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3′-Phosphatase Activity in T4 Polynucleotide Kinase†

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ABSTRACT: The purification of T4 polynucleotide kinase results in the copurification of an activity which will specifically remove the 3′-terminal phosphate from a variety of deoxyribonucleotides and ribonucleotides in the absence of ATP. This phosphatase activity requires magnesium, has a pH optimum of 6.0, and is more active with deoxyribonucleotides than ribonucleotides. T4 polynucleotide kinase and the 3′-phosphatase activity copurify by gradient elution column chromatography on DEAE-cellulose, phosphocellulose, and hydroxylapatite. The two activities are included in and comigrate on Sephadex G-200. Polyacrylamide gel electrophoresis at pH 9.2 results in comigration of the two activities together with the major protein band. The two activities respond in parallel to heat inactivation at 35 °C and ATP, a substrate for the kinase only, protects both activities from heat inactivation. It is therefore suggested that the two activities are functions of the same protein molecule.

Polynucleotide kinase from bacteriophage T_4 infected *Escherichia coli* (EC 2.7.1.78) catalyzes the transfer of the γ phosphate of ATP[1] to the 5′-hydroxyl termini of nucleic acids (Richardson, 1972). Although the biological function of this enzyme has not been established (Chan and Ebisuzaki, 1970), it is widely used for the preparation of ^{32}P-labeled nucleic acids for sequence determination (Szekely, 1972; Maxam and Gilbert, 1977) and for the synthesis of substrates for DNA and RNA ligase (Khorana et al., 1972; Walker et al., 1975). During attempts to use polynucleotide kinase to phosphorylate oligoribonucleotides with a 3′-terminal phosphate (Uhlenbeck and Cameron, 1977), it was discovered that highly purified preparations of the enzyme removed the 3′-phosphate from an oligomer during the time that the 5′-phosphate was added. This phosphatase activity did not require ATP and would specifically remove the 3′-phosphate from a variety of oligoribonucleotides. In an effort to remove this undesirable phosphatase activity, a purification procedure for polynucleotide kinase was followed with assays of both activities. Data were obtained

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; poly(U), poly(uridylic acid); PEI, polyethylenimine; ATP, adenosine triphosphate; pTp, 2′-deoxythymidine 3′,5′-bisphosphate; Tp, 2′-deoxythymidine 3′-monophosphate.

which suggest that the polynucleotide kinase and 3′-phosphatase activities copurify precisely in a variety of chromatographic systems. Additional electrophoretic and heat inactivation data support the contention that both activities are catalyzed by the same protein.

Materials and Methods

Materials. Escherichia coli cells infected by bacteriophage T4 *am* 4314, a mutant in the T4 DNA polymerase gene, and [γ-^{32}P]ATP were gifts of R. I. Gumport. Salmon sperm DNA (Sigma Chemical Co.) was hydrolyzed with micrococcal nuclease to produce a substrate for polynucleotide kinase as described by Richardson (1972). $(Up)_5$ was obtained by limited alkaline hydrolysis of poly(U) (Uhlenbeck and Cameron, 1977). $(pU)_5$ was obtained by a limited hydrolysis of poly(U) with *Neurospora crassa* endonuclease. Poly(U) (5000 A_{260} units) was incubated with 250 units of nuclease (Miles Laboratories, units defined by supplier) in 10 mM $MgCl_2$ and 0.1 M Tris (pH 8.0) at 37 °C for 24 h. The $(pU)_n$ oligomers were separated on a 100-mL DEAE-Sephadex A-25 column with a 2-L linear gradient of 0.2 to 1.0 M triethylammonium bicarbonate. $(pU)_5$ (300 A_{260} units) was recovered and desalted by evaporation. Nucleoside monophosphates were purchased from Sigma Chemical Co. Adenosine 2′,5′-bisphosphate, adenosine 3′,5′-bisphosphate, and pTp were purchased from P-L Biochemicals. [5′-^{32}P]pTp was obtained by incubating Tp with [γ-^{32}P]ATP and polynucleotide kinase under the same conditions described previously for the preparation of [5′-^{32}P]p$(Up)_5$ (Uhlenbeck and Cameron, 1977). In this case the product was purified by descending paper chromatography using a 7:3 (v/v) mixture of 95% ethanol and 1 M ammonium acetate saturated with boric acid at pH 7 (Plesner, 1955).

Polynucleotide Kinase Assay. The procedure developed by Richardson (1972) for the detection of the transfer of radioactivity from [γ-^{32}P]ATP to acid-insoluble DNA was modified slightly. A 50-μL assay mixture contained 2.5 μmol of Tris-HCl (pH 7.6), 0.5 μmol of $MgCl_2$, 0.5 μmol of 2-mercaptoethanol, 4 μg of bovine serum albumin, 1 nmol of [γ-^{32}P]ATP (0.7–1.0 Ci/mol), 60 nmol of DNA substrate, and 0.1 to 1.0 unit of enzyme. After incubation at 37 °C, the reactions were terminated by the addition of 1.5 mL of 5% cold trichloroacetic acid and filtered by suction through cellulose nitrate filters with two 1.5-mL rinses of 5% trichloroacetic acid and one of 95% ethanol. The filters were dried and the radioactivity was measured in a liquid scintillation counter. One unit of polynucleotide kinase activity is defined as 1 nmol of [γ-^{32}P]ATP rendered acid insoluble in 30 min. Slight differences in the substrate concentrations make 1 unit using the above assay conditions equal to 1.5 units using the assay conditions of Richardson (1972).

3′-Phosphatase Assays. Two different types of assays were used to detect the removal of 3′-phosphate groups. Both are patterned after the deoxynucleotidase assays of Becker and Hurwitz (1967). Assay 1 colorimetrically measures the release of inorganic phosphate from Tp. A 25-μL assay mixture contained 2.5 μmol of imidizole hydrochloride (pH 6.0), 0.25 μmol of $MgCl_2$, 0.25 μmol of 2-mercaptoethanol, 0.5 μg of bovine serum albumin, 0.4 μmol of Tp and enzyme. After incubation at 37 °C, the reaction is terminated by the addition of 0.275 mL of cold water. The inorganic phosphate released is measured by adding 0.7 mL of ascorbic acid–ammonium molybdate solution, incubating at 45 °C for 20 min, and reading the absorbance at 820 nm (Chen et al., 1956).

When assaying column fractions, assay 1 could not be used since all columns were eluted with phosphate buffers. Instead, the formation of [5′-^{32}P]pT from [5′-^{32}P]pTp was measured in assay 2. In this case, the 25-μL reaction mixture contained 2.5 μmol of imidazole hydrochloride (pH 6.0), 0.25 μmol of $MgCl_2$, 0.25 μmol of 2-mercaptoethanol, 0.5 μg of bovine serum albumin, 2.5 nmol of [5′-^{32}P]pTp (20 Ci/mol), and enzyme. After incubation at 37 °C, 2-μL aliquots were withdrawn and spotted on 6.6-cm PEI thin-layer chromatography plates (Brinkmann). The plates were developed in 1.0 M ammonium formate and dried and the radioactivity of the pT and pTp regions was determined by liquid scintillation counting.

One unit of 3′-phosphatase activity is defined as 1 nmol of 3′-phosphate removed at 37 °C in 30 min for both assays. Since the substrate identity and concentration are different in the two assays, 1 unit of enzyme measured by assay 2 equals 5 units of enzyme measured by assay 1. Assay 1 is found to be more accurate than assay 2 since only a small fraction of the substrate is hydrolyzed in the assay and a true initial rate may be obtained.

Enzyme Purification. Although the general purification procedure follows Richardson (1972), some changes were made in the early stages so that T4 RNA ligase could be obtained from the same preparation of cells (McCoy, Lubben, and Gumport, in preparation). In addition, all the ion-exchange columns were run with gradients in order to better demonstrate the comigration of the kinase and phosphatase activities. Frozen bacteriophage T4 *am* 4314 infected cells (150 g) were thawed in 0 °C in 400 mL of 50 mM Tris-HCl (pH 7.5), 2.5 mM dithiothreitol, 1 mM EDTA for 12 h. After briefly dispersing the suspension with a blender, the cells were sonicated at less than 6 °C four times in 3-min bursts at 275 W in a Biosonifier cell disrupter. The cell debris was removed by centrifugation at 27 000*g* for 30 min at 4 °C and the supernatant saved. The pellets were resuspended in 150 mL of the sonication buffer, sonicated as before, and centrifuged again. The combined supernatants were diluted to A_{260} of 110 with sonication buffer and brought to 0.7% streptomycin sulfate by the addition of 5% streptomycin sulfate over a period of 20 min at 4 °C. The mixture was centrifuged at 27 000*g* for 10 min and the supernatant was retained as a source of RNA ligase. The streptomycin sulfate pellets were resuspended in 870 mL of 0.1 M potassium phosphate (pH 7.6), 10 mM 2-mercaptoethanol, and 3 mM $MgCl_2$. The suspension was incubated at 37 °C for 1–2 h until 90% of the absorbance at 260 nm became perchloric acid soluble (Richardson, 1972). The suspension was centrifuged at 27 000*g* for 10 min and 370 g of solid ammonium sulfate was added slowly at 4 °C to the 850 mL of supernatant. After stirring at 4 °C for 20 min, the suspension was centrifuged at 27 000*g* for 10 min and the supernatant was discarded. The pellets were suspended in 70 mL of 10 mM potassium phosphate (pH 7.6), 10 mM 2-mercaptoethanol (buffer A) and dialyzed overnight at 4 °C against 6 L of buffer A. The material after dialysis (AS-I, 69 mL, 1235 mg of protein) was the first fraction that could be assayed reliably.

A DEAE-cellulose column (3 × 30 cm) was equilibrated at 4 °C with buffer A and the dialyzed protein fraction applied to it at 1 mL/min. After washing the column with 400 mL of buffer A, a 1-L linear gradient of 10 mM to 100 mM potassium phosphate (pH 7.6) containing 10 mM 2-mercaptoethanol was used to elute the enzyme activity at 4 °C. The active fractions were pooled (DEAE pool, 470 mL, 122 mg of protein), made 0.1 mg/mL in bovine serum albumin, and then precipitated with 70% ammonium sulfate as before. The ammonium sulfate pellets were resuspended in 8.5 mL of 50 mM potassium phosphate (pH 7.6), 10 mM 2-mercaptoethanol, 50 μM ATP (buffer B) and dialyzed overnight at 4 °C against 2 L of buffer B. ATP has been shown to stabilize polynucleotide kinase during purification (Hanggi et al., 1970).

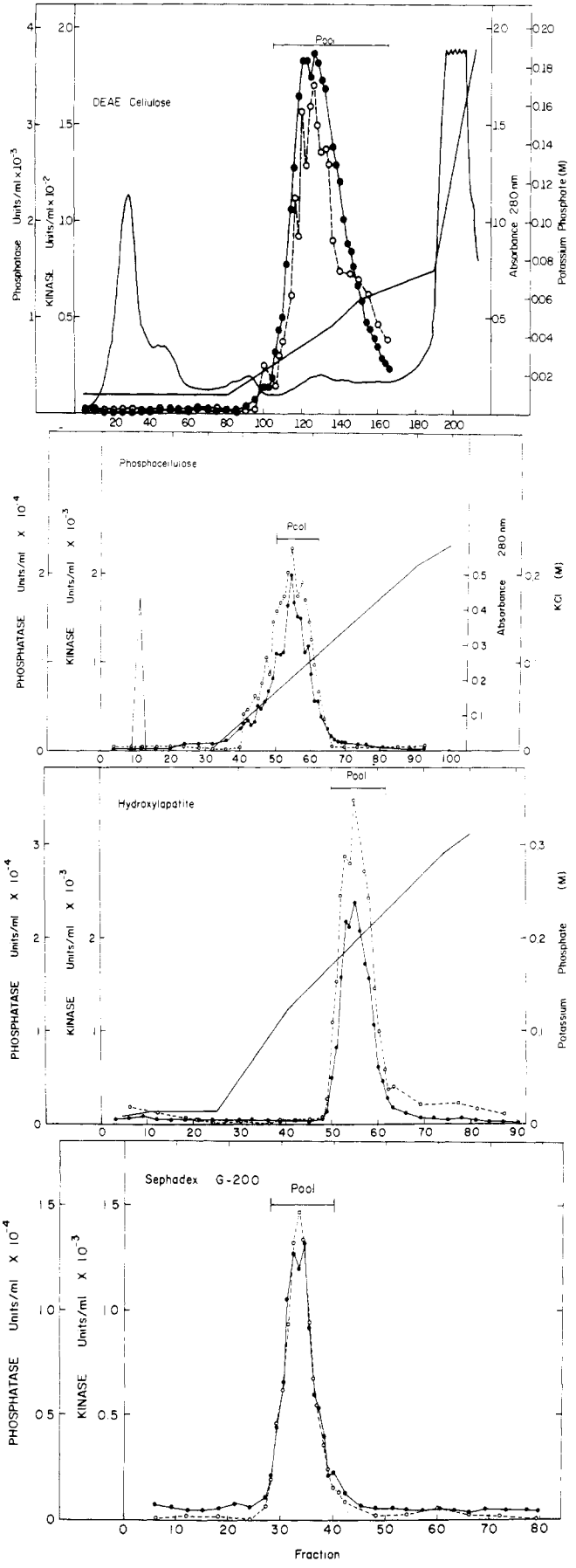

FIGURE 1: Copurification of phosphatase and kinase on (from top to bottom) DEAE-cellulose; phosphocellulose; hydroxylapatite; and Sephadex G-200. In each case, the phosphatase activity measured by assay 2 is marked by open circles and dashed lines and the kinase activity by closed circles and solid lines.

A phosphocellulose column (0.85 × 17 cm) was equilibrated at 4 °C with buffer B and the dialyzed protein fraction applied to it at 15 mL/h. After washing the column with 30 mL of buffer B, a 100-mL linear gradient of 0 to 0.3 M KCl in buffer B was used to elute the enzyme activity. The active fractions were pooled (PC pool, 26 mL) and dialyzed overnight against 4 L of 10 mM potassium phosphate (pH 7.0), 10 mM 2-mercaptoethanol, 50 μM ATP (buffer C). Since very little protein remained, protein concentrations of this fraction and succeeding fractions were not determined.

A hydroxylapatite (Bio-Gel HPT from Bio-Rad Laboratories) column (0.9 × 10 cm) was equilibrated at 4 °C with buffer C and the dialyzed protein fraction applied to it at 15 mL/h. After washing the column with 25 mL of buffer C, a 100-mL linear gradient of 10 mM to 500 mM potassium phosphate (pH 7.0), 10 mM 2-mercaptoethanol, 50 μM ATP was used to elute the enzyme activity. The active fractions were pooled (HAP pool, 16 mL), concentrated 10-fold with Aquacide no. 2 (Calbiochem), and dialyzed overnight against 2 L of 50 mM potassium phosphate (pH 7.0), 10 mM 2-mercaptoethanol, 50 μM ATP (buffer D).

A Sephadex G-200 column (1.8 × 38 cm) was equilibrated at 4 °C with buffer D and the dialyzed protein fraction applied to it at 10 mL/h. The column was eluted in buffer D and the active fractions were pooled (G-200 pool, 15.5 mL), diluted with an equal volume of glycerol, and stored at −20 °C.

Gel Electrophoresis. A 4% acrylamide–0.2% bisacrylamide gel was polymerized in a 6 × 90 mm tube in 0.2 M Tris–borate (pH 9.2) containing 50 μM ATP. Five hundred kinase units of the HAP pool was concentrated to 125 μL and applied to a gel. Electrophoresis was carried out at 4 °C with 2.75 mA per gel for 80 min. The gels were removed from the tubes and either stained with Coomassie blue stain or cut into 2.5-mm slices to assay enzyme activity. Each gel slice was eluted at 4 °C by gently agitating for 12 h in 200 μL of 0.05 M Tris-HCl (pH 7.6), 10 mM 2-mercaptoethanol, and 50 μM ATP.

$NaDodSO_4$-acrylamide gel electrophoresis was carried out as described by Laemmli (1970) in 1 × 100 mm slabs. After staining with Coomassie blue they were scanned with a Helena Quickscan gel scanner.

Results

Copurification of Polynucleotide Kinase and 3′-Phosphatase Activities. Figure 1 shows the elution profiles of the four chromatography columns used in the purification procedure. For each column the polynucleotide kinase activity and the 3′-phosphatase activity using assay 2 were measured. In each of the first three absorption chromatography columns, the polynucleotide kinase activity was found to elute in a single peak at or very near the ionic strength previously reported (Richardson, 1972; Hanggi et al., 1970; Panet et al., 1973; Novogrodsky et al., 1966). The elution position of the kinase activity on G-200 is the same as reported by Panet et al. (1973), and corresponds to a molecular weight of about 150 000. On all four columns, the 3′-phosphatase activity closely comigrates with the kinase activity. The ratio of specific activities is constant across each activity peak within the accuracy of the assays and has approximately the same value of 11–17 phosphatase (assay 2) units per kinase unit. The ratio changes slightly from column to column due to the different amounts of phosphate buffer added with the enzyme to the kinase assay mixture. Phosphate ion is a strong inhibitor of kinase activity (Novogrodsky, 1966) but only a weak inhibitor of phosphatase activity. A better indication of the copurification of the two activities is seen in Table I using pooled, dialyzed fractions and the more accurate assay 1 for phosphatase activity. After the

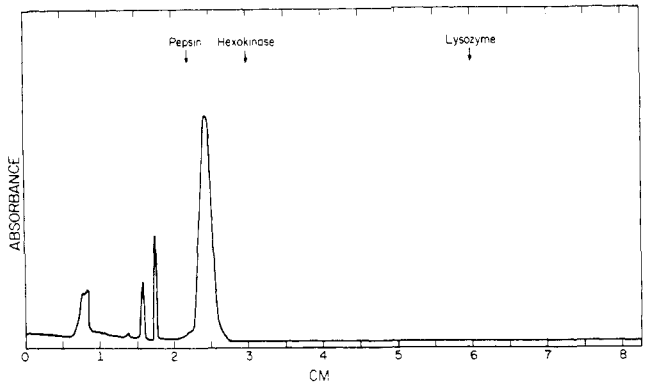

FIGURE 2: A scan of a 15% $NaDodSO_4$-acrylamide slab gel electrophoresis of polynucleotide kinase from the G-200 column.

TABLE I: Enzyme Purification.

Fraction	Units of kinase act.	Units of phosphatase act. (assay 1) × 10^3	Phosphatase to kinase ratio
ASI	35 000	*a*	
DEAE pool	24 000	1900	79
PC pool	8 000	780	97
HAP pool	3 100	300	97
G-200 pool	1 000	77.5	78

[a] 3′-Phosphatase activity contaminated by nonspecific phosphatase activities.

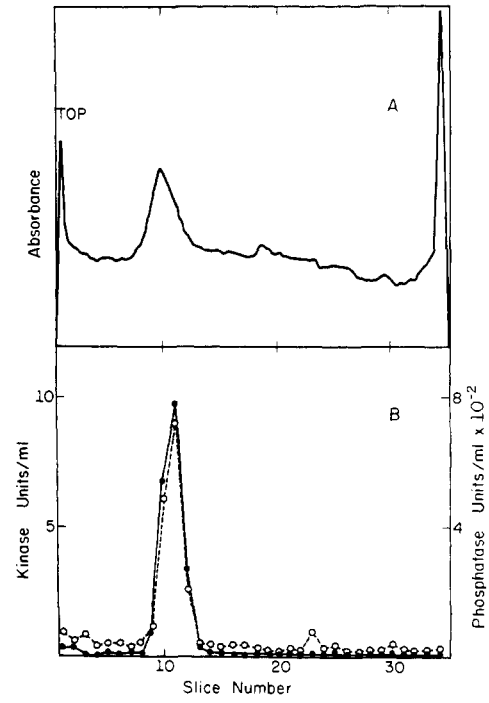

FIGURE 3: Coelectrophoresis of phosphatase and kinase on pH 9.2 polyacrylamide gels. (A) Scan of absorbance of stained gel; (B) kinase activity (closed circles, solid line) and phosphatase activity using assay 1 (open circles, dashed line) eluted from 2-mm slices.

DEAE-cellulose column, the ratio of the two activities remains constant at about 80–90 phosphatase units per unit of kinase. While all the kinase activity which can be recovered from the DEAE column is located in the peak in the gradient, a considerable amount of phosphatase activity also elutes with the 0.5 M potassium phosphate wash applied to the column at the end of the gradient. Further analysis of this phosphatase activity indicates that it is not specific for 3′-phosphate groups.

The purity of the polynucleotide kinase after the G-200 column could be estimated by $NaDodSO_4$-polyacrylamide gel electrophoresis. As can be seen by the scan of a stained gel in Figure 2, a majority of the protein migrates as a single band corresponding to a molecular weight of about 35 000. Three other bands are present in this preparation with apparent molecular weights of 56 000, 49 000, and 45 000. If the stain is proportional to the absorbance, greater than 70% of the total protein is in the major band. Since the minor bands are not seen in all kinase preparations, they are likely to be impurities. Both Panet et al. (1973) and Lillehaug (1977) have reported a molecular weight of 33 000–34 000 daltons on $NaDodSO_4$ gels with purified polynucleotide kinase.

The amount of polynucleotide kinase activity recovered in this purification is quite low. After ammonium sulfate fractionation, Richardson (1972) obtained about 4000 units per g of T4 infected cells. At the corresponding stage in this purification about 350 units per g of T4 infected cells was obtained. The major reason for this difference is that only 0.7% streptomycin sulfate was used to precipitate the kinase activity instead of 0.83% used by Richardson. The low streptomycin concentration was used for two reasons. First, this ensured that T4 RNA ligase remained in the supernatant (McCoy, Lubben, and Gumport, in preparation) which allowed simultaneous purification of the two enzymes. Second, the use of higher concentrations of streptomycin has been shown to result in very poor fractionation on the DEAE cellulose column, especially when frozen cells are used (Hanggi et al., 1970). Subsequent steps in such fractionations are also less effective and the resulting preparations of enzyme are not as pure.

Coelectrophoresis of Kinase and Phosphatase Activities. The enzyme fraction recovered from the hydroxylapatite column is analyzed by acrylamide gel electrophoresis at pH 9.2 in Figure 3. In the upper panel, a scan of the stained gel reveals that the majority (about 80%) of the protein migrates as a single band about one-third of the distance from the anode. In the lower panel slices taken from a parallel gel were eluted and assayed for kinase activity and phosphatase activity using assay 1. The two activities comigrate precisely with one another and have a ratio of 80 phosphatase units per kinase unit similar to that found in Table I. The two activities also closely comigrate with the protein band on the parallel gel. Since the gel electrophoresis experiment was carried out at a pH very different from the pH used for the chromatography columns in the purification, it provides additional evidence that the two activities are present in the same protein molecule.

Heat Inactivation of Kinase and Phosphatase Activities. Polynucleotide kinase has been shown to be extremely sensitive to heat inactivation unless ATP, a substrate of the enzyme, is present to protect the activity (Novogrodsky et al., 1966; Sano, 1976). It was therefore of interest to determine whether the phosphatase activity shows a similar sensitivity to heat inactivation and can also be protected by ATP.

A sample of polynucleotide kinase was dialyzed against 0.4 M NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.05 M Tris-HCl (pH 7.6) for 8 h at 4 °C to remove ATP. The sample was then prepared for heat inactivation by dialyzing for 8 more h at 4 °C against the same buffer without NaCl. Heat inactivation experiments were carried out at 35 °C with and without 4 mM ATP. A control incubation at 0 °C was carried out as well. Aliquots of the three tubes were removed at intervals and immediately assayed for kinase activity and

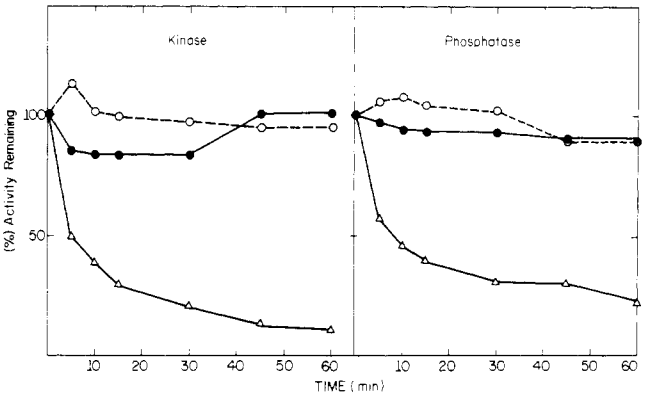

FIGURE 4: Heat inactivation of both activities. A sample of enzyme was incubated either at 0 °C (open circles, dashed line), 35 °C (open triangles, solid line) or 35 °C in the presence of 4 mM ATP (closed circles, solid line). Aliquots of enzyme were removed at various times and assayed for kinase activity (left panel) or phosphatase activity (right panel) using assay 1.

TABLE II: Protection from Heat Inactivation.

Temp (°C)	Oligomer (1 mM)	Kinase act. remaining (%)	Phosphatase act. remaining (%)
0		(100)	(100)
40		8	19
40	$(Up)_5$	55	58
40	$(pU)_5$	4	23
40	$(Up)_4U$	61	49
40	$p(Up)_5$	7	53

phosphatase activity using assay 1. The results of this experiment are shown in Figure 4. Parallel heat inactivation of both activities at 35 °C with a half-life of 5–6 min is observed. The slightly different amounts of residual activity after 60 min are probably due to differential rates of renaturation of the enzyme in the two assay mixtures. ATP (4 mM) protects both activities from heat inactivation at 35 °C. Since ATP is not a substrate for the 3′-phosphatase activity, the ability of ATP to protect that activity from heat inactivation suggests that kinase and phosphatase activities are functions of the same protein. In order to ascertain that ATP was not an undiscovered component of the phosphatase reaction, phosphatase assay 2 was carried out at various concentrations of ATP from 0 to 10 mM. No effect of ATP on either the rate or the extent of the phosphatase reaction was observed. Thus, the similar rates of heat inactivation and the protection of one activity by the substrate of the other provide additional evidence that the two activities are present in the same protein molecule.

Polynucleotide kinase can also be protected from heat inactivation by a wide variety of oligomers with free 5′-hydroxyls (Novogrodsky et al., 1966). In order to examine whether such oligomers will also protect the phosphatase activity from heat inactivation, samples of enzyme prepared as before were incubated at 40 °C for 15 min in the presence of 1 mM oligomer and then assayed for both activities using assay 1 for the phosphatase activity and the standard assay for kinase activity. The results of this experiment are shown in Table II. Under these conditions, $(Up)_5$, a substrate of both the kinase and phosphatase reactions, is effective in protecting both activities from heat inactivation while $(pU)_5$, a product of both reactions, does not protect either activity. Thus, conditions have been chosen such that only substrate protection can occur. Of particular interest, therefore, are those oligomers which are a substrate of one activity and a product of the other. With

TABLE III: Substrate Specificity of 3′-Phosphatase.

Substrate	nmol of P_i released in 60 min
Tp	36
dAp	43
dCp	81
dGp	31
5′-TMP, 3′-UMP, 3′-AMP, 3′-CMP, 3′-GMP, 5′-UMP, 5′-AMP, 5′-CMP, 5′-GMP	>0.5

TABLE IV: Substrate Specificity of 3′-Phosphatase.

	nmol of phosphate released after incubation with	
Oligomer	3′-Phosphatase	Alkaline phosphatase
$(Up)_5$ (40 nmol)	41	44
$(pU)_5$ (40 nmol)	0.5	46
$p(Up)_5$ (40 nmol)	43.5	91

$(Up)_4U$, a substrate of kinase but a product of phosphatase, both activities are protected from heat inactivation. This result is similar to that obtained with ATP, where the kinase substrate protects the phosphatase and again suggests that the two activities are closely related. However, when heat inactivation is attempted in the presence of $p(Up)_5$, only the phosphatase activity is protected. The kinase activity is reduced to a level similar to that obtained when no oligomer is present. Since $p(Up)_5$ is a substrate of the phosphatase and a product of the kinase, the reciprocity in protection of heat inactivation is not observed under these conditions. Kinase substrates can protect phosphatase, but phosphatase substrates do not protect kinase. Thus while the two activities may be closely related physically, it is possible to inactivate one and not the other under specialized circumstances.

Properties of the 3′-Phosphatase Activity. Magnesium ion is required for 3′-phosphatase activity. Replacing the $MgCl_2$ in assay 1 by 10 mM EDTA results in no detectable activity. When increasing amounts of $MgCl_2$ are added to assay 1, the activity reaches a maximum at 3 mM $MgCl_2$ and does not change up to 10 mM $MgCl_2$. Magnesium ion in assay 1 may be replaced by cobalt without significantly altering the rate of reaction, but manganese, zinc, or calcium are unable to substitute for magnesium.

Inorganic phosphate inhibits the 3′-phosphatase activity slightly. Potassium phosphate (5 mM) included in assay 2 reduces the rate of the reaction by about 10%.

When 2′-deoxythymidine 3′-monophosphate is replaced by any one of the other three 2′-deoxynucleotide 3′-monophosphates in assay 1, the reaction occurs at approximately the same rate (Table III). Under these conditions, no detectable release of phosphate was observed for any of the ribonucleotide 3′-monophosphates or the deoxy- and ribonucleotide 5′-monophosphates. However, when the enzyme is incubated with longer oligoribonucleotides with 3′-terminal phosphates, release of phosphate can be detected. Since the 3′-phosphate concentration in assay 1 was prohibitively high for convenient use with oligonucleotides, much lower concentrations were used. In the experiment shown in Table IV, 40 nmol of either $(Up)_5$, $p(Up)_5$, or $(pU)_5$ was incubated in a 50-μL reaction in the buffer used for assay 1 with either 9.5 units of kinase or 2

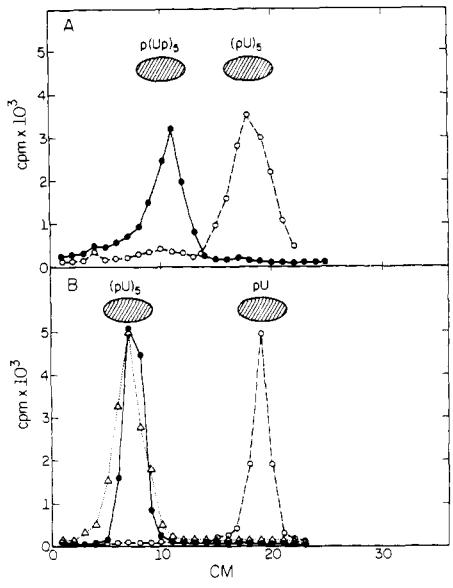

FIGURE 5: Paper chromatography of reaction products. (A) Removal of 3′-phosphate from [5′-^{32}P]p(Up)$_5$ as analyzed by descending paper chromatography. Solid line is before reaction, dotted line after reaction. (B) Hydrolysis of faster moving peak in A with spleen phosphodiesterase (solid line, closed circles) and venom phosphodiesterase (dotted line, open circles). Untreated (dotted line, open triangles).

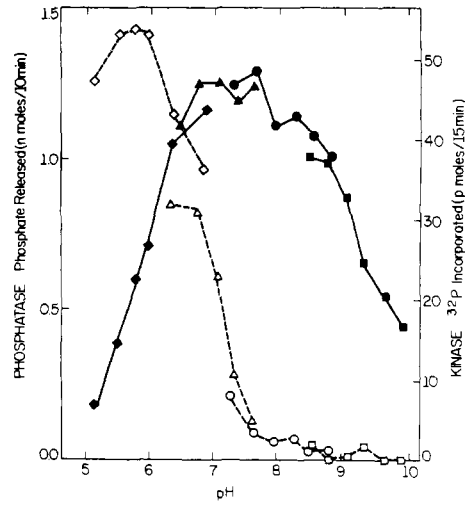

FIGURE 6: pH dependence of both activities. Phosphatase activity with assay 2 is given by open symbols and dashed line and kinase activity by closed symbols and solid line. The assays were identical with those described in Materials and Methods but buffered with 2-(*N*-morpholino)-ethanesulfonic acid (diamonds), imidizole (triangles), tris(hydroxymethyl)aminomethane (circles), and cyclohexylaminoethanesulfonic acid (squares).

μg of bacterial alkaline phosphatase for 2 h at 37 °C. The release of inorganic phosphate was determined colorimetrically as in assay 1. The data show specific quantitative removal of only the 3′-phosphates with the 3′-phosphatase activity and removal of all terminal phosphates with bacterial alkaline phosphatase.

The removal of 3′-phosphates from oligoribonucleotides can also be detected by descending paper chromatography. [5′-^{32}P]p(Up)$_5$ (2.2 nmol) was incubated with 3.8 units of kinase for 1 h in the buffer used for assay 2. The reaction was spotted on Whatman 3 MM chromatography paper. The descending chromatogram was developed for 25 h in a solvent consisting of equal volumes of 1 M NH_4OAc and ethanol. In Figure 5A, the removal of the 3′ phosphate from [5′-^{32}P]p(Up)$_5$ can be detected by an increase in mobility on the chromatogram. The identity of the faster moving peak is confirmed to be [5′-^{32}P]p(Up)$_4$U by the fact that it acquires a sensitivity to snake venom exonuclease as a result of the removal of the 3′-phosphate. The peak (1.5 nmol) was reacted for 30 min with 0.005 units of venom exonuclease in 20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$ and the reaction mixture separated by paper chromatography for 15 h in the same solvent. Figure 5B shows that the oligomer is totally degraded to [5′-^{32}P]pU. Similar treatment with spleen exonuclease gave no hydrolysis, confirming the presence of a 5′-phosphate. Paper chromatography was also used to detect the removal of the 3′-phosphate by the 3′-phosphatase activity from a variety of other oligomers, including (Cp)$_2$Gp, (Cp)$_4$Gp, CpUpGp, CpApGp, and (Ap)$_3$Cp. In these cases, the nonradioactive oligomers were detected on paper by examining under ultraviolet light. The specificity of the enzyme for 3′-phosphates could be demonstrated in this manner as well. When 2 units of kinase was incubated under assay 2 conditions with 0.4 μmol of adenosine 3′,5′-bisphosphate, detectable hydrolysis to pA was observed. Under the same conditions no hydrolysis of adenosine 2′,5′-bisphosphate could be detected.

In Figure 6, the effects of pH on the rate of both enzyme reactions are shown. As reported previously (Richardson, 1972), polynucleotide kinase has a broad activity maximum between pH 6.5 and pH 8.5. The optimum pH for the 3′-phosphatase activity in assay 2 is about pH 5.9. Although comparatively little phosphatase activity remains above pH 8, some removal of 3′-phosphates from oligoribonucleotides may be detected as high as pH 10 (M. Krug, unpublished experiments).

Discussion

Data presented in this paper show that a procedure for purifying T4 polynucleotide kinase leads to the copurification of a 3′-phosphatase activity. Since only a fraction of the total kinase activity in T4 infected cells was recovered by the procedure, this opens the possibility that the purification of a special class of polynucleotide kinase molecules has been effected. However, several reasons may be given which suggest that the polynucleotide kinase obtained in this work is the same enzyme as that obtained by other workers in higher yields. First, although a lower streptomycin sulfate concentration was used, the remainder of the purification procedure is essentially identical with that used by other workers. The activity eluted from each of the three absorption columns at the expected ionic strength and the elution position on Sephadex G-200 was the same as that obtained by others. Second, the subunit molecular weight of 35 000 is within 10% of that obtained previously. Third, the heat inactivation properties of the enzyme correspond closely to those obtained by several workers. Finally, preparations of enzyme purified in this laboratory using higher streptomycin sulfate concentrations and preparations obtained commercially have been found to have a similar ratio of phosphatase to kinase activities. Thus, it is likely that all the polynucleotide kinase activity in T4 infected cells possesses a 3′-phosphatase activity. On the other hand, since additional phosphatase activity was detected on the DEAE-cellulose chromatography column, it is possible that not all the 3′-phosphatase activity in T4 infected cell lysates has kinase activity. However, it was not determined whether this additional phosphatase activity was a result of T4 infection or was due to the 3′-phosphatase activity in *E. coli* (Becker and Hurwitz, 1967).

Becker and Hurwitz (1967) have described a T4-induced

deoxynucleotidase which has many of the properties of the 3′-phosphatase activity described here. In both cases, the enzyme has a pH optimum of about 6, is inhibited by phosphate and rapidly dephosphorylates deoxynucleoside 3′-monophosphates, but does not react with ribonucleoside 3′-monophosphates. However, in contrast to what we describe, the deoxynucleotidase did not remove the 3′-phosphate from $(Up)_4$. Furthermore the purification schemes for the two enzymes are quite different. The relationship between the 3′-phosphatase activity described here and T4 deoxynucleotidase remains to be established.

The presence of an activity in purified preparations of polynucleotide kinase which removes a 3′-phosphate from oligoribonucleotides has been noted previously. Szekely (1972) found that, when using polynucleotide kinase to label oligonucleotides from a T_1 ribonuclease digest of RNA with a [5′-^{32}P]phosphate, a substantial portion of the 3′-phosphates was removed in the process. Although the activity was not further characterized, it was assumed to be a contaminating enzyme in the kinase preparation.

Although not directly demonstrated in this work, it is likely that the 3′-phosphatase activity in polynucleotide kinase will act on DNA as well. Nossal and Hershfield (1971) found that, when DNA digested with micrococcal nuclease to form nicks was reacted with polynucleotide kinase and ATP, the product was a substrate for DNA ligase. Thus, two reactions were performed by the polynucleotide kinase preparation to prepare the DNA to be substrate for DNA ligase. The 3′-phosphate formed by the nuclease had to be removed at the nick and a 5′-phosphate had to be inserted. Although it was assumed by Nossal and Hershfield that the former activity was an impurity in the kinase preparation, the data presented here suggest otherwise. It is possible that a nicked DNA with a 3′-phosphate is the natural substrate of the two activities of polynucleotide kinase. This view would be consistent with the observations that longer oligomers and deoxyribonucleotides are better substrates for the 3′-phosphate activity.

Although the sum of the chromatographic and electrophoretic evidence indicates that the 3′-phosphatase and polynucleotide kinase activities are present on the same protein, the relationship of the two active sites remains to be established. Lillehaug (1977) has shown that the enzyme is a tetramer composed of four identical subunits of 34 000. Thus, presumably both activities must be present on each subunit. A majority of the heat inactivation data suggest that the two activities are closely connected. Both substrates of the kinase can protect the phosphatase from heat inactivation. However, compounds which are only substrates of the phosphatase activity do not protect the kinase activity from heat inactivation. Thus, the active sites are sufficiently independent that one can be inactivated and not the other.

Preliminary genetic evidence suggests that the polynucleotide kinase and the 3′-phosphatase activities are products of the same or closely coupled genes. K. Sirotkin and L. Snyder (personal communication) have measured both activities in cell lysates of a variety of T4 mutants. They find that a number of pseT$^-$ mutants, a gene coding for 3′-phosphatase activity (Depew and Cozzarelli, 1974), are also lacking polynucleotide kinase activity. A more careful examination of the enzymes derived from mutant strains of T4 will be very useful in establishing the relation of the two activities.

The kinase and phosphatase activities of the enzyme may be used independently if desired. By incubating in the absence of ATP, 3′-phosphates can be removed from DNA or RNA without disturbing the 5′-phosphate. If an oligonucleotide with a 3′-phosphate is incubated with the enzyme and ATP at pH 9 or above for limited times, the 5′-phosphate can be introduced without removing a large fraction of the 3′-phosphates. Thus, polynucleotide kinase remains a versatile tool in the enzymatic manipulation of nucleic acid sequences.

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