Characterization of Bone Acid Phosphatase Activity[1] (34768)

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Bone has a characteristically high acid phosphatase activity (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2). Histochemical studies have shown that this activity is concentrated not only in areas of bone resorption, but also in areas of bone formation, including a specific localization in the region where mineralization is initiated in newly formed bone (1). Although bone probably contains an acid phosphatase similar to the major acid phosphatase of the liver lysosomes, several observations, particularly the high activity measured with phenylphosphate as the substrate, suggest that the major acid phosphatase in bone is a different enzyme (2–4). The purpose of the present study was to characterize bone acid phosphatase activity measured with both β-glycerophosphate and phenylphosphate as substrates.

Materials and Methods. Metaphyseal bone samples obtained from femora and tibias of Sprague-Dawley rats (100–200 g) were freed of marrow by rinsing with isotonic saline and crushed in a mortar as previously described (4). The crushed bone samples and samples of liver and spleen were homogenized in 9 vol of distilled water in a VirTis homogenizer. DNA concentration was measured by a modification of the method of Cerotti (4).

Acid phenylphosphatase activity was determined by incubating 0.1 ml of a 2% bone homogenate for 20 min at 37.5° with 2 ml of 10 m*M* phenylphosphate in 0.35 *M* NaCl and 0.1 *M* acetate, pH 5.3. One ml of 0.5 *M* NaOH was added to stop the reaction. After a brief centrifugation, the optical density of the supernatant solution was measured at 290 mμ. Blank values were measured on unincubated samples. Because preliminary studies showed that the activity was increased by the addition of NaCl (optimum at 0.35 *M*), a constant ionic strength was maintained in the reaction medium by reducing the NaCl concentration whenever activators or inhibitors were added. Because ascorbate increased the optical density, sample tubes containing ascorbate were agitated (15–30 min on a vortex mixer after the addition of alkali) to oxidize the ascorbate and thus reduce the optical density.

Cysteine activation was usually carried out by preincubating the homogenized bone samples for 10 min in 1 ml of a solution containing 0.1 *M* cysteine, 0.2 *M* acetate, and 0.7 *M* NaCl, pH 5.3; the reaction was then started by adding 1 ml of 20 m*M* phenylphosphate. Enzyme activity was also measured when cysteine was added directly to the reaction medium. Because cysteine and other sulfhydryl compounds increased the optical density of the reaction medium, the sulfhydryl compound was also added to the blank. Because the sulfhydryl compounds were unstable in solution, the time between the addition of the sulfhydryl compound and measurement of optical density was kept constant for blank and sample tubes.

In the acid β-glycerophosphatase assay the amount of glycerol liberated was measured by the chromotropic acid method (5). The assay was performed usually by incubating 0.3 ml of substrate (0.133 *M* β-glycerophosphate in 0.1 *N* acetate, pH 5.0, equilibrated at 37.5°) with 0.1 ml of 2% bone homogenate for 30 min at 37.5°. The sample tubes were removed from the bath and the reaction was stopped by the addition of 1 ml of freshly prepared 0.01 *M* $NaIO_4$ in 1 *N* sulfuric acid.

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Fifteen min later, 0.1 ml of 1 *N* arsenite was added, and the tubes were allowed to stand for at least 10 min. Four ml of 0.2% chromotropic acid in 19 *N* H_2SO_4 was then added and the mixture was kept at 100° for 30 min. After cooling, the optical density was measured at 570 mμ. In order to limit hydrolysis of substrate by the strong acid used to stop the enzyme reaction, glycerol concentration was measured immediately after incubation; because some hydrolysis does occur, the acid was added to the samples and the blanks at precisely the same time. Although a high cysteine concentration in the reaction medium interfered with glycerol measurement, satisfactory measurements were obtained when the periodate concentration was increased from 0.01 to 0.02 *M*.

Phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) was assayed by measuring the release of inorganic phosphate from casein. The bone homogenates were centrifuged (500*g* for 10 min) to reduce the phosphate content of the homogenate. The resulting supernatant solution (0.1 ml) was incubated for 60 min at 37.5° with 0.3 ml of 5% casein in 0.1 *M* acetate, pH 5.3. The reaction was stopped by the addition of 3 ml of 8% trichloroacetic acid. The samples were then centrifuged, and the free phosphate was measured with the method of Fiske and Subbarow as described by Hawk *et al.* (6).

Results and Discussion. Acid β-glycerophosphatase and acid phenylphosphatase activities of bone were characterized by adding a number of agents to the assay media. The acid β-glycerophosphatase activity was strongly inhibited by tartrate, fluoride, and molybdate and moderately inhibited by mercury (Table I). Because the major lysosomal acid phosphatase in liver has these same properties (7–10), these results are consistent with the presence of this lysosomal enzyme in bone, as has been suggested by Vaes (2).

Fluoride and molybdate were strong inhibitors of the bone acid phenylphosphatase activity as well. Tartrate was only weakly inhibitory, however, and several other agents affected phenylphosphatase activity differently than β-glycerophosphatase activity (Table I). The difference in tartrate inhibition as well as several other differences in the properties of these two activities have been reported previously (2–4). Further, it has been found that vitamin D deficiency increased the acid β-glycerophosphatase activity of bone but decreased the acid phenylphosphatase activity (11). These differences are best explained by the presence of a second acid phosphatase in bone which hydrolyzes phenylphosphate more rapidly than it hydrolyzes β-glycerophosphate.

TABLE I. Effect of Various Agents on Bone Acid Phenylphosphatase and Acid β-Glycerophosphatase Activities.

Agent	Concentration (m*M*)	Activity as % of control: Phenylphosphatase	Activity as % of control: β-Glycerophosphatase
Tartrate	10	83	38
F^-	5	20	7
MoO_4^{2-}	0.1	3	15
Hg^{2+}	0.1	83	55
Cu^{2+}	0.2	53	86
Alloxan	50	116	78
Cysteine	100	894	564
Malonate	50	185	82
Citrate	50	158	67
EDTA	10	155	94

The most striking feature of the bone acid phenylphosphatase activity was a severalfold increase when cysteine was added to the reaction medium (Table I). My finding that this activation also occurred when the bone homogenate was preincubated with cysteine before assaying activity demonstrates that cysteine affects the enzyme itself rather than the enzyme reaction. Two other agents, dithiothreitol and ascorbate, were also strong activators (5- to 10-fold activation at 100 m*M*), but the sulfhydryl binding agents *p*-mercuribenzoate (0.1 m*M*) and *n*-ethylmaleimide (1 m*M*) were without effect. Because ascorbate was a strong activator in addition to the sulfhydryl compounds, cysteine and dithiothreitol, activation was probably due to reduction of some group in the enzyme.

A study of the activation of acid phenylphosphatase activity by preincubation of the bone homogenate with cysteine showed that

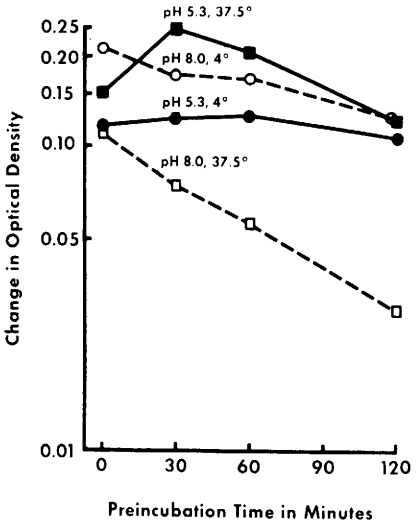

FIG. 1. Effects of preincubation conditions on the activation of bone acid phenylphosphatase activity with 0.1 *M* cysteine. Duplicate portions of a bone homogenate (bone pooled from 3 rats) were preincubated with cysteine at pH 8.0 (no buffer added) or in 0.1 *M* acetate, pH 5.3 at the temperature and for the time indicated before determining acid phenylphosphatase activity. The mean difference in optical density between duplicates was 0.015.

the activation was rapid, occurring at pH 8.0 within the 1-min period required to start the assay, and was dependent on both pH and temperature (Fig. 1). The resultant high activity was not stable but decreased with time, particularly when pH and temperature were high. The activation required a high concentration of cysteine (K_m 0.19 *M* for a 10-min preincubation at pH 5.3 and 37.5°) and was enhanced by the addition of several metal ions, particularly Fe^{2+} (Table II). After activation of the bone enzyme, a 10-fold dilution of the cysteine concentration rapidly lowered activity (33% lower in 10 min at pH 8.0 and 4°), but activity was again increased when more cysteine was added.

Table III shows that the ratio of phenylphosphatase activity to β-glycerophosphatase activity was higher in bone than in liver or spleen. In addition, the acid phenylphosphatase activity of bone was affected differently by malonate and alloxan than was the liver acid phenylphosphatase (Table IV). Both liver and bone acid phenylphosphatase activities were increased by cysteine. Although in the present study malonate inhibited the acid phenylphosphatase activity of the liver homogenates, other workers (10) have reported that both malonate and ascorbate increased the acid phenylphosphatase activity of the mitochondrial-lysosomal fraction of liver homogenates. These findings suggest that the enzyme responsible for the high acid phenylphosphatase activity of bone is present in liver but that it is not the major acid phosphatase there as it is in bone.

The characteristics of the bone acid phenylphosphatase activity are similar to those of phosphoprotein phosphatase. This enzyme has been isolated from spleen (12,

TABLE II. Bone Acid Phenylphosphatase Activity After Preincubation with Metal Ions and Cysteine.[a]

Metal ion	Activity as % of control (no metal ion)	
	Cysteine absent	Cysteine present
None	100	100
Fe^{2+}	99	258
Ni^{2+}	86	144
Cu^{2+}	93	115
Mg^{2+}	86	94

[a] Bone samples were preincubated in 1 ml of pH 5.3 acetate buffer at 37.5° for 10 min. Metal ions (0.1 m*M*) and cysteine (0.1 *M*) were added to the preincubation mixture as indicated. The assay was started by the addition of 1 ml of 20 m*M* phenylphosphate.

TABLE III. Acid Phosphatase Activity of Bone, Liver, and Spleen.

Tissue	Phenylphosphatase (units/mg of DNA[b])	β-Glycerophosphatase (units/mg of DNA[b])	Ratio[a]
Bone	5.17	0.89	5.8
Liver	2.89	2.12	1.3
Spleen	0.43	0.28	1.5

[a] Phenylphosphatase/β-glycerophosphatase.

[b] One unit equals 1 μmole of substrate metabolized/min.

TABLE IV. Effect of Alloxan, Malonate, and Cysteine on the Acid Phenylphosphatase Activity of Liver and Bone.

Agent	Concentration (m*M*)	Activity as % of control	
		Bone	Liver
Alloxan	50	101	76
Malonate	50	342	77
Cysteine[a]	100	797	418

[a] Cysteine was added to the homogenates during a 10-min preincubation at pH 5.3 and 37.5°. The alloxan and malonate were added directly to the reaction medium.

13) but is also present in liver (14). Phosphoprotein phosphatase is strongly inhibited by molybdate and strongly activated by high concentrations of reducing agents, including cysteine. The activation by cysteine is enhanced by the same metal ions that enhanced the cysteine activation of the bone acid phenylphosphatase activity (Table II). Because phosphoprotein phosphatase hydrolyzes phosphoproteins (although much less rapidly than it hydrolyzes phenylphosphate), a bone extract was tested for the ability to hydrolyze the phosphate esters of casein. The bone extract hydrolyzed the casein substrate about one-ninth as rapidly as it hydrolyzed phenylphosphate. Although isolation of the bone acid phosphatases is required to prove their identity, the results of the present study suggest that the high acid phosphatase activity of bone is due to the enzyme, phosphoprotein phosphatase.

Summary. The characteristics of the bone acid phosphatase (EC 3.1.3.2) activity have been studied using both β-glycerophosphate and phenylphosphate as substrates. The acid β-glycerophosphatase activity was strongly inhibited by tartrate, fluoride, and molybdate and moderately inhibited by mercury. These properties are consistent with the presence of an enzyme similar to the major nonspecific acid phosphatase of the liver lysosomes.

The major characteristics of the acid phenylphosphatase activity were strong inhibition by fluoride and molybdate, but not by tartrate; strong activation by cysteine, dithiothreitol, and ascorbate and a moderate activation by malonate. The activation by cysteine was enhanced by the addition of Fe^{2+}, Ni^{2+} or Cu^{2+}. The properties and high activity demonstrated with the acid phenylphosphatase assay indicate the presence of a second enzyme in bone with properties similar to phosphoprotein phosphatase (EC 3.1.3.16).

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