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Streptozotocin-Induced Diabetes: Significant Changes in the Kinetic Properties of the Soluble Form of Rat Bone Alkaline Phosphatase

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ABSTRACT. A soluble form of an alkaline phosphatase, obtained from the osseous plate of streptozotocin-induced diabetic rats, was purified 90-fold with a yield of 26%. The calculated M_r of the purified enzyme was 80,000 by denaturing polyacrylamide gel electrophoresis and 160,000 by gel filtration on Sephacryl S-300, suggesting a dimeric structure for its native form. In the absence of metal ions, the p-nitrophenylphosphatase activity of the purified enzyme was 4223.1 U/mg. Magnesium or calcium ion concentrations up to 2 mM increased the specific activity of the enzyme to 9896.5 and 10,796.2 U/mg, respectively. The enzyme was stimulated to a lesser extent by $MnCl_2$ (5390.1 U/mg) and $CoCl_2$ (5088.2 U/mg). The purified soluble alkaline phosphatase showed a broad substrate specificity, and among the less hydrolyzed substrates were pyrophosphate (1517.6 U/mg) and bis-*p*-nitrophenylphosphate (499.6 U/mg). The enzyme was relatively stable at 45° for periods as long as 180 min, but was denatured rapidly above 50°, following first order kinetics with $T_{1/2}$ values ranging from 3.5 to 57.7 min. The results reported herein suggested that the soluble form of alkaline phosphatase from streptozotocin-induced diabetic rats had its kinetic properties altered, apparently as a consequence of changes in metal-binding properties. BIOCHEM PHARMACOL **58**;5:841–849, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. alkaline phosphatase; osseous plate; endochondral ossification; rat; diabetes; streptozotocin

Alkaline phosphatase often is regarded as an index of bone formation [1–3]. However, there is still no assignment of a simple function for this enzyme [4–8]. Diabetic patients, as well as laboratory animals with experimentally induced insulin deficiency, exhibit alterations in bone and mineral metabolism [9–11], but the mechanisms by which mineral metabolism and bone integrity are altered during diabetes pathogenesis still are poorly understood [9, 12].

It is well known that chronically diabetic rats show a significant increase in serum alkaline phosphatase activity [12–15]. This circulating alkaline phosphatase appears to be of the intestinal type; however, the possibility that it also might arise from bone or liver tissues cannot be excluded [13, 16, 17]. Since the circulating levels of alkaline phosphatase vary with physiological changes [3], conflicting results arise when serum alkaline phosphatase is used as an index of bone-specific isozymes [18].

The bone-forming system described by Reddi and Huggins [19] offers a good model for studying the relationship between diabetes and osseous alkaline phosphatase. This method, which has proven to be highly reproducible, results in a remarkable sequence of cellular transformations. With this system, and using diabetic rats as a model, Rezende *et al.* [20] reported that although bone mineralization apparently is impaired, and bone alkaline phosphatase levels are increased, the sequence of cellular transformations is unaffected.

Alkaline phosphatase is not exclusively present in plasma membranes. It can exist inside vesicles and along non-collagenic fibers [21]. Moreover, callus calcifying cartilage alkaline phosphatase prepared by using non-protease methods exists as two antigenically distinct forms [22]. Although one of these two forms might arise from chondrocytes, and the other from matrix vesicles, it has been suggested that one may represent a soluble form, and the other a membrane-associated enzyme [23, 24]. More direct experimental evidence showing that soluble and membrane-associated forms of alkaline phosphatase are found during endochondral ossification has been provided by us [4, 25, 26].

We report here the purification and the kinetic properties of the soluble form of rat osseous plate alkaline phosphatase that arises during biomineralization, 20 days after s.c. implantation of demineralized bone matrix in streptozotocin-induced diabetic rats. In our opinion, these results represent an improvement in the methodology used

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to study the effects of diabetes on the mineralization process.

MATERIALS AND METHODS

Materials

All solutions were made up by using Millipore MilliQ ultrapure apyrogenic water. Tris, streptozotocin, Sepharose 4B, Phenyl-Sepharose CL-4B, bovine serum albumin, PNPP*, ATP, ADP, TCA, Fast Blue RR salt, bis-PNPP, DTT, EDTA, phenylalanine, PMSF, AMPOL, and α-naphthyl phosphate were obtained from the Sigma Chemical Co. All other materials were of the highest analytical grade commercially available and were used without further purification. Metal ions were used in the form of the metal chloride.

Induction of Diabetes

Experimental diabetes was induced in male Wistar rats (120–150 g) by i.v. administration of a single dose of streptozotocin (60 mg/kg dissolved in 0.01 M citrate buffer, pH 4.5). Wistar rats (120–150 g) injected with the same volume of the buffer were used as controls.

Preparation of Soluble Alkaline Phosphatase

Demineralized bone matrix was implanted in dorsal subcutaneous tissue just after the streptozotocin (or buffer) administration, according to Rezende *et al.* [20]. Briefly, the alkaline phosphatase-rich osseous plaques formed (button-like plaques that ensue after the implantation of demineralized bone matrix and that mimic the processes occurring in the epiphyseal cartilage growth disk) were harvested 20 days after the implantation (the period of maximal PNPPase activity), rinsed in ice-cold 0.9% (w/v) NaCl, and homogenized with 5 mM Tris–HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 150 mM NaCl (3 mL of buffer/g of osseous plate), in a high-speed shearing homogenizer for 5 min at 4°. The homogenate was centrifuged at 15,000 *g* for 20 min, at 4°, and the supernatant (crude extract) was dialyzed overnight against 5 mM Tris–HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 150 mM NaCl.

Purification of Soluble Alkaline Phosphatase

The crude extract was purified initially on a Sepharose 4B column (3 × 83 cm) equilibrated and eluted with 5 mM Tris–HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 150 mM NaCl [25]. Two peaks showing phosphohydrolytic activity were eluted from the column, and only the active fractions corresponding to peak II (the soluble alkaline phosphatase) were concentrated on an Amicon YM-10 ultrafiltration membrane in a stirred cell and adjusted to 1 μM $ZnCl_2$ and 2.7 M NaCl. This fraction (named the S-II fraction) then was applied to a Phenyl-Sepharose CL-4B column (2 × 25 cm) previously equilibrated with 5 mM Tris–HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 1 μM $ZnCl_2$, and 2.7 M NaCl, and the elution proceeded exactly as described by Say *et al.* [26]. Only peak II (named the PS-II fraction), which showed the highest specific activity, was concentrated as described above and then dialyzed overnight against 5 mM Tris–HCl buffer, pH 8.2, containing 4 mM $MgCl_2$ and 22 mM NaCl, at 4°, with two changes of the buffer. Finally, the PS-II fraction was chromatographed on a DE-32 column (2 × 20 cm) previously equilibrated in 5 mM Tris–HCl buffer, pH 8.2, containing 4 mM $MgCl_2$ and 22 mM NaCl. Unbound protein was eluted with the starting buffer, after which the bound proteins were eluted using a stepwise ionic strength NaCl gradient. Seven different protein peaks emerged from the column, and only peak VI (named the DE-VI fraction) was concentrated as described above and dialyzed overnight against 5 mM Tris–HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, at 4°. Aliquots (1.0 mL) of purified enzyme were frozen in liquid nitrogen and stored at −20° for a period no longer than 2 weeks without appreciable loss of activity. These aliquots were allowed to thaw as needed for kinetic studies, placed on crushed ice, and used immediately. Any enzyme not used immediately was discarded.

Enzymatic Activity Measurements

PNPPase activity was assayed discontinuously at 37° in a Varian DMS-80 spectrophotometer by following the liberation of *p*-nitrophenolate ion ($\epsilon_{pH\ 13} = 17{,}600\ M^{-1}\ cm^{-1}$) at 410 nm, according to Ciancaglini *et al.* [27]. Standard assay conditions were 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 1 mM PNPP in a final volume of 1.0 mL. The reaction was initiated by the addition of the enzyme and was terminated with 1.0 mL of 1.0 M NaOH at appropriate time intervals. Bis-PNPP (20 mM) also was used as substrate under the same assay conditions as described above. However, due to the considerable spontaneous hydrolysis of this substrate, continuous kinetic measurements were performed.

For phosphate esters other than PNPP and bis-PNPP, the activity was assayed discontinuously by measuring the amount of inorganic phosphate liberated, according to Heinonen and Lahti [28], adjusting the assay medium to a final volume of 1.0 mL. The reaction was initiated by the addition of the enzyme, stopped with 0.5 mL of cold 30% TCA at appropriate time intervals, and immediately centrifuged at 4000 *g*, at 4°, followed by phosphate determination.

Determinations were carried out in duplicate, and the initial velocities were constant for at least 30 min provided that less than 5% of substrate was hydrolyzed. Controls without added enzyme were included in each experiment to allow for the non-enzymatic hydrolysis of substrate. One

* *Abbreviations*: PNPP, *p*-nitrophenylphosphate; DTT, dithiothreitol; bis-PNPP, bis *p*-nitrophenylphosphate; PMSF, phenylmethylsulfonyl fluoride; AMPOL, 2-amino-2-methyl-propan-1-ol; PNPPase, *p*-nitrophenylphosphatase; and TCA, trichloroacetic acid.

enzyme unit (U) was defined as the amount of enzyme hydrolyzing 1.0 nmol of substrate/min at 37°.

Determination of Protein Concentration

Protein concentrations were determined according to the procedure described by Read and Northcote [29], using bovine serum albumin as standard.

Determination of Blood Glucose

Blood glucose was determined according to King and Garner [30]. Rats that showed serum glucose levels above 200 mg/100 mL of blood were considered to be diabetic.

Molecular Weight Determination

The M_r of the purified soluble alkaline phosphatase was estimated by gel filtration on Sephacryl S-300 and denaturing polyacrylamide gel electrophoresis according to Say et al. [26].

Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out in 6% gels according to Davis [31], using silver nitrate for protein staining. Phosphohydrolytic activity on the gel was detected in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, 0.12% (w/v) Fast Blue RR salt, and 0.12% (w/v) α-naphthyl phosphate, at 37°. When necessary, protein samples were concentrated by centrifugation on Centriflo Amicon cones.

Inhibition Studies

Inhibition studies were carried out using at least four different concentrations of the inhibitor and variable concentrations (from 10 μM to 1 mM) of PNPP. The value of the inhibition constant (K_i) was estimated graphically using secondary plots of the Lineweaver–Burk equation.

Thermal Inactivation Studies

Samples of purified enzyme in 5 mM Tris–HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ were incubated in a water bath at different temperatures for various periods. After the incubation, 100-μL samples were chilled quickly to 4° (to stop the inactivation process), and the residual PNPPase activity was assayed as described above.

Estimation of Kinetic Parameters

Data obtained from substrate hydrolysis studies were fitted on an IBM Pentium microcomputer according to Rezende *et al.* [20] to estimate the values of the kinetic parameters. V, $K_{0.5}$, n, and K_i, which appear in this paper, stand for maximal velocity, apparent dissociation constant, Hill coefficient, and enzyme–inhibitor complex dissociation constant, respectively.

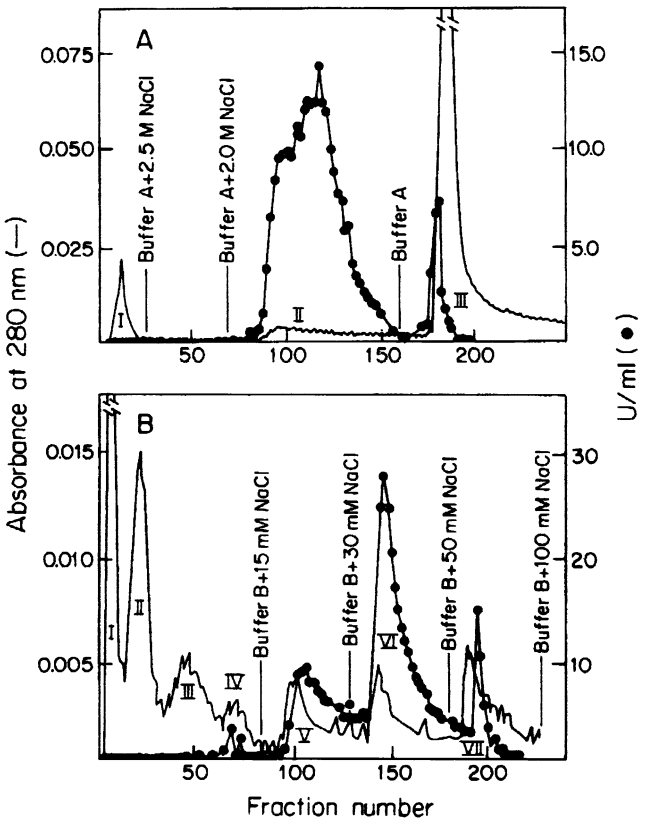

FIG. 1. Purification of a soluble form of osseous plate alkaline phosphatase from streptozotocin-induced diabetic rats. (A) Phenyl-Sepharose CL-4B chromatography. The S-II sample, adjusted to 1 μM $ZnCl_2$ and 2.7 M NaCl, was applied to a column equilibrated with Buffer A (5 mM Tris–HCl, pH 7.5, containing 2 mM $MgCl_2$ and 1 μM $ZnCl_2$) with 2.7 M NaCl. Elution was performed at 4° at a flow rate of 24 mL/hr. Fractions of 4 mL were collected, and 50-μL aliquots were assayed for PNPPase activity as described in Materials and Methods. Fractions corresponding to peak II were pooled and named PS-II fraction. (B) DE-32 chromatography. The PS-II fraction was applied to a DE-32 column equilibrated with Buffer B (5 mM Tris–HCl, pH 8.2, containing 4 mM $MgCl_2$ and 22 mM NaCl), and elution was performed at 4° at a flow rate of 24 mL/hr. Fractions of 4 mL were collected, and 50-μL aliquots were assayed for PNPPase activity as described in Materials and Methods. Fractions corresponding to peak VI were pooled and named the DE-VI fraction.

RESULTS

Of the two active fractions eluted from the Phenyl-Sepharose column (Fig. 1A), only peak II (PS-II fraction) was used, since it showed a good specific activity (1500.3 U/mg) with a significant yield of 58%. Apparently, washing the column with 2.5 M NaCl was essential to remove contaminants. Step-gradient elution of a DE-32 column resolved at least seven protein fractions (Fig. 1B). Four of these showed phosphohydrolytic activity, but only fraction VI (DE-VI fraction), with the highest specific activity (9426.4 U/mg),

TABLE 1. Purification of the soluble form of osseous plate alkaline phosphatase from streptozotocin-induced diabetic rats (SIDR)

Fraction	U/mg		Yield (%)		Purification (fold)	
	SIDR	Control	SIDR	Control	SIDR	Control
S-II	105.6	89.4	100	100	1	1
PS-II	1,500.3	1,221.6	58	41.5	14	13.6
DE-VI	9,426.4	18,399.1	26	22.8	90	206

Assays were carried out in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 1 mM PNPP, at 37°, as described in Materials and Methods.

was homogeneous and was used in this work. This fraction was purified 90-fold with a yield of about 26%. It should be stressed that the extents of purification of the enzymes of both diabetic and control rats were very similar, but the specific activity of the enzyme of controls was 2-fold higher. A summary of a typical purification of the soluble matrix-induced alkaline phosphatase of osseous plate from streptozotocin-induced diabetic rats is shown in Table 1.

Figure 2 shows that the purified soluble alkaline phosphatase from streptozotocin-induced diabetic rats was obtained as a single, somewhat diffuse protein band (Fig. 2A), which was coincident with phosphohydrolytic activity (Fig. 2B). The enzyme purity was greater than 95%, according to densitometric scanning of the gel (not shown).

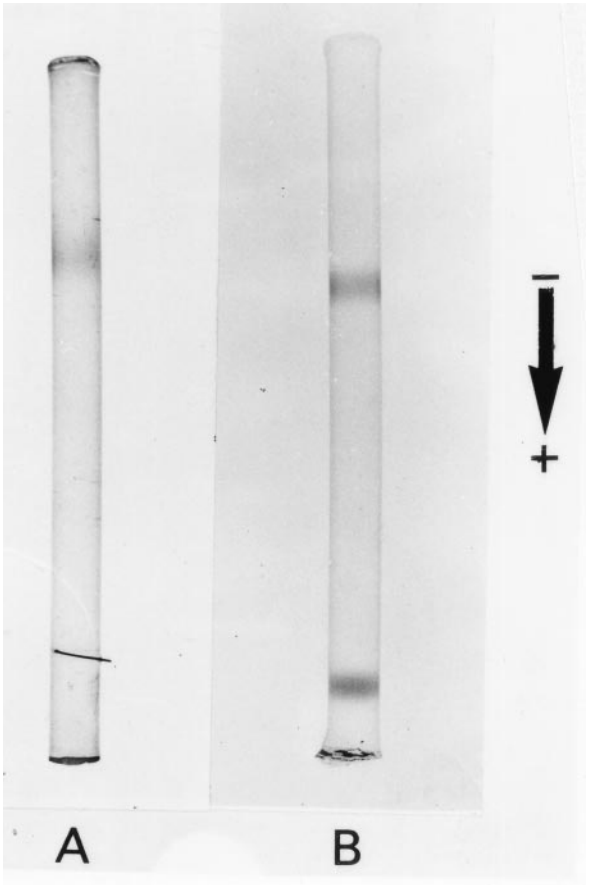

FIG. 2. Polyacrylamide gel electrophoresis of the soluble form of osseous plate alkaline phosphatase from streptozotocin-induced diabetic rats. (A) Silver nitrate staining. (B) Phosphohydrolytic activity.

The M_r of the soluble alkaline phosphatase was estimated to be 160,000 by gel filtration on Sephacryl S-300, but in denaturing conditions the enzyme exhibited an M_r of about 80,000 (not shown). These findings suggested that the soluble enzyme of diabetic rats may exist as a dimer, similarly as observed for controls (not shown).

The effect of PNPP concentration on the PNPPase activity of the soluble alkaline phosphatase of diabetic rats is shown in Fig. 3. In the absence of metal ions, the enzyme followed "Michaelian" kinetics with $K_{0.5}$ = 42.7 μM and a specific activity of 4223.1 U/mg. In the presence of 2 mM $MgCl_2$ or $CaCl_2$, the specific activity of the enzyme increased about 2.5-fold (V = 9426.4 and 9982.2 U/mg for magnesium and calcium ions, respectively), while $K_{0.5}$ remained almost unchanged (33.7 μM for both ions). Furthermore, site–site interactions (n = 1.3) were observed in the presence of these ions. Table 2 summarizes the values of the kinetic parameters estimated for the hydrolysis of PNPP under the different ionic conditions used. Note that the specific activity of the enzyme of diabetic rats was 2-fold lower than that of controls, independent of the ion used,

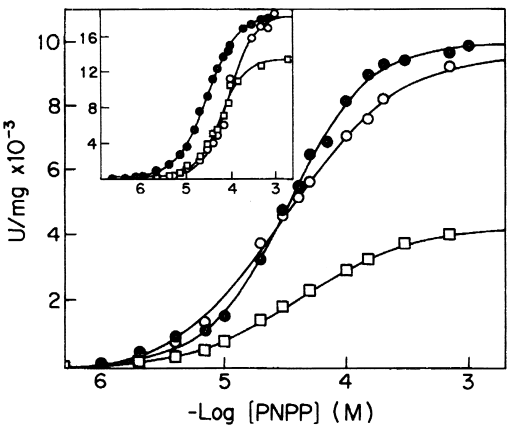

FIG. 3. PNPP concentration dependence on metal ions of the phosphohydrolytic activity of the soluble form of osseous plate alkaline phosphatase from streptozotocin-induced diabetic rats. All values shown in the curves are means of at least three separate preparations, which fell within a range of ± 10% of the indicated value. The curve represented is the theoretical curve giving the best fit with the experimental data. Inset: data corresponding to the enzyme from control rats. The activity was assayed in 50 mM AMPOL buffer, pH 9.4, by using 0.5 μg of purified enzyme, as described in Materials and Methods, in the presence of: (□) no metal ions; (○) 2 mM $MgCl_2$; and (●) 2 mM $CaCl_2$.

TABLE 2. Kinetic parameters for the hydrolysis of PNPP by soluble alkaline phosphatase from streptozotocin-induced diabetic rats in the absence and presence of magnesium and calcium ions

	SIDR			Control		
Conditions	V (U/mg)	$K_{0.5}$ (μM)	n	V (U/mg)	$K_{0.5}$ (μM)	n
No ions	4,233.1 ± 190.5	42.7 ± 2.6	1.0	13,509.2 ± 783.5	52.5 ± 2.0	1.4
$MgCl_2$, 2 mM	9,426.4 ± 603.3	33.7 ± 2.3	1.3	18,427.6 ± 884.5	87.5 ± 6.8	1.5
$CaCl_2$, 2 mM	9,982.2 ± 778.6	33.7 ± 1.6	1.3	18,271.6 ± 986.6	28.8 ± 1.4	1.2

Initial rates were measured at 37°, in 50 mM AMPOL buffer, pH 9.4, containing variable concentrations of PNPP and the metal ion, as described in Materials and Methods. Results represent the means ± SD from at least three separate experiments in duplicate.

but no significant differences in $K_{0.5}$ values were observed. Taken together, these results suggested that magnesium (or calcium) ions did not contribute to the recognition of the substrate at its binding site on the enzyme molecule, similarly as observed for the enzyme from control animals (inset of Fig. 3).

The hydrolysis of several substrates by the soluble alkaline phosphatase from streptozotocin-induced diabetic rats is shown in Table 3. Similarly as for controls, the enzyme of diabetic animals showed a broad substrate specificity, but bis-PNPP and pyrophosphate were hydrolyzed to a minor extent (less than 20%) when compared with the other substrates. Further, it should be noted that the specific activity of the enzyme from diabetic animals was significantly lower than that of controls, independent of the substrate.

The effect of various concentrations of divalent metal ions on PNPPase activity of soluble alkaline phosphatase of diabetic animals is shown in Fig. 4. Comparison of the effects of calcium and magnesium ions on enzyme activity with the effects of manganese and cobalt ions demonstrated that increasing concentrations of magnesium ions resulted in a 2-fold increase in PNPPase activity of the enzyme (V = 9896.5 U/mg) by following "Michaelian" kinetics with $K_{0.5}$ = 520 μM (Fig. 4A). Calcium ions also stimulated PNPPase activity (V = 10,796.2 U/mg) to the levels observed for magnesium ions, but with a $K_{0.5}$ of about 180 μM (Fig. 4B) and showing site–site interactions (n = 1.3). Manganese (Fig. 4C) and cobalt (Fig. 4D) ions stimulated the PNPPase activity of the enzyme by only 25% (5390.1 and 5088.2 U/mg for manganese and cobalt ions, respectively). Although site–site interactions were also observed for these ions, it should be noted that the apparent dissociation constant of the "Michaelian" complex decreased considerably, to values of about 1.0 μM. It should be stressed that manganese and cobalt ions had no effect on the PNPPase activity of the enzyme from controls, whereas a stimulation of about 35% was observed for calcium and magnesium ions (insets of Fig. 4, A and B). Table 4 summarizes the kinetic parameters obtained for the stimulation of the PNPPase activity of the soluble alkaline phosphatase from streptozotocin-induced diabetic rats by divalent metal ions.

Table 5 shows that vanadate (K_i = 4.0 μM), arsenate (K_i = 26.0 μM), and phosphate (K_i = 875.0 μM) ions were competitive inhibitors of the enzyme of diabetic rats,

TABLE 3. Hydrolysis of several substrates by the soluble alkaline phosphatase from streptozotocin-induced diabetic rats

	U/mg	
Substrate	SIDR	Control
PNPP (1 mM)	9,426.4 ± 452.5	18,456.7 ± 738.3
ADP (2 mM)	7,889.6 ± 181.5	14,752.0 ± 560.6
ATP (2 mM)	4,043.7 ± 198.1	5,915.6 ± 295.8
Pyrophosphate (2 mM)	1,517.6 ± 37.9	3,773.8 ± 264.2
bis-PNPP (20 mM)	499.6 ± 29.9	2,485.7 ± 146.6

Initial rates were measured in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and the substrate at the indicated concentration, at 37°, as described in Materials and Methods. Substrate concentrations stand for saturation concentrations. Results represe;nt the means ± SD from at least three separate experiments in duplicate.

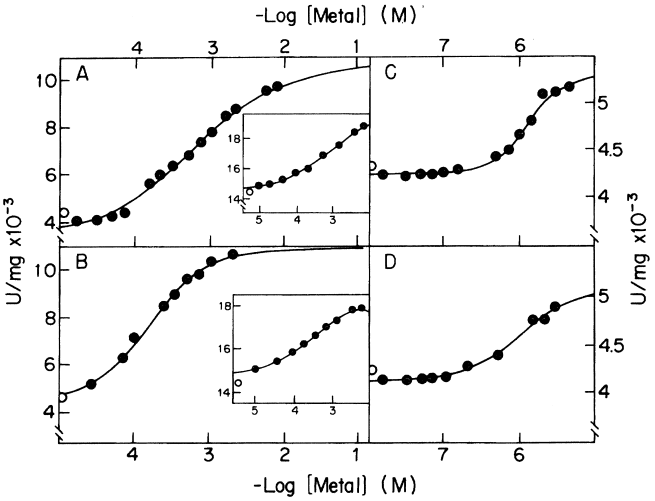

FIG. 4. Stimulation of the phosphohydrolytic activity of the soluble form of osseous plate alkaline phosphatase from streptozotocin-induced diabetic rats by divalent metal ions. The activity was assayed in 50 mM AMPOL buffer, pH 9.4, containing 1 mM PNPP, by using 0.5 μg of purified enzyme, as described in Materials and Methods. Open symbol represents the enzyme activity in the absence of metal ion. (A) $MgCl_2$. (B) $CaCl_2$. (C) $MnCl_2$. (D) $CoCl_2$. All values shown in the curves are the means of at least three separate preparations, which fell within a range of ± 10% of the indicated value. The curve represented is the theoretical curve giving the best fit with the experimental data. Insets: data corresponding to the enzyme from control rats.

TABLE 4. Kinetic parameters for the stimulation of PNPPase activity of soluble alkaline phosphatase from streptozotocin-induced diabetic rats by divalent metal ions

	SIDR			Control		
Metal	**V (U/mg)**	**$K_{0.5}$ (μM)**	***n***	**V (U/mg)**	**$K_{0.5}$ (μM)**	***n***
None	4,233.1 ± 190.5	42.7 ± 2.6	1.0	13,509.2 ± 783.5	52.5 ± 2.0	1.4
$MgCl_2$	9,896.5 ± 445.3	520.0 ± 2.3	1.0	18,622.5 ± 893.9	92.1 ± 4.8	0.7
$CaCl_2$	10,796.2 ± 475.0	180.0 ± 0.8	1.3	17,878.5 ± 1,019.1	293.2 ± 13.1	0.8
$MnCl_2$	5,390.1 ± 188.6	1.1 ± 0.03	1.3			
$CoCl_2$	5,088.2 ± 157.7	0.9 ± 0.03	1.2			

Initial rates were measured in 50 mM AMPOL buffer, pH 9.4, containing 1 mM PNPP and variable concentrations of the metal ion, at 37°, as described in Materials and Methods. Results represent the means ± SD from at least three separate experiments in duplicate.

whereas levamisole (K_i = 32.0 μM) and theophylline (K_i = 210.0 μM) were uncompetitive inhibitors. Zinc ions were a powerful non-competitive inhibitor of the PNPPase activity of the enzyme (K_i = 15.0 μM), whereas DTT, PMSF, and NaF had no effect. EDTA caused a 70% inhibition (versus 30% observed for controls), which was not reversed by an excess of magnesium ions. Further, EDTA-treated enzyme could not be reactivated by any of the divalent metal ions even after dialysis against 5 mM Tris–HCl buffer, pH 7.5 (not shown). Atomic absorption spectrometric analysis revealed the presence of zinc and magnesium ions in samples of EDTA-treated enzyme from both diabetic and control animals (not shown). From these data, it should be observed that there were no striking differences between the effects of such reagents on the PNPPase activity of the soluble alkaline phosphatase from diabetic and control rats.

The enzyme of diabetic animals was less stable than that of controls. A loss of 40% of its original PNPPase activity was observed after 20 days of storage at −20°, while the enzyme of controls remained stable for at least 30 days. On the other hand, thermal denaturation studies showed that purified enzyme from diabetic animals was relatively stable up to 45° for periods as long as 180 min (Fig. 5). However, it was denatured rapidly above 50°, showing first order kinetics with k varying from 0.197 min^{-1} to 0.012 min^{-1} (at 50°) and k = 0.051 min^{-1} (at 55°). Controls assayed under the same conditions gave similar results (inset of the figure).

DISCUSSION

Diabetes currently is considered to be more than just a disease characterized by hyperglycemia. Besides its effects on the liver and other well known insulin-dependent tissues, such as skeletal muscle and adipose tissue, insulin also influences endochondral bone growth and bone formation (for review see Ref. 32). On the other hand, decreased levels of serum vitamin D [33], alterations of collagen metabolism [34, 35], osteopenia [32, 36], changes in calcium and phosphate absorption [9], and alterations of PTH and calcitonin levels [37] are some of the alterations associated with insulin-dependent diabetes that also may affect the mineralization process indirectly.

Previous studies from our laboratory dealing with the relationship between diabetes and osseous alkaline phosphatase have shown that despite impairment of bone mineralization and increase in alkaline phosphatase levels,

TABLE 5. Effect of several reagents on PNPPase activity of soluble alkaline phosphatase from streptozotocin-induced diabetic rats

	SIDR			Control		
Reagent	***Ki* (μM)**	**Type**	**% Initial activity**	***Ki* (μM)**	**Type**	**% Initial activity**
Vanadate	4.0 ± 0.2	C		5.0 ± 0.3	C	
Phosphate	875.0 ± 49.1	C		838.0 ± 42.6	C	
Arsenate	26.0 ± 1.6	C		12.0 ± 0.8	C	
Theophylline	210.0 ± 12.3	U		93.0 ± 7.4	U	
Levamisole	32.0 ± 1.5	U		16.0 ± 0.3	N	
$ZnCl_2$	15.0 ± 1.0	N		10.4 ± 0.8	N	
Streptozotocin (1.5 mM)			98.5 ± 1.7			101.2 ± 8.1
DTT (0.02 mM)			96.4 ± 8.6			93.6 ± 5.2
EDTA (3.0 mM)			31.2 ± 2.5			69.4 ± 2.4
Phenylalanine (6.7 mM)			55.7 ± 3.1			66.2 ± 5.1
PMSF (0.3 mM)			87.1 ± 5.6			89.1 ± 7.6
NaF (5.0 mM)			98.3 ± 7.6			100.9 ± 5.4

Initial rates were measured in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 1 mM PNPP, at 37°, as described in Materials and Methods. Specific activity of 100% corresponds to 9,426.4 and 18,456.7 U/mg for diabetic and control rats, respectively. Results represent the means ± SD from at least three separate experiments in duplicate. C, N, and U stand for competitive, non-competitive, and uncompetitive inhibition, respectively.

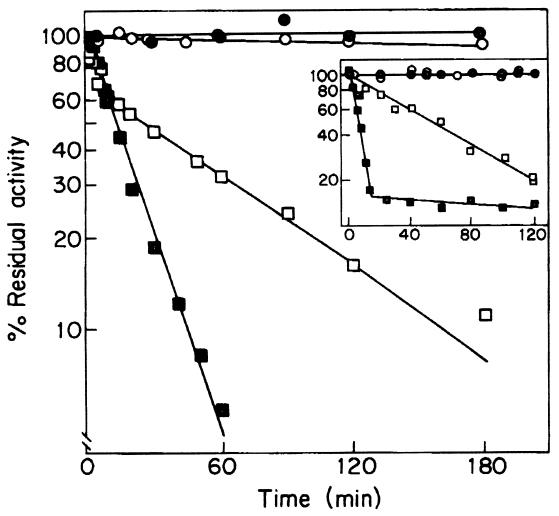

FIG. 5. Thermal denaturation of the soluble form of osseous plate alkaline phosphatase from streptozotocin-induced diabetic rats. The enzyme in 5 mM Tris–HCl, pH 7.5, containing 2 mM $MgCl_2$ was incubated at different temperatures for various times, and the residual activity was estimated in 50 mM AMPOL buffer, pH 9.4, containing 1 mM PNPP and 2 mM $MgCl_2$, by using 0.5 and 0.8 μg of purified enzyme of diabetic and control animals, respectively. All values shown in the curves are the means of at least three separate preparations, which fell within a range of ± 10% of the indicated value. Inset: thermal denaturation of the enzyme from control rats. Key: (●) 37°, (○) 45°, (□) 50°, and (■) 55°.

the sequence of cellular transformations is unaffected [20]. Further, it also has been reported that the structural and catalytic properties of the membrane-bound alkaline phosphatase apparently are not altered by diabetes.

As reported for the soluble enzyme of non-injected normal rats [26], Phenyl-Sepharose CL-4B chromatography, associated with DE-32 chromatography, provided a preparation of homogeneous enzyme from diabetic rats. Despite a yield of 26%, the specific activity of the alkaline phosphatase of diabetic animals (9426.4 U/mg) was half that of the controls and of both human [38] and rat bone [26]. The subunit molecular weight of alkaline phosphatase from diabetic rats, about 80,000, was very similar to that of controls, and was coincident with those reported for the enzyme from other mammalian tissues [26, 38, 39]. Altogether, these data suggested that no major structural changes had occurred in the molecule of soluble alkaline phosphatase during diabetes pathogenesis, as has been reported for the membrane-associated form of the enzyme [20].

Phosphate, arsenate, metavanadate and zinc ions, phenylalanine, theophylline, and levamisole (Table 5) affected the enzyme equally from diabetic and control animals, as reported elsewhere for the osseous plate alkaline phosphatase of non-injected normal animals [4, 26], and other sources [38]. It is well known that alkaline phosphatases, except the placental enzyme, are inactivated irreversibly by EDTA [38, 40, 41]. The greater sensitivity of the enzyme of diabetic rats to EDTA, when compared with that of controls (Table 5), suggested that the divalent metal ions may be crucial for maintaining both the structure and the activity of the enzyme. Attempts to reactivate the EDTA-treated enzyme by incubation with several metal ions, with or without dialysis to remove excess EDTA, were unsuccessful (not shown). The presence of zinc and magnesium ions on EDTA-treated enzyme indicated that the enzyme inactivation was not a consequence of metal depletion by this chelating agent.

Soluble alkaline phosphatase of diabetic rats hydrolyzed several phosphate esters and diesters and pyrophosphate in a similar way as the enzyme of controls (Table 3). However, the initial rate values for the hydrolysis of PNPP and the other substrates were half those of controls. This is in close agreement with the decreased levels of bone alkaline phosphatase activity reported for chronic, streptozotocin-induced insulin-deficient rats [16].

As regards the effects of divalent metal ions, the soluble enzyme of diabetic rats exhibited striking changes when compared with that of controls. Manganese and cobalt ions, reportedly stimulators of other alkaline phosphatases [27, 38, 40], had no effects on the enzyme of controls, but stimulated PNPPase activity of diabetic rats by 35% (see Fig. 4 and Table 4). This is in close agreement with earlier results reported by us for the enzyme of non-injected normal rats [26]. For magnesium and calcium ions, the results were unexpected, since PNPPase activity was stimulated by 150% (see Fig. 4 and Table 4), as compared with the 35% observed for controls. When compared with data reported for other alkaline phosphatases [27, 38, 39, 42–45], and those for the soluble enzyme of non-injected normal rats [26], the data in Table 4 showed a notable increment in the specific activity of the alkaline phosphatase of the diabetic rats in the presence of magnesium and calcium ions. Thus, it seems likely that the depletion of alkaline phosphatase activity in diabetic animals was counterbalanced partially by changes in the affinity of the enzyme for calcium and magnesium ions. However, the physiological significance of these results remains to be elucidated.

Thermal inactivation studies showed no significant differences between the enzymes of diabetic and control rats, which were very similar to that reported by us for non-injected normal rats [26]. However, as also reported for alkaline phosphatases from bone and cartilage [40, 46], the enzyme from diabetic rats was denatured more rapidly than the others at 55° [38].

Although skeletal alkaline phosphatase levels are considered to reflect osteoblastic activity, and therefore can be used as a marker of bone formation [1–3], an elevated level of serum alkaline phosphatase often is taken as an intrinsic feature of this disease, both in human patients and in experimental diabetes [12–15, 38]. As a consequence, this could lead to misinterpretations, since the circulating alkaline phosphatase, but not the skeletal enzyme, generally is correlated with diabetes pathology. Notwithstanding that

the circulating alkaline phosphatase from diabetic rats has been reported to be solely of the intestinal type, bone and/or liver alkaline phosphatase could be present as contaminants increasing the levels of circulating enzyme [13, 16, 17]. Further, bone alkaline phosphatase is difficult to distinguish from other alkaline phosphatase isoforms, since the kidney, liver, and bone isoenzymes are encoded by the same gene and only differ as a result of posttranslational modification of their carbohydrate side chain [47]. These facts and the apparent similarities observed herein for several kinetic properties of the enzymes of diabetic and control animals suggest that data on alkaline phosphatase activity, rather than being conclusively correlated with diabetic pathogenesis, should be interpreted cautiously and not generalized.

Finally, we conclude that our results represent an improved means to study mineralization during diabetes pathology. For instance, the activity of soluble alkaline phosphatase can be measured directly during diabetes pathogenesis, avoiding conflicting results and/or misinterpretation. Considering that the exact cause(s) of the increase in intestinal alkaline phosphatase levels leading to the well-reported diabetes-associated hyperphosphatasemia is still unclear (for review see Ref. 32), the direct assessment of the activity of the soluble enzyme might provide new insights into the mechanisms involved in the mineralization process of diabetes pathogenesis.

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