DIADENOSINE TRIPHOSPHATE SPLITTING BY RAT LIVER EXTRACTS

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SUMMARY: A splitting activity on diadenosine triphosphate has been found in rat liver. One of the products of the cleavage is ADP. A Km of 10 μM has been found. This activity on diadenosine triphosphate seems to be specific as diadenosine tetraphosphate, a nucleotide previously described by others to occur in rat liver at very low concentration, is not a substrate of the reaction. The occurrence of diadenosine triphosphate in rat liver has not been so far reported, but a dinucleoside triphosphate structure has been described at the 5' end of certain mRNAs. The possibility that this enzymatic activity may be involved in the hydrolysis of diadenosine triphosphate or in the processing of mRNAs is suggested.

INTRODUCTION

Recently, our laboratory has been engaged in the study of the metabolism and function of dinucleoside tetraphosphates, i. e., compounds with four inner phosphates linked by phosphodiester bond and joined by their two extremes to the 5' carbon of two nucleosides. Two compounds of this type have been described as present in biological material, diguanosine tetraphosphate (Gp_4G) and diadenosine tetraphosphate (Ap_4A). Gp_4G or diguanosine 5', 5'''-(P^1, P^4 tetraphosphate) is present in Artemia cysts extracts at concentrations of around 10 mM (1); the occurrence of Ap_4A has been described in rat liver, E. coli and ascites tumour cells, in each case at micromolar concentrations (2). An enzyme has been described in Artemia (3, 4) and rat liver extracts (5) which specifically splits dinucleoside tetraphosphates (unpublished results from this laboratory) to the corresponding nucleoside tri and monophosphates. Arguments have been given in favor of

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Gp_4G and Ap_4A being of potential interest in metabolic regulation (5).

Nothing is known on the metabolism and function of dinucleoside triphosphates. Diguanosine triphosphate (Gp_3G) is present in Artemia extracts at concentrations of around 1 mM. The search for a hydrolytic activity on this metabolite, undertaken in this laboratory, has been unfruitful. Although diadenosine triphosphate has not been described as present in rat liver extracts, our previous results on the similarity of the rat liver and Artemia diguanosinetetraphosphatase (EC 3.6.1.17) led us to the search of a splitting activity on dinucleoside triphosphates. The object of this communication is to describe an activity present in liver which splits diadenosine triphosphate, presumably to ADP and AMP.

MATERIAL AND METHODS

White female rats, weighing around 150 g were used. The animals were killed by decapitation. The livers from 4 rats (29 g) were excised and homogenized in a Kontes Duall Grinder with two volumes of 50 mM Tris-HCl buffer pH 7.5, 0.5 mM EDTA. The homogenate was centrifuged at 27,000 x g for 15 minutes, and the resulting supernatant was further centrifuged at 150,000 x g for 60 minutes.

Diadenosine triphosphate was synthesized chemically. The method employed, based essentially on those previously described (6, 7, 8), implied reaction of adenosine 5'-phosphoromorpholidate (2 mmoles) with the triethylamine salt of orthophosphate (1 mmol) in a medium of anhydrous dimethylsulfoxide (35 ml). After 6 days at 30°C, the reaction was diluted three times with glass distilled water and applied to a DEAE-cellulose column (70 x 2.5cm) The products of the reaction were fractionated with 6 l of a linear gradient (0.06-0.25 M) of ammonium bicarbonate pH 8.6. The peak corresponding to diadenosine triphosphate eluted between 0.165-0.185 M ammonium bicarbonate. This peak was concentrated by flask evaporation and characterized as Ap_3A by the following criteria: insensitivity to degradation by alkaline phosphatase, the phosphorus to adenine ratio was 1.5, its treatment with snake venom phosphodiesterase yielded ADP and AMP, the ADP so formed was further degraded to AMP. Experimental details of the treatment of diadenosine triphosphate with snake venom phosphodiesterase are presented in Figure 1.

The concentrations of Ap_3A described below were calculated considering a molar extintion coefficient at neutral pH of 25.8 at 259 nm (results from this laboratory).

RESULTS AND DISCUSSION

Based in part on our previous work with diadenosine tetraphosphate we have developed the following methods to measure the hydrolytic activity on diadenosine triphosphate: a) by evaluation of the inorganic phosphate liberated after incubation of Ap_3A with liver extracts and alkaline phosphatase,

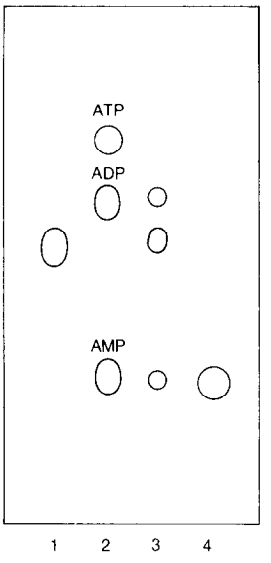

Figure 1. Characterization of diadenosine triphosphate by digestion with snake venom phosphodiesterase. The reaction mixture contained in a final volume of 95 µl, 5 mM Tris-HCl buffer pH 8.0, 5 mM $MgCl_2$, 2.4 A_{260} units of the product and 5 µl of Boehringer's purified snake venom phosphodiesterase diluted five times. Prior to incubation and after incubation at 37°C for 5 min, a 25 µl aliquot was taken and the reaction was stopped by chilling and addition of 10 µl of 0.3 M ammonium acetate pH 3.8. After withdrawing the 5 min aliquot, a further 5 µl of the same phosphodiesterase was added to the remaining mixture and incubation was continued up to 2 hours. An aliquot of 25 µl was then taken and treated as the others. Samples were spotted on Whatman nº1 paper previously washed with 1 N formic acid and with glass distilled water and subjected to electrophoresis in 0.1 M ammonium acetate buffer pH 3.8, 0.1 mM EDTA for 2 hours and 45 min at 30 V/cm. Tracks 1 through 4 correspond to zero time, markers as indicated, 5 min and 2 hours of incubation, respectively.

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similarly as previously described for diadenosine tetraphosphate (5); b) in the hyperchromicity assay we took advantage of the fact that the hydrolysis of Ap_3A is associated with an increase in absorbance of 5.0 A_{259} units/ µ mol; c) in the spectrophotometric coupled method, the hydrolysis of Ap_3A was coupled to alkaline phosphatase and adenosine deaminase and the decrease in absorbance at 265 nm, was followed in a spectrophotometer; the hydrolysis of 1 µmol of Ap_3A implies a decrease in absorbance of 12 A_{265} units (Fig. 2). With the three methods the reaction was linear with both time and amount of extract and was strictly dependent on the addition of Ap_3A. In

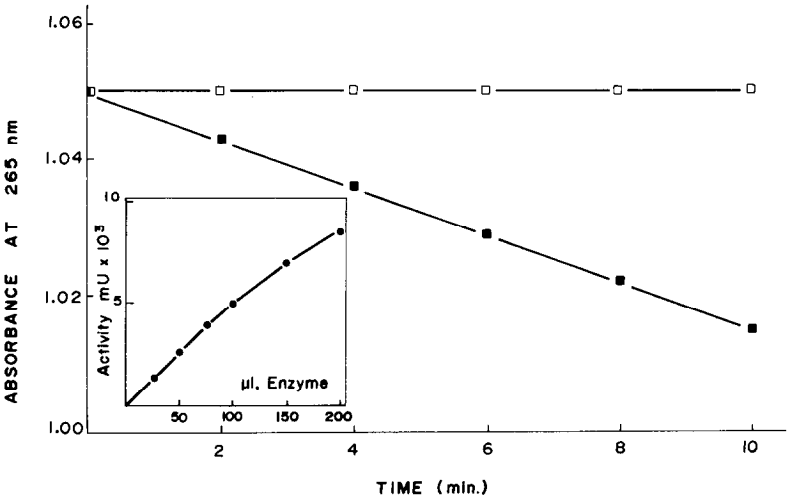

Figure 2. Spectrophotometric coupled method of the hydrolytic activity on diadenosine triphosphate. The reaction mixture contained in a final volume of 1 ml the following components: 50 mM Tris-HCl buffer pH 7.5, 2 mM $MgCl_2$, 0.3 units of alkaline phosphatase, 1 unit of adenosine deaminase and extract. Decrease in absorbance at 265 nm was followed in a spectrophotometer. In the figure, the linearity of the reaction with time and with the amount of extract (inset) is represented. The enzyme used was from the Sephadex G-100 pooled fractions (see Table I). The linearity with time was followed with 50 µl of that preparation.

Fig. 2 the linearity of the reaction using the spectrophotometric coupled method is represented.

The hydrolytic activity on Ap_3A was partially purified as follows. The 150,000 g supernatant was brought up to 30% saturation with ammonium sulphate, stirred during 30 minutes and centrifuged at 27,000 x g during 15 minutes. The precipitate was discarded and the supernatant brought up to 60% saturation with ammonium sulphate and treated as above. The precipitate was resuspended in 20 mM Tris-HCl buffer pH 7.5; 0.5 mM EDTA and applied to a Sephadex G-100 column. Elution was made with the same buffer. The profile of the Sephadex column is presented in Fig. 3. Fractions containing the major portion of diadenosine triphosphate hydrolytic activity were pooled. A summary of a typical purification run is given in Table I.

With the partially purified enzyme preparation, an apparent Km value of 10 µM was obtained for Ap_3A using either the hyperchromicity or the spectrophotometric coupled method. Both methods have the character-

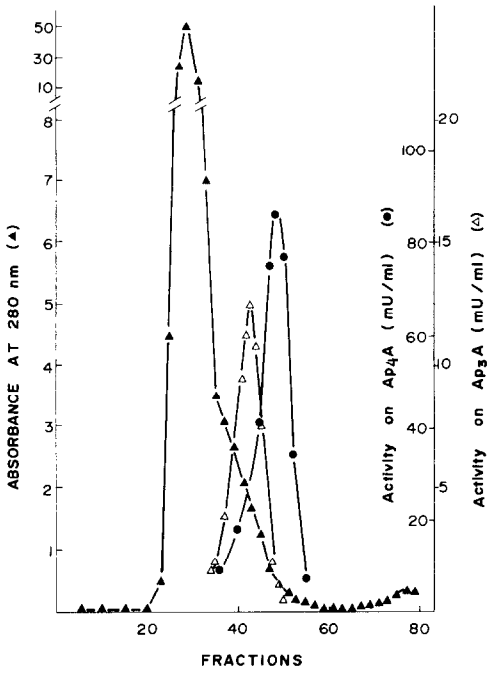

Figure 3. Gel filtration of the 30-60% ammonium sulphate fraction of the 150,000 x g supernatant, 6.8 ml were applied to a column (112 x 2.5 cm) of Sephadex G-100 previously equilibrated with 50 mM Tris-HCl buffer pH 7.5, 0.5 mM EDTA. Elution was made with the same buffer and fractions of 8.5 ml were collected. The hydrolytic activities on Ap_3A and Ap_4A were determined by the hyperchromicity assay. The reaction mixture contained in a final volume of 1 ml the following components, 50 mM Tris-HCl buffer pH 7.5, 2 mM $MgCl_2$, 40 μM of either Ap_3A or Ap_4A and extract. Increase in absorbance at 259 nm was followed in a Gilford model 2400 spectrophotometer.

istics that the splitting activity on Ap_3A can be measured regardless of what the products of the reaction may be. The unmasking of the two bases secondary to the hydrolysis of Ap_3A is, more probably, the parameter measured by the hyperchromicity assay. In the case of the coupled method, the formation of adenosine from the nucleotides resulting from the Ap_3A splitting is evaluated. Adenosine is not a primary product of the reaction as its rate of formation in a reaction mixture whithout added alkaline phosphatase is less than 2% of the rate obtained when the phosphatase is present. Probably, the primary products of the Ap_3A splitting are ADP and AMP as the initial rate of the ADP formation, evaluated with the pyruvate

TABLE I

Partial purification of an enzymatic activity present in rat liver extracts, acting on diadenosine triphosphate

Step	Volume	Protein	Specific activity	Yield per cent
	ml	mg	milliunits/mg	
150,000 g supernatant	49.5	2,613	0.4	100
$(NH_4)_2SO_4$ fractionation	7.3	1,153	0.7	80
G-100 Sephadex chromatography	107.0	160	4.4	70

One unit is the amount of enzyme able to hydrolyze 1 µmol of Ap_3A per minute at 37°C. Proteins were measured by the method of Lowry et al. (9).

kinase-lactate dehydrogenase system, is about half that of the adenosine rate formation when the hydrolysis of Ap_3A is coupled to the alkaline phosphatase. The presence of phosphatases and other unidentified contaminants in the G-100 preparation make it difficult at present to establish unequivocally the stoichiometry of the reaction. Further purification of the Ap_3A splitting activity is currently under way in our laboratory to better characterize this activity. One of our first aims was to investigate whether the activity on Ap_3A here described was different from the one previously described in liver acting on Ap_4A (5). The distinct elution profile, from a Sephadex G-100 column, of the hydrolytic activities on Ap_3A and Ap_4A shows that both activities are indeed different (Fig. 3). Preliminary observations with a further purified Ap_3A hydrolase preparation showed that Ap_4A was not a substrate of the reaction. Conversely, Ap_3A is not a substrate of diguanosinetetraphosphatase from rat liver (5). The Ap_3A hydrolase preparation described here was active on another dinucleoside triphosphate, Gp_3G. When Ap_3A and Gp_3G were assayed as substrates, both at 60 µM,

and using the Pi evaluation method, the velocity of the reaction with Gp_3G was half of that obtained with Ap_3A.

Concerning the physiological role of the Ap_3A hydrolase activity nothing is known at present. As stated in the introduction, this nucleotide has not been so far described in liver extracts, although its synthesis in vitro as a by-product of the reaction of activation of lysine by the lysyl-tRNA synthetase from E. coli has been reported (10). Apart from the action that this enzyme might have on Ap_3A, were this substrate to be present in liver, we would like to suggest the possibility that it could act on RNAs. Very recent reports from several laboratories have shown that at the 5'end of certain viral RNAs, mRNAs from eukaryotic cells and some nuclear RNAs of low molecular weight, there is the sequence mGpppNmpNp (11-15), a methylguanosine linked through its 5'carbon by three phosphates to the 5'carbon of a methyl nucleoside. It is evident that the underlined part of this RNA fragment is similar to a dinucleoside triphosphate of the kind used as substrate in this paper. The possibility that the activity described here is involved in the processing of mRNA, deserves investigation

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