DNA REPAIR

A Laboratory Manual of Research Procedures

Volume 1
(in two parts)

Part A

Edited by
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Chapter 20

PURIFICATION OF THE MAJOR AP ENDONUCLEASE OF ESCHERICHIA COLI

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I. INTRODUCTION

The major AP endodeoxyribonuclease of Escherichia coli is endonuclease VI [1-3], which appears to be the same protein as exonuclease III [3-7]. Another AP endodeoxyribonuclease, named endonuclease IV, has also been purified from the same organism [8].

II. AP SITES AND ASSAYS FOR AP ENDODEOXYRIBONUCLEASE

A. AP Sites: Alkali-Lability

Sites in DNA lacking a purine or a pyrimidine are called AP sites; they are alkali-labile. Exposure to alkaline pH leads to hydrolysis of a neighboring phosphoester bond [9]; the mechanism seems to involve a β elimination cleaving the phosphodiester bridge immediately on the 3' side of the AP site and leaving a 3'-OH and a 5'-phosphate. Schyns et al. [10] found that intact AP sites have a half-life of 37 min at pH 12 and 37°C; they use this half-life to characterize AP sites and distinguish them from other alkali-labile sites. Incubation of DNA in 0.2 M NaOH at 37°C for 15 min is adequate to introduce breaks near most AP sites [1].

B. Preparation of DNA Containing AP Sites

Double-stranded DNA containing AP sites has been prepared in three different ways: acid-depurination, alkylation-depurination, and glycosylase treatment.

1. Acid-Depurination

To DNA in 0.15 M NaCl/0.015 M EDTA,* pH 7.0, acetic acid is added to reach pH 4.0, and the solution is incubated at 37°C. This treatment produces about one AP site per hour per 3000 nucleotides [11].

2. Alkylation-Depurination

To DNA in 0.5 M sodium phosphate (20-40 $\mu g\ ml^{-1}$), methyl methanesulfonate is added to reach 0.3 M final concentration. The mixture is incubated at 37°C for 1 hr, cooled on ice, and dialyzed against 0.15 M NaCl/0.015 M sodium citrate, pH 7.0 (SSC). The alkylated DNA, which contains one methyl group per six to seven nucleotides, is heated at 50°C for 6 hr, then cooled at 4°C, and dialyzed against a suitable buffer. The alkylated-depurinated DNA contains about one AP site per 20 nucleotides [1].

3. Use of DNA Glycosylases

DNA is treated with bisulfite, then with uracil DNA glycosylase [12], or with methyl methanesulfonate and then with 3-methyladenine DNA glycosylase [13].

C. Assays for AP Endodeoxyribonucleases

Several kinds of DNA have been used to prepare a substrate for the AP endodeoxyribonucleases: Escherichia coli, T7 phage, and PM2 phage. They are labeled by growing the microorganisms in the presence of labeled thymidine or ^{32}P-labeled phosphate.

1. E. coli Alkylated-Depurinated Labeled DNA:
Determination of Acid-Soluble Radioactivity [1,2]

The labeled DNA is submitted to alkylation-depurination so as to contain about one AP site per 20 nucleotides. The AP endodeoxyribonuclease activity is followed by the appearance of acid-soluble radioactive products.

To the incubation mixture (40 μl) cooled at 0°C are added 100 μl 15 mM NaCl/1.5 mM sodium citrate, pH 7.0 (0.1 SSC), containing 200 μg calf thymus DNA (carrier), and 500 μl 6.4% $HClO_4$ (to reach 5% final concentration). The tubes are kept on ice for 15 min and centrifuged at 10,000 g for 10 min. A pipette is used to remove 500 μl from the supernatant for radioactivity determination; the result is multiplied by 1.28.

With this technique, the untreated alkylated-depurinated DNA yields an acid-soluble fraction below 1%, whereas, after a treatment with NaOH, the acid-soluble fraction is 30% [1]. One must be aware that the relation between acid-soluble fraction and break frequency is not linear but rather sigmoid. The exact relationship has not yet been worked out.

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*Ethylenediaminetetraacetic acid.

2. T7 Phage Labeled DNA and Sedimentation Analysis [1,3,11]

T7 phage DNA is a linear double-stranded molecule of 2.52×10^7 daltons. For the study of the AP endodeoxyribonucleases, this DNA is most often acid-depurinated. The intact AP sites and the breaks are counted from sedimentation profiles in neutral sucrose gradients [11,14]. The DNA is denatured in two ways: with NaOH or with formamide.

For denaturation with NaOH, the DNA solution is mixed with an equal volume of 0.4 M NaOH/0.015 M EDTA, and the mixture is then incubated at 37°C for 15 min. The solution is neutralized with 1.4 N H_3PO_4.

For denaturation with formamide, the DNA solution is diluted with an equal volume of 0.15 M NaCl/0.015 M EDTA, pH 7.0, then dialyzed at 4°C against 95% formamide [formamide freshly redistilled under reduced pressure/0.015 M EDTA (95:5, vol:vol), adjusted at pH 7.0] with two changes. The temperature is then raised to 37°C for 2 hr. To eliminate the formamide, the solution is dialyzed at 4°C against 0.15 M NaCl/0.015 M EDTA, pH 7.0.

Linear 5-20% wt/vol sucrose gradients (4.8 ml) in 1 M NaCl/0.01 M Tris-HCl*/1 mM EDTA, pH 7.0, are prepared in 5-ml nitrocellulose tubes. A 0.1 ml aliquot of the denatured DNA (0.4 μg) solution is carefully layered on top of the gradient. The tubes are spun for 90 min at 35,000 rpm at 20°C in the SW 50.1 rotor of a Beckman L2-65B centrifuge. The brake is not used during deceleration. The bottom of the tube is perforated, and a 50% sucrose solution is injected at a rate of 0.5 ml min^{-1}; 0.1 ml fractions are collected from the top of the tube and assayed for radioactivity. The average number of breaks per DNA strand (1.26×10^7 daltons) is calculated with a FORTRAN program adapted from Gillespie et al. [15]. The method enables one to measure from 0.1 to 20 breaks per strand [14].

Denaturation with formamide at pH 7.0 leaves the AP sites intact [16]; the breaks, whose number is estimated from the sedimentation profile on the neutral sucrose gradient, were in the DNA before denaturation. Those found by sedimentation after NaOH denaturation are the sum of breaks existing before denaturation and the breaks produced near AP sites. The number of intact AP sites which were in the DNA before denaturation is obtained by subtracting the number of breaks after formamide treatment from the number of breaks after NaOH treatment.

3. PM2 Phage DNA and Sedimentation Analysis [8,13]

This DNA is a closed circular double-stranded molecule; the superhelical and nicked forms, which have sedimentation coefficients of 29S and 21S respectively at an ionic strength of 0.04 [17], can be separated by centrifugation on neutral sucrose gradients. The fraction f of molecules in the superhelical form is related to the average number n of breaks per molecule by Poisson's law:

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*Tris(hydroxymethyl)aminomethane hydrochloride.

$$f = e^{-n} \quad \text{or} \quad n = -\ln f$$

D. Recommended Buffers

Because in Tris-HCl pH 8.0 the AP sites are slowly labile even at 4°C, the DNA substrates are generally kept in 0.15 M NaCl/0.015 M sodium citrate, pH 7.0 (SSC), containing 0.02 M $MgCl_2$. On the other hand, endonuclease VI is in 0.05 M Tris-HCl/0.1 mM β-mercaptoethanol, pH 8.0 (Tris-SH), containing 0.1% bovine serum albumin. To test the enzyme activity, equal volumes of substrate and endonuclease VI solutions are mixed; the pH of the mixture is optimum for endonuclease VI. The enzyme is protected by β-mercaptoethanol and bovine serum albumin, and it is supplied with the necessary Mg^{2+}.

E. The AP Endodeoxyribonuclease Unit

The term enzyme unit, as used in this chapter, is the AP endodeoxyribonuclease activity which—in a medium composed of half SSC/0.02 M $MgCl_2$ and half Tris-SH/0.1% bovine serum albumin—hydrolyzes phosphoester bonds near 1 pmol AP sites min^{-1} (V_{max}).

III. PURIFICATION OF THE MAJOR AP ENDODEOXYRIBONUCLEASE OF E. COLI

The major AP endodeoxyribonuclease of E. coli can be purified in two ways: as endonuclease VI or as exonuclease III.

A. Purification of Endonuclease VI [1,2]

The various steps are followed by the determination of the acid-soluble radioactivity after incubation of the enzyme preparation with alkylated-depurinated labeled DNA. To 20 μl SSC/0.02 M $MgCl_2$ containing 0.3 μg alkylated-depurinated labeled DNA (one AP site per 20 nucleotides) is added 20 μl of the enzyme preparation in Tris-SH/0.1% bovine serum albumin. The acid-soluble radioactivity is measured after a 10 min incubation at 37°C. We previously emphasized that the acid-soluble fraction is not proportional to the frequency of breaks; nevertheless, between 1% and half the acid-soluble fraction produced by NaOH treatment (30%/2 ≈ 15%), there is a near-proportionality between the acid-soluble fraction and the amount of enzyme in the assay.

E. coli B41, a mutant devoid of endonuclease I [18], is used. All manipulations for the enzyme isolation are carried out at 4°C. The bacterial cells (100 g), collected at the end of the exponential phase of growth, are ground in a mortar with 250 g alumina. The thick paste is suspended in 500 ml 0.5 M Tris-HCl/0.1 mM EDTA/0.1 mM β-mercaptoethanol, pH 8.0 (buffer A); the alumina is removed by a 15 min centrifugation at 2500 g and the cell debris

by another 20 min centrifugation at 7000 g. To the 600 ml supernatant, streptomycin sulfate in buffer A is slowly added to reach 0.8% final concentration, and the suspension is centrifuged for 15 min at 10,000 g. Solid ammonium sulfate is added to the supernatant (780 ml; 8.5×10^6 enzyme units; 580 units per mg protein) to reach 50% saturation; after removal of the precipitate by centrifugation, more ammonium sulfate is added to reach 70% saturation. The 50-70% precipitate, collected by centrifugation, is dissolved in 150 ml buffer A and dialyzed against buffer A containing 0.1 M NaCl. This solution (5.8×10^6 enzyme units; 1100 units per mg protein) is applied to a 5 × 40 cm DEAE-cellulose* column equilibrated with buffer A/0.1 M NaCl; after a washing with 100 ml of the same buffer, the elution is carried out with 4 liters of a 0.1-0.3 M NaCl linear gradient in buffer A at a rate of 100 ml hr^{-1}. The fractions containing the AP endodeoxyribonuclease activity, which are eluted between 0.15 and 0.17 M NaCl, are pooled (4.9×10^6 enzyme units; 6100 units per mg protein) dialyzed against 0.04 M sodium phosphate/0.1 mM EDTA/0.1 mM β-mercaptoethanol, pH 6.5 (buffer B), then applied to a 2.6 × 32 cm phosphocellulose column equilibrated with buffer B. The elution is carried out, at a rate of 50 ml hr^{-1}, with 2 liters of a 0-0.25 M NaCl linear gradient in buffer B. The fractions containing the AP endodeoxyribonuclease activity, which are eluted between 0.15 and 0.17 M NaCl—far from the bulk of protein—are pooled (4.1×10^6 enzyme units; 1.64×10^5 units per mg protein). This preparation (250 ml) is concentrated on a small DEAE-cellulose column (1.7 × 2 cm) equilibrated with buffer A: the AP endodeoxyribonuclease activity, which is completely retained, is eluted in 10 ml 0.5 M NaCl in buffer A. This solution is placed on a 2.6 × 92 cm Sephadex G-75 column equilibrated with buffer A; the elution is carried out with buffer A at a rate of 30 ml hr^{-1}, and the enzyme activity is found between 250 and 300 ml. The preparation (50 ml; 4.1×10^6 enzyme units; 1.60×10^6 units per mg protein) is dialyzed against buffer A containing 0.08 M NaCl and applied to a 0.9 × 12 cm DEAE-Sephadex column equilibrated with 0.08 M NaCl in buffer A; the elution is performed at a rate of 8 ml hr^{-1} with 500 ml of a 0.08-0.13 M NaCl linear gradient in buffer A. The AP endodeoxyribonuclease is eluted in 45 ml between 0.106 and 0.114 M NaCl (3.8×10^6 enzyme units; 4.70×10^6 units per mg protein).

The isolated protein appears practically pure on polyacrylamide gel electrophoresis in the native form or after denaturation with urea or sodium dodecyl sulfate. The total yield is approximately 45%, and the purification factor is in the neighborhood of 8000. The molecular weights of the native enzyme estimated by Sephadex G-75 column chromatography and of the denatured enzyme estimated by gel electrophoresis in the presence of sodium dodecyl sulfate are about 32,000 daltons.

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*O-(diethylaminoethyl)cellulose.

The enzyme is kept at -25°C after the addition of 0.1% bovine serum albumin and an equal volume of glycerol. Before use, this solution is diluted with Tris-SH containing 0.1% bovine serum albumin.

B. Purification of Exonuclease III

Weiss [5] has used the method of Richardson and Kornberg [19] with minor modifications. The 3'-phosphatase and the exonuclease activities of the enzyme are followed (see properties of the major endodeoxyribonuclease of E. coli; Sec. IV.A).

E. coli K12 cells (1000 g) are disrupted in 0.05 M glycylglycine pH 7.0, to yield 4500 ml of extract. An equal volume of 0.05 M Tris-HCl/1 mM EDTA, pH 7.5, is added, and enough streptomycin sulfate to precipitate 50% of the enzyme activity. To 5000 ml of the supernatant containing reduced glutathione at 1 mM concentration, at 0°C, is slowly added 325 ml acetone, and the precipitate is discarded; to the new supernatant is added an additional 1425 ml acetone. The second precipitate is dissolved in 1000 ml 0.01 M potassium phosphate/1 mM β-mercaptoethanol, pH 7.5 (buffer A'), and applied to a 10.5 × 18 cm DEAE-cellulose column equilibrated with buffer A'. The elution is carried out, at a rate of 2000 ml hr^{-1}, with 13 liters of a 0.01-0.16 M potassium phosphate linear gradient, pH 7.5, containing 1 mM β-mercaptoethanol. The fractions containing most of the enzyme activity are pooled (1800 ml) and dialyzed against 0.02 M potassium phosphate/1 mM β-mercaptoethanol, pH 6.5 (buffer B'). The dialyzed solution is applied to a 5.6 × 14 cm phosphocellulose column equilibrated with buffer B'; after a washing with 1250 ml buffer B', the elution is performed, at a rate of 750 ml hr^{-1}, with 4.3 liters of a 0.02-0.28 M potassium phosphate linear gradient, pH 6.5, containing 1 mM β-mercaptoethanol. The enzyme is eluted in a sharp peak (about 150 ml). After pressure dialysis against 0.10 M phosphate buffer/1 mM reduced glutathione, pH 7.4, the volume is reduced to about 15 ml.

The isolated protein is practically pure, as judged by polyacrylamide gel electrophoresis in the native form or in the presence of sodium dodecyl sulfate, or by gel filtration on Sephadex G-100. The molecular weights determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, by sedimentation, or by gel filtration, are about 28,000 dal tons. The yield is 6% and the purification factor 1600 [5].

IV. PROPERTIES OF THE MAJOR AP ENDODEOXYRIBONUCLEASE OF E. COLI

A. The Exonuclease III Activity

Exonuclease III has a 3'-phosphatase activity and an exonuclease activity working from the 3' ends of double-stranded DNA in the 3'→5' direction while releasing 5'-deoxyribonucleotides [19,20].

1. Presence of Exonuclease III in Endonuclease VI Preparation

The demonstration of a 3' phosphatase activity is performed in the following way [3,19]: E. coli DNA labeled with ^{32}P (30 μg) and micrococcal nuclease (EC 3.1.4.7.; 1 unit) in 1 ml 0.05 M glycine/0.01 M $CaCl_2$, pH 9.2, are incubated at 37°C for 15 min; to stop the nuclease action, the solution is dialyzed at 4°C against SSC/0.01 M $MgCl_2$ to remove the calcium. To 20 μl of this (3'-phosphate/5'-OH)-nicked [^{32}P]DNA are added 50 μl endonuclease VI preparation (30 units) and 200 μl 0.01 M potassium phosphate/1.5 mM β-mercaptoethanol, pH 7.0; the mixture is incubated 30 min at 37°C before addition of 500 μg calf thymus DNA in 200 μl 0.1 SSC and 500 μl 10% trichloroacetic acid. After 5 min at 0°C, the precipitate is removed by centrifugation, and the radioactivity of the supernatant nonadsorbed on Norit (i.e., the radioactive inorganic phosphate) is measured; it is proportional to the number of nicks introduced in the [^{32}P]DNA by the micrococcal nuclease.

The demonstration of the exonuclease activity can be made on sonicated DNA, but the proof that it is indeed exonuclease III is made on doubly labeled nicked DNA prepared from E. coli DNA labeled with ^{32}P.

The first step in the preparation of this substrate is the introduction of nicks limited by 3'-OH/5'-phosphate: the [^{32}P]DNA (300 μg) and pancreatic deoxyribonuclease (EC 3.1.4.5.; 2 units) in 1.5 ml 0.1 M NaCl/0.01 M sodium citrate/0.017 M Tris-HCl/0.17 mM $MgCl_2$, pH 7.5, are incubated at 37°C for 15 min; the mixture is heated 10 min at 77°C to inactivate the enzyme without denaturing the DNA, then cooled. The second step is the replacement of ^{32}P-labeled nucleotides by ^{3}H-labeled nucleotides, using E. coli DNA polymerase I: 250 μl (3'-OH/5'-phosphate)-nicked [^{32}P]DNA (50 μg), 500 μl 0.4 M glycine/0.04 M $MgCl_2$/6 mM β-mercaptoethanol, pH 9.2, 500 μl of an aqueous solution of dATP/dGTP/dCTP (100 nmol each) and 4.5 μCi [^{3}H]dTTP (20 Ci mmol^{-1}), and 750 μl DNA polymerase I (EC 2.7.7.7.; 4 units) in 0.05 M Tris-HCl/0.01 M β-mercaptoethanol, pH 7.5, are incubated 30 min at 37°C. The doubly labeled DNA is purified by phenol extraction and dialyzed against SSC/0.02 M $MgCl_2$. The DNA segments between the nicks of the double-stranded molecules are thus labeled with ^{32}P at the 5' ends and ^{3}H at the 3' ends.

To 40 μl of solution containing this (3'-OH/5'-phosphate)-nicked doubly labeled DNA (0.6 μg) is added 40 μl endonuclease VI preparation (10 units) in several tubes, which are incubated at 37°C for various times before determination of the ^{32}P and ^{3}H acid-soluble radioactivities. Chromatography on DEAE-Sephadex at pH 7.6 in the presence of 7 M urea [21] shows that the acid-soluble radioactivity is in mononucleotides. On the other hand, ^{3}H is released in the acid-soluble fraction much faster than ^{32}P—which indicates that the exonucleolytic activity proceeds from the nicks in the 3'→5' direction [3].

2. Exonuclease III has an AP Endodeoxyribonuclease Activity

Using the method of Richardson and Kornberg [19], Weiss [5] showed that, during the purification of exonuclease III, there is a constant ratio of 3'-phosphatase, exonuclease, and AP endodeoxyribonuclease activities. Mutations modifying the activity of exonuclease III affect in the same way the AP endodeoxyribonuclease activity [4]. The absence of gene xthA, which codes for exonuclease III, is responsible for the disappearance of 90% of the AP endodeoxyribonuclease activity of E. coli crude extract; the minor AP endodeoxyribonuclease responsible for the remaining 10% of the activity has been called endonuclease IV [6].

Since Verly and Rassart [2] found in the protein which they isolated (endonuclease VI) 45% of the AP endodeoxyribonuclease activity of the crude E. coli extract, it is likely that this protein is the product of gene xthA. The finding of exonuclease III in an endonuclease VI preparation (see Sec. IV.A.1) [3] supports the same conclusion; it must, however, be noticed that the work was carried out, not with the pure enzyme, but with the product of the previous step of purification—which still contained several proteins.

B. Specificity for AP Sites of Endonuclease VI

Endonuclease VI is thus, at the same time, an endonuclease and an exonuclease; the endonucleolytic activity is restricted to AP sites in DNA, and double-stranded DNA is a much better substrate than single-stranded DNA. Endonuclease VI has no action on RNA; it is interesting to note that, on the other hand, pancreatic ribonuclease (EC 3.1.4.22.) is active on AP sites in single-stranded DNA but not in double-stranded DNA [22].

The specificity of endonuclease VI can be shown on heavily modified E. coli labeled DNA relying on the determination of the acid-soluble radioactivity. It can also be shown on lightly modified T7 or PM2 phage labeled DNA by sedimentation analysis; these latter methods have the advantage of not being disturbed by exonuclease activities.

1. On E. coli Labeled DNA

[^{3}H]DNA (1 μg), in 20 μl SSC/0.02 M $MgCl_2$ mixed with 20 μl endonuclease VI (10 units) in Tris-SH/0.1% bovine serum albumin, is incubated in several tubes at 37°C for various times before measurement of the radioactive product soluble in 5% perchloric acid. The DNA is either untreated, alkylated with methyl methanesulfonate (one methyl group per 6-7 nucleotides), or alkylated-depurinated (one AP site per 20 nucleotides) [1-3].

With freshly prepared endonuclease VI, there is practically no activity on untreated DNA. Some action is observed on alkylated DNA and more on alkylated-depurinated DNA; the measured acid-soluble radioactivity tends toward the value observed after an incubation of the same time without enzyme, followed by a treatment with NaOH. Moreover there is no difference

in the observed acid-soluble radioactivity, whether the enzyme is present or not during an incubation which is followed by a treatment with NaOH. These results indicate that the endonuclease VI activity is restricted to the alkali-labile sites, i.e., the AP sites. There is no activity of endonuclease VI on denatured DNA not containing AP sites, and the activity on denatured DNA containing AP sites is much lower than on the double-stranded substrate.

The results are less clear-cut with an aged preparation of endonuclease VI, where the ratio of the activities (exonuclease/endonuclease) is increased. Untreated DNA is slowly hydrolyzed, and the rate of hydrolysis is increased by sonication of the substrate; it can be demonstrated that the acid-soluble radioactivity is in mononucleotides. Incubation with the enzyme of alkylated or alkylated-depurinated [^{3}H]DNA before treatment with NaOH gives a higher acid-soluble radioactivity than the incubation carried out without the enzyme. An exonuclease interferes with the demonstration of the endonuclease VI specificity [3]. One must then resort to the use of a substrate containing few modifications—either T7 or PM2 phage DNA—and study the sedimentation profiles.

2. On T7 Phage DNA

T7 phage [^{3}H]DNA (8 μg) in 500 μl SSC/0.02 M $MgCl_2$ and 500 μl endonuclease VI (1000 units) in Tris-SH/0.1% bovine serum albumin are mixed and incubated at 37°C. Aliquots are taken at various times, denatured with NaOH, and analyzed for breaks by sedimentation on sucrose gradients. With untreated DNA, DNA containing about 150 methyl groups per strand, or acid-depurinated DNA containing 3-10 AP sites per strand, the average number of endonucleolytic breaks per strand is, within experimental error, the same whether the DNA has been incubated with or without the enzyme before the NaOH treatment [1,3]. These results are obtained even with an aged preparation of endonuclease VI, since there is little interference by the exonuclease activity.

The test using PM2 phage DNA, which distinguishes the supertwisted molecules from those containing at least a nick, is insensitive to the presence of an exonuclease.

C. Reduction of AP Sites: No Suppression in Sensitivity of DNA to Endonuclease VI

Equal volumes of alkylated-depurinated [^{3}H]DNA in SSC and 0.2 M sodium borate are mixed (pH 9.5) and cooled at 0°C. To 500 μl of this mixture is added 3.75 mg $NaBH_4$ in 50 μl 0.05 M NaOH. After 30 min at 0°C, 50 μl 1 M H_3PO_4 is added, and the solution is dialyzed against SSC/0.02 M $MgCl_2$.

The acid-soluble radioactivity measured on this reduced alkylated-depurinated [^{3}H]DNA is only slightly greater than that of the starting alkylated-depurinated [^{3}H]DNA—indicating that the reduction procedure introduces only a few additional breaks. On the other hand, a treatment with NaOH,

which raises to 30% the acid-soluble fraction given by alkylated-depurinated [^{3}H]DNA, increases very little the acid-soluble radioactivity given by reduced alkylated-depurinated [^{3}H]DNA—showing that most AP sites have been reduced (reduced AP sites are alkali-resistant).

Alkylated-depurinated [^{3}H]DNA (5 μg), reduced or not, in 200 μl SSC/0.02 M $MgCl_2$, is mixed with an equal volume of endonuclease VI (20 units) in Tris-SH/0.1% bovine serum albumin and incubated at 37°C. The acid-soluble radioactivity of 40-μl aliquots taken at various times is measured. Reduction increases by about 20% the rate of hydrolysis of alkylated-depurinated DNA by endonuclease VI [3].

D. Nature of Ends Resulting from Hydrolysis by Endonuclease VI of Phosphoester Bond near AP Site

1. The 3'-End

When [^{32}P]DNA containing AP sites is incubated with an endonuclease VI/exonuclease III preparation, there is no release of Norit-nonabsorbable radioactivity (i.e., inorganic [^{32}P]phosphate). Since the 3'-phosphatase activity of exonuclease III can be demonstrated on [^{32}P]DNA activated with micrococcal nuclease, which leaves 3'-phosphate/5'-OH ends (Sec. IV.A.1), the conclusion is that the phosphoester bond hydrolyzed by endonuclease VI directly yields a 3'-OH end [3].

2. The 5'-End

Alkylated-depurinated DNA (600 μg; one AP site per 20 nucleotides) in 500 μl SSC/0.02 M $MgCl_2$ is mixed with 500 μl Tris-SH/0.1% bovine serum albumin, containing endonuclease VI (3000 units) or not, and the mixtures are incubated at 37°C for 60 min. The DNA is subsequently denatured with formamide and dialyzed at 4°C successively against SSC containing 6% formaldehyde, then against SSC alone. Four aliquots (200 μl) from each incubation (with or without endonuclease VI) are mixed, in separate tubes, with 165 μl 0.1 M Tris-HCl pH 8.0 containing 1 unit *E. coli* alkaline phosphatase (EC 3.1.3.1.) (two tubes) or not (two tubes), incubated for 30 min at 37°C, then cooled in ice. To all tubes are added 100 μl 0.6 M $MgCl_2$ and 5 μl 1 M β-mercaptoethanol. Two tubes (with and without phosphatase treatment) are supplied with 10 units polynucleotide 5'-hydroxylkinase (EC 2.7.1.78.) in 10 μl 0.05 M sodium phosphate/0.25 M KCl, pH 7.5, whereas the other tubes (with and without phosphatase treatment) receive 10 μl buffer without enzyme; all tubes are finally supplied with 20 μl 0.25 mM [γ-^{32}P]ATP and incubated 60 min at 37°C. After the addition of 10 μg calf thymus DNA in 100 μl 0.1 SSC and 300 μl 0.1 M sodium pyrophosphate, the DNA is precipitated with 2.5 ml 0.4 M trichloroacetic acid containing 0.02 M sodium pyrophosphate and collected on glass fiber disks. After washing with 0.4 M trichloroacetic acid containing 0.02 M sodium pyrophosphate, the disks are dried and counted for radio-

activity. The results obtained with the polynucleotide 5'-hydroxyl-kinase are corrected for controls without enzyme.

Whatever the experimental conditions, there is little radioactivity incorporated in the alkylated-depurinated DNA when there has been no incubation with endonuclease VI. After an incubation with endonuclease VI, the radioactivity introduced by the polynucleotide 5'-hydroxyl-kinase is important only if there has been a phosphatase treatment. The single-stranded breaks introduced near AP sites by endonuclease VI are thus limited by a 5'-phosphate [3].

Additional evidence can be obtained with spleen phosphodiesterase (EC 3.1.4.18.): a treatment with phosphatase is necessary for the degradation by spleen phosphodiesterase of the denatured alkylated-depurinated DNA treated with endonuclease VI (see Sec. IV.E.1) [3].

E. Position of Phosphoester Bond Hydrolyzed by Endonuclease VI

1. Bond on 5' Side of AP Site

This can be shown with [^{32}P]DNA containing AP sites reduced with sodium [^{3}H]borohydride [3].

E. coli acid-depurinated [^{32}P]DNA (one AP site per 100 nucleotides) is dialyzed against 0.01 M sodium borate, pH 9.8. To 2 ml of this solution (200 μg DNA) are added successively 50, 25, and 25 μl 0.05 M NaOH containing sodium [^{3}H]borohydride (10 μg μl^{-1}), and the solution is left at room temperature for 1 hr after each addition; 200 μl 0.05 M NaOH containing 15 mg nonlabeled sodium borohydride is finally added and the solution left for another hour. The excess hydride is destroyed by the addition of 0.2 ml 1 M sodium acetate pH 5, and the DNA purified by Sephadex G-200 chromatography and extensive dialysis against SSC/10 mM EDTA. A control DNA which has not been depurinated is used to show that there is little nonspecific fixation of tritium; the tritium radioactivity found in the reduced depurinated DNA is thus mostly localized in AP sites.

Reduction of AP sites does not prevent the action of endonuclease VI (Sec. IV.C). Acid-depurinated [^{32}P]DNA, reduced with sodium [^{3}H]borohydride (2 μg) in 20 μl SSC/0.02 M $MgCl_2$ and 20 μl endonuclease VI (10 units) in Tris-SH/0.1% bovine serum albumin, is put in several tubes and incubated for various times at 37°C before measurement of the ^{3}H and ^{32}P activities released in the acid-soluble fraction. A small amount of acid-soluble ^{3}H appears, which is quickly at a plateau; it is due to the endonucleolytic activity of the enzyme, since size distribution analysis on DEAE-Sephadex at pH 7.6 in the presence of urea indicates that the radioactivity is in large polynucleotides. By contrast, the amount of acid-soluble ^{32}P increases with time, and most of it is in mononucleotides. These observations suggest that the phosphoester bond hydrolyzed by endonuclease VI is on the 5' side of the AP site, and that exonuclease III, degrading the DNA from the nicks in the 3'→5'

direction, releases ^{32}P-labeled mononucleotides, while leaving the reduced AP site in the DNA molecule [3].

If, when the acid-soluble ^{3}H-labeled material has reached the plateau, 50 μl 0.4 M glycine/0.04 M $MgCl_2$/6 mM β-mercaptoethanol, pH 9.2, 50 μl water containing dATP, dGTP, dCTP, and dTTP (10 nmol each), and 50 μl water containing 1 unit of E. coli DNA polymerase I (EC 2.7.7.7.) are added and the incubation is further carried out at 37°C, the appearance of acid-soluble ^{32}P is only very slightly accelerated, whereas most of the ^{3}H is quickly set free as a mixture behaving like di- and trinucleotides [3].

That endonuclease VI hydrolyzes a phosphoester bond on the 5' side of the AP site can also be shown using snake venom and spleen phosphodiesterases. Samples of acid-depurinated [^{32}P]DNA reduced with sodium [^{3}H]borohydride (10 μg) in 100 μl SSC/0.02 M $MgCl_2$ and 100 μl endonuclease VI (100 units), in Tris-SH/0.1% bovine serum albumin, are put in each of three tubes, incubated for 30 min at 37°C, then heated at 100°C for 10 min and cooled in ice. The first tube is a control. The second, after the addition of 0.4 unit snake venom phosphodiesterase (EC 3.1.4.1.) in 100 μl SSC/0.02 M $MgCl_2$, is incubated 30 min at 37°C. The third tube, after the addition of 2 units E. coli alkaline phosphatase (EC 3.1.3.1.) in 100 μl 0.3 M glycine pH 8.8 is incubated at 37°C for 30 min; 200 μl 0.3 M succinate/0.01 M $MgCl_2$/0.03 M β-mercaptoethanol, pH 6.0, and 2 units spleen phosphodiesterase (EC 3.1.4.18) in 100 μl water are used to complete the mixture before another 30 min incubation at 37°C. Aliquots are taken from all tubes to measure the acid-soluble radioactivity and for size distribution analysis on DEAE-Sephadex at pH 7.6 in the presence of urea. Snake venom phosphodiesterase, which hydrolyzes polynucleotides from 3'-OH ends in the 3'→5' direction, releases ^{32}P in mononucleotides, but very little ^{3}H. Spleen phosphodiesterase, which hydrolyzes polynucleotides from 5'-OH ends in the 5'→3' direction, releases ^{3}H faster than ^{32}P in molecules behaving chromatographically as mononucleotides. It thus appears that denaturation of DNA containing reduced AP sites treated with endonuclease VI produces polynucleotides carrying the reduced AP sites at their 5' ends; the endonuclease VI cleavage thus takes place on the 5' side of the AP site [3].

2. The Hydrolyzed Bond: Immediate Neighbor of AP Site

An elegant demonstration has been given by Clements et al. [23] with the AP endodeoxyribonuclease of Hemophilus influenzae, which has the same properties as E. coli endonuclease VI.

A copolymer poly[d(AT)], in which part of the dTMP is replaced by [5'-^{32}P]dUMP, is successively treated by the E. coli uracil DNA glycosylase to produce AP sites where the base-free deoxyribose is bound to a ^{32}P-labeled phosphate on the 5' side, then with the AP endodeoxyribonuclease. There is no release of Norit-nonadsorbable ^{32}P (as inorganic phosphate);

this release occurs, however, after a treatment with E. coli alkaline phosphatase (EC 3.7.7.7.). This experiment shows that the phosphodiester bond hydrolyzed by the H. influenzae AP endodeoxyribonuclease is the immediate neighbor of the AP site resulting from the departure of uracil. Since the H. influenzae enzyme contains a 3'-phosphatase activity, and since a treatment with a nonspecific phosphatase is necessary to release inorganic ^{32}P-labeled phosphate, the endonucleolytic cleavage cannot yield breaks limited by 3'-phosphate and 5'-OH; it rather produces breaks limited by 3'-OH and 5'-phosphate, as does the E. coli endonuclease VI (Sec. IV.D).

F. Miscellaneous Properties

1. Molecular Weight, Structure, and Amino Acid Composition

The molecular weight of the native enzyme is estimated by Sephadex G-75 column chromatography [2,5] or by sedimentation analysis [5]. Whereas Verly and Rassart [2] reported a value of 32,000 daltons for endonuclease VI, Weiss [5] found 27,400 (±9%) daltons for exonuclease III. The two estimations are not believed to be significantly different. Verly and Rassart [2] and Weiss [5], using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, observed that the molecular weight of the denatured enzyme was not different from that of the native enzyme. E. coli endonuclease VI/exonuclease III thus appears to be a monomeric protein of about 30,000 daltons.

p-Chloromercuribenzoate inhibits the exonuclease and phosphatase activities of exonuclease III [20]. Reduced glutathione or β-mercaptoethanol is used to protect endonuclease VI/exonuclease III during purification, storage, and assay.

The amino acid composition, in molar percent, of purified endonuclease VI (ignoring Cys, Met, and Trp) is: Ala, 7.6; Arg, 3.5; Asp, 11.3; Glu, 15.1; Gly, 16.9; His, 1.8; Ile, 2.8; Leu, 5.4; Lys, 5.6; Phe, 3.9; Pro, 3.5; Ser, 13.0; Thr, 4.6; Tyr, 0.9; and Val, 4.4 [2].

2. Optimum pH

Solutions of 0.05 M Tris/0.01 M $MgCl_2$ are adjusted with HCl to pH values ranging from 6.5 to 9.5. Alkylated-depurinated [^3H]DNA is dialyzed against each of these buffers, whereas aliquots of a concentrated solution of endonuclease VI are diluted 100-fold with each buffer, to which 0.1% bovine serum albumin has been added. Equal volumes of substrate and enzyme solutions of the same pH are mixed and incubated at 37°C, and the appearance of acid-soluble radioactivity is followed as a function of time. The optimum pH found in these conditions for E. coli endonuclease VI is 8.0-8.5 [3]. Richardson et al. [20] observe that the optimum pH of exonuclease III depends on the buffer and the divalent cation concentration.

3. The Divalent Cations

The addition of 10 mM EDTA to the reaction medium inactivates endonuclease VI. The enzyme, extensively dialyzed against SSC/0.1 mM β-mercaptoethanol, when tested in this buffer, is still active without the addition of divalent cations. By contrast, endonuclease VI, extensively dialyzed against Tris-SH/10 mM EDTA, then against Tris-SH alone, is completely inactive when tested on alkylated-depurinated [^{3}H]DNA in the latter medium. The addition of $MgCl_2$ restores the activity, the optimum concentration being 0.02 M; Mn^{2+} or Ca^{2+} cannot replace Mg^{2+} [3]. These results suggest that Mg^{2+} is a necessary cofactor of endonuclease VI and that it is rather firmly bound to the enzyme.

EDTA also inactivates exonuclease III; it is interesting to note that the exonuclease activity can be restored not only with Mg^{2+}, but also with Mn^{2+} [20]. The different effect of Mn^{2+} on the AP endodeoxyribonuclease and the exonuclease activities should be studied. It is reported that Zn^{2+} inhibits the exonuclease and phosphatase activities of exonuclease III [20].

By contrast with endonuclease VI, the minor E. coli AP endodeoxyribonuclease, endonuclease IV, does not need Mg^{2+} to be active; it works at a normal rate in 10 mM EDTA [6,8]. EDTA can thus be used to differentiate between the two enzymes.

4. Thermoresistance

Aliquots (150 μl) of endonuclease VI, in Tris-SH containing 0.1% bovine serum albumin in polypropylene test tubes, are heated for various times at 40°C. The AP endodeoxyribonuclease activity measured on alkylated-depurinated [^{3}H]DNA and the exonuclease activity measured on sonicated [^{3}H]DNA disappear with the same half-life of 7 min [3].

Endonuclease IV, the E. coli minor AP endodeoxyribonuclease, resists heating at 45°C [6,8]. Heating and EDTA treatment are thus different means to differentiate endonuclease IV from endonuclease VI.

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