Endonuclease II of *Escherichia coli* is Exonuclease III*

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Exonuclease III, a phosphatase-exonuclease specific for bibehial 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.

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\[ \text{EXPERIMENTAL PROCEDURE} \]

**Materials**

Exonuclease III—Exonuclease III was purified from 1 kg of *Escherichia coli* K-12 (ATCC 14948) $\%$ 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 (10, 11), human $\gamma$-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 (4). 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 III activity was measured by the release of acid-soluble radioactive material from $^{32}$P- or $^3$H-labeled sonicated bacteriophage T7 DNA (1, 16). The assay for DNA-3'-phosphatase measured the release of Norit-nonadsorbable $^3$P, from uniformly labeled DNA containing 3'-phosphoryl termini (1, 16). The endonuclease II assay measured the release of radionabeled 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° 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 gentle 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 bromophenol 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.

Polyacrylamide 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% polyacrylamide separating gel contained Tris-HCl buffer having a pH of 6.9 at 25° and a pH of 0.5 at 4°, 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 SW50.1 rotor at 48,000 rpm for 48 hours at 4°. 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 ovalbumin 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 KC1, 0.02 M potassium phosphate buffer (pH 7.5), 10-4 M 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 purified enzyme preparation are shown in Table I. The procedure of Richardson and Kornberg 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 III and DNA-3'-phosphatase activities.

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![Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified exonuclease III. A sample (3 µg of protein) was heated with 2-mercaptoethanol and sodium dodecyl sulfate and subjected to electrophoresis in the presence of sodium dodecyl sulfate. Sample preparation, electrophoresis, staining, and spectrophotometric scanning were described under "Materials." Rf is mobility relative to that of bromphenol blue, the tracking dye that was eluted during the staining procedure.](http://www.jbc.org/)
E. coli Endonuclease II-Exonuclease III

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° for 2 days in 0.1 ml of the diluent used for exonuclease III assays (1). Enzymatic activities were then determined on the eluates; recoveries of the three activities were 19 to 23%. x, exonuclease; O, endonuclease II; 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<sub>i</sub>) 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 endonuclease activity on untreated DNA was largely exonuclease; 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 (M₂ = 30,700) and chymotrypsinogen A (M₂ = 25,700). Similarly, its sedimentation rate (Table II) was between that of ovalbumin (M₂ = 43,000) and chymotrypsinogen A (M₂ = 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 that these enzymatic activities were found to be genetically and physically related, both in *Escherichia coli* (7) and in *Hemophilus influenzae* (8). Secondly, the respective enzyme assays are specific enough to avoid significant interference by, and hence subsequent detection of, a second enzymatic activity; as discussed under "Results," the nondepurinated DNA used in the exonuclease assay is a poor substrate for the endonuclease activity, and the glucosylated T4 DNA used in the standard endonuclease II assay is a poor substrate for the exonuclease.

Actually, an association between these activities had been previously discovered but misinterpreted. Friedberg and Goldthwait (3) and Hadi et al. (5) discovered exonuclease activity in highly purified preparations of endonuclease II, but they considered it a contaminant. Although it resembled
Table II

Physical parameters of exonuclease III (endonuclease II)

<table>
<thead>
<tr>
<th>Parameter and method</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stokes radius, a</td>
<td>2.29 nm</td>
</tr>
<tr>
<td>Diffusion coefficient, D</td>
<td>$9.37 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$</td>
</tr>
<tr>
<td>Sedimentation coefficient, $s_{20,w}$</td>
<td>28,500</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>28,000</td>
</tr>
</tbody>
</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 ($\rho$) 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 $\pm$9% into the molecular weight of the native protein.

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 exonuclease 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 suggested 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 depurinized sites in DNA (4). Subsequent 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 terminator? 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'-phosphodiester end groups. The enzyme must recognize, therefore, some other topographical 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 at apurinic sites, and the DNA-3'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
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.

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