Deoxyribonucleic Acid Kinase from Nuclei of Rat Liver: Mechanism, Reversal, and Inhibitors of the Reaction†

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ABSTRACT: The DNA kinase from rat liver nuclei has been shown to use ATP to phosphorylate the 5′-hydroxyl terminus of DNA and longer oligodeoxynucleotides; in contrast to the phage-induced polynucleotide kinase, it is inactive on RNA, low molecular weight oligonucleotides, and nucleoside 3′-monophosphates (Levin, C. J., & Zimmerman, S. B. (1976) *J. Biol. Chem. 251*, 1767–1774). Kinetic measurements indicate that the kinase acts by a sequential mechanism. Protection by substrates against thermal inactivation indicates a random order of enzyme interaction with the substrates. The kinase reaction is readily reversible. Upon incubation with ADP, the terminal phosphate group is stoichiometrically recovered as ATP. The reverse reaction is promoted by a variety of other nucleoside diphosphates but not by ATP. Both native and denatured DNA are substrates for the reverse reaction. The reverse and forward reactions can be employed to label 5′-terminal phosphates without prior dephosphorylation in an exchange reaction. Inorganic sulfate is a relatively strong inhibitor of the kinase; it is competitive with ATP (K_i = 0.2 mM) and noncompetitive with respect to DNA. Dextran sulfate inhibition of the kinase appears competitive with respect to both ATP and DNA.

The DNA kinase purified from nuclei of rat liver has a unique substrate specificity (Levin & Zimmerman, 1976). It phosphorylates 5′-hydroxyl termini in longer oligodeoxynucleotides and DNA and is inactive on ribopolymers or very short oligodeoxynucleotides (eq 1). An otherwise similar

$$\text{ATP} + \text{5′-hydroxyl terminus in DNA} \rightleftarrows \text{ADP} + \text{5′-phosphate terminus in DNA} \quad (1)$$

enzyme from calf thymus appears to have a low activity on RNA in addition to its activity on DNA (Austin et al., 1978). In contrast, polynucleotide kinase from T2-, T4-, or T6-infected *Escherichia coli* is active on all of these materials (Richardson, 1965; Novogrodsky & Hurwitz, 1966; Novogrodsky et al., 1966). The mechanism and reversal of the DNA kinase reaction have not been previously studied. We felt such studies would be of interest in several regards. First, they allow a more detailed comparison between the mammalian DNA kinase and the polynucleotide kinase of phage-infected bacteria. Also, the properties of the reverse reaction influence the use of DNA kinase as a means to label termini. Finally, a number of materials are potent inhibitors of the DNA kinase, and understanding of their mechanism of action may aid in the use of these inhibitors to understand the function of the kinase in vivo.

Experimental Procedures

Where not specified, materials and methods are as previously described (Zimmerman & Levin, 1975; Levin & Zimmerman, 1976).

Materials

Dextran and dextran sulfate were purchased from Pharmacia and chondroitin sulfates A and C were from Miles. DNA kinase (fraction III) was prepared as in Levin & Zimmerman (1976).

Methods

Assay of DNA Kinase Activity in the Forward Direction. The kinase was assayed by the rate of incorporation of ^{32}P from [γ-^{32}P]ATP into an acid-insoluble product (assay A of Levin & Zimmerman, 1976). For measurements of the K_m for DNA, the specific radioactivity of the [γ-^{32}P]ATP was increased to ca. 1 mCi/μmol, and the pellets were subjected to an additional acid precipitation before plating.

Assay of DNA Kinase Activity in the Reverse Direction. The complete system (0.30 mL) contained 7 μg/mL of ^{32}P-labeled nicked DNA (prepared as for ligase assays; Zimmerman & Levin, 1975), 50 mM sodium succinate buffer (pH 5.5), 10 mM $MgCl_2$, 33 μg/mL of bovine plasma albumin, 13 mM β-mercaptoethanol, nucleotides as indicated, enzyme, and water. Enzyme was generally dialyzed as in Levin & Zimmerman (1976). After incubation at 37 °C, the tubes were chilled and calf thymus DNA (0.2 mL of 0.25 mg/mL) and cold trichloroacetic acid (0.5 mL of 10%) were added. After 5 min at 0 °C, the tubes were centrifuged 5 min at 8000*g* and a 0.5-mL aliquot of the supernatant fluids was plated with 1.5 mL of 2 M NH_4OH in stainless steel planchets. Acid-soluble radioactivity is expressed as percent of the total counts (2000–5000 cpm) added to the reaction mixtures. At low extents of reversal, the amount of ^{32}P released was approximately proportional to both time and enzyme. For example, in the presence of 0.1 mM ADP, addition of 0.017, 0.035, or 0.070 unit of kinase released 6, 11, or 19% of the ^{32}P in 5 min and 12, 18, or 30% in 10 min, respectively. With large amounts of kinase (0.35 unit, 40 min), >90% of the label was released.

Kinetic Experiments. The general approach to determining mechanism by kinetic considerations is as described by Cleland (1970) and by Plowman (1972). In the reciprocal plots, all lines are determined by unweighted least squares.

Results

Mechanism of the Reaction. The DNA kinase reaction proceeds by a sequential mechanism, as indicated by several lines of evidence. First, kinetic experiments are consistent with a sequential process and are not consistent with an alternative "ping-pong" mechanism. Secondly, attempts to demonstrate an enzyme–phosphate derivative such as might be involved in a "ping-pong" mechanism by exchange reactions were unsuccessful.

Initial Velocity of the Reaction. Under the normal assay conditions, the K_m for ATP is ca. 1 μM, while the K_m for DNA is <3 μg/mL. For the kinetic experiments to be described, we have added 0.1 M KCl to the standard incubation mixture

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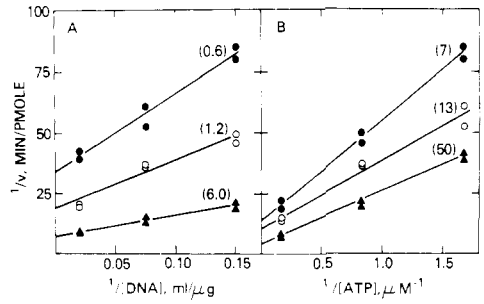

FIGURE 1: Influence of substrate concentration on the velocity of the forward reaction of the kinase. The kinase was assayed as under Methods in the presence of 0.1 M KCl. The numbers in parentheses are the concentrations of ATP (μM) or DNA (μg/mL) in A or B, respectively, for each line. The pairs of points represent the average of triplicate determinations in each of two independent experiments.

Table I: DNA Kinase Is Protected by Either DNA or ATP against Thermal Inactivation[a]

preincubation time (min)	relative kinase activity, substrate present during preincubation at 42 °C		
	none	DNA	ATP
0	(100)[b]	94	98
5	18	40	58
10	4	12	33
20	0	2	10

[a] DNA kinase was preincubated at 42 °C in mixtures containing the normal assay concentrations of succinate buffer, $MgCl_2$, mercaptoethanol, and plasma albumin and, where indicated, of DNA or ATP. At the indicated times, the mixtures were chilled and additions were made so that all tubes contained the normal assay levels of ATP and DNA as well as of all the other components. The completed mixtures were then incubated at 37 °C for 20 min, and the acid-insoluble ^{32}P was estimated by the usual protocol. KCl was present at 0.1 M during both incubations. [b] 8.4 pmol of ^{32}P incorporated.

in order to increase the K_m for DNA into an experimentally measurable range; this addition decreased the maximum velocity of the reaction by <10%. Double-reciprocal plots of initial velocity as a function either of DNA concentration at several ATP levels (Figure 1A) or of ATP concentration at several DNA levels (Figure 1B) yielded nonparallel lines. Such patterns are indicative of a sequential mechanism, whereas parallel lines indicate a "ping-pong" mechanism. The K_m values for ATP and DNA were estimated by replotting the slopes and intercepts from Figures 1A and 1B as functions of the changing fixed substrate. In the presence of 0.1 M KCl, the K_m for ATP is 6 μM and the K_m for DNA is 24 μg/mL.

If the mechanism involved an enzyme–phosphate intermediate, certain exchange reactions should occur. In particular, we examined the UDP-dependent incorporation of radioactivity from [γ-^{32}P]ATP into UTP in the absence of DNA. (UDP is shown below to support the reverse reaction of the kinase.) After the reaction, unlabeled UTP was added to the acid-soluble fraction of the reaction mixture and the UTP was reisolated by successive chromatography on DEAE-cellulose and Dowex 1 columns. The reisolated UTP contained no significant radioactivity. This negative result is consistent with a sequential mechanism.

Substrate Protection against Thermal Inactivation. The kinase was unstable if preincubated under assay conditions (37 °C) but in the absence of its substrates. Such preincubation was also carried out at a slightly elevated temperature (at 42 °C) to increase the rate of inactivation. Either DNA or ATP at their respective assay concentrations provided significant stabilization (Table I). As the assay concentration of either substrate was diminished, the protective effects were maintained essentially unchanged until concentrations of the same order as the K_m values (at 37 °C) were reached, at which point thermal protection was lost. The protection afforded by either DNA or ATP indicates the enzyme can combine independently with either substrate and so is most consistent with a random sequential mechanism.

Table II: Nucleotide Specifity in the Reverse Reaction[a]

nucleotide or other acceptor	acid-soluble ^{32}P formed with given concn of acceptor (pmol/10 min)	
	25 μM	250 μM
ADP (enzyme or $MgCl_2$ omitted)	<0.02	<0.02
ADP	0.33	0.36
dADP		0.41
CDP	0.07	0.28
dCDP	0.10	0.23
UDP	0.13	0.24
dTDP	0.12	0.18
GDP	0.20	0.20
dGDP	0.24	0.23
AMP	<0.02	<0.02
PP_i	<0.02	<0.02
P_i		<0.02

[a] The reverse reaction was assayed as under Methods except that ADP was replaced with nucleotides, PP_i, or P_i as indicated, and $MgCl_2$ was omitted where shown.

Reversal of the Reaction. Reversal of the kinase reaction could be readily demonstrated, although it occurs at a rate several orders of magnitude slower than in the forward reaction. The DNA substrate for the reverse reaction was labeled in its 5′-terminal phosphate groups by the action of the kinase in the forward reaction. The product of the reverse reaction with ADP and [^{32}P]DNA was identified as [^{32}P]ATP by its cochromatography with unlabeled ATP on a DEAE-cellulose column (Chaykin et al., 1965). The radioactivity in the ATP peak was totally adsorbable by charcoal and was acid soluble. Further, the amount of ^{32}P released from DNA is quantitatively accounted for by the amount of [^{32}P]ATP formed; no significant radioactivity was found in inorganic phosphate, AMP, ADP, or adenosine tetraphosphate.

The reverse reaction required the presence of a divalent cation and a suitable nucleotide (Table II). A variety of ribo- and deoxyribonucleoside diphosphates served this function, whereas AMP, PP_i, and P_i were all inactive. ATP does not support the reverse reaction, although at sufficiently high DNA concentrations an apparently ATP-dependent reaction takes place due to formation of ADP in the forward reaction on nonphosphorylated 5′ termini.[1]

The rates of the forward and reverse reactions showed a closely similar dependence upon the pH of the medium (Figure 2). The temperature of incubation had no striking differential effect on the rates in the forward and reverse directions: the rate of reaction in both directions at 37 °C was 3–4-fold higher than at 20 °C and >30-fold higher than at 0 °C. The rate of reaction with native DNA as a substrate is about five times that with denatured DNA (DNA heated 5 min at 100 °C, quenched).

It should be possible to introduce label from [γ-^{32}]ATP without prior dephosphorylation at the nicks. Such an

[1] The rate of the reverse reaction in the presence of ATP was <3% of the rate with ADP when assayed as under Methods but at a DNA concentration of 2 μg/mL. As the DNA concentration was raised, the ATP-dependent rate approached that of the ADP-dependent reaction: 13% at 6.7 μg of DNA/mL and 35% at 20 μg of DNA/mL.

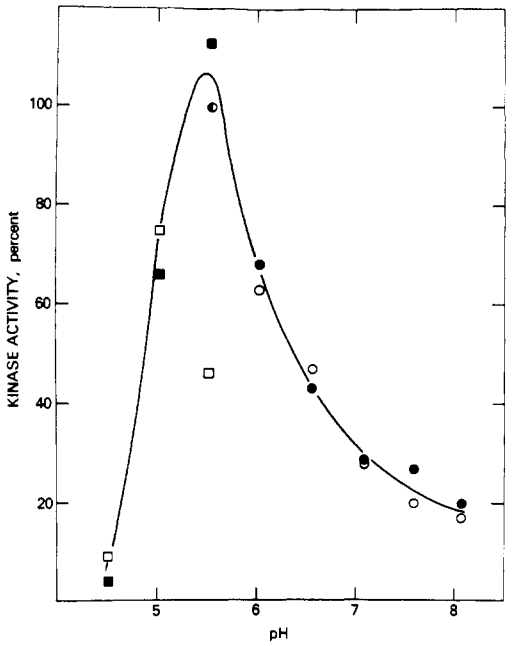

FIGURE 2: Comparison of the effect of pH on the rates of the forward and reverse reaction of the DNA kinase. Forward reaction (closed symbols) and reverse reaction (open symbols) rates were measured as described under Methods except that 0.05 M sodium succinate (□, ■) or Tris–maleate (○, ●) buffer at the indicated pH values was substituted for the usual buffer. The data are normalized to the rate in Tris–maleate buffer, pH 5.6.

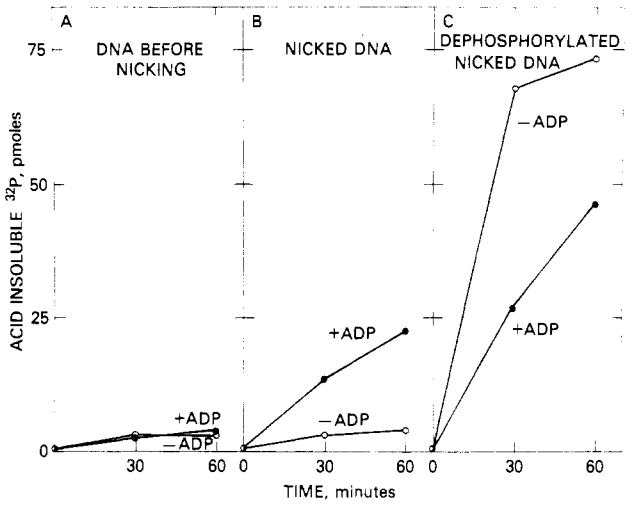

FIGURE 3: ^{32}P incorporation into nicked DNA by an exchange reaction. Assays were carried out as described under Methods for the measurement of the reverse reaction except that [γ-^{32}P]ATP (13 μM, 4300 cpm) was present and unlabeled DNA (30 μg/mL) replaced the labeled DNA. Where indicated, ADP (0.1 mM) was added. Samples of DNA before or after nicking by pancreatic DNase or after nicking and dephosphorylation were prepared as described (Zimmerman & Levin, 1975) for making the ligase substrate; the DNA samples were all similarly treated with phenol, ethanol precipitated, and dialyzed before use.

ADP-dependent exchange reaction is demonstrated in Figure 3. If DNA has not been exposed to pancreatic DNase to generate nicks, only a low-level of radioactivity is incorporated from [γ-^{32}P]ATP, presumably by phosphorylation of already existing 5′-hydroxyl groups (Figure 3A). A prior exposure to pancreatic DNase yields a DNA sample which shows the predicted ADP-dependent incorporation of ^{32}P (Figure 3B). If the nicked DNA is dephosphorylated, then label is introduced more rapidly in the absence of ADP, presumably by the forward reaction (Figure 3C).

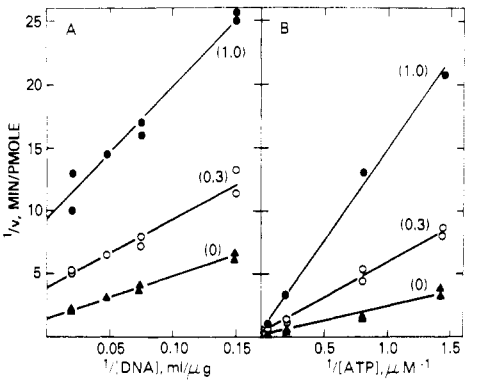

FIGURE 4: Inorganic sulfate inhibition of the forward reaction of the kinase as a function of substrate concentrations. The kinase was assayed as under Methods in the presence of 0.1 M KCl. The numbers in parentheses are the concentrations of Na_2SO_4 (mM) for each line. Duplicate points are from independent experiments.

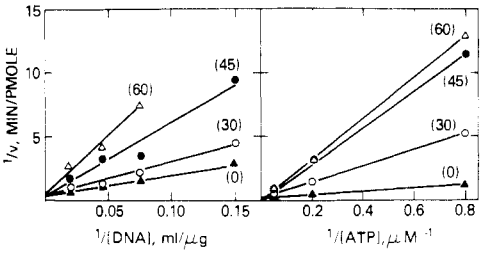

FIGURE 5: Dextran sulfate inhibition of the forward reaction of the kinase as a function of substrate concentrations. The kinase was assayed as under Methods in the presence of 0.1 M KCl. The numbers in parentheses are the concentrations of dextran sulfate (ng/mL) for each line. The points are the averages for from four to seven values.

Inhibitors of the Kinase. Inhibition by Inorganic Sulfate. The kinase was previously shown to be inhibited by very low levels of inorganic sulfate and related compounds (50% inhibition in the forward direction at 0.3 mM Na_2SO_4 under routine assay conditions). The reciprocal plots shown in Figure 4 clearly indicate that sulfate is competitive with respect to ATP binding (K_i = 0.2 mM) and is noncompetitive with respect to DNA. Inhibition by sulfate of the *reverse* reaction requires much higher levels of sulfate (50% inhibition of the reverse reaction at ca. 4 mM Na_2SO_4).

Inhibition by Dextran Sulfate and Heparin. The kinase is very sensitive to inhibition by dextran sulfate or heparin (50% inhibition at ca. 20 ng/mL or 60 ng/mL, respectively, under normal assay conditions). Samples of chondroitin sulfates A or C or dextran did not inhibit, even at much higher levels. Reciprocal plots of dextran sulfate inhibition indicate it is competitive with respect to both ATP and DNA (Figure 5). The forward and reverse reactions are inhibited by similar amounts of dextran sulfate.

Nucleotide Inhibition. Previously we had reported that deoxycytidine nucleotides were relatively strong inhibitors of the kinase. This inhibition has proven to be highly variable in commercial samples and is presumably due to contaminants.

Discussion

The DNA kinase catalyzes a reversible bimolecular group transfer reaction. Mechanisms of such reactions have been divided into two major categories, namely, sequential and ping-pong mechanisms, based upon the order of interaction between the enzyme and its substrates (Cleland, 1970; Plowman, 1972). Sequential reactions are those which demand *all* substrates be bound to the enzyme before any of the substrates are covalently altered. In contrast, in a ping-pong mechanism, one substrate modifies the enzyme covalently (e.g.,

by phosphorylation or adenylylation) and then departs from the surface of the enzyme; upon binding of a second substrate in a subsequent independent reaction, the group originally transferred to the enzyme by the first substrate is covalently joined to the second substrate and unmodified enzyme is regenerated. Our studies on the DNA kinase are fully consistent with its acting by a sequential mechanism. The initial velocity studies as a function of substrate concentration yield reciprocal plots with a series of straight lines which clearly intersect, i.e., a typical sequential pattern. (In contrast, ping-pong mechanisms generally yield a set of parallel lines.) In addition, we were unable to detect an exchange reaction in the absence of DNA which could reasonably be expected to occur if a ping-pong mechanism were operative. Hence, we conclude that the kinase acts without formation of a covalent enzyme–substrate complex. We have not extensively pursued the question of whether there is a preferred order of addition of the substrates or release of the two products. Since either DNA alone or ATP alone affords significant protection to the kinase against thermal inactivation, either substrate can apparently interact with the enzyme in the absence of the other, suggesting that there is no preferred order of binding of substrates. Hence our data are consistent with a random sequential mechanism for the DNA kinase, although it should be noted that we have no information relative to the order of release of products. Most of the kinases whose mechanism have been elaborated fall into this category, although there are examples of kinases which act by ordered sequential or by ping-pong mechanisms (Morrison & Heyde, 1972; see also Boyer, 1973a,b). Of particular interest are studies on the phage-induced polynucleotide kinase since its overall reaction bears the closest resemblance to that of the DNA kinase. Kinetic studies indicate that the T_4 kinase also acts by a sequential mechanism; there is disagreement as to whether the mechanism is ordered or random with respect to the addition of the substrates (Lillehaug & Kleppe, 1975a,b; Sano, 1976).

The DNA kinase reaction is readily reversible. In the presence of ADP, a stoichiometric amount of ATP is formed. A wide variety of nucleoside diphosphates promote the reverse reaction. The reverse reaction of the mammalian DNA kinase differs from that of the phage-induced polynucleotide kinase in a number of respects. For example, the microbial kinase can form adenosine tetraphosphate in an ATP-dependent reverse reaction and also releases a significant fraction of the 5′-phosphate termini as inorganic phosphate (van de Sande et al., 1973); the DNA kinase does not appear to carry out either reaction. The pH optima for the forward and reverse reactions of the microbial kinase occur at ca. pH 9 and 6, respectively, while the pH optimum of the DNA kinase in both the forward and reverse directions occurs at ca. pH 5.5. The reverse reaction of the DNA kinase or of the polynucleotide kinase introduces complications when the enzymes are used to specifically label 5′-hydroxyl termini in the presence of unlabeled 5′-phosphate groups. The latter groups may undergo an exchange reaction with [γ-^{32}P]ATP and lead to an overestimate of the number of 5′-hydroxyl termini. The reverse reaction of the microbial polynucleotide kinase has been diminished relative to the forward reaction by lowering the temperature and by raising the pH and DNA concentrations (Okazaki et al., 1975). In contrast, the relative rates of the forward and reverse reactions carried out by the mammalian DNA kinase are essentially invariant with changes in pH or temperature. The exchange between the γ-phosphate moiety of ATP and 5′-phosphate termini in DNA may be exploited to allow labeling of the termini without an intermediate dephosphorylation. We have demonstrated that the DNA kinase as well as the polynucleotide kinase (van de Sande et al., 1973; Chaconas et al., 1975; Berkner & Folk, 1977) can be used for such labeling. The unique specificity of the DNA kinase for DNA to the exclusion of RNA should offer advantages in the use of this enzyme in labeling termini by exchange or direct phosphorylation.

Inorganic sulfate is a potent inhibitor of DNA kinase. It appears to inhibit by competing with ATP. Certain sulfated polymers are also inhibitors (e.g., dextran sulfate, heparin); the mechanism of their inhibition appears more complex and has not been fully characterized. In any case, both the inorganic and organic sulfate derivatives offer a relatively mild means of terminating kinase activity in vitro.

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