
Partial characterization of an endonuclease activity which appears in nuclease free T4 polynucleotide kinase[†]

Peter C. Loewen

Department of Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2

Received 30 August 1976

ABSTRACT

A nuclease activity has been found to appear in preparations of T4 induced polynucleotide kinase which had originally been nuclease free. The nuclease introduced random nicks into T7 DNA suggesting that it was an endonuclease. Destabilization of the kinase molecule by osmotic shock or by the removal of reducing agents, ATP or salts was shown to stimulate the endonuclease appearance. The molecular weight was found to be $32,000 \pm 10\%$ by gel filtration on G100 Sephadex. The nuclease was active over a wide pH range from pH 5.0 to pH 9.2 in a number of buffer systems and required $MgCl_2$ and reducing agent for maximum activity. Sodium azide did not affect the nuclease appearance.

INTRODUCTION

Since it was first reported in 1965¹, T4 induced polynucleotide kinase has proven to be a very useful tool in nucleic acid research. Polynucleotide synthesis², nucleic acid sequence analysis³⁻⁷, structural studies of λ DNA cohesive ends^{8,9} and analysis of the RNA-DNA junction of Okazaki fragments from DNA replication¹⁰ have all made use of this enzyme.

The absence of contaminating enzymic activities was desired in some of these studies. However, it has been shown that a phosphate exchange reaction could be catalyzed by the kinase¹¹. A more serious problem was the presence of nuclease activities which were occasionally found in some kinase preparations and removed by hydroxylapatite^{12,13} or G100 Sephadex (K. Kleppe - personal communication). More recently the enzyme has been the subject of an extensive study by Kleppe and coworkers^{14,15}.

During the sequence analysis of the 5'ends of T7 DNA, an endonuclease activity was found to reappear in what had

been characterized to be nuclease free kinase preparations ⁷. This work describes the conditions under which the endonuclease appears and a partial characterization of the nuclease activity.

MATERIALS AND METHODS

Nucleotides and DNA [γ -³²P] ATP was prepared by a modification ^{7,16} of the procedure of Glynn & Chappell ¹⁷. [³²P] T7 bacteriophage was prepared as described ¹⁸. The DNA was extracted at 5°C using phenol saturated with 50 mM Tris pH 7.6, 0.1 M NaCl and 10 mM MgCl₂. The DNA, 5×10^4 cpm/ μ g and 250 μ g/ml, was stored for no longer than three days in 10 mM Tris, pH 7.6.

Enzymes Polynucleotide kinase was isolated from T4 am-N82 infected *E. coli* by a modification ¹³ of the method of Richardson ¹. The enzyme was assayed as previously described ¹⁴ and stored in buffer A (50 mM Tris, pH 7.6, 0.1 M KCl, 10 mM mercaptoethanol and 10 μ M ATP) with 50% glycerol at -20°C. Sodium azide, 0.1%, did not affect enzyme activity. Endonuclease was assayed by the method of Weiss et al ¹⁶. [³²P] T7 DNA at 50 μ g/ml was incubated with an enzyme aliquot in 50 mM Tris pH 7.6 (or 50 mM of other buffers described in Table 2), 10 mM MgCl₂, 25 μ M ATP and 10 mM mercaptoethanol for 2 hours at 37°C. The final protein concentration was 4 μ g/ml. The reaction was stopped with 20 mM EDTA and 0.2 M NaOH followed by centrifugation through 5-20% sucrose gradients containing 1 M NaCl, 0.2 M NaOH for 2½ hours at 45,000 r.p.m. in a Beckman SW 50.1 rotor. Fractions of the gradient were collected in 1.5 ml plastic Eppendorf tubes and counted for Cherenkov radiation. The average number of nicks introduced into each DNA molecule was calculated after Litwin et al ¹⁹.

Gel filtration Gel filtration on G-100 Sephadex was carried out as previously described ^{7,20}.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis on 8% SDS (sodium dodecyl sulfate) gels was carried out as described ²¹.

Protein determination The protein concentration was determined by the method of Lowry ²² or the method of Patterson ²³ (for very dilute solutions). This latter method was carried

out after samples were dialyzed against 7 M urea followed by extensive dialysis against 10 mM Tris, pH 7.6. The assay was linear between 1 and 100 $\mu\text{g/ml}$ of protein.

RESULTS

Appearance of an endonuclease activity Several batches of T4 polynucleotide kinase which originally had been characterized as nuclease free were found to contain an endonuclease activity after storage for 1-3 years. Using a microscope, no microorganisms were evident in the enzyme solutions and no bacterial growth occurred when aliquots were incubated on nutrient agar plates at 20°C or 37°C. Purification of any one of these enzyme stocks by gel filtration on G100 Sephadex, removed the endonucleolytic activity from the kinase activity (Figure 1). The purified kinase could be stored in buffer A with 50% glycerol for several months without re-appearance of any nuclease activity.

However, if certain components of the storage buffer were removed or if the kinase was osmotically shocked, the endonuclease activity reappeared. Removal of the reducing agent, mercaptoethanol, or ATP from buffer A with 50% glycerol during dialysis at 5°C for 14 hours resulted in a reappearance of the endonuclease activity. Removal of KCl had only a small effect on the appearance of endonuclease. Osmotically shocking the enzyme solution by successive dialysis against 50% and 5% glycerol in buffer A also facilitated the appearance of nuclease (Figure 1, Table 1). The addition of 0.1% sodium azide did not inhibit the nuclease formation.

The removal of any one of the components from buffer A was found to decrease the kinase activity. For example, solutions of kinase lacking ATP, mercaptoethanol or KCl exhibited 45%, 55% and 26% respectively of the activity in the solution dialyzed with all of the components (Table 1).

Gel filtration separation The separation of the kinase activity from the endonuclease activity was carried out using G100 Sephadex (Figure 2). The molecular weight of the endonuclease activity was determined by comparison of its elution volume to the elution volumes of several proteins of

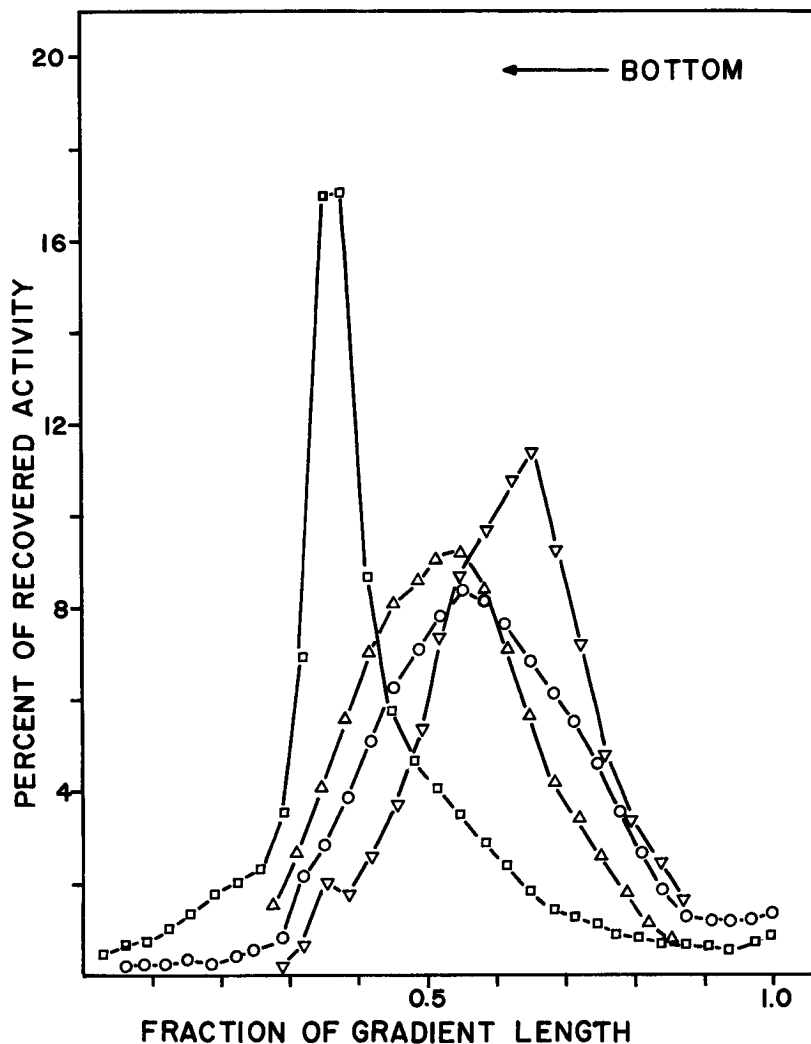


Figure 1. Alkaline sucrose sedimentation analysis of (^{32}P) T7 DNA treated for 2 hours at 37°C with various G100 Sephadex polynucleotide kinase fractions which had been stored in buffer A with 50% glycerol lacking certain components. The enzyme concentrations were all maintained at $4\ \mu\text{g}/\text{ml}$ and the assay buffer is described in Materials and Methods. The following enzyme fractions were studied: a) $20\ \mu\text{l}$ of polynucleotide kinase in complete buffer A with 50% glycerol, \square — \square ; b) $20\ \mu\text{l}$ of polynucleotide kinase in buffer A with 50% glycerol lacking ATP, Δ — Δ ; c) $20\ \mu\text{l}$ of polynucleotide kinase in buffer A with 50% glycerol lacking mercaptoethanol ∇ — ∇ ; d) $20\ \mu\text{l}$ of polynucleotide kinase osmotically shocked as described in Results, \circ — \circ . Untreated DNA was identical to that in (a).

Table 1. Activity of polynucleotide kinase and endonuclease in fractions from G100 Sephadex dialyzed lacking certain components.

<u>Fraction</u>	<u>Kinase activity</u> (units/ml)	<u>Nuclease activity</u> (nicks/DNA molecule)
A. Kinase fraction		
buffer A (complete)	95.0	0
buffer A-ATP	43.0	4
buffer A-mercapto-ethanol	51.6	10
buffer A-KCl	24.8	2
B. Nuclease fraction		
buffer A (complete)	1.8	38
buffer A-ATP	1.8	40
buffer A-mercapto-ethanol	1.7	18
buffer A-KCl	1.1	18

known molecular weight 20 (Figure 3). The kinase has previously been shown to have a molecular weight of $140,000 \pm 10\%$ with four subunits of $33,000 \pm 5\%$ ¹³. Figure 3 shows the molecular weight of the endonuclease to be $32,000 \pm 10\%$. Nuclease fractions isolated whether from lengthy storage or from dialysis without certain components eluted in the same volume. Usually less than 10% of the total protein in the two peaks was present in the nuclease fraction. SDS polyacrylamide gel electrophoresis of 40 μg of kinase containing nuclease activity showed only one band at $34,000 \pm 5\%$.

Endonuclease stability After separation by G100 Sephadex, the effect on the nuclease fraction of the removal of various components from buffer A with 50% glycerol was studied. Removal of either KCl or reducing agent decreased the endonuclease activity while removal of ATP had no effect (Table 1).

The endonuclease level was affected by the removal of certain components from the assay mixture (Table 2). The absence of either MgCl_2 or mercaptoethanol lowered the nuclease activity while ATP had no effect. In addition the nuclease was found to be active over a broad pH range with only a slight enhancement at pH 7.0. The nuclease remained active for a period of up to four hours fragmenting DNA at a linear rate (Table 3). Addition of tRNA (1200 $\mu\text{g}/\text{ml}$) or ATP (200 μM) had no effect on the endonuclease activity.

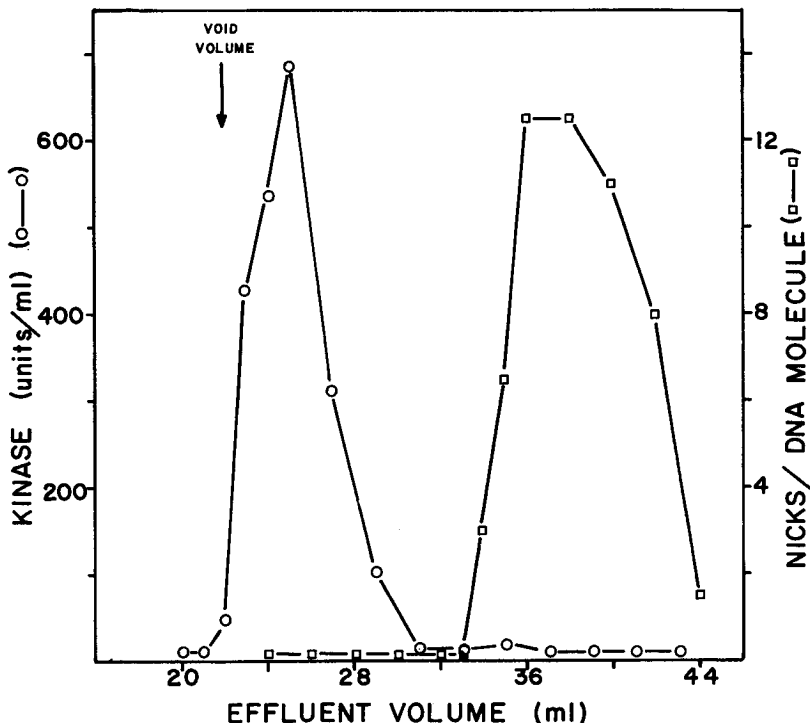


Figure 2. Gel filtration pattern of polynucleotide kinase containing the endonuclease activity. Approximately 4,000 units of polynucleotide kinase were applied to a Sephadex G-100 column (1 x 50 cm) which had been equilibrated with buffer A and 5% glycerol. The column was eluted with equilibrating buffer and aliquots of each fraction were assayed for polynucleotide kinase and endonuclease activity as described under Materials and Methods. Approximately 70% of the kinase activity applied to the column was recovered and approximately 10% of the total protein was recovered in the endonuclease fraction.

Some kinase activity was found in the endonuclease fraction. The level was very low when compared to the kinase activity in the main kinase peak (Table 1). Whether this activity was the result of incomplete separation of the kinase and the endonuclease on the column or of the kinase subunits in the endonuclease fraction could not be determined.

DISCUSSION

This work describes the conditions under which an endonuclease activity can appear in nuclease free fractions of polynucleotide kinase. Several explanations are possible for this

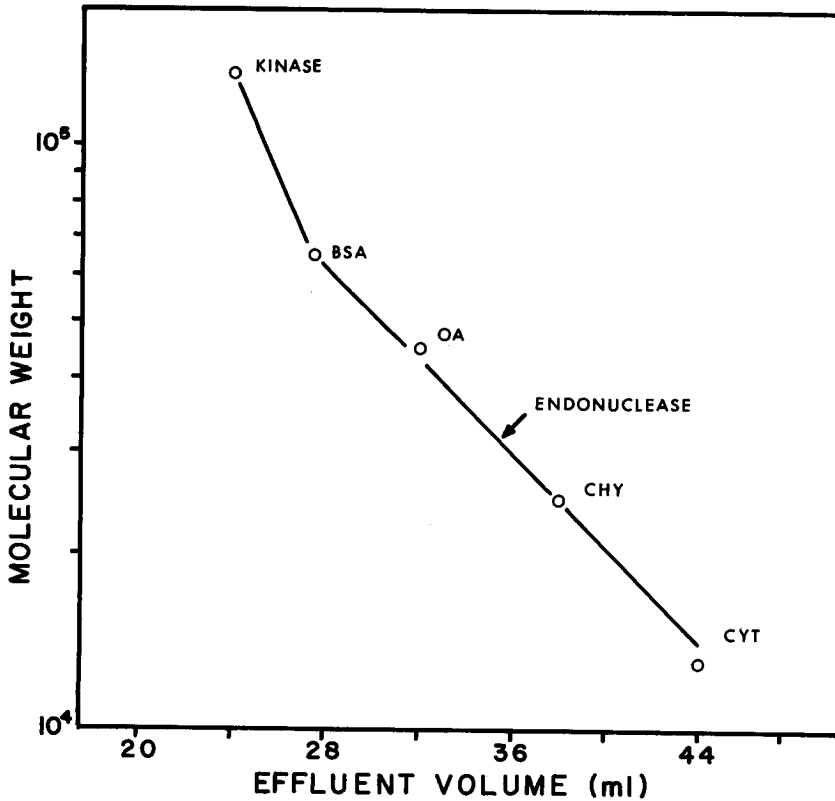


Figure 3. Elution volumes of protein standards used for molecular weight determination on a Sephadex G-100 column (1 x 50 cm): polynucleotide kinase, 140,000 \pm 10%; bovine serum albumin (BSA), 65,000; ovalbumin (OA), 45,000; chymotrypsinogen (CHY), 25,000 and cytochrome c (CYT) 12,400.

Table 2. Endonuclease activity of the nuclease fraction from G100 Sephadex with different components missing from the assay mixture and at different pH's.

Assay mixture	Nuclease activity (nicks/DNA molecule)
1. Mix (complete + Tris pH 7.6)	42
2. Mix (Tris) - ATP	42
3. Mix (Tris) - MgCl ₂	12
4. Mix (Tris) - mercaptoethanol	24
5. Mix + sodium acetate pH 5.0	39
6. Mix + potassium phosphate pH 7.0	51
7. Mix + sodium glycinate pH 9.2	42

Table 3. Rate of nick formation by the nuclease fraction from G100 Sephadex.

Time (minutes)	Nicks/DNA molecule
30	4
60	8
90	12
120	16
150	20
180	24
240	30

phenomenon. 1. There was a bacterial or fungal contamination which produced the endonuclease. 2. There was a pre-existing endonuclease which was identical to and copurified with the polynucleotide kinase and which was active only upon dissociation into subunits. 3. A DNA ligase was present which, until it was inactivated, masked the endonuclease activity. 4. The polynucleotide kinase subunits possessed the unwanted activity upon dissociation.

No bacterial or fungal growth was evident either visually by microscope or during incubation on nutrient agar. Sodium azide in the storage buffer did not inhibit the nuclease appearance. In addition the simple removal of ATP or mercaptoethanol from the dialysis buffer should not stimulate bacterial growth.

Any contaminating proteins would have to have molecular weights and structures similar to polynucleotide kinase. However, the molecular weights of T4 induced ligase and *E. coli* ligase are $68,000 \pm 10\%$ ¹³ and $74,000 \pm 5\%$ ²⁴ respectively. The endonuclease in addition to having an inactive form of 130,000 molecular weight could be active only when dissociated to subunits of 32,000 molecular weight. The data in this paper cannot exclude such a possibility but it seems unlikely.

The explanation that the kinase subunits possess the endonuclease activity also cannot be proved or disproved by this work. The kinase subunit and the endonuclease activity have similar molecular weights, $32,000 \pm 10\%$ for the nuclease and $33,000 \pm 5\%$ for the kinase subunit, as well as similar requirements for KCl, MgCl₂ and mercaptoethanol. More extensive physicochemical studies are necessary.

†Supported by a grant A9600 from the National Research Council of Canada.

REFERENCES

1. Richardson, C. C. (1965) Proc. Nat. Acad. Sci. USA 54:158-165.
2. Khorana, H. G., Agarwal, K. L., Buchi, H., Caruthers, H. M., Gupta, N., Kleppe, K., Kumar, A., Ohtsuka, E., Raj-Bhandary, U. L., van de Sande, S. H., Sgaramella, V. Weber, H., and Yamada, T. (1972) J. Mol. Biol. 72: 209-217 (and succeeding papers).
3. Székely, M. and Sanger, F. (1969) J. Mol. Biol. 43:607-617.
4. Southern, E. M. (1970) Nature 227:794-798.
5. Murray, K. (1973) Biochem. J. 131:569-583.
6. Simsek, M., Ziegenmeyer, J. Heckman, J. and Raj Bhandary, U. L. (1973) Proc. Nat. Acad. Sci. USA 70:1041-1045.
7. Loewen, P. C. (1975) Nucl. Acids. Res. 2:839-852.
8. Wu, R. and Kaiser, A. D. (1968) J. Mol. Biol. 35:523-537.
9. Richardson, C. C., Masamune, Y., Live, T. R. Jacquemin-Sablon, A., Weiss, B. and Fareed, G. E. (1968) Cold Spring Harbor Symp. Quant. Biol. 33:151-164.
10. Pigiet, V., Eliasson, R. and Reichard, P. (1974) J. Mol. Biol. 84:197-216.
11. van de Sande, J. H., Kleppe, K. and Khorana, H. G. (1973) Biochemistry 12:5050-5055.
12. Jacquemin-Sablon, A. and Richardson, C. C. (1970) J. Mol. Biol. 47:477-493.
13. Panet, A., van de Sande, J. H., Loewen, P. C. Khorana, H.G., Raae, A. J., Lillehaug, J. R. and Kleppe, K. (1973) Biochemistry 12:5045-5050.
14. Lillehaug, J. R. and Kleppe, K. (1975) Biochemistry 14: 1221-1225.
15. Lillehaug, J. R. and Kleppe, K. (1975) Biochemistry 14: 1225-1229.
16. Weiss, B., Live, T. R. and Richardson, C. C. (1968) J. Biol. Chem. 243:4530-4542.
17. Glynn, J. M. and Chappell, J. B. (1964) Biochem J. 90: 147-149.
18. Kozinski, A. W. and Syzbalski, W. (1959) Virology 9 260-274.
19. Litwin, S., Shahn, E. and Kozinski, A. W. (1969) J. Virol. 4:24-30.
20. Andrews, P. (1964) Biochem. J. 91: 222-233.
21. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244: 4406-4412.
22. Lowry, O. H., Rosebrough, N. J., Farr, L. and Randall, R. J. (1951) J. Biol. Chem. 193:265-275.
23. Patterson, D. S. P. (1964) Biochem. Biophys. Acta 86:405-407.
24. Modrich, P., Anraku, Y. and Lehman, I. R. (1973) J. Biol. Chem. 248:7495-7501.