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INHIBITION OF T4 POLYNUCLEOTIDE KINASE BY THE ATP ANALOG, β, γ-IMIDOADENYLYL 5′-TRIPHOSPHATE

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Summary

The inhibition of T4 polynucleotide kinase by β,γ-imidoadenylyl 5′-triphosphate has been investigated. It was found that the ATP analog was a competitive inhibitor with regard to ATP and a noncompetitive inhibitor with regard to DNA possessing a 5′-hydroxyl group. At pH 8.0, the K_i values were 3 and 11 mM, respectively. β,γ-imidoadenylyl 5′-triphosphate was not a substrate in the forward reaction, but would replace ADP and ATP in the reverse reaction. The reverse reaction was also used to make β,γ-imidoadenylyl 5′-tetraphosphate.

Introduction

In order to obtain a good knowledge of the mechanism by which an enzyme exerts its catalytic function, the study of the effect of specific inhibitors are of crucial importance. It has previously been reported that T4 polynucleotide kinase under certain conditions followed a Bi-Bi Ordered mechanism [1]. At high ionic strength or in the presence of spermidine, the mechanism changed to a rapid random equilibrium type [2,3]. This change in reaction mechanism could be explained by structural alterations of the protein [2,4]. The enzyme probably switched from a square planar to a tetrahedral conformation when an assay system of high ionic strength ($I = 0.1$) was employed.

We have previously reported that inorganic phosphate and pyrophosphate were inhibitors of T4 polynucleotide kinase [1]. Inorganic phosphate was found to be a competitive inhibitor of ATP at pH 8.0. The inhibitor constant being approx. 30 mM. Also monovalent metal ions cause inhibition at high concentrations.

Abbreviations: HO-DNA, DNA possessing a 5′-hydroxyl group; P-DNA, DNA possessing a 5′-phosphate group.

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Narang et al. [5] reported that oligonucleotides which contained a phosphotriester internucleotidic group next to the 5′-nucleotide were not substrates of the kinase. Van de Sande and Bilsker [6] showed that blocking groups attached to the sugar of the 5′-nucleotide did not significantly interfere with the T4 polynucleotide kinase reaction. Since only mononucleotides with a 3′-phosphate group are substrates for the enzyme, it is suggested that the 3′-phosphate group of the terminal nucleotide is essential in the binding of the second substrate to the enzyme, while the sugar moiety does not play a similar essential role. The fact that nucleosides are not substrates for T4 polynucleotide kinase strengthens this assumption.

The Michaelis-Menten coefficient (K_m) for the second substrate, ATP, is relatively high ($1.5 \cdot 10^{-4}$ M) [1] and the K_{GTP} is of the same order of magnitude. Novogrodsky et al. [7] reported that T2 polynucleotide kinase was able to use all four NTPs as phosphate donors. One may, therefore, argue that polynucleotide kinase has an NTP-binding site which does not discriminate strongly against the base moiety. On the assumption that it is the phosphate chain which is the main recognition site, the inhibition of T4-polynucleotide kinase activity caused by β,γ-imidoadenylyl 5′-triphosphate has been investigated.

Materials and Methods

Enzyme. T4-polynucleotide kinase was prepared according to the method of Panet et al. [8] and the purity was as stated in that paper.

DNA and ATP preparations. Calf thymus DNA and oligo dpT(pT)9 were from Sigma Chemical Co. dC-C-G-G-T-T-C-G-A-T-T (DNA I) and dG-C-A-T-C-A-G-C-C-A (DNA II), which are segments of the gene coding for yeast alanine tRNA, was a gift from Professor H.G. Khorana, M.I.T., Boston, Mass., U.S.A. The 5′-phosphate groups were removed from the DNAs as described previously [1]. γ-^{32}P-ATP was prepared by the method of Glynn and Chappell [9] and the specific activity was approx. 5 Ci/mol. β,γ-imidoadenylyl 5′-triphosphate was obtained from Sigma Chemical Co.; β,γ-imido[8-^{3}H]adenosine 5′-triphosphate (20.3 Ci/mol) was from the Radiochemical Centre, Amersham, U.K. Other chemicals used were of highest purity.

Assay systems. The assay systems employed were as described previously [1,10]. Details are given in the figure legends.

Results

The substrate analog, β,γ-imidoadenylyl 5′-triphosphate, was found to inhibit T4 polynucleotide kinase (Fig. 1). The initial reaction rate of phosphorylation was significantly affected by the inhibitor. In Fig. 1B, the inhibition of initial rates is given as a function of the β,γ-imidoadenylyl triphosphate concentration. Approx. 3 mM of the inhibitor was required to cause 50% inhibition. The above experiments were performed at pH 8.0, using calf thymus DNA as phosphate-accepting substrate. When the inhibition effect was measured at pH 9.2 in glycylglycine/HCl buffer, almost identical results were obtained. The inhibition of initial reaction rate did not depend on the kind of DNA used as the phosphate accepting substrate since essentially identical results were found

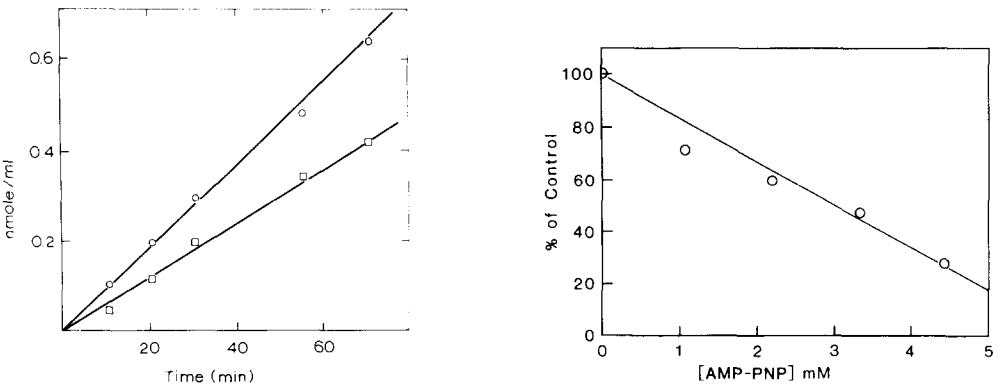


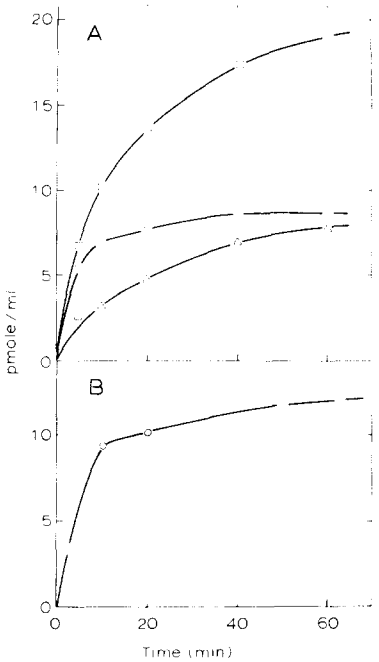
Fig. 1. Time course of phosphorylation by T4 polynucleotide kinase. The assay mixture contained 66 mM Tris · HCl pH 8.0, 0.23 mM dephosphorylated calf thymus DNA, 65 μM [γ-^{32}P]ATP, 15 mM 2-mercaptoethanol, 7 mM $MgCl_2$, and 0.7 units/ml of enzyme. The temperature was 35°C. (A) —○—, control; —□—, 2.4 mM β,γ-imidoadenylyl 5′-triphosphate added. (B) Inhibition of initial reaction rates by β,γ-imidoadenylyl 5′-triphosphate. The inhibitor concentration varied as indicated. Each point is the mean of two separate experiments and the incubation time was 10 min.

when single-stranded DNAs of defined sequences were employed (unpublished data). However, using single-stranded DNAs as substrates, DNA I and II, the addition of 1.9 mM β,γ-imidoadenylyl 5′-triphosphate resulted in a 28% decrease of the final degree of phosphorylation. This result suggested that β,γ-imidoadenylyl 5′-triphosphate interfered with the T4-polynucleotide kinase in such a way that it stimulated the reverse reaction to occur. In Fig. 2A, the effect of β,γ-imidoadenylyl 5′-triphosphate on the reverse reaction is shown. After 60 min at 35°C, a 100% increase in the amount of dephosphorylated 5′-P-DNA was observed. The addition of 0.1 M KCl resulted in an approx. 50% decrease in the initial reaction rate. From the above experiment it was not possible to conclude whether or not β,γ-imidoadenylyl 5′-triphosphate served as a substrate for T4 polynucleotide kinase in the reverse reaction. To clarify this the experiment was also done in the absence of ADP (Fig. 2B). The results were consistent with the assumption that β,γ-imidoadenylyl 5′-triphosphate did act as a substrate in the reverse reaction. However, numerous attempts, under different conditions, to show that β,γ-imidoadenylyl 5′-triphosphate was a substrate for T4 polynucleotide kinase in the forward reaction were not successful, either at pH 8.0 or at pH 9.2. The possible reaction intermediate, AppNpDNA, was not detectable under any assay conditions employed (unpublished data).

The expected product from the reverse reaction using β,γ-imidoadenylyl 5′-triphosphate as a substrate should be β,γ-imidoadenylyl tetraphosphate. To test this, the reaction products from the reverse reaction were separated on a Sephadex G-25 column according to the method of Van de Sande et al. [11] (Fig. 3). The peak corresponding to adenosine tetraphosphate was pooled and the compound was tested in two ways:

(1) Using sensitivity towards phosphatase treatment [11]. More than 95% of the labeled phosphate was removed by this treatment.

(2) The compound was also sensitive to the phosphatase activity of myosin when tested according to the method of Small and Cooper [12]. Aliquots were



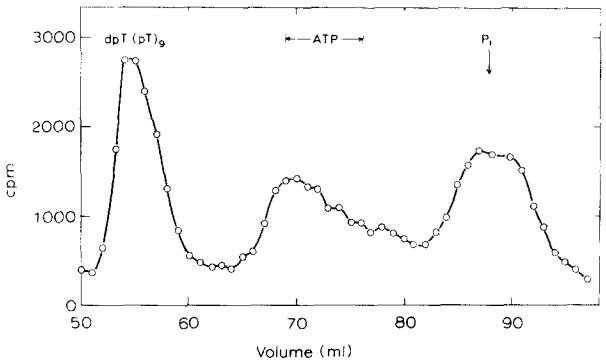


Fig. 2. Time course of the reverse reaction of T4-polynucleotide kinase. The assay mixture contained 66 mM imidazole/HCl (pH 6.8), 20 μM ADP, 7 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 0.1 μM ^{32}P-dpT-(pT)9 (5'-hydroxyl ends) and 1 unit/ml enzyme. (A) —○—, control; —□—, 2.4 mM β,γ-imidoadenylyl 5'-triphosphate added, —△—, 2.4 mM β,γ-imidoadenylyl 5'-triphosphate and 0.1 M KCl added. (B) Assay mixture as above except that 2.4 mM β,γ-imidoadenylyl 5'-triphosphate replaced ADP.

Fig. 3. Gel filtration of the reaction products from T4 polynucleotide kinase reaction. The reaction conditions were as in Fig. 2B. The reaction mixture was incubated for 20 min at 35°C then made 30 mM in EDTA, mixed with unlabeled ATP, and passed through a column of Sephadex G-25 (1.1 × 92 cm). The column was eluted at 4°C with 50 mM triethylammonium bicarbonate. Aliquots of the fractions were assayed for ^{32}P. The ATP marker was detected by measuring absorbance of the fractions at 260 nm.

chromatographed on DEAE-cellulose ion exchange paper (DE-81), as described previously [1]. After 60 min at 25°C, approx. 75% of the radioactivity was transferred to inorganic phosphate. The remaining 25% did not move from the origin, suggesting that it could be bound to the proteins in the reaction mixture.

Since β,γ-imidoadenylyl triphosphate was not a substrate for T4 polynucleotide kinase in the forward reaction, it can be considered to be a "dead-end inhibitor". The mode of action of this inhibitor was analyzed in two ways. In Fig. 4A, the T4 polynucleotide kinase activity has been plotted according to Lineweaver-Burk (ATP being the variable substrate) and in the presence and absence of β,γ-imidoadenylyl 5'-triphosphate. The Michaelis-Menten constant for ATP was determined to be $2 \cdot 10^{-4}$ M which is close to our earlier calculation [2]. In the presence of 1.9 or 3.8 mM inhibitor, a significant inhibition was obtained and the inverse plots were consistent with competitive inhibition. From the straight line obtained from the replot of slope against concentration of inhibitor, it is suggested that the β,γ-imidoadenylyl 5'-triphosphate caused linear competitive inhibition of the T4 polynucleotide kinase activity, ATP

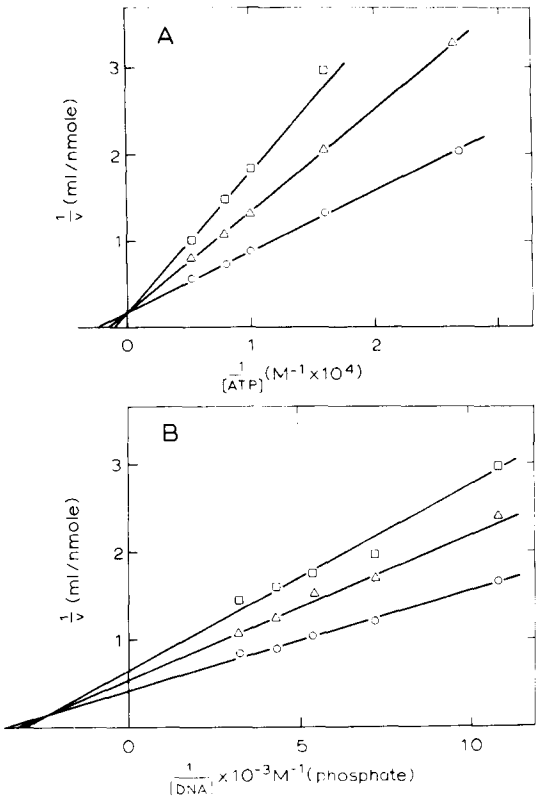


Fig. 4. (A) Double reciprocal plot of the effect of ATP concentration on T4 polynucleotide kinase in the presence of β,γ-imidoadenylyl 5'-triphosphate. Assay conditions were as described in Fig. 1, except that ATP concentration varied as indicated. —○—, control; —△—, 1.9 mM; —□—, 3.8 mM β,γ-imidoadenylyl 5,-triphosphate added. (B) Double reciprocal plot of the effect of calf thymus DNA concentration on T4 polynucleotide kinase in the presence of β,γ-imidoadenylyl 5'-triphosphate. Assay conditions were as described in Fig. 1 except that the calf thymus DNA concentration varied as indicated. —○—, control; —△—, 1.9 mM; —□—, 3.8 mM β,γ-imidoadenylyl 5'-triphosphate added.

being the variable substrate. On this assumption the inhibitor constant, K_i, was determined to be approx. 3 mM. The inhibition pattern with regard to calf thymus DNA as the variable substrate is shown in Fig. 4B. The inhibition was non-competitive and, assuming a linear non-competitive inhibition pattern (replot slope vs. concentration of inhibitor, inset, Fig. 4B), the inhibition constant was calculated to be approx. 11 mM. The inhibition patterns did not depend on pH, since experiments performed at pH 9.2 in glycylglycine/HCl buffer gave the same general results as reported above. The K_i was for both substrates approx. 5 mM at pH 9.2. However, at this pH using single-stranded DNAs as the variable substrate a strong substrate inhibition was observed. This inhibition was unaffected by the presence of β,γ-imidoadenylyl triphosphate (unpublished data).

Discussion

In an attempt to study the effect of inhibitors on T4 polynucleotide kinase, several substrate analogs were initially tested and shown not to interfere signifi-

icantly with the enzymatic activity of the protein. Among the components tested were: α,β-methyleneadenosine 5′-triphosphate, β,γ-methyleneadenosine 5′-triphosphate, NAD, NADH, HS-CoA and the protein synthesis inhibitor, puromycin. β,γ-methyleneadenosine 5′-triphosphate was the only compound which showed some inhibitory effect on the initial rate. At 2.5 mM β,γ-methyleneadenosine 5′-triphosphate 5—10% inhibition was repeatedly recorded. Furthermore, α,β-methyleneadenosine 5′-triphosphate seemed to resemble ATP closely, since the inhibition observed with this analog paralleled the dilution of the ^{32}P-labeled ATP.

In the present report, the effect of β,γ-imidoadenylyl triphosphate on the T4 polynucleotide kinase activity has been studied. The ATP analog was a weak competitive inhibitor with regard to ATP and a noncompetitive inhibitor when HO-DNA was the variable substrate. The K_i found when ATP was the variable substrate was approx. 20 times the K_{ATP}. This large difference cannot be properly explained at present; however, it may be suggested that the Mg · β,γ-imidoadenylyl 5′-triphosphate differs significantly from the Mg · ATP complex. Only a fraction of the former being of the same configuration as the latter complex [15]. Thus, the true inhibitor concentration would be lower than indicated. The inhibition patterns obtained for the ATP analog confirmed our previous results that T4 polynucleotide kinase, under the assay conditions employed, followed an Ordered Bi-Bi reaction mechanism, with HO-DNA binding to the enzyme first.

As would be expected from previous reports, β,γ-imidoadenylyl 5′-triphosphate was not a substrate for T4 polynucleotide kinase in the forward reaction. The only enzyme reported to split off the γ-phosphate is *Escherichia coli* alkaline phosphatase [13]. The finding that β,γ-imidoadenylyl 5′-triphosphate was a substrate in the reverse reaction offered evidence that the analog bound to the enzyme in a similar manner to ATP, since ATP (in addition to ADP) is a substrate in the reverse reaction [11]. It is concluded that the conformation of the phosphate chain plays an important role in the binding of ATP to the active enzyme. Further evidence in support of this came from the observation that β,γ-methyleneadenosine 5′-triphosphate (which differs from ATP both with regard to charge and the angle between the β,γ-phosphate groups) was only a weak inhibitor of T4 polynucleotide kinase. Less than 10% inhibition was measured in the presence of 2.5 mM methylene analog (unpublished data). The Mg · β,γ-methylene-adenosine 5′-triphosphate complex caused some reverse reaction to occur; however, replacing $MgCl_2$ with $MnCl_2$ abolished this effect.

It is also of interest to notice that the reverse reaction can be used to produce AMP-PNPP, the tetraphosphate analog of ATP, from β,γ-imidoadenylyl 5′-triphosphate. This analog has been shown to be of potential use in the study of myosin ATPase [14].

Acknowledgments

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