Sensitivity of Intestinal Alkaline Phosphatase to L-Homoarginine and Its Regulation by Subunit-Subunit Interaction

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ABSTRACT—The inhibitory effect of levamisole and L-homoarginine on alkaline phosphatases (ALP, orthophosphoric monoester phosphohydrolase, EC. 3. 1. 3. 1) in various tissues was studied to characterize differences in the mechanism of inhibition of ALP isoenzymes. The ALP activity from the placenta, kidneys, and a clonal osteogenic cell line, MC3T3-E1, was hyperbolically inhibited with a dependency on the concentration of levamisole ($K_{i0.5} = 10-12 \mu\text{M}$) or L-homoarginine ($K_{i0.5} = 1 \text{ mM}$), but the activity of intestinal ALP was little inhibited by $240 \mu\text{M}$ levamisole and sigmoidally inhibited by L-homoarginine ($K_{i0.5} = 13 \text{ mM}$). Hill plot analysis of the L-homoarginine inhibition data showed that the Hill coefficient values of the placenta, kidney, and osteogenic cells were around 0.9–1.0 and that of the intestine was 1.8. The effect of sodium dodecyl sulfate (SDS), which may decrease the subunit-subunit interaction, on the L-homoarginine inhibition of the intestinal ALP was tested. The L-homoarginine concentration-dependent inhibition curve changed from sigmoidal to hyperbolic, and the Hill coefficients decreased with increasing concentrations of SDS. These results suggest that the differences in inhibition by L-homoarginine and affinity changes of intestinal ALP for L-homoarginine are caused by the subunit-subunit interaction of oligomers.

Keywords: Alkaline phosphatase inhibition, L-Homoarginine, Sodium dodecyl sulfate, Positive cooperativity, Subunit-subunit interaction

Alkaline phosphatases (ALPs) are widely distributed enzymes that hydrolyze orthophosphoric-monoester at alkaline pH optima. There are three distinct forms of ALP in mammalian tissue: placental, intestinal, and liver/bone/kidney (L/B/K), which are distinguished by differences in molecular weight, heat stability, sensitivity to inhibitors (1, 2), and recently also cDNA analysis (3–5). Although ALPs have been studied for the last seventy years, little is known about their physiological functions (1).

Inhibition studies have been done to characterize each of the ALP isoenzymes and to estimate their physiological functions. The inhibitions of isoenzymes by levamisole and L-homoarginine were studied by several groups, but the results were not always consistent (6–8). The purposes of this paper are to confirm the organ-specific inhibition and to study the kinetics of inhibition by levamisole and L-homoarginine. We demonstrate that the inhibition of intestinal ALP by levamisole and L-homoarginine is different from its inhibition by other drugs, and its unique sensitivity to L-homoarginine may be regulated by a subunit-subunit interaction.

MATERIALS AND METHODS

ALPs

Bone-type ALP was obtained from a cultured clonal osteogenic cell line, MC3T3-E1 cells (9, 10), which was donated by Dr. Kodama (Ozu University) and Dr. Kuboki (Hokkaido University). Cell cultures were grown in a minimum essential medium supplemented with 10% (v/v) fetal bovine serum (Boehringer Mannheim-Yamanouchi, Tokyo). They were maintained at 37°C in a 95% air–5% CO₂ atmosphere.

When the cells were inoculated at 5×10^4 cells/60-mm plastic dish containing 4 ml of medium, they usually reached a confluent monolayer after 4 to 5 days, and then the ALP activity increased. The cells were harvested at 10 days after confluence and then were ultrasonicated.

The cell homogenate was centrifuged at $5,500 \times g$ for 20 min to sediment nuclei, large fragments, and most mitochondria. The supernatant was centrifuged at $100,000 \times g$ for 45 min. The resultant pellet was used as a microsomal fraction.

The ALP was mainly recovered in the microsomal frac-

tion (11), and it was used for bone-type ALP determinations. The kidney-type (from calf kidney) ALP was purchased from Funakoshi Co. (Tokyo), and intestinal ALP (from bovine intestine, type VII-S) and placental ALP (from porcine placenta, type XVI) were from Sigma (St. Louis, MO, USA).

Enzyme assay

ALP activity was assayed by the method of Bessey et al. (12) with a slight modification (11). The concentrations of the various components in the 0.5-ml reaction mixture were 1–20 μ g of enzyme from various sources, 12.5 μ mol of sucrose, 0.8 μ mol of MgCl_2 , 100 μ mol of tris-HCl at pH 9.5, and 0.8 μ mol of *p*-nitrophenyl phosphate (*p*NPP) with or without levamisole or L-homoarginine. The reaction was started by the addition of *p*NPP at 37°C and terminated with 0.5 ml of cold 15% (w/v) trichloroacetic acid after 20 to 45 min. The amount of *p*-nitrophenol liberated was measured spectrophotometrically at 420 nm after the addition of 0.6 ml of 1 N NaOH to develop color. Protein concentration was estimated by the method of Bradford (13) with bovine plasma albumin as a standard.

Fig. 1. Effect of levamisole concentration on inhibitions of alkaline phosphatase isoenzymes. The activities of intestine (○), E1-cell (●), placenta (△) and kidney (□) ALPs in the presence of different concentrations of levamisole were measured; each 0.5-ml reaction mixture contained 1–20 μ g of ALP isoenzymes; 100 μ mol tris-HCl, pH 9.5; 12.5 μ mol sucrose; 0.8 μ mol MgCl_2 ; 0.8 μ mol *p*NPP; and 0, 1.9, 3.8, 7.5, 15, 30, 60 or 120 nmol levamisole. The reaction was started by the addition of *p*NPP and terminated after 30 min at 37°C by the addition of 0.5 ml of 15% (w/v) trichloroacetic acid; 0.6 ml of 1 N sodium hydroxide was added to make the solution basic (11), and the optical density at 420 nm was measured. The activity in the absence of levamisole was taken as the 100% value.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 7.5% (w/v) polyacrylamide gel overlaid with a 3% (w/v) polyacrylamide stacking gel as described by Laemmli (14). Non-SDS-PAGE was basically performed according to Davis' method (15).

RESULTS

The inhibition of ALPs by levamisole

The inhibition of ALPs from MC3T3-E1 cells, placenta, kidney, and intestine by levamisole was tested (Fig. 1). The ALP activities of MC3T3-E1 cells, placenta and kidney decreased hyperbolically with the concentration of levamisole ($K_{i0.5} = 10\text{--}12\text{ }\mu\text{M}$). However, intestinal activity was almost unchanged until the concentration was increased to 240 μM levamisole.

The inhibition of ALPs by L-homoarginine

The ALP activities of MC3T3-E1 cells, placenta, and kidney decreased hyperbolically with the concentrations of L-homoarginine ($K_{i0.5} = 1\text{ mM}$) (Fig. 2). However, intestinal ALP activity was stable until the concentration of 1 mM L-homoarginine, and then decreased sigmoidally ($K_{i0.5} = 13\text{ mM}$). About 40% of the activity was not inhibited even in the presence of 40 mM L-homoarginine.

Fig. 2. Effect of L-homoarginine concentration on inhibitions of alkaline phosphatase isoenzymes. The activities of intestine (○), E1-cell (●), placenta (△) and kidney (□) ALPs in the presence of different concentrations of L-homoarginine were measured. Assay conditions were as in Fig. 1 except that 0, 0.3, 0.6, 1.3, 2.5, 5, 10 or 20 μ mol L-homoarginine replaced levamisole.

Hill plot analysis of the inhibition data by levamisole and L-homoarginine

A sigmoidal concentration-dependency suggests that there is more than one binding site and that there is cooperation among these sites (16, 17). Hill plot analysis was done using the data obtained in Figs. 1 and 2 to examine whether binding sites to levamisole or L-homoarginine show cooperativity. When $\log [v/(V_{\max} - v)]$ was plotted against $\log [\text{levamisole}]$ or $\log [\text{L-homoarginine}]$, with v , the enzyme activity in the presence of various concentrations of levamisole or L-homoarginine, and $V_{\max}$, the activity without the inhibitor, straight lines were obtained (data not shown for levamisole and for L-homoarginine, data shown in Fig. 3). The slopes of these lines are equal to the n value, the Hill coefficient, which is the apparent number of inhibitor molecules combining with one molecule of enzyme assuming only one active site per molecule or which shows the extent of negative or positive cooperativity between inhibitor binding sites (17). The Hill coefficients obtained from the data of Fig. 1 were around 0.9–1.0 for bone-type, placental and kidney ALP isoenzymes (data not shown). Figure 3 shows the Hill plot using the inhibition data by L-homoarginine obtained in Fig. 2. The Hill coefficients for bone-type, placental and kidney ALPs were also 0.9–1.0. However the Hill coefficient for intestinal ALP was 1.8.

Fig. 3. Hill plot analysis of the data in Fig. 2. $\log [v/(V_{\max} - v)]$ plotted against $\log [\text{L-homoarginine}]$ for intestinal ($\circ$), placental ($\triangle$), E1-cell ($\bullet$) and kidney ($\square$) ALPs inhibited by L-homoarginine. The enzyme activity in the presence of various concentrations of L-homoarginine is v , and $V_{\max}$ is the activity of the enzyme in the absence of L-homoarginine. Hill coefficients were calculated from the slopes, and the $K_{i0.5}$ value was calculated from the intercept of the slope and the X axis.

The effect of SDS treatment on the inhibition of intestinal ALP by L-homoarginine

SDS is often used to decrease the subunit-subunit interaction and to dissociate subunits. The intestinal ALP was treated with SDS to test whether cooperativity among the L-homoarginine binding sites is caused by a subunit-subunit interaction. First, the effect of SDS on ALP activity was tested. The activity decreased to about 50%, with a dependency on the SDS concentration ($K_{i0.5} = 0.1\%$ w/v SDS), and the remaining 50% activity was maintained in the presence of 1 to 10% (w/v) SDS (data not shown). Figure 4 shows the effect of different concentrations of SDS on the inhibition of intestinal ALP by L-homoarginine. The L-homoarginine concentration-dependent inhibition curve clearly changed from sigmoidal to hyperbolic, and the apparent $K_{i0.5}$ values for L-homoarginine decreased when the concentration of SDS increased.

The effects of SDS concentration on Hill coefficients, $K_{i0.5}$ and remaining ALP activity

A Hill plot was made with the data in Fig. 4, and the calculated Hill coefficients and $K_{i0.5}$ values are summarized in Table 1. The Hill coefficients decreased from 1.8 without SDS to 0.7 with 0.8% SDS. The $K_{i0.5}$ values also decreased from 13.1 mM to 1.2 mM with increasing SDS. These changes of the Hill coefficients and $K_{i0.5}$ values were observed in the presence of 0.03% SDS where there was no loss of ALP activity.

Fig. 4. Effect of SDS on L-homoarginine inhibition of intestinal ALP. Experiments similar to those in Fig. 2 were performed without ($\circ$) or with a final SDS concentration of 0.03% (w/v) ($\square$), 0.1% ($\bullet$), 0.5% ($\triangle$), or 0.8% ($\blacktriangle$). The values without L-homoarginine at each SDS concentration was the 100% value.

Table 1. Hill coefficients and $K_{i0.5}$ values calculated with Fig. 4

SDS (%)	Hill coefficient n	$K_{i0.5}$ (mM)	Remaining activity without L-homoarginine (%)
0	1.8	13.1	100
0.03	1.5	10.4	100
0.1	1.2	4.1	55
0.5	0.7	3.1	41
0.8	0.7	1.2	40

The Hill plot used the L-homoarginine dependent inhibition data with SDS (Fig. 4), and the Hill coefficients and the $K_{i0.5}$ values were calculated as described in the legend to Fig. 3. The remaining activity without L-homoarginine is also shown.

Apparent molecular weight of intestinal ALP in the absence or presence of SDS

We assumed that the changes in Hill coefficients and the affinity for L-homoarginine were caused by dissociation of the subunits in the presence of SDS. We tested whether the apparent molecular weight of intestinal ALP changes in the presence of SDS. Figure 5 shows the results of polyacrylamide gel electrophoresis. The apparent molecular weight of intestinal ALP in the presence of 1% SDS was 60 K daltons, with no higher molecular weight band. The result was not affected by the presence or absence of 1% 2-mercaptoethanol. On the other hand, a band was detected between 140 K and 443 K daltons with non-SDS-PAGE.

Fig. 5. Apparent molecular weight of intestinal ALP by PAGE performed in the presence or absence of SDS. SDS-PAGE (left) was performed by the Laemmli method (14) using a 7.5% (w/v) polyacrylamide separating gel overlaid with a 3% (w/v) polyacrylamide stacking gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R 250. The standard markers were myosin (200,000), β -galactosidase (116,248), bovine serum albumin (66,267) and aldolase (42,400). The apparent molecular weight of intestinal ALP was about 60,000. The PAGE on the right was without SDS (15) and silver-stained (Bio-rad silver stain kit). The standard markers were thyroglobulin (669,000), ferritin (443,000), lactate dehydrogenase (139,850), bovine serum albumin (66,267) and soy bean trypsin inhibitor (20,100).

DISCUSSION

Alkaline phosphatase isoenzymes are widely distributed in mammalian tissue, but their physiological roles are not clear (1, 18–20). It is important to characterize the enzymatic activity of each of the ALP isoenzymes to estimate their physiological functions, and a characterization of isoenzymes is also useful for determining the source of ALP isoenzymes appearing in the serum to allow a differential diagnosis of diseases.

One approach to distinguish ALP isoenzymes is to compare the sensitivity to ALP inhibitors, and inhibition studies have been done with L-homoarginine (6), levamisole (7, 8), L-phenylalanine (8, 21), vanadate (22, 23) and other inhibitors (1, 8). The results reported were not always consistent and have been inadequate to characterize the isoenzymes. Belle reported that the inhibition of intestinal ALP by several inhibitors was clearly different from the inhibitions of kidney, bone, liver, placenta and mammary tumor ALPs (8), whereas Lin and Fishman reported that intestinal and placental ALPs are similar but different from bone and liver isoenzymes with respect to L-homoarginine inhibition (6). Our results (Figs. 1 and 2) are consistent with those obtained by Belle (8). The discrepancies may be due to different tissue species or differences in experimental conditions. It is necessary to study the mechanism of inhibition in detail to better characterize the inhibition of isoenzymes.

In the present study, the inhibition of intestinal ALP by levamisole and L-homoarginine is different from that of placental, kidney, and bone-type ALP (Figs. 1 and 2). Intestinal and placental ALPs have amino acid sequences that are closely related, as deduced from cDNA analyses, as compared with the sequences of the L/B/K ALPs (5). This suggests that the different inhibitions of isoenzymes by levamisole and L-homoarginine are not related to differences in the primary structures of the ALP isoenzymes. Our results suggest that the different sensitivity of intestinal ALP to L-homoarginine is caused by cooperation between L-homoarginine binding sites brought about by subunit-subunit interaction, because 1) The inhibition curve showed a sigmoidal dependency on the L-homoarginine concentration (Fig. 2), and the Hill plot analysis suggested the existence of positive cooperativity between the L-homoarginine binding sites (Fig. 3); and 2) SDS decreased the Hill coefficients, indicators of the extent of cooperativity, from 1.8 to 0.7 (Fig. 4 and Table 1), suggesting that the positive cooperativity for L-homoarginine binding to the intestinal ALP decreased with increasing SDS concentration.

The inhibition curves for bone-type, placental and kidney ALPs were hyperbolic, and the Hill coefficients were around 0.9–1.0. However, the Hill coefficient for intes-

tinal ALP was 1.8 (Figs. 2 and 3). We may explain these results as follows: In the case of bone-type, placental, and kidney ALP, there is only one L-homoarginine binding site per active ALP molecule and the sites on each subunit are independent and equivalent to each other. On the other hand, there is more than one binding site per active intestinal ALP molecule and positive cooperativity caused by subunit-subunit interaction between these sites. This positive cooperativity stimulates the binding of one L-homoarginine molecule to the site on one subunit to the L-homoarginine binding on the other subunit. The L-homoarginine binding results in a sigmoidal inhibition curve. When subunit-subunit interaction was decreased or lost by SDS treatment, the positive cooperativity disappeared, and the inhibition curve was hyperbolic (16, 17). The cooperative binding of oxygen to hemoglobin was explained by subunit-subunit interaction (24).

Fosset et al. reported that calf intestinal ALP is a dimer composed of two similar subunits of 69,000 molecular weight and is dissociated into subunits on exposure to SDS (25). Although the apparent molecular weight in SDS-PAGE (Fig. 5) is smaller than theirs, the results shown in Fig. 5 did not contradict their results and supported the above explanation.

The inhibition of intestinal ALP by levamisole is also different from that of bone-type, placental and kidney ALPs (Fig. 1). The ALP activities of MC3T3-E1 cells, placenta and kidney decreased hyperbolically with the levamisole concentration, and the Hill coefficients were around 0.9–1.0, which suggest that approximately one molecule of levamisole combines with one active site of bone-type, placental, and kidney enzymes and that there is no cooperativity between the levamisole binding sites. However intestinal ALP activity was almost unchanged until 240 μ M levamisole. These results may also be explained by subunit-subunit interaction, but this has not yet been experimentally established.

The $K_{0.5}$ values decreased from 13.1 mM to 1.2 mM with increasing SDS concentration (Table 1). This indicates that the apparent affinity of intestinal ALP for L-homoarginine increased 10 times by SDS treatment. If the association and the dissociation of the subunits of oligomeric enzyme can regulate the sensitivity to drugs ten times, such a system could offer an excellent candidate for regulating drug sensitivity. SDS is not a physiological substance, but there may be an as-yet-undescribed physiological system for regulating subunit-subunit interaction. The ALPs are thought to be membrane bound enzymes (1). In the case of Na,K-ATPase, which is also a membrane bound and oligomeric enzyme, it is suggested that enzyme activity is affected by the composition of phospholipids or aging of the biomembrane (26). These factors may affect the state of membrane bound and oligomeric enzymes.

Acknowledgments

We thank Dr. H. Kodama of Ohu University and Dr. Y. Kuboki of Hokkaido University for giving us the clonal cell line, MC3T3-E1 cells. This work was supported by The Akiyama Foundation, Japan.

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