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Selective Elimination of the Exonuclease Activity of the Deoxyribonucleic Acid Polymerase from *Escherichia coli* B by Limited Proteolysis*

H. Klenow and I. Henningsen

UNIVERSITETETS BIOKEMISKE INSTITUT B, KØBENHAVN, DENMARK

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Abstract. Purification of DNA polymerase from *E. coli* B has in two cases each time led to the isolation of two separate polymerase activities, enzyme A and enzyme B. Enzyme A was in contrast to enzyme B almost completely devoid of exonuclease activity. Each of the two enzymes yielded a single symmetrical activity peak in gel filtration chromatograms. From the elution volumes the molecular weights were estimated to be about 70,000 for enzyme A and about 150,000 for enzyme B.

Treatment of enzyme B with subtilisin led to an increase of about 30 per cent of the polymerase activity while the exonuclease activity almost completely disappeared. The product of the subtilisin treatment (enzyme C) gave rise to a single symmetrical polymerase activity peak in a gel filtration chromatogram. The elution volume was identical to that obtained with enzyme A. It is concluded that enzyme A and enzyme C are formed by limited proteolysis of enzyme B.

Introduction. The DNA polymerase from *E. coli* B has been isolated by Jovin *et al.*[1] as a homogeneous protein. It has been shown to consist of a single polypeptide chain with a molecular weight of 1.09×10^5. It contains one disulfide bond and a single reactive sulfhydryl group.

The enzyme catalyzes both the extension of polydeoxyribonucleotides from the 3′—OH end, the exchange of inorganic pyrophosphate with the terminal pyrophosphate groups in deoxyribonucleoside triphosphates, and an exonucleolytic degradation of polydeoxyribonucleotides from the 5′-phosphate end group and the 3′-hydroxyl end group leading to deoxyribonucleoside monophosphates. A considerable amount of information on the physical-chemical properties as well as about the mode of action of the enzyme has recently been obtained.[2–6]

In the present communication it is shown that the DNA polymerase may be modified by limited proteolysis leading to an almost complete loss of the exonuclease activity, while the polymerase activity is retained. The modified enzyme has a molecular weight of about 70,000 according to gel filtration data under the experimental conditions used.

Experimental Procedure. Materials: Unlabeled 2′-deoxyribonucleoside triphosphates were obtained from Sigma Chemical Co. (St. Louis) and [^{3}H]-labeled 2′-deoxyribosylthymine triphosphate (dTTP) (about 10 Ci/mmole) from Schwarz Bio-

Research Inc. (Orangeburg, N.Y.). "Activated" calf thymus DNA was prepared according to Aposhian and Kornberg[7] by treatment of the sodium salt of calf thymus DNA with crystalline pancreatic deoxyribonuclease (Worthington Biochemical Corp.). DEAE-Sephadex, Sephadex, and blue dextran were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Pancreatic ribonuclease was obtained from Sigma Chemical Co., USA, ovalbumin and subtilisin (type Carlsberg) were gifts from Prof. M. Ottesen, Carlsberg Laboratory, Denmark, yeast alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase were obtained from Boehringer, Mannheim, Germany, and bovine plasma albumin from Armour Pharmaceutical Co., England.

DNA polymerase was purified from *E. coli* strain B. The procedure of purification of DNA polymerase was that of Jovin *et al.*,[1] with the exception that DEAE-Sephadex was used instead of DEAE cellulose at step 4.

Poly d(A-T) was kindly supplied by Dr. P. Reichard, Stockholm, and by Dr. T. Jovin, Göttingen. [^{3}H]-poly d(A-T) was prepared by incubation at 37° of poly d(A-T) (6 nmoles), dATP (200 nmoles), [^{3}H]-dTTP (200 nmoles, 125 μCi μmole^{-1}), $MgCl_2$ (2.5 μmoles), potassium phosphate buffer pH 7.4 (25 μmoles) and enzyme A (9 μg protein) in a total volume of 600 μl. After 5 hr the amount of acid-insoluble radioactivity corresponded to the formation of close to 400 nmoles of [^{3}H]-poly d(A-T) and the incubation mixture was heated at 75° for 6 min and cooled in ice. This solution was passed into a column of Sephadex G-25 (1.0 × 9.5 cm) previously equilibrated with 0.02 *M* potassium phosphate pH 6.5, 0.01 *M* mercaptoethanol. The column was eluted with the same buffer and the 4 fractions (800 μl each) with highest acid-insoluble radioactivity were pooled.

Enzyme assays: DNA polymerase assay: The assay measures the incorporation of [^{3}H]-activity from [^{3}H]-dTTP into an acid-insoluble product. The incubation was performed at 37° in plastic tubes and in the standard assay the incubation mixture contained "activated" calf thymus DNA (1.20 μmoles deoxyribose per ml), dATP, dGTP, dCTP, [^{3}H]-dTTP (each 16 μM; [^{3}H]-dTTP had a specific activity of 100 Ci/mole), 3 mM $MgCl_2$, 1.3 mM dithiothreitol, enzyme (0.2–0.5 μg protein ml^{-1}), Tris-HCl buffer, pH 8.4, at 37° (32 mM), and KCl (167 mM).

The assay was performed with the filter paper technique according to Bollum.[8] The disks were counted in a liquid scintillation spectrometer. The determinations of enzyme activities in fractions from column chromatography were based on two time samples (10 and 20 min). Other determinations were based on four time samples (7, 14, 21, and 28 min). The four points followed a straight line.

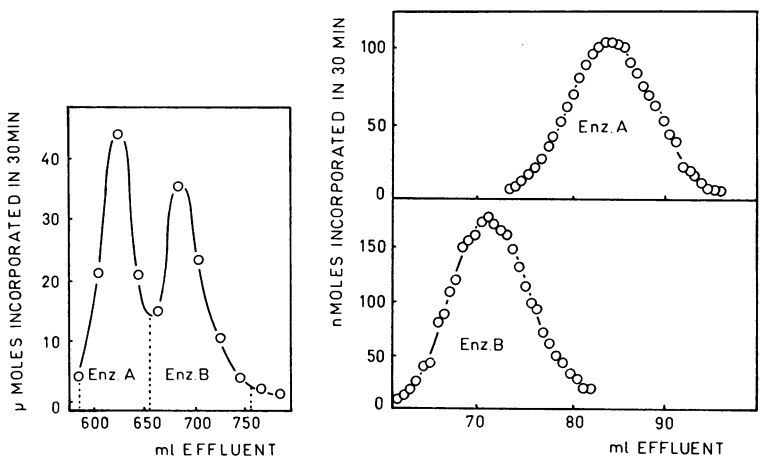
One DNA polymerase unit corresponds to the incorporation in 30 min of 10 nmoles of total 2′-deoxyribonucleoside monophosphates into the acid-insoluble fraction. It was assumed that the individual nucleotides were incorporated in the same molar proportion as that of the bases in thymus DNA, i.e. $(A + T)/(G + C) = 0.57$.

Exonuclease assay: The assay measures the formation of acid-soluble radioactivity formed from ^{3}H-labeled poly d(A-T). In the standard assay (37°) the incubation mixture contained ^{3}H-labeled poly d(A-T) (58 μM, 62 μCiμmole^{-1}), $MgCl_2$ (7 mM), dithiothreitol (1.3 mM), glycine-KOH buffer pH 9.1 (66 mM), and enzyme (0.1 to 14 μg protein ml^{-1}). At 3, 6, 9, and 12 min after addition of enzyme samples of 70 μl were transferred to an ice cold solution of DNA (100 μl, 2 mg ml^{-1}). To this mixture was added 150 μl of ice cold 0.5 *M* perchloric acid and after 5 min the precipitate was removed by centrifugation. An aliquot (300 μl) of the supernatant solution was transferred to 10 ml of a liquid scintillator and counted in a liquid scintillation spectrometer. One exonuclease unit corresponds to the formation of 10 nmoles of acid-soluble nucleotides in 30 min.

Other methods: Analysis by gel filtration chromatography was performed with Sephadex G-200. The gel was equilibrated with buffer (0.1 *M* ammonium sulfate, 0.1 *M* potassium phosphate pH 7.0, 0.01 *M* mercaptoethanol) and packed into a column (1.5 × 81 cm). The enzymes and proteins analyzed were dissolved in the buffer used to equilibrate the gel and applied in a volume of 0.5 ml. The column was

eluted with the same buffer at a rate of about 3.6 ml hr^{-1} and fractions of 620 μl were collected. The concentration of marker proteins in the collected fractions was determined by the absorbancy at 280 nm. Protein in solution of DNA polymerase was determined according to Lowry *et al.*[9]

Results. Isolation of two molecular species of DNA polymerase from *E. coli* B: An elegant procedure for preparing DNA polymerase as a homogeneous protein has recently been published by Jovin *et al.*[1] The main purification is obtained by chromatography on a column of phosphocellulose after removal of residual nucleic acids by treatment with DEAE-cellulose. In our hands the phosphocellulose chromatography has in two instances given rise to two DNA polymerase activity peaks (peaks A and B, Fig. 1) instead of one. The fractions corresponding to each activity peak were pooled separately and carried through the final fractionation step of the procedure. This step consists of chromatography on a column of Sephadex G-100, and the activity peak obtained with enzyme A eluted after 0.39 column volumes while that obtained with enzyme B eluted after 0.34 column volumes. The specific DNA polymerase activities of the two enzymes were 4.3×10^3 and 2.4×10^3 units mg^{-1} for enzymes A and B, respectively. The specific exonuclease activities were about 2.0 units mg^{-1} and 1.1×10^3 units mg^{-1} for enzymes A and B, respectively. Thus, the ratio of polymerase units to exonuclease units was about 2200 for enzyme A while it was



(*Left*) FIG. 1.—*Phosphocellulose column chromatography of fraction 5 of the procedure for preparation of DNA polymerase.*[1] A sample of fraction 5 (59 ml, 430 mg protein) was dialyzed with stirring against running buffer (0.02 *M* potassium phosphate, pH 6.5, 0.01 *M* mercaptoethanol) for $3^1/_2$ hr at 2–4°C. After centrifugation the dialysate had a conductivity corresponding to 0.03 *M* buffer and was passed into a column (2.7×21.5 cm) of phosphocellulose equilibrated with 0.02 *M* buffer. The column was eluted with a linear concentration gradient of potassium phosphate buffer pH 6.5 (500 ml 0.02 *M* buffer, 0.01 *M* mercaptoethanol, and 500 ml 0.3 *M* buffer 0.01 *M* mercaptoethanol). The elution rate was 43 ml hr^{-1} and fractions of 10 ml were collected. Samples of 2 μl of the column fractions were assayed for DNA polymerase activity.

(*Right*) FIG. 2.—*Analysis of enzyme A and enzyme B by gel filtration chromatography.* Samples containing 128 units of enzyme A and 206 units of enzyme B were chromatographed separately on a column of Sephadex G-200. Samples of 30 μl of the column fractions were assayed for DNA polymerase activity. The elution volume of blue dextran 2000 was 45.0 ml.

only 2.2 for enzyme B. For a sample of DNA polymerase prepared by Dr. T. Jovin this ratio was found to be 1.8.

Enzymes A and B were analyzed by gel filtration chromatography on a column of Sephadex G-200. In each case a single almost symmetrical DNA polymerase activity peak appeared. Enzyme A had an elution volume of 83 ml and enzyme B an elution volume of 71 ml (Fig. 2). A sample of DNA polymerase prepared by Dr. T. Jovin was found to have an elution volume identical to that of enzyme B. When the elution volumes from the same column of marker proteins were plotted against the logarithm of respective molecular weights according to Andrews,[10] most experimental points followed a straight line. Assuming the polymerase to follow the same relationship as the marker proteins, the molecular weight of enzyme B was found to be about 150,000 and that of enzyme A about 70,000 (see Fig. 3 and refs. 11–15).

Proteolytic conversion of enzyme B: In the two preparations of DNA polymerase that gave two polymerase activity peaks (enzymes A and B) in the phosphocellulose chromatogram the ratio between the size of two peaks was different. The reason for that is not quite clear but it suggested uncontrolled variations in the isolation procedure.

The above findings suggested the possibility that in our case a DNA polymerase almost devoid of exonuclease activity, enzyme A, may have been formed by limited proteolysis of enzyme B at step 3 or another step in the preparation. No proteolysis may have occurred in the original preparations leading to a single species of DNA polymerase with exonuclease activity physically inseparable from the polymerase activity.[1] The correctness of this assumption was supported by treatment of enzyme B with the proteolytic enzyme subtilisin (type Carlsberg). It appears from Figure 4 that incubation of enzyme B with subtilisin caused the exonuclease activity to disappear almost completely, while the DNA polymerase activity simultaneously increased by about 30 per cent followed by a slight decrease. In the same period of time but in the absence of subtilisin both the exonuclease activity and the DNA polymerase activity of enzyme B decrease about 15 per cent. Treatment of a sample of DNA polymerase prepared by Dr. T. Jovin with subtilisin resulted in a corresponding 30 per cent increase in DNA polymerase activity and a rapid loss in exonuclease activity.

The DNA polymerase (enzyme C) formed by subtilisin treatment of enzyme B has been analyzed by gel filtration. Enzyme B was treated with subtilisin in

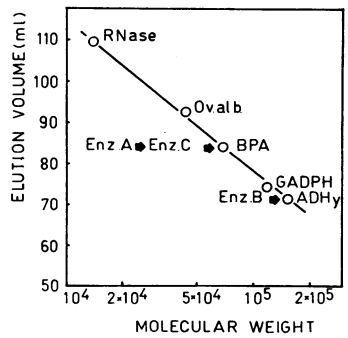


FIG. 3.—*Calibration with marker proteins of a column of Sephadex G-200.* The marker proteins were the following: Pancreatic ribonuclease (RNase), molecular weight 13,700;[11] ovalbumin (Ov.alb.), molecular weight 43,000;[12] bovine plasma albumin (BPA), molecular weight 68,000;[13] glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (GAPDH), molecular weight 117,000;[14] alcohol dehydrogenase from yeast (ADHY), molecular weight 150,000.[15] The elution volumes for the enzymes A, B, and C are indicated by arrows.

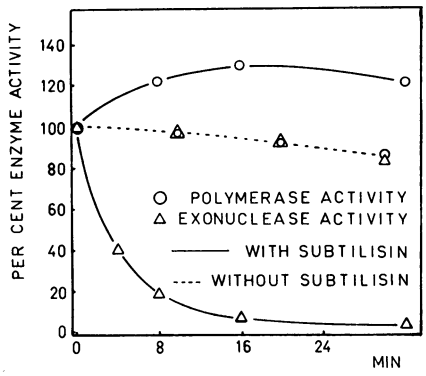


FIG. 4.—*Elimination of the exonuclease activity of enzyme B by treatment with subtilisin.* Enzyme B (1.1 μg protein) was incubated at 37° in 140 μl of a solution containing potassium phosphate buffer, pH 6.5 (67 mM), mercaptoethanol (0.01 *M*), bovine plasma albumin (40 μg), and subtilisin (0.012 μg). At time intervals samples of 10 μl were transferred to ice cold reaction mixtures for exonuclease assay without [^{3}H]-dTTP (300 μl) and DNA polymerase assay without triphosphates (270 μl), respectively. When all time samples of the subtilisin digest had been transferred to the assay mixtures, these were prewarmed for 5 min at 37° and the assays were started by addition of [^{3}H]-poly d(A-T) to the exonuclease assay mixtures and of deoxyribonucleoside triphosphates to the DNA polymerase assay mixtures as described under enzyme assays.

phosphate buffer (pH 6.5, 0.13 *M* for 1 hr) (Fig. 5*A*). A sample was chromatographed on a column of Sephadex G-200 and a single symmetrical polymerase activity peak was obtained at an elution volume identical to that found for enzyme A (Fig. 5*B*). For preparative purpose the main part of the subtilisin digest was chromatographed on a column of Sephadex G-100 (1.35 ml of solution on a column 2.5 × 26 cm). The collected fractions (2 ml) which contained polymerase activity were pooled and saturated with ammonium sulfate. The precipitate was dissolved in 50 per cent glycerol and 50 per cent potassium phosphate

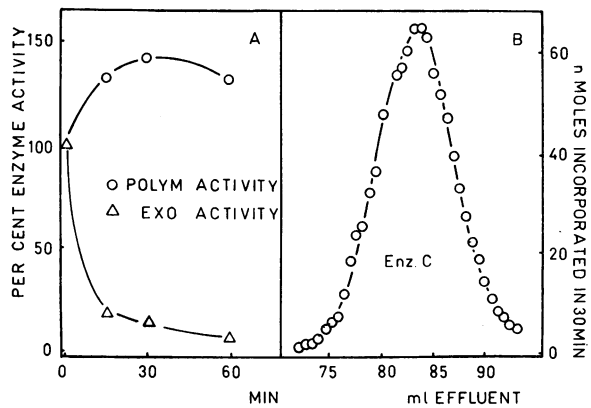


FIG. 5*A*. *Formation of enzyme C.* Enzyme B (0.6 mg protein) was incubated at 37° in 1500 μl of a solution containing phosphate buffer pH 6.5 (0.13 *M*), mercaptoethanol (0.01 *M*), bovine serum albumin (0.39 mg), and subtilisin (0.6 μg). At time intervals samples of 10 μl were transferred to ice cold solutions of albumin (190 μl, 10 mg ml^{-1}). Samples of 10 μl of these solutions were assayed for exonuclease activity and for polymerase activity, respectively, as described under *Enzyme Assays.*

B. Analysis of enzyme C by chromatography on Sephadex G-200. After incubation for 1 hr of the reaction mixture described under A, a sample of 100 μl was passed into a column of Sephadex G-200. The column was eluted as described under **Materials and Methods** and 30 μl of the column fractions were analyzed for DNA polymerase activity.

buffer (pH 7.0, 0.1 *M*). The ratio of polymerase units to exonuclease units in this preparation was about 260. The Sephadex treatment separates the modified DNA polymerase from subtilisin since the latter enzyme has a molecular weight of about 27,000.[16]

Discussion. The following findings suggest that enzyme B and the enzyme described by Jovin *et al.*[1] are identical. The elution volumes obtained for the two enzymes on a column of Sephadex G-200 are identical, the ratio of exonuclease activity to polymerase activity is almost the same for the two enzymes, and they are both modified in the same way by treatment with subtilisin, resulting in increased apparent polymerase activity and in a drastic decrease in exonuclease activity. This possibility would mean that the exonuclease activity present in enzyme B is identical with exonuclease II. The latter activity has been found to be physically inseparable from the enzyme studied by Jovin *et al.*[1] In the following the enzyme characterized by Jovin *et al.*[1] and enzyme B are assumed to be identical and are termed native DNA polymerase.

The molecular weight of native DNA polymerase has been determined by sedimentation equilibrium analysis by Jovin *et al.* to be 109,000.[1] The molecular weight as estimated in the present paper from gel filtration data according to Andrews[10] is significantly larger (150,000). Stokes radius for the native polymerase may be calculated from the data of Jovin *et al.*[1] to be about 32 Å while Stokes radius as estimated in the present paper from gel filtration data is almost identical to that of yeast alcohol dehydrogenase. For the latter enzyme Stokes radius may be calculated from the diffusion coefficient to be about 46 Å.[15] The significantly higher values for molecular weight and Stokes radius estimated from gel filtration experiments as compared to those determined by sedimentation equilibrium experiments may be ascribed to an unusual conformation of the native enzyme or to the difference in ionic conditions used in the two types of procedures. The gel filtration experiments were performed in a solution of ammonium sulphate (0.1 *M*), potassium phosphate (0.1 *M*, pH 6.5), and mercaptoethanol (0.01 *M*), while the equilibrium sedimentation experiments were performed in a solution of potassium chloride (0.1 *M*), potassium phosphate (0.0025 *M*) and mercaptoethanol (0.01 *M*). A pronounced specific binding of sulfate ions to the native polymerase may possibly account for the different results obtained.

The finding that the exonuclease activity of the native polymerase may be specifically removed by limited proteolytic digestion without loss of polymerase activity indicates that a part of the enzyme molecule is particularly susceptible for proteolytic attack. This part of the molecule is apparently indispensable for the exonuclease activity but not for polymerase activity. The finding that the subtilisin treatment gives rise to a polymerase activity which according to gel filtration data is associated with a single molecular species of reduced size suggests that the proteolysis under the experimental conditions used progresses only to a certain point. Since subtilisin is a rather unspecific proteolytic enzyme,[17] this phenomenon may be ascribed to a pronounced flexibility or a partial unfolding of the subtilisin-susceptible part of the native DNA polymerase in contrast to the rest of the molecule. The increase in polymerase activity which accompanies

the proteolytic modification of the native enzyme indicates that the apparent polymerase activity per active site increases under the experimental conditions used. It is unlikely, therefore, that the concomitant decrease in molecular weight causes a drastic decrease of the constants for binding of substrates and primer to the active sites.

Besides the native enzyme from *E. coli* B the DNA polymerases from T4 and T5 infected coli cells have also been characterized.[18] Their molecular weights have been found to be 112,000[19] and 96,000,[20] respectively. Both enzymes are like the native enzyme from *E. coli* B associated with an exonuclease activity which is physically inseparable from the polymerase activity. In contrast, it has been found that the enzyme from *B. subtilis* has only barely detectable exonuclease activity.[21] In view of the present findings it seems possible that the *B. subtilis* enzyme as isolated is not a native form but a modified enzyme formed during the preparation by limited proteolytic digestion by subtilisin present in large amounts in extracts of *B. subtilis*.

It is possible that other proteolytic degradation products of the native polymerase different from enzymes A and C and with retained polymerase activity may be formed under experimental conditions different from those that led to formation of enzymes A and C. This possibility might explain part of the findings by Lezius *et al.*[22] and by Cavalieri and Carrol[23] that the DNA polymerase from *E. coli* B may exist in several different forms with molecular weights ranging from about 24,000 to about 300,000.

Note added in proof: After the submission of the present work, experiments leading to similar conclusions have been published by Brutlag *et al.* (*Biochem. Biophys. Res. Commun.*, **37**, 982 (1969)). We have found that subtilisin-treatment of native DNA polymerase in the presence of DNA may lead to cleavage of the enzyme into a polymerase unit and an exonuclease unit with estimated molecular weights of 70,000 and 35,000, respectively (Klenow, H., and K. Overgaard-Hansen, *FEBS Letters*, in press).

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Code number of enzymes mentioned in the text: DNA polymerase: DNA deoxyribonucleotidyltransferase (EC 2.7.7.7.) Subtilisin: Subtilopeptidase (EC 3.4.4.16).

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