DIADENOSINE TETRAPHOSPHATE SPLITTING BY RAT LIVER EXTRACTS

Carmen G. Vallejo, José M. Infante, Jaime Renart and Antonio Sillero

Instituto de Enzimología del C.S.I.C.,
Facultad de Medicina de la Universidad Autónoma,
Madrid-34, Spain.

Received January 18,1973

SUMMARY: A splitting activity on diadenosine tetraphosphate, a compound reported by others to occur in liver at a concentration of 10^{-7} - 10^{-8} M, has been found in rat liver extracts. One of the products of the cleavage is ATP. A Km of 6 μmolar has been found. This low Km favors the view that the activity here described may act on this substrate in vivo.

INTRODUCTION

In the course of studies on the mechanism of activation of amino acids, Zamecnik and collaborators discovered diadenosine tetraphosphate (Ap_4A) as a by product of the reaction of activation of lysine by the lysyl-tRNA synthetase from E.Coli (1). The formation of the complex AMP-lysine-synthetase takes place according to reaction (a). In an in vitro assay, ATP may compete with PPi in the back reaction, with the formation of Ap_4A, as shown in reaction (b). The formation of Ap_4A was strictly dependent on the presence of lysine in the reaction mixture.

$$\text{ATP} + \text{lysine} + \text{E} \rightleftharpoons \text{AMP-lysine-E} + \text{PPi} \qquad \text{(a)}$$

$$\text{AMP-lysine-E} + \text{ATP} \rightleftharpoons Ap_4A + \text{lysine} + \text{E} \qquad \text{(b)}$$

Thereafter, Ap_4A was shown to be present in liver at a concentration of roughly 10^{-7} - 10^{-8} M (2). Adenosine tetraphosphate had been previously synthesized chemically by Verheyden et al (3).

To our knowledge nothing is known on the degradation of Ap_4A in liver or in liver extracts. The object of this communication is to describe an activity present in liver which splits diadenosine tetraphosphate, presumably to ATP and AMP.

MATERIAL AND METHODS

While male rats, weighing around 150 g were used. The animals were

killed by decapitation. The livers from 3 rats (16.7 g) were excised and homogenized in a Kontes Duall Grinder with two volumes of 0.035 M Tris-HCl buffer, pH 7.7, 0.07 M KCl, 0.009 M $MgCl_2$, 0.25 M sucrose. The homogenate was centrifuged at 27,000 g for 15 minutes. The supernatant was further centrifuged at 150,000 g for 60 minutes and dialyzed against 4 liters of 0.035 M Tris-HCl buffer, pH 7.7, 0.05 M KCl, 0.009 M $MgCl_2$ during 5 hours followed by an overnight dialysis against 6 liters of the same buffer. The resulting dialysate was subjected to the purification procedure shown in Results and Discussion.

Diadenosine tetraphosphate was synthesized chemically. The method employed, based essentially on those previously described by Moffatt et al (3,4,5), implied reaction of adenosine 5'-phosphoromorpholidate with the trietylamine salt of pyrophosphate in a medium of anhydrous dimetilsulfoxide. The products of the reaction were applied to a DEAE cellulose column and separated with a linear gradient of ammonium bicarbonate. This synthesis shall be described in detail elsewhere (6). The synthesized diadenosine tetraphosphate was characterized by the following criteria (7): insensitivity to degradation by alkaline phosphatase, the phosphorus to adenine ratio was 1.81, the treatment with snake venom phosphodiesterase yield ATP and AMP; the ATP so formed is further degraded to AMP. Experimental details of the treatment of diadenosine tetraphosphate with the snake venom phosphodiesterase are presented in Figure 1.

The concentrations of Ap_4A described below were calculated considering a molar extinction coefficient at neutral pH of 25.4 at 260 nm (7).

RESULTS AND DISCUSSION

Preliminary experiments showed that the diadenosine tetraphosphate was acted upon by some kind of enzymatic activity presumably hydrolytic present in liver extracts, with liberation of compounds sensitive to alkaline phosphatase. This property was used to measure this activity. The standard assay conditions were: 50 mM Tris-HCl buffer pH 8.0, 2 mM $MgCl_2$, 0.3 units of alkaline phosphatase (Boehringer), liver extract, and 0.33 mM Ap_4A in a total volume of 0.25 ml. The mixtures were incubated for 15 minutes at 37ºC and the reaction stopped by adding 1 ml of 10% trichloroacetic acid. The samples were centrifuged and the inorganic phosphate was determined in the supernatants by the method of Fiske and SubbaRow (8). Liver extract was added to the reaction mixture to produce from 0.05 to 0.1 μmol of inorganic phosphate.

TABLE I

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

Step	Volume	Protein	Specific activity	Yield per cent
	ml	mg	mU/mg	
150,000 g supernatant	31.0	790	3.6	100
$(NH_4)_2SO_4$ fractionation	8.2	508	4.4	78
G-100 Sephadex chromatography	90.0	45	34.0	54
Concentrate	3.3	17	45.0	28

One unit is the amount of enzyme able to hydrolize 1 μmol of Ap_4A per minute at 37ºC. Proteins were measured by the method of Lowry et al. (9).

In these conditions the reaction was linear with respect to both time and amount of extract, and was strictly dependent on the addition of Ap_4A (results not shown).

The hydrolytic activity on Ap_4A was partially purified as follows. The dialysate was brought up to 30% saturation with ammonium sulphate, stirred during 30 minutes and centrifuged at 27,000 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 the dialysis buffer and applied to a column of Sephadex G-100. Elution was made with the dialysis buffer. The profile of the Sephadex column is presented in Figure 2. The fractions that contained enzymatic activity were pooled, concentrated with ammonium sulphate and dialyzed overnight. The dialysate, slightly turbid, was centrifuged at 27,000 g for 15 min and the precipitate discarded. The supernatant is the concentrate shown in Table I. All operations were carried out at about 4ºC. The purification procedure is summarized in Table I.

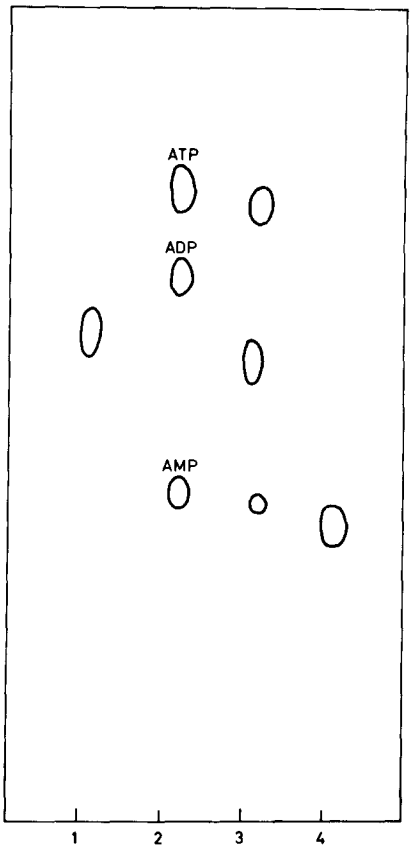

Fig. 1.

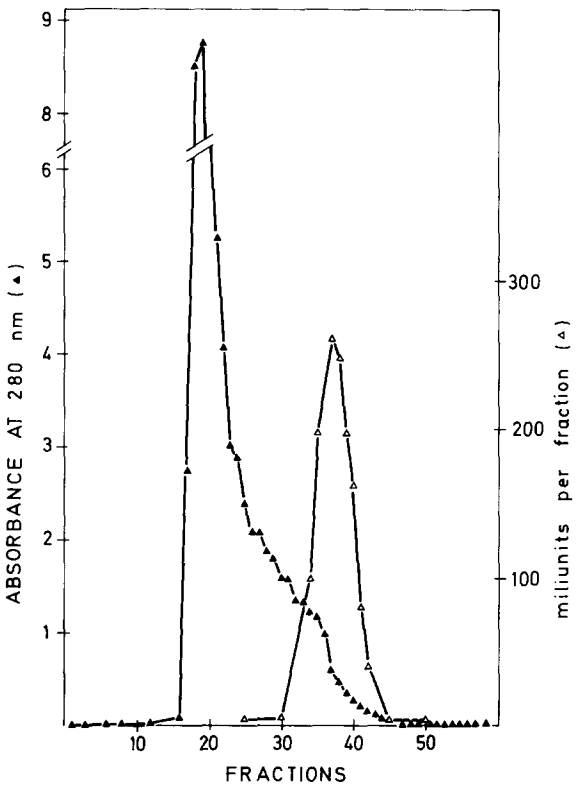

Fig. 2.

Figure 1. Characterization of diadenosine tetraphosphate by digestion with snake venom phosphodiesterase. The reaction mixture contained in a final volume of 80 µl, 5 mM Tris-HCl buffer, pH 8.0, 5 mM $MgCl_2$, 1.5 A_{260} units of the product and 5 µl of Worthington's purified snake venom phosphodiesterase diluted twelve times. Prior to incubation and after incubation at 37ºC for 0.5 min, a 25 µl aliquot was taken and the reaction was stopped by chilling and addition of 10 µl of 60 mM citrate pH 5.0. After withdrawing the 0.5 min aliquot, a further 5 µl of the same phosphodiesterase was added to the remaining mixture and incubation was continued up to 30 minutes. An aliquot of 25 µl was then taken and treated as the others. Samples were spotted on Whatman nº1 paper and subjected to electrophoresis in 0.02 M citrate buffer, pH 5.0, for 2 hours at 30 V/cm. Tracks 1 through 4 correspond to zero time, markers as indicated, 0.5 and 30 minutes of incubation, respectively.

Figure 2. Gel filtration of the 30-60% ammonium sulphate fraction of the 150,000 g rat liver supernatant. 8.2 ml were applied to a column (2.8 x 90 cm) of Sephadex G-100 previously equilibrated with the dialysis buffer, and fractions of 9 ml were collected. The hydrolytic activity on Ap_4A was determined as described in the text.

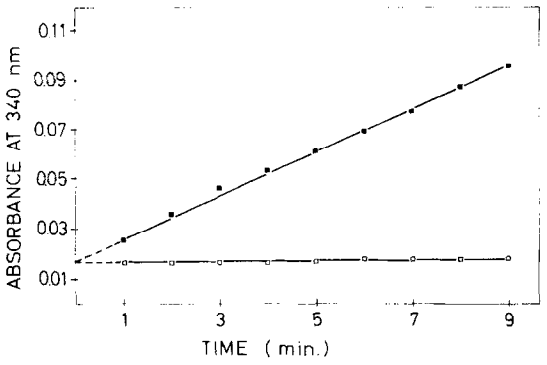

Fig. 3.

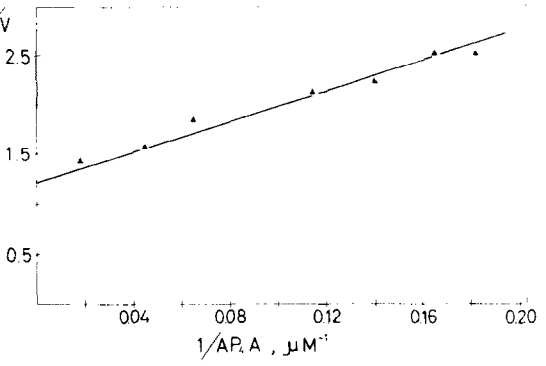

Fig. 4.

Figure 3. Spectrophotometric assay of the hydrolytic activity on diadenosine tetraphosphate, in a partially purified rat liver extract. The hydrolysis of Ap_4A was coupled to the hexokinase-glucose 6-phosphate dehydrogenase system as described in the text. The assay was carried out with 25 μl of the concentrate shown in Table I. Filled circles, complete system with μ M Ap_4A. Open circles, blank without Ap_4A.

Figure 4. Effect of the concentration of diadenosine tetraphosphate on the initial rate of the hydrolytic activity present in liver extracts. The assays were carried out with 25 μl of the concentrate described in Table I, using a Gilford model 2400 spectrophotometer.

The purification was followed by measuring the hydrolytic activity with the method described above using alkaline phosphatase as auxiliary enzyme. It is clear that this method of evaluation presents difficulties for kinetic studies. Therefore, a spectrophotometric method was developed to measure this activity. We reasoned that the hydrolysis of Ap_4A could be symmetric and/or asymmetric. In the first case one mol of Ap_4A would give rise to two mols of ADP. An asymmetric cleavage would produce one mol of ATP and one mol of AMP from one mol of Ap_4A. We tried then to couple the hydrolysis of Ap_4A to the hexokinase-glucose 6-phosphate dehydrogenase system. The reaction mixture contained in a final volume of 1 ml the following components: 50mM Tris-HCl buffer pH 7.5, 5 mM $MgCl_2$, 2 mM glucose, 0.3 mM NADP, 0.7 units of hexokinase, 1.4 units of glucose 6-phosphate dehydrogenase and varying amounts of both purified extract and Ap_4A. Increase in optical density at 340 nm was followed in a spectrophotometer. When assayed with this method, the hydrolytic activity on Ap_4A present in partially purified liver extract was linear with time and dependent on the presence of Ap_4A (Figure 3). This result shows that at least there is an asymmetric cleavage of Ap_4A

yielding ATP and AMP. An apparent Km of about 6 μM was obtained using the same method (Figure 4). Preliminary observations with a further purified myokinase-free preparation seems to indicate the absence of significant symmetric splitting of diadenosine tetraphosphate.

Due to technical difficulties, it is not known exactly the concentration of Ap_4A in liver. Zamecnik has reported values of about 10^{-7} - 10^{-8} M (2). However, unless special precautions are taken in handling the samples, the actual concentration may be somewhat higher considering the presence of this hydrolytic activity in liver extracts. The Km found for this activity on Ap_4A favors the view that it may act on this substrate in vivo.

Studies are in progress to further purify the activity here described. This shall allow characterization of the products of the reaction, and to establish whether this activity is specific for Ap_4A.

ACKNOWLEDGEMENTS

This work was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research to A.Sillero and M.A.G.Sillero. C.G.Vallejo is a postdoctoral fellow of the Fundación Juan March, Madrid. J.Renart is a predoctoral fellow of the Ministerio de Educación y Ciencia. We are indebted to Dr. A.Sols for helpful criticism. We thank Mr. H.Baudach (Pharma Waldhof) for a generous gift of AMP, Miss M.C.Langa and Mr. L.Seguido for skillful technical assistance. We also wish to thank Dr. M.A.G.Sillero for useful discussion and help in the characterization of Ap_4A.

REFERENCES

(1) Zamecnik P.C., Stephenson M.L., Janeway C.M., and Randerath K., Biochem.Biophys.Res.Commun., 24, 91 (1966)

(2) Zamecnik P.C., Cold Spring Harbor Symp., Quant.Biol., 34, 1 (1969)

(3) Verheyden D.L.M., Wehrli W.E., and Moffatt J.G., J.Am.Chem.Soc., 87, 2257 (1965)

(4) Wehrli W.E., Verheyden D.L.M., and Moffatt J.G., J.Am.Chem.Soc., 87, 2265 (1965)

(5) Adam A., and Moffatt J.G., J.Am.Chem.Soc., 88, 838 (1966)

(6) Infante J.M., Renart J., Sillero M.A.G., and Sillero A., to be published

(7) Randerath K., Janeway C.M., Stephenson M.L., and Zamecnik P.C., Biochem.Biophys.Res.Commun., 24, 98 (1966)

(8) Fiske C.H., and SubbaRow Y., J.Biol.Chem., 66, 375 (1925)

(9) Lowry O.H., Rosebrough N.J., Farr A.L., and Randall R.J., J.Biol.Chem., 193, 265 (1951)