Bis-(5'-guanosyl) tetrphosphatase in rat tissues

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(Received 30 September 1981/Accepted 5 October 1981)

The occurrence and distribution of bis-(5'-guanosyl) tetrphosphatase activity towards dinucleoside tetrphosphates between the 27000g supernatant and sedimented fraction were studied in liver, kidney, brain, muscle and intestinal mucosa from rat. The P^1P^4 -bis-(5'-guanosyl) tetrphosphate-hydrolysing activities found in total homogenates were 0.77, 1.44, 0.39, 0.36 and 2.14 units ($\mu\text{mol/min}$)/g respectively. The activities found in the 27000g-sedimented fractions were 74, 49, 11, 4 and 96% of those present in the homogenates respectively. The properties of the soluble enzymes were investigated. All of them have low K_m values for P^1P^4 -bis-(5'-guanosyl) tetrphosphate (from 2 to 50 μM), are competitively inhibited by guanosine 5'-tetrphosphate with K_i values from 10 to 160 nM, have molecular weights of about 21000, require Mg^{2+} or Mn^{2+} and are inhibited by Ca^{2+} . These properties show that bis-(5'-guanosyl) tetrphosphatase (EC 3.6.1.17), an enzyme previously characterized in *Artemia salina* and rat liver [Warner & Finamore (1965) *Biochemistry* **4**, 1568–1575; Vallejo, Sillero & Sillero (1974) *Biochim. Biophys. Acta* **358**, 117–125; Lobatón, Vallejo, Sillero & Sillero (1975) *Eur. J. Biochem.* **50**, 495–501], is present in all the rat tissues examined. The inhibition of the enzyme by Ca^{2+} could be related to the effect of P^1P^4 -bis-(5'-adenosyl) tetrphosphate as a trigger of DNA synthesis [Grummt, Wärtl, Jantzen, Hamprecht, Huebscher & Kuenzle (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6081–6085].

Two dinucleoside tetrphosphates have been described in biological materials: Ap_4A and Gp_4G . The former is present at very low concentration in ascites-tumour cells, rat liver and *Escherichia coli* (Zamecnik, 1969). On the other hand Gp_4G is abundant in *Artemia salina* encysted embryos (Finamore & Warner, 1963; Sillero & Ochoa, 1971). Recent evidence points to a regulatory role for these compounds. Gp_4G is an activator, at nanomolar concentration, of GMP reductase from *Artemia* and erythrocytes (Renart *et al.*, 1976; Spector *et al.*, 1979). The amount of Ap_4A present in mammalian cells varies in parallel with the proliferative activity of the culture (Rapaport & Zamecnik, 1976), and it has been proved that the synthesis of DNA is elicited by Ap_4A in cells arrested in G_1 phase (Grummt, 1978). This effect is possibly a consequence of a direct action of Ap_4A on DNA polymerase α , since Grummt *et al.* (1979)

have demonstrated that the calf thymus enzyme specifically binds to that compound. Recently, similar results showing a tight association between the DNA polymerase and Ap_4A -binding activities have been obtained by Rapaport *et al.* (1981) working with HeLa cells.

Bis-(5'-guanosyl) tetrphosphatase (EC 3.6.1.17) has been described in *Artemia* (Warner & Finamore, 1965; Vallejo *et al.*, 1974) and rat liver (Lobatón *et al.*, 1975). This enzyme specifically splits Ap_4A and/or Gp_4G to the corresponding nucleoside tri- and mono-phosphates, shows a very low K_m value for both tetrphosphates and is strongly inhibited by p_4A and p_4G . The purpose of the present study was to investigate the properties of the soluble enzyme from different rat tissues. The inhibition of the enzyme by Ca^{2+} is here described for the first time.

Materials and methods

Preparation of extracts

White female rats weighing about 200g and fed on a stock diet *ad libitum* were used. The animals

Abbreviations used: Ap_4A , P^1P^4 -bis-(5'-adenosyl) tetrphosphate; Gp_4G , P^1P^4 -bis-(5'-guanosyl) tetrphosphate; p_4A , adenosine 5'-tetrphosphate; p_4G , guanosine 5'-tetrphosphate.

were killed by decapitation. Liver, kidney, brain, muscle and intestinal mucosa were quickly removed, cooled at 4°C, weighed and, separately, finely minced with scissors and homogenized in a motor-driven Potter S apparatus with 2, 4, 4, 5 and 5 vol. of 50 mM-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5, respectively. Typical weights of tissue samples were about 4, 1.5, 1.5, 4 and 3 g respectively. The extracts were centrifuged at 27000 g_{av} for 1 h at 2°C in a Sorvall model RC-5B centrifuge, with the SS-34 rotor. For the enzyme assays in crude extracts, samples from the total homogenates and from the 27000 g precipitates and supernatants were taken and dialysed overnight at 4°C against approx. 200 vol. of the same buffer as cited above.

Assay methods

As previously known (Lobatón *et al.*, 1975), the enzyme is specific for dinucleoside tetraphosphates, and either Ap_4A or Gp_4G could be used as substrate for the reaction. In the present work Gp_4G was almost exclusively employed to measure the activity of the enzyme. Two methods were used. The pH values quoted for the Tris/HCl buffers were measured at the temperature at which they were used.

(1) *Alkaline phosphatase coupled method.* This method is based on the fact that Gp_4G is not a substrate for alkaline phosphatase but the products (GTP + GMP) of its hydrolysis by the tetraphosphatase are. The standard assay conditions were as follows: 50 mM-Tris/HCl buffer, pH 8.0, 5 mM- $MgCl_2$, 0.35 unit of alkaline phosphatase (Sigma Chemical Co.), 0.6 mM- Gp_4G and extract, in a volume of 100 μ l. The mixtures were incubated at 37°C and the reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid. The samples were centrifuged, and the P_i was determined in the supernatants by the method of Fiske & SubbaRow (1925).

(2) *Hyperchromicity assay.* Unless otherwise stated, the reaction mixture contained, in a final volume of 1 ml, the following components: 50 mM-Tris/HCl buffer, pH 7.5, 5 mM- $MgCl_2$, 40 μ M- Gp_4G and extract. Increase in absorbance was followed at 252 nm in 1 cm-light-path cuvettes maintained at 37°C. The hydrolysis of 1 μ mol of Gp_4G is associated with an increase of 1.1 A_{252} units. In every case 1 unit is defined as the amount of enzyme able to transform 1 μ mol of substrate/min at 37°C.

Identification of the reaction products

For these experiments, the bis-(5'-guanosyl) tetraphosphatase from each tissue was partially purified on a Sephadex G-100 column (see the Results section). Two different methods were used to identify the products of the reaction. In one of them, a 70 μ l portion of the pooled fractions was incubated, in a

final volume of 90 μ l, with 1.6 mM- Gp_4G and 5 mM- $MgCl_2$. Samples (15 μ l) were taken from the reaction mixture before incubation and at the times indicated for each tissue: kidney and brain (10 and 20 min), muscle (45 and 70 min), intestinal mucosa (90 and 130 min). The reaction was stopped by immersion of the samples in a boiling-water bath for 1 min. Then they were analysed by poly(ethyleneimine)-cellulose t.l.c. The plates were purchased from Macherey-Nagel, and they were washed with water and dried again immediately before use. Four samples were spotted on each plate. One of them contained a mixture of GMP, GDP and GTP; the others corresponded to the three samples taken from the reaction mixtures employed for the assay of each tissue. The elution was accomplished with 1.2 M- KH_2PO_4 , adjusted at pH 3.8 with HCl. The plates were dried when the solvent had run to 12 cm ahead of the origin, and they were inspected under u.v. light (254 nm). In these conditions Gp_4G does not migrate, but p_4G , GTP, GDP, guanosine and GMP run with increasing mobilities.

Coupled enzymic assays were also used to measure the products of the hydrolysis of either Gp_4G or Ap_4A by the tetraphosphatase from the different tissues examined. For that, the following nucleotides were measured with the enzymic systems indicated in parentheses: GDP or ADP (pyruvate kinase and lactate dehydrogenase); GTP (phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase); ATP and AMP (adenylate kinase, pyruvate kinase and lactate dehydrogenase). Precise details of these methods were as previously described (Lobatón *et al.*, 1975).

Source of nucleotides

Gp_4G was obtained from *Artemia* cysts as reported previously (Vallejo *et al.*, 1974). The other nucleotides were purchased from Sigma Chemical Co.

Results

Distribution of the enzymic activity between the soluble and particulate fractions from rat tissues

A preliminary study of the subcellular distribution of the Gp_4G -hydrolysing activity in different rat tissues showed that it was almost exclusively located in the cytosol and nuclear pellets (5000 g precipitate). Accordingly, the occurrence and distribution of that activity in the 27000 g supernatant and sedimented fraction were examined in the selected tissues. That centrifugal force is appropriate for separation, in one run, the nuclear and membranous pellet from the cytosol, thus facilitating the processing of the homogenates. The microsomal vesicles that still may be present in the 27000 g

supernatant neither contain bis-(5'-guanosyl) tetraphosphatase nor interfere with the enzymic assay.

Liver, kidney, brain, skeletal muscle and intestinal mucosa were homogenized as described in the Materials and methods section, and the breis were centrifuged at 27000g for 1h. The Gp₄G-hydrolytic activity was measured with the alkaline phosphatase coupled assay. This is the more reliable method available when the activity on dinucleoside tetraphosphates is to be measured in crude extracts. Nevertheless, the samples must be extensively dialysed to eliminate nucleotides, which yield a very high background of P_i in control assay mixtures without Gp₄G. The results of these experiments are presented in Table 1. The total Gp₄G-hydrolytic activity varies between 2.1 units/g (intestinal mucosa) and 0.36 unit/g (muscle). Concerning the subcellular distribution, most of the enzyme present in the intestinal mucosa is in the 27000g-sedimented fraction, whereas the activity is preferentially located in the supernatant fractions of brain and muscle. The distribution of the activity is more nearly equal between the two fractions in liver and kidney. As shown below, in the present paper we deal exclusively with the soluble enzyme.

Properties of the enzyme from the 27000g supernatant

As stated in the introduction, the enzyme from rat liver had been previously studied by Lobatón *et al.* (1975), and in the present paper it is used as a reference. The occurrence and properties of the enzyme from other mammalian tissues were not known. The approach followed was as indicated. Non-dialysed supernatants of all the tissues were chromatographed in a column (1cm × 90cm) of Sephadex G-100 equilibrated with 50mM-Tris/HCl/0.5mM-EDTA buffer, pH 7.5. Samples of volume 2ml were applied for every tissue extract. In the

cases of brain, muscle and intestinal mucosa, the 27000g supernatants were put into Minicon B-15 concentrators (from Amicon Corp.) so as to give, in a volume of 2ml, bis-(5'-guanosyl) tetraphosphatase activities of 180, 425 and 1260 munits respectively. The large amount of the intestine enzyme applied to the column was because of the elevated rate of inactivation during the chromatographic run. Elution was accomplished with the above-cited buffer at 10ml/h, and 2.7ml fractions were collected. The tetraphosphatase activity from all tissues was eluted as a single peak between fractions 15 and 25, which were pooled and used in the following experiments, unless otherwise stated.

Two methods were followed to investigate the products of the reaction. In one of them Gp₄G was incubated with bis-(5'-guanosyl) tetraphosphatase, and the products were analysed by t.l.c. as described in the Materials and methods section. With the enzymic preparations from the four tissues examined (brain, muscle, kidney and intestinal mucosa), the hydrolysis of Gp₄G was accompanied by the appearance of two u.v.-absorbing spots with the same mobilities as GTP and GMP. No spot corresponding to GDP was observed. The products of the reaction were also analysed spectrophotometrically with coupling of the hydrolysis of Gp₄G to the phosphoglycerate kinase/glyceraldehyde 3-phosphate dehydrogenase system to measure GTP. In the cases of brain, kidney and muscle, 1 mol of GTP was formed per mol of Gp₄G hydrolysed. Accurate results could not be obtained with the enzymic preparation from intestinal mucosa, owing to the high increase in A₃₄₀ observed in the absence of Gp₄G. In order to confirm the t.l.c. results obtained with the intestinal enzyme (see above), and since no other method was available for the assay of GTP and/or GMP, the enzymic activity was assayed with Ap₄A as substrate. When the hydro-

Table 1. *Distribution of the Gp₄G-hydrolysing activity between the 27000g supernatant and 27000g-sedimented fraction in rat tissues*

The hydrolytic activities are presented as means ± S.D. obtained from three to five independent experiments, each of them corresponding to one animal, except those performed with intestinal mucosa, which were performed with two rats. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Further experimental details are given in the text.

	Activity (munits/g of fresh tissue)			Specific activity (munits/mg of protein)		
	Homogenate	27000g supernatant	27000g-sedimented fraction	Homogenate	27000g supernatant	27000g-sedimented fraction
Liver	768 ± 206	223 ± 28	566 ± 211	4.3 ± 1.2	3.0 ± 0.1	4.5 ± 1.7
Kidney	1444 ± 393	680 ± 193	710 ± 108	9.7 ± 2.8	12.9 ± 3.0	7.2 ± 2.9
Brain	390 ± 26	254 ± 77	43 ± 5	3.1 ± 0.2	10.1 ± 2.6	0.5 ± 0.1
Skeletal muscle	363 ± 124	199 ± 112	15 ± 9	2.1 ± 0.6	6.1 ± 2.6	0.12 ± 0.07
Intestinal mucosa	2143 ± 538	190 ± 54	2061 ± 508	40 ± 16	8.0 ± 2.0	64 ± 25

lysis of Ap_4A was coupled to the adenylate kinase/pyruvate kinase/lactate dehydrogenase system. 2 mol of ADP was formed per mol of Ap_4A hydrolysed. Nevertheless, when the adenylate kinase was omitted in the reaction mixture, no formation of ADP was observed. This result shows that 1 mol of Ap_4A is split to 1 mol of ATP and 1 mol of AMP by the intestinal enzyme. In a similar manner, no production of GDP was observed when the hydrolysis of Gp_4G was coupled to the pyruvate kinase/lactate dehydrogenase system, with the enzymic preparations from the four tissues here examined. Altogether, the results above presented indicate that the enzymic activities here described split dinucleoside tetraphosphates to the corresponding nucleoside tri- and mono-phosphates, as with the similar enzyme previously reported in liver (Lobatón *et al.*, 1975).

The effects of substrate and p_4G concentrations on bis-(5'-guanosyl) tetraphosphatase initial velocity were assayed with the continuous spectrophotometric method at 252 nm. The results were plotted in double-reciprocal form, and K_m (Gp_4G) and K_i (p_4G) values were calculated (see Table 2). Kidney, muscle and brain enzymes displayed K_m values within the range 2–7 μM , but three independent experiments performed with the intestinal enzyme yielded 10-fold higher K_m values. Inhibition by p_4G was competitive in every case with nanomolar K_i values, the lowest and the highest ones being obtained with the kidney and intestine enzymes respectively.

The hydrolysis of Gp_4G by the Sephadex G-100 eluates obtained from all the tissues was strictly dependent on the presence of Mg^{2+} or Mn^{2+} in the assay mixtures. Higher activities were always ob-

Fig. 1. Inhibition of bis-(5'-guanosyl) tetraphosphatase by Ca^{2+}

Initial velocities, v , were measured with the hyperchromicity method, as described in the text, with various concentrations of Gp_4G , both in the absence (Δ) and in the presence of two different (○, lower; ●, higher) concentrations of CaCl_2 : liver, 0.11 and 0.27 mM; kidney, 0.12 and 0.24 mM; brain, 0.12 and 0.24 mM; muscle, 0.15 and 0.30 mM; intestinal mucosa, 0.75 and 1.5 mM. Further experimental details are given in the text.

Table 2. *Properties of bis-(5'-guanosyl) tetraphosphatase from different rat tissues*

All the data are from the present work, except the results from liver, which were taken from the literature (Lobatón *et al.*, 1975; Vallejo *et al.*, 1976) and are included here for comparison. All the measurements were performed with the hyperchromicity assay. Further comments and experimental details are given in the text. V is the highest velocity obtained with various concentrations of either MgCl_2 or MnCl_2 , as indicated, at constant concentration of Gp_4G .

	K_m (Gp_4G) (μM)	K_i (p_4G) (nM)	Optimum [Mg^{2+}] (mM)	Optimum [Mn^{2+}] (mM)	V (Mn^{2+}) V (Mg^{2+})	Molecular weight	Optimum pH
Liver	2	13	5	0.5	0.15	22 000	8–8.5
Kidney	1.4–2.4	9	2–3	0.35	0.5	20 000	8
Brain	4–7	52	2–5	1.5–2.5	0.6	21 200	7.5
Skeletal muscle	2	17	2	0.7	0.3	21 900	7.5
Intestinal mucosa	20–50	160	5	1	0.6	19 900	7–7.5

tained with MgCl_2 , the optimal concentration range being 2–5 mM; the optimal concentration range for MnCl_2 was lower (see Table 2). Maximum velocity with Mn^{2+} was never more than 60% of that obtained with Mg^{2+} . Ca^{2+} , at concentrations of up to 10 mM, could not substitute for these ions. Indeed, a decrease in the activity of rat Mg^{2+} -dependent bis-(5'-guanosyl) tetraphosphatase was observed when CaCl_2 was added to the reaction mixture. When initial velocities were measured at constant 5 mM- MgCl_2 , both in the absence and in the presence of CaCl_2 , and various concentrations of Gp_4G , the results shown in Fig. 1 were obtained. Line-weaver–Burk plots clearly show that the inhibition of the tetraphosphatase by Ca^{2+} is of mixed type for the liver, kidney, brain and muscle enzymes. As defined by Dixon & Webb (1979), this type of inhibition causes the reciprocal plots to intersect anywhere to the left of the $1/v$ axis, except in the $1/s$ axis. In contrast, in the plot for the intestinal enzyme (Fig. 1), the lines tend either to intersect under the $1/s$ axis or to be parallel, corresponding to mixed or to uncompetitive inhibition respectively, according to the definitions given by Dixon & Webb (1979). K_i calculations have not been attempted in any case.

The bis-(5'-guanosyl) tetraphosphatase activity was measured at different pH values, with Tris/HCl and Tris/acetate buffers. Maximum velocities were observed between pH 7.0 and 8.5 (Table 2).

Molecular weights were determined by gel-filtration procedures as follows. Samples (1 ml) from the 27 000g supernatants from rat tissues were applied to a column (1 cm $\times$ 90 cm) of Sephadex G-75 equilibrated with 50 mM-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5. The elution was performed with the same buffer at a constant flow of 16.5 ml/h, and 0.99 ml fractions were collected. Elution volume was calculated from the elution profile of bis-(5'-guanosyl) tetraphosphatase, and molecular weights were extrapolated from a calibration graph, which was obtained passing the following markers

(1 ml each) through the column: cytochrome *c* (mol.wt. 12 400) (3 mg/ml), myoglobin (mol.wt. 17 000) (3 mg/ml), adenylate kinase (mol.wt. 21 300) (0.1 mg/ml), chymotrypsinogen (mol.wt. 25 000) (3 mg/ml) and ovalbumin (mol.wt. 45 000) (3 mg/ml). The apparent molecular weights obtained for the bis-(5'-guanosyl) tetraphosphatase of the tissues considered are in Table 2.

Discussion

From Table 1 it is apparent that the five tissues examined have a significant hydrolytic activity towards dinucleoside tetraphosphates but that there are quantitative differences among them. The highest total activity is shown by intestinal mucosa, whereas muscle and brain exhibit the lowest one. There are also differences in the subcellular distribution, with muscle and brain activities being predominantly in the 27 000g supernatant, and the intestinal-mucosa and liver activities being mainly in the 27 000g-sedimented fraction. The same picture emerges if specific activity (units/mg of protein) is considered instead of the activity/g of tissue, though some of the dissimilarities among the tissues are enhanced; e.g., the specific activity of the mucosa enzyme (total or particulate) is very markedly greater than the values for the other tissues. On the other hand, the activity present in the 27 000g supernatants seems to be more constant, with values fairly near to 200 munits/g in all the tissues examined, except kidney, which exhibits significantly higher activity. The properties found for the soluble enzyme of kidney, brain and muscle are very similar to those previously described for the bis-(5'-guanosyl) tetraphosphatase from liver (Lobatón *et al.*, 1975; Vallejo *et al.*, 1976). Very low K_m values for Gp_4G and Ap_4A (2 μM both), competitive inhibition by p_4G ($K_i = 10 \text{ nM}$), a molecular weight of about 22 000 and a strict requirement for Mg^{2+} or Mn^{2+} are characteristic properties of the enzyme.

This can be presented as evidence for the existence of a bis-(5'-guanosyl) tetraphosphatase similar to the hepatic one in those tissues. The enzyme from the intestine displays higher K_m and K_i values for Gp_4G and p_4G respectively, and also a different pattern of Ca^{2+} inhibition (Fig. 1), which suggests the presence of a different enzyme form in the intestinal mucosa. However, a definitive conclusion is not feasible because of the instability of the enzyme from that tissue (a feature not shared by the enzyme activities from the other tissues studied). The extent of inactivation was about 50% and 80% after 1 and 4 days of incubation at 4°C respectively. The enzyme was stable during the first 20 min, but had lost 35% of its activity after 60 min of incubation at 37°C. In contrast, the bis-(5'-guanosyl) tetraphosphatase activity from liver, kidney, muscle and brain did not decay during considerably longer periods at either 4°C or 37°C. Thus the possibility that the kinetic properties observed *in vitro* for the mucosa enzyme do not agree with those prevailing *in vivo* must be seriously considered.

Inhibition of bis-(5'-guanosyl) tetraphosphatase by Ca^{2+} deserves a special comment, since this ion participates in the control of the cell cycle in mammals, both at mitotic and interphase stages (Dedman *et al.*, 1979; Means & Dedman, 1980; Whitfield, 1980). As has been shown for Ap_4A (Grummt *et al.*, 1979), a role for Ca^{2+} has also been implied in the initiation of DNA synthesis (Whitfield, 1980). Ap_4A seems to be an activator of DNA polymerase α (Grummt *et al.*, 1979). The mechanism of Ca^{2+} action is not known, but possibly Ca^{2+} could influence the cell cycle through inhibition of the tetraphosphatase and subsequent increase in the concentration of Ap_4A . In this context, the cytosolic concentration of free Ca^{2+} is between 0.1 and 10 μM (Whitfield, 1980), whereas the total concentrations of Ca^{2+} during our assays were between 0.1 and 1 mM. Nevertheless the effect of Ca^{2+} on bis-(5'-guanosyl) tetraphosphatase is enhanced when the assay is performed in the presence of lower Mg^{2+} concentration. When the kidney tetraphosphatase was assayed in the presence of 20 μM - Ap_4A and 0.5 mM- $MgCl_2$, a 50% inhibition was elicited by 25 μM - $CaCl_2$. This value of total Ca^{2+} approaches to the upper limit of the free Ca^{2+} concentrations in cells. On the other hand, the free concentration of Mg^{2+} inside the cell has been reported to be about 1 mM (Peck & Ray, 1971; Williams, 1974). Consequently we consider that Ca^{2+} could be an important factor in the regulation of dinucleoside tetraphosphate metabolism and that, in turn, bis-(5'-guanosyl)

tetraphosphatase could be a link between Ca^{2+} and Ap_4A effects on DNA synthesis.

This investigation was supported by grants from Diputación Foral and Caja de Ahorros Provincial de Alava, Ayuntamiento and Caja de Ahorros Municipal de Vitoria, Fondo Nacional para el Desarrollo de la Investigación Científica and Fundación Juan March. We are also especially indebted to Miss Asunción Marquinez and Mr. Juan Manuel Rodriguez for their excellent technical assistance.

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