Endonuclease II, apurinic acid endonuclease, and exonuclease III
(chemical carcinogens/DNA repair/Escherichia coli endonucleases and exonucleases)

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ABSTRACT An endonuclease of Escherichia coli active on a DNA treated with methylmethane sulfonate has been separated from an endonuclease active on depurinated sites. The former enzyme is designated here as endonuclease II, while the latter enzyme is designated as apurinic acid endonuclease. Endonuclease II is also active on DNA treated with methylnitrosourea, 7-bromomethyl-12-methylbenz[a]anthracene, and \( \gamma \)-irradiation. A third fraction which contains activities for both depurinated and alkylated sites needs further study. Endonuclease II, molecular weight 33,000, has been purified 12,500-fold and does not have exonuclease III activity. Apurinic acid endonuclease, molecular weight 31,500, has been purified 11,000-fold and does not have exonuclease III activity. Exonuclease III, molecular weight 28,000, has been purified 2300-fold and does not have endonucleolytic activity at depurinated reduced sites or at alkylated sites in DNA. Therefore, these are three separate proteins. Exonuclease III can produce, presumably by its exonucleolytic activity, double-strand breaks in heavily alkylated DNA under conditions where it does not make single-strand endonucleolytic breaks at either depurinated-reduced or alkylated sites.

The first purpose of this paper is to define endonuclease II of Escherichia coli as an activity different from the apurinic acid endonuclease of E. coli. Strauss and Robbins first described an endonucleolytic activity in extracts of Bacillus subtilis that recognized alkylated DNA (1). In this laboratory, an enzyme in extracts of E. coli, active on heavily alkylated DNA, was partially purified, characterized, and designated endonuclease II of E. coli (2, 3). The substrate used for these experiments was DNA that was entrapped in a polyacrylamide gel and then alkylated with methylmethane sulfonate [Me\( \text{SO}_3 \text{OMe} \) (MMS)] at an Me\( \text{SO}_3 \text{OMe} \) to-nucleotide ratio of 6000 to 1. A partially purified preparation of endonuclease II was also found to have an endonucleolytic activity on depurinated reduced DNA (4), and this activity was thought to be due to the same enzyme that was active on Me\( \text{SO}_3 \text{OMe} \) treated DNA. However, Verly et al. (5, 6), using the purification procedure originally described in this laboratory, obtained an enzyme that was active in depurinated DNA but not on alkylated DNA. Subsequently, we succeeded in separating the activity on depurinated sites in DNA from the activity on Me\( \text{SO}_3 \text{OMe} \) treated DNA (7, 8). The former we designate as the apurinic acid endonuclease of E. coli, while the latter we designate as endonuclease II of E. coli. Endonuclease II of E. coli is also active on DNA treated with methylnitrosourea, 7-bromomethyl-12-methylbenz[a]anthracene, and \( \gamma \)-irradiation (7–11).

The second purpose of this paper is to demonstrate that endonuclease II, the apurinic acid endonuclease, and exonuclease III are separate proteins. Originally, Yajko and Weiss (12) demonstrated that a number of E. coli mutants deficient in exonuclease III were also deficient in "endonuclease II" and vice versa. The "endonuclease II" activity was measured with heavily alkylated DNA in acrylamide gel (2), as noted above.

They found that the two activities copurified and a thermosensitive mutant of one activity was thermosensitive for the other activity. Furthermore, in a revertant of one of the mutants, the levels of both activities increased. This evidence suggested that the two enzyme activities were due to the same protein. Weiss has recently purified a protein of molecular weight 28,000 to homogeneity (13) and showed that it has the activity expected of exonuclease III and also has an activity on the heavily alkylated DNA entrapped in polyacrylamide gel. He has concluded that endonuclease II of E. coli is exonuclease III. However, we have shown that the three enzymes, endonuclease II, exonuclease III, and apurinic acid endonuclease, are separate proteins. Furthermore, we have noted that a purified preparation of exonuclease III can make double-strand breaks in heavily alkylated DNA under conditions where it is unable to make single-strand breaks at alkylated or depurinated sites.

METHODS

Assays. The assays for the endonucleases involved either DNA immobilized in acrylamide gel or DNA examined on sucrose gradients. \[^3^H\]Thymidine-labeled T4 DNA was prepared as described (2). Preparations of DNA had specific activities ranging from approximately 1 to 5 × 10⁶ cpm/nmol. Endonuclease assays, using either the DNA-gel or sucrose gradients, and the exonuclease assay are described in the legends to Figs. 1 and 2.

Enzymes. The E. coli strain used for small-scale enzyme purification was AB 1157. This was grown in a fermentor to mid-logarithmic phase in a modified EM-9 medium (16) supplemented with L-leucine, L-proline, L-histidine, L-threonine, and L-arginine, each at 20 \( \mu \)g/ml. For the initial fractionation, see legend of Fig. 1.

For a large-scale preparation of the apurinic acid endonuclease and endonuclease II, E. coli JC4583 (endo I, his- \( ^{-} \), F\( ^{-} \), gal\( ^{-} \), Sm\( ^{-} \), B1\( ^{-} \)) was grown at the New England Enzyme Center, and 800 g were used. The purification steps will be described in future publications. Although each purified endonuclease preparation, when examined by sodium dodecyl sulfate (NaDodSO₄)-gel electrophoresis, showed only a single band, it could not be concluded unequivocally that the preparations were homogeneous because large amounts of enzyme were not available to look for small amounts of contaminant protein.

Exonuclease III was purified from 200 g of E. coli (JC 4583) by a modification of the procedure of Richardson and Kornberg (15), which included ammonium sulfate fractionation and DEAE-cellulose, phosphocellulose, Sephadex G-100, and hydroxyapatite column chromatography. The enzyme purification was 2900-fold when the 5' phosphatase activity in fraction III due to exonuclease III was used for the calculation. The preparation was not homogeneous by NaDodSO₄ gel electrophoresis. Dr. C. C. Richardson very kindly provided a sample of his purified exonuclease III for comparison with this preparation.
The apurinic acid endonuclease can be separated from endonuclease II by DEAE-cellulose chromatography (Fig. 1). When fraction III, a 45–70% ammonium sulfate precipitate, was applied to a DEAE-cellulose column, the apurinic acid endonuclease (peak I) was eluted with 0.1 M NaCl prior to the gradient. This activity was assayed by the gel method using DNA with very few depurinated-reduced sites. This enzyme has been purified 11,000-fold in this laboratory, to a single band on NaDodSO₄ gel electrophoresis. It has a molecular weight by gel filtration of 31,500. Verly and Rassart have also purified this enzyme 9450-fold to homogeneity (17) and have found a molecular weight of 32,000 by gel filtration and 33,000 by NaDodSO₄ gel electrophoresis.

Endonuclease II, defined as the enzyme that recognizes DNA treated with MMs, methylthiorosourea, 7-bromomethyl-12-methylbenz[a]anthracene, or γ-irradiation, is eluted with 0.25 M NaCl as peak III (Fig. 1). It is this fraction and not the apurinic acid endonuclease which recognizes the specific damage (other than apurinic or apyrimidinic sites) in these substrates (7, 8). Endonuclease II has been purified in this laboratory over 12,500-fold to a single band on NaDodSO₄ gel electrophoresis. Its molecular weight by gel filtration is 33,000 and by NaDodSO₄ gel is 34,500.

Peak II (Fig. 1) contains both the apurinic acid endonuclease activity and the endonuclease II activity. The molecular weight of the material obtained from the DEAE-cellulose column was approximately 58,000. The nature of this material is not clear. A control assay, not shown in Fig. 1, involved native DNA entrapped in the gel and incubated in the presence of 8-hydroxyquinoline. There was no release of native DNA from the gel in peaks I and III, but there was some activity overlapping peak II with maximum activity in fraction number 145. Thus,
that weight molecular overlapped, passedapurinic acid endonuclease that activity. The either release represent either exonuclease or endonuclease III. Separation of endonuclease III isomer of T4 DNA was also observed when 10% 1000 fold. Centrifugation was at 30,000 rpm for 3 hr at 20°C. (C) Neutral sucrose gradient fractionation of heavily alkylated DNA. The DNA was alkylated at a molar ratio of 800:1, MeSO_2OMe to DNA nucleotide, as described (2). The reaction mixture contained 23.3 nmol of DNA (4.7 × 10⁵ cpm/nmol) and enzyme as indicated. The sedimentation in the neutral gradient was at 28,000 rpm for 3 hr.

described on DEAE-cellulose is able to separate the major apurinic acid endonuclease activity, peak I, from the major endonuclease II activity, peak III.

Separation of exonuclease III from apurinic acid endonuclease and endonuclease II

Exonuclease III is an enzyme which can be separated from the apurinic acid endonuclease and from endonuclease II. Therefore, it is not the same enzyme as the apurinic acid endonuclease or endonuclease II (13). On DEAE-cellulose chromatography, exonuclease III elutes in an area overlapping the apurinic acid endonuclease in peak I (Fig. 1). A purified preparation of exonuclease III provided by Dr. Richardson was eluted in the same position. It is apparent that the exonuclease III activity does not coincide exactly with the apurinic acid endonuclease. Pₐ release was also observed in the area of peak II, but does not represent either exonuclease III activity or alkaline phosphatase activity. The nature of this activity is unknown.

Exonuclease III could be partially separated from the apurinic acid endonuclease in early stages of purification because of a difference in molecular weights. When a fraction of the apurinic acid endonuclease that had been purified 500-fold was passed through a Sephadex G-100 column, the two activities overlapped, but did not coincide. In this experiment, the molecular weight of the exonuclease III was 25,500, as opposed to that of the apurinic acid endonuclease of 31,500.

Exonuclease III was purified 2500-fold. The evidence that this was exonuclease III was as follows: (i) the major purification steps of the published procedure were followed. (ii) The enzyme preparation possessed a 3' -phosphatase activity, as well as the ability to release acid-soluble fragments from labeled DNA. (iii) The Pₐ released by this exonuclease III preparation was comparable to the Pₐ released from the same substrate by alkaline phosphatase. (iv) The enzyme activity was inhibited 95% by 3 × 10⁻⁵ M ZnCl₂. (v) Dr. C. C. Richardson kindly provided us with a sample of exonuclease III for comparison with our preparation. The ratio of Pₐ release to acid-soluble nucleotide release was the same for the two preparations. (vi) The chromatographic behavior of both preparations on the DEAE-cellulose column was similar. (vii) The molecular weight by gel filtration of the exonuclease III purified in this laboratory was 25,500 to 26,000, while the molecular weight of Dr. Richardson's preparation was 26,000. Therefore, although the preparation purified in this laboratory was not homogeneous, we can conclude that it is exonuclease III by the above criteria.

Exonuclease III purified in this laboratory had no endonuclease activity on either apurinic acid sites or alkylated sites in DNA (Fig. 2A and B). T4 DNA was depurinated and then reduced with NaBH₄ to prevent alkali-catalyzed phosphodiester bond hydrolysis (4). After incubation with or without exonuclease III or the apurinic acid endonuclease, samples were examined in alkaline sucrose gradients. Fig. 2A shows that exonuclease III was unable to recognize depurinated sites that were
recognized by the apurinic acid endonuclease. Likewise, exonuclease III was unable to recognize sites in the DNA due to alkylation with MeSO₂OMe (Fig. 2B). These sites were recognized by endonuclease II.

The apurinic acid endonuclease purified 11,000-fold has negligible exonuclease III activity, as measured by Pᵢ release (Table 1). Thus, the apurinic acid endonuclease activity can be shown not to coincide with the exonuclease III activity on DEAE-cellulose and Sephadex G-100 columns, and the purified endonuclease does not have significant levels of exonuclease III. The low activity observed with alkylated DNA (Table 1) may be due to depurinated sites in this substrate. Endonuclease II activity, isolated from the DEAE-cellulose column, did not have any contaminating exonuclease III activity (Fig. 1, peak III). Also, in the preparation purified 12,500-fold there was no significant exonuclease III 3'-phosphatase activity (Table 1). The low activity observed with depurinated reduced DNA seems to be an intrinsic property of the enzyme.

Exonuclease III, therefore, has no endonuclease activity directed against depurinated or alkylated sites, and as noted above, the purified apurinic acid endonuclease and the endonuclease II preparations have no significant exonuclease III activity.

False-positive assays of endonucleolytic activities by exonuclease III

One basis of the claim that exonuclease III and endonuclease II were the same protein (13) was the use of the DNA-gel assay. The conclusion was that a homogeneous protein with a molecular weight of 28,000 had exonuclease, 3'-phosphatase, and endonuclease activity (13). If DNA is released from the gel by exonucleolytic activity, then an erroneous conclusion could be drawn. To measure the "endonuclease II" activity of the purified protein (13) the method that was originally described in our laboratory was used in which DNA is entrapped in a polyacrylamide gel and then alkylated heavily with MeSO₂OMe (2). We show here that exonuclease III can make double-strand breaks in heavily alkylated DNA (Fig. 2C) under conditions where there is no endonuclease activity (Fig. 2A and B). T₄ DNA was treated at the high MeSO₂OMe to nucleotide ratio and then used as a substrate. After incubation with the enzyme, the DNA was examined in neutral sucrose gradients to look for double-strand breaks (Fig. 2C). It is apparent that double-strand breaks occur with the exonuclease III preparation, which does not make single-strand breaks at either apurinic (Fig. 2A) or alkylated (Fig. 2B) sites. We suspect that the heavily alkylated DNA can undergo chemical depurination, and at some of these depurinated sites, β-elimination with phosphodiester bond hydrolysis occurs. This creates sites for exonucleolytic action. If two sites are near but on opposite strands, exonucleolytic action will result in a double-strand break. Double-strand breaks are required to release DNA from a polyacrylamide gel (14). Therefore, we feel that the endonucleolytic activity observed to be associated with the exonucleolytic activity (13) was actually due to the exonucleolytic action of the purified exonuclease III, which was able to produce double-strand breaks without making single-strand breaks.

A false assay was also observed in this laboratory with depurinated DNA. Exonuclease III, lacking the apurinic acid endonuclease activity (Fig. 2A), was chromatographed on Sephadex G-100 and the fractions were examined both by the Pᵢ release assay for exonuclease III and by the gel assay using DNA with a small number of depurinated sites. Both activities paralleled each other but the endonucleolytic activity on the gel was very low compared to the Pᵢ release and very low compared to the usual release by the apurinic acid endonuclease (see also Table 1). The experiments described in this section indicate that exonuclease III does not recognize apurinic or alkylated sites in DNA, but does give false-positive reactions, especially when heavily alkylated DNA is used.

**DISCUSSION**

The confusion regarding the nomenclature of the phosphodiesterases of *E. coli* that recognize depurinated sites and alkylated sites has been extensive. The term endonuclease II was originally used in this laboratory to define an enzyme active on DNA treated with MeSO₂OMe (2, 3). We then concluded erroneously that the activity on depurinated DNA was due to the same enzyme (4). However, Verly et al. (5, 6, 17) demonstrated that the apurinic acid endonuclease had no activity on alkylated DNA. The two activities have now been separated, and each has been purified to a single band on NaDODSO₄-gel electrophoresis. This is a third fraction, as yet not purified extensively, which contains both activities. We propose that the enzyme that recognizes depurinated sites be called apurinic acid endonuclease and that the enzyme that recognizes alkylated DNA as well as other substrates (8–11) be called endonuclease II.

Endonuclease II has both a phosphodiesterase and an N-glycosidase activity (9, 10). Both of these activities parallel each other during chromatography of fractions that are purified 12,500-fold (Kirtikar, unpublished observations). The suggestion that these are separate enzymes, an N-glycosidase and a phosphodiesterase, that act sequentially (8) cannot be correct since kinetic experiments with alkylated DNA and with depurinated DNA show that the rate of phosphodiester bond breakage with depurinated DNA is far too slow to account for the rate of phosphodiester bond breakage with alkylated DNA (Kirtikar, unpublished observations).

Mutants, isolated by others, have been shown in this laboratory to lack either the apurinic acid endonuclease or endonuclease II. The mutant isolated by Yajko and Weiss, BW 2001 (12), lacks the apurinic acid endonuclease in peak I when grown at 42°, but has a normal level of activity for MeSO₂OMe-treated DNA in peak III (8). A mutant, AB3027, isolated by Howard-Flanders (19), has no endonuclease II activity (peak III), but has
a normal apurinic acid activity in peak I (8). These observations provide further evidence that these are separate proteins coded for by separate genes.

Exonuclease III has been claimed to be endonuclease II (as defined by an activity on heavily alkylated DNA-gel) on the basis of genetic evidence (12), and also on the basis of a homogeneous protein, purified 1600-fold, which showed both activities (13). We have provided the following evidence to prove that exonuclease III is not endonuclease II: (i) the apurinic acid endonuclease has been purified 11,000-fold and has no significant 3'-phosphatase activity characteristic of exonuclease III. Its molecular weight is approximately 31,500. (ii) Endonuclease II has been purified 12,500-fold and also has no significant 3'-phosphatase activity. Its molecular weight is approximately 33,000. (iii) Exonuclease III has been purified 2300-fold and has no endonuclease activity on depurinated or alkylated sites in DNA. Its molecular weight is approximately 26,000. The probable explanation for the conclusion that exonuclease III was the same enzyme as endonuclease II (13) lies in the ability of the exonuclease to make double-strand breaks in heavily alkylated DNA under conditions where the enzyme is unable to make single-strand breaks at depurinated-reduced or alkylated sites. It should be emphasized that results such as those shown in Fig. 1 were obtained with DNA-gel assays in which the DNA was either depurinated sparingly or alkylated lightly and the gels were used for testing immediately after preparation. Purified exonuclease III has little activity on this depurinated gel and no activity on this alkylated gel.

However, these results do not close the issue. Yajko and Weiss claimed that their thermosensitive mutant, BW 2001, was missing both exonuclease III and endonuclease II (12). We have examined the chromatographic pattern of this mutant, grown at elevated temperature, and shown that it is missing both the apurinic acid endonuclease as well as exonuclease III. They also claimed that AB3027 was missing both exonuclease III and endonuclease II. We have observed that the 3'-phosphatase activity of exonuclease III as well as the endonuclease II activity on MeSO2OMe-treated DNA (peak III) are both missing in this mutant but the apurinic acid endonuclease is normal. Furthermore, a deletion mutant, BW 9109, is missing all three enzymes. These data suggest that the genes for endonuclease II, apurinic acid endonuclease, and exonuclease III are all clustered in the region at 38.2 min on the revised map (20), as determined by Weiss for exonuclease III (21). The data also suggest that AB 3027 and BW 2001 are double mutants, but this requires further investigation. There is no evidence to support the hypothesis that these enzymes arise from a larger molecule by proteolytic cleavage.

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