AN ACTIVE FRAGMENT OF DNA POLYMERASE PRODUCED BY PROTEOLYTIC CLEAVAGE*

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Summary

DNA polymerase from Escherichia coli was cleaved by limited proteolytic action into two fragments of 76,000 and 34,000 molecular weight. The cleaved enzyme is still an active polymerase but has a reduced 5'⟶3' nuclease activity. The fragments were separated by gel filtration. The isolated larger fragment retains the polymerizing activity and the 3'⟶5' nuclease activity present in the native enzyme, but not the 5'⟶3' nuclease. This specific proteolytic cleavage was catalyzed most effectively by an extract from Bacillus subtilis or by trypsin.

DNA polymerase from Escherichia coli, a single polypeptide chain of 109,000 molecular weight, catalyzes several reactions at a single active center (Kornberg, 1969). At the 3' end of a DNA primer, 3'⟶5' exonucleolytic activity, pyrophosphate exchange, and pyrophosphorolysis are closely related to the polymerizing reaction (Deutscher and Kornberg, 1969a). Binding of the deoxyribonucleoside triphosphate substrate is thought to be adjacent to another enzyme site which catalyzes a 5'⟶3' exonucleolytic degradation of a nicked DNA (Deutscher and

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Kornberg, 1969b; Cozzarelli et al., 1969). This report describes a cleavage of DNA polymerase by proteolytic action into fragments of 76,000 and 34,000 molecular weight, the larger of which retains the polymerizing activity and 3'⟶5' nuclease but not the 5'⟶3' nuclease function.

Results

Evidence that DNA polymerase of E. coli is a single polypeptide chain with a molecular weight of 109,000 includes SDS-polyacrylamide[1] gel electrophoretic analysis (Jovin et al., 1969) (Fig. 1A). Further confirmation was obtained by performing the analysis after reduction with dithiothreitol in 6 M guanidine and alkylation with iodoacetate. However, a sample of enzyme removed from liquid nitrogen storage and kept in HEPES buffer for 4 weeks at 0° yielded two bands upon SDS-polyacrylamide gel electrophoresis (Fig. 1B). The principal band was at a molecular weight position of 76,000 with a fainter band at 34,000. The aged enzyme still showed full polymerase activity with a DNA primed assay (Richardson et al., 1964). During poly d(A-T) synthesis with the aged enzyme the polymerization reaction went to completion and the product d(A-T) was not degraded as with the normal enzyme (Schachman et al., 1960). This indicated the absence of a nuclease function in the aged polymerase.

The absence of subunits in the native enzyme and the appearance of lower molecular weight fragments upon aging suggested that proteolysis had occurred, perhaps as the result of bacterial contamination. Several proteases studied for their effect on the enzyme included a B. subtilis extract, trypsin, chymotrypsin, and subtilisin BPN' (Fig. 1C, D, E, and F). Whereas cleavage of polymerase occurred in each case, the treat-

[1] Abbreviations used are: SDS (sodium dodecyl sulfate) and HEPES (N-hydroxyethyl piperazine N'-ethanesulfonic acid), and TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone).

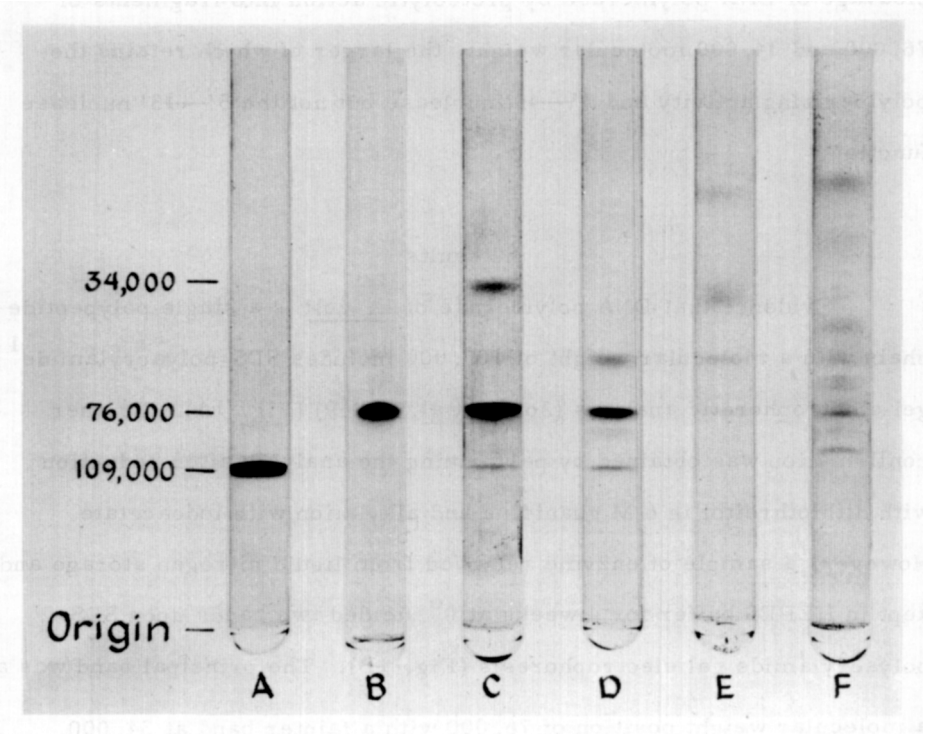

Fig. 1. SDS-polyacrylamide gel electrophoresis of DNA polymerase. Electrophoresis and molecular-weight determinations were performed as described by Shapiro et al. (1967) with the modifications of Weber and Osborn (1969), using a 5% polyacrylamide gel. Electrophoresis was for 4 hours at 8 ma. per gel. DNA polymerase, bovine serum albumin, and the dimer of bovine serum albumin were used as molecular-weight markers.

A, Polymerase (5 μg) dialyzed versus 0.05 M HEPES buffer (pH 7.4).

B, Polymerase (5 μg) aged 4 weeks at 0°. A sample of polymerase (0.5 ml, 4.8 mg/ml) stored in liquid nitrogen was dialyzed against 0.05 M HEPES buffer and kept at 0° for 4 weeks.

C, Polymerase (3 μg) treated with an extract of B. subtilis (SB 19). The extract was prepared as described by Okazaki and Kornberg (1964) except that glutathione was omitted. 4.27 mg of DNA polymerase was incubated with 0.44 mg of extract (total protein) in 1 ml of 0.05 M potassium phosphate buffer (pH 7.4) for 130 min at 37°. The reaction mixture was chilled and 10 μl of 1 M β-mercaptoethanol was added.

D, Polymerase (5 μg) treated with trypsin. 27 μg of polymerase was incubated with 18 ng of trypsin (Worthington, 2x crystallized, treated with TPCK) in 50 μl of 0.1 M TRIS-HCl (pH 7.5), 0.1 mM β-mercaptoethanol at 37° for 30 min.

E, Polymerase (5 μg) treated with chymotrypsin. 27 μg of polymerase was incubated with 250 ng of chymotrypsin (Worthington, 3x crystallized) in 50 μl of 0.1 M TRIS-HCl (pH 7.5), 0.1 mM β-mercaptoethanol at 37° for 30 min.

F, Polymerase (5 μg) treated with subtilisin BPN'. 18 μg of polymerase was incubated with 1 ng of subtilisin BPN' (Nagase) in 20 μl of 0.05 M HEPES buffer (pH 7.4) at 37° for 20 min.

ment with the B. subtilis extract most closely resembled the degradation pattern seen in the aged polymerase. More than 90% of the polymerase was cleaved to yield 76,000 and 34,000 molecular weight fragments and a trace of an intermediate sized product (about 53,000). Trypsin also yielded a 76,000 molecular-weight fragment as the principal product, but a larger amount of the 53,000 molecular-weight material. Chymotrypsin and subtilisin BPN' were less effective in producing a limited cleavage although the 76,000 molecular-weight fragment could be identified among the degradation products.

TABLE I

Polymerase	Mol. wt.	Polymerase activity[a]		3'⟶5' Nuclease activity[b]		5'⟶3' Nuclease activity[c]	
		Rate	%	Rate	%	Rate	%
Normal	109,000	145	(100)	10.0	(100)	40.0	(100)
Cleaved[d]	---	350	240	11.7	117	8.4	21
Seph. I	76,000	322	220	9.8	98	1.8	<5
Seph. II	34,000	0	0	0.0	<5	0.0	<5

[a] Assay with activated DNA (as in Richardson et al., 1964, except 0.05 M potassium phosphate buffer (pH 7.4) was used). Rate is expressed as molecules of nucleotide incorporated per molecule of enzyme per min.

[b] Release of ^{3}H-dTMP from 2.0 nmoles of ^{3}H-poly d(T)$_{300}$ (16,000 cpm/nmole) in a 200 µl reaction mixture containing 0.05 M HEPES buffer (pH 7.4) and 5 mM $MgCl_2$ (Kelly et al., 1969). Rate is expressed as molecules of nucleotide released per molecule of enzyme per min.

[c] Release of ^{3}H-dTMP from 1.8 nmoles of ^{3}H-poly d(T)$_{300}$-ddTMP (a polymer of thymidylate terminated with a dideoxythymidylate residue at the 3' end (Atkinson et al., 1969)) in a 200 µl reaction mixture containing 2.4 nmoles of poly d(A)$_{4000}$, 0.05 M HEPES buffer (pH 7.4) and 5 mM $MgCl_2$ (Kelly et al., 1969). Rate is expressed as for 3'⟶5' nuclease activity.

[d] Treated with B. subtilis extract (Fig. 1C).

The cleaved enzyme showed an augmented polymerase activity and a decrease in the 5'⟶3' nuclease activity (Table I). Treatment with trypsin also caused an increase in polymerase activity (up to 1.5 fold) which decreased with longer treatment as the 76,000 molecular-weight fragment was itself degraded.

The products of polymerase cleavage by B. subtilis extract were separated by gel filtration (Fig. 2). Electrophoresis of Fraction I showed it to be a single band (>90%) with a molecular weight of 76,000. The polymerase and the 3'⟶5' nuclease functions reside in the 76,000 molecular weight fragment (Table I). No 5'⟶3' nuclease activity was detected in either of the separated fractions.

The cleaved polymerase and the separated Fraction I were compared with the normal polymerase in the kinetics of primed d(A-T)

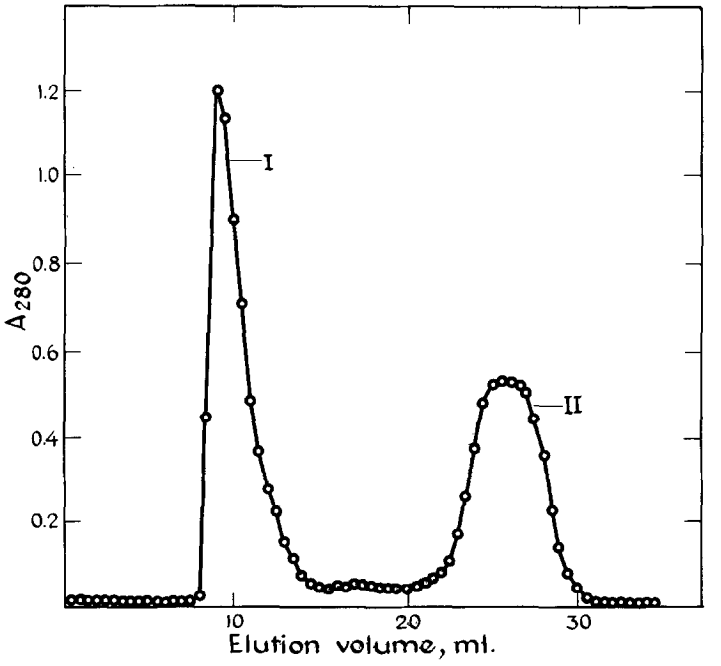

Fig. 2. Gel filtration of cleaved polymerase. DNA polymerase was treated with B. subtilis extract (see Fig. 1C) and the products were layered on a column of G-75 Sephadex (fine, 38 cm x 0.65 cm^2, 8.8 ml void volume) and eluted with 0.05 M potassium phosphate buffer (pH 7.4), 1 mM β-mercapto ethanol at 4°. Pooled fractions under each peak are designated I and II.

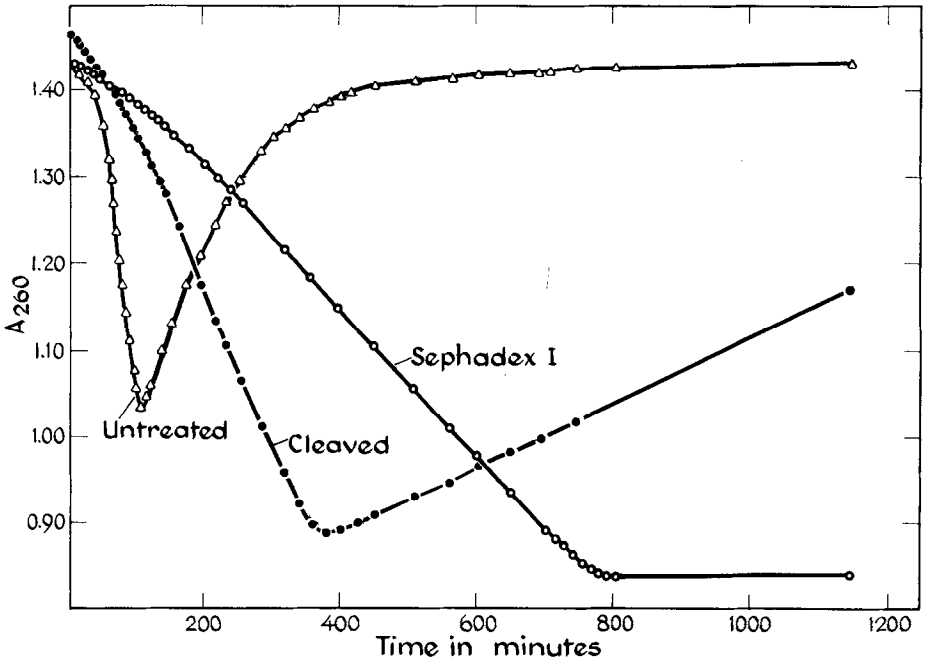

Fig. 3. Synthesis of poly d(A-T). 150 nmoles of dTTP, 150 nmoles of dATP, and 2.4 nmoles of poly d(A-T) were incubated at 37° in 0.5 ml of 67 mM potassium phosphate buffer (pH 7.4), 6.7 mM $MgCl_2$, 1 mM β-mercaptoethanol with enzyme additions as follows: a) untreated polymerase (20 μμmoles), b) cleaved polymerase (7.6 μμmoles, see Fig. 1C), c) Sephadex fraction I (11 μμmoles).

synthesis (Fig. 3, Schachman et al., 1960). The normal enzyme showed an early period of decrease in optical density during which d(A-T) synthesis predominated, followed by an increase in optical density when the product d(A-T) was degraded by the nuclease function. Synthesis by the cleaved polymerase was less autocatalytic and the rate of nuclease action was also diminished. The isolated Fraction I showed a nearly linear period of synthesis and, in contrast to normal and cleaved polymerase, no detectable breakdown of product.

Discussion

There is a region of the DNA polymerase which is susceptible to proteolytic action. Loss of one third of the polymerase molecule by cleavage in this region results in selective loss of the 5'⟶3' nuclease

activity. The small amount of 5'⟶3' nuclease present in the cleaved polymerase (21%) may represent the continued association of the two fragments since over 90% of the polymerase had been cleaved as judged by electrophoresis. The basis for the increase in the rate of polymerization by Fraction I remains to be investigated. The apparent correlation of the 5'⟶3' nuclease activity and the capacity for autocatalytic d(A-T) synthesis suggests that this nuclease activity generates new primer ends by the release of oligonucleotides (Kelly et al., 1969). The absence of enzymatic activities in Fraction II must be considered tentative since this fraction appears to be degraded further by proteolytic action.

The existence of several forms of E. coli DNA polymerase of varying molecular weights and enzymatic properties has been reported (Cavalieri and Carroll, 1968; Lezius et al., 1967). These forms may prove to be degradation products of a single polypeptide of 109,000 molecular weight. The DNA polymerase isolated from B. subtilis was found to have little nuclease activity (Okazaki and Kornberg, 1964) and a lower molecular weight (Falaschi and Kornberg, 1966; Op den Kamp, Atkinson, Bertsch, and Kornberg, unpublished results). This polymerase deserves further study to determine whether it may have undergone a proteolytic cleavage during isolation.

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