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ENZYMATIC CHARACTERIZATION OF THE CHONDROCYTIC ALKALINE PHOSPHATASE ISOLATED FROM BOVINE FETAL EPIPHYSEAL CARTILAGE *

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Summary

Purified chondrocytic alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) from bovine fetal epiphyseal cartilage hydrolyzes a variety of phosphate esters as well as ATP and inorganic pyrophosphate. Optimal activities for *p*-nitrophenyl phosphate, ATP and inorganic pyrophosphate are found at pH 10.5, 10.0 and 8.5, respectively. The latter two substrates exhibit substrate inhibition at high concentrations. *p*-Nitrophenyl phosphate demonstrates decreasing pH optima with decreasing substrate concentration. Heat inactivation studies indicate that both phosphorolytic and pyrophosphorolytic cleavage occur at the same site on the enzyme. Mg^{2+} (0.1—10.0 mM) and Mn^{2+} (0.01—0.1 mM) show a small stimulation of *p*-nitrophenyl phosphate-splitting activity at pH 10.5.

Levamisole, P_i, CN^-, Zn^{2+} and L-phenylalanine are all reversible inhibitors of the phosphomonoesterase activity. P_i is a competitive inhibitor with a K_i of 10.0 mM. Levamisole and Zn^{2+} are potent non-competitive inhibitors with inhibition constants of 0.05 and 0.04 mM, respectively. The chondrocytic alkaline phosphatase is inhibited irreversibly by Be^{2+}, EDTA, EGTA, ethane-1-hydroxydiphosphonate, dichloromethane diphosphonate, L-cysteine, phenyl-

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methylsulfonyl fluoride, *N*-ethylmaleimide and iodoacetamide.

NaCl, KCl and Na_2SO_4 at 0.5—1.0 M inhibit the enzyme.

At pH 8.5, the cleavage of inorganic pyrophosphate (pyrophosphate phosphohydrolase, EC 3.6.1.1) by the chondrocytic enzyme is slightly enhanced by low levels of Mg^{2+} and depressed by concentrations higher than 1 mM. Ca^{2+} show only inhibition. Similar effects of Mg^{2+} and Ca^{2+} on the associated ATPase (ATP phosphohydrolase, EC 3.1.6.3) activity were observed.

Arrhenius studies using *p*-nitrophenyl phosphate and AMP as substrates have accounted for the ten-fold difference in V in terms of small differences in both the enthalpies and entropies of activation which are 700 cal/mol and 2.3 cal/degree per mol, respectively.

Introduction

Studies in our laboratory have permitted the isolation and purification of two distinct forms of alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) from bovine fetal epiphyseal cartilage [1]. It was readily demonstrated that alkaline phosphatase activity exists in both the chondrocyte and matrix vesicle fractions [1,2]. Moreover, recent evidence indicates that these separate activities are associated with different molecular entities [1]. Structural analysis [1] of the two enzymes suggests that the matrix vesicle alkaline phosphatase is derived from the chondrocytic enzyme in harmony with cytochemical and microscopic evidence which indicates that both enzymes are membrane bound [3] and that the matrix vesicles may be derived from the chondrocyte membrane by budding. The matrix vesicle enzyme (M_r = 310 000) is composed of two distinct subunits, one of which (M_r = 141 000) is identical to the chondrocyte alkaline phosphatase [1].

Arsenis et al. [4] have described the separation, purification and characterization of two separate alkaline phosphatase activities from rabbit fracture callus calcifying cartilage.

The present paper describes the partial enzymatic characterization of the chondrocytic alkaline phosphatase from bovine fetal epiphyseal cartilage. The enzyme's associated alkaline phosphatase, ATPase (ATP phosphohydrolase, EC 3.6.1.3) and inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) activities have been investigated with respect to their pH optima, divalent cation effects, reversible and irreversible inhibitors, as well as the temperature dependence of the catalytic activity.

Experimental

Materials

Chondrocytes were prepared by the method of Ali et al. [2]. The chondrocyte alkaline phosphatase was isolated and purified by the method of Fortuna et al. [1]. All preparations used possessed a specific activity of 900 μmol *p*-nitrophenyl phosphate hydrolyzed/min per mg protein and were chromatographically homogeneous on columns of Sepharose 6B and DE-52 cellulose

(Whatman). Non-SDS gel electrophoresis [1,5] showed a single band of protein with associated alkaline phosphatase activity [6].

Methods

p-Nitrophenyl phosphate assay for alkaline phosphatase activity. Orthophosphate-releasing activity was routinely measured spectrophotometrically at pH 10.5 with 2-amino-2-methyl-1-propanol-HCl as buffer. Details are described elsewhere [1].

Assay for ATPase and inorganic pyrophosphatase activities. Enzymatic activity towards ATP and inorganic pyrophosphate was measured by a method modified from Martin and Doty [7].

Assay for activity towards alcohol and nucleoside phosphomonoesters. The hydrolysis of acid-stable phosphate esters was followed by measuring the phosphate released according to a modification of the method of Chen et al. [8]. Color was developed in tubes containing 1.24% ammonium molybdate and 0.018% ascorbic acid in 2.4 N perchloric acid. The solutions were read at 750 nm after standing for 15 min at room temperature. The assay is sensitive to inorganic phosphate concentrations of 20 μM.

Initial rate measurements were limited to 10% hydrolysis and were measured at 6—8 substrate concentrations. The data were analyzed by a weighted least-squares program to give V and K_m.

Heat inactivation studies. 1 ml of enzyme solution containing 80—100 ng protein was incubated at 45°C for 3 h. Aliquots of 150 μl were taken at 0, 15, 30, 45, 90 and 180 min and cooled in ice until assayed. Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate at pH 10.5. Inorganic pyrophosphatase and ATPase activities were assayed at pH 8.5 and 10.0, respectively.

Inhibitor studies. Alkaline phosphatase activity in the presence of reversible inhibitors was examined at pH 10.5 with *p*-nitrophenyl phosphate. K_m and V were obtained by a weighted least-squares analysis of initial velocities at 6—8 substrate concentrations in the presence of constant concentrations of inhibitor.

Inhibition was determined to be irreversible if after dialysis against an inhibitor-free buffer over night at 4°C, the inhibition remained. Irreversible inhibitors were incubated for 15—30 min at pH 7.45 with the purified enzyme. Alkaline phosphatase activity was then assayed at pH 10.5 with *p*-nitrophenyl phosphate.

Results

Substrate specificity

The alkaline phosphatase activity is linear with time and with the amount of added enzyme. 2 ng of purified enzyme is required for the routine assay.

K_m and V values were determined for 23 phosphomonoesters in addition to ADP, ATP, UDP and PP_i and the results are listed in Table I. The standard error for K_m was 3% and that for V was 1%.

pH optima

Fig. 1 gives the pH optima for the hydrolysis of *p*-nitrophenyl phosphate,

TABLE I

MAXIMUM VELOCITIES AND MICHAELIS CONSTANTS FOR THE HYDROLYSIS OF A VARIETY OF PHOSPHOMONOESTERS AND PYROPHOSPHATE SUBSTRATES BY PURIFIED CHONDROCYTE ALKALINE PHOSPHATASE FROM BOVINE FETAL EPIPHYSEAL CARTILAGE

All assays were carried out at pH 10.5 in 0.12 M 2-amino-2-methyl-1-propanol-HCl with the exceptions noted. Inorganic pyrophosphatase activity was followed at pH 8.5 in 0.12 M Tris-HCl. ATPase activity was measured at pH 10.0 in 0.12 M 2-amino-2-methyl-1-propanol-HCl. Details are discussed under Experimental.

Substrate	Specific activity (μmol/min per mg)	Michaelis constant (mM)
5′AMP	85.2	6.5
3′AMP	76.5	5.4
2′AMP	113.0	4.9
2,′5′dAMP	108.0	17.75
ADP	91.2	4.8
ATP (pH 10.0)	45.0	1.5
3′GMP	83.9	13.4
2′GMP	61.5	8.66
2,′5′dGMP	43.2	18.9
5′UMP	85.2	32.0
3′UMP	111.0	10.7
2′UMP	86.1	4.35
2,′5′dUMP	111.0	32.7
UDP	60.2	5.5
5′CMP	81.6	27.2
3′CMP	112.0	10.2
2′CMP	109.0	8.04
2,′5′dCMP	93.3	30.9
5′TMP	142.0	36.7
Glucose 1-phosphate	190.0	135.0
Glucose 6-phosphate	53.9	61.8
Phosphoethanolamine	51.2	31.7
2-Phosphoglycerate	2.6	30.1
α-Glycerophosphate	33.5	21.1
β-Glycerophosphate	115.0	30.1
p-Nitrophenyl phosphate	900.0	0.80
PP_i (pH 8.5)	37.0	0.79

ATP and PP_i. The pH optima are 10.5, 10.0, and 8.5, respectively. The pH optimum of the alkaline phosphatase activity is a function of substrate concentration. At a *p*-nitrophenyl phosphate concentration of 6 mM, the pH optimum is observed at pH 10.5, whereas at a concentration of 20 μM, the pH optimum falls to 9.5.

K_m and V values were determined from pH 7.5 to 10.5 for *p*-nitrophenyl phosphate and ATP, and from 7.5 to 9.5 for PP_i (cf. Fig. 2). For *p*-nitrophenyl phosphate and ATP, pK_m vs. pH is linear beteeen pH 8.0 and pH 10.5, with a slope of −1.2. For *p*-nitrophenyl phosphate, K_m decreases from 0.8 mM at pH 10.5 to 1 μM at pH 7.5, an 800-fold change, while V decreases 33-fold from 2115 to 63.5 μmol/s per mol protein (M_r = 141 000). Plots of log k_3 vs. pH demonstrate a positive non-integer slope, approx. 0.65, for *p*-nitrophenyl phosphate and ATP in the region of pH 7.5—9.5.

Heat inactivation studies

The inactivation of the ATPase, inorganic pyrophosphatase and alkaline

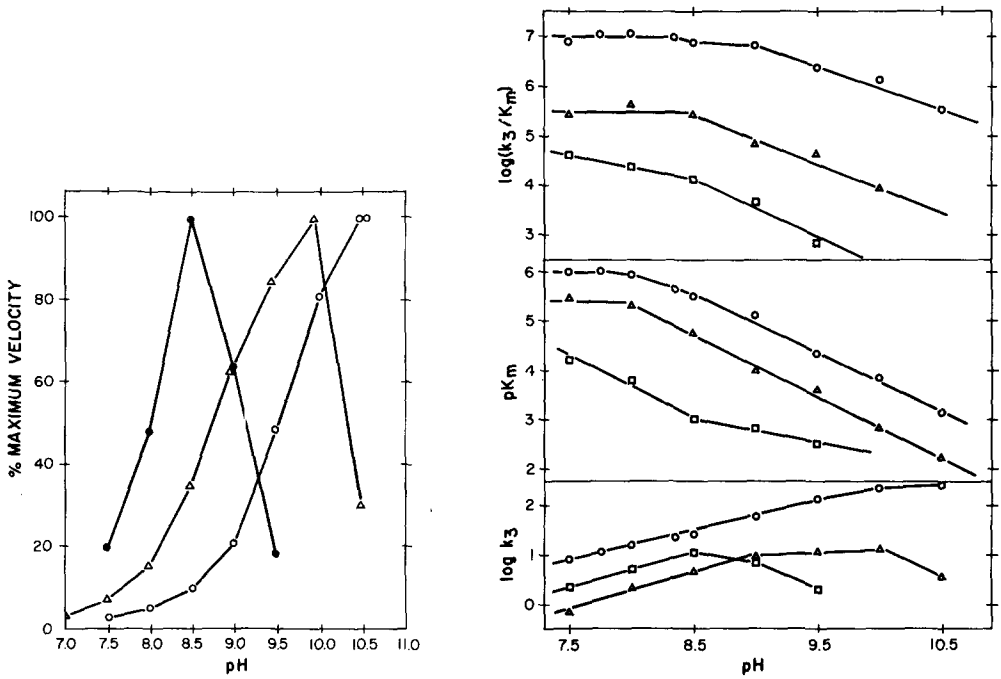

Fig. 1. Plots of the percent maximal velocity vs. pH for the chondrocyte alkaline phosphatase-catalyzed hydrolysis of: ○, *p*-nitrophenyl phosphate, ●, inorganic pyrophosphate and △, ATP. Activities are expressed as a percentage of the maximal velocity exhibited at the pH optimum. The values of the optimum pH and V are: *p*-nitrophenyl phosphate, 10.5 and 900 μmol/min per mg purified enzyme; inorganic pyrophosphate, 8.5 and 37.0 μmol/min per mg purified enzyme; ATP, 10.0 and 45 μmol/min per mg purified enzyme.

Fig. 2. The effect of pH on pK_m, log k_3 and log (k_3/K_m) for the chondrocyte alkaline phosphatase-catalyzed hydrolysis of: ○———○, *p*-nitrophenyl phosphate; △———△, ATP and □———□, PP_i. The linear portions of each curve were analyzed by least squares. The slopes of the linear regions and their respective errors are: *p*-nitrophenyl phosphate: pK_m, −1.19 ± 0.11; log k_3, +0.59 ± 0.05; log (k_3/K_m), −0.83 ± 0.02; ATP: pK_m, −1.24 ± 0.10; log k_3 (pH 7.5—9.0), 0.62 ± 0.05; log (k_3/K_m) (pH 8.5—10.0), −0.94 ± 0.02, and PP_i: pK_m (pH 7.5—8.5), −1.11 ± 0.22; pK_m (pH 8.5—9.5), −0.59 ± 0.01; log k_3 (pH 7.5—8.5), 0.70 ± 0.03; log k_3 (pH 8.5—9.5), −0.73 ± 0.26; log (k_3/K_m) (pH 7.5—8.5), −0.49 ± 0.01; log (k_3/K_m) (pH 8.5—9.5), −1.32 ± 0.25. The term k_3 is V/E_0, where E_0 is the molar concentration of active sites and V, the maximum velocity, is expressed as M/s substrate hydrolyzed. The values of M_r are taken as 141 000 and there are eight identical subunits/mol enzyme, each of which is assumed to contain an active site [1].

phosphatase activity was followed for 3 h at 45°C and was found to decrease with time in essentially identical patterns (cf. Fig. 3).

Inhibitor studies

Zn^{2+} and levamisole were found to be the strongest inhibitors and were of the non-competitive type (cf. Table II). Fig. 4 illustrates the non-competitive inhibition pattern for levamisole.

EDTA, EGTA and L-cysteine are potent irreversible inhibitors of the alkaline phosphatase (cf. Table III). Ethane-1-hydroxydiphosphonate and dichloromethanediphosphonate are also irreversible inhibitors of this enzyme, presumably on the basis of their ability to chelate metal ions. *N*-Ethylmaleimide and

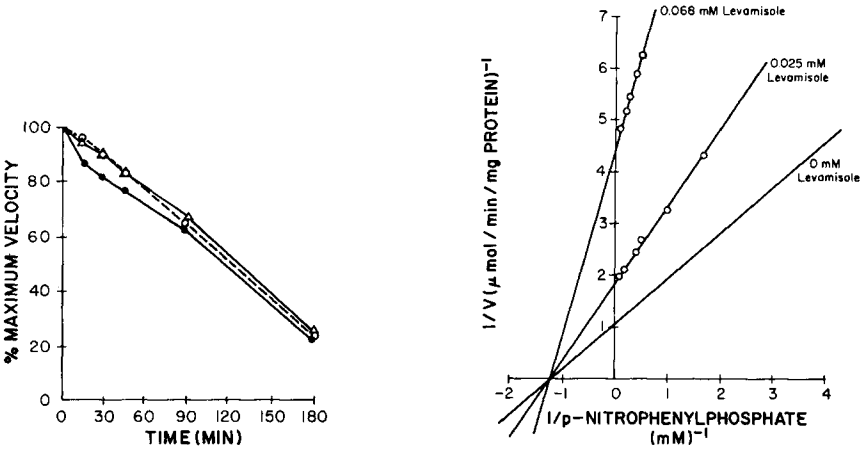

Fig. 3. Heat inactivation profile for: ○- - - - -○, the chondrocytic alkaline phosphatase; △———△, inorganic pyrophosphatase and ●———●, ATPase activities. Activity is expressed as a percentage of the initial activity present before incubation at 45° C.

Fig. 4. The inhibition of chondrocyte alkaline phosphatase by levamisole. Assays were carried out according to the method outlined in Table II.

iodoacetamide at 10 mM concentrations show only a small reduction in the alkaline phosphatase activity after 30 min.

Effect of cations

The effects of mono- and divalent cations on the chondrocytic alkaline phosphatase activity were examined at pH 10.5 using *p*-nitrophenyl phosphate as substrate. Fe^{2+}, Ba^{2+} and Ca^{2+} show neither stimulatory nor inhibitory effects in the mM range. Mn^{2+} at 0.1 mM and Mg^{2+} at 5.0 mM give 30% and 15% increases in activity, respectively. No further enhancement of the Mg^{2+}-stimulating effect by Ca^{2+} is observed. Na^{+} and K^{+} at concentrations of 10 mM activate the phosphomonoesterase activity, 11% and 18%, respectively. Higher concentrations (0.5—1.0 M) inhibit the enzyme approx. 40%.

TABLE II

REVERSIBLE INHIBITORS OF CHONDROCYTIC ALKALINE PHOSPHATASE

Alkaline phosphatase activity in the presence of reversible inhibitors was examined at pH 10.5 utilizing *p*-nitrophenyl phosphate as substrate. Apparent Michaelis constants and maximum velocities were obtained from estimates of initial velocities at six to eight different substrate concentrations and fixed inhibitor concentration. The data were analyzed by weighted least-squares analyses of Lineweaver-Burk transformations of the data. Errors in the estimation of the inhibition constants range from 8 to 12%.

Inhibitor	Type	Inhibitor constant (mM)
Inorganic phosphate	Competitive	10.0
Levamisole	Non-competitive	0.05
Sodium cyanide	Non-competitive	0.9
Zinc chloride	Non-competitive	0.04
L-Phenylalanine	Uncompetitive	23.0

TABLE III

EFFECT OF IRREVERSIBLE INHIBITORS ON THE CHONDROCYTE ALKALINE PHOSPHATASE

Residual enzymatic activity was measured after preincubation with the inhibitor for the indicated times followed by dialysis against inhibitor-free buffer for 24 h at 4°C. Activity measurements were carried out at pH 10.5 using *p*-nitrophenyl phosphate as substrate.

Inhibitor	Concentration (mM)	Incubation period (min)	% residual activity
EDTA	1.0	15	1.0
EGTA	1.0	15	1.0
Ethane-1-hydroxydiphosphonate	1.0	30	19.8
Dichloromethanediphosphonate	0.9	30	42.2
L-Cysteine	3.0	30	6.6
$BeSO_4$	0.1	15	42.2
Phenylmethylsulfonyl fluoride	1.0	180	67.7
N-Ethylmaleimide	10.0	30	80.7
Iodoacetamide	10.0	30	74.0

ATPase and inorganic pyrophosphatase activities

Inhibition by excess substrate is demonstrated by both ATP and PP_i and is shown in Fig. 5. K_m and V values were determined by extrapolation from the linear portion of the relationship and are indicated in Table I. Substrate inhibition by both ATP and PP_i is observed at all pH values for which K_m and V were determined.

The effect of Mg^{2+} and Ca^{2+} concentrations of 0.1—10 mM on the inorganic

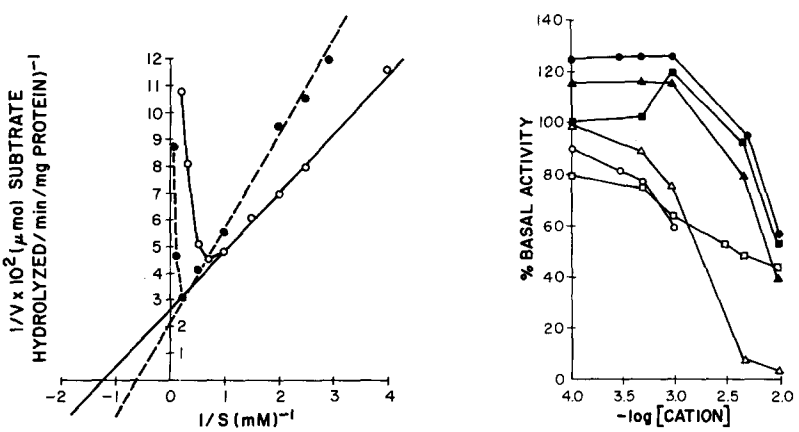

Fig. 5. Lineweaver-Burk plots of $1/v$ vs. $1/S$ for the hydrolysis of ATP at pH 10.0 (○———○) and PP_i at pH 8.5 (●- - - - -●), by the purified chondrocytic alkaline phosphatase.

Fig. 6. The effect of the divalent cations, Mg^{2+} and Ca^{2+}, on the chondrocyte-associated pyrophosphatase activity at pH 8.5 and the ATPase activity at pH 10.0 and 7.5. The curves illustrated are: ●———●, Mg^{2+}-ATPase, pH 10.0; ▲———▲, Mg^{2+}-inorganic pyrophosphate, pH 8.5; ■———■, Mg^{2+}-ATPase, pH 7.5; ○———○, Ca^{2+}-ATPase, pH 10.0; △———△, Ca^{2+}-inorganic pyrophosphatase, pH 8.5, and □———□, Ca^{2+}-ATPase, pH 7.5. Activities are expressed as percentages of basal levels defined as that activity present in the absence of any added cations. The following substrate concentrations were used: ATPase (pH 10), 5 mM ATP; inorganic pyrophosphatase (pH 8.5), 1 mM PP_i; ATPase (pH 7.5), 200 μM ATP.

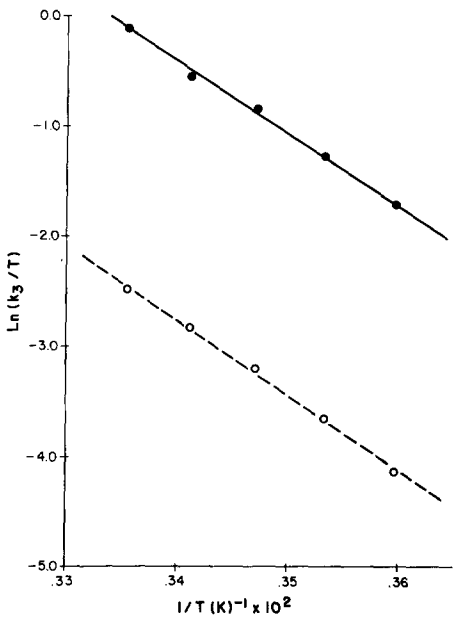

Fig. 7. The effect of temperature on V for the hydrolysis of p-nitrophenyl phosphate (●——●) and 5′-AMP, (○- - - - -○) by the chondrocyte alkaline phosphatase. ln (k_3/T) is plotted versus $1/T$ in the transition-state plot. The value of k_3 in units of s^{-1} is defined as the mol of substrate hydrolyzed/s per mol of active sites. The M_r equals 141 000 [1] and the enzyme is assumed to contain eight identical subunits, each of which contains a functioning active site.

pyrophosphatase activity at pH 8.5 and ATPase activity at pH 10.0 and 7.5 was examined and the results are graphed in Fig. 6. Mg^{2+} between 0.1 mM and 1 mM stimulates the inorganic pyrophosphatase activity 16%. At higher concentrations, inhibition results. Ca^{2+} shows almost complete inhibition at 3 mM. No addition enhancement occurs when Ca^{2+} is added to the magnesium-stimulated enzyme. At pH 10 0.1—1 mM Mg^{2+} increase the ATPase activity 25%, while higher concentrations cause inhibition. Calcium exhibits no effect between 0.1 and 1.0 mM. At pH 7.5 (cf. Fig. 6) 1 mM Mg^{2+} stimulates the ATPase activity to 120% of basal levels. At higher concentrations, inhibition occurs. Ca^{2+} causes no effect between 0.1 and 10 mM while inhibiting the activity to 50% of basal levels at a concentration of 10 mM. No (Ca^{2+} + Mg^{2+})-stimulated ATPase activity exists at either pH 7.5 or 10.0.

Arrhenius studies

The temperature dependence of V at pH 10.5 using p-nitrophenyl phosphate and 5′-AMP as substrates appears in Fig. 7. Enthalpies of activation for p-nitrophenyl phosphate and 5′-AMP are 12 840 ± 770 and 13 530 ± 810 cal/mol, respectively. Values for the entropy of activation are —4.37 ± 0.09 and —6.67 ± 0.13 cal/degree per mol, respectively.

Discussion

V values for all substrates except p-nitrophenyl phosphate and 2-phosphoglycerate are essentially similar, comparable to what is observed for *Escherichia*

coli [9] and mammalian alkaline phosphatases [10] from other tissues. This implies that a common intermediate is transformed in the rate-determining step, although evidence against this concept has been reported [11]. K_m values increase in the order 2′-NMP < 3′-NMP < 5′-NMP. K_m values for the nucleoside monophosphates are all comparable within their respective isomer series with the exception of 3′- and 5′-AMP. This suggests that the structure of the base component is not important, but that the position of the phosphate on the ribose or deoxyribose ring can influence the binding and phosphorylation steps in the overall kinetic mechanism.

ADP, UDP, ATP and PP_i have K_m values below 6 mM at the pH optimum in contrast to many phosphomonoesters which exhibit K_m values in excess of 20 mM. These effects may be due to either an increased binding as a result of additional negative charge in the region of the phosphate groups or to a greater relative ease of phosphorylation of the enzyme by the enzyme substrate complex. Non-nucleosidic phosphomonoesters exhibit elevated K_m values suggesting an R group binding site favoring the adsorption of aromatic, non-polar residues.

Phosphatase I enzyme of Arsenis et al. [4] from rabbit callus cartilage is considered derived from chondrocytes and compared directly to the bovine fetal chondrocyte alkaline phosphatase. V for *p*-nitrophenyl phosphate for the rabbit enzyme is similar to that for other substrates, whereas, in the bovine fetal enzyme, V is ten-fold greater than that observed for other substrates. Substrate inhibition was not observed with *p*-nitrophenyl phosphate in this study. Inhibition with this substrate has been observed in other mammalian alkaline phosphatases [4] at concentrations up to 75 mM.

Above pH 10.6, the chondrocyte alkaline phosphatase is irreversibly and rapidly denatured. This contrasts with the findings of Gottlieb and Sussman [12] who showed that human placental phosphatase is fully active toward *p*-nitrophenyl phosphate at pH 11.5.

The pK_m vs. pH curves for *p*-nitrophenyl phosphate and ATP (cf. Fig. 2) indicate that one or more groups with an apparent pK_a of 7.75 are important in the binding and/or phosphorylation steps suggesting the involvement of a hydrated zinc complex [13,14]. The sensitivity of the enzymatic activity to EDTA and EGTA and the resultant structural instability which leaves the enzyme irreversibly denatured support the contention that a divalent metal ion is involved in maintaining the structural and enzymatic integrity of the chondrocyte alkaline phosphatase. The pK_m vs. pH plots in Fig. 3 show that the slopes for ATP and *p*-nitrophenyl phosphate, between pH 8.0 and 10.0, have comparable non-integer values of approximately −1.2 suggesting that a common group or groups are involved in binding and/or phosphorylation. The log k_3 vs. pH curves for ATP and *p*-nitrophenyl phosphate give positive non-integer slopes (approx. 0.60) in the range of pH 7.5—9.5. The change in slope observed near pH 9.5—10.0 in these curves may reflect the requirement of a basic group in the rate-determining step. This group may be the conjugate base of the serine side chain which becomes phosphorylated in the catalytic mechanism.

The more precipitous reduction in the K_m value for *p*-nitrophenyl phosphate compared to that for V upon lowering the pH accounts for the fact that the

enzyme is a more efficient catalyst at pH 7.5 than at 10.5 at low values of the substrate concentration. Concentrations of *p*-nitrophenyl phosphate below 24 μM and ATP below 33 μM would be hydrolyzed with greater overall velocity at pH 7.5 than at pH 10.5 and 10.0, respectively. The pH of bovine fetal matrix fluid is near 7.5 and the intramatrix concentrations of ATP, PP_i and phosphomonoesters are probably in the μM range [15] and, therefore, the chondrocytic alkaline phosphatase may be ideally suited to a role in which it maintains the intramatrix concentration of inorganic phosphate.

The bovine chondrocyte alkaline phosphatase is typical of other mammalian alkaline phosphatases in that its monoesterase activity is only slightly stimulated by Mg^{2+} and Mn^{2+}, not being effected by Ca^{2+} or a combination of Ca^{2+} and Mg^{2+} [16]. Arsenis et al. [4] found a different response of their phosphatase I preparation to Mg^{2+} and Ca^{2+}. They observed a 25-fold stimulation of activity in the presence of 1 mM Mg^{2+}, whereas the bovine chondrocyte enzyme demonstrates only a 7% increase in activity at 1 mM Mg^{2+}. Ca^{2+} at 10 mM is capable of stimulating phosphatase I from rabbit callus cartilage threefold. There is no enhanced ($Ca^{2+} + Mg^{2+}$) activity in the rabbit or bovine chondrocyte enzyme.

Heat inactivation studies suggest that the same active site is responsible for the hydrolysis of *p*-nitrophenyl phosphate, ATP and PP_i. The similarity of V values for ATP, PP_i and nucleoside and non-nucleoside phosphomonoesters also indicates a common site.

The uncompetitive inhibition by L-phenylalanine suggests that decomposition of the phosphoryl enzyme may be rate determining. This has been interpreted to imply that bound L-phenylalanine blocks the acceptor site for hydrolysis of the phosphorylated enzyme [17]. Non-competitive inhibition by such ligands as Zn^{2+}, CN^- and levamisole, in which K_m remains unaltered, suggests that in these cases phosphorylation of the enzyme by the enzyme-substrate complex is rate determining. Levamisole and Zn^{2+} are among the most potent inhibitors of the chondrocytic alkaline phosphatase. CN^- is known to form complexes with transition metal ions. Inhibition by CN^- is consistent with the inhibition by divalent metal ion-chelating agents such as EDTA and EGTA. P_i, a competitive inhibitor, possesses a K_i value similar to K_m values for low K_m phosphomonoester substrates.

The first five irreversible inhibitors of Table III undoubtedly exert their effects through divalent metal ion chelation. Attempts to reactivate the enzyme by adding back Zn^{2+}, Ca^{2+}, or Mg^{2+}, following dialysis to remove excess EDTA were unsuccessful, contrary to observations by Harkness [10] for the crystalline placental phosphatase. The inability to restore activity to the chondrocyte enzyme suggests that a divalent metal ion is required for the structural integrity of the protein. The inhibition by *N*-ethylmaleimide and also by iodoacetamide suggests that a slowly reacting SH-group may be involved in either maintenance of structure or the catalytic mechanism directly. The very slow inactivating reaction of phenylmethylsulfonyl fluoride with chondrocyte alkaline phosphatase is reminescent of the reactivity of diisopropylphosphonylfluoridate with other mammalian phosphatase enzymes [18,19] in that, in contrast to serine proteases, the reactive serine which is phosphorylated is relatively unresponsive to these reagents.

$\Delta G^{\ddagger}$ for 5′-AMP is 15 520 cal/mol, some 1380 cal/mol greater than that for the substrate, *p*-nitrophenyl phosphate. This difference is sufficient to account for the ten-fold variation in k_3 values for *p*-nitrophenyl phosphate, 263.8 s^{-1} and 5′-AMP, 25.8 s^{-1}. One-half of the difference, 690 cal/mol, is attributable to the lower enthalpy of activation for *p*-nitrophenyl phosphate. This is consistent with the idea that *p*-nitrophenyl phosphate is an inherently less stable substrate. The entropy of activation is more negative by 2.3 cal/degree per mol for 5′-AMP. This unfavorable entropy term may arise due to steric crowding in the transition-state relative to the ground state. The greater negative $\Delta S^{\ddagger}$ for 5′-AMP relative to *p*-nitrophenyl phosphate would then be associated with its greater steric bulk and the lack of accomodation in the transition state of the adenosine moiety compared to the *p*-nitrophenyl group.

The inhibition of the ATPase and inorganic pyrophosphatase activity by excess substrate correlates with the observation that high concentrations of PP_i and ATP tend to inhibit calcification whereas lower concentrations tend to activate the process [20,21]. It suggests strongly that these activating and inhibitory effects may be mediated directly in the calcifying cartilage by the chondrocyte enzyme, thus providing a direct role for these enzymes in calcification [20].

The effect of divalent cations on the ATPase and inorganic pyrophosphatase activity of the chondrocyte alkaline phosphatase is complicated by several factors. Both Mg^{2+} and Ca^{2+} complex the substrates and could cause inhibition by reducing the concentration of the true substrates, free ATP and PP_i. Secondly, because both ATP and PP_i exhibit strong substrate inhibition at high concentrations, an artifactual stimulation of enzyme activity might be observed if the initial ATPand PP_i concentrations were in the inhibitory range and the added Mg^{2+} and Ca^{2+} complexed the substrates. To insure that the later possibility could not arise, substrate concentrations were carefully selected in the non-inhibitory range.

In conclusion, chondrocytic alkaline phosphatase from bovine fetal epiphyseal cartilage exhibits many characteristics typical of mammalian alkaline phosphatases with a few notable exceptions such as the irreversible alkaline and EDTA denaturations. Unlike matrix vesicles, chondrocytes do not calcify, and although chondrocyte and matrix vesicle phosphatases are derived, at least in part, from the same source [1,22], a different biological role for the chondrocyte enzyme may be rationalized other than that proposed for the enzyme isolated from matrix vesicles [22]. Because of its wide specificity and high efficiency at physiological pH values, chondrocytic alkaline phosphatase may serve to enrich the intramatrix fluid with inorganic phosphate at the expense of organic phosphate esters such as 5′-AMP. An additional role may involve destruction of excess ATP and PP_i in the matrix thus preventing buildup of concentrations of these compounds sufficient to block calcification [23].

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