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A RAPID PURIFICATION OF T4 POLYNUCLEOTIDE KINASE USING BLUE DEXTRAN-SEPHAROSE CHROMATOGRAPHY

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Summary

A rapid batch procedure is described for purification of T4 polynucleotide kinase (ATP:5′-dephosphopolynucleotide 5′-phosphotransferase, EC 2.7.1.78) to near homogeneity using Blue Dextran-Sepharose chromatography. The enzyme preparation is sufficiently free of contaminating endonuclease and alkaline phosphatase activities to be suitable for radioactively labeling nucleic acids in vitro. Kinetic measurements indicate that the chromophore of Blue Dextran, Cibacron Blue F3GA, inhibits the activity of T4 polynucleotide kinase competitively with respect to single stranded DNA substrate and non-competitively with respect to the rATP substrate.

Introduction

T4 polynucleotide kinase (ATP:5′-dephosphopolynucleotide 5′-phosphotransferase, EC 2.7.1.78) is an enzyme encoded by the bacteriophage T4 which catalyzes the transfer of the γ-phosphoryl group from rATP to the free 5′-hydroxyl groups of ribo- or deoxyribonucleotides [1]. It is the major enzyme used in radioactively labeling the 5′-termini of polydeoxynucleotides in vitro prior to the determination of nucleotide sequences by the Maxam and Gilbert method [2]. Existing purification procedures for the enzyme [3—7] involve multiple chromatographic steps which are time-consuming and laborious. Using the traditional procedure for the preparation of T4 infected cell extracts, we have found that T4 polynucleotide kinase binds very tightly to Blue Dextran-Sepharose and can be batch-eluted with 1 M KCl after the other T4 coded proteins have been removed with lower KCl concentrations. Using this procedure the enzyme can be prepared from a frozen cell past in 2 days.

Materials and Methods

Bacteria and Bacteriophage

Escherichia coli B06, CR63 (SUI+) and bacteriophage T4 BL292 (gene 55$^-$)

were provided by Dr. Thomas Conway. T4 BL292 lysates were prepared by growing *E. coli* CR63 at 33°C in HB broth to an A_{600} of 0.6, infecting with T4 BL292 at a multiplicity of 0.1 phage/bacterium, and then allowing the culture to lyse spontaneously (6—8 h). Cell debris was removed by centrifugation at 10 000 × *g* for 10 min.

Infection of the nonpermissive host, *E. coli* B06, was as described by Richardson [4]. Approx. 25 g of infected cell paste were obtained from a 20-l culture.

Some enzyme purifications were done with T4 XF-1 infected *E. coli* B cells purchased from P-L Biochemicals.

Preparation of T4 extracts

T4 infected *E. coli* extracts were prepared as described by Richardson [4] and by Panet et al. [6] using batches of frozen cells. The $(NH_4)_2SO_4$ precipitated product was resuspended in and dialyzed against 10 mM Tris-HCl (pH 7.4)/10 mM 2-mercaptoethanol and stored at −20°C. This material is referred to as fraction IV.

Blue Dextran-Sepharose purification of T4 polynucleotide kinase

Blue Dextran-Sepharose, prepared according to the procedure of Ryan and Vestling [8], was equilibrated with 10 mM Tris-HCl buffer (pH 7.4)/7 mM $MgCl_2$/10 mM 2-mercaptoethanol, termed buffer A.

2 ml fraction IV material from infected cells, 25 ml buffer A, and 24 ml wet Blue Dextran-Sepharose were stirred at 0°C for 10 min. The Blue Dextran-Sepharose was recovered by filtration over a scintered glass funnel and washed in situ with 200 ml buffer A, followed by 2000 ml buffer A/0.45 M KCl. Less than 0.1% of the applied T4 polynucleotide kinase activity eluted in the 0.45 M KCl wash. The T4 polynucleotide kinase was then recovered by washing four times with 25-ml aliquots of buffer A/1 M KCl/0.05 mM rATP and concentrated to 2 ml by precipitation with $(NH_4)_2SO_4$, 50% saturation. The enzyme was dialyzed into buffer A containing 0.5 mM rATP and 50% glycerol and subsequently stored at −20°C. The ATP was added to stabilize the enzyme after batch elution from the Blue Dextran-Sepharose [6,9].

Enzyme assays

T4 polynucleotide kinase was assayed as described by Richardson [4]. The reaction volume of 300 µl contained 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.066 mM [γ-^{32}P]ATP (1—10 · 10^7 cpm/µmol), 50 µg micrococcal nuclease digested calf thymus DNA and 20-µl samples. Incubation was at 37°C for 30 min. The reaction was terminated by the addition of 1 ml 0.1 M $Na_2P_2O_7$, 50 mM trisodium ethylenediaminetetraacetate, 50 µg/ml calf thymus DNA and 2 ml cold 10% trichloroacetic acid. After the precipitate was collected on a Whatman GF/A glass fiber filter and washed with an additional 30 ml cold 10% trichloroacetic acid, the filter was placed in 5 ml of Bray's solution and the radioactivity measured in a Beckman LS-100 scintillation counter. One unit of activity is defined as the incorporation of 1 nmol ^{32}P into acid precipitated DNA in 30 min at 37°C [4].

T4 endonuclease activity was monitored by observing the conversion of

supercoiled plasmid DNA molecules to the open circular and linear forms. Each assay contained 50 μl 50 mM Tris-HCl, (pH 7.4), 10 mM $MgCl_2$, 5 mM dithiothreitol, 3 μg plasmid 82-8B, a circular plasmid DNA of 11.9 kilobases (a gift of Dr. James Hartley) and a volume of polynucleotide kinase containing 10 units of enzyme activity. After incubation at 37°C for 60 min, the reaction was stopped by the addition of 5 μl 0.1 M trisodium ethylenediaminetetraacetate, 0.5% Bromphenol Blue and 60% sucrose. A 25-μl sample was applied to a 0.85% agarose slab gel containing 2 μg/ml ethidium bromide and subjected to electrophoresis as described previously [20]. Since the endonuclease activity was found to be inhibited by the high salt concentrations introduced from fractions containing 0.45 M and 1.0 M KCl, these samples were concentrated and dialyzed overnight prior to assay.

Alkaline phosphatase activity was measured as described by Garen and Levinthal [11]. Each assay (1 ml) contained 0.67 M Tris-HCl (pH 8)/1 mM *p*-nitrophenylphosphate (Sigma) and 25 μl concentrated sample. Product formation was measured by following the increase in absorbance at 410 nm. One unit of phosphatase activity is defined as the production of 1 μmol *p*-nitrophenol per min at 25°C. Commercial *E. coli* alkaline phosphatase (Worthington) was used as standard.

Preparation of the DNA substrate for enzyme assays

Calf thymus DNA (100 mg; from Sigma) was dissolved in 50 ml H_2O at 100°C, cooled and sonicated for 5 min in a Branson sonicator. The solution was made to 50 mM glycine-NaOH buffer (pH 9.5)/10 mM $CaCl_2$. 5 μg micrococcal nuclease (Sigma) was added and the digestion was allowed to proceed until 30% of the A_{260} material was soluble in 0.6 M $HClO_4$. The reaction was terminated by the addition of trisodium ethylenediaminetetraacetate to 25 mM. The digested DNA was dialyzed against 40 vol. 1 M KCl for 24 h, then dialyzed against three changes (40 vol. each) of H_2O. The DNA was precipitated with ethanol and resuspended to a concentration of 0.5 mg/ml.

The average DNA fragment size was determined by maximally phosphorylating the DNA fragments using [γ-^{32}P]rATP and a purified preparations of T4 polynucleotide kinase [12]. From the specific activity of the [γ-^{32}P]-rATP and the DNA concentration, the extent of phosphorylation was calculated to be approx. 1 μmol ^{32}P/150 μmol nucleotides. Therefore the length of the DNA fragments was assumed to be an average of 150 nucleotides.

Other methods

[γ-^{32}P]rATP was prepared according to Schendel and Wells [13] and then diluted to the desired specific activity. Protein determinations were conducted as described by Bradford [14]. 10% Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis was conducted according to Laemmli [15] and the proteins stained with Coomassie Brilliant Blue R.

Results

Purification of T4 polynucleotide kinase using Blue Dextran-Sepharose

Preliminary measurements using a small column of Blue Dextran-Sepharose

indicated that (i) the crude extract must be diluted at least 20-fold with buffer A to obtain efficient binding of polynucleotide kinase activity, (ii) only approx. one-third of the bound enzyme can be eluted with buffer A containing either 10 mM rATP or 10 mM deoxythmidine 3'-monophosphate, and (iii) the enzyme can be nearly quantitatively eluted with buffer A containing 1 M but not 0.45 M NaCl. However, polynucleotide kinase eluted with 1 M NaCl had a low specific activity, 812 units/mg protein, and the eluant contained at least 10 different polypeptide chains as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis. By contrast, purification of the enzyme through the ammonium sulfate fractionation (fraction IV) of the standard purification procedure [4] prior to a larger scale, Blue Dextran-Sepharose chromatography resulted in a homogeneous preparation of high specific activity as shown in Table I and Fig. 1. The mobility of the purified protein in the sodium dodecyl sulfate gel corresponds to polypeptide having a molecular weight of $3.3 \cdot 10^4$ daltons in good agreement with the reported [6,16] subunit molecular weight of T4 polynucleotide kinase. Similarly, the specific activity of the enzyme purified by Blue Dextran-Sepharose chromatography is comparable to that obtained by previous more lengthy purification procedures [7] using micrococcal nuclease treated DNA as substrate.

The various eluates from Blue Dextran-Sepharose were examined for endonuclease activity using a mixture of super-coiled and relaxed open-circle plasmid DNA having 11.9 kilobases as shown in Fig. 2. Crude extract (track 2) and fraction IV (track 3) contain extensive amounts of endonuclease activity as shown by the presence of degraded oligonucleotides in track 2 and the absence of any material stained by ethidium bromide in track 3. The buffer A wash (track 4) has less endonuclease activity than does a concentrated and dialized sample of the 0.45 M KCl wash (track 5). Track 6 shows the assay of endonuclease activity when 10 units of kinase was incubated with 3 μg of DNA at 37°C for 1 h. The sensitivity of this assay is sufficient to detect a single nick in the 11.9 kilobase plasmid which would convert the super-coiled DNA molecules to relaxed open-circle DNA molecules. The intensity of the upper band in track 6 when compared to track 1 indicates that approx. 30% more of the super-coiled molecules were converted to open-circles. This corresponds to the introduction of one nick in every 40 kilobases of DNA during the 1 h incubation. No further identification or characterization of the endonuclease activities was attempted. No alkaline phosphatase activity could be detected in the purified T4 polynucleotide kinase preparation. From standard assays for phosphatase [11] this means than less than $4 \cdot 10^{-6}$ units of phosphatase were present per unit of kinase.

TABLE I

PURIFICATION OF T4 POLYNUCLEOTIDE KINASE FROM T4 INFECTED *E. COLI* CELLS

Fraction and step	Total protein (mg)	Specific activity	Yield (%)
I Crude extract	477	46	100
II Streptomycin	260	138	160
III Autolysis	116	164	85
IV Ammonium sulfate	39	270	47
V Blue Dextran-Sepharose	0.087	11 800	40

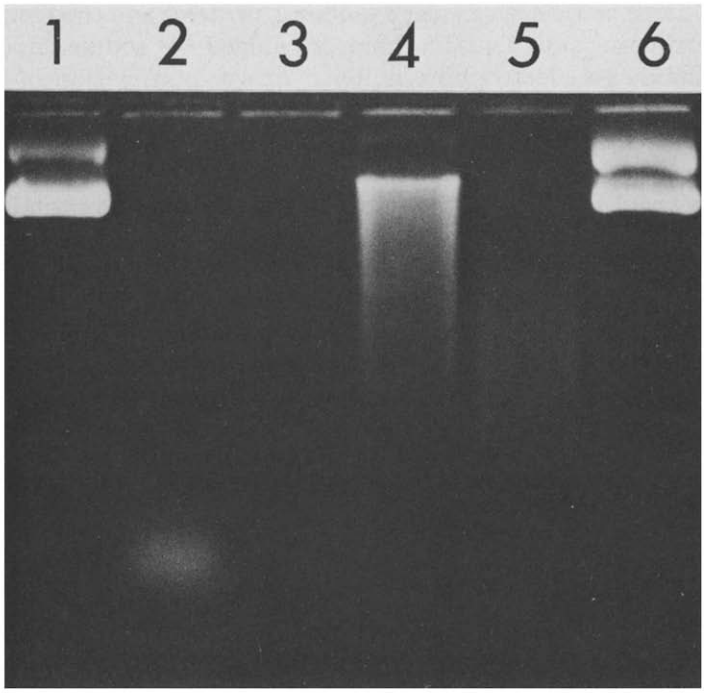

Fig. 1. An SDS polyacrylamide slab gel electrophoresis of a pooled dialyzed and concentrated 1 M KCl eluate from Blue Dextran-Sepharose chromatography containing 10 μg of protein applied to the top of the gel as shown.

Fig. 2. An agarose gel for analysis of endonuclease activity. The sample in track 1 was 1 μg of untreated control plasmid DNA which contains supercoiled DNA (bottom band) and nicked open circle DNA (upper band). All other tracks contained 1 μg of this plasmid DNA incubated with dialyzed and concentrated samples of crude extract (track 2), Fraction IV (track 3), Buffer A wash of the Blue Dextran resin after application of Fraction IV (track 4), the 0.45 M KCl wash of the resin (track 5) or the 1 M KCl wash containing the polynucleotide kinase activity (track 6).

Inhibition of T4 polynucleotide kinase activity by Cibacron Blue F3GA

The dependence of the observed catalytic rate on the concentrations of the two substrates rATP and DNA was studied by varying systematically the concentration of one substrate in the presence of different fixed concentrations of the other substrate. Such measurements produced linear double reciprocal plots each having a common intersection point to the left of the abscissa as previously reported [12,17] and is characteristic [18] of a sequential bisubstrate mechanism. Our measurements give apparent K_m values for rATP and 5'-polynucleotide terminii (DNA) of 65 μm and 6.5 μm, respectively, in agreement with previously reported values [12,17].

Fig. 3 shows a double reciprocal plot of the reaction kinetics in the presence of the free dye, Cibacron Blue F3GA, with a constant saturating rATP concentration and variable concentrations of 5′-hydroxyl groups on single stranded DNA fragments having an average length of 150 nucleotides. A competitive inhibition pattern is obtained, which when replotted as slope vs. dye concentration, produces a parabolic dependence on blue dye concentration. As shown in the insert to Fig. 3, the replot of slope vs. the square of the dye concentrations produces a linear relationship.

Fig. 4 shows the equivalent double reciprocal plot in which the 5′-hydroxyl groups are held at a constant saturating level and rATP concentration is varied. The data demonstrate non-competitive inhibition when the reactions were conducted in the presence of 0.1 M KCl. When these measurements with variable rATP concentrations were repeated in the absence of KCl, hyperbolic double reciprocal plots were obtained as previously reported [12] which are not readily interpretable.

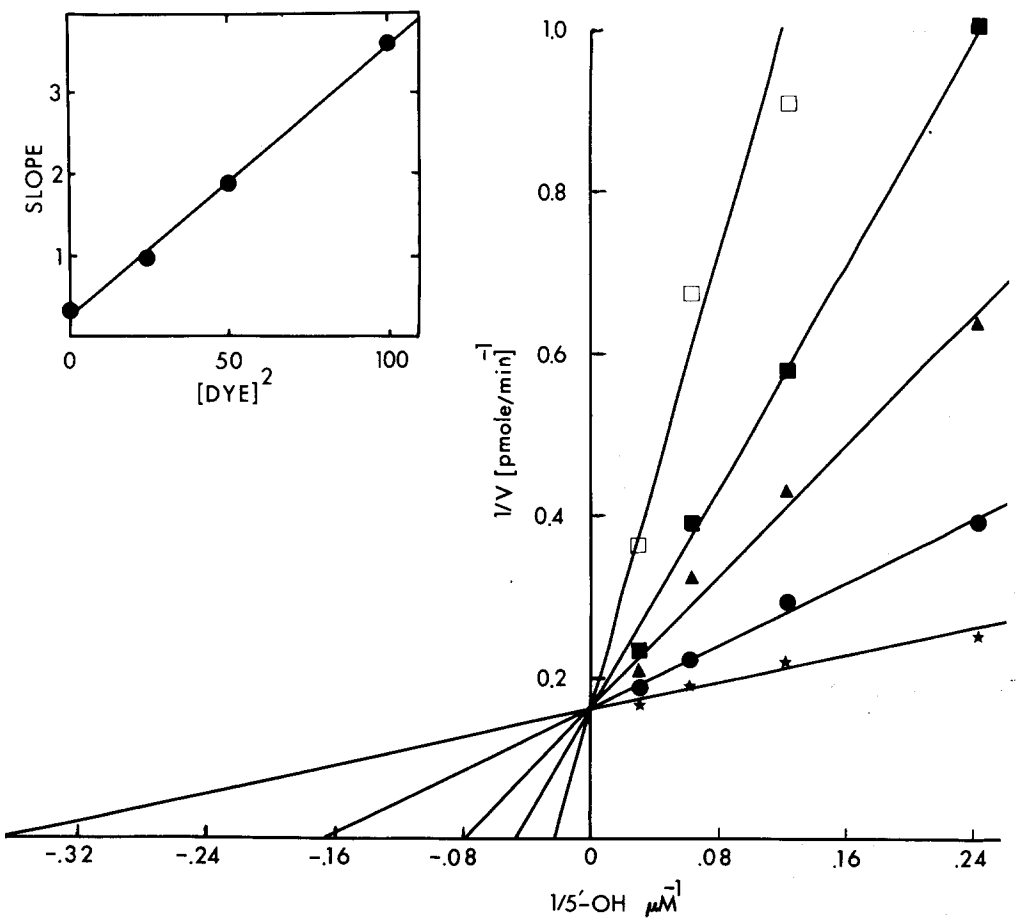

Fig. 3. A double reciprocal plot showing the inhibition of T4 polynucleotide kinase by Cibacron Blue F3GA with respect to 5′-hydroxyl termini. The rATP concentration was held constant at 400 μM and the dye concentrations were 0 (★), 5 μM (●), 7.5 μM (▲), 10 μM (■) and 15 μM (□), No KCl was present in the assay solutions. The lines are least squares fits of the data points. The insert shows a replot of the slopes of the lines versus the square of the dye concentration.

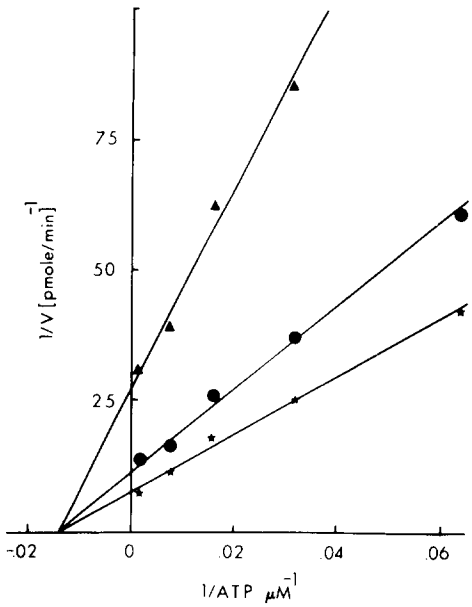

Fig. 4. A double reciprocal plot showing th inhibition of T4 polynucleotide kinase by Cibacron Blue F3GA with respect to ATP concentration in the presence of 0.1 M KCl. The concentration of the 5'-hydroxyl termini was held constant at 50 μM and the dye concentrations were 0 (★), 5 μM (●) and 10 μM (▲). The lines are least squares fits of the data points.

Discussion

The binding of the dye Cibacron Blue F3GA to a variety of dehydrogenases and kinases has been attributed [19] to the structural similarity of the dye to both NAD and ATP and has been utilized in the purification of enzymes which bind these ligands. Accordingly, we anticipated that T4 polynucleotide kinase would bind to Blue Dextran-Sepharose and be selectively eluted with solvents containing rATP. While the enzyme was observed to bind to Blue Dextran-Sepharose, rATP was found to be a poor eluant at a concentration which rapidly elutes other kinases with excellent recoveries. Subsequent catalytic measurements (Fig. 3 and 4) indicated that the dye is a competitive inhibitor for the single stranded DNA binding site and not the rATP site. Similar results have been obtained with the enzymes polynucleotide phosphorylase and TRP-tRNA synthetase [20] whereby the dye binds to the polynucleotide and not the mononucleotide site. However, linearization of the replot of the dye inhibition with respect to DNA concentration (insert of Fig. 3) required the dye concentration to be squared. This indicates either that there is more than one form of active kinase as suggested by Lillehaug and Kleppe [21] and that alternative enzyme forms have different dye affinity or that there is only one form of active kinase and that it has more than one dye binding site.

Elution of the kinase with buffer A containing 1 M but not 0.45 M KCl indicates that the polyanionic dye interacts with a strongly cationic site on the kinase, a conclusion in keeping with the dye occupancy of the DNA site. The

very high KCl concentration required to elute the kinase accounts for separation from other T4 enzymes, particularly the endonuclease, which are eluted by lower KCl concentrations. However, it is important that the Blue Dextran-Sepharose be washed with at least 40 column bed volumes of buffer A containing 0.45 M KCl to elute the majority of the endonuclease activity prior to elution of the kinase with buffer A containing 1 M KCl. The remaining endonuclease activity which then coelutes with the kinase is capable of introducing only about one nick per 40 kilobases per h at 37°C. This level of endonuclease activity does not interfere with the convential reaction used to label DNA fragment for sequence analysis [2]. If only functional purity of the kinase is required, i.e., no greater endonuclease contamination, Blue Dextran-Sepharose chromatography of crude extract rather than Fraction IV may be used with a considerable saving of time and effort.

Acknowledgements

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