Dinucleoside polyphosphates stimulate the primer independent synthesis of poly(A) catalyzed by yeast poly(A) polymerase

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Novel properties of the primer independent synthesis of poly(A), catalyzed by the yeast poly(A) polymerase are presented. The commercial enzyme from yeast, in contrast to the enzyme from *Escherichia coli*, is unable to adenylate the 3′-OH end of nucleosides, nucleotides or dinucleoside polyphosphates (Np_N). In the presence of 0.05 mM ATP, dinucleotides (at 0.01 mM) activated the enzyme velocity in the following decreasing order: Gp4G, 100; Gp3G, 82; Ap6A, 61; Gp2G, 52; Ap3A, 51; Ap2A, 41; Gp3G, 36; Ap5A, 27; Ap2A, 20, where 100 represents a 10-fold activation in relation to a control without effector. The velocity of the enzyme towards its substrate ATP displayed sigmoidal kinetics with a Hill coefficient (n_H) of 1.6 and a K_m(S,3) value of 0.308 ± 0.120 mM. Dinucleoside polyphosphates did not affect the maximum velocity (V_max) of the reaction, but did alter its n_H and K_m(S,3) values. In the presence of 0.01 mM Gp4G or Ap4A the n_H and K_m(S,3) values were (1.0 and 0.063 ± 0.012 mM) and (0.8 and 0.170 ± 0.025 mM), respectively. With these kinetic properties, a dinucleoside polyphosphate concentration as low as 1 μM may have a noticeable activating effect on the synthesis of poly(A) by the enzyme. These findings together with previous publications from this laboratory point to a potential relationship between dinucleoside polyphosphates and enzymes catalyzing the synthesis and/or modification of DNA or RNA.

*Keywords:* Ap4A; Gp4G; dinucleoside polyphosphates; yeast poly(A) polymerase.

We have recently shown that *Escherichia coli* poly(A) polymerase adenylates the 3′-OH end of nucleosides, nucleotides and dinucleotides of the type nucleoside (5′) oligophospho (5′) nucleosides (Np_N) [1]. This novel property of *E. coli* poly(A) polymerase moved us to analyze whether these compounds were also substrates of eukaryotic yeast poly(A) polymerase. The yeast enzyme is involved in the processing of the 3′-OH end of mRNA [2,3], forming a complex with two cleaving factors and a polyadenylation factor [4,5]. The core yeast poly(A) polymerase appears to have a molecular mass of around 63 kDa [3]. Separated form the complex, the core yeast enzyme catalyzes the addition of poly(A) tails to a variety of RNAs or poly(A) of different lengths [3]. The experiments described below were carried out with a commercial preparation obtained from an *E. coli* strain containing a clone of the yeast poly(A) polymerase gene [6]. In principle, it can be assumed that this preparation corresponds to pure poly(A) polymerase with no contaminating cleaving factors.

While using this preparation, we observed that primer independent poly(A) synthesis was activated by dinucleoside polyphosphates. The findings reported here could open new views both on the catalytic properties of yeast poly(A) polymerase and on the intracellular role of dinucleoside polyphosphates, a family of compounds of increasing metabolic and regulatory interest [7–11].

**MATERIALS AND METHODS**

**Materials**

Poly(A) polymerase from yeast was from Amersham Pharmacia Biotech (Code 7422SZ, lot numbers: 109217; 109899; 110378; 111182. One unit of enzyme is the amount that incorporates 1 nmol of ATP (as AMP) into an acid insoluble form in 1 min at 37 °C. These preparations contained 761 U·mL⁻¹ (1522 U·mg⁻¹ protein). When required, the enzyme was diluted in 0.25% bovine serum albumin (BSA). Shrimp alkaline phosphatase (EC 3.1.3.1) was from Roche Molecular Biochemicals and phosphodiesterase (from *Crotalus durissus*, EC 3.1.4.1) was from Boehringer Mannheim. [α-32P]ATP (3000 Ci mmol⁻¹) was from Dupont NEN. TLC silica-gel fluorescent plates were from Merck. X-ray films were from Konica Corporation. Radioactively labeled nucleotides were quantified by the use of an InstantImager (Packard Instrument Co.) HPLC was carried out in a Hewlett Packard chromatograph (model 1090), with a diode array detector, commanded by an HPLC ChemStation. The Hypersil ODS column (2.1 × 100 mm) was from Hewlett Packard.
METHODS

Enzyme assays

Unless indicated otherwise the reaction mixtures contained 20 mM Tris/HCl pH 7.0, 50 mM KCl, 0.7 mM MnCl₂, 0.2 mM EDTA, 100 μg/mL acetylated bovine serum albumin (acetylated BSA), 10% (v/v) glycerol, ATP, MgCl₂, yeast poly(A) polymerase and, where indicated, other nucleotides or dinucleotides. After incubation at 30 °C or 37 °C the reaction mixtures were analyzed by TLC or HPLC. When indicated, the reaction mixtures were treated with 20 mM phosphodiesterase for 1 h at 37 °C, and after inactivation of the phosphatase, by heating at 90 °C for 5 min, treated further with 20 μg/mL phosphodiesterase for 1 h at 37 °C.

TLC

The reaction mixtures (usually 0.01–0.02 mL) contained (0.02 mM)[γ⁻32P]ATP (20 μCi/mL⁻¹). Aliquots (0.0015 mL) of the reaction were taken, spotted on silica gel plates, and developed in dioxane/ammonium hydroxide/water 6 : 1 : 6 (v/v/v). Nucleotide spots were localized with a 253-nm wavelength light and the radioactivity measured by autoradiography and/or with an InstantImager.

HPLC

Aliquots (0.01 mL) of the reaction mixtures (usually in a volume of 0.035 mL) were transferred into 0.1 mL of water and kept at 95 °C for 1.5 min. After chilling, the mixtures were filtered (using a Millipore HA, 0.45 μm nitrocellulose membrane) and a 0.05-mL aliquot injected into a Hypersil ODS column. Elution was performed at a flow rate of 0.5 mL/min with a 20-min linear gradient (5–30 mM) of sodium phosphate (pH 7.5), in 20 mM tetrabutylammonium bromide/20% methanol (v/v) (buffer A) followed by a 10-min linear gradient (30–100 mM) of sodium phosphate (pH 7.5) in buffer A.

RESULTS

Comparison of poly(A) polymerase from E. coli and yeast

As stated in the Introduction, E. coli poly(A) polymerase, in the presence of micromolar concentrations of ATP, adenylates the 3’-OH residues of most of the nucleosides, nucleotides and dinucleotides tested and, under our experimental conditions, is unable to catalyze the synthesis of a poly(A) chain in the absence of a primer [1]. In order to explore whether the yeast enzyme also exhibited the same properties we assayed, in parallel, the activity of both enzymes on guanosine, GDP and diguanosine tetraphosphate (Gp₄G), in the presence of 0.02 mM [γ⁻32P]ATP. While confirming the adenylylation of guanosine, GDP or Gp₄G, the yeast enzyme. In the absence or presence of these compounds, labeled ATP was transformed mainly into a radioactive spot retained at the origin of the TLC plate, a position that could correspond to poly(A) chain(s). In addition, the chromatographic pattern of the radioactive synthesized products changed in the presence of Gp₄G (see below). From these results, it seemed of interest to explore the effect of diguanosine polyphosphates (GpₙG) on yeast poly(A) polymerase.

Effect of diguanosine polyphosphates on yeast poly(A) polymerase

The enzyme was incubated with 0.2 mM ATP and in the absence or presence of Gp₂G, Gp₃G, Gp₄G and the reaction products analyzed by HPLC after 30, 60 and 120 min incubation. In the absence of dinucleotides, the amount of ATP decreased slowly along the incubation time, with no concomitant increase of any ATP derivative (Fig. 1A). In the presence of diguanosine polyphosphates (Gp₂G, Gp₃G or Gp₄G), ATP consumption was strongly stimulated, but again, formation of potential products of the reaction was not observed. The results obtained after 30 min incubation are represented in Fig. 1B. The apparent loss of ATP was assumed to be due to the formation of a product, probably poly(A), that could be retained by the column.

To test this assumption, the enzyme was incubated with 0.2 mM ATP, under the same experimental conditions as in Fig. 1, for 60 min at 37 °C. A control without enzyme was also carried out. The complete reaction mixture was then divided into equal parts and one of them treated with phosphodiesterase. The three samples involved were analyzed by HPLC. The amount of ATP in the control, indicates the ATP present at the start of the reaction (Fig. 2A); the ATP that was consumed after incubation with the polymerase (Fig. 2B), was totally recovered as AMP (Fig. 2C), when the reaction mixture was treated with phosphodiesterase before analysis by HPLC. From these results (Figs 1 and 2), it can be concluded that poly(A) was synthesized from ATP, in the absence of primer, and that Gp₂G, Gp₃G, and Gp₄G stimulated that synthesis.

Stimulation of poly(A) synthesis as a function of diguanosine diphosphate (Gp₂G) concentration

The concentration of dinucleoside polyphosphate needed to stimulate the synthesis of poly(A) was analyzed using Gp₂G as effector. Yeast poly(A) polymerase was incubated with 0.02 mM [γ⁻32P]ATP, in the absence and presence of three different concentrations of Gp₂G (0.001, 0.010, or 0.050 mM). After 5, 10 and 20 min incubation, aliquots of the reaction mixture were analyzed by TLC. The results corresponding to the 5-min incubation are shown in Fig. 3. No appreciable stimulation of poly(A) (spot at the origin) was observed in the absence of Gp₂G, whereas in its presence the ATP spot decreased, increasing consecutively the radioactivity at the origin. In the presence of 0.01 or 0.050 mM Gp₂G, almost no ATP was left in the assay after 5 min incubation. These results show that a concentration as low as 0.001 mM Gp₂G stimulates, under these conditions, the synthesis of poly(A) catalyzed by yeast poly(A) polymerase around sixfold.
Relative activity of GpₙGs as effectors of the synthesis of poly(A)

Based on the above results, the effect of several diguanosine polyphosphates on the synthesis of poly(A) was comparatively studied. The enzyme was incubated for 10 min with 0.05 mM [α-³²P]ATP, and in the absence or presence of Gp₂G, Gp₃G, Gp₄G, Gp₅G (0.01 mM each). Aliquots of the reaction mixture were applied to a TLC plate. Under these conditions, the maximum ATP consumed was less than 50% (Fig. 4A). Formation of poly(A) (spots at the origin) was clearly seen in the samples containing dinucleotides, but scarcely visible in the control reaction (with enzyme and without GpₙG) carried out in duplicate (lanes C). The reaction mixtures were treated further with alkaline phosphatase and (after inactivation of the phosphatase) with phosphodiesterase and analyzed by TLC as above (Fig. 4B). The results round off those presented in Figs 4A i.e. AMP, representing the amount of ATP incorporated into poly(A), appears preferentially in the reaction mixtures containing the effectors (Fig. 4B). From the radioactivity present in the AMP spot, the relative capacity of diguanosine polyphosphates to stimulate the synthesis of poly(A), considering a media of four experiments, was: Gp₄G, 100; Gp₃G, 82; Gp₂G, 52; Gp₅G, 36, where 100 represents a 10-fold activation in relation to a control without effector.

Effect of diadenosine polyphosphates on poly(A) polymerase

Previous experiments had shown that diadenosine polyphosphates also stimulated the synthesis of poly(A) catalyzed by yeast poly(A) polymerase. The relative activity of diadenosine polyphosphates as effectors of the poly(A) synthesis was assayed as in Fig. 4, using 0.05 mM [α-³²P]ATP as substrate, in the absence or presence of 0.01 mM Ap₄As. The relative efficiency of diadenosine polyphosphates to stimulate the synthesis of poly(A), considering a media of four experiments, was: Ap₆A, 61; Ap₄A, 51; Ap₃A, 41; Ap₅A, 27; Ap₃A, 20 (results not shown). These values were calculated relative to the maximal activation (100) considered for Gp₄G (see above).

Dinucleoside polyphosphates diminish the Kₘ(S₀.₅) value for ATP in the primer independent synthesis of poly(A)

In order to understand why dinucleoside polyphosphates activated the primer independent synthesis of poly(A), the effect of 0.01 mM Gp₄G or Ap₄A on the synthesis of poly(A) was analyzed at different ATP concentrations (0, 0.025, 0.05, 0.1 and 0.2 mM). Samples were taken after 10 min incubation (a time at which the velocity of the reactions were linear, as tested in previous assays) spotted on TLC plates and the rate of synthesis of poly(A) as a function of ATP concentration determined as in Fig. 4. Moreover, in these conditions less than 30% of the ATP was consumed in the case of the reaction mixtures containing effectors and the lowest concentration of substrate. The Michaelis-Menten (Fig. 5A), Lineweaver-Burk (Fig. 5B) and Hill (Fig. 5C) plots of the results showed that the enzyme presented a sigmoidal kinetics that tended to hyperbolic in the presence of Gp₄G or Ap₄A. From these plots, maximum velocity (V_max) and Kₘ(S₀.₅) values were determined. In the absence of effector, the enzyme presented a Hill coefficient of around 1.6 that decreased to around 1.0 and 0.8 in the presence of 0.01 mM Gp₄G or Ap₄A, respectively. The Kₘ(S₀.₅)
values for ATP were 0.308 ± 0.120 mM \((n = 5)\), 0.063 ± 0.012 mM \((n = 3)\) and 0.170 ± 0.025 mM \((n = 3)\) in the absence or presence of Gp\(_2\)G or Ap\(_4\)A, respectively. The \(V_{\text{max}}\) value determined for the primer independent synthesis of poly(A) was about the same in the absence or presence of dinucleotides, i.e. around 500 U mL\(^{-1}\) [equivalent to a rate \((k_{\text{cat}})\) of AMP incorporation of 1 s\(^{-1}\)] a value close to that determined in the presence of poly(A) as a primer, as stated by the manufacturer.

**DISCUSSION**

Some experimental aspects can be considered firstly, in relation to the methods currently used by others to assay poly(A) polymerases. As noted previously [1], the labeled RNA-(A)\(_n\) products synthesized by polymerases are usually determined by acid precipitation or phenol extraction and ethanol precipitation. The amount of radioactivity determined in those precipitates is the parameter used to determine the poly(A) polymerase activity [12–18]. Potential reaction products that do not precipitate with these procedures may pass unnoticed.

Adenylation of nucleosides, nucleotides and dinucleotides by \(E.\) coli poly(A) polymerase [1] was detected using TLC and HPLC methods, the same two methods used in this work to study the yeast enzyme. The TLC procedure involves spotting aliquots of the complete reaction mixture onto a plate and analysis of all the potential reaction products synthesized during incubation. In the HPLC procedure, the reaction mixture is heated and filtered (see Materials and methods). All the poly(A) products synthesized from ATP passed through this filter, but were retained by the precolumn or column. The enzyme activity could be followed either, by measuring the decrease of the ATP content in the reaction mixture or by treating the reaction mixture first with alkaline phosphatase (to hydrolyze residual adenosine 5’-phosphates to adenosine) and then with phosphodiesterase to hydrolyze the synthesized poly(A) to AMP. According to our results, the amount of

![Fig. 2. ATP consumption catalyzed by yeast poly(A) polymerase.](image)

![Fig. 3. Effect of different concentrations of Gp\(_2\)G on the synthesis of poly(A) catalyzed by yeast poly(A) polymerase.](image)
AMP so obtained was equimolar to the ATP consumed during the enzyme reaction.

The difference between the enzyme from E. coli and yeast concerning their substrate specificity towards nucleosides, nucleotides and dinucleotides is also worth noting. The yeast poly(A) polymerase, contrary to the E. coli enzyme, is apparently unable to adenylate the 3'-OH end of those compounds. However, the primer independent activity of the yeast enzyme is strongly activated by dinucleoside polyphosphates. Commercial yeast poly(A) polymerase presented, in the absence of primer, a sigmoidal kinetics towards its substrate ATP, with a Hill coefficient ($n_H$) of around 1.6. The presence of Gp$_4$G or Ap$_4$A changed the kinetic from sigmoidal to hyperbolic, decreasing the $K_m$ ($S_{0.5}$) value from 0.308 ± 0.120 mM to 0.063 ± 0.012 mM and 0.170 ± 0.025 mM in the presence of Gp$_4$G or Ap$_4$A.

Fig. 4. Effect of diguanosine polyphosphates (Gp$_2$G, Gp$_3$G, Gp$_4$G, Gp$_5$G) on the synthesis of poly(A) catalyzed by yeast poly(A) polymerase. The reaction mixture (0.02 mL) contained: 0.05 mM ATP, 0.4 μCi [α-32P]ATP, 0.01 mM Gp$_n$G, 0.19 units of the enzyme and other conditions as described in Materials and methods. After 10 min incubation at 30 °C, aliquots were taken, and spotted on a TLC plate (Part A). The rest of the reaction mixture was treated with shrimp alkaline phosphatase and phosphodiesterase as described in Materials and Methods and analyzed as above (Part B). Lane (– E): control without enzyme; lanes (C): complete reaction with no added dinucleotide; other lanes (1–4) with added Gp$_n$G (0.01 mM) as indicated.

Fig. 5. Influence of ATP concentration on the primer independent synthesis of poly(A) catalyzed by yeast poly(A) polymerase. Effect of Ap$_4$A or Gp$_4$G. The reaction mixture (0.02 mL) contained: variable concentrations of [α-32P]ATP (0.025–0.2 mM) specific activity: 320 μCi/mmol, 0.2 mM MgCl$_2$, 0.1 units of the enzyme, 0.01 mM Ap$_4$A or Gp$_4$G where indicated and other conditions as described in Materials and methods. After 10 min incubation at 30 °C, the reaction was stopped by heating 2 min at 90 °C, treated with alkaline phosphatase and phosphodiesterase and analyzed by TLC. ($v$, is expressed as μmol of AMP incorporated min$^{-1}$mL$^{-1}$).
respectively. This would result in an activation of the polymerase at low ATP concentrations.

We have as yet no clue on either the mechanism of action or the physiological significance of these findings. Nevertheless, the following points could be raised.

The occurrence of ApG, at (sub)micromolar concentrations has been described in all prokaryotic and eukaryotic systems examined [19] and the presence of millimolar concentrations of GpG and GpG in Artemia and other crustacea is well documented [20,21]; in addition, many members of the GpG, ApA and ApG families have been found in human blood platelets [22,23].

About the presence of these compounds in the nucleus, ApG is a dinucleotide specifically described to be present in that organelle [24,25], but due to the pore size of the nuclear envelope it can be considered that the (d)inucleotide content in the nucleus may be similar to that in whole cells [26]. An additional, and still unsolved problem, is the question of how much of the (d)inucleotide content in nuclei is free or ligated to nuclear structures [27] or present in the environment in which the poly(A) polymerase is located. This may have an influence on the enzyme as it seems to be a relationship between the enzyme activity and the concentration of both ATP and dinucleotides: poly(A) polymerase displays a sigmoidal kinetics that becomes hyperbolic in the presence of dinucleotides, a behavior that greatly enhances the enzyme activity particularly at low ATP concentrations; for dinucleotides, a behavior that greatly enhances the enzyme activity and the concentration of both ATP and dinucleotides. The sigmoidal kinetics displayed by the enzyme favors the formation of a sequence of adenylate units from adenosine triphosphate. Adenine dinucleotides are more active than adenine dinucleotides with even number of inner phosphates, but due to the low number of inner phosphates tend to be more efficient than those with odd number of phosphates.

We are aware that poly(A) polymerase has been described as a multienzyme complex that may have in vivo different, or additional, properties to those reported here. In any event, yeast poly(A) polymerase, as supplied by the manufacturer, is strongly activated by μmolar concentrations of dinucleotides, preferentially at low ATP concentrations. The physiological significance of these findings deserves further exploration.

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