Isolation and Characterization of Two Mutant Forms of T4 Polynucleotide Kinase*

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The purification of polynucleotide kinase from *Escherichia coli* infected by two different mutants in the T4 polynucleotide kinase (pseT) gene is described. The pseT1 enzyme has virtually no 3' specific phosphatase activity and normal polynucleotide kinase activity. The pseT47 enzyme has very little phosphatase activity and no kinase activity. However, enzyme isolated from a pseT1+pseT47 mixed infection appears to contain heterodimers with considerably more phosphatase activity. Thus, the pseT47 mutation partially inactivates the phosphatase and totally inactivates the kinase. A study of the action of polynucleotide kinase on plasmid DNAs nicked to give a 3'-phosphate and a 5'-hydroxyl indicates that although the enzyme can catalyze both the removal of the 3'-phosphate and the insertion of a 5'-phosphate, there is no evidence for a concerted reaction involving both activities on the same polypeptide chain.

The enzyme encoded by the T4 pseT gene catalyzes two different reactions. It is a polynucleotide kinase which transfers the γ-phosphate from ATP to the 5'-hydroxyl terminus of a DNA or RNA molecule (1) and it is a phosphatase which specifically removes 3'-terminal phosphates from DNA or RNA (2). Although the biochemical properties of both activities have been relatively well characterized (3-5), their role in T4 infection is not understood. Presumably, the physiological function of this enzyme would provide a rationale for having both activities on the same polypeptide chain.

Analysis of mutants in the pseT gene has provided useful information about the possible function of the enzyme. While pseT mutants grow normally on most strains of *Escherichia coli*, two restrictive hosts have been described. One of these, *E. coli* CTr5X, is a derivative of a clinical isolate (6) and the other is a mutation of *E. coli* K-12 called lit (7). When pseT mutants infect either of these hosts, no progeny phage are made, presumably due to a reduction in the rate of phage DNA replication and the absence of any T4 late proteins (8).

While most pseT mutations reduce both the kinase and phosphatase activities, two mutants have been reported to inactivate only one of the two activities. The partially purified pseT1 enzyme has been shown to have normal levels of kinase activity and less than 0.1% of the wild type phosphatase activity (9). From assays in cell extracts, the pseT47 mutant appears to have no kinase, but some phosphatase activity (8).

Since both pseT1 and pseT47 phage show the mutant phenotype when infected in CTr5X, this suggests that both activities are required for growth of T4. Surprisingly, when CTr5X is infected simultaneously with pseT1 and pseT47 phage, the infection is not productive even though both activities should be present (8). From this result, it would appear that both the kinase and phosphatase are required to be active on the same polypeptide chain for this enzyme to perform its function in T4 infection.

Since the two activities of this enzyme function at opposite ends of a polynucleotide chain, it is not clear why both activities should be required to be on the same polypeptide chain for physiological function. However, a single-stranded break or nick in duplex DNA which has a 3'-phosphate and a 5'-hydroxyl is one situation where the substrates for both activities would be adjacent. The two activities of polynucleotide kinase should be capable of removing the 3'-phosphate and introducing a 5'-phosphate, thereby making the nick a substrate for T4 DNA ligase (10). If these two reactions could occur more efficiently in a concerted mechanism when the two activities are on the same polypeptide chain, an explanation of the mixed infection data would be achieved.

This proposal that polynucleotide kinase acts to "shuttle" a phosphate across a nick in DNA forms the basis of the experiments in the paper. The purification of T4 polynucleotide kinase from the pseT1 and the pseT47 mutants of T4 is described. The enzymatic and physical properties of these two enzymes are compared to those of the enzyme isolated from wild type T4 and from a mixed infection with both pseT1 and pseT47 phage. In addition, the action of polynucleotide kinase on nicked plasmid substrates is tested.

**MATERIALS AND METHODS**

**Biochemicals**—Deoxythymidine 3'-monophosphate, β,γ-imidoadenosine 5'-triphosphate, ribonuclease A, and salmon sperm DNA were purchased from Sigma. Lysozyme, bacterial alkaline phosphatase, deoxyribonuclease I, and micrococcal nuclease were purchased from Worthington. Deoxythymidine 3',5'-bisphosphate was obtained from P-L Biochemicals and bovine serum albumin from Calbiochem. [γ-32P]ATP was purchased from New England Nuclear or synthesized by the method of Johnson and Walseth (11) using 32P-inorganic phosphate. T4 DNA ligase was a gift from R. Gumpourt, University of Illinois.

**Bacteriophage-infected Cells**—T4 bacteriophage amN81 pseT1 and amN81 pseT47 were constructed by L. Snyder, Michigan State University (8). T4 1083 (amE10 X345) was provided by J. Wiberg, University of Rochester. *E. coli* strains B40Su and CTr5X were obtained from H. Boyer, University of California, San Francisco.

Bacteriophage T4 amN81, pseT1 were propagated on the amber-suppressing *E. coli* strain B40Su in a 12-liter fermentor using modified L broth (13). The pH of the media was maintained near 7.0 during growth by adding 5 M KOH. Cells were infected at a multiplicity of 0.05 at a cell density of 2 × 10^7 cells/ml. The cell density leveled off 90 min after infection, but the cells did not usually lyse even as

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long as 4 h after infection. The addition of 0.5% chloroform failed to
stimulate lysis of the cells; however, once cells and cell debris were
removed by centrifugation, approximately 109 phage/ml remained in
this cleared lysate for use in the preparation of phage-infected cells.
Phage were checked for the frequency of revertants in the amber and the
pseT 1 mutations by plating on E. coli BB and C3TRY (6), respectively. For
the pseT 1 mutation, the reversion frequency was less than 10-4. The reversion frequency for the amber mutation, however, increased during the propagation of the phage from 10-4 to 10-2 after sufficient phage had been obtained. The accumulation of amber revertants during the propagation of the phage was a property of both the pseT 1 and pseT 47, but not of the phage containing a wild type polynucleotide kinase gene. There were no revertants of different amber mutations and the use of other E. coli amber-suppressing strains did not significantly change the amber reversion frequency.

T4 amN81, pseT 1 infected cells were obtained by growing 190 liters of E. coli BB in modified L broth to 1.6 x 10^9 cells/ml and then
infecting at a multiplicity of 0 by adding approximately 10 liters of
the cleared lysate described above. The pH of the culture was again
maintained near pH 7.0 with 5 mM KOH. Three hours after infection,
the cells (970 g) were harvested by centrifugation and stored at
-70°C.

The same procedure was used for obtaining cells infected with T4
1083 (amE10 X345) or T4 amN81, pseT 47. For cells infected simultaneous
ly with T4 amN81, pseT 1, and T4 amN81, pseT 47, the multiplicity of infection was five for each phage strain and only 30 g
of phage-infected cells were made in 12 liters of culture.

Assays—Polynucleotide kinase activity was determined essentially as
described by Richardson (14). Sodium acetate was replaced by
micrococal nuclease to an average chain length of 80 nucleo
tides. The precise concentration of 5'-hydroxyl termini of this DNA
substrate was determined by phosphorylating it to completion with an excess of [γ-32P]ATP of known specific activity. A standard 50-μl
assay contained 2.5 μmol of Tris-HCl (pH 7.6), 0.5 μmol of MgCl2, 0.25 mmol of dithiothreitol, 4 μg of bovine serum albumin, 5 nmol of
[γ-32P]ATP (10-20 Ci/mol), and 1 nmol of 5'-hydroxyl termini of
DNA. Reactions were stopped by adding 1.5 ml of cold 5% trichloro
acetic acid and then filtered by suction through cellulose nitrate filters. The filters were rinsed with four 1.5 ml aliquots of 5% trichlo
roacetic acid and one of cold 95% ethanol. After the filters were dried,
the radioactivity was measured in a liquid scintillation counter. A unit
of kinase activity is defined as 1 nmol of phosphate incorporated
into acid-insoluble material in 30 min at 37°C. Since the ATP concen
tration in this assay is 4 times higher than that used by Richardson (14)
in order to bring it well above the estimated Km for ATP (15), a unit
of activity in this assay is equal to 0.7 using the assay conditions
described by Richardson (14).

Assays performed to determine the apparent kinetic parameters for
polynucleotide kinase were carried out with enzyme that contained no
ATP. The same assay was used in the presence of the enzyme when
passing the enzyme sample through a 0.5-ml DEAE-Sephadex A-25
column equilibrated with 50 mM potassium phosphate, pH 7.6, 2.5 mM
dithiothreitol, and 50% glycerol at 4°C. Enzyme samples without
ATP were used without delay since they lost activity rapidly above
4°C.

In the assays to determine the apparent Km and Vmax for DNA, the
ATP concentration was 500 μM and six concentrations of 5'-hydroxyl
develops of DNA were between 1 and 50 μM used. To determine the
apparent Km and Vmax for ATP, the concentration of 5'-hydroxyl ends
develops of DNA was between 20 μM and 1 mM. Five concentrations
were carried out under the conditions described above. 5 μg of each type of enzyme were used in each 50-
μl assay to obtain convenient initial velocities.

Both assays described by Cameron and Uhlenbeck (2) were used
to detect and measure 3'-phosphatase activity. Assay 1, a colorimetric
assay detecting the release of inorganic phosphate from deoxythymi
dine 3'-monophosphate, was used in most cases. A unit of 3'-phos
phatase activity is defined as 1 nmol of phosphate released at
37°C in 30 min. Assay 2, a chromatographic assay detecting the
change in mobility of [5'-32P]deoxythymidine-3'-32P by as it is
treated to [5'-32P]deoxythymidine-5'-32P, was only used for the elution
of the 5'-32P containing eluates where inorganic phosphate in the eluting buffer was too high to use Assay 1.

To determine the apparent kinetic parameters of the 3'-32P
phosphatase activity, Assay 1 was used with 100 mM sodium succinate (pH 5.3) as the buffer instead of 100 mM imidazole-HCl (pH 6.0) as
previously described (2). The concentration of deoxythymidine 3'-
monophosphate was varied from 0.5 to 90 mM for the wild type enzyme and from 1 to 60 mM for the pseT 1.47 enzyme. 10 μg of wild
type or 540 ng of pseT 1.47 enzyme were used in each 25-μl
assay to obtain convenient initial velocities.

Enzyme Purification—Polynucleotide kinase was purified from
300 g of E. coli infected with T4 amN81 pseT 1. The early steps of
sonication, streptomycin sulfate precipitation, autolysis, and ammo
nium sulfate precipitation were identical to those used previously (2) to
purify wild type polynucleotide kinase except that the volumes
were doubled to account for the greater amount of cells. The ammo
nium sulfate pellets were resuspended in 80 ml of 50 mM potassium
phosphate (pH 7.6), 10 mM 2-mercaptoethanol (Buffer A) and di
dithiothreitol against 508 g of bovine serum albumin. The active
fractions were pooled (Fraction IV) and dialyzed twice against
12-liter volumes of 10 mM potassium phosphate (pH 7.0), 10 mM 2-
mercaptoethanol (Buffer B).

A hydroxyapatite (Bio-Gel HPT from Bio-Rad) column (1.4 x 7
cm) was equilibrated with Buffer B. The dialyzed fraction was applied
to the column at 0.15 ml/min, and the enzyme was eluted with a
400-
ml linear gradient of 10 to 500 mM potassium phosphate (pH 7.0), 100
mM 2-mercaptoethanol. The active fractions were pooled (Fraction V) and dialyzed against
12 liters of wash buffer (50 mM potassium phosphate (pH 7.0), 100 mM
2-mercaptoethanol (Buffer B). A DEAE-Sephadex A-25 column (6.4 x 35.5 cm) was equilibrated with Buffer A. The
outlet of the A-25 column was connected to the inlet of the phosphocellulose column and the protein sample applied to the A-25
column at 1 ml/min. The connected columns were then washed with
4 liters of Buffer A. Under these conditions, polynucleotide kinase activity passes through the A-25 column and is retained by the
phosphocellulose resin. The columns were disconnected and a 3.2-
liter linear gradient from 0 to 0.3 M KCl in Buffer A was used to elute
the enzyme from the phosphocellulose column at 0.7 ml/min. The
active fractions were pooled (Fraction III) and dialyzed twice against
12-liter volumes of 10 mM potassium phosphate (pH 7.0), 10 mM 2-
mercaptoethanol (Buffer B).

A hydroxyapatite (Bio-Gel HPT from Bio-Rad) column (1.4 x 7
cm) was equilibrated with Buffer B. The dialyzed fraction was applied
to the column at 0.15 ml/min, and the enzyme was eluted with a
400-
ml linear gradient of 10 to 500 mM potassium phosphate (pH 7.0), 100
mM 2-mercaptoethanol. The active fractions were pooled (Fraction V) and dialyzed against
12 liters of wash buffer (50 mM
potassium phosphate (pH 7.0), 100 mM 2-mercaptoethanol (Buffer B). A DEAE-Sephadex A-25 column (6.4 x 35.5 cm) was equilibrated with Buffer A. The
outlet of the A-25 column was connected to the inlet of the phosphocellulose column and the protein sample applied to the A-25
column at 1 ml/min. The connected columns were then washed with
4 liters of Buffer A. Under these conditions, polynucleotide kinase activity passes through the A-25 column and is retained by the
phosphocellulose resin. The columns were disconnected and a 3.2-
liter linear gradient from 0 to 0.3 M KCl in Buffer A was used to elute
the enzyme from the phosphocellulose column at 0.7 ml/min. The
active fractions were pooled (Fraction III) and dialyzed twice against
12-liter volumes of 10 mM potassium phosphate (pH 7.0), 100 mM 2-
mercaptoethanol (Buffer B).

A Sephadex G-100 column (2 x 85 cm) was equilibrated with 50
mM potassium phosphate (pH 7.0), 10 mM 2-mercaptoethanol (Buffer
C), and the concentrated protein fraction applied to it at 0.5 ml/min.
The enzyme was eluted with Buffer C, and the active fractions were pooled (Fraction IV) and dialyzed overnight against 6 liters of 50 mM
Tris-HCl (pH 7.6), 10 mM 2-mercaptoethanol, 5% glycerol (Buffer
D). A DEAE-Sephadex A-25 column (3.2 x 100 cm) was equilibrated with
Buffer D and the dialyzed fraction was applied to it at 0.6 ml/min. After
the column was washed with 75 ml of Buffer D and then with 75 ml of 0.3
mM KCl in Buffer D, the enzyme was eluted with a linear 200-ml
gradient from 0.3 to 2.3 M KCl in Buffer D. The active fractions were pooled (Fraction V), and the enzyme was purified to homogeneity.

To determine the apparent kinetic parameters of the 3'-phosphatase activity, Assay 1 was used with 100 mM sodium succinate (pH 5.3) as the buffer instead of 100 mM imidazole-HCl (pH 6.0) as
previously described (2). The concentration of deoxythymidine 3'-
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agarose columns since it was determined that it was not retained by the resin. After elution from the G-100 column and concentrating with Aquacide no. 1, the pseT 47 enzyme was dialyzed and stored as above.

Reaction of Nicked pBR322 DNA With Polynucleotide Kinase—E. coli strain 294 containing pBR322 (12) was grown on L broth with ampicillin and the plasmid was amplified with chloramphenicol. Plasmid DNA was isolated from 19 g of cell paste using the procedure described by Davis et al. (18). To remove any contaminating RNA, the DNA was incubated in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE buffer) for 2 h at 37 °C with 25 μg/ml of RNase A which had been boiled to inactivate any DNase. After phenol extraction, the sample was passed over a Bio-Beads SM-2 column (Bio-Rad) equilibrated in TE buffer to remove small oligonucleotides. The covalently closed circular pBR322 DNA was then repurified by a second CsCl-ethidium bromide equilibrium density centrifugation and stored in TE buffer.

Covalently closed circular pBR322 DNA was treated with pancreatic DNase I in the presence of ethidium bromide to introduce single chain scission/plasmid DNA molecule with a 5'-phosphate and a 3'-hydroxyl (19). The reaction mixture was identical to that described (19) except that 8.3 μg/ml of DNase I was used. After incubation for 30 min at 37 °C, the reaction was terminated by extracting 3 times with 1/1(v/v) phenol-chloroform saturated with 0.1 M Tris-HCl (pH 8.0). The DNA was recovered by ethanol precipitation and stored in TE buffer. This substance was referred to as the —OH—P—plasmid.

pBR322 with a single chain scission with a 5'-hydroxyl and 3'-phosphate (—P—HO—P—plasmid) was prepared in a similar way using micrococcal nuclease. The reaction contained 10 mM Tris-HCl (pH 8.0), 2 mM CaCl2, 1 mM MgCl2, 1 mM EDTA, 0.1 mg/ml of bovine serum albumin, 33 μg/ml of ethidium bromide, 9 μg/ml of pBR322, and 1 ng/ml of micrococcal nuclease. Incubation and recovery of DNA was by the same procedure as for the —OH—P—plasmid.

Plasmid DNA with a single chain scission with both 5'- and 3'-hydroxyls (—OH—P—plasmid) was prepared by treating DNase I cut plasmid with bacterial alkaline phosphatase. The reaction mixture contained 10 mM Tris-HCl (pH 8.2), 0.5 mM MgCl2, 20 mM NaCl, 9 μg/ml of DNA 1 cut pBR322, and 0.15 mg/ml of alkaline phosphatase. After incubation at 65 °C for 60 min, the DNA was phenol-extracted, concentrated, and resuspended as described above.

The purity of the intact and nicked plasmid DNA was confirmed by both neutral and alkaline agarose gel electrophoresis. Horizontal slabs (0.3 × 11.78 cm) of 1.4% (w/v) agarose were prepared at 20 V/cm. After the electrophoresis was completed, the gel was rinsed in water to remove the excess ethidium bromide and the DNA bands were visualized by illuminating the gel from below with a short wavelength ultraviolet light. Alkaline denaturing agarose gel electrophoresis (21) was performed using the same agarose concentration and gel size as for the non-denaturing gels. Gels were rinsed in the running buffer used for the nondenaturing gels and then stained in 5 μg/ml of ethidium bromide.

Reactions of polynucleotide kinase with plasmid substrates contained 66 mM Tris-HCl (pH 7.6), 6.6 mM MgCl2, 10 mM dithiothreitol, 2.7 μg/ml of DNA, and 6-16 μg/ml of enzyme containing no ATP in the storage buffer. 1 mM ATP or γ-phosphoadenosine 5'-triphosphate was added to some reactions. After incubation at 37 °C for 1 h, the reactions were heated to 85 °C for 5 min to inactivate the polynucleotide kinase. Each reaction was then incubated with 100 μM ATP and 5 μg/ml of T4 DNA ligase for 37 °C for 1 h. Prior to application to the gel, each reaction was phenol-extracted.

RESULTS

Enzyme Purification—The elution profiles of the four chromatography columns used in the purification of pseT 1 polynucleotide kinase are shown in Fig. 1. Only the kinase activity was measured during the isolation procedure since it had been shown that the mutant enzyme lacked the 3'-phosphatase activity (9). The early stages of this isolation follow the purification procedure described by Richardson (14). However, instead of DEAE-cellulose, a DEAE-Sephadex A-25 column was used and the enzyme was not adsorbed onto the resin. The kinase activity eluted from the phosphocellulose and hydroxylapatite columns at ionic strengths similar to those previously reported (2, 14, 16, 22).

In the enzyme preparation described here, and in every other one tested, the native molecular weight of the enzyme required gel filtration on Sephadex G-100 instead of the Sephadex G-200 used previously. The elution position of the enzyme (Fig. 1c) is similar to that of bovine serum albumin (molecular weight: 68,000) which is about one-half the previously reported value of 140,000 (2, 22). This difference may be explained by the dissociation of an α4 tetramer to an α2 dimer. Lillehaug (23) has shown that an equilibrium between the monomeric, dimeric, and tetrameric forms of the enzyme can exist.

The addition of the ATP-agarose chromatography step to the purification procedure increases the specific activity of the enzyme. The improved purification is the result of the separation of both contaminating proteins and inactive polynucleotide kinase from the active enzyme. This latter observation is based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples from the eluent of the ATP-agarose column. Samples eluted at 0.3 M KCl were found to contain a protein of the size of polynucleotide kinase but no activity. In addition, homogeneous samples of pseT 1 polynucleotide kinase purified several months previously were applied to the ATP-agarose column and inactive enzyme was detected in the 0.3 M KCl fraction. Thus, the ATP-agarose column is capable of separating active from inactive forms of pseT 1 polynucleotide kinase.

A summary of pseT 1 polynucleotide kinase recovery and its specific activity at different stages in the purification is given in Table 1. Assays at early stages of the purification were not performed because of their low reliability. The recovery of pseT 1 polynucleotide kinase using this procedure is slightly better than that previously reported by Richardson (14) for the wild type enzyme. Work by ourselves and others using a variety of RNA and DNA substrates indicates that polynucleotide kinase prepared in this fashion is free of contaminating nuclease activities. Attempts to simplify the purification procedure were not successful since all the steps are needed to remove contaminating 3'-exonuclease and T4 topoisomerase activities.

The purification of polynucleotide kinase from T4 1083 infected cells was very similar to the pseT 1 purification described above. The wild type enzyme eluted from the phosphocellulose, hydroxylapatite, and ATP-agarose columns at slightly different salt concentrations than the pseT 1 enzyme. This could be due to a difference in the charge of the two proteins, but may also be the result of small differences in column dimensions and flow rates. The wild type enzyme also eluted from the Sephadex G-100 column at a position corresponding to a molecular weight of 68,000.

A slight change in the purification procedure was required for the isolation of polynucleotide kinase from E. coli infected with T4 amN81 pseT 47. The pseT 47 enzyme eluted from the phosphocellulose and hydroxylapatite columns at only slightly different salt concentrations than the pseT 1 enzyme. This also eluted from the Sephadex G-100 column at a molecular weight of 68,000. However, the pseT 47 was not applied to the ATP-agarose column since it was found that this enzyme eluted from ATP-agarose at 0.3 M KCl which results in no further purification. This result implies that the pseT 47 enzyme cannot bind ATP which is consistent with its lack of polynucleotide kinase activity. During the purification of the pseT 47 enzyme, the amount of active enzyme recovered at each step was found to decrease substantially. A number of changes in the purification procedure, including adding ATP and glycerol to the column buffers, were not successful in improving the stability of the enzyme. No polynucleotide
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case, the solid points and line indicate the absorbance at 280 nm and the open points and
dotted line indicate the kinase activity. The horizontal lines over the activity peaks indicate the fractions pooled. On the Sephadex G-100
column the elution positions of bovine serum albumin (BSA) and carbonic anhydrase (CA) are indicated.
eliminate the phosphatase activity, it is highly selective for reducing one activity and leaving the other unchanged. This suggests that the kinase and phosphatase active sites are likely to be separate from each other.

The pseT 47 enzyme has no detectable kinase activity and very little phosphatase activity. Since considerable losses of phosphatase activity were observed during the purification and no affinity purification step was used for this enzyme, it is likely that the pseT 47 enzyme contains a large proportion of inactive enzyme molecules. Nevertheless, it appears that the pseT 47 mutation affects both activities, but reduces the kinase activity to a much greater extent.

The specific activities for kinase and phosphatase obtained when the enzyme is purified from cells infected with both the pseT 1 and pseT 47 bacteriophage differ substantially from when the enzymes are purified from cells infected with the individual mutants. Thus, the phosphatase-specific activity for the pseT 1,47 enzyme is 100 times greater than that of the pseT 47 enzyme alone even though the pseT 1 mutant contributes no phosphatase activity. The kinase-specific activity for the pseT 1,47 enzyme is about one-third of the pseT 1 enzyme. If it is assumed that each mutant in the mixed infection contributes an equal amount of gene product and the subunits associate randomly, the resulting population of proteins would be one-half heterodimers and one-fourth of each homodimer. However, since the purification of the pseT 1,47 enzyme includes an ATP affinity step, the pseT 47 homodimers are not expected to be present in the final preparation. Therefore, the pseT 1,47 enzyme might be expected to contain one-third pseT 1 homodimers and two-thirds heterodimers. The increased phosphatase activity of the pseT 1,47 enzyme with respect to the pseT 47 enzyme can be explained if the pseT 1 subunit in the heterodimer stabilizes the pseT 47 subunit from inactivation. Attempts to prepare pseT 1-pseT 47 heterodimers in vitro by mixing preparations of the two mutant enzymes were not successful since no increase in the phosphatase activity was detected. Presumably, the purified pseT 47 enzyme is already largely inactive.

The kinase-specific activity of the pseT 1,47 enzyme is not as easily explained. Although the presence of one-third pseT 1 homodimers could neatly explain the reduced specific activity of the pseT 1,47 kinase, this would imply that the pseT 1 subunit in the heterodimer has no kinase activity. This appears unlikely in view of the affinity of the entire pseT 1,47 enzyme preparation to ATP-agarose. Thus, either the formation of heterodimers is favored or a select population of enzyme molecules was purified.

**Enzyme Kinetics**—The apparent kinetic parameters for three of the purified enzymes are presented in Table III. No parameters were determined for the pseT 47 enzyme since it had too little activity. The values shown were calculated from Eadie-Hofstee plots (26) of the data and are accurate to ± 20% for each number in the table. The constant DNA concentration used when the ATP concentration was varied was sufficiently high (20 μM) to saturate the enzyme. However, when the DNA concentration was varied, the ATP concentration that was used (500 μM) was only 3-fold higher than the Kₘ. Thus, the kinetic parameters reported should be regarded as apparent parameters although the true Kₘ (ATP) estimated from the extrapolation of kinetic data (15) agrees closely with the apparent value we have determined here.

It is interesting to note that the Kₘ of 3 mM for the hydrolysis of TMP is much greater than the Kₘ of 20 μM reported for the same substrate in the polynucleotide kinase reaction (15). Although this might suggest that the two activities have entirely different functions since they act in such different concentration ranges, a broader range of substrates for the phosphatase reaction needs to be tested.

The Kₘ and Vₘₐₓ for both ATP and DNA obtained for the pseT 1 enzyme kinase reaction are essentially identical to the values obtained for the wild type enzyme. It was not possible to determine the kinetic parameters for the phosphatase reaction needs to be tested.

**Table II**

| Enzyme | Kinase | Phosphatase |
|--------|--------|-------------|
|        | units/mg | units/mg |
| Wild type | 170,000 | 1.56 × 10⁻³ |
| pseT 100 | 210,000 | 0.86 |
| pseT 47 | <0.2 | 125 |
| pseT 1,47 | 69,000 | 18,000 |

**Table III**

| Enzyme | Kinase activity | Phosphatase activity |
|--------|-----------------|----------------------|
|        | Kₘ | Vₘₐₓ | Kₘ | Vₘₐₓ | Kₘ | Vₘₐₓ |
| DNA (μM) | DNA (μM) | DNA (μM) | DNA (μM) |
| Wild type | 2.7 | 8.8 | 188 | 11.8 | 3.3 | 41.5 |
| pseT 1 | 3.1 | 12.3 | 152 | 16.2 | |
| pseT 1,47 | 4.5 | 7.7 | 171 | 10.1 | 15.2 | 0.46 |
to obtain kinetic parameters for the phosphatase activity of the \( pseT 1 \) enzyme due to the low activity. Thus, the \( pseT 1 \) mutation only affects the phosphatase activity, supporting the hypothesis that the two activities are carried out at independent sites on the enzyme.

The kinetic parameters for the kinase activity obtained with the \( pseT 1 \) enzyme are quite similar to those determined for the \( pseT 1 \) and the wild type enzymes. The similar \( K_m \) values are expected since the \( pseT 1 \) enzyme contains a substantial proportion of \( pseT 1 \) homodimers. Since the \( V_{max} \) values are nearly two-thirds of those of the \( pseT 1 \) enzyme, it suggests that the kinase activity \( pseT 1 \) subunit in the heterodimer can act independently.

Since the phosphatase activity of the \( pseT 1 \) enzyme is entirely due to the \( pseT 1 \) subunit in the heterodimer, the substantial proportion of \( pseT 1 \) homodimers. Since the one of the activities.

Collision parameters for the phosphatase presumably reflect the true effect of the \( pseT 1 \) mutation. The increased \( K_m \) and greatly decreased \( V_{max} \) suggest that the \( pseT 1 \) mutation has a substantially altered phosphatase activity; thus, it is not analogous to the \( pseT 1 \) mutation which independently alters one of the activities.

**Amino Acid Analyses**—The results of the amino acid analyses of the wild type and \( pseT 1 \) enzymes are shown in Table IV. The two enzymes have very similar amino acid compositions which was expected since the \( pseT 1 \) enzyme is a point mutant of the wild type enzyme (6). These data, however, differ substantially from the previously reported amino acid analysis of wild type polynucleotide kinase for at least six amino acids (23).

**Action of Polynucleotide Kinase on Nicked Plasmid Substrates**—In order to determine whether the polynucleotide kinase and phosphatase activities can “shuttle” a phosphate across a nick in DNA, a coupled assay using singly nicked plasmid DNA and T4 DNA ligase was employed. A DNA molecule nicked to leave a 3′-phosphate and a 5′-hydroxyl (–P HO–) cannot be sealed by T4 DNA ligase, whereas a nick with a 3′-hydroxyl and a 5′-phosphate (–OH P–) can be sealed (10). If a –P HO– plasmid substrate is incubated with polynucleotide kinase, the sealing of this nicked substrate by DNA ligase would indicate that a phosphate had been removed from the 3′ side of the nick and a phosphate introduced at the 5′ side of the nick. Thus, the sequential action of polynucleotide kinase and DNA ligase would convert the –P HO– nicked plasmid substrate from an open circular to a covalently closed circular form. These two plasmid conformations are well separated in an agarose gel containing ethidium bromide.

The results of an experiment examining the action of polynucleotide kinase phosphatase on –P HO– nicked plasmid DNA under varying conditions are shown in Fig. 3. Two DNA ligase control reactions are displayed in Lanes 3 and 10. If –P HO– plasmid is incubated with DNA ligase, the nick is not sealed and the substrate remains as an open circle (Lane 3). However, if a –OH P– plasmid is incubated with DNA ligase, the nick is sealed and the plasmid substrate is converted into a covalently closed circle which migrates more rapidly in the gel (Lane 10). This indicates that the DNA ligase concentration used is sufficient to seal the available nicks.

When wild type polynucleotide kinase was incubated with –P HO– plasmid in the presence of ATP and then reacted with DNA ligase, covalently closed circular plasmid is formed (Lane 4). If ATP is omitted (Lane 5) or the nonhydrolyzable \( \beta,\gamma \)-imidoadenosine 5′-triphosphate (27) is substituted for ATP (Lane 6), no reaction occurs. These experiments show that polynucleotide kinase can quantitatively transfer a phosphate from the 3′ side to the 5′ side of a nick in DNA and that ATP hydrolysis is required for this to occur. This strongly suggests that a mechanism involving the transfer of the same

**TABLE IV**

| Amino acid             | No. of residues/34,000 dalton subunit |
|------------------------|--------------------------------------|
|                        | Wild type | \( pseT 1 \) |
| Aspartic acid and asparagine | 29        | 32          |
| Threonine              | 16        | 18          |
| Serine                 | 17        | 15          |
| Glutamic acid and glutamine | 35        | 36          |
| Proline                | 12        | 13          |
| Glycine                | 25        | 25          |
| Cysteine               | 16        | 15          |
| Valine                 | 23        | 24          |
| Methionine             | 11        | 11          |
| Isoleucine             | 17        | 18          |
| Leucine                | 18        | 17          |
| Tyrosine               | 11        | 11          |
| Phenylalanine          | 8         | 9           |
| Histidine              | 5         | 5           |
| Lysine                 | 26        | 28          |
| Arginine               | 17        | 17          |
| Tryptophan             | 3         | 3           |
phosphate across the nick can be ruled out. The 5'-phosphate is introduced from the γ-phosphate of ATP in the normal polynucleotide kinase reaction.

The polynucleotide kinase concentration required for efficient phosphate transfer in the reaction of Lane 4 of Fig. 3 is quite high. Experiments varying the enzyme concentration under the same reaction conditions showed that at least 500 units/ml of enzyme were required for high yields of covalently closed circular plasmid. This concentration is at least 10 times higher than is generally needed for the phosphorylation of DNA termini and confirms previous experiments indicating that a nick in DNA is a poor substrate of polynucleotide kinase (28).

It also appears that the presence of the 3'-phosphate at the nick does not greatly stimulate the 5'-phosphorylation. This was shown with -OH HO— nicked plasmid prepared by dephosphorylating DNase 1 nicked DNA with bacterial alkaline phosphatase. It was found that the concentration of polynucleotide kinase necessary to efficiently convert the -OH HO— plasmid to covalently closed circular plasmid DNA was also about 500 units/ml. Thus, if a concerted mechanism involving the simultaneous removal of the 3'-phosphate and insertion of a 5'-phosphate occurs, it does not lead to an increased rate of reaction.

To determine if the phosphate transfer reaction requires both activities on the same polypeptide chain, -P HO— plasmid substrates were incubated with enzymes or mixtures of enzymes possessing only one of the two activities. Ideally, this experiment would be carried out with combinations of the pseT 1 enzyme, the pseT 47 enzyme, and the pseT 1,47 enzyme. However, due to the high concentration of enzyme required for the assay and the very low phosphatase activity of the pseT 47 and pseT 1,47 enzymes, these experiments were not successful.

Instead, a tryptic fragment of wild type polynucleotide kinase phosphatase which only has the phosphatase activity (29) was used in conjunction with the pseT 1 enzyme, which only has the kinase activity. As can be seen in Lanes 7 and 9 of Fig. 3, either one of these enzymes alone cannot convert the -P HO— nicked plasmid to a form that can be sealed by DNA ligase. However, when both enzymes are present (Lane 8), a successful reaction is seen. This experiment clearly shows that phosphate transfer can occur in two independent steps catalyzed by different proteins. Since the amount of phosphatase and kinase activity added when two enzymes are used is no greater than when the wild type enzyme is used, there appears to be no kinetic advantage for having both activities on the same polypeptide chain.

We have therefore shown that polynucleotide kinase can convert a -P HO— nick to a -OH P— nick on a plasmid DNA. Both activities and the hydrolysis of ATP are required for this reaction to occur. However, the reaction is catalyzed in two steps and can be carried out by two different proteins. Furthermore, no increase in the efficiency of the reaction is observed when the reaction is carried out with both activities present on the same polypeptide chain. Thus, there is no evidence for a concerted mechanism with both activities operating at a nick in DNA.

**DISCUSSION**

We have improved and extended the purification procedure for polynucleotide kinase described by Richardson (14). By introducing an ATP affinity column, homogeneous enzyme that lacks contaminating activities can be obtained. The purification of the enzymes from *E. coli* infected with the T4 mutants pseT 1 and pseT 47 as well as from cells infected with both pseT 1 and pseT 47 was found to be very similar to the wild type enzyme.

The physical and enzymatic properties of the pseT 1 enzyme are identical to the wild type polynucleotide kinase phosphatase except that virtually no phosphatase activity is observed. Since pseT 1 is believed to be a point mutation (6), a single amino acid change can almost totally eliminate the phosphatase activity without altering the kinase activity. This also suggests that the two active sites are enzymologically distinct from one another. In the following paper, additional evidence is presented to support this point. The pseT 1 enzyme is extremely useful in applications where polynucleotide kinase activity is needed in the absence of 3'-phosphatase activity (9).

The enzyme isolated from cells infected by the pseT 47 mutant is totally without polynucleotide kinase activity. In this case, however, the purified pseT 47 enzyme has less than 1 part in 10⁶ of the wild type phosphatase activity as well. In addition, the pseT 47 phosphatase activity is much less stable during purification by the standard methods. This suggests that the pseT 47 point mutation not only inactivates the kinase activity, but also destabilizes the protein. The phosphatase activity of the pseT 47 enzyme can be examined to some extent by isolating the enzyme from a mixed infection with both pseT 1 and pseT 47 phage. The pseT 1,47 enzyme isolated from this infection has 100 times more phosphatase activity than the pseT 47 enzyme. Since the two activities coelute in all steps of the purification including the ATP-agarose column, it is likely that the pseT 1 and pseT 47 subunits form a heterodimer and stabilize the phosphatase activity in the pseT 47 subunit. It is not clear whether the still relatively low phosphatase activity is due to the pseT 47 mutation or inactive heterodimers.

The extremely low levels of phosphatase activity found in preparations of the pseT 1,47 enzyme could explain the failure of the pseT 1-pseT 47 mixed infection to grow on the nonpermissive host CTX5. Even in the cell extract of the mixed infection, the total phosphatase activity is greatly reduced from the levels seen in a wild type infection. Thus, it appears that the failure of the mixed infection to grow on CTX5 is due to the low levels of phosphatase activity. This also implies that the lack of growth may not be caused by a requirement for both activities on the same polypeptide chain.

We have hypothesized that a possible physiological function of polynucleotide kinase phosphatase is to act on a nick in DNA which has a 3'-phosphate and 5'-hydroxyl. The role of this T4 enzyme would be to transfer the phosphate from the 3' side of a nick to the 5' side, thus making it a substrate for DNA ligase. This hypothesis came from the observation that DNA replication in nonpermissive infections is poor (6) and the clear preference of the phosphatase activity for deoxyribonucleotide sequences.

We have shown that T4 polynucleotide kinase can mediate phosphate transfer at the appropriate nick in DNA. This reaction proceeds by a mechanism requiring ATP in which the two activities of the enzyme are independent. The 3'-phosphate at the nick is removed by the phosphatase activity and released to the surrounding buffer, and the 5'-hydroxyl is phosphorylated through hydrolysis of ATP by the kinase activity. This indicates that the reaction does not involve the transfer of the same phosphate across the nick. There is also no indication that the reaction proceeds more efficiently when both activities are present on the same polypeptide chain. Thus, there is no biochemical evidence to support this model.

¹ L. Snyder, personal communication.
Two Mutant Forms of T4 Polynucleotide Kinase

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REFERENCES
1. Richardson, C. C. (1965) Proc. Natl. Acad. Sci. U. S. A. 54, 138-165
2. Cameron, V., and Uhlenbeck, O. C. (1977) Biochemistry 16, 5120-5126
3. Kleppe, K., and Lillehaug, J. (1979) in Advances in Enzymology (Meister, A., ed) Vol. 48, pp. 245-275, (John Wiley & Sons, Inc., New York
4. Folk, W. R. (1981) in Gene Amplification and Analysis, Vol. II, Analysis of Nucleic Acid Structure by Enzymatic Methods (Chirikjian, J. R., and Papas, T. S., eds) pp. 300-311, Elsevier/North-Holland, New York
5. Engler, M. J., and Richardson, C. C. (1981) in The Enzymes (Boyer, P., ed) Vol. 15, pp. 1-29, Academic Press, New York
6. Depew, R. E., and Cozzarelli, N. R. (1974) J. Virol. 13, 888-897
7. Cooley, W., Sirotkin, K., Green, R., and Snyder, L. (1979) J. Bacteriol. 140, 83-91
8. Sirotkin, K., Cooley, W., Runnels, J., and Snyder, L. R. (1978) J. Mol. Biol. 123, 221-233
9. Cameron, V., Saltis, D., and Uhlenbeck, O. C. (1978) Nucleic Acids Res 5, 825-833
10. Lehman, I. R. (1974) Science (Wash. D. C.) 186, 790-797
11. Johnson, R. A., and Walaeth, T. F. (1979) Adv. Cyclic Nucleic Res. 10, 135-167
12. Boyer, H. W., Betlach, M., Bolivar, F., Rodriguez, R. L., Heyneker, H. L., Shine, J., and Goodman, H. M. (1977) in Recombinant Molecules: Impact on Science and Society (Beers, R. F., and Bassett, E. G., eds) pp. 9-20, Raven Press, New York
13. Baltz, R. H., and Drake, J. W. (1972) Virology 49, 462-474
14. Richardson, C. C. (1972) Proc. Nucleic Acid Res. 2, 815-828
15. Lillehaug, J. R., and Kleppe, K. (1975) Biochemistry 14, 1221-1225
16. Hanggi, U. J., Streeck, R. E., Voigt, H. P., and Zachau, H. G. (1970) Biochim. Biophys. Acta 217, 278-293
17. Matsudaira, P. T., and Burgess, D. R. (1978) Anal. Biochem. 87, 386-396
18. Davis, R. W., Botstein, D., and Roth, J. R. (1980) Advanced Bacterial Genetics, pp. 116-119, Cold Spring Harbor Laboratory, New York
19. Hsieh, T., and Wang, J. C. (1975) Biochemistry 14, 5120-5126
20. Loening, U. E. (1969) Biochem. J. 113, 131-138
21. McDonnell, M. W., Simon, M. N., and Studier, F. W. (1977) J. Mol. Biol. 110, 119-146
22. Panet, A., van de Sande, J. H., Loewen, P. C., Khorana, H. G., Rase, A. J., Lillehaug, J. R., and Kleppe, K. (1973) Biochemistry 12, 5045-5050
23. Lillehaug, J. R. (1977) Eur. J. Biochem. 73, 499-506
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
25. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685
26. Eadie, G. S. (1942) J. Biol. Chem. 146, 85-93
27. Lillehaug, J. R. (1978) Biochim. Biophys. Acta 525, 357-363
28. Lillehaug, J. R., Kleppe, R. K., and Kleppe, K. (1976) Biochemistry 15, 1866-1865
29. Saltis, D. A., and Uhlenbeck, O. C. (1982) J. Biol. Chem. 257, 11340-11345