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NUCLEOTIDASE ACTIVITIES IN THE SOLUBLE FRACTION OF RAT LIVER HOMOGENATE

PARTIAL PURIFICATION AND PROPERTIES OF A 5'-NUCLEOTIDASE WITH pH OPTIMUM 6.3*

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SUMMARY

$(NH_4)_2SO_4$ fractionation of the nonsedimentable fraction of rat liver homogenates indicated that two 5'-nucleotidases (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) with pH optima around 6 were present. One of the enzymes had a broader specificity for 5'-nucleotides than the other, which acted mainly on dTMP and dUMP.

A procedure is described for purifying the former enzyme to the stage where it is essentially free of nonspecific phosphatases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.1 and EC 3.1.3.2). The method involves $(NH_4)_2SO_4$ fractionation and precipitation by dialysis at pH 6.0. Chromatography on DEAE-cellulose and partial heat inactivation indicated that only one 5'-nucleotidase was present in the preparation. The enzyme had a pH optimum at 6.3, it was Mg^{2+} dependent, and had a lower K_m value for IMP (K_m = 0.2 mM) than for any of the other nucleotides tested. Evidence is presented indicating that the enzyme is identical with the 5'-nucleotidase isolated recently from rat liver acetone powder by ITOH, MITSUI AND TSUSHIMA (*J. Biochem. Tokyo*, 63 (1968) 165). The enzyme is localized in the soluble cell cytoplasm and has properties different from the 5'-nucleotidases detected previously in particulate structures.

INTRODUCTION

Our search for nucleotidases with a pH optimum at around 6 (ref. 1) in the soluble fraction of rat liver homogenates led to the detection of a specific 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) which had not been recorded previously in the literature. Some properties of the enzyme were described in the preliminary note[1] referred to above. In the present communication further information concerning the purification and properties of the enzyme is presented.

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When these studies were in progress ITOH *et al.*[2] reported the isolation from rat liver acetone powder of a 5′-nucleotidase with an optimum pH at 6.5. The present work clearly indicates that this enzyme is identical with our 5′-nucleotidase.

MATERIALS AND METHODS

Chemicals

All the nucleotides and β-glycerophosphate were purchased from Sigma Chemical Co.

DEAE-cellulose, a generous gift from Dr. B. Haber, City of Hope Medical Center, Calif., was washed thoroughly with 0.2 M methanolic KOH, 0.2 M methanolic HCl and distilled water before use.

All chemicals not specified were commercial products of the highest purity available.

Preparation of soluble fraction

Livers from 6–7 female rats were homogenized for 0.5 min in 0.25 M sucrose with a Teflon–glass homogenizer, and the 105 000 × *g* supernatant (soluble fraction) was prepared as described previously[3]. Aliquots were stored at −20° for analysis of protein and enzyme activity. No significant change in the phosphohydrolase activities could be detected after 1 week in the frozen state. The bulk of the supernatant was used immediately for enzyme purification, as described in the text.

Enzyme assay

Unless otherwise stated, the standard assay mixture for the nucleotidase contained in a total volume of 0.5 ml: 0.01 M substrate, 0.1 M $MgCl_2$, 0.05 M Tris–maleate buffer (pH 6.3) and enzyme preparation. After 30 min of incubation at 37° the reaction was stopped by adding 0.25 ml of 25% (w/v) trichloroacetic acid. Inorganic phosphate released was determined by the method of FISKE AND SUBBAROW[4].

Assay conditions optimal for dTMP dephosphorylation by the soluble fraction of liver[3] have been used in some of the experiments. The procedure is similar to that described above except that incubation was carried out for 20 min with 0.02 M substrate, 0.05 M $MgCl_2$ and at pH 6.0.

Assay of protein

Protein was determined according to the method of LOWRY *et al.*[5]. Crystalline bovine serum albumin was used as the standard.

RESULTS

Distribution of 5′-nucleotidase activities in different fractions of the nonsedimentable liver supernatant

The soluble fraction of liver (50 ml) was diluted with 20 ml of 0.1 M Tris–maleate buffer (pH 6.3) and 30 ml of water to give a final concentration of about 10 mg of protein per ml. The solution was brought to 30% satn. with $(NH_4)_2SO_4$ by dropwise addition of 42.9 ml of saturated $(NH_4)_2SO_4$ solution with stirring over a 15-min period, followed by a further 10-min period before the precipitate was collected

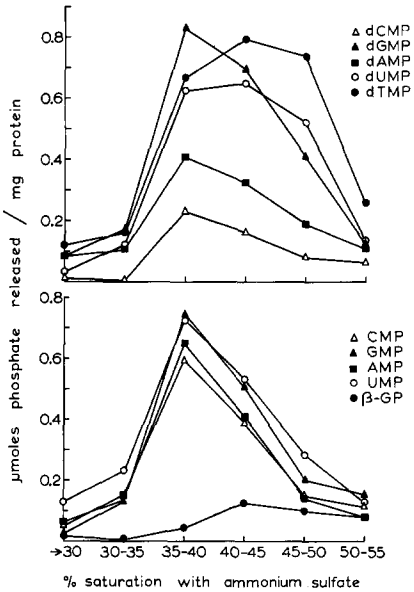


Fig. 1. Distribution of 5′-nucleotidase activities in preparations obtained by $(NH_4)_2SO_4$ fractionation of the soluble fraction of liver. Fractionation was carried out with saturated (at 0°) $(NH_4)_2SO_4$ solution adjusted to pH 6.3 with NH_4OH and containing 2 mM EDTA. Substrate dephosphorylation was measured under conditions optimal for dTMP dephosphorylation by the soluble fraction of liver. β-GP, β-glycerophosphate.

by centrifugation. Five additional protein fractions were prepared from the supernatant by increasing the degree of $(NH_4)_2SO_4$ saturation stepwise by 5% from 30% up to 55% by successive additions of the proper amount of saturated $(NH_4)_2SO_4$ solution. The precipitate formed in each step was collected by centrifugation, dissolved in 0.005 M Tris–HCl buffer (pH 8.8) containing 0.001 M EDTA and 0.001 M β-mercaptoethanol, and dialyzed overnight against the same buffer. The dephosphorylating activities of the dialyzed preparations were measured with a series of ribo- and deoxyribonucleotides and with β-glycerophosphate as substrates. The results are recorded in Fig. 1. The profile of the curves indicates that the ribonucleotides were dephosphorylated by an enzyme which precipitated mainly at 35–40% satn. with $(NH_4)_2SO_4$. It can be further seen that the enzyme was also active towards the deoxyribonucleotides. Apparently, the enzyme is a 5′-nucleotidase, since the acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) activity, measured with β-glycerophosphate as substrate, was small and showed no peak in this range. Moreover, only small activities similar to those of the acid phosphatase were found with 2′- and 3′-ribonucleotides as substrates (not shown).

The profile of the curves for dTMP and dUMP dephosphorylation indicates that these nucleotides were also dephosphorylated by an enzyme which precipitates at 40–50% saturation with $(NH_4)_2SO_4$. The lack of a corresponding profile of the curves obtained with the other nucleotides suggests that the enzyme is largely specific for dTMP and dUMP. The purification and properties of this enzyme, which may be termed a deoxythymidylate 5′-nucleotidase, will be reported in a separate communication.

Purification of 5'-nucleotidase

The enzyme was purified to the stage where it was essentially free of β-glycerophosphatase activity. All operations were carried out at 0–4°. The saturated $(NH_4)_2SO_4$ solution used was as described in Fig. 1. Centrifugations were carried out at 10 000 × *g* for 10 min. The steps of purification are summarized in Table I.

TABLE I

PURIFICATION OF 5'-NUCLEOTIDASE

Enzyme activities were measured at pH 6.0 under the conditions optimal for dTMP dephosphorylation by the soluble fraction of liver. UMP was used as substrate for the 5'-nucleotidase. 1 unit of enzyme is defined as the amount which catalyzes the dephosphorylation of 1 μmole of substrate per 20 min. β-GP, β-glycerophosphate.

Fraction	*Protein (mg/ml)*	*Total activity for UMP (units)*	*Specific activity (units/mg protein) for*		
			UMP	*dTMP*	*β-GP*
I. Soluble fraction of liver	23.5	623	0.27	0.32	0.049
II. $(NH_4)_2SO_4$, 34–40% satn.	13.9	125	0.80	0.65	0.044
III. Precipitate, dialysis at pH 6	3.7	81	1.72	1.23	0.016
IV. Soluble part, dialysis at pH 6	2.9	19	0.52	1.02	0.190

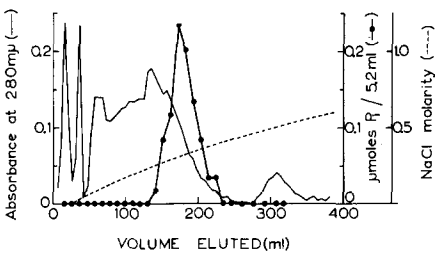
$(NH_4)_2SO_4$ fractionation. The soluble fraction of liver (100 ml) was diluted with 40 ml of 0.1 M Tris–maleate buffer (pH 6.3) and 60 ml of water and brought to 34% satn. with $(NH_4)_2SO_4$ by dropwise addition of 103 ml of saturated $(NH_4)_2SO_4$ solution with stirring over a 15-min period followed by a further 10-min period before the precipitate was removed by centrifugation. The supernatant solution (298 ml) was brought to 40% satn. by the addition of 29.8 ml of $(NH_4)_2SO_4$ in the same manner. The precipitate was collected by centrifugation and dissolved in 0.005 M Tris–maleate buffer (pH 6.0) containing 0.001 M EDTA and 0.001 M β-mercaptoethanol (Fraction II, 11.3 ml).

Dialysis at pH 6. Fraction II (10 ml) was dialyzed for 18 h against 2 l of the above buffer. The precipitate formed was collected by centrifugation and dissolved in 0.005 M Tris–HCl buffer (pH 8.8) containing 0.001 M EDTA and 0.001 M β-mercaptoethanol (Fraction III, 11.3 ml). Table I shows that the 5'-nucleotidase, measured with UMP as substrate, accumulated in the precipitate. Different preparations have shown specific activities ranging from 1.81 to 1.03. The β-glycerophosphatase activity was negligible. The enzyme preparation was used to determine properties of the 5'-nucleotidase. It could be kept for 1 week at either 0° or −20° without loss of activity.

dTMP phosphohydrolase activity. The enzyme activities of the soluble part of the dialyzed sample (Fraction IV) are included in Table I for comparison. It can be seen that the β-glycerophosphatase activity accumulated in this fraction. The fact that the preparation had a 5 times higher activity with dTMP as substrate reflects the presence of the deoxythymidylate 5'-nucleotidase. Fig. 1 indicates that this enzyme may be prepared advantageously from the enzyme fraction which precipitates at 39–49% satn. with $(NH_4)_2SO_4$.

Purity of the 5'-nucleotidase

Table I shows that the enzyme preparation (Fraction III) was essentially free of β-glycerophosphatase activity. The fact that the activity above pH 8.5 was negligible (Fig. 3) indicates that alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) and nucleotidases with alkaline pH optimum were not present in the enzyme preparation to a significant extent. Chromatography of the preparation on DEAE-cellulose (Fig. 2) showed that the activity towards UMP was eluted as a single peak, indicating that UMP was dephosphorylated by only one enzyme. Moreover, the eluted enzyme, which had a 4-fold higher specific activity



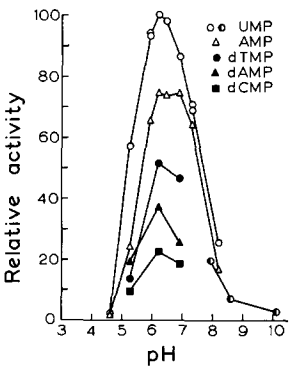


Fig. 2. Chromatography on DEAE-cellulose. Fraction III (9 ml) was applied to a DEAE-cellulose column (1.5 cm × 15 cm) equilibrated with 0.005 M Tris–HCl buffer (pH 7.5) containing 0.001 M EDTA and 0.001 M β-mercaptoethanol. Elution was carried out at a rate of 0.5 ml/min with the same buffer containing an NaCl concentration gradient. The gradient was set up with 400 ml of buffer in the mixing vessel (constant volume) and 1 M NaCl, dissolved in the same buffer, in the other container. Effluent fractions of 5.2 ml were collected and analyzed for protein and 5'-nucleotidase activity with UMP as substrate.

Fig. 3. Effect of pH on the activity of 5'-nucleotidase. Activity was measured under standard assay conditions, except that pH was varied as indicated. pH was adjusted to the desired value with NaOH and was measured at 20° in the complete reaction mixture. ◐, glycine buffer.

with UMP as substrate than the preparation (Fraction III) applied to the column, had practically unchanged relative activities towards dGMP, UMP, and dTMP (100, 83, and 39, respectively, as compared to 100, 83, and 43 for Fraction III). Partial inactivation of the enzyme preparation by heat treatment at 50° for various periods of time further showed that the activities towards UMP and dTMP had a parallel sensitivity to heat, providing additional support for the suggestion that only one enzyme was responsible for the dephosphorylation of the two substrates. On the basis of these findings the enzyme preparation (Fraction III) was considered to be sufficiently pure for characterization of the 5'-nucleotidase.

Properties of the 5'-nucleotidase

Effect of pH. Fig. 3 shows that the enzyme has a sharp pH optimum at 6.2 with UMP, dTMP, dAMP or dCMP as substrate. With AMP the activity has an optimum between pH 6.2 and 6.9.

Effect of Mg^{2+}. Fig. 4 shows that the enzyme was completely inactive in the

TABLE II

SUBSTRATE SPECIFICITIES OF 5′-NUCLEOTIDASE

Relative enzyme activities were measured under standard assay conditions. The dephosphorylation of 5′-dGMP was assigned a value of 100; other substrate activities are given relative to that of 5′-dGMP. The K_m values were determined from Lineweaver–Burk plots (Fig. 5) of the reaction velocities at varied substrate concentrations. The substrate concentration ranges used were 0.25–2.0 mM for IMP, GMP and dGMP; 2.5–20 mM for UMP, AMP, CMP, and dUMP; 5–40 mM for dTMP and dAMP; and 10–80 mM for dCMP. Incubations were carried out in duplicate for 4 and 8 min with IMP, GMP and dGMP, and for 7.5 and 15 min with the other nucleotides. The amount of enzyme added was adjusted so that less than 25% of the substrates was dephosphorylated. Under these conditions the phosphate released was proportional to time over the assay period.

Substrate	*Relative activity*	K_m *(mM)*
5′-dGMP	100	0.41
5′-GMP	97	0.30
5′-IMP	94	0.21
5′-UMP	83	2.2
5′-AMP	62	6.8
5′-CMP	59	4.6
5′-dUMP	57	6.3
5′-dTMP	43	9.4
5′-dAMP	31	12.2
5′-dCMP	19	29.7
2′-, 3′-UMP*	1.5	
2′-, 3′-AMP*	1.0	
2′-, 3′-GMP*	0.8	
2′-, 3′-CMP*	0.3	
Ribose 5-phosphate	2.4	
β-Glycerophosphate	0.1	

* Mixed isomers.

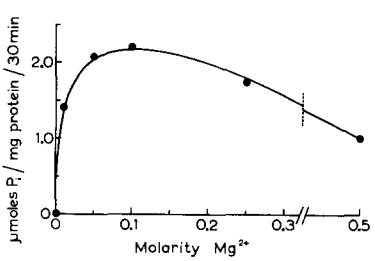


Fig. 4. Effect of Mg^{2+} on the activity of 5′-nucleotidase. Enzyme activity was measured with UMP as substrate under standard assay conditions, except that the concentration of $MgCl_2$ was varied as indicated.

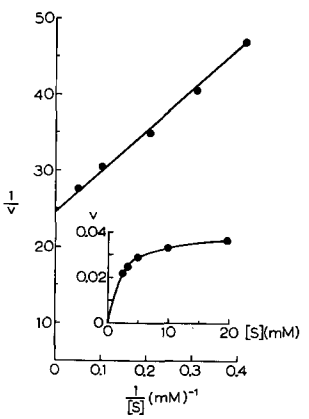


Fig. 5. Effect of UMP concentration on enzyme activity. The activities were measured under the standard assay conditions except that the substrate concentration was varied as indicated, and incubations were carried out in duplicate for 7.5 and 15 min. The phosphate released was strictly proportional with time over the assay period.

absence of Mg^{2+} and that optimal activity was attained at 0.1 M Mg^{2+}. Higher concentrations inhibited the enzyme activity.

Substrate specificities. The relative activities of the enzyme towards a number of nucleoside monophosphates and other phosphate esters are shown in Table II. It can be seen that the enzyme has a high specificity for 5'-nucleotides. dGMP GMP, and IMP were dephosphorylated more rapidly than any of the other substrates tested. The enzyme also showed relatively high activity towards UMP, whereas the activity towards dTMP was only 43% of that with dGMP as substrate.

The apparent K_m values (Table II) were estimated from Lineweaver–Burk plots of the reaction velocities at various concentrations of the 5'-nucleotides. The activities with all the nucleotides tested followed linear Lineweaver–Burk kinetics (see Fig. 5) in the substrate concentration ranges used. With IMP, GMP, and dGMP inhibition occurred at substrate concentrations greater than 5 mM. The 5'-nucleotidase has the lowest K_m values for IMP, GMP, and dGMP. The K_m values for UMP and dTMP are 10 and 45 times higher, respectively, than for IMP.

Comparison of the 5'-nucleotidase with the enzyme isolated by ITOH *et al.*[2]

The 5'-nucleotidase isolated recently from rat liver acetone powder by ITOH *et al.*[2] has a pH optimum at 6.5, is Mg^{2+} dependent and has K_m values of 0.4 mM for IMP and 0.8 mM for GMP. The data are in close agreement with those of our enzyme. According to our observation, pH 6.3 measured in the complete reaction mixture corresponds to pH 6.5 of the pure buffer. The discrepancy between the K_m values given by ITOH *et al.* and those of our enzyme (Table II) may be explained in the light of the decreasing reaction rates which occur during incubation with low IMP and GMP concentrations if the incubation time and amount of enzyme added are not properly adjusted.

In order to compare other properties of the two enzymes the relative rates of dephosphorylation of different substrates and the effect of inhibitors on the 5'-nucleotidase activity were determined under the conditions given by ITOH *et al.*[2]. Data for both enzyme preparations are recorded in Table III for comparison. It can be seen that the relative activities of the two enzyme preparations toward the sub-

TABLE III

COMPARISON OF 5'-NUCLEOTIDASE ACTIVITIES UNDER CONDITIONS GIVEN BY ITOH *et al.*[2]

The reaction mixture contained 0.003 M substrate, 0.01 M $MgCl_2$, 0.1 M Tris–maleate buffer (pH 6.5) and enzyme preparation (Fraction III). Total volume, 0.5 ml; incubation time, 20 min; temp., 37°. Final concn. of inosine was 3 mM and of NaF 1 mM.

Substrate	*Addition*	*Relative activity*	*Data taken from* ITOH *et al.*[2]
IMP		100	100
dGMP		98	90
UMP		50	38
AMP		36	17
dTMP		16	—
IMP	Inosine	51	58
IMP	NaF	15	36

strates are in reasonably good agreement, and the activity of both preparations is inhibited to about the same extent by inosine and NaF. The data as a whole seem to prove the identity of the enzymes.

DISCUSSION

Among the 5′-nucleotidases of rat liver recorded in the literature[2,3,6–8], one has been identified in the nonsedimentable fraction of the liver homogenates[3]. The present work describes properties of an additional 5′-nucleotidase present in this fraction. Its intracellular localization in the soluble cytoplasm is indicated by its rapid appearance in the nonsedimentable fraction during homogenization, by the difference in properties of this enzyme and the 5′-nucleotidases associated with particulate structures[6–8], and finally by the fact that the lysosomes are not significantly disrupted under the homogenization procedure used[3]. The present study further indicates the presence of a deoxythymidylate 5′-nucleotidase in the same subcellular fraction. The purification and specificities of this enzyme will be reported in a separate communication.

The data of the present 5′-nucleotidase clearly indicate that the enzyme is identical with the 5′-nucleotidase purified recently by ITOH *et al.*[2] from rat liver acetone powder. By using the same purification procedure the authors showed that the enzyme is also present in the livers of frog, pig[2] and chicken[9]. The significance of the enzyme is not yet clear, but its seemingly wide distribution in animal species and its low K_m values for IMP and guanine nucleotides suggest that it has a particular function in purine metabolism.

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