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Effect of calcium on rat intestinal alkaline phosphatase activity and molecular aggregation

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Abstract

Two fractions of rat intestinal alkaline phosphatase (IAP) were detected by Western blot: 168 ± 6 and 475 ± 45 kDa. The low molecular weight fraction constitutes 43% of the isolated proteins exhibiting 82% of the enzymatic activity, and a heavier fraction constitutes 57% of the isolated proteins and has 18% of the enzymatic activity. Calcium produced an increase of the 475-kDa form to the detriment of the 168-kDa form. This work also describes the kinetic and structural changes of IAP as a function of calcium concentration. With $[Ca^{2+}] < 10$ mmole/L, the Ca^{2+}-IAP interaction fitted a binding model with 7.8 ± 4.4 moles of Ca^{2+} /mole of protein, affinity constant = 19.1 ± 8.4 L/mmole, and enzymatic activity increased as a linear function of $[Ca^{2+}]$ ($r = 0.946$ $p < 0.01$). On the other hand, with $[Ca^{2+}] > 10$ mmole/L the data did not fit this model and, the enzymatic activity decreased as a function of $[Ca^{2+}]$ ($r = -0.703$ $p < 0.05$).

Keywords: *Calcium, alkaline phosphatase, binding, rat, intestine, inhibition*

Introduction

The experiments reported in this paper were induced by experiments investigating the metabolism of monofluorophosphate (MFP) [1,2,3]. Most of this drug administered orally is hydrolyzed to phosphate and fluoride by intestinal alkaline phosphatase (IAP). The fraction of MFP absorbed without hydrolysis, increases by co-administration with calcium and binds to α2-macroglobulin (α2M) [1,3]. The metabolism of the complex α_2M-MFP presumably plays an important role in the bioavailability of MFP [2]. High calcium concentrations in the intestinal lumen inhibit IAP non-competitively [3], increase the half life of MFP and enhance its absorption.

This paper reports data on the interaction between calcium and IAP from rat, the biphasic effect of the cation on enzyme activity and on the aggregation of the protein. The concentrations of calcium at which these phenomena have physiological relevance are close to those occurring at the proximal intestinal lumen after the intake of a tablet (containing 250–750 mg of calcium) as a dietary supplement.

Materials and methods

Animals and reagents

Twenty four hour-fasted female inbred rats IIM/FcM strain (subline "m"), 180–200 g of body weight, fed with balanced food (Cargill, Buenos Aires, Argentine) and tap water *ad libitum* were used [4] according to the principles stated in the NIH guide [5].

Purification of IAP from intestinal mucosa

Ten mL of fresh intestinal mucosa [6] were mixed with an equal volume of buffer Tris-HCl 20 mmole/L, $MgCl_2$ 1 mmole/L, pH 8.2 and homogenized at 4°C. The purification process [7] involved: lipid extraction with butanol, gel filtration, and chromatography on DEAE-cellulose with a NaCl gradient. The enzyme

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was recovered by precipitation with acetone at −20°C, and dissolved in Tris-HCl buffer. IAP activity and protein concentration [8] were measured at the end of each purification step. The purification factor in the last step was 58, and the IAP solution was adjusted to 0.4 mg of protein/mL.

Production of primary Guinea Pig anti-rat IAP antibodies

Antiserum against rat IAP was raised in Guinea Pigs. The enzyme, purified as stated above was further purified by gel electrophoresis. The fraction of gel containing approximately 200 μg of active alkaline phosphatase was washed with phosphate buffer saline (PBS) pH 7.4, and the protein extracted by grinding the gel. Aliquots of this solution were mixed with complete Freund's adjuvant, and injected subcutaneously and intradermically. Antibody production was boosted by the administration of 200 μg of enzyme with incomplete Freund's adjuvant at 1-month intervals. The animals were bled 20 days after each boost, and the presence of the antibody in the serum was detected by radial immunodiffusion [9].

Measurement of alkaline phosphatase

Enzymatic activity in solutions. This was measured by a kinetic method [10] with p-nitrophenylphosphate (pNPP) as substrate. Activity was expressed as pmoles of pNPP $s^{-1}L^{-1}$.

Enzymatic activity in gels. After electrophoresis, gels were washed with Tris-HCl buffer and then incubated in a reaction mixture of β-glycerophosphate 0.5 mole/L, and $Co(NO_3)_2$ 0.5 mole/L, pH 8.2. IAP activity was detected by a purple precipitate of $Co_3(PO_4)_2$ [11]. A digital image of the gel was obtained. The integrated optical density (IOD) was measured with the aid of GelPro Analyzer 3.0 software (Media Cybernetics, Silver Spring MD, USA). IOD is a linear function of IAP activity ($r = 0.989$, $p < 0.001$). The method had a within-run coefficient of variation of 2.6% and a between-run coefficient of variation of 11.3%.

Amount of IAP by Dot Blot [12]. One half microlitre samples containing IAP standards or unknowns were applied to a nitrocellulose membrane (Hybond™-C extra, Amersham Life Science). After washing with PBS pH 7.4, the nitrocellulose membrane was incubated overnight with PBS containing Tween 0.1 ml/dL and defatted milk powder 5 g/dL. Membranes were then incubated with Guinea Pig anti-rat IAP primary antibody in PBS. Filters were then washed with PBS and incubated with the secondary antibody (rabbit anti-Guinea Pig IgG (whole molecule) peroxidase conjugate). Filters were washed with PBS and developed with 3-amino-9-ethylcarbazole at pH 5, and hydrogen peroxide. Digital images of dots were obtained, which were analyzed with GelPro Analyzer 3.0. Concentration of samples were calculated with the parameters of the regression curve obtained with standards. Known amounts of alkaline phosphatase were processed in the same way to obtain a calibration curve. IOD followed a linear function of IAP concentration in the range 0.0025–0.025 μg/μL, $IOD = 17450 \times [IAP]$, $r = 0.926$ $p < 0.0001$. The method has a within-run coefficient of variation of 6% at 0.015 μg/μL and 37% at 0.0025 μg/μL.

Kinetics of binding of calcium to IAP

The time required to obtain a stable inhibitory effect of calcium on IAP activity was investigated. At 20°C, IAP solutions were mixed with Ca^{2+} solution to attain 50 mmole/L. Immediately after the addition and subsequently at 20 s intervals, 10 μL aliquots were removed, mixed with pNPP reagent and the initial rate of hydrolysis recorded. Stable readings were obtained after 3–4 min. The data from four separate experiments were used to calculate the time needed to attain 50% of the initial value immediately after Ca^{2+} addition.

Measurement of calcium-IAP binding parameters

Triplicates of 200 μL of purified rat IAP solution were incubated for 5 min at room temperature with calcium (0–100 mmole/L) in the presence of 740 kBq of $^{45}Ca^{2+}$ (New England Nuclear, Research products, Boston, MA, USA). Ten μL of the mixture were employed for the measurement of total radioactivity. One hundred μL aliquots of these solutions were ultrafiltered through Ultrafree-MC Millipore 30-kDa cut-off membranes (Millipore Corporation, Bedford, MA, USA), and radioactivity was measured in the ultrafiltered solutions. Counts per min (cpm) were recorded until counting error was inferior to 2%.

With the data of total and ultrafiltered radioactivity, calcium and IAP concentrations, the molar ratio Ca^{2+}/ IAP (r) and free calcium (c) in the solution were calculated. The ratio "r/c" was plotted as function of "r" according to Scatchard [13] to estimate the number of binding sites (n) and affinity constant (K).

Dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Four per cent stacking and 5% separating gels were used [14]. Electrical supply was controlled with an EPS 3500 power supply (Pharmacia Biotech, Uppsala, Sweden). After electrophoresis, lanes containing molecular-weight markers, (phosphorylase

b cross-linked, Sigma Co, St. Louis, MI, USA), were fixed and dyed with Coomasie Brilliant Blue R.

Western Blot

After electrophoresis, proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond ™-C extra, Amersham Life Science). After transference, reaction with primary and secondary antibodies was done as described above. The amount of protein was expressed as the amount in each band, in relation to the amount in each lane (Rel Abs).

SDS-PAGE of IAP in the presence of 50 mmole/L $CaCl_2$

SDS-PAGE was run under the conditions stated above. Ten μg of IAP were electrophoresed in each lane. In one control lane IAP without calcium was run. Three kinds of experiments were carried out after electrophoresis.

1- Detection of active IAP. Proteins were transferred to a nitrocellulose membranes (four replicates), and then they were incubated for 30 min with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at pH 9.5 [15]. The site of phosphatase activity is revealed by a blue chromophore.

2- Molecular weight measurement and amount of protein in each lane by Western blot (four replicates).

3- Measurements of radioactivity in gels (four replicates): Ten μg of IAP and $^{45}Ca^{2+}$ were electrophoresed. Portions of the gel, where bands were detected by Western Blot, were cut, ground, and mixed with scintillation counting solution. Radioactivity was measured as stated above. Radioactivity was negligible in places where IAP was not detected by immunoblotting. Radioactivity of a given band was expressed as a percentage of total radioactivity in the lane.

Gel filtration of IAP on Sephadex® G200

Aliquots of IAP with and without Ca^{2+}50 mmole/L were filtered through a column of Sephadex® G-200 (Pharmacia Fine Chemical-Uppsala, Sweden). Elution was done with buffer Tris HCl pH 8.2 with a chromatographic equipment (Pharmacia Biotech.-Uppsala, Sweden). Fractions of 0.75 ml were collected and IAP activity and concentration were measured as stated above.

Results

Kinetics of the interaction calcium-IAP

In aqueous solutions, the presence of Ca^{2+}50 mmole/L, decreased IAP activity following a one phase exponential decay function of time. At 20°C, half-life of this phenomenon was 62.6 ± 2.0 s, $n = 4$. In the following experiments, IAP was previously incubated with Ca^{2+} for 5 min.

Activity of IAP as a function of calcium concentration

Activity of IAP displayed a biphasic behaviour as a function of calcium concentration in the incubation medium. At Ca^{2+} concentrations lower than 10 mmole/L, enzyme activity increased linearly as a function of calcium concentration ($r = 0.946$ $p < 0.01$). With calcium concentrations greater than 20 mmole/L the aforementioned relationship is reversed ($r = -0.703$ $p < 0.05$). Figure 1A displays the activity data obtained in solution with the purified enzyme and pNPP as substrate.

Figure 1B displays the data obtained in the gel electrophoresis experiments, where activity was measured by the amount of $Co_3(PO_4)_2$ precipitated *in situ* at 168 kDa. The data parallels that obtained with pNPP. At calcium concentration lower than 10 mmole/L, $r = 0.721$, $p < 0.05$. At calcium

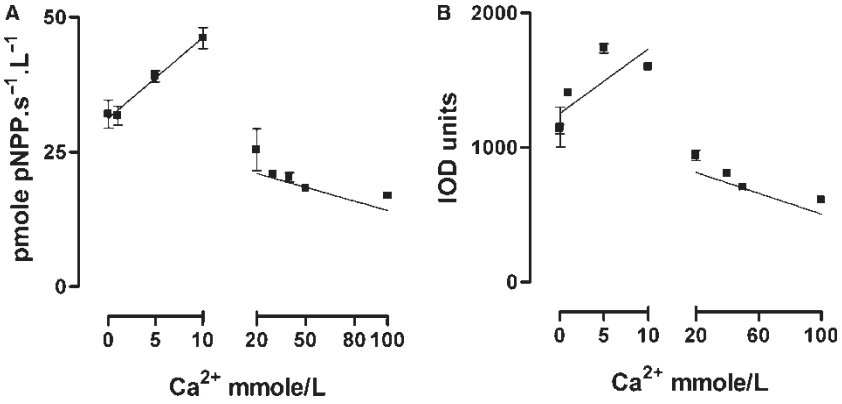

Figure 1. IAP activity as a function of calcium concentration. Panel A: Enzymatic activity in solution. Panel B: Enzymatic activity measured over polyacrylamide gel after electrophoresis. IOD: Integrated Optical Density. See text for experimental details. Each point indicates the mean ± SEM of four replicates.

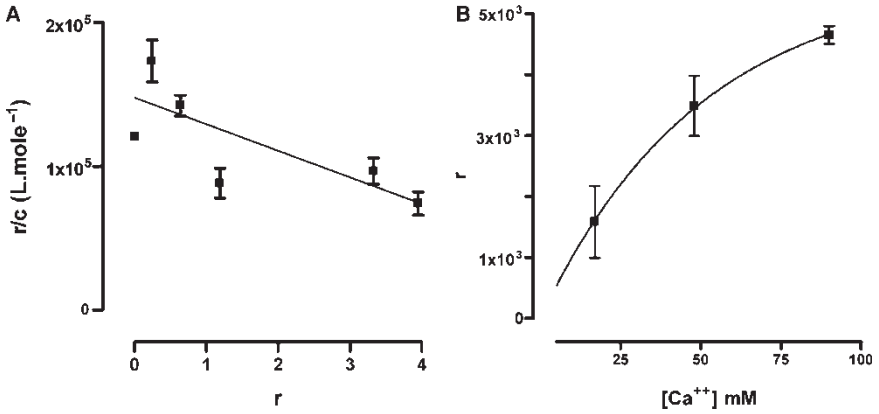

Figure 2. Calcium binding to purified IAP. Panel A: Scatchard plot, calcium concentration below 10 mmole/L. Panel B: kinetics of calcium binding with calcium concentrations above 20 mmole/L. Each point represents the mean ± SEM of three replications of the experiment.

concentrations exceeding 10 mmole/L, $r = -0.773$, $p < 0.05$. At 475 kDa IAP activity was negligible.

Measurement of calcium-IAP binding parameters

At calcium concentration lower than 10 mmole/L, the relationship between r/c as a function of "r", strongly suggested that calcium binds to IAP following a binding model of "n" independent sites with the same affinity (Figure 2A). The affinity constant for the interaction was 19.1 ± 8.4 L/mmole and IAP had 7.8 ± 4.4 sites for calcium binding. With Ca^{2+}20–100 mmole/L the kinetics did not fit the Scatchard model, and "r" followed a hyperbolic function of calcium concentration. IAP appeared to bind up to 6300 ± 1300 ions per mole of protein, assuming a molecular weight of 168 kDa.

The distribution of radioactivity ($^{45}Ca^{2+}$) in the resolution gel at 0 or 50 mmole/L Ca^{2+} agrees with these results (Table I).

Gel filtration of IAP on Sephadex® G200

Gel filtration of IAP in the absence of calcium displayed two peaks (Figure 3), with elution volume (Ve) = 14.4 ± 1.1 mL and Ve = 21.7 ± 0.9 mL. The former peak accounts for 57% of the proteins and 18% of the enzymatic activity. Its enzymatic specific activity was estimated with the area under the curve of activity and μg of protein as 0.15 pmoles of pNPP/s.μg of protein.

Table I. Distribution of $^{45}Ca^{2+}$ bound to the two fractions of IAP in SDS-PAGE.

Calcium, mmole/L	Percentage of radioactivity in bands without IAP activity	Percentage of radioactivity in bands with IAP activity
0	0 ± 0.2	100 ± 8.1
50	89 ± 5.4	11 ± 2.4

The lighter one accounted for 43% of total proteins and 82% of enzymatic activity. Its specific enzymatic activity was estimated as 1.74 pmoles of pNPP/s.μg of protein.

When the same experiments were repeated in the presence of Ca^{2+}50 mmole/L, the amount of IAP of the heavier fraction increased without modification in elution volume, while the concentration of the lighter fraction decreased.

Polyacrylamide gel electrophoresis of IAP

Western blot of IAP revealed two bands reacting with the primary anti-IAP antibody (Figure 4A). Bands 1 and 2 had a relative molecular weight of 475 ± 45 kDa and 168 ± 6 kDa (4 experiments). Measurement of Rel Abs indicated that when no Ca^{2+} was added, the enzyme distributed between bands 1 and 2 in the proportion of 0.38 ± 0.03 and 0.23 ± 0.07 Rel Abs units, respectively. With Ca^{2+}50 mmole/L, on the other hand, the enzyme distributed between bands 1 and 2 in the proportion of 0.52 ± 0.04 and 0.08 ± 0.01 Rel Abs units, respectively. Only the fraction with lower molecular weight exhibited IAP activity as shown by reaction with BCIP (Figure 4 B).

Discussion

Several proteins participate in the mechanism of intestinal calcium absorption namely, calcium binding protein D_{9K}, epithelial calcium channel TRPV6, Na^+-Ca^{2+} exchanger, Ca^{+2}-ATPase and alkaline phosphatase. With the exception of the latter, they have known roles in calcium transport across the epithelium [16]. Intestinal alkaline phosphatase is a brush border protein of the enterocyte as TRPV6 and the recently discovered calcium-sensing receptor [17]. This paper reports changes in activity and molecular mass of IAP by its interaction with calcium.

Recent studies have shown the existence of two mRNA encoding the rat IAP [18,19,20]. Research on

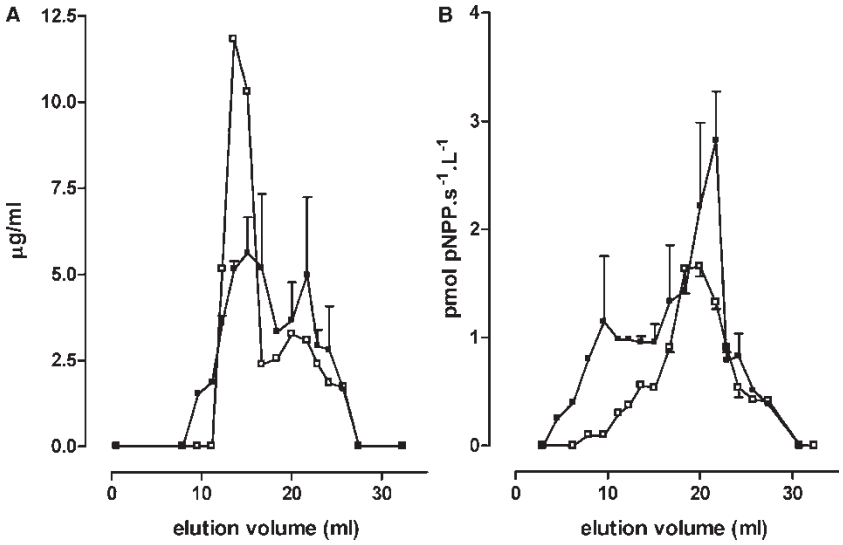

Figure 3. Gel filtration experiments. Eighty micrograms of IAP were filtered through Sephadex G200 without (solid squares) or with 50 mmole/L calcium in the elution buffer (open squares). Panel A describes the elution pattern of proteins measured with an specific antibody by the dot blot technique (see text for experimental details). Panel B describes the elution pattern of enzymatic activity in aliquots of each fraction. Each point represents the mean ± SEM of three replications of the experiment.

the differences in fine structure of these isozymes has already been initiated [21]. Several phenomena have been reported that further explain the molecular heterogeneity of the enzyme: Post-translational modifications [22], proteolysis of the molecule, that modifies enzymatic activity and molecular weight [23], and molecular association [23].

Good correlations between calcium transfer and IAP activity have been reported in *in vivo* [24,25,26] and *in vitro* models [27,28]. Calbindin-D_{9K} and IAP are significantly decreased in spontaneously hypertensive rats [29]. These findings further extend the association between the stimulatory effect of vitamin D and IAP [30,31] with transepithelial calcium transport.

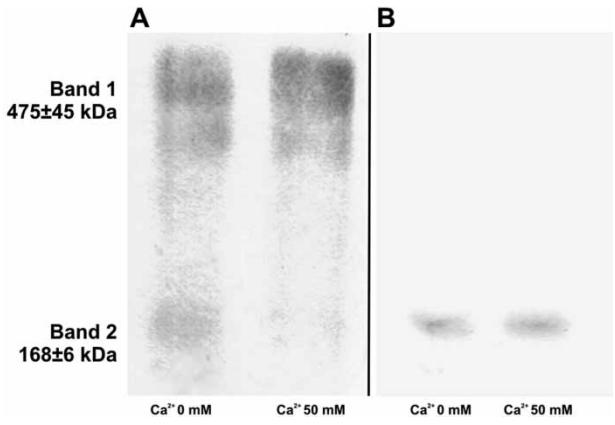

Figure 4. Panel A: Western Blot of IAP revealed two bands reacting with the primary anti-IAP antibody. Band 1 and 2 had a relative molecular weights of 475 ± 45 and 168 ± 6 kDa (four replicates). Measurement of Rel Abs indicated that when no Ca^{2+} was added, the enzyme distributed 62% in band 1 and 38% in band 2. With Ca^{2+} 50 mmole/L, the enzyme distributed 87% in band 1 and 13% in band 2. Panel B: Only the fraction with lower molecular weight exhibited IAP activity as shown by reaction with BCIP.

Cell membranes are characterized by high affinity and capacity for binding calcium ions. Thus, uptake of calcium by brush border vesicles will be determined in part by binding as well as transmembrane transport. Some of the experiments [32] are consistent with the concept that a major component of calcium uptake is a movement across the brush border vesicles membranes.

Membrane transport of calcium depends in part on membrane lipid fluidity [33]. Deliconstantinos et al. [34], working with rat intestinal brush border membranes, studied the effect of Ca^{2+} on the physicochemical properties of the membranes. Ca^{2+} addition decreased lipid fluidity and increased the energy of activation of IAP. Saini & Posen [35] have reported that the high post-prandial phosphatase activity of rat sera was partially sensitive to antibodies obtained with IAP.

We are not aware of studies on the binding ability of IAP to Ca^{2+}. The calcium binding ability of a 52-kDa alkaline phosphatase of calcifing and precalcifing cartilage has been demonstrated with non-homogenous preparations [36] and with purified proteins from bovine scapulae [37]. This protein binds 25 calcium ions/ mole of protein, with a dissociation constant of 0.31 µmole/L. The effect of calcium on the enzyme activity of the enzyme was not investigated.

Deliconstantinos et al. found a rapid inhibitory effect of Ca^{2+}(0 to 10 mmole/L) on membrane-bound and detergent solubilized activity of brush border rat IAP [34]. With the same calcium concentrations, we report that calcium binds to IAP following a model of

independent sites and simultaneously increases enzymatic activity. With high Ca^{2+} concentration (50 mmole/L) we observe the increase of a high-molecular weight form and simultaneously, a decrease in enzymatic activity and the amount of low-molecular weight IAP. These conclusions are supported by SDS-PAGE, Western Blot and gel filtration experiments. The decrease in radioactivity in the area of the gel with the 168-kDa form IAP when IAP was treated with calcium and $^{45}Ca^{2+}$ and the proportional increase of radioactivity in the area with the 475-kDa form, strongly suggest that Ca^{2+} binding induces an important modification in the molecular mass of the enzyme. The ratio of molecular weights of these fractions is close to 3 when analyzed by Western Blot. Further experiments already under course are required to demonstrate whether the adduct Ca^{2+}-phosphatase may act in the transepithelial transport of calcium.

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