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Diguanosine 5′,5‴-P^1,P^4-tetraphosphate and other purine nucleotides inhibit endoribonuclease VI from *Artemia*

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The activity of the endoribonuclease VI from *Artemia* is sensitive to several purine nucleotides. The enzyme is non-competitively inhibited by diguanosine tetraphosphate ($K_i = 75$ μM), a nucleotide abundant in *Artemia* encysted gastrulae and located in the same particulate fraction as the gastrular ribonuclease. Diguanosine triphosphate and diadenosine tetraphosphate are less efficient inhibitors ($K_i \simeq 200$ μM). The ribonuclease is non-competitively inhibited by 5′-AMP ($K_i = 10$ μM) and 5′-GMP ($K_i = 50$ μM) but is insensitive to the corresponding 5′-phosphates of cytosine and uridine. Other purine mononucleotides inhibit the enzyme activity less efficiently. The modulation of the enzyme activity by these nucleotides is discussed in relation with the changes in ribonuclease activity during early development of *Artemia*.

Diguanosine tetraphosphate; Purine nucleotide; RNase; (*Artemia*)

1. INTRODUCTION

The activity of endoribonuclease VI from *Artemia,* a very specific enzyme for cleaving UpN bonds [1,2], is controlled during the development of *Artemia* by mechanisms that are poorly understood [1,2]. Previous studies [3,4] showed that the appearance of enzymatic activity occurs in a two-step process: an initial burst concomitant with hatching of the nauplii, followed by a second increase which is dependent on protein biosynthesis but independent of mRNA transcription [4]. Based on these observations the hypothesis was advanced [3,4] that the increase in enzymatic activity could be due in part to an unmasking process. Its dependence on protein biosynthesis could be a consequence of the need for some protein that is required for activation of a pro-enzyme or inactivation of an inhibitor acting on the gastrular enzyme. In line with the second possibility we initiated a search for effectors that could modulate the ribonuclease activity. Previously, we found that 2′-CMP was a strong and specific inhibitor of this enzyme [5]. To our knowledge, there is no evidence of the existence of this compound in *Artemia,* although an enzyme able to hydrolyze 2′,3′-cyclic nucleotides to give the corresponding 2′-isomers has been observed in several tissues [6]. Consequently, we directed our attention to regulatory compounds present in *Artemia* as possible modulators of the ribonuclease activity. Among these compounds, diguanosine 5′,5‴-P^1,P^4-tetraphosphate (Gp_4G) is the most abundant [7,8], exerts a regulatory effect on enzymes of very different catalytic properties [9–15], and its levels decrease at the time of the expression of ribonuclease activity [3,8,15]. Moreover, Gp_4G is located in the $700 \times g$ heavy subcellular fraction consisting mainly of nuclei and yolk granules as occurs with the gastrular ribonuclease [3,8,15].

Here, we present evidence showing that Gp_4G and, less efficiently, Gp_3G are inhibitors of *Artemia* endoribonuclease VI. Other purine nucleotides are also inhibitors with even greater efficiency than the diguanosine nucleotides. The significance of these levels of inhibition is discussed in relation with the control of this enzyme activity during development of *Artemia*.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Mono, di- and polynucleotides were obtained from Sigma (St. Louis, MO).

2.2. *Enzymes*

The *Artemia* endoribonuclease VI used in this work is a partially purified preparation (step IV) [2].

Alkaline phosphatase and snake venom phosphodiesterase were from Boehringer. Rabbit muscle adenylic acid deaminase (ADA) (EC 3.5.4.6) and myokinase (MK) (EC 2.7.4.3) were from Sigma. Ribonuclease activity was determined as in [5] with 10 mM $CaCl_2$ replacing the magnesium acetate unless otherwise stated.

Digestion of Gp_4G (0.4 mM) and Gp_3G (1 mM) with alkaline phosphatase (12 U) and snake venom phosphodiesterase (3 mU) was performed in incubation mixtures (0.4 ml) containing 50 mM Tris-HCl (pH 8), 6 mM $Mg(CH_3COO)_2$ and the enzymes either alone or in combination. After incubation overnight at 30°C the mixtures were heated to 90°C and appropriate aliquots were added to reaction mixtures for the ribonuclease assay indicated above, to reach the final concentrations of nucleotides listed in table 1.

3. RESULTS AND DISCUSSION

Gp_4G is abundant in *Artemia* encysted gastrulae and is located in the same ($700 \times g$) heavy subcellular fraction as endoribonuclease VI [16]. The effect of increasing the Gp_4G concentration on the initial velocity of *Artemia* endoribonuclease VI is shown in fig.1. Half inhibition is achieved at around 75 μM, which is within the range of concentrations of Gp_4G present in the cytosol of *Artemia* cysts [8]. The inhibition produced by Gp_4G is stronger with Ca^{2+} than with Mg^{2+} at equimolar concentrations and is less apparent in crude enzyme preparations. Gp_3G is also an inhibitor although less efficient than Gp_4G ($K_i \simeq 200$ μM). In order to confirm that the observed levels of inhibition were in fact due to Gp_4G and Gp_3G, we performed the experiments described in table 1. The nucleotides were subjected separately to the action of alkaline phosphatase and snake venom phosphodiesterase alone or in combination. Gp_4G and Gp_3G are insensitive to the former but are hydrolyzed by the phosphodiesterase to GMP plus GTP or GDP, respectively. These digestion products are sensitive to the action of alkaline phosphatase. As shown in table 1, the inhibition by Gp_4G and Gp_3G is completely abolished after treatment with the two enzymes in combination, but does not disappear after treatment of the

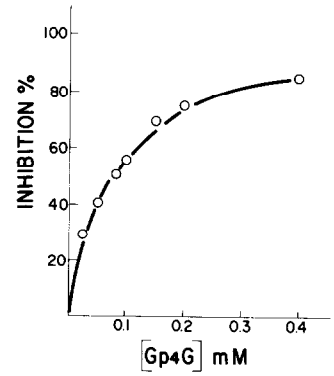

Fig.1. Effect of Gp_4G on ribonuclease activity. Reactions were carried out as described in section 2 with 10 mM $CaCl_2$ and different concentrations of Gp_4G as indicated.

dinucleotides with either enzyme separately. These results demonstrate that the observed levels of inhibition are in fact due to Gp_4G and Gp_3G and that the digestion products of these dinucleotides by the venom phosphodiesterase are also inhibitors of the endoribonuclease. That this is so is confirmed by the experiment in fig.2 which shows the differential effect of increasing concentrations of GMP, GDP and GTP on the initial velocity of the enzyme. The apparent affinity of the ribonuclease for GMP ($K_i = 50$ μM) is even higher than that for Gp_4G or Gp_3G. Changing guanine for adenine results in an increase in efficiency of the nucleotide at inhibiting ribonuclease activity. Half inhibition is attained at 10 μM 5'-AMP. The results in table

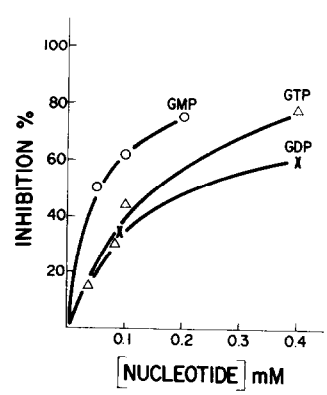

Fig.2. Effect of guanosine nucleotides on ribonuclease activity. Reactions were carried out as described in section 2 with the indicated concentrations of each nucleotide.

Table 1

Sensitivity of the inhibition produced by Gp_4G and Gp_3G to digestion by alkaline phosphatase and snake venom phosphodiesterase

Conditions	Activity (A_{260}/30 min)	Inhibition (%)
(1) No additions	1.6	–
Gp_4G, 250 μM (A)	0.7	58
(A) + phosphatase	0.9	56
(A) + phosphodiesterase	0.3	81
(A) + phosphatase + phosphodiesterase	1.6	–
(2) No additions	1.5	–
Gp_3G, 200 μM (B)	0.7	54
(B) + phosphatase	0.8	47
(B) + phosphodiesterase	0.7	54
(B) + phosphatase + phosphodiesterase	1.7	–

Digestion of Gp_4G and Gp_3G and determination of the ribonuclease activity were as described in section 2

Table 2

Inhibition of the ribonuclease by 5′-AMP

Conditions	Activity (A_{260}/20 min)	Inhibition (%)
(1) No additions	0.9	–
AMP, 50 μM (A)	0.2	78
(A) + ADA	0.8	8
(2) No additions	1.3	–
ADP, 50 μM (A)	1.1	–
ADP, 100 μM (B)	1.1	–
(A) + MK	0.7	47
(B) + MK	0.5	62

When indicated (Expt 1) AMP (24 nmol) was deaminated by incubation at 30°C for 3 h, with 10 U adenylic acid deaminase (ADA) in reaction mixtures (0.4 ml) containing 125 mM Hepes (pH 7.5), 12.5 mM $Mg(CH_3COO)_2$, 1.25 mM EDTA and 62.5 mM KCl. 80 μl of this preincubated mixture were mixed with 2 A_{260} units of poly(U) and appropriate amounts of ribonuclease in a final volume of 0.1 ml and the reaction was followed as indicated in section 2. In expt 2, AMP was synthesized from ADP (5 and 10 nmol) with myokinase (MK, 90 U) in reaction mixtures (80 μl) containing 50 mM Hepes (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM EDTA, 50 mM KCl. After 5 min at 30°C appropriate amounts of ribonuclease and 2 A_{260} units of poly(U) were added to these preincubated mixtures (final volume, 0.1 ml) and the ribonuclease activity was followed as described in section 2

2 demonstrate that the inhibition is in fact due to 5′-AMP. Synthesis of AMP by incubation of ADP (which is not inhibitory up to 200 μM) with myokinase results in the appearance of an inhibitory effect. Conversely, deamination of AMP with adenylic acid deaminase results in loss of the inhibition produced by AMP (table 2). The observed inhibition by nucleotide 5′-monophosphates is base-specific. None of the pyrimidine mononucleotides 5′-CMP, 5′-IMP and 5′-UMP are inhibitors at 100 μM (table 3). These findings contrast with those previously obtained for the 2′-nucleotide monophosphates in that only the pyrimidine nucleotide 2′-CMP, and none of the other base isomers, was a potent inhibitor of the ribonuclease [5]. The results in table 3 summarize the relative efficiencies of several nucleotide mono- and polyphosphates as inhibitors. Among the polyphosphates tested, Gp_4G is the most efficient. AMP and GMP are the best inhibitors among the 5′-monophosphates. The affinity of the ribonuclease for these 5′-nucleotide monophosphates is one order of magnitude lower than that observed for 2′-CMP ($K_i = 1$ μM) [5]. The levels of inhibition produced by the nucleotides shown in table 3 are non-competitive with respect to the substrate.

From this and previous work [5], it follows that the activity of *Artemia* endoribonuclease VI can be modulated in vitro by several nucleotides. The identification of the mode of operation of these mechanisms in the cell needs further work and will become more apparent when the physiological role of this enzyme has been elucidated. However, it could be envisaged that the sensitivity to some of these compounds may contribute to the changes in enzyme activity observed during early development of *Artemia*. For example, inhibition by Gp_4G could contribute to the low activity found in en-

Table 3

Nucleotide specificity of inhibition of the ribonuclease

Nucleotide	K_i (μM)
5′-AMP	10
ADP, 2′-AMP, Ap_4A	≥200
ATP, 3′-AMP, cAMP	–
5′-GMP	50
Gp_4G	75
GDP, GTP, Gp_4, Gp_3G	≥200
5′-UMP, 5′-CMP, 5′-IMP	–

(–) Less than 20% inhibition at 100 μM

cysted gastrulae [3], as Gp_4G is present at high concentrations (10 mM) in the same particulate fraction as the gastrular ribonuclease [8,15]. The change in enzyme location upon emergence of the nauplii to the cytosol [3] where the concentration of Gp_4G is much lower (0.1 mM) [8] results in an increase in enzymatic activity as demonstrated [3]. The implication of this ribonuclease in mRNA processing has been suggested based on its narrow substrate specificity to cleave UpU and UpA bonds [1,2] together with the existence of internal poly(U) sequences that are mostly processed during the maturation of mRNAs [20]. *Artemia* encysted gastrulae contain stored mRNAs that are translated during the early stages of development [18,19] at the time of increase in the activity of this endoribonuclease.

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