Polyphosphates strongly inhibit the tRNA dependent synthesis of poly(A) catalyzed by poly(A) polymerase from *Saccharomyces cerevisiae*

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Abstract **Polyphosphates of different chain lengths (P_3, P_4, P_{15}, P_{35}), (1 μM) inhibited 10, 60, 90 and 100%, respectively, the primer (tRNA) dependent synthesis of poly(A) catalyzed poly(A) polymerase from *Saccharomyces cerevisiae*. The relative inhibition evoked by p_4A and P_4 (1 μM) was 40 and 60%, respectively, whereas 1 μM Ap_4A was not inhibitory. P_4 and P_{15} were assayed as inhibitors of the enzyme in the presence of (a) saturating tRNA and variable concentrations of ATP and (b) saturating ATP and variable concentrations of tRNA. In (a), P_4 and P_{15} behaved as competitive inhibitors, with K_i values of 0.5 μM and 0.2 μM, respectively. In addition, P_4 (at 1 μM) and P_{15} (at 0.3 μM) changed the Hill coefficient (n_H) from 1 (control) to about 1.3 and 1.6, respectively. In (b), the inhibition by P_4 and P_{15} decreased V and modified only slightly the K_m values of the enzyme towards tRNA.**

Key words: Poly(A) polymerase; Polyphosphates; Dinucleoside polyphosphates; *Saccharomyces cerevisiae*; P_4; P_{15}

1. Introduction

We have recently described novel properties for the poly(A) polymerases from *Escherichia coli* [1] and from *Saccharomyces cerevisiae* [2]. The first, but not the second enzyme, adenylylates the 3′-hydroxyl residues of nucleosides, nucleoside 5′-phosphates and nucleoside(5′)oligophospho(5′)nucleosides or dinucleoside polyphosphates (Np_nN). Dinucleoside polyphosphates stimulate the primer independent synthesis of poly(A) catalyzed by the yeast enzyme, activation not observed using the enzyme from *E. coli* [1]. In the absence of primer, the velocity of the enzyme from *S. cerevisiae* towards its substrate ATP displayed sigmoidal kinetics with a Hill coefficient of around 1.6 and a K_m ($S_{0.5}$) value of around 0.3 mM. In the presence of Gp_4G or Ap_4A, the kinetics became hyperbolic and the K_m ($S_{0.5}$) value for ATP decreased to 0.06 and 0.170 mM, respectively. These findings may have physiological implications since concentrations of dinucleoside polyphosphates as low as 1 μM have a noticeable activating effect on the primer independent synthesis of poly(A) catalyzed by the yeast enzyme [2].

Searching for other possible effectors of the primer independent synthesis of poly(A) we observed that polyphosphates of several chain lengths were strong inhibitors of yeast poly(A) polymerase, either in the absence or presence of primer tRNA. This inhibition is analyzed here, and the general physiological roles of polyphosphates are also discussed.

2. Materials and methods

2.1. Materials

Yeast poly(A) polymerase was from Amersham Pharmacia Biotech (Code 74225Z, lot numbers: 111182-015 and 111875-016. One unit (U) 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 600 U/ml (1.522 U/mg 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. Adenosine 5′-tetraphosphate (p_4A), P^1,P^4-di(adenosine-5′)tetraphosphate (Ap_4A), pyrophosphate (P_2) tripolyphosphate (P_3), tetrapolyphosphate (P_4), and the linear-chain polyphosphates with an average chain length of 15 ± 3 or 35 ± 4 phosphates were from Sigma, St. Louis, MO, USA. [α-^{32}P]ATP (3000 Ci/mmol) and [2,8-^{3}H]ATP (40 Ci/mmol) were from Dupont NEN and Amersham, respectively. TLC silica-gel fluorescent plates were from Merck, ion exchange DEAE paper-circles (DE81) from Whatman (Cat No. 3658 023) and X-ray films from Konica Corporation. Radioactively labeled nucleotides were quantified by the use of an InstantImager (Packard Instrument Co.).

2.2. Enzyme assays

Unless indicated otherwise the reaction mixtures contained 20 mM Tris–HCl pH 7.0, 50 mM KCl, 0.7 mM $MnCl_2$, 0.2 mM EDTA, 100 μg/ml acetylated BSA, 10% (v/v) glycerol, 0.05 mM ATP, 0.2 μM tRNA, polyphosphates and yeast poly(A) polymerase as indicated. After incubation at 30°C the reaction mixtures were analyzed by thin layer chromatography (TLC) or by ion exchange.

2.2.1. TLC. The reaction mixtures (0.02 ml) contained (0.05 mM) [α-^{32}P]ATP (10 μCi/ml). Aliquots (1.5 μl) of the reaction were taken, spotted on silica gel plates, and developed in dioxane/ammonium hydroxide/water 6:1:6 (v/v). The radioactivity in [α-^{32}P]AMP or [α-^{32}P]AMP incorporated into poly(A) was measured by autoradiography and/or with an InstantImager.

2.2.2. Ion exchange. The reaction mixture (0.05 ml) contained (0.05 mM) [^{3}H]ATP (20 μCi/ml). At different times of incubation, the RNA products synthesized were measured as follows: aliquots of 0.01 ml were spotted on DE81 circles (pretreated with 10 mM sodium pyrophosphate, ethanol washed, and dried) and processed as previously described [3]; papers were dropped immediately into a wash solution of 0.3 M ammonium formate (pH 7.8), 10 mM sodium pyrophosphate, and 0.1% SDS, gently stirred at intervals for at least 1 h, washed three additional times for 5 min, twice with 95% ethanol and once with diethyl ether, dried and counted.

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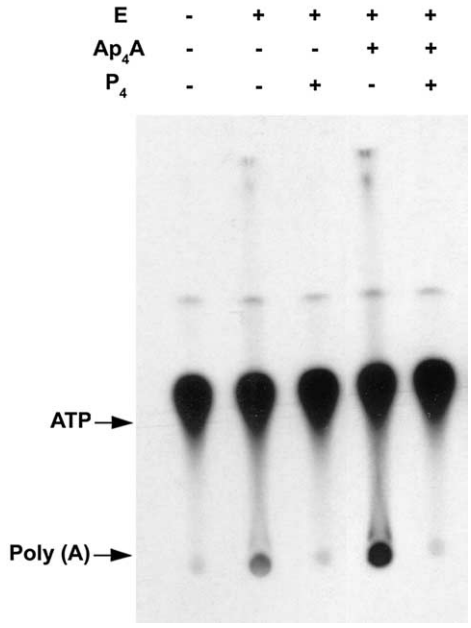

Fig. 1. Inhibition by P_4 of the primer independent synthesis of poly(A) catalyzed by yeast poly(A) polymerase. The reaction mixture (0.02 ml) contained (in the absence of tRNA): 0.05 mM ATP, 0.2 μCi [α-^{32}P]ATP, 10 μM Ap_4A or 5 μM P_4, as indicated, 0.15 U of enzyme (E) and other conditions as described in Section 2. After 40 min incubation at 30 °C, aliquots were taken and spotted on a TLC plate.

3. Results

3.1. Effect of polyphosphates on the synthesis of poly(A) catalyzed by yeast poly(A) polymerase

While searching for activators, other than dinucleoside polyphosphates, of the primer independent synthesis of poly(A) catalyzed by yeast poly(A) polymerase, we observed that P_4

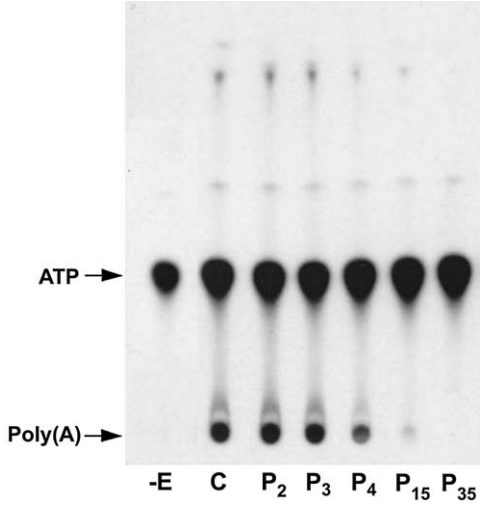

Fig. 2. Effect of P_2, P_3, P_4, P_{15} or P_{35} on the synthesis of poly(A) catalyzed by yeast poly(A) polymerase. The reaction mixture (0.02 ml) contained: 0.05 mM ATP, 0.2 μCi [α-^{32}P]ATP, 0.2 μM tRNA, 0.023 units of enzyme, 1 μM of P_2, P_3, P_4, P_{15} or P_{35} when indicated; other conditions as described in Section 2. After 7 min incubation at 30°C, aliquots were taken, and analyzed by TLC. Lanes: (-E) control without enzyme; (C) complete reaction with no added polyphosphates; other lanes with added P_n as indicated.

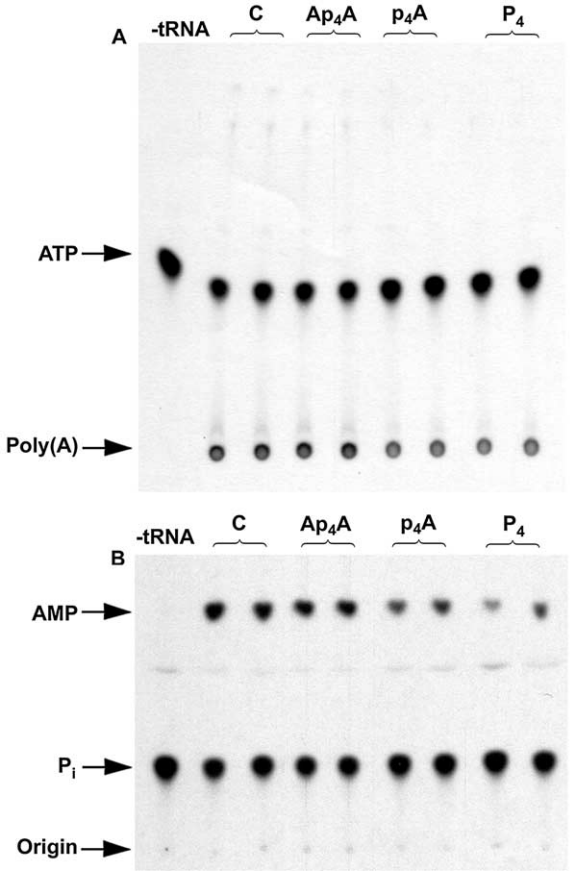

Fig. 3. Effect of Ap_4A, p_4A or P_4 on the synthesis of poly(A) catalyzed by yeast poly(A) polymerase. A: The reaction mixture (0.02 ml) contained: 0.1 mM ATP, 0.2 μCi [α-^{32}P]ATP, 0.2 μM tRNA, 1 μM of Ap_4A, p_4A or P_4 when indicated, and 0.024 units of enzyme and other conditions as described in Section 2. After 10 min incubation at 30°C, aliquots were taken, and spotted on TLC plates. B: Thereafter an aliquot (0.01 ml) of the reaction mixture was treated with 20 U/ml shrimp alkaline phosphatase for 1 h at 37°C, heated at 90°C for 5 min to inactivate the phosphatase and treated further with 20 μg/ml phosphodiesterase for 1 h at 37°C. Aliquots were spotted on TLC and analyzed as described in Section 2. Lanes: (-tRNA) control without primer; lanes (C) complete reaction with no added effectors; other lanes with added Ap_4A, p_4A or P_4 as indicated.

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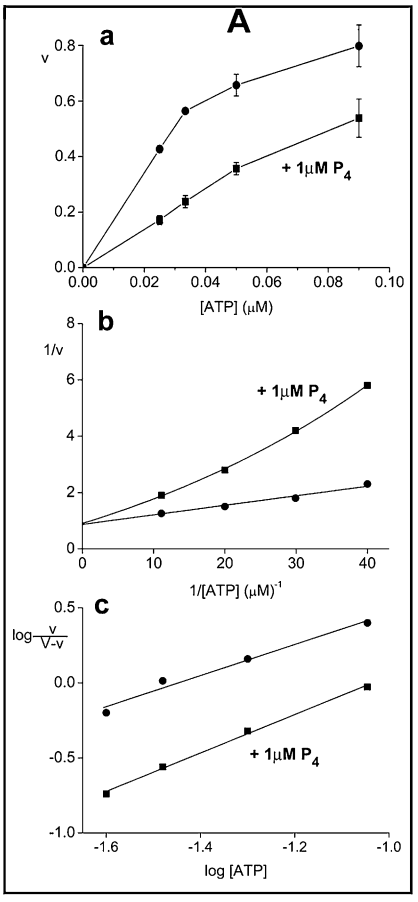
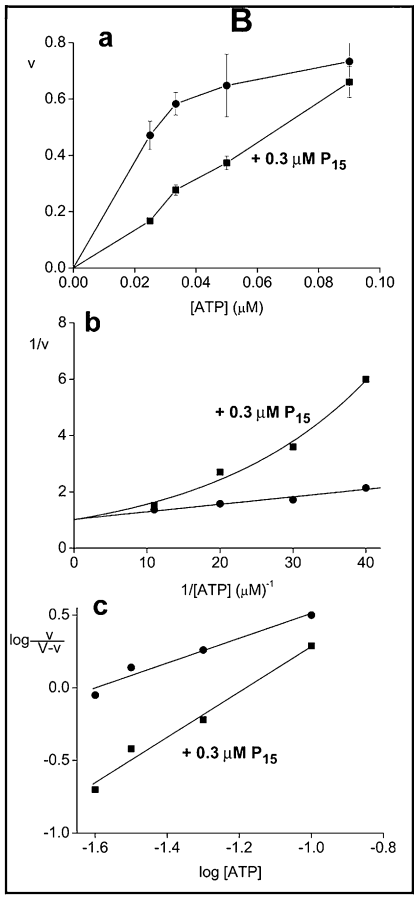
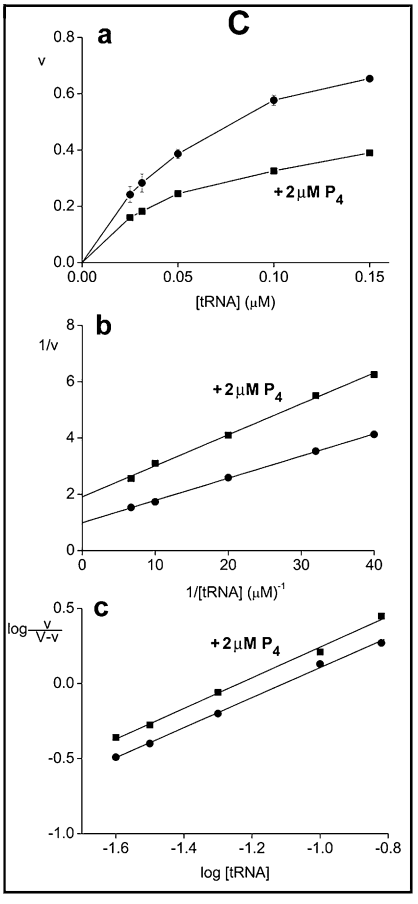
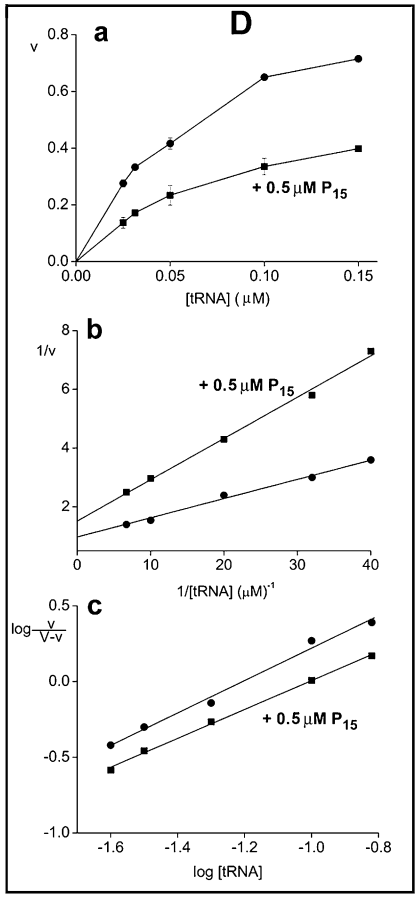
Fig. 4. Effect of P_4 or P_{15} on yeast poly(A) polymerase in the presence of variable concentrations of ATP or tRNA. Parts A and B: The reaction mixtures (0.02 ml) contained 0.02 units of enzyme, a fixed concentration of tRNA (0.2 μM) and variable concentrations of ATP ([α-^{32}P]ATP (0.025–0.09 mM); specific activity: 565 μCi/μmol). In addition the mixtures contained 1 μM P_4 (A) or 0.3 μM P_{15} (B). After 7 min incubation at 30°C, aliquots were taken and analyzed by TLC as described in Section 2. Parts C and D: The reaction mixtures (0.02 ml) contained, 0.024 U of enzyme, a fixed concentration of [α-^{32}P]ATP (0.1 mM); (specific activity: 200 μCi/μmol) and variable concentrations of tRNA (0.025–0.15 μM). In addition the mixtures contained 2 μM P_4 (C) or 0.5 μM P_{15} (D). After 10 min incubation at 30°C, aliquots were taken and analyzed by TLC. v, is expressed as μmol of AMP incorporated min^{-1} ml^{-1} into poly(A). In A, B, C, and D, the results are depicted as Michaelis–Menten (a), Lineweaver–Burk (b) and Hill (c) representations.


A
a
v
+ 1μM P4
[ATP] (μM)
b
1/v
+ 1μM P4
1/[ATP] (μM)-1
c
log v/(V-v)
+ 1μM P4
log [ATP]


B
a
v
+ 0.3 μM P15
[ATP] (μM)
b
1/v
+ 0.3 μM P15
1/[ATP] (μM)-1
c
log v/(V-v)
+ 0.3 μM P15
log [ATP]


C
a
v
+2μM P4
[tRNA] (μM)
b
1/v
+2μM P4
1/[tRNA] (μM)-1
c
log v/(V-v)
+2μM P4
log [tRNA]


D
a
v
+ 0.5 μM P15
[tRNA] (μM)
b
1/v
+ 0.5 μM P15
1/[tRNA] (μM)-1
c
log v/(V-v)
+ 0.5 μM P15
log [tRNA]

(a chain of tetraphosphates) was a strong inhibitor of the primer independent synthesis of poly(A). The addition of 5 μM P_4 to a standard reaction mixture containing 0.05 mM [α-^{32}P]ATP [2], totally inhibited the primer independent synthesis of poly(A), even in the presence of the activator Ap_4A (Fig. 1).

The effect of P_4 was also tested on the primer (tRNA) dependent synthesis of poly(A). The addition of 1, 4 and 10 μM P_4, to reaction mixtures containing 0.05 mM [2,8-^{3}H]ATP and 0.9 μM tRNA, caused an inhibition of around 70, 90 and 100%, respectively, reaching half maximal inhibition at around 0.6 μM P_4 as determined by the ion-exchange method (results not shown). Similar experiments carried out with poly(A) polymerase from *E. coli*, showed that concentrations as high as 30 μM P_{15} (in the presence of 0.05 mM ATP and 7 μM tRNA) had no appreciable effect on the synthesis of poly(A) (results not shown).

The specificity of the inhibition by polyphosphates was tested in two ways: (i) with polyphosphates of different chain lengths. In this case, the enzyme was incubated for 7 min (with 0.2 μM tRNA and 0.05 mM [α-^{32}P]ATP), and in the absence or presence of 1 μM P_2, P_3, P_4, P_{15} or P_{35}; the residual activity was around 100, 90, 40, 10 and 0%, respectively (Fig. 2); (ii) with a tetraphosphate-chain flanked by two adenosines (Ap_4A), with a tetraphosphate-chain located at the 5′-end of one adenosine (p_4A), or with a single tetraphosphate chain. The experimental conditions were as in (i), except that the reaction mixtures were treated with alkaline phosphatase to eliminate residual ATP followed by snake venom phosphodiesterase to degrade poly(A) to AMP (Fig. 3B). The activity obtained in the presence of Ap_4A, p_4A of P_4 (1 μM each) was around 100, 60 and 40% in relation to the velocity obtained in the absence of effectors (Fig. 3A,B).

3.2. Kinetics of enzyme inhibition by polyphosphates

The influence of a fixed concentration of two representative polyphosphates (P_4 or P_{15}) on the velocity of the reaction was assayed in the presence of variable concentrations of ATP or tRNA. The inhibition elicited by 1 μM P_4 or 0.3 μM P_{15} in the presence of a saturating concentration of tRNA and variable concentrations of ATP are depicted in Fig. 4A,B in the form of Michaelis–Menten (a), Lineweaver–Burk (b) and Hill (c) representations. In the absence of effectors the saturation curves are hyperbolic; the inhibition by both P_4 and P_{15} tend to be competitive and in both cases the presence of the polyphosphates changes the saturation curve from hyperbolic to sigmoidal, what results more evident in the case of P_{15}. The K_m ($S_{0.5}$) value and the Hill coefficient (n_H) calculated in the absence of polyphosphates were 0.038 mM ± 0.009 and 1.0, respectively. The equivalent values obtained in the presence of 1 μM P_4 or 0.3 μM P_{15} were (0.111 ± 0.034 mM and 1.3) and (0.065 ± 0.011 mM and 1.6), respectively (mean of three determinations). The calculated apparent K_i values for P_4 and P_{15} were 0.5 μM and 0.2 μM, respectively.

In the similar way the influence of a fixed concentration of P_4 (2 μM) or P_{15} (0.5 μM) on the velocity of the reaction was assayed in the presence of a saturating concentration of ATP and variable concentrations of tRNA (Fig. 4C,D). The presence of P_4 or P_{15} decreased the V value and modified only slightly the K_m value (0.063 ± 0.008 μM) determined for tRNA in the absence of effectors. The calculated n_H values were around 1 in the presence or absence of inhibitors.

4. Discussion

The occurrence of polyphosphates has been described in many organisms including bacteria, fungi, plants, insects and vertebrates [4–9]. The amount present varies from mM to (sub)μM concentrations. Although the exact subcellular location remains elusive, particularly in case of their presence at (sub)μM concentration (as in mammals), there are reports both on the presence of polyphosphates in nuclei [9] and on their synthesis by these organelles [10].

Some of the studies on polyphosphates have been carried out in *S. cerevisiae*. Here, polyphosphates are mainly located in vacuoles [11,12] and in lesser amount surrounding the plasma membrane [8]. Probably it is difficult to asses the exact location of polyphosphates in other subcellular fractions, such as cytosol, mitochondria and nuclei, due to their potential contamination, during the purification processes, from the relatively huge amount of polyphosphates present in the vacuoles.

The concentration of polyphosphates is controlled by enzymes involved in their synthesis and degradation such as polyphosphate kinase (EC 2.7.4.1); polyphosphate-glucose phosphotransferase (EC 2.7.1.63); 3-phosphoglyceroyl-phosphate-polyphosphate phosphotransferase (EC 2.7.4.17); endopolyphosphatase (EC 3.6.1.10) and exopolyphosphatase (EC 3.6.1.11) [4–6,8]. Polyphosphates have been implicated in several cellular processes and in the control of the level of ATP and pyrophosphate, both the center of two very important metabolic crossroads [5,6,13,14].

In our view, these, and other potential effects, carried out by polyphosphates (in yeast and in other organisms) can be considered under two different, but complementary aspects: either due to macromolecules present at high concentration and frequently condensed in organelles or due to their presence, in different cellular compartments, at low concentration (as free or partially bound molecules). Most of the roles so far assigned to polyphosphates are related with the first aspect. However, as systematically stated in different reviews on polyphosphates, there is a common concern about the possibility that polyphosphates could play more general and universal functions in biology, including their possible specific role in each cell compartment [4–9]. Being this so, these specific effects would entail the action of polyphosphates at (sub)μM concentration, the concentration prevailing in many of the living species and tissues examined.

In this regard, the activation by polyphosphates of the cytosolic IMP-GMP 5′-nucleotidase from rat brain has been recently reported in our laboratory [15]. The calculated K_a for P_{18}, P_{19} and P_{20} were around 3, 0.9 and 1.3 μM, respectively. Polyphosphates have been previously described in rat brain [9] and its effect on 5′-nucleotidase could accelerate the hydrolysis of AMP to adenosine, a well-characterized neurotransmitter [16].

As shown above polyphosphates seem to act on yeast poly(A) polymerase in a complex way: they compete with ATP, with a K_i value in the order of (sub)μM, changing simultaneously the kinetics of the reaction from hyperbolic to sigmoidal. These results seem to indicate that the effect of polyphosphates on the enzyme is rather specific, competing mainly with the terminal triphosphate located at the 5′-end of ATP. In this context, p_4A was less inhibitory than P_4, and Ap_4A had no effect on the primer dependent synthesis of poly(A). Curi-

ously, Ap_4A is a strong activator of the primer independent synthesis of poly(A) catalyzed by the same (yeast) enzyme and, a concentration as high as 30 μM of P_{15} had no appreciable effect on the poly(A) polymerase from *E. coli*. Inhibition of restriction enzymes, T4 DNA ligase and *Taq*I DNA polymerase by polyphosphates, have also been reported [17].

In summary we have presented evidence of the inhibitory effect of polyphosphates on yeast poly(A) polymerase and, based on the low concentration at which this inhibition takes place, a physiological role in the processing of mRNA can be suggested.

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