[Chem. Pharm. Bull. 32(1) 198—204 (1984)]

A Role of Alkaline Phosphatase in Phosphate Transport[1)]

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(Received April 21, 1983)

In order to elucidate the physiological function of intestinal alkaline phosphatase, the characteristics of alkaline phosphatases from rat and human small intestine were compared under optimal and physiological pHs. The K_m values of these enzymes towards *p*-nitrophenylphosphate at the physiological pH were lower by two orders of magnitude than those at the optimal pH. At the physiological pH, phosphate, arsenate and vanadate competitively inhibited alkaline phosphatase activity, as they did at the optimal pHs, and the K_i values of these inhibitors at the physiological pH were also lower by two orders of magnitude than those at the optimal pHs. The effects of various inhibitors and antiserum to rat intestinal alkaline phosphatase on the transport of phosphate into everted rat intestine were investigated. The results obtained from the present study indicate that a phosphate transport system operating at physiological pH exists in the upper part of the small intestine where the alkaline phosphatase is maximally concentrated. It was also found that the phosphate transport was affected by various inhibitors and antiserum to rat intestinal alkaline phosphatase, but L-homoarginine and ouabain had no effect. From the above findings, it is suggested that alkaline phosphatase may function not only as a hydrolytic enzyme of phosphomonoesters but also as a phosphate transporter in the physiological state.

Keywords——alkaline phosphatase; rat intestine; human intestine; physiological function; hydrolytic enzyme; phosphate transporter

Alkaline phosphatase is known to catalyze the hydrolysis of various phosphate compounds. Characterization of this enzyme is generally performed at the optimal pH and using synthetic phosphomonoester as a substrate. Alkaline phosphatase is found in abundance at the absorption site of various materials,[2–4)] and in a preliminary experiment, we found that rat intestinal alkaline phosphatase might function not only as a hydrolytic enzyme of phosphoester but also as a phosphate transporter in the physiological state.[5)] In the present work, we studied the effects of various inhibitors on alkaline phosphatase activities from rat and human small intestine at a physiological pH (7.4), and compared the results with those at the optimal pHs. Several investigators have proposed hydrolysis of phosphoesters,[6)] phosphate transferase activity[7,8)] and transport of phosphate[9,10)] as functions of alkaline phosphatase, although others have reported that inhibitors of this enzyme are without effect on its function in intestine and kidney.[11–13)] In most mammalian species, intestinal alkaline phosphatase is maximally concentrated on the microvilli of the small intestine.[14,15)] This site is important in the transport of phosphate from the external to the internal milieu.[9)] Therefore, a phosphate transporter with an ability to pick up phosphate and transport it clearly exists at that site. Although alkaline phosphatase was discovered more than 80 years ago, there is no

positive evidence regarding its physiological function. In this paper, we now offer evidence of a possible role of intestinal alkaline phosphatase as a phosphate transporter in the physiological state.

Experimental

Materials——All reagents used were of analytical reagent grade. Carrier-free [^{32}P]orthophosphate was obtained from New England Nuclear Corp. Male Wistar rats, weighing between 180 and 200 g, were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals. The animals were provided with a standard laboratory diet and water.

Preparation of Intestinal Alkaline Phosphatases——Alkaline phosphatases were extracted from human and rat intestinal mucosa by the modified butanol method of Morton.[16] The supernatant was dialyzed against 10 mM Tris-HCl buffer (pH 7.4) containing 10 μM $MgCl_2$ and $ZnCl_2$ and was used as the enzyme solution. This enzyme was used for experiments to determine the kinetic parameters.

Assay of Alkaline Phosphatase Activity——Alkaline phosphatase activity was measured in 50 mM glycine–KCl–KOH buffer containing 0.5 mM $MgCl_2$ at the optimal pH and also in 20 mM Tris-HEPES buffer (pH 7.4) containing 0.1 M NaCl and 0.1 M mannitol using *p*-nitrophenylphosphate as a substrate.[17] One ml of 10 mM substrate solution and 3 ml of the above buffer were preincubated at 37 °C. One hundred μl of the enzyme solution was added and the enzyme reaction was carried out at 37 °C for 15 min. The reaction was stopped by the addition of 2 ml of 0.2 N NaOH and the absorbance was determined at 405 nm. One unit of alkaline phosphatase activity was defined as the amount of enzyme which produced 1 μmol/min of *p*-nitrophenol. The kinetic parameters (K_m and V_{max}) were estimated from Lineweaver–Burk plots, and inhibition constants (K_i) were calculated from Dixon plots or Lineweaver–Burk plots.

Assay of Protein——The everted intestine was homogenized with 4 volumes of distilled water in a Polytron homogenizer, and the homogenate was centrifuged ($10000 \times g$, 20 min). In the soluble fraction, protein was determined by the method of Lowry *et al.*[18] using bovine serum albumin as a standard. In addition, the absorbance was measured at 280 nm.

Measurement of Phosphate Transport into Everted Rat Intestine——An intestinal segment (about 7 cm) was quickly removed from a male Wistar rat, washed gently with 20 mM Tris-HEPES buffer (pH 7.4) containing 0.1 M NaCl and 0.1 M mannitol, and everted by means of a glass tube. Two glass tubes were inserted into both ends of the everted intestine and tied securely with thread.[19] The glass tubes were connected with Tygon tubes. The intestinal lumina were immersed in 1 mM disodium hydrogen phosphate in the above buffer maintained at 37 °C and saturated with 95% O_2 and 5% CO_2. Six ml of the above buffer was circulated through the serosal lumen of the everted intestine with roller pump at 0.5 ml/min. Phosphate contents in the circulating buffer were determined by the method of Fiske and SubbaRow.[20]

Antiserum——Antiserum to rat intestinal alkaline phosphatase was obtained from a rabbit immunized with the purified rat intestinal alkaline phosphatase in the same manner as described in the previous paper.[21] The inhibition of rat intestinal alkaline phosphatase activity by antiserum was investigated according to Lehmann.[22] Five μl of anti-rat intestinal alkaline phosphatase antiserum precipitated up to 60% (0.6 U) of the original activity.

Binding of Human Intestinal Alkaline Phosphatase and [^{32}P]-Orthophosphate——The purified human intestinal alkaline phosphatase was prepared according to the previous paper[23] and the crude enzyme was extracted from intestinal mucosa by the method of Morton.[16] These enzymes were labelled with [^{32}P]-orthophosphate under the following conditions. The purified enzyme (1 mg protein) or crude enzyme (10 mg protein) was mixed with 5 mCi carrier-free [^{32}P]orthophosphate in 50 mM acetate buffer (pH 5.0) containing 10 μM $MgCl_2$ and $ZnCl_2$. The mixture was applied to a Sephadex G-200 column (1.5 × 100 cm) equilibrated with the above buffer. Alkaline phosphatase activity was determined by the method of Kind and King.[24] Radioactivity in the solutions was determined in an Aloka model LSC-651 liquid scintillation spectrometer (Aloka Instrument Co., Tokyo, Japan). Samples (50 μl) were dissolved in 10 ml of Oxifluor®–H_2O (New England Nuclear Corp., Boston, Mass.) in glass counting vials and shaken vigorously before counting.

Results and Discussion

Kinetic Parameters of Intestinal Alkaline Phosphatase at the Optimal and Physiological pHs

The optimal pH of rat intestinal alkaline phosphatase was investigated. Rat and human intestinal alkaline phosphatase had optimal pHs of 9.5 and 10.5,[25] respectively. Table I shows comparative data on the K_m and V_{max} of rat and human intestinal alkaline phosphatases towards *p*-nitrophenylphosphate as a substrate at both the optimal and physiological pHs. It is evident that both parameters are markedly decreased at pH 7.4. These data indicate that

TABLE I. Kinetic Parameters of Intestinal Alkaline Phosphatase at Optimal and Physiological pHs

Source	Optimal pH		Physiological pH	
	K_m (μM)	V_{max} (μmol/min/mg)	K_m (μM)	V_{max} (μmol/min/mg)
Rat	790	180	6.1	33
Human	880	740	4.7	87

The conditions for measurement of these parameters were as described in the text.

TABLE II. Summary of Inhibition Data for Intestinal Alkaline Phosphatase by Various Inhibitors at Optimal and Physiological pHs

Inhibitor	Optimal pH		Physiological pH	
	Rat	Human	Rat	Human
	Apparent K_i (mM)			
Phosphate	2.8	13	0.15	0.46
Arsenate	0.24	1.6	0.0048	0.0050
Vanadate	0.029	0.21	0.0033	0.0038
L-Phenylalanine	9.1	2.5	4.6	1.5
KCN	1.9	2.0	3.3	2.8

The conditions for measurement of these parameters were as described in the text.

TABLE III. Relation between Rat Intestinal Alkaline Phosphatase Activity and Phosphate Transport

Segment number	Phosphate transport (nmol/mg protein)	Alkaline phosphatase activity (U/mg protein)
1	110±1.9	2.6±0.11
2	76±7.4	1.7±0.12
3	61±1.9	1.0±0.020
4	33±6.5	0.5±0.070

This distance from the duodenum to the ileum is denoted by the segment numbers 1—4. The values are represented as the amount of transported phosphate after 90 min of incubation. Alkaline phosphatase activity was measured at the optimal pH (9.5). Mean value±S.D. derived from three experiments are given. Other conditions were as described in the text.

while the affinity of the substrate to the enzyme is greatly increased, hydrolytic activity towards the substrate is nevertheless suppressed. The effects of various inhibitors for both enzymes were also investigated at the optimal and physiological pHs. As shown in Table II, although all these compounds were powerful inhibitors of the hydrolytic activity, the K_i values of competitive inhibitors (phosphate, arsenate and vanadate) at pH 7.4 were lower by two orders of magnitude than those at the optimal pHs in both rat and human intestinal alkaline phosphatases. Therefore, the binding of phosphate to the molecule of rat or human intestinal alkaline phosphatase at physiological pH is likely to be much tighter than that at the optimal pH and this should be advantageous for picking up phosphate. At the physiological pH, however, the K_i values of L-phenylalanine as an uncompetitive inhibitor and potassium

TABLE IV. Effect of pH on the Phosphate Transport into Everted Rat Intestine

pH	Phosphate transport (nmol/mg protein)
6.5	57.4±3.9
7.0	59.0±3.2
7.4	84.5±7.4
8.0	54.8±2.9

The phosphate transport was investigated at the indicated pH values. The data show the amounts of transported phosphate after 60 min of incubation. Mean values±S.D. derived from three experiments are given. Other conditions were as described in the text.

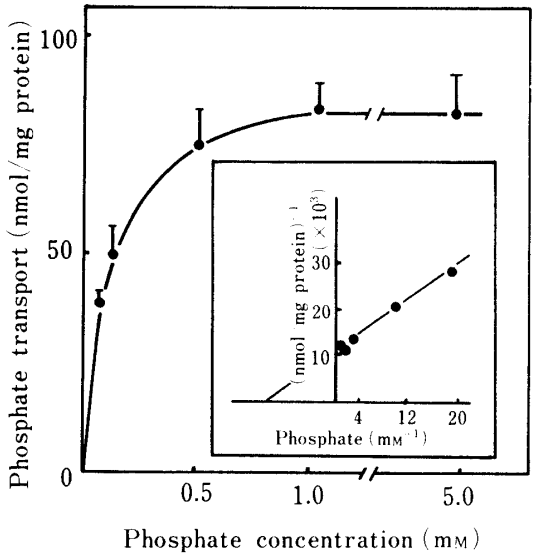

Fig. 1. Effect of Phosphate Concentration on Phosphate Transport

The conditions were as described in the text. The values are represented as the amount of transported phosphate after 60 min of incubation. The inset shows the Lineweaver–Burk plot of the data. Mean values±S.D. derived from three experiments are given.

cyanide as a chelator removing zinc were not significantly different from those at the optimal pHs. On the other hand, these enzymes were not affected by either L-homoarginine (an inhibitor of liver alkaline phosphatase) or ouabain (an inhibitor of sodium- and potassium-dependent adenosine triphosphatase (ATPase).

Phosphate Transport into Everted Rat Intestine

Some functions of alkaline phosphatase have been proposed by several investigators.[6-10] The high optimal pH of the enzyme phosphohydrolase activity would appear to be inconsistent with this being the primary function at neutral pH in the body.

Utilizing rat intestine, the relation between intestinal alkaline phosphatase activity and phosphate transport was investigated. As shown in Table III, the amount of phosphate transported into the serosal lumen of everted rat intestine was maximal at the upper part of the jejunum. This site (segment number 1) was used in the standard system for the investigation of phosphate transport. The correlation between the enzyme activity and the amount of transported phosphate was excellent ($r=0.990$). The results indicate that there is a phosphate transport system in the upper part of the jejunum and that alkaline phosphatase, localized at this site, may be involved in this transport. The sites of intestinal absorption of most materials are not all at the upper part of the jejunum. For example, lipids, bile acids, vitamin B_{12} and glucuronated drugs are absorbed at the ileum.[28] Therefore, these findings indicate that phosphate is preferentially transported at the upper part of the jejunum.

The effect of pH change from 6.5 to 8.0 in the mucosal fluid on phosphate transport was

TABLE V. Effects of Various Inhibitors on Phosphate Transport into Everted Rat Intestine

Inhibitor or substrate	Phosphate transport (nmol/mg protein)
Control	110± 1.9
Disodium phenylphosphate[a)]	106±12
Vanadate (1 mM)	83± 4.5
KCN (1 mM)	71± 2.0
L-Phenylalanine (10 mM)	64± 7.7
Arsenate (1 mM)	49± 8.7
Anti-rat intestinal alkaline phosphatase antiserum	45± 8.4
Ouabain (1 mM)	110± 9.7
L-Homoarginine (10 mM)	110± 3.9

a) Disodium phenylphosphate at 4.2 mM instead of 1 mM disodium hydrogen phosphate in the control.

The incubation medium consisted of 20 mM Tris-HEPES (pH 7.4), 0.1 M NaCl, 0.1 M mannitol, 1 mM disodium hydrogen phosphate and various inhibitors as indicated. The data show the amounts of transported phosphate after 90 min of incubation. Mean values ±S.D. derived from three experiments are given. Other conditions were as described in the text.

also investigated. As shown in Table IV, the amount of phosphate transported into the serosal lumen of everted rat intestine was maximal at pH 7.4, which is the physiological pH. Also, as shown in Fig. 1, the amount of phosphate tranported through everted rat intestine showed a saturation curve which followed Michaelis–Menten kinetics, and the apparent K value for phosphate was calculated to be 1.0×10^{-4} M. This value is in good agreement with the K_i (1.5×10^{-4} M) calculated from the inhibition of the activity of rat intestinal alkaline phosphatase by phosphate at pH 7.4. The amount of phosphate transported into the serosal lumen of everted rat intestine in the presence of various inhibitors or anti-rat intestinal alkaline phosphatase antiserum was also examined. The inhibitors presented in Table V significantly suppressed the phosphate transport. It was found that the phosphate transport was affected by various inhibitors of rat intestinal alkaline phosphatase activity, while L-homoarginine and ouabain had no effect on the transport of phosphate under these conditions. In particular, anti-rat intestinal alkaline phosphatase antiserum also suppressed the phosphate transport, whereas normal rabbit serum did not. The binding of antibody to the enzyme has no influence on the enzyme activity.[26,27)] This fact may be attributed to the inhibition of conformational change induced by the binding of phosphate.[30)] On the other hand, L-homoarginine and ouabain, which are inhibitors of liver alkaline phosphatase and sodium- and potassium-dependent ATPase, respectively, had no effect on the transport of phosphate under these conditions. From these results, it is suggested that rat intestinal alkaline phosphatase has the ability to pick up phosphate and to act as a phosphate transporter in the physiological state. Moreover, when disodium phenylphosphate was used instead of disodium hydrogen phosphate as a substrate, the phosphate liberated from the substrate was transported into the serosal lumen of everted rat intestine as shown in Table V.

The above results strongly suggest that alkaline phosphatase may function not only as a hydrolytic enzyme of phosphomonoesters but also as a phosphate transporter in the physiological state. Moreover, the direction of phosphate transport is probably controlled mainly by pH.

However, the ability of competitive inhibitors to suppress the phosphate transport was not well accounted for by the kinetic parameters, the K_i values of these inhibitors. This discrepancy may be explained on the basis that the kinetic parameters of the hydrolytic activity of alkaline phosphatase cannot be applied to the phosphate transport activity. The

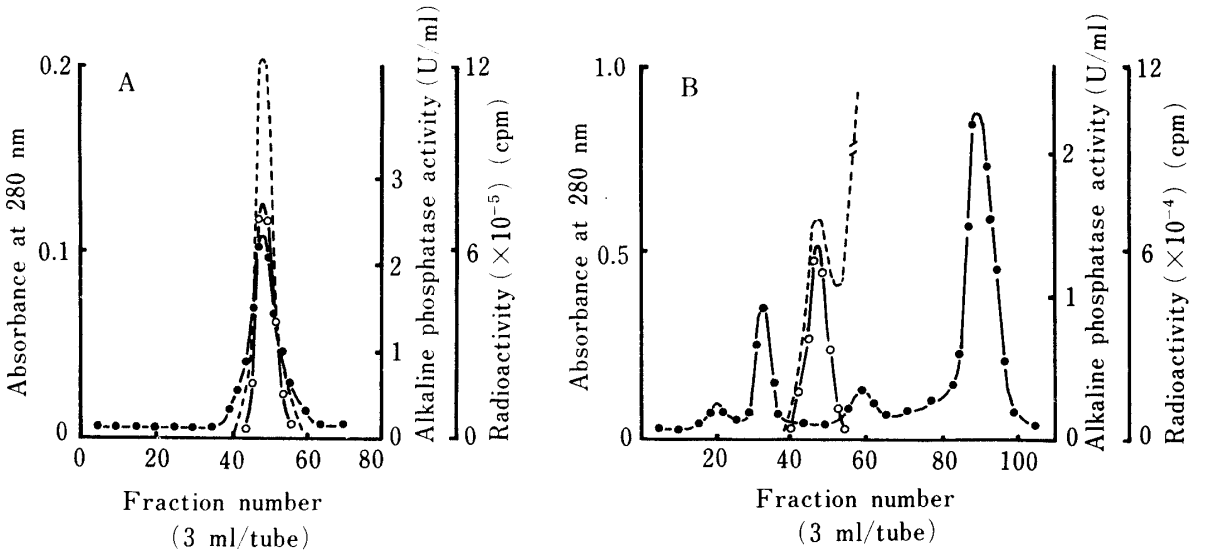

Fig. 2. Elution Patterns of Human Intestinal Alkaline Phosphatase Bound with [^{32}P]Orthophosphate

—●—, protein; ---○---, alkaline phosphatase activity; - - - -, radioactivity.
A, purified human intestinal alkaline phosphatase; B, crude human intestinal alkaline phosphatase. The conditions were as described in the text.

phosphate transport was not suppressed by these inhibitors at concentrations corresponding to the K_i values. Indeed, the velocity of phosphate transport (1.45×10^{-9} mol·mg^{-1}·min^{-1}) was in a ratio of 1:23000 compared with that (3.30×10^{-5} mol·mg^{-1}·min^{-1}) of the hydrolytic activity of rat intestinal alkaline phosphatase towards *p*-nitrophenylphosphate.

Sodium- and potassium-dependent ATPase was shown recently to be phosphorylated by inorganic phosphate.[29] However, in the presence of ouabain, the phosphate transport was not affected. It is not known whether 5′-nucleotidase could be phosphorylated by orthophosphate.[9]

Binding of Phosphate to Human Intestinal Alkaline Phosphatase

It is difficult to carry out experiments on phosphate transport using everted human intestine. No significant differences between rat and human intestinal alkaline phosphatase have been found in enzymic properties, including the behavior towards substrate and inhibitors at the optimal pH. In particular, the substrate or phosphate affinity of human intestinal alkaline phosphatase was greatly increased at physiological pH, as was that of rat intestinal alkaline phosphatase. Therefore, the binding of [^{32}P]orthophosphate to human intestinal alkaline phosphatase was investigated by gel filtration. As shown in Fig. 2, the purified enzyme specifically bound [^{32}P]orthophosphate. In similar experiments with the crude enzyme, the radioactivity appeared in the fraction containing the enzyme activity. The elution pattern of [^{32}P]orthophosphate bound to human intestinal alkaline phosphatase was the same with both purified and crude enzymes. It was considered that the large peak of radioactivity following this peak was due to [^{32}P]orthophosphate bound to the albumin-rich fraction, because the elution volume coincided with that of albumin. The results suggest that human intestinal alkaline phosphatase may also take part in phosphate transport as a phosphate transporter in the physiological state.

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