The Ultraviolet Endonuclease of Bacteriophage T4

FURTHER CHARACTERIZATION*

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SUMMARY

The T4 ultraviolet endonuclease was previously shown to produce single strand incisions (nicks) in ultraviolet-irradiated DNA on the 5' side of thymine dimers. The present studies demonstrate that the purified endonuclease creates 3'-OH and 5'-P termini at the sites of nicking. Photoreactivation of ultraviolet-irradiated DNA in vitro results in a loss of endonuclease-sensitive sites, thereby demonstrating directly that dimers are required for substrate sites in DNA. The endonuclease has a molecular weight of approximately 18,000 and attacks ultraviolet-irradiated single-stranded Escherichia coli and M-13 DNA.

The mutant bacteriophage T4v1 is defective in the v gene and is abnormally sensitive to ultraviolet radiation (1). In addition, no excision of thymine dimers is observed following infection of Escherichia coli with this phage (2). The v gene has been shown to be the structural gene for an endonuclease that in vitro specifically introduces single strand breaks (nicks) into ultraviolet-irradiated double-stranded DNA (3-8). Unirradiated DNA is unaffected by incubation with the enzyme (3-8). We and others previously have purified and partially characterized this enzyme. It has been shown that the endonuclease activity has no requirement for divalent cation and is active in 10 mM EDTA. It has a broad pH optimum between 7.0 and 8.0 and is insensitive to inhibition by SH group inhibitors, caffeine, or tRNA (3-8). In the present study we have determined the chemistry of the termini created by endonuclease incision of ultraviolet-irradiated DNA. In addition, we present evidence that pyrimidine dimers are recognized as substrate sites by the enzyme in single-stranded DNA. Finally, a molecular weight for the purified enzyme has been determined.

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acrylamide and 0.26% \( N, N' \)-methylenebisacrylamide in 375 mM Tris-HCl buffer, pH 9.0. Both gels contained 0.1% sodium dodecyl sulfate. Fraction V of the T4 ultraviolet endonuclease (2.5 ml) was lyophilized to dryness and resuspended in 0.1 ml of distilled water, 0.01 ml of 10% sodium dodecyl sulfate, 0.01 ml of glycerol, 0.01 ml of \( \beta \)-mercaptoethanol, 0.01 ml of bromphenol blue, and 0.01 ml of 0.06 ml of Tris-HCl buffer, pH 7.2. Of this, 0.01 ml was layered under the running buffer into a well in the stacking gel. Bovine serum albumin (molecular weight 69,000), ovalbumin (molecular weight 43,000), and cytochrome c (molecular weight 11,700) were used as molecular weight markers. A constant potential difference of 125 volts was applied across the gel until the tracker dye reached the bottom of the gel. The gels were then removed and stained with Coomassie brilliant blue. Destaining was by diffusion at 37°C in 5% methyl alcohol and 7.5% acetic acid. The molecular weight of the T4 ultraviolet endonuclease was determined from a standard curve constructed from the RF of the marker proteins run in different wells of the same gel. The RF values were taken from densitometer tracings of the gels performed with a RFT scanning densitometer (Transidyne General Corporation, Ann Arbor, Mich.).

**Sephadex Gel Filtration**

A column measuring 60.0 x 1.0 cm (diameter) was packed with preswollen Sephadex G-75 and equilibrated in 10 mM Tris-HCl buffer, pH 8.0. Molecular weight markers used were myoglobin (17,000), ovalbumin (43,000), and bovine serum albumin (69,000). The markers, together with blue dextran dye and phenol red dye were applied to the column in a volume of 0.5 ml using a Mariott flask. The flow rate of the column was 6.0 ml per hour. Fractions (0.5 ml) were collected at 4°C until the phenol red marker was through the column. Absorbance at 280, 340, and 410 nm was read in a Unicam SP 500 spectrophotometer. The column was re-equilibrated with 10 mM Tris-HCl buffer, pH 8.0, and 1,000 units of T4 ultraviolet endonuclease (Fraction IV) were applied in a volume of 1.0 ml. Fractions (0.5 ml) again were collected and assayed for endonuclease activity as previously described (4). The molecular weight of the endonuclease was determined from the position of the peak of enzyme activity relative to the peak positions of the protein markers.

**Calculation of Pyrimidine Dimer Content of Ultraviolet-irradiated M-13 DNA**

The fraction of total thymine in ultraviolet-irradiated M-13 [\( ^{3}H \)]DNA in pyrimidine dimers, was measured directly by two-dimensional thin layer chromatography as described previously (17). As an estimate of the distribution of \( T \) and \( C \) in M-13 DNA, values experimentally determined for \( H. influenzae \) DNA (38) were used since the absolute and relative amounts of \( T \) and \( C \) are very similar in these two DNAs (H. influenzae DNA: \( T = 31.0\% \), \( C = 18.5\% \); M-13 DNA: \( T = 35.8\% \), \( C = 19.8\% \)).

If the measured fraction of thymine in pyrimidine dimers is \( A \), then the fraction of total thymine in pyrimidine dimers is \( \frac{T}{\overline{T}} = A \frac{\overline{T}}{\overline{T} + C/2} = \frac{71}{71 + 24/2} = A \times 0.885. \) Since thymine is 35.8% of M-13 DNA:

\[
\begin{align*}
\text{the fraction of total nucleotide in} & = \frac{A \times 0.855}{A \times 0.306} = A \times 0.290. \\
\text{the fraction of total nucleotide in} & = \frac{A \times 0.290}{A \times 0.290} = A \times 1.000 \\
\text{the fraction of total nucleotide in} & = \frac{A \times 1.000}{A \times 0.306} = A \times 3.250 \\
\text{two nucleotides. Therefore the total number of pyrimidine dimers per} & = \frac{A \times 0.422}{3,000 \times \frac{1}{2}} = A \times 0.646. \end{align*}
\]

**Measurement of Endonucleolytic Incisions in Ultraviolet-irradiated DNA**

**Thymine Dimers in DNA Are Substrate Sites**

In an attempt to demonstrate directly that pyrimidine dimers are required to produce substrate sites in ultraviolet-irradiated DNA, the following experiment was carried out. Phage T7 DNA was ultraviolet-irradiated at a fluence of 40 J per m². This DNA was incubated in the presence and absence of *Escherichia coli* photoreactivating enzyme and the thymine dimer content of an aliquot of each was determined. Under the incubation conditions described in the legend to Table I, 60% of the thymine dimers were monomerized.

The rest of the DNA then was incubated with T4 ultraviolet endonuclease and sedimented in alkaline sucrose density gradients. As shown in Table I the reduction in endonuclease-sensitive sites in reasonable agreement with the reduction in thymine dimer content of the DNA after photoreactivation.

**Termini Produced by Endonucleolytic Incision of Ultraviolet-irradiated Double-stranded DNA**

The results of incubating specifically nicked ultraviolet-irradiated T7 DNA with purified *E. coli* DNA polymerase I under DNA polymerizing conditions are shown in Fig. 1. The rate of incorporation of [\( ^{3}H \)]TMP into acid-

**Results**

| Thymine dimer content of DNA | Before photoreactivation | After photoreactivation | Per cent reduction |
|-----------------------------|--------------------------|-------------------------|-------------------|
| Single strand breaks per T7 DNA molecule | 0.14 | 0.055 | 60.8 |

**Table I**

**Photoreactivation of endonuclease-sensitive sites**

[\( ^{3}H \)T7 DNA] was ultraviolet-irradiated at 40 J per m². Incubation with *Escherichia coli* photoreactivating enzyme was carried out in 0.20 ml containing: DNA, 15.0 nmol (as nucleotide); potassium phosphate buffer, 20.0 mM, pH 7.2; EDTA, 1.0 mM; diethiothreitol, 0.1 mM, photoreactivating enzyme (918 units). Incubation was at 37°C for 90 min under a 250-watt General Electric spot lamp maintained 5.0 cm above the water bath. A control tube was incubated under identical conditions without photoreactivating enzyme. The reaction was terminated by removing the source of photoreactivating light and 0.05 ml of each sample was removed. To this was added 0.05 ml of 5% bovine serum albumin and 0.05 ml of cold 20% trichloroacetic acid. Following centrifugation at 6000 \( X \) g for 20 min, the acid-precipitable fraction was hydrolyzed in 97% formic acid and thymine in thymine dimers was measured as described previously (17). The rest of the DNA was divided into 0.06-ml aliquots which were incubated with or without T4 ultraviolet endonuclease. Incubations (0.17 ml) contained: T7 DNA, 4.0 nmol; Tris-HCl buffer, 50.0 mM, pH 8.0; \( \beta \)-chloromercuri-phenylsulfonic acid, 0.6 mm. Incubation was at 37°C for 60 min following which 0.025 ml of each sample was layered onto a 5 to 20% alkaline sucrose gradient containing 0.8 M NaCl and 0.2 M NaOH. Sedimentation was at 38,000 rpm for 225 min at 20° in a SW 56 rotor. Tubes were drained from the bottom by collecting 8-drop fractions directly into scintillation vials. Water, 1.0 ml, was added to each, followed by 10.0 ml of a toluene-based scintillation mixture consisting of two parts Omniflour (5.04 g per liter of toluene) and one part Triton X-100. Radioactivity was measured in a Beckman LS-250 liquid scintillation spectrometer. The average number of single strand breaks per DNA molecule was computed from the sedimentation profiles using the nomogram of Litwin et al. (21).
The DNA was ultraviolet-irradiated at 50 J per m² and 41.25 nmol (as nucleotide) was incubated at 37° for 60 min with or without the addition of 190 units of T4 ultraviolet endonuclease (Fraction V) in a total volume of 0.5 ml. Both nicked and unnicked DNA (8.25 nmol) were preincubated with or without bacterial alkaline phosphatase (0.7 units) in a total of 0.2 ml. Incubation was at 45° for 60 min. Each reaction tube then was supplemented with N-2-hydroxyethylpiperaziner-N′-2-ethanesulfonic acid (Hepes) buffer (each at 0.05 mM), and [H]TTP (0.5 μCi) and purified Escherichia coli DNA polymerase I (1.8 units). The final volume of the reaction mixtures was 0.3 ml. Control samples containing no DNA polymerase were included. Incubation was at 37° for the times indicated. Reactions were terminated by the addition to each tube of 1.0 ml of calf thymus DNA (0.5 mg per ml) in 0.1 m sodium pyrophosphate. Tubes were boiled for 2 min, quenched on ice and 1.5 ml of 10% cold trichloroacetic acid were added. The suspension was filtered through 2.4-cm GF/C glass filter discs which were washed with 5% trichloroacetic acid and then with 95% ethanol. Radioactivity was determined by counting the dried discs for 3'-OH termini at the sites of nicking by T4 ultraviolet endonuclease. These experiments indicate the presence of 3'-OH sites produced by endonuclease treatment.

As seen in the figure, the rate of degradation of ultraviolet-irradiated DNA by exonuclease I of Escherichia coli. T7 [TH]DNA was prepared as described in the text. The DNA was at a specific radioactivity of 2.25 × 10⁶ cpm per μg. DNA was ultraviolet-irradiated at 200 J per m² and treated with T4 ultraviolet endonuclease (Fraction V) as follows. Incubation mixtures (0.8 ml) contained 5.4 nmol of DNA (as nucleotide), 10 mM Tris-HCl buffer (pH 8.0), 1.0 mM EDTA, and 96 units of endonuclease or an equivalent volume of T4 HCl buffer. Incubation was at 37° for 90 min following which the reaction tubes were boiled for 10 min and immediately quenched in ice. To 0.1 ml of each mixture containing 0.075 nmol of DNA the following were added: glycine buffer, pH 9.2, 50 mM; MgCl₂, 5.0 mM; and 3.5 units of bacterial alkaline phosphatase or an equivalent volume of water. The final volume of this reaction mixture was 0.255 ml. Incubation was at 37° for 30 min, following which 55.4 units of exonuclease I of E. coli were added for the times indicated. Reactions were terminated by the addition of 0.1 ml of 1% bovine serum albumin and 0.4 ml of cold 10% trichloroacetic acid. The tubes, including controls with no exonuclease addition were centrifuged at 5,000 × g and the acid-soluble fraction saved. Radioactivity was determined by counting 0.4 ml of the acid-soluble fraction with 10.0 ml of the scintillation mixture described in the legend to Table I in a liquid scintillation spectrometer. Circles, ultraviolet-irradiated DNA plus endonuclease; triangles, ultraviolet-irradiated DNA without endonuclease; closed symbols, bacterial alkaline phosphatase added; open symbols, no bacterial alkaline phosphatase.

Precipitable DNA is significantly increased compared to unnicked ultraviolet-irradiated DNA. This result is unaffected by preincubation with bacterial alkaline phosphatase. These data indicate that 3'-OH termini are produced at the sites of endonucleolytic incision. Previous studies (26) have shown that incubation of nicked T7 ultraviolet-irradiated DNA with DNA polymerase I results in excision of thymine dimers. Thus the 5'-terminus apparently is digested rather than displaced in this reaction. Further confirmation of the 3'-OH terminus is provided by the results shown in Figs. 2, 3, and 4. Fig. 2 demonstrates that the rate of degradation of specifically nicked and then denatured T7 DNA by purified exonuclease I of E. coli is significantly greater than that of unnicked ultraviolet-irradiated denatured DNA. This result, too, is essentially unaffected by preincubation with alkaline phosphatase. Since exonuclease I of E. coli specifically requires 3'-OH termini in single-stranded DNA, these data confirm the presence of 3'-OH sites produced by endonuclease treatment of the DNA. Fig. 3 shows the results of an experiment designed to demonstrate 5'-P termini in specifically nicked ultraviolet-irradiated DNA. Bovine spleen phosphodiesterase degrades denatured DNA with 5'-OH termini. DNA degradation is, however, inhibited by the presence of pyrimidine dimers (22, 23). As seen in the figure, the rate of degradation of ultraviolet-irradiated, nicked, and then denatured T7 DNA by spleen phosphodiesterase, is increased only when the DNA has been incubated with both photoreactivating enzyme and bacterial alkaline phosphatase to remove pyrimidine dimers and terminal phosphate groups. In control experiments T7 DNA that had been preincubated with micrococcal nuclease (which produces 5'-OH termini) and then denatured was extensively degraded by bovine spleen phosphodiesterase. These experiments indicate the presence of 5'-P termini at the sites of nicking by T4 ultraviolet endonuclease and confirm that the nicks occur on the 5' side of the dimers.
phosphodiesterase was present in all incubations. Incubations (0.5 ml) contained T7 DNA (50 nmol), EDTA (10 mM), p-chloromercuri phenyl sulfonic acid (1.0 mM), and endonuclease (120 units). Incubation was at 37° for 60 min, following which the DNA was extracted twice in cold buffered phenol and dialyzed extensively against 20.0 mM potassium phosphate buffer pH 7.2. A second incubation with or without photoreactivating enzyme was carried out in reaction volumes of 0.25 ml containing nicked or uncleaved DNA (12.0 nmol), potassium phosphate buffer (20.0 mM, pH 7.2), EDTA (1.0 mM), dithiothreitol (0.1 mM), and photoreactivating enzyme of Escherichia coli (918 units or an equivalent volume of bacterial alkaline phosphatase). The tubes then were boiled for 10 min and rapidly quenched in ice. Each of the four DNA samples (treated with or without photoreactivating enzyme and alkaline phosphatase) was divided into 0.04-ml aliquots to which were added 0.15 ml of 0.2 M sodium acetate buffer pH 8.0, 0.05 ml of 1% bovine serum albumin, and 0.6 ml of cold 10% trichloroacetic acid. The tubes were centrifuged at 6,000 × g and the supernatant was discarded. The thymine dimer content of the precipitate was measured as previously described (17), and it was determined that incubation with photoreactivating enzyme caused monomerization of 87% of the thymine dimers. The DNA samples were dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and incubated at 60° for 45 min with or without the addition of 3.5 units of T4 ultraviolet endonuclease. Further experiments (data not shown) have been obtained without photoreactivation, indicating that T4 polynucleotide ligase can seal nicks adjacent to pyrimidine dimers at 20°. These results confirm the presence of 3'-OH and 5'-P termini. Future studies are aimed at detailing the kinetics of the joining reaction in the presence and absence of dimers in the DNA.

Degradation of Single-stranded DNA by T4 Ultraviolet Endonuclease—Evidence is presented that purified T4 ultraviolet endonuclease attacks ultraviolet-irradiated but not unirradiated single-stranded DNA. Fig. 5 shows the sedimentation profiles of denatured E. coli DNA and demonstrates that ultraviolet-irradiated DNA incubated with T4 ultraviolet endonuclease sediments at a significantly slower rate than such DNA without endonuclease incubation. Further experiments (data not shown) demonstrate a linear relationship between total ultraviolet

Finally, Fig. 4 demonstrates that phosphodiester bond breaks created in ultraviolet-irradiated double-stranded DNA by incubation with T4 ultraviolet endonuclease can be rejoined by incubation with T4 polynucleotide ligase. The data shown in the figure are from an experiment in which the nicked DNA was pre-treated with photoreactivating enzyme in order to monomerize dimers. Calculation of the weight average molecular weight of the DNA from the sedimentation profiles indicates that approximately 50% of the endonucleolytic incisions were rejoined in the presence of DNA ligase. Qualitatively similar results (data not shown) have been obtained without photoreactivation, indicating that T4 polynucleotide ligase can seal nicks adjacent to pyrimidine dimers at 20°. These results confirm the presence of 3'-OH and 5'-P termini. Future studies are aimed at detailing the kinetics of the joining reaction in the presence and absence of dimers in the DNA.

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fluorescence to the DNA and the extent of endonucleolytic degradation.

Although *E. coli* DNA was denatured in alkali and then neutralized in low ionic strength in order to facilitate incubation with enzyme, we were concerned about the possibility that sufficient renaturation may have occurred so that pyrimidine dimers attacked by the endonuclease were actually in regions of DNA with a duplex conformation. In order to minimize this potential problem, we carried out experiments using M-13 DNA from *H*-labeled purified phage. The DNA was maintained and incubated at a maximal ionic strength of 50 mM, under which conditions it is believed to exist primarily in the single-stranded form (24). Fig. 6 shows that unirradiated M-13 DNA incubated with T4 ultraviolet endonuclease does not undergo degradation detectable by sedimentation in cesium chloride velocity gradients, while ultraviolet-irradiated DNA does.

Further evidence supporting the ability of the T4 ultraviolet endonuclease to recognize pyrimidine dimers in single-stranded DNA is provided by the data shown in Table II. The table shows a correlation between the number of endonucleolytic incisions calculated from the fraction of *P* label in M-13 DNA rendered acid-soluble during incubation with endonuclease, and the calculated number of pyrimidine dimers in *H*-labeled DNA, as a function of increasing ultraviolet fluence. In order to be sure that reactions were carried to completion, M-13 DNA irradiated at the highest fluence used in Table II was incubated under identical conditions for periods between 10 to 120 min. The release of *P* following incubation with bacterial alkaline phosphatase reached a maximum at 30 min of incubation. These results indicate that all pyrimidine sites in ultraviolet-irradiated M-13 DNA are attacked by the T4 ultraviolet endonuclease. Thus, even if some degree of double-stranded structure exists in M-13 DNA under our experimental conditions, it is unlikely that all dimers are located in these regions.

**Molecular Weight Determination**—Electrophoresis of a lyophilized preparation of Fraction V of the T4 ultraviolet endonuclease was carried out in polyacrylamide gel containing sodium dodecyl sulfate. The gel showed a single major band at a molecular weight calculated at 17,700 relative to the standard proteins used (Fig. 7). A single extremely faintly staining band at a
3'-OH sites can allow for the initiation of repair resynthesis of 3'-OH and 5'-P termini at the sites of incision. The presence of pyrimidine dimers in the DNA. In addition, the present studies have demonstrated that the endonuclease creates incisions in the 5' → 3' direction (Fig. 7, inset). Molecular weight determination of purified T4 ultraviolet endonuclease. Details of polyacrylamide gel electrophoresis in sodium dodecyl sulfate and gel filtration on Sephadex G-75 are provided in the text. The arrows indicate the position of the stainable band in the gel and the peak of enzyme activity off Sephadex. B.S.A., bovine serum albumin; CYTO.C, cytochrome c.

### Table II

| Ultraviolet fluence (J/m²) | Acid-soluble %P | Calculated number of nicks per molecule M-13 DNA | Thymine dimers in pyrimidine dimers | Calculated number of pyrimidine dimers per M-13 DNA molecule |
|---------------------------|-----------------|-----------------------------------------------|----------------------------------|-------------------------------------------------|
| (J/m²) × 10^-4          |                 |                                               |                                  |                                                 |
| 1                        | 0.20            | 5.9                                           | 0.60                             | 3.9                                             |
| 3                        | 0.47            | 14.1                                          | 1.84                             | 11.9                                            |
| 6                        | 1.05            | 31.5                                          | 3.44                             | 22.2                                            |
| 13                       | 1.90            | 44.9                                          | 0.14                             | 40.0                                            |

DNA in vivo without further modification of the termini. Indeed, our results show that in vitro, E. coli DNA polymerase I will utilize nicks for DNA synthesis in the 5' → 3' direction. These studies also demonstrate that the T4 ultraviolet endonuclease has a molecular weight of approximately 18,000, with no evidence for subunit structure.

A question of considerable interest with respect to this endonuclease is its substrate specificity. Previous studies have demonstrated a stoichiometric relationship between the calculated number of dimers and the number of endonucleolytic incisions in DNA, thereby providing indirect evidence that pyrimidine dimers provide substrate sites in DNA (4, 7). The present studies have approached this question more directly. Thus, if the concentration of thymine dimers in DNA is reduced by incubation with photoreactivating enzyme, the number of endonucleolytic incisions in the DNA is reduced. The only known catalytic activity of E. coli photoreactivating enzyme is the splitting of the cyclobutane ring covalently linking two pyrimidines in ultraviolet-irradiated DNA (27). Similar results have been obtained by introducing purified T4 endonuclease into Brij-treated ultraviolet-irradiated E. coli (28). In this case all endonuclease sensitive sites measurable by sedimentation velocity of the DNA are removed by exposing the cells to photoreactivating light.

Given that pyrimidine dimers are necessary to produce substrate sites for the T4 ultraviolet endonuclease, the possibility...
exists that the enzyme recognizes a localized conformational distortion in the secondary structure of the DNA rather than the dimer directly. It has been previously demonstrated with Form I SV 40 DNA containing an average of 1 pyrimidine dimer per molecule, that the endonuclease makes only single strand breaks in ultraviolet-irradiated double-stranded DNA (7). Thus, one needs to consider the possibility that either the dimer-containing strand or the opposite strand at a dimer site is attacked. Evidence available at this time indicates that only the dimer-containing strand is attacked.

1. The present studies demonstrate that the enzyme apparently recognizes thymine dimers in ultraviolet-irradiated single-stranded DNA, suggesting that it is not dependent on a conformational distortion of the secondary structure of the DNA, but rather recognizes the dimer directly.

2. As shown by the results presented in Fig. 4, photoreactivation of nicked DNA is required to facilitate degradation at 5’ termini by bovine spleen phosphodiesterase. This suggests that all sub-strate sites created for the phosphodiesterase are close to pyrimidine dimers rather than opposite them. In a similar vein it has previously been shown that when ultraviolet-irradiated T7 DNA incised with T4 ultraviolet endonuclease is incubated with a partially purified extract of T2-infected E. coli that contains a phage-coded dimer excision activity, 80 to 90% of the thymine dimers can be excised with a loss of only 10 to 20 nucleotides per dimer into the acid-soluble phase, suggesting that at least 80 to 90% of the endonucleolytic incisions are close to pyrimidine dimers (20).

We believe that these experiments rule out the possibility of an equally probable attack at either strand at a dimer site in duplex DNA; however, attack at the strand opposite a dimer may occur at a low frequency. We are currently investigating this question by constructing DNA duplexes in which only one strand contains pyrimidine dimers. Further studies also are being carried out to determine the relative efficiency with which the enzyme attacks ultraviolet-irradiated single- and double-stranded DNA.

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