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Mg-Dependent, Zn-ATPase: Enzymatic Characteristics, Ion Specificities and Tissue Distribution

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Abstract. Mucosal crude microsomes, prepared from proximal rat small intestine, exhibited significant Mg-dependent, Zn-ATPase activity; V_{max} = 23 μmoles Pi/mg protein/hr, K_m = 160 nM, and Hill Coefficient, n = 1.5. Partial purification (~10-fold) was achieved by detergent extraction, and centrifugation through 250 mM sucrose: V_{max} = 268 units, K_m = 1 nM, and n = 6. In partially purified preparations, the assay was linear with time to 60 min, and with protein concentration to 1 μg/300 μl. Activities at pH 8 and 8.5 were higher than at pH 7.2. The ATP K_m was 0.7 mM, with an optimal ATP/Mg ratio of ~2. Ca elicited ATPase activity but did not augment the Zn-dependent activity. In partially purified preparations, the homologous salts of Co, Cd, Cu, and Mn exhibited no detectable activity. Vanadate inhibition studies yielded two component kinetics with a K_i of 12 μM for the first component, and 96 μM for the second component, in partially purified preparations. Tissue distribution analyses revealed gradients of activity. In the proximal half of the small intestine, Mg/Zn activity increased progressively from crypt to villus tip. In long axis studies, this activity decreased progressively from proximal to distal small bowel.

Key words: Zinc — ATPase — intestine — mucosa

Introduction

A variety of P-type ATPases mediate energy-dependent cation transport in all species, including bacteria, studied to date (Pedersen & Carafoli, 1987; Fagan & Saier, 1994; Lutsenko & Kaplan, 1995). The recent discovery of putative P-type ATPases involved in copper transport in the intestine and liver, added two new closely related isoforms to this extensive gene family (Tanzi et al., 1993; Petrukhin et al., 1994; Solioz & Vulpe, 1996; Payne & Gitlin, 1998).

Copper and zinc share a number of chemical and biochemical properties; they are the prominent trace divalent heavy metals intrinsic to the structures and bioactivities of a variety of enzymes, including mixed function oxygenases. They share similar mechanisms of incorporation in protein structures via chelation by adjacent cysteine residues (Rotilio et al., 1972; Buhler & Kagi, 1974). And, deficiencies or excess accumulations of either of these trace metals result in serious and at times lethal toxic states (Prasad, 1993; Tanzi et al., 1993; Petrukhin et al., 1994; Cox, 1995; Cuajungco & Lees, 1997). The existence of an active transport system capable of precise regulation of intestinal absorption of copper, prompted us to explore the existence and properties of an analogous Zn-specific ATPase in the rat small intestine.

A search of the literature yielded three relevant earlier studies. Kowarski and Schachter (1973), reported significant Zn-dependent, ATPase activity in rat duodenal mucosal particulates, with a Zn K_m of 0.7 mM, and shortly thereafter documented metabolically-dependent net mucosal to serosal flux of Zn^{65} against the potential gradients in everted rat jejunal sacs (Kowarski, Blair-Stanek & Schachter, 1974). Both Zn-ATPase activity and active Zn transport were in part Vitamin D-dependent. Ronquist (1988) recorded significant divalent metal ion stimulation of ATPase activity in membrane particulates prepared from human semen. The order of activation was Zn > Mn > Cd > Ba > Sr. Orthovanadate inhibited Zn-ATPase activity with a K_i of 0.5 mM.

We now report on the partial purification and enzymatic properties of an intestinal, Mg-dependent, Zn-

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activated ATPase, that is vanadate sensitive. Further characterization included divalent cation specificity, and distribution of activity within the mucosa of the proximal small intestine, and in the mucosa down the long axis of the small intestine.

Materials and Methods

Tissue Preparation

Male Sprague-Dawley rats, 300–350 g, were used throughout the study. The rats were fed ad libitum overnight and then killed by instantaneous decapitation. The proximal half of the small intestine was removed by sharp dissection, and cut into 4 to 5, 12–15 cm sections from the proximal duodenum to the mid-jejunum. Each section was flushed once with 50 ml of ice-cold Hanks' balanced salt solution (HBSS) to remove the lumen contents. Separate intestinal villus and crypt cell fractions of V_w, V_1, V_2, V_3, and crypt were isolated by a slight modification of the method described by Flint et al (1991).

Preparation of Crude Microsomes

Fractions V_w and V_1 were pooled (V_{w+1}), as were V_2 and V_3 (V_{2+3}). To remove surface mucous, V_{w+1}, V_{2+3}, and crypt fractions were suspended separately in ice-cold 50 mM Tris HCl (pH 7.3), 5 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1 mM PMSF and centrifuged at 1000 × g for 5 min. This step was repeated 3 times, followed by an additional 3 washes with this solution, except that the EDTA concentration was reduced to 1 mM. The cells were homogenized in 10–15 ml of 250 mM sucrose-30 mM Tris HCl (pH 7.2), with 20 strokes in a glass homogenizer fitted with a Teflon pestle, at 1000 rev./min. The homogenates were centrifuged at 1300 × g for 10 min. The sediment was resuspended by homogenization in the original volume of sucrose-Tris HCl and centrifuged again at 1300 × g for 10 min. The combined supernatants were then centrifuged at 66,000 × g for 30 min. The fluffy layer was carefully aspirated from the top of the pellet, resuspended in sucrose-Tris HCl, pH 7.2–7.4, and stored at −20°C. Protein concentrations of all fractions were determined by Peterson's modification of the micro-Lowry method (Peterson, 1977).

The intestinal whole mucosal preparations were obtained by cutting the entire small intestine into three approximately equal lengths, proximal, middle and distal, from the proximal duodenum to the end of the ileum. Each segment was flushed twice with 50 ml of ice-cold Hanks' balanced salt solution (HBSS) to remove the lumen contents, and was sliced open longitudinally with scissors and unfolded onto an ice chilled glass plate. The entire mucosa was scraped gently with a microscope slide and collected into a 50-ml centrifuge tube. To remove the surface mucus, the scrapings were washed separately exactly as described for the V_{w+1}, V_{2+3} and crypt fractions. Crude and partially purified mucosal preparations of proximal, middle and distal intestinal segments were prepared as described for V_{w+1}, V_{2+3} and crypt except that only 1.0 mg SDS/mg protein ratio was used in the extraction procedure (*see* below).

Partial Purification of Zn/Mg-ATPase

Magnesium-dependent, Zinc adenosine triphosphatase (Zn/Mg-ATPase) was purified by a modification of the Jorgensen Na, K-ATPase method (1988). Crude microsomes (1.4 mg/ml) were incubated in (in mM) 3 ATP (Tris salt), 2 EDTA, 30 Tris HCl, pH 7.5 and various concentrations of sodium dodecyl sulfate (SDS), for 30 min at 20°C in a shaking water bath. After incubation the samples were then transferred into 6- to 10-fold volumes of 250 mM sucrose-30 mM Tris HCl (pH 7.2), in centrifuge bottles of the Ti-70 Beckman fixed angle rotor and centrifuged through the sucrose-Tris HCl solution at 120,000 × g for 30 min at 4°C. The pellets were resuspended in sucrose-Tris HCl and stored at −20°C.

ATPase Assay

The activity was measured by a modification of the colorimetric ATPase assay of Cortas and Walser (1971). Ten μg of crude and 1.0 μg of partially purified enzyme were added to a final volume of 300 μl containing 30 mM Tris HCl buffer (pH 7.2), 0–2.0 μM of stepwise increments of $ZnCl_2$, 10 mM KCl, 5 μg/ml Oligomycin, in the absence and presence of 3 mM $MgCl_2$ and pre-incubated at 37°C for 5 min. The reaction was started by the addition of ATP (Tris salt) to a final concentration of 3 mM, incubated at 37°C, and stopped after 30 min by addition of ice-cold 10% trichloroacetic acid (TCA) and immersion in ice. The amount of P_i released was then measured colorimetrically. In the whole mucosal preparations from the proximal, middle, and distal segments, 40 mM Tris-HCl, pH 8, and 2 mM $MgCl_2$ were used in the assay. The activity of Zn/Mg-ATPase was expressed as μmoles P_i/mg protein/hr corrected for basal Mg-ATPase activity. Zn/Mg-ATPase kinetics, i.e., V_{max}, K_m and n (Hill coefficient), were estimated by curve fitting with the Ultrafit software package of Biosoft.

Mg Specificity

To assess the need for Mg in the assay, activities were measured in the presence of Zn alone, Mg alone, Ca alone, and various combinations of these salts. The concentrations used were 1 μM $ZnCl_2$, (in mM) 3.2 $CaCl_2$ and 3 $MgCl_2$, in medium containing (in mM) 40 Tris-HCl, pH 8, 3 ATP (Tris salt), 10 KCl, and 5 μg/ml oligomycin.

Zn Specificity

A critical issue in assessing the significance of a cation-sensitive ATPase, is the uniqueness of the metal ion driving the reaction. The divalent metal ions, Mn^{++}, Co^{++}, Cd^{++}, and Cu^{++}, were chosen to evaluate the specificity of the response to Zn^{++} in partially purified preparations. The reaction medium was the same as in the Ca^{++} vs Mg^{++} experiments listed above, which contained varying concentrations of $MnCl_2$, $CoCl_2$, $CdCl_2$, $CuCl_2$, and $ZnCl_2$.

Vanadate Inhibition

Aliquots of partially purified enzyme (final concentration, 1 μg protein/300 μl) in 1 μM $ZnCl_2$, 3 mM $MgCl_2$, 5 μg/ml Oligomycin, 10 mM KCl, and 30 mM Tris HCl, pH 7.2, and 0–200 μM Na_3VO_4 were vortexed and incubated at 37°C for 20 min in a water bath. The ATPase reactions were then started by addition of ATP (Tris salt); Zn/Mg-ATPase activity was estimated, as described above. The vanadate experiments were carried out in the presence and absence of Mg. The vanadate K_i values for Zn/Mg-ATPase and Mg-ATPase were calculated by fitting Double Exponential Decay using Ultrafit software of Biosoft.

Materials

The conventional chemical reagents, all reagent grades or the highest purity available, were purchased from Sigma, J.T. Baker, and Fisher

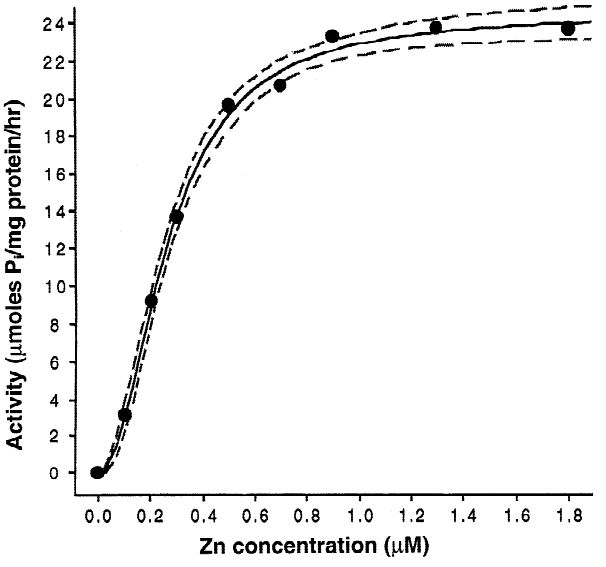

Fig. 1. Zinc-dependence of ATPase activity in crude V_{w+1} microsomes. Crude V_{w+1} microsomes were prepared as described in the text. Zn dependence was determined at 9 concentrations of $ZnCl_2$, as shown in the Fig., in the presence of 3 mM $MgCl_2$, 3 mM ATP (Tris salt), 10 mM KCl, 5 μg/ml oligomycin in 30 mM Tris HCl, pH 7.2. ATPase activity was corrected for the baseline in the absence of Zn and in the presence of 3 mM $MgCl_2$. The best fit was obtained with the Hill equation in the Ultrafit package. V_{max} = 24.4 ± 1.1 μmoles P_i/mg protein/hr, K_m = 0.07 ± 0.04 μM, n = 2.0 ± 0.3. Dashed lines are the 95% confidence lines.

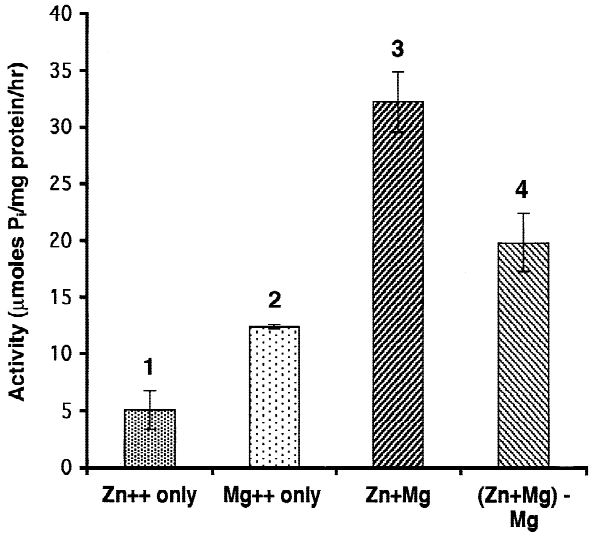

Fig. 2. Summary of Zn/Mg-ATPase activity of Crude V_{w+1} microsomes. The enzyme assays were performed in the standard medium (*see* legend to Fig. 1). Column *1, Zn^{++} only* denotes the presence of $ZnCl_2$ (2 μM) in the absence of Mg^{++}. Column *2, Mg^{++} only* denotes the presence of $MgCl_2$ (3 mM) in the absence of Zn^{++}. Column *3, Zn+Mg* denotes the presence of both $ZnCl_2$ (2 μM) and $MgCl_2$ (3 mM). Column *4, (Zn+Mg)-Mg* represents ATPase activity corrected for the Mg^{++} background, by subtracting (*2*) from (*3*). Each bar and vertical line is the mean ± SEM of three different preparations.

Scientific. The Protein Assay Kit (Peterson's modification of the micro-Lowry method), the tris salt of adenosine 5′-triphosphate (<0.1% Na), and the sodium orthovanadate (purity >95%) were supplied by Sigma. Manganese chloride, cobalt chloride, cupric chloride, cadmium chloride and zinc chloride were the highest purity available and purchased from Sigma. Calcium chloride (0.0002% heavy metals, 0.005% Mg) was supplied by Mallinckrodt.

Results

To set the standard assay conditions for quantitative evaluation of Zn-dependent ATPase activity, a series of pilot studies were conducted on crude microsomes of the V_{w+1} fraction. Preliminary experiments included the concentrations of Zn and Mg needed to elicit the reaction, the concentration of oligomycin that minimized Zn-free ATPase (i.e. background) activity, and the concentrations of KCl and Tris buffer that augmented the reaction (*data not shown*). These findings were the basis for using 10 mM KCl, 5 μg/ml of oligomycin, and 30 mM Tris buffer in the standard assay medium. Tris-ATP rather than Na-ATP, was chosen as the substrate to avoid superposition of Na/K-ATPase activity on the Zn-dependent activity. Assessment of pH dependence, and linearity with time and protein concentration was deferred pending partial purification of the enzyme. As shown in Fig. 1, in the presence of 3 mM $MgCl_2$, a $ZnCl_2$ concentration range of 0.1–1.8 μM yielded significant ATPase activity. The curve gave a better statistical fit to the Hill equation than the Michaelis-Menten equation. The Hill coefficient (n = 2.0 ± 0.3) implies a positively cooperative reaction, and the K_m of 70 nM is consistent with the expected very low intracellular concentration of Zn, since it is a trace metal.

To define Mg dependence, and to correct for baseline Mg-dependent activity, ATPase assays were performed in the presence of $ZnCl_2$ and no $MgCl_2$, or in the presence of $MgCl_2$ and no $ZnCl_2$, or in the presence of both. V_{max} of Zn + Mg was ~6-fold greater than with Zn only, and 2.5-fold greater than with Mg only (Fig. 2). Subtracting the activity elicited by Mg only from that of Zn + Mg, gave net Zn, Mg-ATPase activity of 19.8 ± 2.6 μmoles P_i/mg protein/hr. This estimate was not corrected for Mg-independent, Zn activity (Zn-only) since this may represent residual activity of the specific Zn system. The nature of the Mg-only activity was not addressed. Some of it may represent partial activation of the Zn-dependent enzyme. We decided to attempt partial purification of the enzyme before extending its characterization.

Previous experience with integral membrane proteins, such as the Na/K-dependent ATPase, indicated that incubation in chelators and detergents, followed by density gradient centrifugation yielded purification close to

Table 1. Effect of SDS/Protein ratio on V_{max}, K_m, and Hill coefficient of Zn/Mg-ATPase

SDS/Protein Ratio[1]	V_{max}[2] (mean ± SEM)	K_m[3] (mean ± SEM)	Hill coefficient (mean ± SEM)
0	23.3 ± 1.3	0.16 ± 0.06	1.5 ± 0.2
0.2	32.6 ± 1.6	0.08 ± 0.04	2.0 ± 0.3
0.4	68.3 ± 2.9	0.09 ± 0.04	3.2 ± 0.5
0.6	93.2 ± 3.4	0.09 ± 0.03	3.3 ± 0.4
0.8	115.4 ± 2.5	0.17 ± 0.03	4.7 ± 0.4
1.0	262.3 ± 4.0	0.006 ± 0.003	4.1 ± 0.5
1.2	268.4 ± 3.5	0.001 ± 0.001	5.9 ± 0.6
1.6	244.8 ± 9.8	0.04 ± 0.03	4.3 ± 0.8

[1] Unit = mg SDS/mg Protein
[2] Unit = μmoles P_i/mg protein/hr
[3] Unit = μM
Two separate V_{w+1} crude membrane fractions were incubated at 20°C for 30 min in 3 mM ATP, 2 mM EDTA, 30 mM Tris HCl, pH 7.5, and varying concentrations of SDS as shown; followed by centrifugation through 250 mm sucrose, 30 mM Tris HCl, pH 7.2 for 30 min at 120,000 × g. Each enzyme assay used 9 concentrations of $ZnCl_2$ (0–2.0 μM), and the two curves were collapsed into a single curve by point-to-point averaging at each concentration of Zn. The means and SEMs were computed with the Hill equation in the Ultra-fit curve-fitting program.

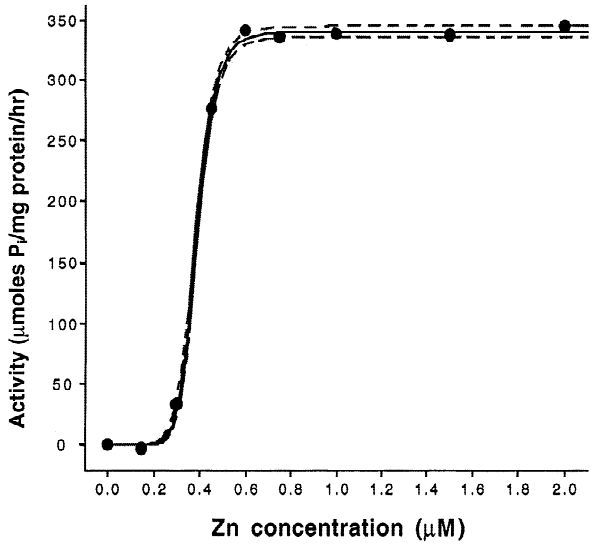

Fig. 3. Zn/Mg-ATPase activity of a partially purified preparation. The V_{w+1} preparation was incubated in 1.2 mg SDS/mg protein, for 30 min at 20°C and centrifuged through 250 mM sucrose, 30 mM Tris HCl for 30 min at 120,000 × g. The enzyme assay and curve fitting were done as described in the legend to Fig. 1. V_{max} = 340.3 ± 4.6 μmoles P_i/mg protein/hr, K_m = 0.2 ± 0.1 nM, n = 9.2 ± 1.0. Dashed lines are the 95% confidence lines.

homogeneity (Jorgensen, 1988). Accordingly, crude V_{w+1} microsomes were incubated with or without ATP in EDTA, with a range of SDS concentrations, and then centrifuged through discontinuous sucrose or glycerol density gradients. These attempts yielded only modest increases in enzyme-specific activity of protein recovered from interfaces in the gradients or the pellets (*data not shown*). Preparations incubated in 3 mM ATP and 2 mM EDTA without SDS, and centrifuged through sucrose-Tris HCl for 30 min, increased Zn/Mg-ATPase activity by only 20%, compared to untreated crude preparations (*data not shown*). In contrast, incubation of crude preparations with SDS at 0.1–1.6 mg SDS/mg protein (with EDTA and ATP), followed by centrifugation through sucrose-Tris HCl, gave progressive increases in enzyme-specific activity of the pellets (Table 1). The optimum SDS/protein ratio was 1.0–1.2, which yielded more than a 10-fold increase in V_{max}. This increase was accompanied by lowering of the K_m to less than 10 nM, and increases in the Hill coefficient to as high as 6. To optimize for time of centrifugation, we tried 15, 20, 30, 45, 60, and 90 min, at 120,000 × g. Peak activity was achieved with centrifugation for 20 and 30 min. After 30 min, the longer the centrifugation time, the lower the enzyme activity. Compared to 30 min, samples centrifuged for 45 min, 60 min, and 90 min gave 95.5%, 85%, and 79% of the specific activity, respectively. The fall in specific activity on centrifugation for longer than 30 min may result from pelleting of extraneous protein. Subsequent use of the optimal conditions for partial purification yielded preparations with even higher specific activities, lower K_ms and remarkably high Hill coefficients. An enzyme activity graph of one such preparation is shown in Fig. 3: V_{max} of 340 μmoles P_i/mg protein/hr, K_m of 0.1 nM, and n of 9.

Two partially purified preparations (i.e. preincubation in either 0.8 or 1.0 mg SDS/mg protein) of the V_{w+1} fraction, with varying specific activities, at an enzyme protein concentration of 1–2μg/300 μl and under V_{max} conditions, were used to assess linearity of the enzyme assay with time of incubation. The assay was precisely linear, up to 1 hr, under standard conditions. All the points fell within the 95% confidence limits for a straight line (*data not shown*).

Another requirement for validation of quantitative enzyme assays is linearity with protein concentrations. Under V_{max} conditions, a partially purified V_{w+1} preparation gave a precisely linear dependence on enzyme protein concentration, within the range 0–1.0 μg/300 μl. All of the points fall within the 95% confidence limits for a straight line (*data not shown*).

Two V_{w+1} preparations purified by incubation in two different concentrations of SDS were analyzed over a pH range from 7.2–8.5 (Fig. 4). The activities at pH 8 were 1.3- to 1.4-fold greater than at pH 7.2, and marginally greater than at pH 8.5.

In earlier experiments on crude membrane preparations, Zn-dependent ATPase activity was substantially augmented by 3 mM $MgCl_2$. To define the Mg optimum, activities of a partially purified V_{w+1} preparation (at an

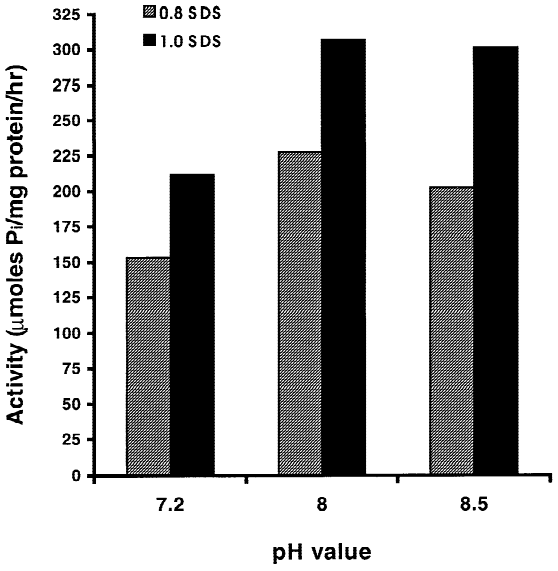

Fig. 4. pH dependence of Zn/Mg-ATPase activity. V_{w+1} preparations were partially purified by incubation with (▨) 0.8 or (■) 1.0 mg SDS/mg protein and processed as described in the legend to Fig. 3. The enzyme reactions contained 2.5 μM $ZnCl_2$, 3 mM $MgCl_2$, 3 mM ATP (Tris salt). pH of reaction medium was adjusted to the range, 7.2–8.5, as shown, by titration of Tris base with HCl.

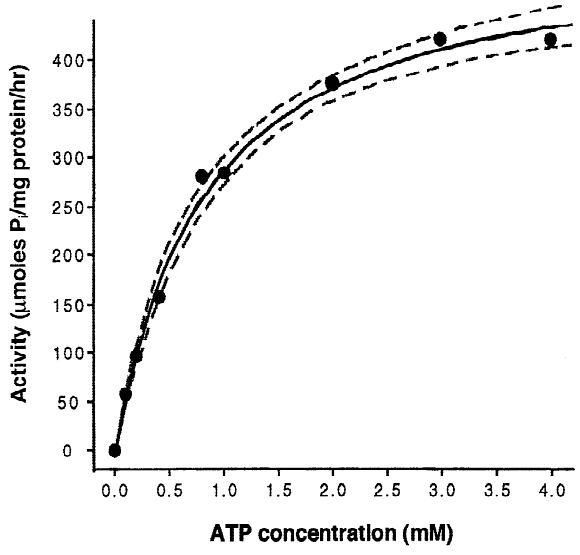

Fig. 5. ATP-dependence of Zn/Mg-ATPase activity. V_{w+1} preparations were partially purified by incubation in 1.0 mg SDS/mg protein and processed as described in the legend to Fig. 3. The enzyme assays contained 1 μM $ZnCl_2$, 2 mM $MgCl_2$, Tris buffer (pH 8) and ATP (Tris salt) concentrations of 0–4 mM. The enzyme reactions for ATP range, 0.2–0.8 mM, and the range, 1–4 mM, were run separately. Values corresponding to range 0.2–0.8 mM of ATP are the mean of two different preparations and to range 1–4 mM of ATP are the mean of four different preparations. The dashed lines are the 95% confidence lines, in fitting the curve to the Michaelis-Menten equation.

SDS/protein ratio of 1.0) were measured in the presence of 1 μM $ZnCl_2$ and $MgCl_2$ of 0–4 mM. The low levels of activity recorded in the absence of Mg were subtracted from all of the results, which adjusted the activity to zero in the absence of Mg. A peak of activity was obtained between 1–2 mM, with modest declines at 3–4 mM (*data not shown*). At 3 mM ATP concentration the optimum ATP/Mg ratio was ~2.

To explore the relationship between ATP-dependence of the reaction and the Mg concentration further, and to define the K_m for ATP, V_{w+1} preparations (partially purified by incubation at a 1.0 mg SDS/mg protein ratio) were assayed in 1 μM $ZnCl_2$, 2 mM $MgCl_2$, at pH 8, and 0 to 4 mM Tris ATP. Optimal activity was achieved between 3–4 mM ATP, at ATP/Mg ratios of ~2 (Fig. 5). A single curve was obtained by averaging results at each ATP concentration (*see* legend to Fig. 5). These data gave a best fit to the Michaelis-Menten equation, as indicated by the closeness of the dashed lines (95% confidence limits). The ATP K_m was 0.7 ± 0.3 mM.

To evaluate the specificity of Mg dependence, Ca was chosen for comparison, since it is closely related to Mg in the alkali earth metal series. Accordingly, Zn-ATPase activity was measured in partially purified preparations, in the presence and absence of $CaCl_2$, under the same conditions as with $MgCl_2$ (Fig. 6). The activity at V_{max} obtained with Ca (3.2 mM) plus Zn (1 μM) was less than one-fourth that obtained with Mg (3.0 mM) plus Zn (1 μM). In contrast to the marked augmentation obtained with Mg, the Ca plus Zn activity was simply the sum of the activities obtained with Zn alone and with Ca alone. The Mg-dependence of the response to Ca implies that a separate Ca-ATPase was present, as a minor component, in this preparation, and that Mg is required for optimum Zn-ATPase activity. The possibility that Ca could compete for the Mg-specific mechanism, as an inhibitor, was evaluated by measuring Zn/Mg-ATPase activity under standard conditions, in the presence or absence of 3.2 mM $CaCl_2$. As shown in Fig. 6, total ATPase activity was fractionally higher in the presence of both Mg and Ca. If Ca-ATPase was simply additive to the Zn/Mg activity the predicted yield would be 622 (165 + 457). The experimental yield was 529. Thus, Ca may be a weak inhibitor of the activity.

Vanadate is a potent inhibitor of P-type ATPases (Cantley, Cantley & Josephson, 1978; Pedersen & Carafoli, 1987; Fagan & Saier, 1994; Solioz & Vulpe, 1996). To explore the specificity and sensitivity of vanadate inhibition, varying concentrations of vanadate (0–200 μM) were pre-incubated with preparations that were partially purified by pre-treatment with 1.0 mg SDS/mg protein. In control experiments, low level ATPase activity, in the absence of Mg, was resistant to inhibition by vanadate even at concentrations of 160 μM (*data not shown*). In contrast, Zn/Mg-ATPase was significantly inhibited by vanadate in the partially purified preparation in the presence of Mg (Fig. 7). Inhibition was characterized by

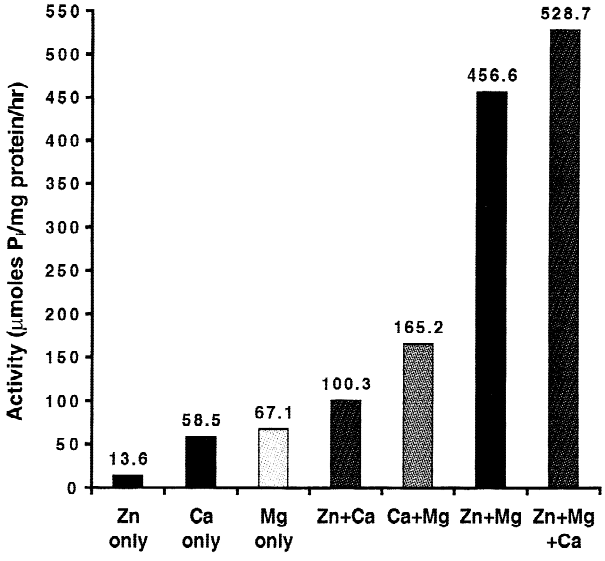

Fig. 6. Ca- vs Mg-dependence of Zn-ATPase activity. Crude microsomes of the V_{w+1} preparation were partially purified by incubation in 1.0 mg SDS/mg protein and processed as described in the legend of Fig. 3. The enzyme reaction was carried out in the presence of 1 μM $ZnCl_2$, and 3.2 mM $CaCl_2$, or 3 mM $MgCl_2$, in 40 mM Tris buffer (pH 8) containing 3 mM ATP (Tris salt), 10 mM KCl, 5 μg/ml oligomycin. Enzyme activities were corrected for the baseline in the absence of Zn, Ca, and Mg. Numbers above bars are the activities.

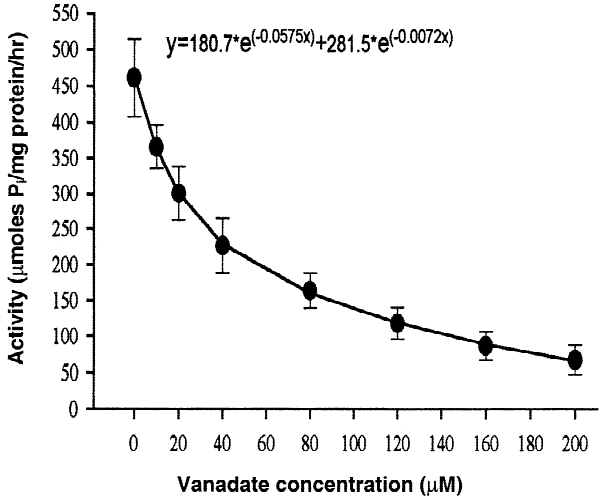

Fig. 7. Effect of vanadate on Zn/Mg-ATPase activity. V_{w+1} partially purified microsomes were incubated with $ZnCl_2$ (1 μM), $MgCl_2$ (3 mM) and varying concentrations (0–200 μM) of sodium vanadate for 20 min at 37°C. Partial purification was achieved by incubation in 1.0 mg SDS/mg protein and processing as described in the legend of Fig. 3. The composition of the assay medium and enzyme activities were as described in the Methods section. The curve was derived by applying the double exponential decay equation in the Ultrafit package. K_i was computed from the decay constants, λ_n, from: $K_{i(n)} = 0.695/\lambda_n$. In this experiment, $K_{i(1)} = 12.1$ μM and $K_{i(2)} = 96.3$ μM. Each point and vertical line represents the mean ± SEM of 3 different preparations, assayed separately.

a two component exponential decay; K_i for the first component, 12 μM, for the second component, 96 μM. Based on the ratio of the intercepts, the highly sensitive component constituted 39% of total activity.

Standard assays were carried out on partially purified V_{w+1} preparations (extracted with 1.0 mg SDS/mg protein), with a series of homologous, divalent cations; Co^{++}, Cd^{++}, Cu^{++}, Mn^{++} and Zn^{++}. At isomolar concentrations, 0–2.0 μM, as chloride salts, no significant activity was detected for Co^{++}, Cd^{++}, Cu^{++}, and Mn^{++} (Fig. 8). These results indicate that the Zn activity is highly specific.

To explore the intestinal distribution of the enzyme, two sets of studies were undertaken; the activity yield within the mucosa, from tip to crypt of the proximal ½ of the small intestine, and the yield from duodenum to the terminus of the ileum, along the long axis. The mucosal epithelium of the small intestine is infolded in the form of villi, with absorptive activity maximal at the tips and cellular replication maximal in the crypts (Van Corven, De Jong & Van Os, 1986; Flint et al., 1991; Ferraris & Diamond, 1993). Thus, there may be a gradient in the distribution of Zn/Mg-ATPase activity which in future studies may correlate with a transport capacity gradient. Accordingly, the mucosa was divided into three fractions; V_{w+1}, V_{2+3}, crypt. These three fractions were partially purified by extraction with 0 to 1.2 mg SDS/mg protein, and assayed under V_{max} conditions (Fig. 9). Extraction with 1.0 and 1.2 mg SDS/mg protein yielded more than 10-fold increases in V_{max} in all three layers. The V_{w+1} preparation exhibited two-fold higher activity than V_{2+3}. The lowest activity was recorded with the crypt preparation. The Hill coefficients were essentially the same in all three partially purified villus layers.

To explore the distribution of Zn/Mg-ATPase activity along the long axis, the small intestine was divided into three approximately equal segments. The proximal segment included the entire duodenum as well as a part of the adjacent jejunum, the middle segment consisted mainly of the jejunum, and the distal segment consisted chiefly of the ileum. In these experiments, the entire mucosa was assayed. As summarized in Table 2, in partially purified mucosal preparations (i.e., preincubation in 1.0 mg SDS/mg protein), V_{max} of the proximal segment was 256 units. V_{max} of the mid-segment was 57% and that of the distal segment only 9% of that in the proximal segment. No differences were noted in the K_m for Zn or the Hill Coefficients between the three segments. These experiments gave lower Hill Coefficients (2.5–3.8) than in partially purified preparations of the V_{w+1} fraction (*compare* Tables 1 and 2).

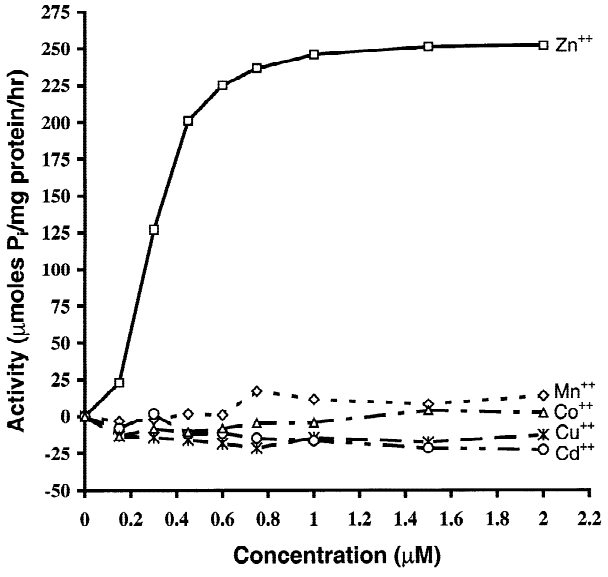

Fig. 8. Divalent cation specificity of Zn/Mg-ATPase The V_{w+1} preparation was partially purified by incubation in 1.0 mg SDS/mg protein and processed as described in the legend to Fig. 3. The enzyme assays were carried out under standard conditions (see legend to Fig. 1) except for Tris buffer (pH 8), in the presence of the indicated concentrations of $ZnCl_2$ or one of the other divalent cation compounds: $MnCl_2$, $CoCl_2$, $CuCl_2$, $CdCl_2$. Enzyme activities were corrected for the baseline in the presence of Mg and in the absence of Zn or other divalent cations. In partially purified preparations Zn yielded a V_{max} of 251 ± 3.16 μmoles P_i/mg protein/hr, a K_m of 0.02 ± 0.01 μM and a Hill coefficient of 3.2 ± 0.23. None of the other divalent cations gave any detectable activity.

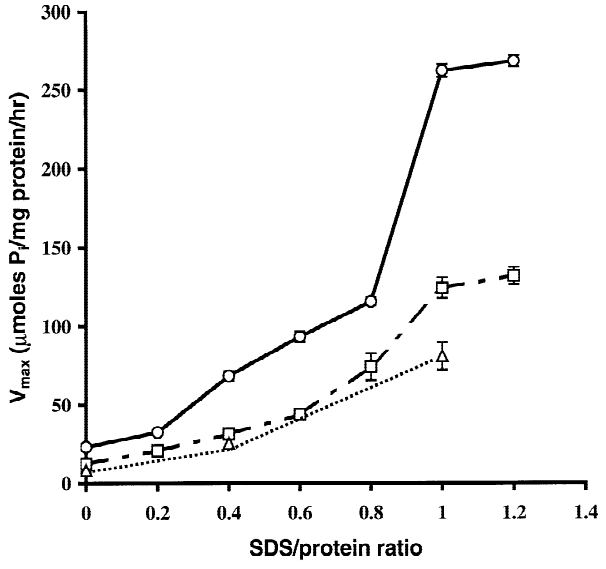

Fig. 9. Zn/Mg-ATPase activity in partially purified preparations from three mucosal levels. V_{w+1} (○), V_{2+3} (□), and crypt (△) preparations were partially purified by extraction with 0–1.2 mg of SDS/mg of protein and assayed under standard conditions (see legend to Fig. 1). Each point is the mean ± SEM of two independent preparations derived from two curves that were collapsed into a single curve by point-to-point averaging at each concentration of $ZnCl_2$.

Table 2. V_{max}, K_m, and Hill coefficient of Zn/Mg-ATPase in partially purified mucosal preparations along the long axis of the small intestines.

Segments	V_{max} [1]	K_m [2]	Hill coefficient
Proximal	256.0 ± 24.9	0.08 ± 0.08	2.5 ± 0.9
Middle	151.7 ± 17.2	0.72 ± 0.27	2.5 ± 0.5
Distal	23.4 ± 2.1	0.49 ± 0.23	3.8 ± 1.0

[1] Unit = μmoles P_i/mg protein/hr
[2] Unit = μM
Mucosal preparations were partially purified and assayed as described in Methods. Each value is the mean ± SEM of three different preparations.

Discussion

Zinc is a crucially important trace element in biological systems with a range of functions; structural, catalytic and regulatory, as an intrinsic component of oxygenases, proteases, and transcription factors, as well as others (Angel et al., 1987; Cuajungco & Lees, 1997). The potential toxicity of Zn as a heavy metal, and the essential role it plays in a multiplicity of pathways dictates the need for precise regulation of absorption of dietary Zn, as well as its distribution among body compartments. Over the last 40 years, Na/K, H/K, and Ca transport ATPases (P-type), have been extensively described at the molecular and physiological levels (Pedersen & Carafoli, 1987; Fagan & Saier, 1994; Lutsenko & Kaplan, 1995). Recently two variants of a Cu-specific, P-type ATPase were discovered by genetic analysis of Menkes' Disease, a Cu absorption defect, and Wilson's Disease, a Cu excess disorder (Tanzi et al., 1993; Petrukhin et al., 1994; Solioz & Vulpe, 1996; Payne & Gitlin, 1998). A distinguishing feature of the Menkes' and Wilson's type Cu-ATPases is the tandem binding of 5/6 atoms of Cu by Cys-4 chelating mechanisms, near the amino-terminus (Petrukhin et al., 1994; Payne & Gitlin, 1998). The similarities in some of the chemical and biochemical properties of Cu and Zn prompted us to explore the possible presence of a Zn-specific ATPase in intestinal epithelia.

Bacterial cation-specific, P-type ATPases have been described recently with specificities for Zn (*ZnT* A), Cd (Cad A), and Cu (Cop A, Cop B) (Rensing, Mitra & Rosen, 1997). A common feature is the presence of 1–6 N-terminal GXXCXXC motifs that are putative metal binding domains (Solioz & Vulpe, 1996; Rensing et al., 1997). The Zn-enzyme, *ZnT* A, was identified as a translocator by ATP-dependent, vanadate-inhibitable Zn efflux from everted vesicles prepared from *E. coli* (Rensing et al., 1997). Evolutionary relationships among Cop A, Cop B, Cad A, and the mammalian Menkes' and Wilson's Cu-ATPases are indicated by the following ho-

mologies, (*i*) putative heavy metal ion binding sites in the polar N-terminal region, (*ii*) a unique number of transmembrane helices, (*iii*) a conserved intramembranous CPC or CPx motif that is a distinguishing feature of heavy metal transport ATPases, and (*iv*) the conserved sequence of the phosphorylation domain, DKTGT, which is common to all P-type ATPases (Odermatt et al., 1993; Fagan & Saier, 1994; Solioz & Vulpe, 1996). A similar comparison of *ZnT* A with mammalian Zn-ATPase awaits cloning and sequencing of the latter.

In the earlier studies of Schachter and colleagues (Kowarski & Schachter, 1973; Kowarski et al., 1974), and Ronquist (1988), K_ms for Zn of 0.5 mM and 0.4 mM, respectively, were reported. These values are surprisingly high in view of the trace concentrations of Zn in mammalian cells (Cuajungco & Lees, 1997). In our studies, the crude preparations gave a Zn K_m of 0.16 μM, and in partially purified preparations, a value of 1 nM. This 1,000-fold decrease in K_m with partial purification may be a consequence of extraction of bound zinc during purification. The fall in K_m was accompanied by marked increases in the apparent Hill coefficient, which also is consistent with exposure of more Zn binding sites during purification. The tandem Cu-binding sites in the Menkes'/Wilson's Cu-ATPases (Petrukhin et al., 1994; Lutsenko et al., 1997; Payne & Gitlin, 1998) raise the possibility of the existence of similar, multiple Zn binding sites in the Mg-activated, Zn-ATPase, detected in our preparations. The exceptionally high Hill coefficients (*n* with a mean value of 6, in the combined group) after extraction with high concentrations of SDS, is consistent with the existence of a multiplicity of Zn binding sites. An alternative explanation could be the presence of a large number of interacting subunits in an enzyme complex.

A gene family, widely expressed in mammalian organs and tissues, has recently been identified as Zn transporters and designated ZnT-1, -2, -3, and -4 (Palmiter & Findley, 1995; Palmiter, Cole & Findley, 1996a; Palmiter et al., 1996b; Huang & Gitschier, 1997). Their respective roles in Zn transport have been inferred from a variety of studies. All of the Zn transporters are highly homologous in DNA sequence to each other, and to two yeast proteins, ZRC1 and COT2, and a bacterial protein, czcD. It is unlikely that the Zn transporters mediate active rather than facilitated transport. None have been shown to have Zn-dependent, ATPase activity, and their sequences do not exhibit the major structural features of P-type ATPases.

The divalent metals, Co^{++}, Cd^{++}, Cu^{++}, Mn^{++}, and Zn^{++} constitute a homologous series, with similar chemical properties (Williams, 1989). These salts were used as a stringent test of the specificity of Zn^{++} as the substrate for the ATPase activity. The complete absence of activity with isomolar concentrations of Co^{++}, Cd^{++}, Cu^{++}, and Mn^{++} in the partially purified preparations attests to the metal ion specificity of Zn in the activation of this ATPase.

Four features in our study are consistent with the possibility that the Zn enzyme is a P-type ATPase: stable integration in membranes, Mg-dependence, specificity of Zn, and vanadate inhibition. Stable integration is indicated by the resistance to extraction with SDS/protein ratios as high as 1.6. The specificity of Mg-dependence (i.e., comparison to dependence on Ca) is documented in the present study. Zn is the unique activator in comparison to a panel of other divalent heavy metals. Vanadate inhibition is a common feature of P-type ATPases, typically in μmolar concentrations (Cantley et al., 1978; Fagan & Saier, 1994; Solioz & Vulpe, 1996). In partially purified preparations, vanadate inhibition was characterized by two components with K_is of 12 μM, and 96 μM, respectively. Although the basis for the heterogeneity in sensitivity to vanadate is unresolved, these results are in accord with the properties of cation-transporting ATPases (Fagan & Saier, 1994; Solioz & Vulpe, 1996). Vanadate inhibition also exhibited biphasic kinetics in the prostasome experiments, with an overall half-maximal inhibition at 500 μM (Ronquist, 1988).

The mucosa of the proximal small bowel consists of replicating crypt cells that migrate to the surface of the villi, and in the process mature into more highly absorptive epithelial cells, at the tip (Van Corven et al., 1986; Flint et al., 1991; Ferraris & Diamond, 1993). In accord with the expectation of highest Zn absorptive activity at the tip, Zn-ATPase activity, at V_{max}, was highest in the outer fraction, intermediate in the middle fraction and least in the crypt fraction. The properties of these activities were indistinguishable in terms of K_ms for Zn, Hill coefficients, and fold-increases in activities on partial purification. If in future studies the intra-villus distribution of the Zn transport capacities of these cell layers proves to be proportionate to the gradient of Zn enzyme activity, it would contribute to its identification as a transport system.

Our results document decreasing Zn enzyme activities, in partially purified whole mucosal preparations, along the long axis of the small intestine. No differences in the kinetic properties of the enzyme were noted in these assays, implying only differences in abundance. Earlier studies on the sites of zinc absorption in the small intestine in both animals and human, however, reported conflicting results. Methfessel and Spencer (1973), Davies (1980), and Jackson et al. (1981) found that in the rat, zinc absorption was significantly higher in the duodenum or the upper small intestine than in distal segments. But Antonson et al. (1979) reported that zinc absorption was ~3-fold higher in the ileum than in the jejunum or the duodenum of the rat. In humans, Lee et al. (1989) noted that the proximal jejunum had the high-

est rate of zinc absorption, followed in decreasing order by the duodenum, and the ileum. Until the basis for these discrepancies in rates of zinc absorption have been elucidated or resolved, no meaningful comparisons can be made to our estimates of Zn enzyme activity along the long axis of the small intestine.

In all of the preparations divided from tip to crypt, normal to the mucosal surface, partial purification of the zinc enzyme resulted in marked increases in the Hill coefficient, generally as high as 6. This was not the case, however, with full mucosal thickness preparations, in the long axis experiments. These high Hill coefficients may reflect multiple in tandem Zn binding sites, by analogy to the Menkes- and Wilson-type Cu-ATPases (Petrukhin et al., 1994; Lutsenko et al., 1997; Payne & Gitlin, 1998). If so, our failure to record similar increases in the Hill coefficients in the long axis studies, with full thickness mucosal preparations, may be a consequence of lesser degrees of extraction of bound zinc during purification, as compared to the fractionated preparations.

Earlier studies by Davies (1980) and Jackson et al. (1981) are consistent with a two-step mechanism of intestinal Zn absorption, possibly passive entry across the apical surface and active extrusion by a basal-lateral Zn pump. The Mg-dependent, Zn-ATPase may prove to be the enzymatic expression of a cation-transporting ATPase.

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