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DINUCLEOSIDASETETRAPHOSPHATASE IN RAT LIVER AND *ARTEMIA SALINA*

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Summary

A comparative study of an enzymatic activity present in *Artemia salina* and rat liver which specifically splits dinucleoside tetraphosphates is presented. All the purine and pyrimidine dinucleoside tetraphosphates tested, i.e. diadenosine, diguanosine, dixanthosine and diuridine tetraphosphates, were substrates of both enzymes with similar maximum velocities and K_m values, (around 10 μM). The inhibition by nucleotides of the enzyme from the two sources is also similar. Particularly relevant is the strong inhibition caused by nucleoside tetraphosphates which have K_i values in the nanomolar range. The *Artemia* enzyme has a slightly lower molecular weight (17 500) than the liver enzyme (21 000) and is more resistant to acidic pH. Based on previous findings, the enzyme from *Artemia salina* was named diguanosinetetraphosphatase (EC 3.6.1.17) by the Enzyme Commission. The results presented in this paper show that the liver and *Artemia* enzymes are similar, and we propose to name this enzyme as dinucleosidetetraphosphatase or dinucleoside-tetraphosphate nucleotidehydrolase.

Introduction

Diadenozine tetraphosphate (Ap_4A) is present in rat liver at concentrations of approx. 10^{-7} M [1]. An enzyme has recently been purified from liver extracts [2] which splits Ap_4A to ATP and AMP. This enzyme is very similar to another one, previously described in *Artemia salina* cysts [3,4] which splits diguanosine tetraphosphate (Gp_4G) to GTP and GMP. The latter enzyme has been named diguanosinetetraphosphatase (EC 3.6.1.17) by the Enzyme

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Abbreviations: Ap_4A, diadenosine tetraphosphate; Gp_4G, diguanosine tetraphosphate: Up_4U, diuridine tetraphosphate; Xp_4X, dixanthosine tetraphosphate; p_4G, guanosine 5′-tetraphosphate; p_4A, adenosine 5′-tetraphosphate.

Commission, and by extension the same name has been applied to the liver enzyme [2]. The liver enzyme has been shown to be specific for Ap_4A and Gp_4G [2] and the *Artemia* enzyme is known to hydrolyze at least Gp_4G [3,4].

Previous results from this laboratory were consistent with the active site of the liver enzyme having two loci for each of the two terminal bases (nucleosides or nucleotides) of the Ap_4A or Gp_4G molecule. These two loci would be separated by the appropriate distance to fit the four inner phosphates [2]. However, it was not known whether the nature of the base (purine, pyrimidine) was essential for the catalytic action of the enzyme. In this paper we show that dixanthosine tetraphosphate (Xp_4X) and diuridine tetraphosphate (Up_4U) are good substrates for the liver and *Artemia* enzymes. Some differences between the enzymes are also reported. Based on the results presented here, we propose to name the liver and *Artemia* enzyme as dinucleosidetetraphosphatase or dinucleoside-tetraphosphate nucleotidehydrolase.

Materials and Methods

Preparation of substrates

Diuridine tetraphosphate was synthesized through reaction of uridine 5′-phosphoromorpholidate (0.54 mmol) with the triethylamine salt of pyrophosphate (0.35 mmol) in a medium of anhydrous pyridine (10 ml). After standing 5 days at 30°C, pyridine was eliminated from the reaction mixture by flask evaporation, and the resulting material was resuspended in 8 ml of glass-distilled water, applied to a DEAE-cellulose column (37.5 × 2.6 cm) and fractionated with 3.2 l of a linear gradient (0.06—0.25 M) of ammonium bicarbonate pH 8.6. The peak eluting between 0.17—0.19 M ammonium bicarbonate was characterized as Up_4U by the following criteria: insensitivity to alkaline phosphatase, phosphorus to base ratio and analysis of the products of hydrolysis (UTP and UMP), after treatment with phosphodiesterase I, by electrophoresis in citrate buffer, pH 5.0.

Dixanthosine tetraphosphate was obtained through deamination of diguanosine tetraphosphate. 36 mg of Gp_4G were dissolved in 3 ml of 2 M HCl, and 1 ml of 4 M $NaNO_2$ was added slowly with stirring. The mixture, after standing for 10 min at room temperature, was applied to a Sephadex G-10 column (39 × 2 cm) previously equilibrated with glass-distilled water. Fractions of 1.5 ml were collected. An ultraviolet absorbing peak eluted in the fractions 26—41, clearly separated from the rest of the components of the reaction mixture. This peak was characterized as Xp_4X by the following criteria: (a) ultraviolet absorption spectrum (λ_{max} at 248 and 276 nm at pH 9; λ_{max} at 236 and 259 nm at pH 1) similar to that of commercial xanthosine 5′-monophosphate (XMP); (b) extensive treatment of the nucleotide with phosphodiesterase I and subsequent electrophoresis of the products of the reaction in ammonium citrate, pH 5, gave a spot at the ultraviolet light region with the same electrophoretic mobility as XMP; no detectable spot was observed in the position corresponding to GMP.

Diadenosine tetraphosphate was synthesized as previously described [2] and diguanosine tetraphosphate was isolated from *Artemia salina* cysts [4] The rest of the nucleotides used were purified as previously described [4].

Source of enzyme

Unless otherwise stated, *Artemia* dinucleosidetetraphosphatase was from the concentrate of the Sephadex G-100 step [4], and the liver enzyme, from the DEAE-Sephadex step [2].

Enzymatic assays

Dinucleosidetetraphosphatase activity was determined by two procedures.

1. Hyperchromicity assay. In this method, advantage is taken of the fact that the hydrolysis of 1 μmol of Ap_4A or Gp_4G implies an increase in optical density of 5.4 A_{259} or 1.1 A_{252} respectively. This method is not applicable when either Up_4U or Xp_4X are used as substrates since in our experimental conditions the hydrolysis of these nucleotides is nor accompanied by a detectable change in optical density. The reaction mixture contained in a final volume of 1 ml the following components: 50 mM Tris · HCl buffer, pH 7.5; 5 mM $MgCl_2$; substrate, and enzyme. Increase in absorbance was followed in a Varian Techtron model 635 or in a Gilford model 2400 spectrophotometers.

2. Coupled assays. The hydrolysis of Gp_4G, Ap_4A, Up_4U and Xp_4X is coupled to the phosphoglycerate kinase/glyceraldehyde 3-phosphate dehydrogenase system. The reaction mixture contained in a final volume of 1 ml the following components: 50 mM Tris · HCl buffer, pH 7.5; 7 mM $MgCl_2$; 2 mM EDTA; 12 mM 3-phosphoglycerate; 0.2 mM NADH; dinucleosidetetraphosphatase; substrate and auxiliary enzymes. When Gp_4G, Ap_4A or Xp_4X were used as substrates, 1 unit of phosphoglycerate kinase and 2 units of glyceraldehyde 3-phosphate dehydrogenase were added. In the case of Up_4U, 3 units of both enzymes were used. Decrease in absorbance at 340 nm was followed in a spectrophotometer.

The hydrolysis of Ap_4A was coupled, when indicated, to the hexokinase/glucose 6-phosphate dehydrogenase system. The reaction mixture contained in a final volume of 1 ml the following components: 50 mM Tris · HCl buffer, pH 7.5; 5 mM $MgCl_2$; 2 mM glucose; 0.3 mM $NADP^+$; Ap_4A; 0.7 units of hexokinase; 1.4 units of glucose 6-phosphate dehydrogenase and dinucleosidetetraphosphatase. Increase in absorbance was followed at 340 nm in a spectrophotometer.

In every case, one unit is the amount of enzyme able to transform one μmol of substrate per min at 37°C.

Results and Discussion

Substrate specificity

It was previously known that Gp_4G was a substrate of the *Artemia* enzyme [3,4] and Gp_4G and Ap_4A were substrates of the liver enzyme [2]. Dinucleoside triphosphates were not substrates of both enzymes [2,4]. The existence of four inner phosphates in the substrate was therefore essential for the catalytic action of the enzyme to take place. As outlined in the introduction, it was not known, however, whether the nature of the nucleotidic base could influence the velocity of the reaction. To test this possibility, two more dinucleoside tetraphosphates were synthesized in our laboratory, a dipyrimidine nucleotide (Up_4U), and another dipurine nucleotide (Xp_4X). The results presented in Table

I indicate that Up_4U and Xp_4X are almost as good substrates as Gp_4G and Ap_4A for both enzymes. Based on the data presented in Table I and some similarities (see below) between the *Artemia* and liver enzymes, it seems to us that both activities correspond to the same enzyme, that could be named as dinucleoside-tetraphosphatase rather than diguanosinetetraphosphatase.

Inhibition by nucleotides

The inhibition of the dinucleosidetetraphosphatase by nucleoside tetraphosphates had been previously reported [2,4]. A systematic exploration of the effect of guanosine and adenosine mono- , di- , tri- and tetraphosphates on both enzymes when either Gp_4G or Ap_4A were used as substrates is presented in Table II. In the case of the *Artemia* enzyme, guanosine 5′-tetraphosphate (p_4G) is a specially potent inhibitor (K_i = 0.006 μM) when Gp_4G is used as substrate; adenosine 5′-tetraphosphate (p_4A) is less inhibitory than p_4G (K_i = 0.13 μM) when either Gp_4G or Ap_4A are substrates of the reaction. The inhibitory effects of p_4A and p_4G on the liver enzyme were rather similar. Nucleoside mono- , di- and triphosphates were almost equally inhibitory regardless of being adenine or guanine nucleotides, and their K_i values were 2—3 orders of magnitude higher than those of nucleoside tetraphosphates. The inhibition caused by a nucleoside triphosphate (50 μM ATP) was not increased by further addition of the same concentration of either P_i or PP_i, thus showing that the integrity of the molecule of nucleoside tetraphosphate is necessary for its specific inhibitory effect to take place.

Stability at acidic pH and molecular weight

The liver and *Artemia* enzymes are different in their stability at acidic pH. Dinucleosidetetraphosphatase from liver loses 95% of its activity after 1 min of incubation at pH 4.1, whereas the *Artemia* enzyme retained 100 and 75% of its activity after incubation during 4 and 24 h at the same pH, respectively. The different stability at pH 4.1 is not due to the presence of acidic proteases in the liver preparation as after incubation of a mixture of the liver and *Artemia* enzyme at that pH, during 5 min, an enzymatic activity equivalent to that of the *Artemia* enzyme added, was recovered. This difference in stability suggested

TABLE I

KINETIC PROPERTIES OF DINUCLEOSIDETETRAPHOSPHATASE FROM RAT LIVER AND *ARTEMIA SALINA*

K_m and V values of the enzyme for dinucleoside tetraphosphates were determined as described in Material and Methods.

Substrate	Rat liver		*Artemia salina*	
	K_m (μM)	V (%)	K_m (μM)	V (%)
Gp_4G	2	100	5	100
Ap_4A	2	62	2	88
Up_4U	10	33	11	30
Xp_4X	16	78	18	123

TABLE II

INHIBITION OF DINUCLEOSIDETETRAPHOSPHATASE FROM RAT LIVER AND *ARTEMIA SALINA* BY NUCLEOTIDES

K_i values (μM) were determined from Lineweaver-Burk plots using in each case the enzyme, the substrate and the inhibitor shown in the Table. The enzymatic assay was carried out by the hyperchromicity method except in the cases indicated with (*), in which the coupled method involving hexokinase and glucose-6-phosphate dehydrogenase was used. Some of the K_i values presented here have been previously published (marked with ‡) [2,4].

Inhibitor	Substrates			
	Rat liver		*Artemia salina*	
	Gp_4G	Ap_4A	Gp_4G	Ap_4A
p_4G	0.013	0.014 ‡	0.006 ‡	0.013 *
p_4A	0.018	0.048 *‡	0.130 ‡	0.137 *
GTP	11	14 ‡	14 ‡	13
GDP	7	7	56 ‡	7
GMP	24	35	24 ‡	73
ATP	12	31 ‡	30 ‡	33
ADP	21	33 ‡	67	52
AMP	51	85 *‡	55	98 *

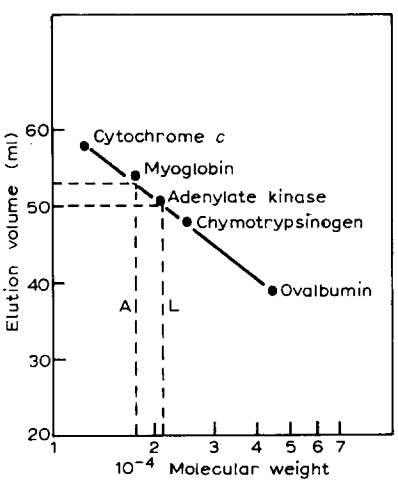

Fig. 1. Molecular weight determination of dinucleosidetetraphosphatase by Sephadex G-75 gel filtration. The column (0.8 X 145 cm) was previously equilibrated in 20 mM Tris · HCl, pH 7.5, 0.5 mM EDTA. The bed volume was 42.5 ml. The following samples in a final volume of 0.65 ml of equilibrating buffer were applied and run successively: (a) ovalbumin (3 mg), adenylate kinase (0.025 mg, 5 units), cytochrome *c* (3 mg) and *Artemia* dinucleosidetetraphosphatase (4 mg, 93 mU); (b) ovalbumin (3 mg) and myoglobin (3 mg); (c) adenylate kinase (0.025 mg, 5 units), chymotrypsinogen (3 mg) and cytochrome *c* (3 mg); (d) adenylate kinase (0.025 mg, 5 units), chymotrypsinogen (3 mg) and rat liver dinucleosidetetraphosphatase (0.03 mg, 15 mU). The elution profiles of the enzymes were determined by suitable assays; those of ovalbumin and chymotrypsinogen by absorbance at 280 nm and those of myoglobin and cytochrome *c* by absorbance at 400 nm. Fractions of 1.01 ml were collected at a flow rate of 0.15 ml/min. An estimated molecular weight of 17 500 and 21 000 were calculated for the *Artemia* and rat liver dinucleosidetetraphosphatase respectively. L and A refer to the liver and *Artemia* dinucleosidetetraphosphatase, respectively.

then a different primary structure. Accordingly, the molecular weight of dinucleosidetetraphosphatase from the two sources was determined by filtration through Sephadex G-75, and an estimated molecular weight of 17 500 and 21 000 was calculated for the *Artemia* and rat liver enzymes respectively (Fig. 1).

The results presented in this paper show the presence of an enzyme in *Artemia* and rat liver which specifically splits dinucleoside tetraphosphates to the corresponding nucleoside tri- and monophosphates. Both enzymes show similar kinetic properties and present a slight difference in molecular weight. These similarities together with those in K_m and V values towards the dinucleoside tetraphosphates tested, seem to us valid arguments in favour of the functional identity of the enzyme from the two sources and for naming it as dinucleosidetetraphosphatase. Although so far this enzyme has been described only in *Artemia* and rat liver it may be reasonably expected to be ubiquitous since one of its substrates, Ap_4A, apparently is [1] and Gp_4G has been described as present in *Artemia salina*, *Daphnia magna* and several fungi [5—7].

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