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KINETIC STUDIES ON THE REACTION CATALYZED BY POLYNUCLEOTIDE KINASE FROM PHAGE T_4-INFECTED *ESCHERICHIA COLI*

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Summary

Kinetic properties of polynucleotide kinase (EC 2.7.1.78) isolated from *Escherichia coli* cells infected with phage T_4 were investigated. The reaction depends on the concentration of MgATP, while free ATP or free Mg^{2+} have neither inhibitory nor accelerating effect. The initial reaction velocity was plotted against variable concentrations of ATP as the phosphate donor at various fixed concentrations of 5′-hydroxyl-DNA or -oligo(rA) as the phosphate acceptor in the presence or absence of products. The double reciprocal plot analysis of the data suggested that the reaction obeys the random sequential mechanism. Various constants were determined and the reaction mechanism was discussed.

Introduction

The general aspect of the phosphorylation catalyzed by kinases is thought to occur through a nucleophilic attack by the acceptor molecule on the γ-phosphoryl group of ATP, requiring indispensable divalent metal ions such as Mg^{2+} and Mn^{2+} [1]. For example, in the case of creatine kinase, the true substrate, as phosphate donor, was found to be a nucleosidetriphosphate-metal complex with free ATP and free Mg^{2+} showing inhibitory effects [2].

Abbreviations used: $_{OH}$DNA, DNA with free 5′-hydroxyl group; pDNA, DNA with free 5′-phosphomonoester group; $_{OH}(rA)_{25}$, oligoriboadenylic acid of average chain length of 25 with free 5′-hydroxyl group; $p(rA)_{25}$, oligoriboadenylic acid of average chain length of 25 with free 5′-phosphomonoester group;

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Polynucleotide kinase isolated from *Escherichia coli* cells infected by phage T_2 or T_4 transfers the γ-phosphate of nucleosidetriphosphate to the 5′-hydroxyl termini of mono- and polynucleotides [3]. The molecular weight of this enzyme was estimated to be 140 000 by Sephadex G-200 gel filtration. The enzyme consists of four similar subunits as revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis [4]. The enzyme depends on Mg^{2+} for the reaction and reacts equally well with ATP, UTP, GTP and CTP as phosphate donor with an apparent K_m of around $2 \cdot 10^{-5}$ M [5]. The overall reaction pathway can be expressed as

$$_{OH}\text{DNA (or RNA)} + \text{XTP} \overset{Mg^{2+}}{\rightleftharpoons} {}_{p}\text{DNA (or RNA)} + \text{XDP}$$

The optimal pH's for forward and reverse reactions were found to be 9.0 and 6.5, respectively [6]. Because of its highly specific catalysis of phosphate transfer, the enzyme has become a very useful tool in nucleic acid research. The purpose of the present study is to elucidate the role of magnesium ions and to determine the kinetic mechanism. In this communication, we report evidence that the true substrate as phosphate donor for polynucleotide kinase is a nucleosidetriphosphate-metal complex and the reaction obeys random sequential mechanism.

Materials and Methods

[γ-^{32}P] nucleosidetriphosphate

[γ-^{32}P] ATP, -GTP and -CTP were prepared by a modified method according to Glynn and Chappell [7]. A 0.4-ml reaction mixture contained 1.5 mM 3-phosphoglyceraldehyde, 4.2 mM nucleosidetriphosphate, 1 mM β-mercaptoethanol, 10 mM $MgCl_2$, 50 mM Tris · HCl, pH 8.0, 0.1 mg phosphoglycerate kinase (Boehringer, 15745), 0.1 mg glyceraldehyde-3-phosphate dehydrogenase (Boehringer, 15146) and 5—10 mCi carrier-free ^{32}P (Amersham, Buchler) and incubated at 36° C. For analysis, 1 μl aliquot was taken and mixed with 1 ml of 20% Norit solution containing 0.5 mg bovine serum albumine. After centrifugation for 5 min at 4000 × *g*, the supernatant was counted for ^{32}P-radioactivity. After 30 min incubation, 70% of inorganic phosphate was converted into Norit adsorbed material in the case of ATP and GTP, and 40% in the case of CTP. The reaction mixture was diluted 2-fold with 0.05 M triethylammonium bicarbonate buffer, pH 7.5, and applied to a DEAE cellulose column (0.6 cm × 5 cm) equilibrated with the same buffer. After washing the column with the starting buffer and 0.25 M triethylammonium bicarbonate buffer, the nucleosidetriphosphates were eluted with 1 M triethylammonium bicarbonate buffer. The materials were dried by evaporation and dissolved in 0.2 ml water. The specific activities (cpm/μmol) were 10^{10} cpm for ATP, $4.3 \cdot 10^9$ cpm for GTP and $3 \cdot 10^9$ cpm for CTP. Further purification by Dowex 50 (Cl^-) column had no influence on the behaviour of nucleosidetriphosphate in the standard assay.

Nicked DNA and oligo(rA)

Nicked DNA (calf thymus) was prepared by the method described by

Richardson [3]. The average numbers of 5'-OH terminal group in the DNA was estimated to be one to every 50 nucleotides from the ratio of optical density to ^{32}P-incorporated by the polynucleotide kinase reaction.

Oligo(rA) was prepared by the modified method according to Martin et al. [8]. 50 mg poly(rA) (Boehringer, 15127) was dissolved in 10 mM Tris · HCl, pH 7.2, made to 0.4 M KOH and incubated for 22 min at 37°C. After neutralization with 1 M HCl, the solution was made to 0.1 M HCl and incubated further for 1 h at 37°C to open cyclic 2',3'-phosphate. The solution was neutralized with 1 M NaOH and solid urea was added to give a final concentration of 7 M. The solution was loaded on a DEAE-Sephadex A-25 column (3 cm × 10 cm) equilibrated with 20 mM Tris · HCl, pH 7.4, containing 0.1 M NaCl and 7 M urea. After being washed with the starting buffer, the materials were eluted by linear gradient from 0.1 M to 0.6 M NaCl in 20 mM Tris · HCl, pH 7.4, and 7 M urea. Appropriate fractions were collected, dialyzed extensively against water and lyophylized. The resulting material was dissolved in 1 ml water and used for assay. The average chain length of the peak fraction used in this study was estimated to be 25, based on the ratio of optical density to ^{32}P-radioactivity incorporated by the reaction with polynucleotide kinase.

Preparation of polynucleotide kinase

Bacteriophage T_4, amber mutant 46,47 (DNAase minus) was propagated on *E. coli*, strain Su-7 with final yield of $2.6 \cdot 10^{11}$ p.f.u. per ml. A 200-liter culture of *E. coli* strain B-41 was grown on M-9 medium containing 0.5% casamino acid to a cell density $1 \cdot 10^9$ cells per ml. L-tryptophane to a final concentration of 30 μg per ml and T_4—46,47 at a MOI (multiplicity of infection) of 3 were added. Cells were collected 30 min after the infection, packed in a 50 g batch, frozen and stored at −50°C until use. Polynucleotide kinase was purified by a modified method according to the description by Richardson [3]. All procedures were carried on in a cold room at 4°C. Centrifugation was usually at 10 000 rev./m in the SS-1 or SS-34 rotor of Sorvall Centrifuge, RC2-B, for 20 min. 50 g of cells were thawed in 400 ml 50 mM Tris · HCl, pH 7.6, containing 1 mM glutathione buffer and homogenized with the aid of 100 g glass beads in a Waring blendor (Sorvall Omnimixer) for 10 min (or sonicated for 15 min by Sonifier, B-12, Branson). After centrifugation, the supernatant was adjusted to an absorbance of 60 at 260 nm by adding glutathione buffer and mixed with streptomycine to a final concentration of 1%. The precipitate was centrifuged down, suspended in 100 ml glutathione buffer, autolyzed in the presence of 4 mM $MgCl_2$ for 2 h at 37°C, and centrifuged. The supernatant was treated with $(NH_4)_2SO_4$ and the precipitate between 0.2 and 0.8 saturation was collected by centrifugation, resuspended in 60 ml glutathione buffer and dialyzed overnight against 10 mM potassium phosphate buffer containing 1 mM β-mercaptoethanol. After this step, the buffer (pH 7.2) contained 1 mM β-mercaptoethanol throughout the procedure. The dialysate was loaded on a DEAE-cellulose column (3 cm × 10 cm) equilibrated with the dialysis buffer, and eluted with 50 mM phosphate buffer. Fractions showing polynucleotide kinase activity as determined by ^{32}P-incorporation into nicked DNA were collected, applied to a phospho-cellulose column (3 cm × 6 cm) equilibrated with 50 mM phosphate buffer. Active fractions were eluted with

the buffer containing 0.3 M KCl, combined, diluted 5-fold with 20 mM phosphate buffer and applied on a hydroxyapatite column (2 cm × 2 cm) equilibrated with 20 mM phosphate buffer. Active fractions were eluted with 0.3 M phosphate buffer, combined, dialyzed against solid sucrose for concentration, then against 10 mM Tris · HCl, pH 7.6, and finally against Tris · HCl (10 mM, pH 7.6)/60% glycerol and stored at −20°C. The specific activity of the final preparation was approx. 6600 units per mg protein (1 unit is defined as 1 nmol ^{32}P-incorporated per 30 min at 37°C [3]).

Assay for polynucleotide kinase activity

A 50-μl reaction mixture contained 0.1 M Tris · HCl, pH 8.0, 6 mM dithiothreitol, appropriate concentrations of $MgCl_2$, phosphate acceptor and [γ-^{32}P]ATP as listed in the figure legends and 0.2—0.7 unit of polynucleotide kinase. ATP was selected as phosphate donor because ATP, CTP and GTP were equally accepted by the enzyme with similar apparent K_m value of $4 \cdot 10^{-4}$ M in the presence of 10 mM $MgCl_2$ and 0.1 mM $_{OH}(rA)_{25}$ (figure not shown). The incubation temperature was 30°C according to the experiment which showed the optimal temperature for the reaction between 30°C and 35°C. The values for Q_{10} and apparent activation energy were calculated to be 2.0 and 11 890 cal. per mol, respectively. For the standard assay when $_{OH}(rA)_{25}$ was the phosphate acceptor, a 10-μl aliquot was withdrawn at time intervals of 0, 5, 10 and 20 min and spotted on a chromatography paper (Carl Schleicher, 2040—b) of which starting area was treated with 0.4 M EDTA and dried previously. The high voltage electrophoresis was carried out in pyridine-acetate, pH 3.5, at 3600 V for 45 min in the Savant apparatus, Model No. LT 20 A. The origin was cut out and counted for radioactivity in toluol-based scintillator. The reaction velocity was estimated from the time-course graph and expressed as the amount of ^{32}P incorporated per 10 μl reaction mixture per 5 min. For the standard assay when $_{OH}$DNA was the phosphate acceptor, a 50-μl reaction mixture was mixed with 0.1 ml of 0.1% bovine serum albumine and 0.1 ml of 0.18 M $Na_4P_2O_7$ and acid insoluble materials were collected on the membrane filter (Sartorius, 11306, pore size 0.45 μ), which was dried and counted for radioactivity in toluol-based scintillator. The reaction velocity is expressed as the amount of ^{32}P incorporated per 50 μl reaction mixture per 5 min.

Results

Effect of magnesium concentrations

The first problem for the kinetic studies on polynucleotide kinase reaction was to know the relationship of magnesium and ATP. For example, if the enzyme accepts only MgATP as the phosphate donor, then free ATP and/or free Mg^{2+} have either inhibitory or no effect on the reaction velocity. To solve these questions, three experiments were carried out. Fig. 1-A shows the effect of various concentrations of $MgCl_2$ at a fixed concentration of ATP. The reaction velocity reaches to the plateau at 1 mM $MgCl_2$ at any concentrations of ATP, and no inhibition was seen up to 50 mM $MgCl_2$. Based on the dissociation constant for MgATP of $1.4 \cdot 10^{-5}$ M at pH 8.0 [9], more than 1 mM $MgCl_2$ is necessary to bind all free ATP. The results suggest that the enzyme accepts

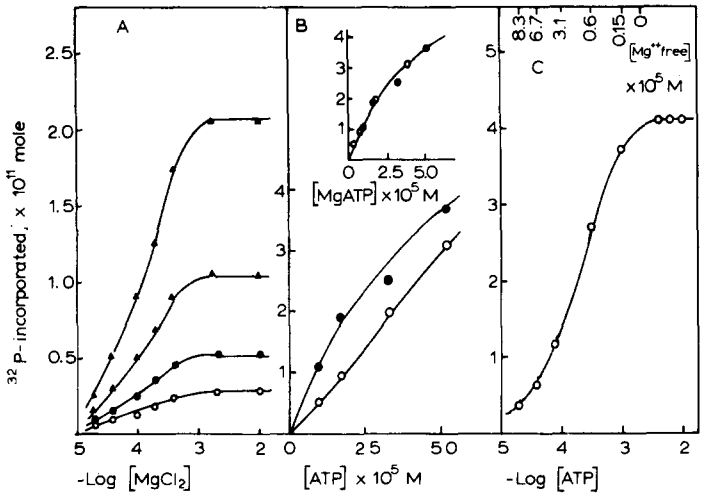

Fig. 1. Effect of concentrations of $MgCl_2$ on the initial reaction velocity. (A) Effect of total concentrations of $MgCl_2$ at different fixed concentrations of ATP, 10 μM (○), 20 μM (●), 40 μM (△) and 80 μM (▲). The concentration of $_{OH}(rA)_{25}$ was 20 μM. (B) Effect of ATP concentration varied in equimolar ratio with $MgCl_2$ (○) and in the presence of 10 mM excess $MgCl_2$ (●). The insert shows the same data plotted as velocity vs [MgATP], the concentration of which was calculated according to a dissociation constant of 14 μM as described in the text. (C) Effect of total ATP concentration at fixed concentration of $MgCl_2$ (0.1 mM). The concentration of $_{OH}(rA)_{25}$ was 40 μM. The concentrations of free Mg^{2+} calculated are also shown in the figure.

only MgATP as the substrate and free Mg^{2+} has no effect on the reaction, which was confirmed by the second experiment (Fig. 1-B). When the reaction velocity was examined at various ATP concentrations varied in equimolar ratio with $MgCl_2$, the plots showed a sigmoidal curve, whereas addition of 10 mM $MgCl_2$ changed the curve to a normal hyperbolic one. If the MgATP concentration was calculated from the same data and plotted against velocity, the two curves coincided completely (Fig. 1-B, inserted figure). Fig. 1-C shows the effect of ATP concentrations on the reaction velocity at a fixed concentration of total $MgCl_2$. ATP enhances the reaction as far as free Mg^{2+} remains, but when all Mg^{2+} is bound by ATP to form MgATP, the reaction velocity reaches to the plateau where free ATP has no effect anymore. We can conclude from these results that the enzyme requires MgATP as the phosphate donor, and free ATP and free Mg^{2+} have nothing to do with the reaction. For further kinetic experiments, $MgCl_2$ concentration was fixed at 10 mM, where ATP and ADP form a completely corresponding magnesium complex (the dissociation constant of MgADP was taken to be 0.25 mM [9]).

Effect of substrate concentrations

The second step for the kinetic studies was to examine the effect of variations in the concentrations of phosphate-donor and -acceptor. As the phosphate acceptor, oligo-riboadenylate and nicked DNA were selected since DNA and RNA were supposed to behave differently. The initial reaction velocity was determined at variable concentrations of one substrate at different fixed concentrations of the other substrate. Fig. 2 shows the results of experiments when

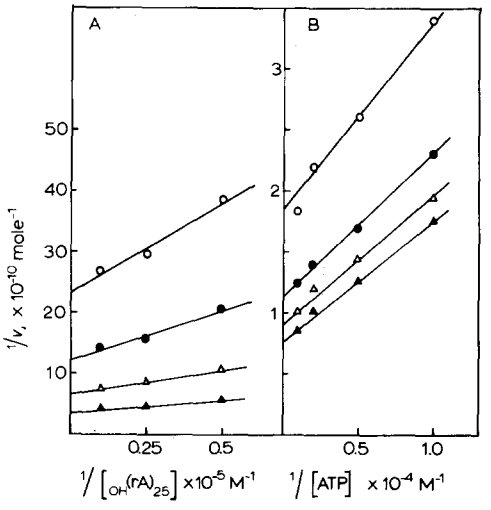

Fig. 2. Double reciprocal plots of the effect of substrate concentrations on the initial reaction velocity at fixed concentration of $MgCl_2$ (10 mM). (A) Effect of $_{OH}(rA)_{25}$ concentration at different fixed concentrations of ATP, 10 μM (○), 20 μM (●), 40 μM (△) and 80 μM (▲). (B) Effect of ATP concentration at different fixed concentrations of $_{OH}$DNA, 0.8 μM (○), 2 μM (●), 4 μM (△) and 8 μM (▲).

$_{OH}(rA)_{25}$ (A) and $_{OH}$DNA (B) are offered as the phosphate acceptor. In both cases, double reciprocal plots show non-parallel, straight lines which meet at a point below the horizontal axis. The results suggest that the reaction proceeds sequentially and because of straight lines, the mechanism seems to be either ordered Bi-Bi or rapid equilibrium random Bi-Bi [10].

Effect of product concentrations

The third step is to determine the kinetic mechanism. For this purpose, product-inhibition studies are most suitable, because the product-enzyme complex certainly has some inhibitory effects on enzyme-substrate complex formation and by examining inhibition patterns, we can obtain valuable information to determine the kinetic mechanism [11]. The product-inhibition study is usually rather complicated to interprete because of the reverse reaction. But in the case of polynucleotide kinase reaction, the situation would be much simpler since, at pH 7.6, the reverse reaction is less than 2% of the forward reaction [6]. This means that both ADP and $_{p}$DNA would be dead-end inhibitors, i.e. the enzyme which binds the product cannot contribute to the overall reaction any more under the conditions of the experiment (pH 8.0). The results of inhibition studies are presented in Fig. 3 (phosphate acceptor, $_{OH}(rA)_{25}$) and Fig. 4 (phosphate acceptor, $_{OH}$DNA). ADP is a competitive inhibitor against ATP (Fig. 3-A, Fig. 4-A), and a non-competitive inhibitor against $_{OH}$DNA (Fig. 4-B) and $_{OH}(rA)_{25}$ (Fig. 3-B). $_{p}$DNA is a competitive inhibitor against $_{OH}$DNA (Fig. 4-C) and a non-competitive inhibitor against ATP (figure not shown). Similarly, $_{p}(rA)_{25}$ is a competitive inhibitor against $_{OH}(rA)_{25}$ (figure not shown) and non-competitive inhibitor against ATP (Fig. 3-C). The apparent inhibition constants (K_i) were calculated and stated in Table I. We obtained two competitive and two non-competitive inhibition patterns for each

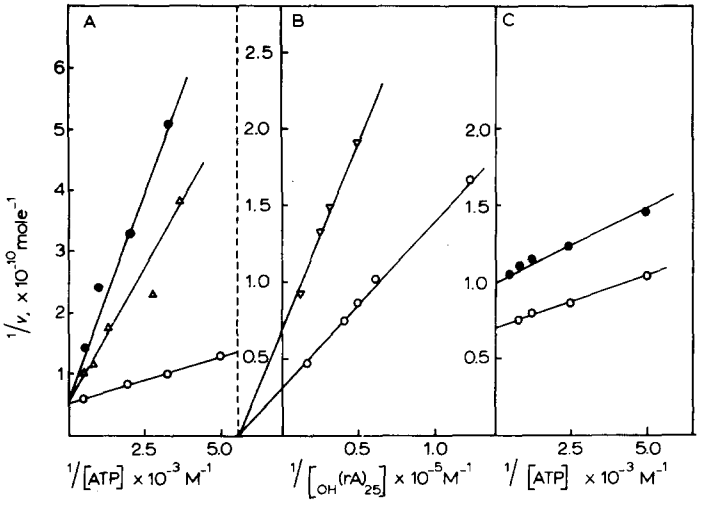

Fig. 3. Double reciprocal plots of the effect of products on the initial reaction velocity with $_{OH}(rA)_{25}$ as the phosphate acceptor. $p(rA)_{25}$ was prepared by phosphorylation of $_{OH}(rA)_{25}$ with cold ATP and polynucleotide kinase. The resulting sample was found to be almost totally phosphorylated since it did not accept ^{32}P any more by the polynucleotide kinase reaction with [γ-^{32}P]ATP. (A) Inhibition by ADP at variable concentrations of ATP. The concentrations of $_{OH}(rA)_{25}$ and $MgCl_2$ were 40 μM and 10 mM, respectively. The concentrations of ADP were 2 mM (△) and 4 mM (●). The plot in the absence of ADP is shown as a control (○). (B) Inhibition by 1 mM ADP (▽) at variable concentrations of $_{OH}(rA)_{25}$ in the presence of 1 mM ATP and 10 mM $MgCl_2$. The plot in the absence of ADP is shown as a control (○). (C) Inhibition by 30 μM $p(rA)_{25}$ at variable concentrations of ATP (●) in the presence of 40 μM $_{OH}(rA)_{25}$ and 10 mM $MgCl_2$. The plot in the absence of $p(rA)_{25}$ is shown as a control (○).

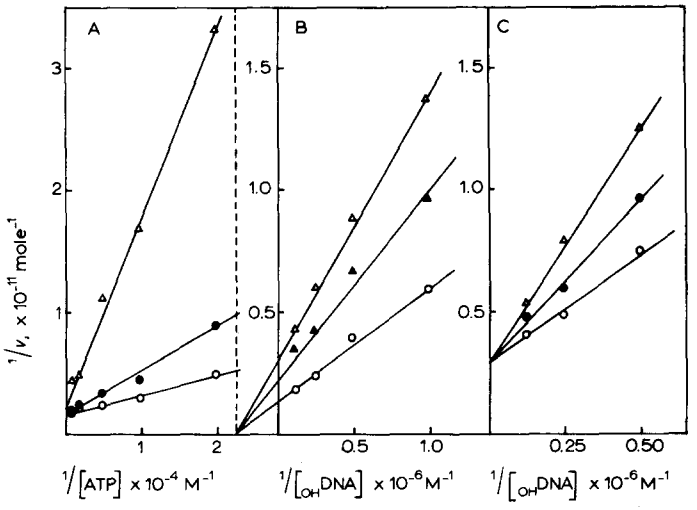

Fig. 4. Double reciprocal plots of the effect of products on the initial reaction velocity with $_{OH}DNA$ as the phosphate acceptor. pDNA was prepared by the phosphorylation of $_{OH}DNA$ with cold ATP and polynucleotide kinase. (A) Inhibition by ADP at variable concentrations of ATP in the presence of 8 μM $_{OH}DNA$ and 10 mM $MgCl_2$. The concentrations of ADP were 0.1 mM (●) and 1 mM (△). The plot in the absence of ADP is shown as a control (○). (B) Inhibition by ADP at variable concentrations of $_{OH}DNA$ in the presence of 0.5 mM ATP and 10 mM $MgCl_2$. The concentrations of ADP were 0.5 mM (▲) and 1 mM (△). The plot in the absence of ADP is shown as a control (○). (C) Inhibition by pDNA at variable concentrations of $_{OH}DNA$ in the presence of 0.5 mM ATP and 10 mM $MgCl_2$. The concentrations of pDNA were 7 μM (●) and 21 μM (△). The plot in the absence of pDNA is shown as a control (○).

TABLE I

PATTERN OF INHIBITION AND APPARENT INHIBITION CONSTANTS FOR PRODUCTS (M)

Variable substrate	Product						Fixed substrate
	ADP		pDNA		$p(rA)_{25}$		
ATP	C	$1.5 \cdot 10^{-4}$	NC	$8.2 \cdot 10^{-5}$	—	—	8 μM $_{OH}$DNA
ATP	C	$1.2 \cdot 10^{-4}$		—	NC	$6 \cdot 10^{-5}$	40 μM $_{OH}(rA)_{25}$
$_{OH}$DNA	NC	$6.3 \cdot 10^{-4}$	C	$1.8 \cdot 10^{-5}$		—	0.5 mM ATP
$p(rA)_{25}$	NC	$8 \cdot 10^{-4}$		—	C	$1.7 \cdot 10^{-5}$	1.0 mM ATP

Abbreviations: C, competitive, NC, non-competitive.

ADP and $_{p}$DNA (or $_{p}(rA)_{25}$). The best interpretation for the results would be that the reaction obeys the rapid equilibrium random Bi-Bi mechanism [9—11].

ATP-binding test

If the reaction mechanism is random Bi-Bi, the enzyme should bind to ATP in the absence of $_{OH}$DNA, and $_{OH}$DNA in the absence of ATP. A direct binding experiment would be difficult since it is improbable that a stable intermediate complex is formed. However, T_2-induced polynucleotide kinase was reported to be completely inactivated by 10 min incubation at 38°C in the absence of substrate, while the presence of phosphate donor or acceptor pro-

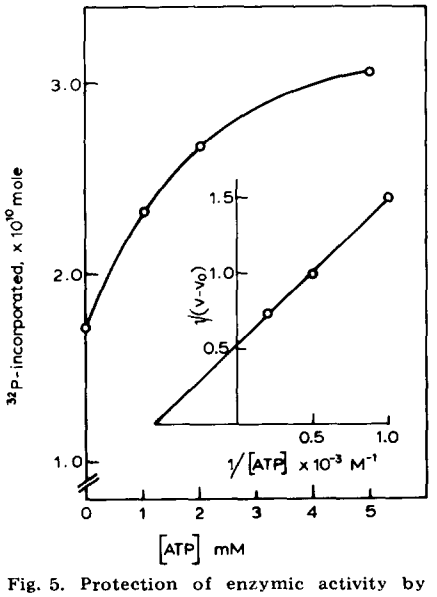

Fig. 5. Protection of enzymic activity by ATP against heat inactivation. A 50-μl reaction mixture contained 0.1 M Tris · HCl, pH 8.0, 2.5 units polynucleotide kinase and various concentrations of ATP, and incubated at 38°C. A 20-μl aliquot was withdrawn at time 0 and 30 min after the start of incubation and assayed for the remaining activity. The reaction mixture contained 1 unit heat-treated enzyme, 2 mM [γ-^{32}P] ATP, 6 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 M Tris · HCl, pH 8.0 and 0.1 mM $_{OH}(rA)_{25}$, and incubated at 30°C. A 10-μl aliquot was assayed for the activity as described in the text. Double reciprocal plots are also shown to estimate the protection constant.

tected it almost totally against the inactivation [5]. A similar result was obtained with T_4-induced polynucleotide kinase and ATP as shown in Fig. 5, although the conditions of the experiment did not cause the enzyme complete inactivation. The protection constant was estimated to be $1.8 \cdot 10^{-3}$ M [13]. These results support the theory that the enzyme is able to bind one substrate to the corresponding catalytic site in the absence of the other substrate.

Discussion

The present study suggests that phosphate transfer reaction by polynucleotide kinase obeys a rapid-equilibrium random Bi-Bi mechanism. The reaction process in the absence of products can be written as:

$$E + A \rightleftharpoons EA \qquad K_1 = [E][A]/[EA]$$
$$EA + B \rightleftharpoons EAB \qquad K_2 = [EA][B]/[EAB]$$
$$E + B \rightleftharpoons EB \qquad K_3 = [E][B]/[EB]$$
$$EB + A \rightleftharpoons EAB \qquad K_4 = [EB][A]/[EAB]$$

where K represents a constant, A and B ATP and $_{OH}$DNA (or $_{OH}(rA)_{25}$), respectively, and E is the enzyme. The rate equation will be:

$$v = \frac{V[A][B]}{K_1K_2 + K_2[A] + K_4[B] + [A][B]} \qquad \text{(i)}$$

All the values of K and V were calculated graphically from the secondary replots of the primary plots shown in Fig. 2, and stated in Table II [10]. If we assume that these constants represent the affinity of the substrates for the enzyme, then DNA seems to have much higher affinity than RNA. The first substrate binding to the enzyme seems to reduce the affinity of the second substrate about two to three times. This suggests that the first substrate binding may cause a certain change in the enzyme structure [12]. Considering the fact that polynucleotide kinase consists of four subunits, this may be an interesting question for further investigation. The individual rate constant is not possible to determine except the breakdown step of the ternary complex, which is equal to the turnover number ($V/[E]_{total}$). We could not ontain the exact concentration of the enzyme solution used, but by comparing the specific activity with that of 65 000 per mg protein (reported by Panet et al., who purified the

TABLE II

KINETIC CONSTANTS FOR POLYNUCLEOTIDE KINASE REACTION (M)

Kinetic constants	Substrate	
	$_{OH}$DNA	$_{OH}(rA)_{25}$
K_1	$4.4 \cdot 10^{-5}$ M	$1 \cdot 10^{-4}$ M
K_2	$1.6 \cdot 10^{-6}$ M	$3 \cdot 10^{-5}$ M
K_3	$7 \cdot 10^{-7}$ M	$1 \cdot 10^{-5}$ M
K_4	$1.2 \cdot 10^{-4}$ M	$2 \cdot 10^{-4}$ M
V	$4.25 \cdot 10^{-13}$ (mol/s)	$3.3 \cdot 10^{-13}$ (mol/s)

enzyme to show a single band on sodium dodecyl sulfate gel electrophoresis [4]), we calculated the turnover number to be roughly 6—10 s^{-1} for both $_{OH}$DNA and $_{OH}(rA)_{25}$ as the phosphate acceptor.

For the inhibition study, we assumed that both ADP and $_{P}$DNA (or $_{P}(rA)_{25}$) served as a dead-end inhibitor. In this case, the rate equation should be multiplied by the factor of $(1 + [I]/K_i)$ where the inhibitor can bind any form of the enzyme [11]. Therefore, the rate equation (i) in the presence of ADP should be:

$$v = \frac{V[A][B]}{K_1(1 + [I]/K_i)K_2 + K_2[A] + K_4(1 + [I]/K_i)[B] + [A][B]} \quad \text{(ii)}$$

in reciprocal forms,

$$\frac{1}{v} = \left(\frac{1}{[A]}\right)\left(\frac{K_1K_2}{V[B]} + \frac{K_4}{V}\right)\left(1 + \frac{[I]}{K_i}\right) + \left(\frac{1}{V}\right)\left(\frac{K_2}{[B]} + 1\right) \quad \text{(iii)}$$

or

$$\frac{1}{v} = \left(\frac{1}{[B]}\right)\left(\frac{K_1(1 + [I]/K_i)K_2}{V[A]} + \frac{K_2}{V}\right) + \left(\frac{1}{V}\right)\left(\frac{K_4(1 + [I]/K_i)}{[A]} + 1\right) \quad \text{(iv)}$$

where K_i is the inhibition constant and [I] is the concentration of ADP. Thus when $1/v$ is plotted against 1/[ATP] in the presence of ADP, the pattern would be competitive (eqn. (iii)). When $1/v$ is plotted against $1/[_{OH}$DNA] in the presence of ADP, the pattern would be non-competitive (eqn. (iv)). Similar equations are obtained for the reaction in the presence of $_{P}$DNA and $_{P}(rA)_{25}$. These equations can well satisfy the experimental data. Although absolute K_i values were not obtained, the apparent K_i values can still give us some information about the ternary complex formation (Table I). For example, pairs which lack phosphate (ADP vs. $_{OH}$DNA) or pairs which have two phosphates ($_{P}$DNA vs. ATP) show a 5 to 6-fold decrease in affinity for the enzyme. This suggests the importance of the special site where the phosphate should be located on the enzyme.

So far, few kinetic studies on the polynucleotide kinase reaction have been published. One important report was made by Lillenhaug and Kleppe [14]. Based on the inhibition studies, it concluded that the enzyme reacts according to the ordered sequential mechanism, i.e., the enzyme reacts first with $_{OH}$DNA and then ATP. In this case, ATP is not bound by the enzyme in the absence of $_{OH}$DNA. This assumption seems to be contrary to the fact that the enzyme and ATP can form a complex detected by protection experiments. Furthermore, van de Sande et al. reported that the enzyme catalyzed the formation of adenosine tetraphosphate from ATP in the reverse reaction [6]. This suggests that the binding site for ATP and ADP is identical, i.e., they bind to the same catalytic form of the enzyme. Therefore, it seems to be a suitable explanation for the polynucleotide kinase reaction to assume the random Bi-Bi mechanism. However, we cannot rule out the possibility that the enzyme behaves differently depending on conditions such as pH and the presence of activators or inhibitors [15]. Especially if an individual subunit has different

catalytic properties from others, the situation would become much more complicated, and the results reported here may be only one aspect of the polynucleotide kinase reaction among numerous others.

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