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ORIGINAL

Tyrosine phosphatase-like activity of bone-type alkaline phosphatase at neutral pH range

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Abstract: We aimed to determine physiological substrate and optimal pH for bone-type alkaline phosphatase (ALP, EC. 3. 1. 3. 1). Bone-type ALP was purified over 900-fold from clonal osteoblastic cells. MC3T3-E1, by butanol treatment, and chromatographies of anion exchange, Con A affinity and gel filtration. The purified enzyme showed a single protein band of molecular weight 76,000 on SDS-polyacrylamide gel electrophoresis. The purified enzyme hydrolyzed *p*-nitrophenyl phosphate (*p*NPP) and dephosphorylated tyrosine-phosphorylated myelin basic protein (Tyr-P-MBP), but not serine- and threonine-phosphorylated MBP. Both activities were inhibited by levamisole, an ALP inhibitor, but not by okadaic acid or dephostatin (protein phosphatase inhibitors). The optimal pH ranges for *p*NPP and Tyr-P-MBP activities were 9.4 to 10.4 and 8.3 to 8.7, respectively. The ratios of activity at optimal pH to the one at pH 7 were 32.2 and 1.6 for *p*NPP and Tyr-P-MBP, respectively. Tyr-P-MBP dephosphorylation was competed by *p*NPP. These results suggest that bone-type ALP displays tyrosine phosphatase-like activity at neutral pH range and this activity may play an important role in physiological function of bone-type ALP.

Keyword: alkaline phosphatase, protein tyrosine phosphatase, osteoblastic cell, purification

Introduction

Alkaline phosphatases (ALPs) are present in many mammalian tissues and abundant in the cells associated with hard tissue formation. Bone-specific ALP is used as an index of cell differentiation into osteoblast and of the activity of bone formation in many studies [1-3]. It is also used in clinical chemistry as a marker enzyme, which reflects the dynamic phase of the bone [4,5]. Many efforts have been made to determine the physiological functions of the enzymes present in hard tissue, but no specific character of the enzymes to explain the function of hard tissue has yet been described. Physiological substrates and pH for ALPs also remain to be determined. When artificial substrates, for example pNPP, are used to measure the ALP activities, alkaline pH, which is far from physiological condition, is optimal for the activity [4-6]. A question arises here whether ALP can work at alkaline pH for physiological substrates. Several reports have suggested that ALP has protein phosphatase-like activity [7-12], but the physiological substrate for ALP has not been established. We have investigated the possibility that ALP in the cells associated with hard tissue formation might act as protein phosphatase at physiological pH range and participate in the regulation of tissue formation. This is the first study to report that ALP of osteoblastic cells has tyrosine phosphatase-like activity at neutral pH.

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Materials and Methods

1. Cell culture

A mouse clonal osteoblastic cell line, MC3T3-E1 [13,14], was donated by Dr. Kodama (Ohu University) and Dr. Kuboki (Hokkaido University). The cells were seeded in the dishes of 100 mm diameter, and grown in a a-minimum essential medium (α-MEM) supplemented with 10 % (v/v) fetal bovine serum (FBS) (F.Hoffmann-La Roche Ltd., Basel, Switzerland) and kanamycin sulfate in a 5 % CO_2-95 % air atmosphere at 37°C. The medium in each dish was changed every 2 or 3 days and the cells were harvested at 37 days after confluence, ultrasonicated and then used for butanol treatment.

2.Butanol treatment

The cell homogenate was suspended in 10 mM tris-HCl buffer (pH 7.4) containing 0.2 mM $MgCl_2$, 0.02 mM $ZnSO_4$ and 1 mM phenylmethylsulfonylfluoride (buffer A), and then the same volume of butanol was added to the homogenate and the mixture was agitated for 3 hours at room temperature. The mixture was centrifuged at 12,000 rpm for 30 minutes and water layer was recovered. It was concentrated with the use of Centriprep-30 (Sartorius AG, Goettingen, Germany) and then buffer was changed to 10 mM tris-HCl buffer (pH 7.4) containing 0.2 mM $MgCl_2$ and 0.02 mM $ZnSO_4$ (buffer B) by using HiPrep 26/10 (Amersham Pharmacia Biotech, Uppsala, Sweden). The crude extract was kept at 4°C

3.Column chromatography

Column chromatography was performed using FPLC system (Amersham Pharmacia Biotech). Purification of ALP was performed basically according to the method described by Toen et al. [15]

1) Anion exchange column chromatography

Crude extract was applied to POROS 50D column (PerSeptive Biosystems GmbH, Wlesbaden, Germany) (0.46 × 10 cm) which had been equilibrated with buffer B. The column was washed with buffer B until the absorbance at 280 nm returned to the base line, and then the column was eluted with a linear gradient of NaCl (0-1 M) in buffer B. The active fractions were pooled and the buffer was changed with the use of PD 10 column (Amersham Pharmacia Biotech) equilibrated with 10 mM tris-HCl buffer (pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.2 mM $MgCl_2$, 0.02 mM $ZnSO_4$ and 500 mM NaCl (buffer C).

2) Affinity chromatography

Active fractions were applied to HiTrap Con A column (0.7 × 2.5 cm) (Amersham Pharmacia Biotech) which had been equilibrated with buffer C. The column was washed with buffer C, and then eluted with a linear gradient of methyl-α-D-mannoside (0-100 mM) in buffer C. The active fractions were pooled and concentrated with Centrisalt I (Cut-off 20,000) (Sartorius AG).

3) Gel filtration chromatography

The concentrated active fractions were applied to Superdex 200 HR 10/30 gel filtration chromatography column (1.0x30 cm) (Amersham Pharmacia Biotech) which had been equilibrated with 10 mM tris-HCl buffer (pH 7.4) containing 0.2 mM $MgCl_2$, 0.02 mM $ZnSO_4$ and 500 mM NaCl. (buffer D). The column was eluted with the same buffer; fractions with peak activity were pooled and kept at -80°C until use. The molecular weight of ALP was estimated by eluting the gel filtration standards, i.e. thyroglobulin, gamma globulin, ovalbumin, myoglobin and vitamin B-12 (Bio Rad, Hercules, CA) on the same condition.

4. pNPP hydrolysis

pNPP hydrolysis by the enzyme fraction was measured as described previously [16]. Namely, basic reaction mixture contained 5 ml of enzyme fraction, 25 μmol of carbonate, 12.5 mmol of sucrose and 1 mmol of $MgCl_2$ in 400 μl (pH 9.7) and the reaction was started by the addition of 100 μl of 10 mM pNPP at 37°C

Protein concentration was measured with Bio-Rad DC protein assay kit (Bio Rad) using bovine serum albumin as standard.

5. Protein phosphatase activity

Tyrosine- or serine/threonine-phosphorylated proteins were prepared basically by the methods described by Tonks et al. [17] and Swarup et al. [11], respectively. Myeline basic protein (MBP) (Upstate Biotechnology, Lake Placid, NY) and reduced, carboxymethylated and maleylated lysozyme (RCM-lysozyme) (Sigma, St. Louis, MO) were phosphorylated with [γ-^{32}P] ATP catalyzed by Abl protein tyrosine kinase (New England Biolabs Inc. UK). MBP and type ‡UA histone (Sigma) were phosphorylated with [γ-^{32}P] ATP catalyzed by cAMP-dependent protein kinase, a catalytic subunit (Sigma). The concentrations of tyrosine-phosphorylated MBP (Tyr-P-MBP), RCM-lysozyme (Tyr-P-RCM-lysozyme), serine/threonine-phosphorylated MBP (Ser/Thr-P-MBP) and histone (Ser/The-P-histone) were 5.47, 0.59, 13.25 and 4.95 μM, respectively. The assay was based on the measurement of released [^{32}P] Pi from labeled substrates. The concentrations of the various components in 40 μl reaction mixture were 1 to 5 μl of enzyme fraction, 1.25 μmol of sucrose, 0.1 mmol of $MgCl_2$ and 2.5 μmol of tris-acetate or carbonate of different pH levels. The reaction was started by the addition of 10 μl

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of phosphorylated proteins at 30°C, and then terminated by 200 μl of cold 20 % trichloroacetic acid after appropriate reaction time. The mixture was vortexed, placed on ice for 10 minutes and centrifuged at 12,000 g for 5 minutes. The radioactivity of [^{32}P] Pi recovered in the supernatant was counted with a liquid scintillation counter.

6. Hydrolysis of phosphate compounds

For measurement of the hydrolysis of ATP, β-glycerophosphate, pyridoxal phosphate and phosphoethanolamine, concentrations of the components in 250 μl reaction mixture were fixed as follows: 1 to 5 μl of enzyme fraction, 7.5 μ mol of sucrose, 0.6 μmol of $MgCl_2$ and 15 μmol of tris-acetate or carbonate. The reaction was started by the addition of 50 ml of phosphate compounds at 37°C, and was terminated by addition of 300 ml of 12 % SDS after appropriate reaction time. Inorganic phosphate released by the enzyme reaction was measured colorimetrically according to method of Chifflet et al.[18]. The activity observed in the presence of levamisole was subtracted from the one without levamisole for measurement of the activity catalyzed by ALP.

7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a 7.5 % (w/v) polyacrylamide gel overlaid with a 3 % (w/v) stacking gel as described by Laemmli [19] and the gel was visualized with the use of PlusOne Protein silver staining kit (Amersham Pharmacia Biotech).

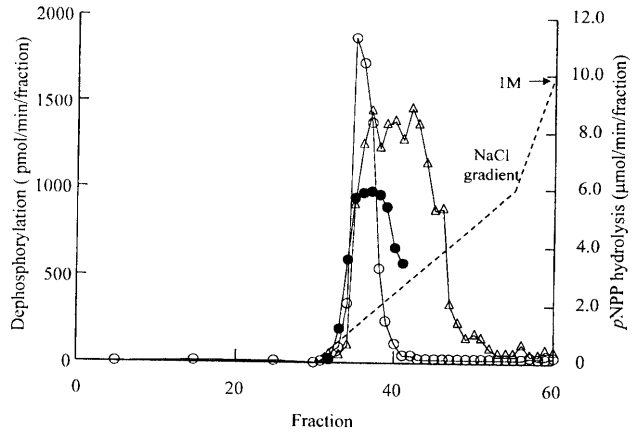

Fig. 1. Typical elution profiles of the POROS 50D anion exchange chromatography of bone-type ALP. The POROS 50D anion exchange chromatography was performed as described in Materials and Methods. Fractions were assayed for *p*NPP hydrolysis (○), Ser/Thr-P-MBP (△) and Tyr-P-MBP dephosphorylation (●).

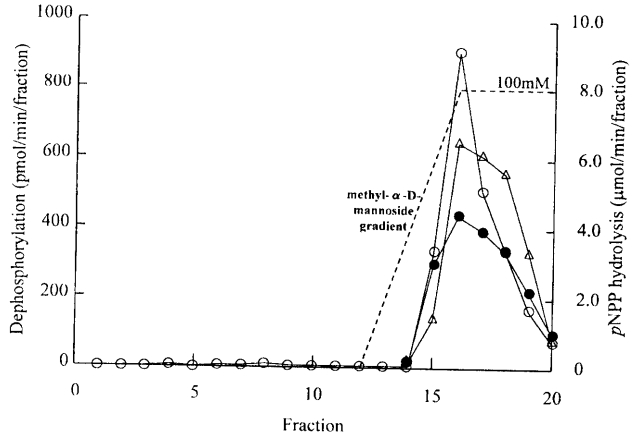

Fig. 2.Typical elution profiles of the HiTrap Con A affinity chromatography of bone-type ALP. Con A affinity chromatography was performed as described in Materials and Methods. Fractions were assayed for *p*NPP hydrolysis (○), Ser/Thr-P-MBP (△) and Tyr-P-MBP dephosphorylation (●).

Results

1. Purification of ALP from osteoblastic cell line, MC3T3-E1, by column chromatography

For characterization of bone-type ALP, clonal osteoblastic cells, MC3T3-E1, were cultured and ALP was purified from the cell extract as described in Material and Methods. During the purification steps including butanol treatment, anion exchange chromatography (Fig. 1), Con A affinity chromatography (Fig. 2), and gel filtration chromatography (Fig. 3), ALP activities were monitored with pNPP as a substrate, which is commonly used for ALP assay. ALP was purified over 900-fold from cell extract (Table 1) and the enzyme after gel filtration gave a single protein band on SDS-PAGE with silver staining (Fig. 4). Apparent molecular weight of the purified enzyme esti-

Table 1 . Purification of alkaline phoshatase from MC3T3-E1 osteoblastic cells

purification step	total ALP activity (μmol/min)	total protein (mg)	ALP-specific activity ((μmol/min/mg protein)	purification ratio	yield %
cell extract	104.67	720.89	0.15	1.0	100.0
anion exchange	29.90	12.66	2.36	16.3	28.6
Con A affinity	14.13	1.50	9.42	64.9	13.5
gel filtration	2.37	0.02	113.67	906.8	2.3

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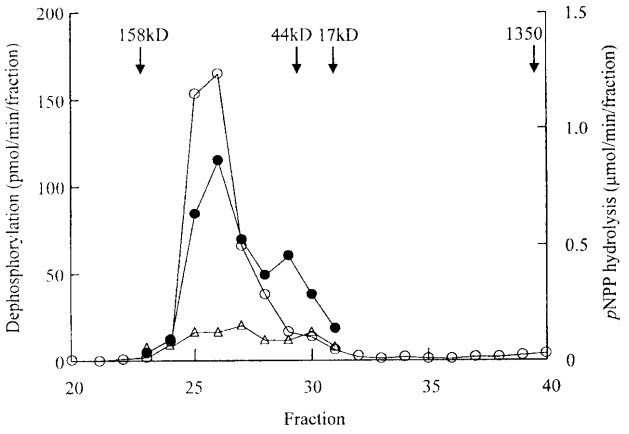

Fig. 3. Typical elution profiles of the Superdex 200 HR gel filtration chromatography of bone-type ALP. Gel filtration chromatography was performed as described in Materials and Methods. Fractions were assayed for *p*NPP hydrolysis (○), Ser/Thr-P-MBP (△) and Tyr-P-MBP dephosphorylation (●). The gel filtration column was standardized with thyroglobulin, gamma globulin, ovalbumin, myoglobin and vitamin B-12. The molecular weight of ALP was estimated by the log Mr vs. Kav plot.

mated from gel filtration was 78.000 and 76.000 from SDS-PAGE. For the optimal pH for pNPP hydrolysis, activities after anion exchange, Con A affinity and gel filtration chromatographies were measured at different pH values (Fig. 5). The optimal pH for pNPP hydrolysis was decided to be around 9.4 to 10.4 for all samples.

2. Protein phosphatase-like activity of MC3T3-E1 cells during purification process of ALP

We examined dephosphorylation of phosphorylated proteins by the fractions during purification steps to determine whether ALP of MC3T3-E1 cells could act as a protein phosphatase (Figs. 1-3). Dephosphorylation of Tyr-P-RCM-lysozyme or Ser/Thr-P-histone was not detected after Con A affinity chromatography (data not shown). Most of Ser/Thr-P-MBP dephosphorylation was

Table 2: Effects various chemicals on *p*NPP hydrolysis and tyrosine-phosphorylated MBP dephosphorylation.

inhibitor	IC_{50} (mM)	
	*p*NPP	Tyr-P-MBP
levamisole	0.45	0.25
okadaic acid	n.i.	n.i.
dephostatin	n.i.	n.i.
sodium metavanadate	< 0.01	< 0.01
L-homoarginie	15	>20

pNPP hydrolysis(pH9.70) and Tyr-P-MBP dephosphorylation (pH8.77) were assayed in the presence of different concentrations of inhibitors, and concentrations for half maximal inhibition (IC50) were calculated.(ni.,not inhibited.)

separated from pNPP hydrolytic fraction after anion exchange chromatography (Fig. 1) and was hardly detected after gel filtration (Fig. 3). On the other hand, fractions active for Tyr-P-MBP dephosphorylation mostly corresponded to the active fractions during purification steps (Figs. 1-3).

3. Characterization of Tyr-P-MBP dephosphorylation

We tested the pH dependency of the dephosphorylation of Tyr-P-MBP using the enzyme after gel filtration (Fig. 5). Fig. 5 shows a typical result of 4 independent experiments. The highest activities were obtained in the pH range of 8.3-8.7, but about 62 % of maximal activities were kept at neutral pH value. We tested the dependency of activity on the concentration of Tyr-P-MBP (data not shown). Dephosphorylation was proportional to the concentration of 0.2 to 2.2 μM Tyr-P-MBP. Then we tested competition of pNPP with 2.2 μM Tyr-P-MBP dephosphorylation. *p*NPP inhibited Tyr-P-MBP dephosphorylation depending on its concentration and half maximal

Table 3: Substrate specifivity and optimal pH range ob bone-type ALP.

substrate	optimal pH	apparent Km (mM)	activity ratio (at optimal pH/pH7)
pNPP	9.70 (9.43-10.42)	0.30	32.2
ATP	9.22 (8.64-9.70)	1.10	7.6
b-glycerophosphate	8.97 (8.11-9.22)	0.35	6.3
pyridoxal phosphate	9.11 (8.76-9.56)	0.27	159.7
phosphoethanolamine	9.38 (8.87-9.75)	0.84	24.1
Tyr-P-MBP	8.67 (8.33-8.70)	n.d.	1.6

Levamisole-sensitive hydrolytic activities of active fractions MC3T3-E1 for phosphate compounds were measured using cell extract at different concentrations or at different pH values. Apparent Km values were calculated by double reciprocal prots. (n.d.,not determined.)

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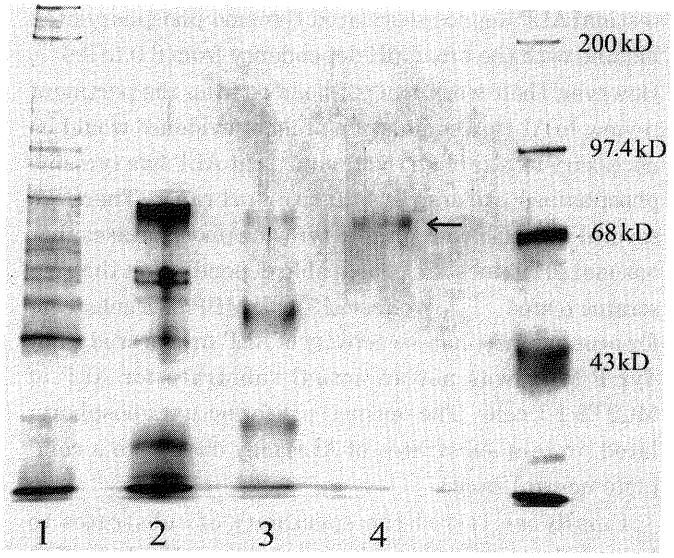

Fig. 4. SDS-PAGE analysis of the samples from various stages of ALP purification of MC3T3-E1 cells. SDS-PAGE was performed as described in Materials and Methods and protein bands were stained by silver staining. The samples were: Lane 1, cell extract; Lane 2, POROS 50D anion exchange; Lane 3, Con A affinity; Lane 4, Superdex 200 gel filtration. Molecular weight standards included myosin (200 kD), phosphorylase B (97.4 kD), bovine serum albumin (68 kD) and ovalbumin (43 kD).

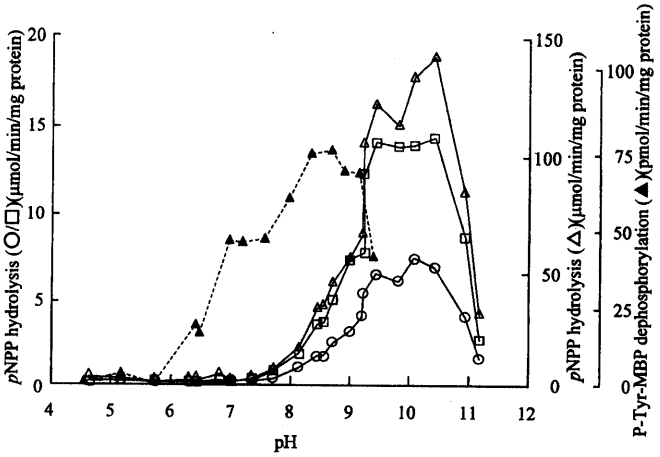

Fig. 5.Effects of pH values on pNPP hydrolysis and Tyr-P-MBP dephosphorylation. pNPP was hydrolyzed by ALP fractions after POROS 50D anion exchange (○), Con A affinity (□) and Superdex 200 gel filtration (△) column chromatography at various pH values. Tyr-P-MBP was also dephosphorylated by active fractions after Superdex 200 gel filtration (▲, dotted line). Tris-acetate buffer (pH 4.67-9.20) and carbonate buffer (pH 9.22-11.17) were used for the reaction

inhibition was obtained at 100 μM *p*NPP.

4. Effects of agents on pNPP hydrolysis and Tyr-P-MBP dephosphorylation

We tested the effects of several agents on both *p*NPP hydrolysis and Tyr-P-MBP dephosphorylation (Table 2). Levamisole inhibited both activities, but okadaic acid, an inhibitor of protein serine/threonine phosphatases, and dephostatin, an inhibitor of some protein tyrosine phosphatases did not inhibit either of activities. Vanadate inhibited both activities at low concentrations. L-homoarginine, an inhibitor of ALP, inhibited both activities, too. Since ALP requires magnesium ions for its activity, we tested the effects of EDTA on both activities. EDTA, of which concentration was higher than the added magnesium, inhibited both activities (data not shown).

5. Substrate specificity of levamisole-sensitive phosphatase activities

For evaluation of substrate specificity of ALP, concentration dependency of levamisole-sensitive phosphatase activities of several phosphate compounds and pH dependency of their activities were tested using cell extract. The optimal pH range, apparent Km values and the ratio of activities at optimal pH to those at pH 7 were summarized in Table 3. All samples tested including Tyr-P-MBP gave broad activity peak at alkaline pH (parentheses in Table 3). The ratios of activities at optimal pH to those at pH 7 were calculated and the value for Tyr-P-MBP was the smallest.

Discussion

ALPs are widely distributed enzymes in mammalian tissues, which hydrolyze orthophosphoric-monoester at alkaline pH optima. Although ALPs have been studied for the last seventy years, little is known about their physiological functions, substrates and pH optima. ALPs are abundant in the cells associated with hard tissue formation; they are thought to play an important role in hard tissue formation, but the detail remained to be elucidated. We have studied the relation between ALP and hard tissue formation using a clonal osteoblastic cell line, MC3T3-E1 [16]. MC3T3-E1 cells were established from neonatal mouse calvaria, and selected for high ALP activity in the confluent stage. The cells have the capacity to differentiate into osteoblasts and osteocytes, and to form calcified bone tissue in vitro. MC3T3-E1 are thought to be excellent cells in which the function of ALP during the process of hard tissue formation can be studied and thus there are many reports of measuring ALP activities in the cell culture [20-22]. It has been reported that ALP activity increases in accordance with the progress of calcification and thus can be an indicator of calcification. An important question is raised here. When ALP activities are assayed, artificial substrates, for example pNPP, phenyl phosphate and β-glycerophosphate, are usually used at alkaline pH. On the other hand, MC3T3-E1 cells are cultured with a medium of which pH is usually 7.2 to 7.4 and ALP activity with those artificial substrates is

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very low at these pH values, as already known and we too have demonstrated it in this study (Fig. 5 and Table 3). Namely, ALP works inefficient at neutral pH for those substrates [4,6,28]. What is the physiological substrate and pH for ALP? To understand the physiological function of ALP, it is important to know its physiological substrates and working pH. Hence we purified ALP from MC3T3-E1 to test its substrate specificity and to determine its optimal pH for different substrates.

By butanol treatment and three-column chromatography steps (Figs. 1-3), we purified ALP over 900-fold from cell extract, which showed a single band with silver staining (Table 1 and Fig. 4). Molecular weight estimated from SDS-PAGE and gel filtration was similar to the one previously reported for bone-type ALP [23,24]. We used *p*NPP as substrate to monitor ALP activity during purification, and found that the optimal pH values for *p*NPP hydrolysis were 9.4 to 10.4 for all samples after each purification step. The activities were inhibited by levamisole [25,26], L-homoarginine [16,27] and also by EDTA. As the purified enzyme had a typical character of ALP, we tested the substrates and pH dependency using the enzyme.

Many phosphate compounds have been tested as candidates of physiological substrates for ALP, but none of them has been recognized as true substrate [4,25,28,29]. We tested the possibility that ALP might work as protein phosphatase at neutral pH. We prepared MBP and RCM-lysozyme phosphorylated at tyrosine residues [17,30]. We also prepared MBP and histone phosphorylated at serine and threonine residues [17]. Among these substrates, active fraction after gel filtration dephosphorylated only Tyr-P-MBP (Fig. 3), suggesting the presence of substrate specificity for phosphorylated proteins. This dephosphorylation was inhibited by ALP inhibitors (Table 2) and EDTA, but not inhibited by protein phosphatase inhibitors, okadaic acid [31] and dephostatin [32]. Both *p*NPP hydrolysis and Tyr-P-MBP dephosphorylation were inhibited by vanadate[6]. Moreover, dephosphorylation for Tyr-P-MBP was competed by *p*NPP with a half maximal inhibition concentration close to the apparent Km value of *p*NPP hydrolysis. These results suggest that ALP from osteoblastic cells selectively dephosphorylate Tyr-P-MBP.

Then we tested pH dependency of dephosphorylation of Tyr-P-MBP by active fraction. As shown in Fig. 5, the activity showed broad peak at pH 8.3-8.7, which shifted to 9.4-10.4 with *p*NPP, and was accompanied by a shoulder till pH 6.9. In this neutral pH range, about 62 % of maximal activity was kept. The results suggest that bone-type ALP dephosphorylates Tyr-P-MBP at neutral pH range and may be able to work at neutral pH for physiological substrates. Swarup et al. reported that calf intestinal ALP dephosphorylated tyrosine-phosphorylated histone with the broad pH dependency from 6.0 to 9.0 [11]. However, their work has not been cited in the pertinent review to ALP. Accumulation of more evidence would be necessary to establish the finding that ALP has tyrosine phosphatase-like activity at neutral pH range. There are several reports about protein tyrosine phosphatases that optimal pH values are changeable depending on the substrates tested [9,10,12]. We tested Tyr-P-MBP as a substrate for protein phosphatase activity of ALP in this study, but Tyr-P-MBP was not an actual substrate for ALP of MC3T3-E1 cells. The optimal pH for actual phosphorylated protein substrates of ALP may change to a complete neutral range.

Finally we tested the specificity of substrates to levamisole-sensitive phosphatase activities (Table 3). ATP, pyridoxal phosphate and phosphoethanolamine were tested as a candidate of physiological substrates [4,28,29], but optimal pH values were alkaline for all substrates and the ratio of activity at optimal pH to one at pH 7 was higher than that of Tyr-P-MBP. If ALP works at neutral pH range physiologically, these substrates must be inefficient for ALP.

From the above results, we conclude that ALP of osteoblastic cells displays dephosphorylation of tyrosine-phosphorylated protein at neutral pH range. And this activity may play an important role in physiological function of bone-type ALP.

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