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ALKALINE PHOSPHATASE OF CHICKEN LIVER MICROSOMES

II. ACTIVITY WITH AMP AND OTHER SUBSTRATES

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SUMMARY

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) was purified approx. 430-fold from the microsomal fraction of chicken liver. During the course of purification, 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity and alkaline phosphatase activity were not separated from each other.

The hydrolysis of phenylphosphate by the purified enzyme preparation was competitively inhibited by AMP.

Adenine and cytosine mononucleotides were more active substrates of this enzyme than the other nucleotides tested. ADP and ATP were also suitable substrates of this enzyme and hydrolyzed most rapidly at 1 mM Mg^{2+}. Mixed substrate experiments suggested that alkaline phosphatase had organic pyrophosphatase activity.

INTRODUCTION

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) and 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) of animal liver are known as membrane-bound enzymes and the latter is used as a common marker for hepatic plasma membrane during cell fractionation[1,2].

In our previous paper[3], we assumed that 5'-nucleotidase was not present in a significant amount, if it existed in the microsomal fraction of chicken liver, and that alkaline phosphatase played a relatively important role in the dephosphorylation of nucleotides.

Substrate specificity of alkaline phosphatase has been extensively studied indicating that various phosphomonoesters can be substrates for this enzyme. However, there have only been few studies on the hydrolysis of nucleotides by the alkaline phosphatase of animal livers[4].

This paper reports further studies on the kinetic properties of the purified alkaline phosphatase with special reference to substrate specificity. In the present

experiments, it was confirmed that adenine and cytosine nucleotides were active substrates among the nucleotides tested and that phenylphosphate and AMP were hydrolyzed at the same active site. The organic pyrophosphatase activity of this enzyme is also described.

MATERIALS

TEAE-cellulose was purchased from Serva Entwicklungslabor and Sepharose 6B was from Pharmacia Fine Chemicals. Disodium phenylphosphate and sodium β-glycerophosphate were obtained from E. Merck, the sodium salt of the nucleotides and Tris were from the Sigma Chemical Co. All other chemicals were of reagent grade or of the highest quality available.

METHODS

Preparation of alkaline phosphatase

Chicken liver microsomes and purified enzyme were prepared according to the methods previously described[3] with minor modifications. Briefly, 1 vol. of *n*-butanol was added to 2 vol. of microsomal suspensions and incubated at 37 °C for 10 min. After centrifugation at 17 000 × *g* for 20 min, the aqueous phase was collected and fractionated with acetone at 60% and $(NH_4)_2SO_4$ between 0.3 and 0.6 saturation. The enzyme preparation was further purified on a TEAE-cellulose column with a linear gradient of 0.0-0.4 M NaCl and gel filtration on a column (2.5 cm × 40 cm) of Sepharose 6B. A summary of the purification is shown in Table I. Starting from the microsomes a purification of about 430-fold was obtained. About 3-fold higher purification was achieved as compared with the method previously described. The recovery of the initial activity was 19%. In each purification step, the recoveries of 5′-nucleotidase and alkaline phosphatase activities were similar and the ratio of the two activities was constant as reported in the preceding paper[3].

Assay of enzyme activity

Unless otherwise stated, the standard assay conditions were as previously described. The assay mixture for 5′-nucleotidase activity in a total volume of 1.0 m contained: 15 mM AMP, 10 mM $MgCl_2$, 100 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.5)

TABLE I

PURIFICATION OF ALKALINE PHOSPHATASE FROM CHICKEN LIVER MICROSOMES

Steps	*Total protein (mg)*	*Total activity (units × 10⁻²)*		*Specific activity (units/mg protein)*		*Ratio of activity*
		AMP	*Phenylphosphate*	*AMP*	*Phenylphosphate*	
Microsomes	25 000	1150	3090	4.6	12.4	2.69
n-Butanol extract	1 570	644	1732	41	110	2.69
Acetone ppt (0–60%)	555	635	1680	114	302	2.65
$(NH_4)_2SO_4$ ppt (0.3–0.6 satn)	327	597	1630	183	498	2.73
TEAE cellulose	66	351	945	536	1442	2.69
Sepharose 6B	11.2	225	591	2010	5280	2.63

and enzyme preparation. The assay mixture for alkaline phosphatase activity contained 45 mM disodium phenylphosphate, 10 mM $MgCl_2$, 100 mM Na_2CO_3–$NaHCO_3$ buffer (pH 10.0) and enzyme in a total volume of 1.0 ml. The inorganic phosphate released was determined by the method of Fiske and SubbaRow[5] and of Chen *et al.*[6]. The liberated phenol was measured by a modification of the King–Armstrong method by Chang and Moog[7]. A unit of enzyme is defined as the amount of enzyme which hydrolyzed 1 μmole of phosphate ester per h and specific activity is estimated in units per mg of protein.

Protein determination

Protein was determined according to the method of Lowry *et al.*[8]. Crystalline bovine serum albumin was used as standard.

RESULTS

Effect of AMP on the hydrolysis of phenylphosphate

Although the experimental results reported previously suggested the existence, in the purified alkaline phosphatase preparation, of a single enzyme protein[3], a possibility that the purified enzyme, having a relatively large molecular size, existed as an enzyme complex of alkaline phosphatase and 5′-nucleotidase still remained to be excluded. In order to exclude this possibility we measured the hydrolysis of phenylphosphate in the presence of varying amounts of the AMP. If both substrates were hydrolyzed by the same enzyme, then each should interfere with the hydrolysis of the other in a predictable manner on the basis of their apparent K_m values. As shown in Fig. 1, hydrolysis of phenylphosphate was competitively inhibited by AMP. For an inhibitor which is used as an alternate substrate by the same enzyme one would predict that the apparent K_m would be equal to the apparent K_i. The apparent K_i for AMP was found to be about 4.64 mM, virtually the same as the apparent K_m for AMP as measured in the absence of phenylphosphate.

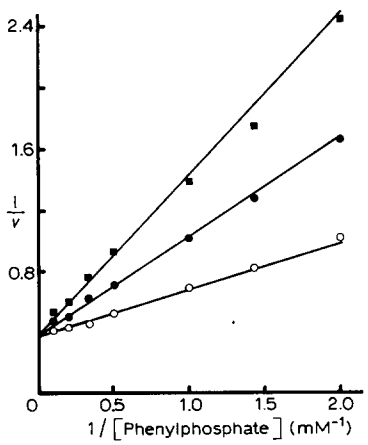

Fig. 1. Effect of AMP the on hydrolysis of phenylphosphate (Lineweaver–Burk plot). The reaction mixture (1 ml) contained 100 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.4), 10 mM $MgCl_2$, 0.5–10 mM phenylphosphate, 0, 5, and 10 mM AMP and enzyme preparation. After incubation for 5 min at 37 °C the amount of phenol formed was measured. ■, with 10 mM AMP; ●, with 5 mM AMP; ○, without AMP.

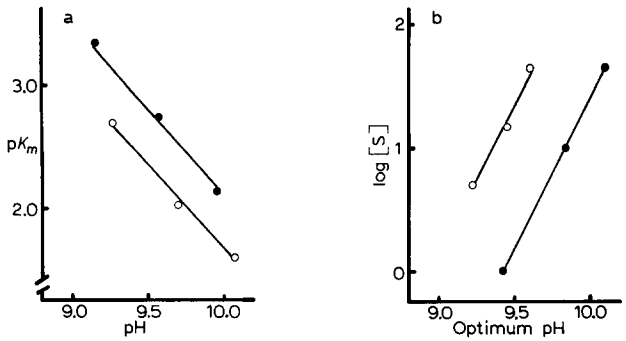

Fig. 2. Studies on optimum pH and apparent K_m. (a) Plots of pK_m against pH for the hydrolysis of AMP (○—○), and phenylphosphate (●—●). The reaction mixtuie contained 100 mM Na_2CO_3–$NaHCO_3$ buffer, 10 mM $MgCl_2$, various amounts of substrates and enzyme preparation. (b) Relationship between log of substrate concentration and optimum pH for hydrolysis of AMP (○—○) and phenylphosphate (●—●). The assay conditions are as described in (a). Ohter details were described in the text.

Optimum pH and apparent K_m

Plots of pK_m against pH and of log [S] against optimum pH for the alkaline phosphatase and 5′-nucleotidase activities are given in Fig. 2. The curves for alkaline phosphatase activity show the same slopes as the curves for 5′-nucleotidase activity over the effective pH range of Na_2CO_3–$NaHCO_3$ buffer. In the pH range tested, maximum and minimum K_m values were 25 mM at pH 10.07 and 2 mM at pH 9.25, respectively, for AMP, and 7.2 mM at pH 9.95 and 0.44 mM at pH 9.15 for phenylphosphate. Different buffer systems also affected the apparent K_m values. At pH 9.25, K_m values for AMP were 2 mM with Na_2CO_3–$NaHCO_3$, 0.36 mM with Tris–HCl and 0.34 mM with ammediol–HCl buffer. The concentration of each buffer was 100 mM. Fig. 2b shows that pH optima shift from 9.6 to 9.22 and from 10.1 to 9.42 with decreasing concentrations of AMP from 48 to 5.4 mM and of phenylphosphate from

TABLE II

SUBSTRATE SPECIFICITY AND MICHAELIS CONSTANTS OF ALKALINE PHOSPHATASE

All rates are referred to as relative to the hydrolysis of AMP which is taken as 100. The reaction mixture contained 100 mM Na_2CO_3–$NaHCO_3$ buffer, 10 mM $MgCl_2$, 10 mM substrate and enzyme preparation.

Substrate (10 mM)	*Optimum pH*	*K_m (mM)*	*Relative activity*
AMP	9.4	3.9	100
CMP	9.3	6.9	79
GMP	9.3	7.9	60
UMP	9.2	4.1	48
IMP	9.3	5.4	39
2′,(3′)-AMP	9.5		116
dAMP	9.3		100
dCMP	9.3		109
TMP	9.2	5.5	65
dGMP	9.2		60
Phenylphosphate	9.8	3.1	233
β-Glycerophosphate	9.4	5.7	111

TABLE III

RELATIVE RATE OF HYDROLYSIS OF ADENINE NUCLEOTIDES BY ALKALINE PHOSPHATASE AT VARIOUS CONCENTRATIONS OF Mg^{2+}

Relative rates are referred to hydrolysis of AMP (in the presence of 10 mM Mg^{2+})as 100. Other details are described in the text.

Nucleotide (10 mM)	*Optimum pH*	*Concentration of $MgCl_2$*		
		0 mM	*1 mM*	*10 mM*
AMP	9.4	23	96	100
ADP	9.4	22	98	87
ATP	9.2	4	19	14

45 to 1 mM, respectively. The apparent K_m and optimum pH of 5'-nucleotidase acivity were always higher and more acidic than those of alkaline phosphatase activity.

Substrate specificity

The relative rates of hydrolysis of the various mononucleotides at 10 mM concentration and apparent K_m values for these substrates were determined at respective optimum pH. As seen in Table II, adenine and cytosine nucleotides were hydrolyzed more rapidly than others and the purified enzyme exhibited similar affinity for those substrates tested. Inorganic and organic pyrophosphates were also suitable substrates of this enzyme. Inorganic pyrophosphatase activity was determined in the mixture containing 10 mM sodium pyrophosphate, 1 mM $MgCl_2$, 100 mM ammediol-HCl buffer and enzyme preparation. Optimum pH was at around pH 8·8 and at this pH, 10 mM phenylphosphate was hydrolyzed about 4-fold more rapidly than pyrophosphate in similar assay conditions. Table III shows the relative rates of hydrolysis of adenosine 5'-mono-, di- and triphosphate and the effect of Mg^{2+}. Each rate determination was made at optimum pH with 10 mM substrate concentration and 10 mM $MgCl_2$. AMP and ADP are hydrolyzed at a similar rate and more rapidly than ATP. With increasing concentration of Mg^{2+}, the rate of hydrolysis of AMP increased as mentioned previously[3]. Maximum organic pyrophosphatase activity was obtained with a 1 mM concentration of Mg^{2+}.

TABLE IV

HYDROLYSIS BY PURIFIED ENZYME PREPARATION OF PAIRS OF SUBSTRATES

The reaction mixture contained 100 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.3), 1 mM $MgCl_2$, each 5 mM substrate and enzyme preparation.

Nucleotides (each 5 mM)	*Observed (units)*	*Expected for independent hydrolysis (units)*
AMP	5.58	
ADP	6.52	
ATP	1.26	
AMP + ADP	6.58	12.10
AMP + ATP	3.08	6.84

Mixed substrate experiments

To obtain further evidence that alkaline phosphatase has organic pyrophosphatase activity, mixed substrate experiments were carried out in which the rate of dephosphorylation from a pair of substrates present together was compared with that of dephosphorylation from a single substrate. With a pair of substrates, AMP and ADP or AMP and ATP, the reaction rate was lower than the additive rate expected for independent hydrolysis of each substrate, indicating that the dephosphorylation of ADP and ATP is catalyzed by alkaline phosphatase (Table IV).

DISCUSSION

Alkaline phosphatase was purified approx. 430-fold from the microsomal fraction of chicken liver. About a 3-fold higher purification was achieved with the slightly modified procedure as compared with the method previously employed[3]. No crucial evidence, indicating the existence of two enzymes, 5′-nucleotidase and alkaline phosphatase, was obtained. From the experimental results with the purified enzyme preparation, it was confirmed that the hydrolysis of AMP and phenylphosphate was catalyzed at the same active site of the one enzyme.

Earlier kinetic studies of alkaline phosphatase by several workers have demonstrated the marked variation of apparent K_m with pH and also the pronounced shift of optimum pH to more acidic values with decreasing substrate concentration[9]. It was also the case reported here. For this reason, the relative rates of hydrolysis of various nucleotides were determined in the assay condition of 10 mM substrate and optimum pH at this substrate concentration. Adenine and cytosine nucleotides which have an amino group at Position 6 in the purine and pyrimidine bases are hydrolyzed at a faster rate than other nucleotides tested. This substrate specificity of the alkaline phosphatase from chicken liver microsomes is different from that of the human liver and placental alkaline phosphatase which hydrolyze UMP at similar rate to AMP[4,10]. Moreover, it is interesting that 5′-nucleotides having a keto group at Position 6 in the purine base, *e.g.* IMP, GMP and XMP, are the most active substrates of 5′-nucleotidase reported by Itoh *et al.*[11] which are located in the soluble fraction of chicken liver. 5′-Nucleotodase was assumed not to be present in a significant amount, if it was detected, in a microsomal fraction of chicken liver as mentioned in our earlier work[3]. It seems probable that this alkaline phosphatase catalyzes the hydrolysis of nucleotides at a more neutral pH, at which this enzyme has a low apparent K_m. There are few significant variations in the K_m values among the substrates tested. Alkaline phosphatase of chicken liver microsomes is also capable of hydrolyzing inorganic and organic pyrophosphates. Our results are in agreement with the observations made by Eaton and Moss[4] on human alkaline phosphatase. Although human liver alkaline phosphatase hydrolyzes ADP at a slower rate with 10 mM Mg^{2+} as compared with AMP[4], our enzyme preparation hydrolyzed ADP at almost the same rate as AMP. Such a result may be responsible for the relatively high concentration of substrate employed in the present experiment. Mixed substrate experiments also support the view that alkaline phosphatase has organic pyrophosphatase activity.

Two types of 5′-nucleotidase have been reported in the soluble and menbranous fraction of rat liver[1,2,12–14], whereas no crucial evidence showing the existence

of membrane-bound 5′-nucleotidase in chicken liver was obtained. We do not as yet know whether nucleotide phosphohydrolase activities of chicken liver microsomes are only due to alkaline phosphatase. Sanford and Rosenberg[15] have recently reported that the embryonic chicken liver plasma membrane did not exhibit significant 5′-nucleotidase activity nor alkaline phosphatase activity. The physiological significance of the difference in the distribution of 5′-nucleotidase in the subcellular fractions between uricotelic and ureotelic animals remains to be elucidated.

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