 1, 10, 100, 500 and 1000 μM, while the temperature dependence of Zn(II) uptake was examined over the three lower Zn(II) concentrations. These experiments were identical in nature to control experiments at 37 °C except preincubation, and incubation was performed at temperatures of 0 or 18 °C. This range of temperatures allowed examination of the Q_{10} effect. This is a ratio of the rate of a physiological reaction at two temperatures, standardised to a 10° increase in temperature. Q_{10} values were calculated as follows:

$$Q_{10} = (k_2/k_1)^{10/(T_2-T_1)} \quad (1)$$

where k refers to the reaction rate and T is the reaction incubation temperature.

For all manipulations, the stop/wash buffer contained 0.5 mM EGTA. This membrane-impermeant Zn(II) chelator removes extracellularly bound Zn(II) that could confuse measures of true Zn(II) uptake [26]. The importance of this Zn(II) pool was examined by a set of experiments (at Zn(II) concentrations of 1, 100 and 1000 μM Zn(II)), in which EGTA was removed from the stop/wash buffer.

To test the influence of histidine–Zn(II) complexes upon Zn(II) uptake, a range of chelated histidine–Zn(II) species and concentrations were achieved by the addition of L- or D-histidine (Analytical grade; Fluka) to the Zn(II)-containing extravesicular medium at concentrations of 50 or 500 μM (see Table 1). In another set of experiments, an inorganic Zn(II) chelator, bicarbonate ($NaHCO_3$; 1 mM), was added to the extravesicular medium. Intravesicular medium was identical to that in control (Zn(II) alone) experiments.

2.6. Calculations

Zn(II) uptake was calculated according to the following formula:

$$U = (\text{cpm}/\text{SA})/[p] \quad (2)$$

where cpm is the counts per minute, SA is the specific activity (cpm $nmol^{-1}$), and $[p]$ is the protein concentration in μg μl^{-1}. This gives a Zn(II) uptake (U) expressed as nmol μg $protein^{-1}$. Initial uptake rates were calculated in an identical manner except the uptake was divided by a time factor (0.5 min) resulting in an uptake rate expressed as nmol μg $protein^{-1}$ min^{-1}. All data were background-corrected for nonspecific Zn(II) binding to membrane filters, determined under each set of conditions tested.

Data are expressed as means ± S.E. Significant effects ($P < 0.05$) of treatments were determined using analysis of

Table 1
Marker enrichment and recovery for brush-border membrane vesicles prepared from rainbow trout intestine

Marker	Enrichment[a]	Recovery[b] (%)	n
Alkaline phosphatase	9.69 ± 4.92	5.21 ± 0.76	10
Succinate dehydrogenase	0.21 ± 0.05	0.84 ± 0.38	6
Na^+, K^+-ATPase	1.07 ± 0.41	1.39 ± 0.48	4
Protein		2.02 ± 0.67	10

Data are expressed as mean ± S.E.

[a] Enrichment is the ratio of enzyme specific activity in the brush-border membrane vesicle fraction to that in the initial homogenate.

[b] Recovery is the proportion of total activity in the initial homogenate recovered in the brush-border membrane fraction, where total activity is the specific activity multiplied by the total protein.

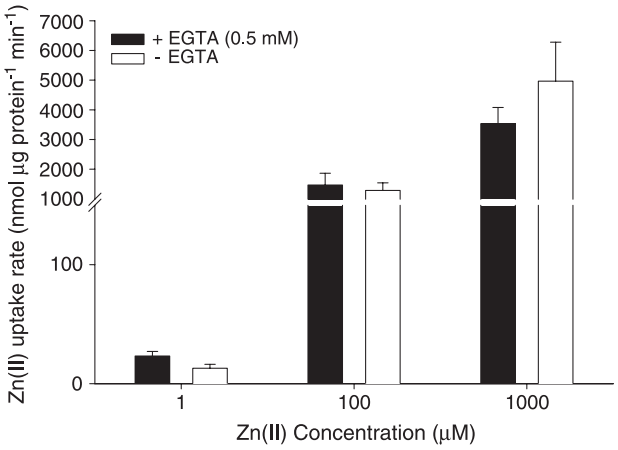

Fig. 1. Effect of EGTA removal from the wash buffer on Zn(II) uptake rate into intestinal BBMVs. Initial uptake rates (nmol µg protein^{-1} min^{-1}) were determined at the stated Zn(II) concentration, after 30-s incubations at 37 °C. Values represent means ± S.E. for 4 (−EGTA), 5 (1000 µM, +EGTA) or 10 (1, 10 µM+EGTA) experiments. Zn(II)/EGTA ratio for black bars is 0.002, 0.2 and 2 at 1, 10 and 1000 µM, respectively.

variance (ANOVA), followed by a post-hoc least significant differences (LSD) test.

3. Results

3.1. Characterisation of the BBMV preparation

Table 1 shows that the methods used for isolation of the brush-border membrane fraction were successful in preparing a highly enriched (9.7-fold) preparation, relatively free from contamination by mitochondrial (0.21% enrichment) and basolateral membranes (1.07% enrichment). These results correspond closely to previous investigations. Enrichment factors of 2.5 to 18.2 for brush-border; 0.15 to 3.7 for mitochondrial; and 1.3 to 3 for basolateral fractions have been noted previously for fish intestinal brush-border preparations [16,17,21]. Analysis of alkaline phosphatase activity before and after permeabilisation revealed the vesicles were oriented right-side out (111 ± 16%; mean ± S.E.). This also corresponds to previous findings for tilapia intestinal BBMV preparations [17].

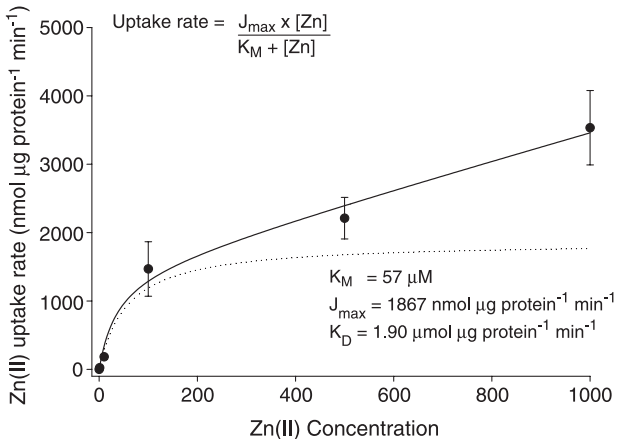

Fig. 2. Zn(II) uptake rate (nmol µg protein^{-1} min^{-1}) into intestinal BBMVs, after a 30-s incubation at 37 °C, as a function of extravesicular Zn(II) concentration. Kinetic parameters were calculated directly from the graph using SigmaPlot (SPSS, Inc.). Plotted points represent the means ± S.E. of 5 (500, 1000 µM) to 10 (1–100 µM) experiments. Dotted line represents the saturable component alone.

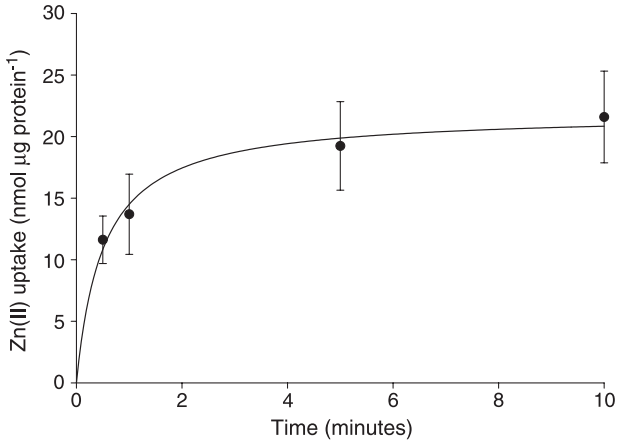

Fig. 3. Zn(II) uptake (nmol µg protein^{-1}) into intestinal BBMVs as a function of incubation time, at an extravesicular Zn(II) concentration of 1 µM. Plotted values represent the means ± S.E. of 8–10 data points.

To further confirm vesiculisation, the osmotically sensitive space was probed by incubating vesicles in a range of mannitol concentrations. These results (not shown) exhibited a preparation in which Zn(II) accumulation was inversely correlated to osmolarity ($r^2 = 0.92$), i.e. positively correlated to intravesicular space. This suggested that the BBMVs were sealed. Extrapolation to infinite osmolarity

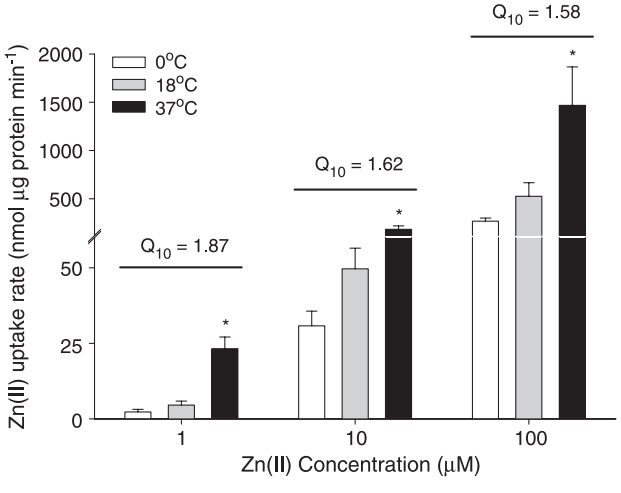

Fig. 4. Zn(II) uptake rate (nmol µg protein^{-1} min^{-1}) into intestinal BBMVs as a function of incubation temperature (0, 18 or 37 °C), following a 30-s incubation. Q_{10} ratios were calculated as described in the text, on the ratio between uptake rates at 0 and 37 °C. Plotted values represent the means ± S.E. for 3 (0, 18 °C) and 10 (37 °C) experiments. Uptake rates that were statistically different ($P < 0.05$) at 37 °C compared to the lesser temperatures (*) were determined by ANOVA.

Table 2
Zn(II) speciation in the experimental histidine solutions

Experimental conditions	Zn(II) concentration (μM)	Zn(II) species (% of total Zn(II))		
		Zn^{2+}	$Zn(His)^{+}$	$Zn(His)_2$
Zn(II) alone	1	77.5	0	0
	10	77.5	0	0
	100	77.4	0	0
50 μM histidine	1	14.1	67.3	14.5
	10	16.7	66.5	12
	100	46.4	38.6	1.4
500 μM histidine	1	0	31.1	68.1
	10	0	31.7	67.4
	100	1.2	39.3	59.2

Zn(II) speciation was calculated using the geochemical speciation program MINEQL+ (Version 4.01; Environmental Research Software). The log K values used in the calculation of speciation were 7.3 and 13.0 for $Zn(His)^{+}$ and $Zn(His)_2$, respectively. Where percentages do not add to 100%, the remainder was comprised of minor Zn(II) species (primarily $Zn(OH)^{+}$, $ZnCl_2$, and $ZnCl^{+}$).

(zero intravesicular space) yielded a considerable membrane Zn(II) binding component (553 nmol μg protein^{-1}). The Zn(II) binding component was present independent of EGTA in the stop/wash buffer. These results show that EGTA did not appear to remove any more Zn(II) than did EGTA-free buffer (Fig. 1).

3.2. Zn(II) uptake by BBMVs

Zn(II) uptake consisted of two components (Fig. 2). Passage across the brush-border membrane was best described by a saturable component, superimposed upon a linear uptake mechanism that dominated at higher concentrations. The saturable component was analysed by Michaelis–Menten kinetics, which revealed a maximal Zn(II) uptake rate (J_{max}) of 1867 ± 296 nmol μg protein^{-1} min^{-1}, with uptake half-saturated (K_M) at a Zn(II) concentration of 57 ± 17 μM. The diffusive component was expressed by a constant (K_D) with a magnitude equal to 1.90 μmol μg protein^{-1} min^{-1}. Uptake components were modelled concurrently over the entire Zn(II) concentration range using SigmaPlot 7.0 software (SPSS Inc.).

Fig. 3 illustrates a time course of Zn(II) association with membrane vesicles at 1 μM of Zn(II). Zn(II) accumulation follows a hyperbolic pattern, with a rapid, initial uptake phase over the first minute, with steady-state achieved within 10 min of exposure to extravesicular Zn(II). BBMVs incubated for extended periods (>10 min) exhibited lowered Zn(II) uptake, suggesting a loss of membrane integrity, or significant Zn(II) efflux activity (data not shown).

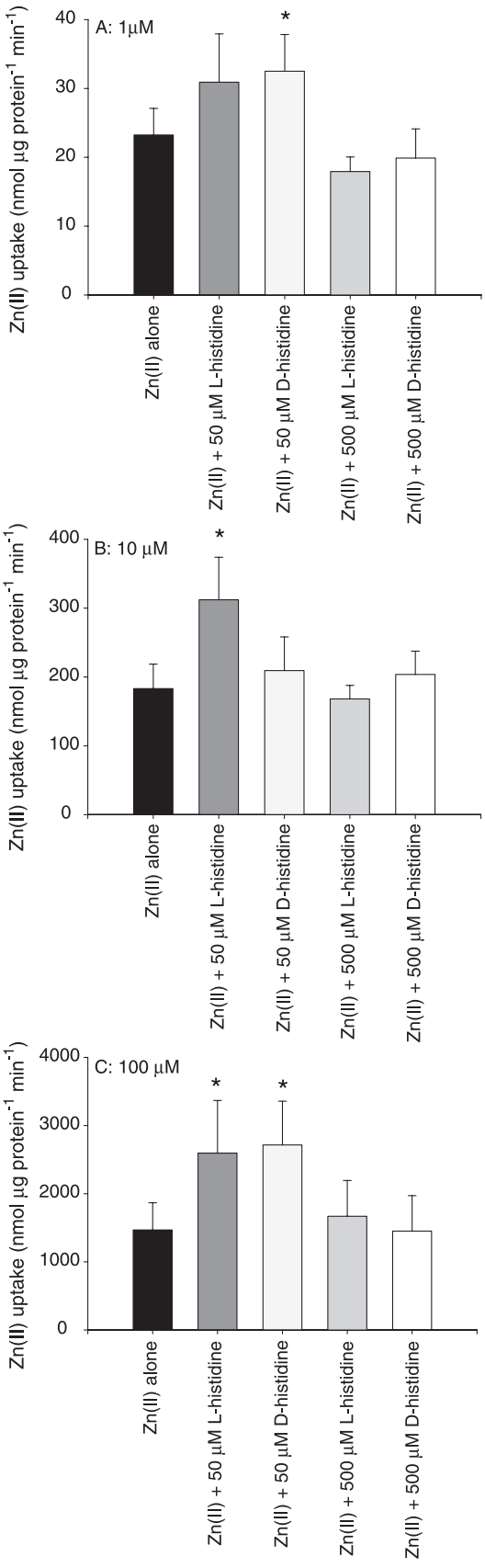

Fig. 5. (A–C) Influence of 50 or 500 μM L- or D-histidine upon Zn(II) uptake rate (nmol μg protein^{-1} min^{-1}) into intestinal BBMVs following a 30-s incubation at Zn(II) concentrations of 1 (A), 10 (B) or 100 μM (C) at 37 °C. Plotted points represent the means ± S.E. of 4 (all histidine treatments) or 10 (Zn(II) alone) experiments. Statistical differences (P<0.05) between amino acid treatments and Zn(II) alone (*) were determined by ANOVA, followed by post-hoc LSD analysis.

Temperature dependence of Zn(II) uptake was also examined (Fig. 4). Statistically significant increases in Zn(II) uptake were noted at all concentrations when the incubation temperature was increased from 0 to 18 °C and from 18 to 37 °C. Q_{10} values ranging from 1.58 to 1.87 for 100 and 1 μM, respectively, were discerned, indicative of a process not solely dependent on the physicochemical properties of the substrates.

3.3. Influence of extravesicular histidine on Zn(II) uptake

The presence of histidine greatly altered the speciation of Zn(II) in the extravesicular medium (Table 2). At the histidine concentrations investigated (50 and 500 μM), a wide range and proportion of Zn(II) species were discerned over the range of Zn(II) concentrations examined. The relative proportions of each Zn(II) species were calculated using a geochemical speciation modelling program (MINEQL+, Version 4.01; Environmental Research Software). A mono-chelated histidine species ($Zn(His)^+$) was the dominant form of Zn(II) at 50 μM histidine, while the bis-chelated form ($Zn(His)_2$) was most prominent at 500 μM histidine. Free ionic Zn^{2+} varied from 77.5% in the histidine-free buffers to absent when extravesicular histidine concentrations were 500 μM. Analysis of Zn(II) speciation in bicarbonate experiments showed that at all Zn(II) concentrations tested, free, ionic Zn^{2+} was absent.

The influence of histidine upon Zn(II) uptake is exhibited in Fig. 5A–C. Histidine at 50 μM stimulated initial Zn(II) uptake. The magnitude of this stimulation was as much as 1.8-fold (at 100 μM Zn(II) for D-histidine), and was statistically significant for at least one of the stereoisomers at each Zn(II) concentration examined. Conversely, 500 μM histidine had no statistically significant action upon Zn(II) uptake at any of the concentrations. At all concentrations, over all of the examined time points (not shown) there were no significantly different effects on Zn(II) absorption between L- and D-stereoisomers of histidine. Subsequently, for correlation analysis (below) results from D- and L-isomers were collated.

Table 3
Correlation of Zn(II) speciation to Zn(II) uptake in the presence of histidine

Zn(II) concentration (μM)	Zn(II) species	Pearson correlation coefficient
1	Zn^{2+}	0
	$Zn(His)^+$	0.469*
	$Zn(His)_2$	0
	$Zn^{2+} + Zn(His)^+$	0.343*
	$Zn^{2+} + Zn(His)_2$	0
10	Zn^{2+}	0
	$Zn(His)^+$	0.588*
	$Zn(His)_2$	0.044
	$Zn^{2+} + Zn(His)^+$	0.113
	$Zn^{2+} + Zn(His)_2$	0
100	Zn^{2+}	0
	$Zn(His)^+$	0.432*
	$Zn(His)_2$	0
	$Zn^{2+} + Zn(His)^+$	0.193
	$Zn^{2+} + Zn(His)_2$	0

Correlation coefficients represent predicted Zn(II) species concentrations (calculated using the geochemical speciation program MINEQL+ (Version 4.01; Environmental Research Software)) versus Zn(II) uptake in the presence of 0, 50 and 500 μM histidine. D-Histidine and L-histidine data were pooled. Negative correlations are recorded as 0. Where indicated (*), significance was tested at the $\alpha = 0.05$ level, with n values ranging from 24 to 26 for each correlation analysis.

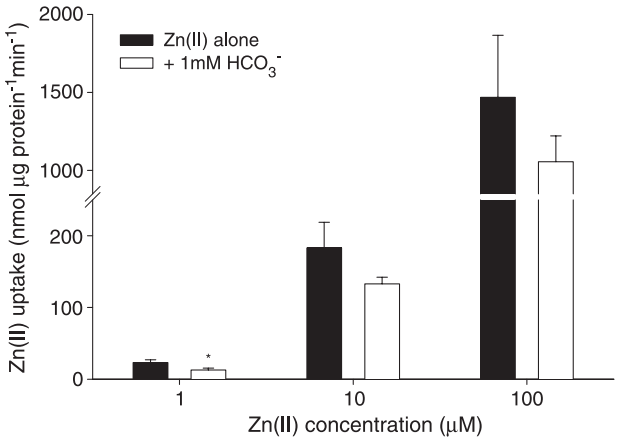

Fig. 6. Zn(II) uptake rate (nmol μg protein^{-1} min^{-1}) into intestinal BBMVs in the presence of 1 mM extravesicular HCO_3^-. Uptake rates were determined at the stated Zn(II) concentrations, after 30-s incubation at 37 °C. Plotted values represent the means ± S.E. for 4 (HCO_3^-) and 10 (Zn(II) alone) experiments. Statistically different (*) uptake rates ($P < 0.05$) were determined by ANOVA, followed by post-hoc LSD analysis.

The variation in Zn(II) speciation coupled with the considerable influence of histidine upon Zn(II) uptake permitted an analysis of the potentially important species involved in Zn(II) uptake (Table 3). The altered pattern of Zn(II) uptake with histidine concentration was related to accompanying changes in Zn(II) speciation, by correlation analyses. At all Zn(II) concentrations, the mono-chelated species ($Zn(His)^+$) was significantly correlated ($P < 0.05$) with Zn(II) uptake. This correlation was strongest at 10 μM Zn(II), with the variation in $Zn(His)^+$ levels explaining 59% of the variation in Zn(II) uptake.

In bicarbonate experiments, where Zn(II) was also complexed, the pattern of uptake was very different to that observed in the presence of histidine (Fig. 6). Extravesicular bicarbonate, at a concentration of 1 mM, significantly inhibited initial Zn(II) uptake at 1 μM Zn(II). Control levels of 23.2 nmol μg protein^{-1} min^{-1} were reduced to 12.9 nmol μg protein^{-1} min^{-1}. This effect may have persisted at higher Zn(II) concentrations, but was not statistically significant.

4. Discussion

4.1. Characterisation of Zn(II) uptake across the intestinal brush-border membrane

The incubation of Zn(II) with a purified, brush-border enriched membrane vesicle preparation resulted in a con-

centration-dependent association of Zn(II) with the vesiculated membrane fraction. This association was likely due to transport because it was markedly dependent on temperature as well as on intravesicular volume. Furthermore, EGTA in wash buffers did not remove more zinc from the vesicle surface than did EGTA-free buffers. EGTA binds Zn(II) more strongly (log $K_1 = 13.19$) than most biological ligands (e.g. Zn(II)-cysteine: log $K_1 = 11.0$), suggesting that there is a very high affinity of Zn(II) for the membrane, or that the Zn(II) associated with the BBMV after the wash was intravesicular [27]. The results from the osmolarity experiment delineated a considerable membrane-binding (osmotically independent) component.

The passage of Zn(II) across the intestinal brush-border membrane of freshwater rainbow trout was described by two mechanisms. At low Zn(II) concentrations uptake appeared saturable. This component was superseded by a linear uptake pathway at Zn(II) concentrations above 500 μM. The zinc uptake characteristics correspond to those discerned for intestinal Zn(II) transport in mammals and in marine teleosts [5–7].

The affinity of the saturable Zn(II) uptake component described here ($K_M = 57$ μM), is amongst the highest reported for intestinal zinc uptake in any organism. Raffaniello et al. [28] reported an affinity constant of 41 μM for apical transfer of Zn(II) across the mammalian Caco-2 intestinal cell line, while Blakeborough and Salter [29], in porcine intestinal BBMVs, measured a Zn(II) uptake constant of 67 μM. Other reports detail affinity constants from in vitro and cell culture experiments in the range of 220–490 μM [30–32].

Compared to Zn(II) uptake across the gill, however, the intestine is a low affinity absorptive pathway. Branchial affinity for Zn(II) uptake in freshwater fish is in the order of 10-fold higher than that detailed here (K_M of 3.6–7.8 μM; see Ref. [3] and references therein). These results confirm previous findings [8], where Zn(II) was described as a low affinity, high capacity route for Zn(II) absorption. Differences in Zn(II) uptake affinity between the data detailed here and that described by Glover and Hogstrand [8] were attributed to the distinct techniques used. In vivo Zn(II) absorption was best described by a single saturable component with a K_M of 309 μM, an affinity fivefold less than that discerned in the present investigation. This discrepancy is likely the result of Zn(II)-induced mucus secretion in vivo. As little as 5 μM of Zn(II) in the intestinal fluid provoked a significantly increased mucus production. Mucus was found to act as a moderator of Zn(II) uptake, facilitating absorption at lower Zn(II) concentrations while limiting uptake at higher levels. It seems likely that luminal mucus present in the intact animal obstructs the diffusive uptake pathway that was present at high Zn(II) levels in the current study. The higher affinity for Zn(II) measured directly across the brush-border membrane compared to the apparent affinity across the epithelial surface as a whole suggests, however, that mucus overlying the apical membrane would act as an effective donor of Zn(II). This may be a mechanism that allows mucus to act as a promoter of Zn(II) absorption at low luminal Zn(II) concentrations [8].

A caveat to note is that the vesicle experiments were performed at 37 °C, a temperature considerably in excess of both the acclimation temperature (11–15 °C), and that likely to be encountered in the natural environment. However, this temperature has been successfully used in previous studies to study ion transport across epithelial cell membranes in rainbow trout [17,23] and was selected to optimise transmembrane Zn(II) transport and provide a comparison of uptake with mammalian studies which are conducted at 37 °C. Therefore, the rate of apical Zn(II) uptake reported here is likely to exceed that of the fish in vivo. A consequence of this protocol is likely to be an alteration in membrane fluidity. Enhanced membrane fluidity has been shown to influence the J_{max} but not the K_M of Zn(II) transport in intestinal BBMVs of rats [33].

4.2. Histidine facilitated Zn(II) uptake

The presence of histidine in the transport medium promoted Zn(II) absorption across the brush-border membrane. This effect was highly dependent upon Zn(II) speciation, with $Zn(His)^+$ dominating explainable uptake (see Table 3). This suggests that Zn(II) uptake was facilitated, at least in part, by the formation of a $Zn(His)^+$ species. Mono-histidine chelates of Zn(II) have been proposed as Zn(II) transport facilitators in mussels [13], rat liver hepatocytes [34] and across mammalian blood–brain barrier [35]. In contrast, histidine was shown to inhibit Zn(II) uptake across rat intestinal brush-border membrane [32]. This may be a consequence of the much shorter incubation times in the study of Teillet et al. [32]. Initial uptake rates over 2 s were used, as opposed to the 30 s of the current analysis. Thus, although there are some inconsistencies between results obtained by different investigators, the existence of zinc uptake associated with histidine chelation appears to be a phylogenetically conserved process.

It is tempting to suggest that this histidine-stimulated Zn(II) transport is mediated by a histidine transporter in the brush-border membrane. However, an alternative explanation is that the stimulatory effect of histidine may be mediated by a more generalised ligand exchange reaction. In this scenario the histidine would facilitate the passage of Zn(II) to a transporter on the epithelial surface. Similar suggestions have been made by others for amino acid-stimulated Zn(II) uptake (e.g. Refs. [36,37]). An attempt was made to resolve which one of the above mechanisms for histidine-stimulated Zn(II) uptake is valid by using L- and D-stereoisomers for Zn(II). The rational for this was that mammalian intestinal histidine transport is far greater for L- than for D-histidine [38]. The data presented here do not support a stereospecific action of histidine upon apical Zn(II) uptake. In fact, there was little difference between histidine stereoisomers in their effects on BBMV Zn(II)

transport (Fig. 5). Similarly, in vivo analysis of intestinal Zn(II) absorption in trout discerned no stereospecific effects of histidine on accumulation of Zn(II) in the epithelium [9]. While lack of stereospecificity would suggest that histidine-stimulated zinc uptake is the result of ligand exchange, whereby histidine donates Zn(II) to a Zn(II) transporter, the results are not conclusive. Histidine transport has not specifically been examined in fish, but piscine transport of amino acids appears more promiscuous with regard to stereospecificity than that of mammals [39]. Thus, the lack of differential Zn(II) uptake as a function of the respective histidine stereoisomer chelate may simply have reflected a low stereospecificity of rainbow trout amino acid transporters. Consequently, either a ligand exchange or the transport of an intact histidine–Zn(II) chelate across the brush-border membrane may explain the stereochemical flexibility of the findings exhibited here. Alternative mechanisms such as a direct structural effect of histidine on membrane structure or an indirect influence of histidine on zinc-binding to the membrane vesicles cannot be excluded either until further analysis is performed.

In the intact rainbow trout intestine, the inclusion of histidine in the intestinal fluid increased the accumulation rate of Zn(II) in the subepithelium of the intestine, but had no significant effect on Zn(II) accumulation elsewhere in the body [9]. Based on the results of the present study, it can be proposed that this stimulatory effect was a consequence of Zn(II) uptake via a transporter located in the brush-border membrane. One conundrum is that in the intact animal the presence of a $Zn(His)_2$ complex was more effective in mediating Zn(II) uptake than the $ZnHis^+$ complex [9], whereas in the present study histidine-stimulated zinc uptake was attributed to $ZnHis^+$. The reason for this difference is not clear, but could well be related to the more complex system involved in the previous in vivo study [9].

In the present study, no stimulatory effect of bicarbonate was discerned. In red blood cells there is evidence suggesting that the uptake of Zn(II) may be linked to an anionic complex involving bicarbonate (e.g. Refs. [18,40]). More recent research suggests that the human Zn(II) transporter, hZIP2, is stimulated by bicarbonate and can mediate cellular Zn(II) uptake [41]. In contrast, the closely related human Zn(II) importer, hZIP1, is insensitive to bicarbonate [42] as is another potential Zn(II) import protein (hZLT1) of the Cation Diffusion Facilitator family [43]. It is clear from the results of the present study that whatever protein is mediating Zn(II) uptake in the fish intestine, it is not stimulated by the presence of bicarbonate in *cis* position with Zn(II).

4.3. Summary

The multiplicity of potential Zn(II) uptake pathways is becoming increasingly obvious at a molecular level. In mammalian systems numerous Zn(II) transporting entities have been described (see above, for review see Ref. [15]). These transporters appear to be highly conserved amongst organisms. The results presented in the current study highlight this multiplicity at a physiological level in the intestine of rainbow trout. It is apparent that Zn(II) uptake across the apical intestinal membrane may be achieved by both saturable and diffusive pathways, the latter of which may be linked to potassium efflux (C.N. Glover., unpublished data). Furthermore, Zn(II) uptake may be mediated by the free ion (Zn^{2+}), or by association with a chelated amino acid ($Zn(His)^+$).

Acknowledgements

The research described herein was supported by United States Environmental Protection Agency Grant (R826104) awarded to CH, while CG was partially funded by the Society of Environmental Toxicology and Chemistry (SETAC) Doctoral Fellowship in Environmental Science, sponsored by Proctor and Gamble Company. The animal care and handling was performed in accordance with, and with approval given under, the Animal (Scientific Procedures) Act (UK) 1986.

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