Endonuclease II of *Escherichia coli* is Exonuclease III*

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Exonuclease III, a phosphatase-exonuclease specific forbibical DNA, was purified to >98% homogeneity from *Escherichia coli* K-12. Of equal purity in the preparation was endonuclease II, an activity specific for DNA that has been partially depurinated by treatment with methyl methanesulfonate. The two activities, which could not be separated by electrophoresis, by sedimentation, or by gel filtration, were associated with a single monomeric protein of 28,000 daltons. To explain how a relatively small protein could have such diverse activities, it is proposed that one site on the enzyme can recognize interstrand spaces created either by depurination or by spontaneous terminal unwinding of a DNA duplex.

**EXPERIMENTAL PROCEDURE**

**Materials**

Endonuclease III—Endonuclease III was purified from 1 kg of *Escherichia coli* K-12 (ATCC 19498) log phase cells supplied by the Grain Processing Corp. The purification procedure was that of Richardson and Kornberg (1), with the following minor modifications. (a) *E. coli* K-12 was used rather than *E. coli* B; (b) the cells were disrupted with a Manton-Gaulin homogenizer (9); and (c) enough streptomycin was added to precipitate 50% of the enzyme, rather than 15%, resulting in a correspondingly lower yield (6% rather than 15%). It is not known to what extent these modifications may have influenced the purity of the final product.

Protein Standards—The molecular parameters of exonuclease III were measured against protein standards with known molecular weights, Stokes radii, or sedimentation coefficients. The physical values used were those cited in the following references: bovine serum albumin (11, 12), human γ-globulin (10, 11), *E. coli* alkaline phosphatase (12), ovalbumin (10, 11), bovine chymotrypsinogen A (10, 11), bovine pancreatic DNase I (13), and horse heart cytochrome c (14). In some cases, Stokes radii were calculated from known diffusion coefficients (15). Pancreatic DNase and alkaline phosphatase were purchased from Worthington Biochemical Corp. and others from Schwarz/Mann.

**Methods**

Enzyme Assays—Exonuclease activity was measured by the release of acid-soluble radioactive material from 32P- or 3H-labeled sonicated bacteriophage T7 DNA (1, 16). The assay for DNA-3'-phosphatase measured the release of Norit-nonsorbable 32P, from uniformly labeled DNA containing 3'-phosphoryl termini (1, 16). The endonuclease II assay measured the release of radiolabeled DNA fragments from bacteriophage T4 DNA that had been entrapped in beads of a polyacrylamide gel and treated with methyl methanesulfonate (3, 7). Enzyme units were as previously defined (7, 16).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Protein samples were reduced and denatured by heating to 100°C in the presence of 2-mercaptoethanol and sodium dodecyl sulfate and then subjected to disc electrophoresis in a 10% gel containing 0.1% sodium dodecyl sulfate (17). In attempts to recover enzyme activity from the gels, a gentler denaturation procedure (11) and a photopolymerized stacking gel (18) were used with the same results. The gels were
stained overnight with Coomassie blue (11), the position of the tracking dye bromphenol blue was measured, and the gels were then destained by transverse electrophoresis in a Canalco gel destainer. Each gel was scanned at 550 nm with a Gilford recording spectrophotometer equipped with a linear transport device.

**Polycrylamide Gel Electrophoresis of Native Protein**—The gel dimensions were those of Laemmli (17) and the formulations were those of Jovin et al. (18). The 7.5% polycrylamide separating gel contained Tris HCl buffer having a pH of 6.9 at 25°C and a pH of 9.5 at 4°C, the operating temperature. The current was 3 mA/tube. Staining, destaining, and spectrophotometric scanning were performed as for the sodium dodecyl sulfate gels.

**Sedimentation Analysis of Proteins**—Proteins in a total volume of 0.1 ml were layered on a linear sucrose gradient (4.8 ml) containing 50 mM potassium phosphate (pH 7.5), 0.1 mM dithiothreitol, and 5 to 20% sucrose (Harshaw). Centrifugation was performed in a Beckman SW50L rotor at 48,000 rpm for 48 hours at 4°C. Each tube was punctured at the bottom, and 38 eight-drop fractions were collected. Ovalbumin and chymotrypsinogen were located by their absorbances at 215 nm at the bottom, and 38 eight-drop fractions were collected. Ovalbumin and chymotrypsinogen were located by their absorbances at 215 nm (19), and exonuclease III was detected by enzymatic assay.

**Gel Filtration**—Sephadex G-100, fine mesh, was equilibrated with a solution containing 0.2 M KCl, 0.02 M potassium phosphate buffer (pH 7.5), 10 mM dithiothreitol, and 10% glycerol. The column (0.8 x 60 cm) was operated by downward flow at a pressure head of 10 cm. Protein mixtures were applied in a total volume of 0.2 ml. For the determination of Stokes radius by the method of Ackers (14), the following amounts were used: exonuclease III, 617 μg; pancreatic DNase, 10 μg; other proteins, 0.2 mg. Fractions of 0.53 ml were collected. Pancreatic DNase was detected by enzyme assay (20), exonuclease III by exonuclease activity, cytochrome c by its absorbance at 410 nm, and other proteins by their absorbances at 280 nm.

**Protein Determinations**—Unless otherwise stated, protein concentration was determined by the method of Lowry et al. (21) with bovine serum albumin as a standard.

### RESULTS

**Purity of Enzyme Preparation**—Exonuclease III was purified by the method of Richardson and Kornberg, with only minor modifications (see "Materials"). The enzyme was prepared from *Escherichia coli* K-12 rather than the traditional B strain because the former had been used as the source for endonuclease II (3-6) and had served in the genetic studies on the two enzymes (7). Results of intermediate purification steps were similar to those of Richardson and Kornberg (1) and therefore are not repeated here, but the characteristics of the purified enzyme preparation are shown in Table I. Purification of exonuclease III activity resulted in an equal purification of endonuclease II and exonuclease III, as previously noted (7), again suggesting the enzyme activities are physically associated.

A sample of the purified enzyme was examined by sodium dodecyl sulfate-polycrylamide gel electrophoresis under denaturing conditions (Fig. 1). Greater than 98% of the protein migrated in a single band. Using the renaturation procedure of Weber and Kitter (99), I was unable to demonstrate directly that this protein is the enzyme; exonuclease III activity could not be recovered from a sodium dodecyl sulfate solution either before or after electrophoresis. Experiments described below, however, will demonstrate that the enzymatic activities are associated with this homogeneous protein, the yield of which was about 2 mg/kg (wet weight) of cells.

**Alternative Purification Schemes**—The standard purification procedure for endonuclease II also results in co-purification of an exonuclease (5), but the final preparation is heterogeneous, both physically and enzymatically (5, 23); therefore, it was not used here. Jovin, et al. (9) have devised an alternative purification method for exonuclease III in which the enzyme is purified from a streptomycin precipitate instead of the supernatant used in the standard procedure. A sample of their original preparation, generously provided by Dr. Paul Englund, was subjected to analytical disc gel electrophoresis at pH 9.5. The preparation was heterogeneous; about 50% of the protein migrated in a band with an Rf similar to that of homogeneous exonuclease III, but the rest was distributed in four or five bands of slower mobility. This purification procedure, however, used an autolysis step to degrade the nucleic acids in the streptomycin precipitate. Because accompanying proteolysis might have contributed to the observed heterogeneity, I tried to substitute a polyethylene glycol extraction procedure (24) for the autolysis. The resulting preparation was equally homogeneous, however, and its electrophoretic profile resembled that of the previous preparation. Nevertheless, these latter two purifications resulted in co-purification of endonuclease II and exonuclease III activities in ratios similar to that in Table I. The procedure of Richardson and Kornberg produced the purest protein in the fewest steps, and the resulting purified preparation (Table I) was used in all the experiments described below.

**Inability to Separate Endonuclease II from Exonuclease III**—Samples of the purified enzyme preparation were subjected to polyacrylamide gel electrophoresis at pH 9.5 (Fig. 2), to centrifugation in a sucrose density gradient (Fig. 3), and to gel filtration chromatography (Fig. 4). In each experiment, fractions containing peak endonuclease II activity also contained peak exonuclease and DNA-3'-phosphatase activities.

### Table I

<table>
<thead>
<tr>
<th>Activity measured</th>
<th>Specific activity</th>
<th>Purification factor*</th>
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<tbody>
<tr>
<td>Exonuclease</td>
<td>128,000</td>
<td>1,600</td>
</tr>
<tr>
<td>DNA-3'-phosphatase</td>
<td>30,000</td>
<td>700</td>
</tr>
<tr>
<td>Endonuclease II</td>
<td>96,000</td>
<td>1,600</td>
</tr>
</tbody>
</table>

*The purification factor is the ratio of the specific activity of the purified enzyme to that of the crude cell extract.

*Measurements were affected by the nonlinearity of the assay and by interference from 5'-nucleotidase in the crude extract (16).
FIG. 2. Polyacrylamide gel electrophoresis of purified native exonuclease III. Nondenatured samples (13 μg of protein) were run in parallel on 7.5% polyacrylamide gels. One gel was stained for protein with Coomassie blue and then scanned with a spectrophotometer. The other gel was cut into 1.5-mm slices and soaked at 4°C for 2 days in 0.1 ml of the diluent used for exonuclease III assays. Enzymatic activities were then determined on the eluates; recoveries of the three activities were 19 to 23%.

x, exonuclease; o, endonuclease; o, DNA-3'-phosphatase.

FIG. 3. Sedimentation analysis of purified exonuclease III. A sample containing 3.4 μg of protein was applied to the sucrose gradient. After centrifugation, from 30 to 40% of each enzyme activity was recovered.

x, exonuclease; o, endonuclease II; o, DNA-3'-phosphatase.

FIG. 4. Gel filtration chromatography of purified exonuclease III. A sample containing 0.13 mg of protein was applied to a column of Sephadex G-100, and 60 fractions were collected, totaling 1.06 column volumes. Recoveries of each enzyme activity and of protein were 84 to 95%.

x, exonuclease; o, endonuclease II; o, DNA-3'-phosphatase; o, protein.

Similar results were obtained in the last step (Sephadex G-100) of the modified procedure of Jovin et al. described above; the elution profiles (not shown) of all three activities coincided and resembled those in Fig. 4. The results (Figs. 2 to 4) suggested that the enzymes are identical. Furthermore, in the electrophoresis and gel filtration experiments (Figs. 2 and 4), sufficiently large samples were applied to permit subsequent detection of protein in the fractions. In each case, the protein traveled in a single peak corresponding in position to that of the enzymatic activities; therefore, this apparently homogeneous protein (also seen in Fig. 1) is almost certainly the single protein that catalyzes both exonuclease III and endonuclease II reactions.

At this point, it is necessary to discuss the nature and specificity of the enzyme assays and to provide evidence that the assayed enzymes are indeed exonuclease III and endonuclease II. The exonuclease assay measured the release of acid-soluble material (mononucleotides) from radioactively labeled sonicated T7 bacteriophage DNA, whereas DNA-3'-phosphatase was measured through the release of Norit-nonadsorbable radioactivity (P) from 32P-labeled DNA that had acquired 3'-phosphoryl termini by partial digestion with micrococcal nuclease (1, 2). This latter activity is a unique identifying characteristic of exonuclease III; it is co-purified with the exonuclease (2), has a similar heat inactivation curve (2), and is affected by mutations altering exonuclease III activity (16). We may conclude that the exonuclease measured in Table I and in Figs. 2 to 4 is indeed exonuclease III because it was associated with DNA-3'-phosphatase activity and because it was purified by the scheme first used to isolate exonuclease III.

The endonuclease II assay measured the release of radioactively labeled fragments from methyl methanesulfonate-treated T4 phage DNA entrapped in a polyacrylamide gel (3, 7). Specificity was enhanced by the use of T4 DNA that, because of its glucose content, is relatively resistant to diges-
tion by exonuclease III activity (25). It was demonstrated previously (7) that the endonuclease associated with exonuclease III is endonuclease II; a standard purification procedure for endonuclease II yielded an altered enzyme from a mutant having an altered exonuclease III activity. The identity of endonuclease II in the peak fractions of Figs. 2 to 4 was confirmed by finding that it was an endonuclease specific for methyl methanesulfonate-treated DNA. The enzyme was 15 to 20 times more active on methyl methanesulfonate-treated than on untreated DNA-gel preparations, and of the radioactive material released from the former substrate, 86 to 93% was acid-insoluble. This specificity for methyl methanesulfonate-treated DNA was even greater than it first appeared because even the small amount of apparent endonucleaseolytic activity on untreated DNA was largely exonucleaseolytic; of the material released from the untreated gel substrate, 55 to 100% was acid-soluble. These collective results confirm that each of the three assays measures a separate activity of a common protein.

Molecular Weight and Quaternary Structure—After the enzyme preparation had been heated in the presence of sodium dodecyl sulfate and 2-mercaptoethanol, gel electrophoresis in the presence of sodium dodecyl sulfate revealed a single protein band (Fig. 1). This procedure should have dissociated the subunits of an oligomeric protein and fractionated them according to their molecular weights (11). Because only one major band was seen, the protein must contain either a single polypeptide or else several polypeptides of identical molecular weight. It was possible, for example, that the protein comprised two subunits of equal molecular weight, one having exonuclease III activity and the other having endonuclease II activity. If so, then the molecular weight of the native protein should be twice that of the denatured protein. To examine this possibility, the experiments of Figs. 1, 2, and 4 were repeated with proteins of known molecular weight as internal standards. The molecular weight of the sodium dodecyl sulfate-denatured enzyme protein (Fig. 5) was found to be 28,000 under denaturing conditions adequate to dissociate the subunits of two other oligomeric proteins, namely, γ-globulin and alkaline phosphatase. Properties of the native enzyme were also compatible with this molecular weight. During Sephadex gel filtration (Fig. 6) its elution volume was between that of pancreatic DNase (M, 30,700) and chymotrypsinogen A (M, 25,700). Similarly, its sedimentation rate (Table II) was between that of ovalbumin (M, 43,000) and chymotrypsinogen A (M, 25,700). A molecular weight of 27,400 (+9%) was calculated for the native enzyme from the sedimentation and gel filtration data (Table II). Thus, the molecular weights of the denatured protein and of the native enzyme agree, indicating that the enzyme contains a single polypeptide chain. Endonuclease II and exonuclease III are therefore identical.

DISCUSSION

In the studies above, exonuclease III was purified to near homogeneity. Endonuclease II activity was co-purified and subsequently could not be separated from exonuclease III by electrophoresis, centrifugation, or gel filtration. The molecular weights of the native and denatured proteins agreed, indicating that a single polypeptide catalyzes both types of reactions. An obvious question is that if these enzymes are identical, why has this fact escaped detection for so long? First of all, there was no readily apparent reason why enzymes with such diverse activities should be identical; thus, it was purely by accident

![FIG. 5. Determination of molecular weight by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (11, 17). The proteins were reduced and denatured by heating in the presence of 2-mercaptoethanol and sodium dodecyl sulfate, after which they were subjected to electrophoresis in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Mobilities were measured relative to the tracking dye bromphenol blue. BSA, bovine serum albumin; γ-H and γ-L, heavy and light chains, respectively, of γ-globulin; BAP, bacterial (Escherichia coli) alkaline phosphatase half-mer; OVA, ovalbumin; EXO III, exonuclease III; CT, chymotrypsinogen A.](http://www.jbc.org/)

![FIG. 6. Determination of molecular radius by gel filtration (14). This calibration curve was constructed by plotting known values for the Stokes radius of various proteins against experimentally determined values for a/r; the ratio of Stokes radius to the mean effective pore radius of the Sephadex G-100 gel. Values for a/r were calculated (14) from the relative elution volumes of the proteins. Numbers in parentheses are molecular weights. CYT c, cytochrome c; CT, chymotrypsinogen A; DNase I, pancreatic DNase; EXO III, exonuclease III; OVA, ovalbumin.](http://www.jbc.org/)
exonuclease III in its specificity for duplex DNA and in its poor ability to degrade T4 DNA, it was thought not to be exonuclease III because it was not as sensitive to inhibition by Zn$^{2+}$ as exonuclease III had been reported to be. Hadi et al. (5) subjected a purified but physically heterogeneous preparation of endonuclease II to isoelectric focusing and found that (a) the endonuclease activity was resolved in a single symmetrical peak corresponding to the major peak of endonuclease activity, and (b) the rest of the endonuclease activity was located in multiple overlapping peaks devoid of measurable exonuclease activity. Their first finding is compatible with the results in this paper, and the second may be explained by a later discovery (23) that their enzyme preparation contained more than one endonuclease. In addition, the degree of physical heterogeneity they observed suggests partial proteolysis or denaturation of the enzyme(s) in their preparation.

Verly and Paquette (27) have identified an endonuclease of E. coli specific for apurinic sites in DNA. Its identity with endonuclease II was questioned (27, 28) because of the reported ability of the latter enzyme to act on alkylated as well as depurinated sites in DNA (4). Subsequently preparations of endonuclease II were found to contain an activity that converts alkylated bases to apurinic sites (29) and possibly a second endonuclease (23). The enzyme of Verly and Paquette, a monomeric protein of about 39,000 daltons, was purified to near homogeneity (30), and it was postulated as the major endonuclease in endonuclease II preparations. This identity has not been established with certainty, and their homogeneous enzyme was not tested for exonuclease III activity.

The molecular weight of exonuclease III, 28,000, is surprisingly low for a multifunctional enzyme of DNA metabolism. For example, DNA polymerase I of E. coli, an enzyme catalyzing both polymerase and 5'→3' exonuclease reactions, has a molecular weight of 109,000 (9). The low molecular weight of exonuclease III suggested that, unlike DNA polymerase I, a single active site catalyzes all of its enzymatic reactions. If so, how does an apurinic site resemble a chain terminus? We already know that exonuclease III recognizes the ends of DNA in a manner different from that of many other enzymes. Most polymerases and exonucleases recognize substrates unique to ends, e.g. 3'-hydroxyl groups, but exonuclease III will degrade DNA chains from their 3'-ends whether they are terminated by 3'-hydroxyl or by 3'-phosphoryl end groups. The enzyme must recognize, therefore, some other topological feature of the ends of a duplex, a feature that might be similar to an apurinic site. I propose that the exonuclease recognizes a space created by the unwinding of a terminal base pair that occurs because of reduced base-stacking forces at the ends of duplexes. Then, in each of its three characteristic substrates, the enzyme may recognize a space created by displacement or removal of a base; the exonuclease substrate has spaces left by displaced (unwound) terminal bases, the endonuclease substrate has spaces at apurinic sites, and the DNA phosphatase substrate has spaces corresponding to missing 3'-terminal nucleoside residues. The model is presented in more detail in Fig. 7.

The model leads to a number of useful predictions, some of which have already been fulfilled. First of all, exonuclease III should remove a 3'-terminal nucleotide even if it is mispaired, and it does (31). Secondly, when attacking partially depurinated DNA, it should create depurinated deoxyribose 5'.monophosphate (pdR) end groups. As yet, there is only indirect evidence for this latter conclusion based on the inability to label these termini with the help of phosphatase and polynucleotide kinase (5). Thirdly, the exonuclease should be less active at single strand breaks than at the ends of duplexes because there are stacking forces between the base pairs bracketing a nick (32). Accordingly, Donelson and Wu (33) found that under conditions where exonuclease III removed no

<table>
<thead>
<tr>
<th>Parameter and method*</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Stokes radius, α</td>
<td>2.29 nm</td>
</tr>
<tr>
<td>Gel filtration (14)</td>
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<tr>
<td>Diffusion coefficient, D</td>
<td>(9.37 \times 10^{-9} \text{cm}^2\text{s}^{-1})</td>
</tr>
<tr>
<td>Sedimentation coefficient, (s_{20,\text{w}}) (26)</td>
<td>3.02 S</td>
</tr>
<tr>
<td>Ovalbumin (3.55 S) as standard</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsinogen A (2.58 S) as standard</td>
<td>2.82 S</td>
</tr>
<tr>
<td>Average</td>
<td>2.92 S</td>
</tr>
<tr>
<td>Fractional ratio, (f_0/f_2)</td>
<td></td>
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<tr>
<td>Sedimentation, diffusion coefficients</td>
<td>1.15</td>
</tr>
<tr>
<td>Molecular weight</td>
<td></td>
</tr>
<tr>
<td>Sodium dodecyl sulfate gel electrophoresis (11, 17)</td>
<td>27,400</td>
</tr>
<tr>
<td>Average</td>
<td>28,500</td>
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</table>

*Methods of calculation are described in references cited or in Ref. 15. Some of the data is from Figs. 5 and 6.

**The partial specific volume \(\bar{v}\) was approximated with an assumed value of 0.725. Because the value for most proteins is between 0.70 and 0.75 (10, 26), the estimate introduced a probable uncertainty of ±8% into the molecular weight of the native protein.

![Fig. 7. Model for how endonuclease II and exonuclease III activities might share a single catalytic site. The enzyme (shaded area) is shown attacking DNA duplexes, cleaving phosphoester bonds at the arrows. The enzyme (A) has three domains: one, the active site, recognizes and cleaves phosphoesters; a second recognizes duplex structure, and for schematic purposes only, it is represented as recognizing a deoxyribonucleoside residue on the opposite strand; the third site recognizes a space. The space may be (B) that which was previously occupied by a base at an apurinic site, (C) that beyond the end of a strand, or (D) that created by displacement or unwinding of a 3'-terminal base that is not as constrained by stacking forces as a nonterminal base.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Recognition sites</th>
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<tr>
<td>P-ester (active site)</td>
<td></td>
</tr>
<tr>
<td>space</td>
<td></td>
</tr>
<tr>
<td>duplex</td>
<td></td>
</tr>
<tr>
<td>3' - Phosphatase</td>
<td></td>
</tr>
<tr>
<td>(A) Enzyme:</td>
<td></td>
</tr>
<tr>
<td>(B) Endonuclease</td>
<td></td>
</tr>
<tr>
<td>(C) 3'-Phosphatase</td>
<td></td>
</tr>
<tr>
<td>(D) Exonuclease</td>
<td></td>
</tr>
</tbody>
</table>
more than six terminal nucleotides from each chain, no nucleotides were released from nicks. A fourth prediction, yet to be tested, is of considerable biological significance: the enzyme might act as an endonuclease on the unpaired or mispaired regions of heteroduplexes, thus cleaving deletion or substitution loops or acting in the excision repair of mismatched bases.

Exonuclease III (endonuclease II) is believed to function in the excision repair of lesions in DNA caused by chemically induced or spontaneous depurination (7). Therefore, teleologists will point out that the cleavage site suggested in the model (Fig. 7) is on the “wrong” side of the depurinated nucleotide because it leaves it at a 5’ terminus, unable to be removed directly by the 3’ → 5’ action of exonuclease III. The model infers, however, that the endonuclease and exonuclease activities may be related through a common mechanism of action rather than a common purpose. That this mechanism may be widespread is suggested by the presence of a similar exonuclease III-endonuclease II enzyme in a distantly related organism, *H. influenzae* (8).

**REFERENCES**

Endonuclease II of Escherichia coli is exonuclease III.
B Weiss


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