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T4 Endonuclease Involved in Repair of DNA

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Abstract. An enzyme activity, named T4 endonuclease V, was purified from T4-infected *Escherichia coli*. The enzyme induces single-stranded breaks in ultraviolet-irradiated DNA but does not act on native or heat-denatured DNA. The enzyme activity is dependent on the dose of ultraviolet irradiation, and the number of the breaks formed is approximately equal to the number of pyrimidine dimers present in the DNA. Denatured DNA, which has been exposed to ultraviolet light, is also attacked by the enzyme although the extent of the reaction is greater with irradiated native DNA. The enzyme shows optimal activity at pH 7.5 and does not require added divalent ions. When the enzyme-treated, irradiated DNA is subjected to stepwise degradation by spleen phosphodiesterase, dimers are released more rapidly than thymine into the acid-soluble fraction, suggesting that the enzyme induces a break at the 5′-side of a pyrimidine dimer. The enzyme, whose formation is controlled by the v^+ gene of T4, appears to be responsible for the first step of excision repair.

The existence of excision repair and its role in the recovery of organisms from radiation-induced damage is now well established. It has been demonstrated that ultraviolet (UV)-induced pyrimidine dimers are excised by wild type bacteria, *in vivo*, in the form of acid-soluble oligonucleotides from the DNA; however, some UV-sensitive mutants lack this activity.[1,2] This type of repair mechanism has been found in many biological systems, including human skin cells.[3,4]

In spite of the biological importance of excision repair, its precise mechanism still remains obscure, partly because of the complexity of the reactions. It is supposed that the process involves many enzyme-mediated steps, including those of the recognition of damage, excision, insertion of new bases, and the final joining of single-stranded interruptions in the polynucleotide chain.

We have shown[5–7] that an extract of *Escherichia coli* infected with bacteriophage T4 contains an enzyme activity that specifically releases nucleotides containing pyrimidine dimers from UV-irradiated DNA. Little or no activity is found in an extract of uninfected *E. coli;* the activity appears to be induced by infection with T4. Furthermore, it was shown that v_1 and v_2, T4 mutants having increased sensitivity to UV radiation, are unable to induce such an activity, suggesting that the enzyme, the formation of which is controlled by the v^+ gene of T4, is involved in repair of UV-damaged DNA *in vivo*.[5,7–9] This paper describes the isolation and properties of the v^+ gene-controlled activity, which has turned out to be an endonuclease specific for UV-irradiated DNA.

Materials and Methods. Bacteria and bacteriophage: *E. coli* strain 1100, a mutant of K12 lacking endonuclease I,[10] was furnished by Dr. H. Hoffmann-Berling and used throughout these experiments. Bacteriophage T4B was provided by Dr. S. Benzer.

Chemicals: [2-^{14}C]thymine was obtained from the Daiichi Pure Chemicals Co., Ltd. Chromatographically purified *E. coli* alkaline phosphatase and snake (*Crotalus adamanteus*) venom phosphodiesterase were purchased from Sigma Chemical Co. and further purified by DEAE-cellulose and Dowex-50 chromatography, respectively.[11,12] Purified bovine spleen phosphodiesterase, and pancreatic deoxyribonuclease were obtained from Worthington Biochemical Co. and Sigma Chemical Co.

Preparation of DNA: [^{14}C]thymine-labeled T4 and *E. coli* DNA were prepared as described previously.[6] ^{32}P-labeled T4 DNA was prepared similarly from T4B, grown in Tris–casamino acid medium containing [^{32}P]P_i. DNA was dissolved in 0.02 M NaCl at a concentration of 50 μg/ml, and irradiated with a 15-W germicidal lamp (Toshiba) at room temperature. The dose rate was determined by a Toshiba germicidal UV-meter.

Determination of pyrimidine dimers: DNA and the acid-soluble fraction were hydrolyzed by heating with 6 N perchloric acid at 100°C for 3 hr. The amount of thymine and thymine-containing dimers was determined by Dowex-1 column chromatography.[6]

Sedimentation analysis: Sucrose gradient centrifugations were performed by layering a 0.1-ml sample containing 1–2 μg of labeled DNA onto a 5–20% linear sucrose gradient (4.7 ml) prepared in 0.02 M K_3PO_4–0.05 M EDTA (final pH, 12.4) or 0.15 M NaCl–0.015 M sodium citrate (pH 7.0), and centrifuging at 15°C in the RPS40 rotor of the Hitachi 55P centrifuge. 10-drop fractions were collected from the bottom of each tube, and the radioactivity was determined in a liquid scintillation counter.

Assay of endonuclease activity: The standard assay measures the liberation of phosphomonoester groups produced by incision of DNA. The reaction mixture (0.25 ml) contained 16 nmol of ^{32}P-labeled T4 DNA that had been exposed to UV-light (3.4×10^4 ergs/mm^2), 10 μmol of Tris·HCl (pH 7.5), 2.5 μmol of $MgCl_2$, and enzyme. After incubation at 37°C for 60 min, 0.03 ml of 50% trichloroacetic acid was added and the mixture was centrifuged in the cold. The precipitate was dissolved in 0.1 ml of 0.1 M NaOH and neutralized by adding 0.01 ml of 2 M Tris·HCl (the final pH was 8.0). 0.3 unit of *E. coli* alkaline phosphatase and 1.2 μmol of EDTA were added, and each reaction mixture was incubated for 30 min at 45°C. At the end of incubation, 0.1 ml of carrier DNA (calf thymus), 0.05 ml of 1 M $MgCl_2$, and 0.04 ml of 50% trichloroacetic acid were added in succession. After centrifuging at $1000 \times g$ for 10 min, the supernatant fluid was treated with charcoal to remove contaminating small DNA fragments; 0.1 ml of 5% charcoal suspension was added to the supernatant fluid and the mixture was shaken for 5 min and then centrifuged. The charcoal treatment was repeated once, and the radioactivity in the final supernatant solution was determined.

One unit of the enzyme is defined as the amount catalyzing the conversion of 1 nmol of ^{32}P from UV-irradiated DNA into phosphomonoesterase-sensitive forms in 60 min under the standard conditions. The activity is proportional to enzyme concentration until 0.4% of the total ^{32}P in the DNA is converted.

Purification of endonuclease: (*a*) *Assay:* The v^+ gene-controlled activity was determined by the conversion of UV-irradiated, ^{32}P-labeled, T4 DNA into acid-soluble forms. Since more than one enzyme is involved in the reaction, the assay was performed in the presence of an extract of T4v_1-infected cells, which contains all of the components except the v^+ gene product.[7] The reaction mixture (total volume of 0.5 ml) contained 16 nmol of ^{32}P-labeled T4 DNA, irradiated at 3.4×10^4 ergs/mm^2, 20 μmol of Tris·HCl (pH 7.5), 5 μmol of $MgCl_2$, 200 μg of protein from an extract of T4v_1-infected cells, and enzyme (to be assayed). The reaction was performed at 37°C for 60 min, and the radioactivity released into acid-soluble form was determined.

(*b*) *Preparation of extracts: E. coli* 1100 was grown to 5×10^8 cells/ml at 37°C in broth and infected with T4B at a multiplicity of 5. After 15 min of infection, the culture was chilled and cells were collected in a refrigerated centrifuge. The cells were suspended

in 10 mM Tris·HCl–1 mM 2-mercaptoethanol–10% ethylene glycol (pH 7.5) at a concentration of 10^{11} cells/ml, and disrupted in an ultrasonic disintegrator. After centrifugation at 10,000 × *g* for 20 min, the supernatant fluid was taken as the extract.

(*c*) *Purification:* To 45 ml of the supernatant were added 5.2 ml of 20% (w/w) Dextran-500, 14.5 ml of 30% (w/w) polyethylene glycol-6000, and 7.5 g of NaCl with continuous stirring. After stirring in the cold for 1 hr the mixture was centrifuged at 1000 × *g* for 10 min. A clear supernatant fluid was dialyzed against the extraction buffer and applied to a DEAE-cellulose column (2 × 20 cm) equilibrated with the same buffer. The column was eluted with 120 ml of buffer. Fractions that contained the v^+ gene-controlled activity were pooled, dialyzed against 10 mM potassium phosphate–1 mM 2-mercaptoethanol–10% ethylene glycol (pH 6.5), and then applied to a phosphocellulose column (1 × 15 cm) equilibrated with the same buffer. The column was washed with 30-ml portions of the phosphate–mercaptoethanol–ethylene glycol buffer containing 0.1, 0.2, or 0.3 M KCl. Of the activity applied, 75% was obtained in 40 ml of the 0.3 M eluate.

The phosphocellulose fraction was purified about 280-fold over the extract and contained 15% of the activity initially present. The fraction was used in most of the present experiments. For further purification of the enzyme, a concentrated phosphocellulose fraction was applied to a Sphadex G-100 column (1 × 58 cm) and eluted with the buffer. The activity eluted with a pattern similar to that of whale myoglobin (molecular weight: 17,800), indicating that the enzyme is a relatively small protein.

Results. The v^+ gene-controlled activity as an endonuclease specific for UV-irradiated DNA: The v^+ gene-controlled activity was purified from an extract of T4-infected cells. The purified enzyme liberates little or no acid-soluble ^{32}P from normal or UV-irradiated DNA labeled with ^{32}P, suggesting that degradation is endonucleolytic rather than exonucleolytic.

^{32}P-labeled T4 DNA was treated with the purified enzyme, denatured, and then incubated with *E. coli* alkaline phosphatase. As shown in Fig. 1, ^{32}P was released from irradiated DNA, but not from normal DNA treated in the same manner. This indicates that the T4 enzyme induces endonucleolytic cleavage specifically in UV-irradiated DNA. The release of ^{32}P from irradiated DNA terminates when about 0.4% of the total ^{32}P is liberated. This value is close to 0.45%, the ratio of pyrimidine dimers to total bases in the DNA; thus, the result is consistent with the idea that an incision is made for every dimer.

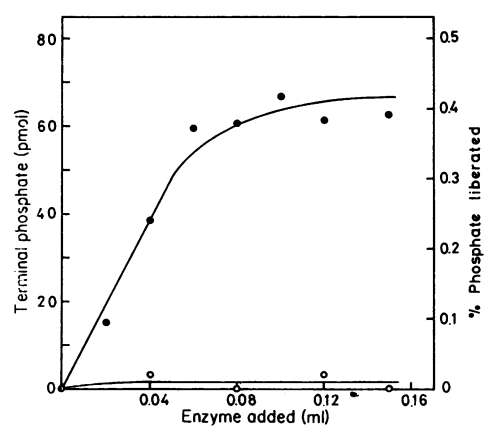

FIG. 1. The selective breakdown of UV-irradiated DNA by T4 endonuclease V. ^{32}P-labeled T4 DNA, exposed to UV (3.4 × 10^4 ergs/mm^2) or not, was treated with various amounts of T4 endonuclease V; the amount of phosphate converted to phosphomonoesterase-sensitive form was determined. ●, irradiated DNA; ○, non-irradiated DNA.

Fig. 2 indicates that the amount of ^{32}P released is almost proportional to the dose of ultraviolet irradiation (up to 10^4 ergs/mm^2). When irradiated DNA was heated in a boiling-water bath for 10 min and treated in the same manner, a significant amount of radioactivity was released from the DNA, although the extent of the reaction was less than with nonheated DNA irradiated with the same dose of UV. The hyperchromicity of the DNA reveals that it is denatured under these conditions (shown in Fig. 2*a*), and thus it is likely that the T4 enzyme attacks both double- and single-stranded DNA provided that the DNA has been irradiated. Essentially similar results were obtained with DNA that had been denatured and then exposed to UV.

The enzyme showed optimal activity at pH 7.5 in 0.04 M Tris-chloride or Tris-maleate buffer. The activity is not totally dependent on the presence of added divalent ions, although it is stimulated by Mg^{++}. The activity in the presence of 10 mM EDTA was about 70% of that in the presence of 10 mM $MgCl_2$.

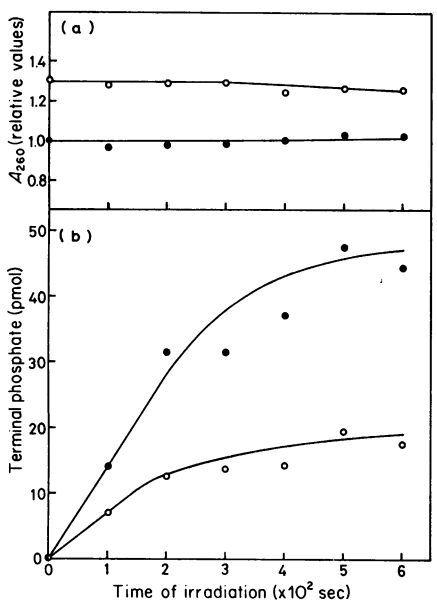

FIG. 2. The action of T4 endonuclease V on native and denatured DNA, both of which had been irradiated with various doses of UV. ^{32}P-labeled T4 DNA (160 nmol/ml in 0.15 M NaCl) was irradiated with various doses of UV and divided into two portions, one of which was heated in a boiling-water bath for 10 min while the other was kept at room temperature. (*a*) Absorbance at 260 nm of the heated and unheated DNA samples was measured and corrected for the volume changes with radioactivity of each sample. (*b*) Heated and unheated DNA, after irradiation, were treated with an excess of endonuclease V (0.08 units/reaction tube) under the standard conditions. ●, native DNA; ○, heated DNA.

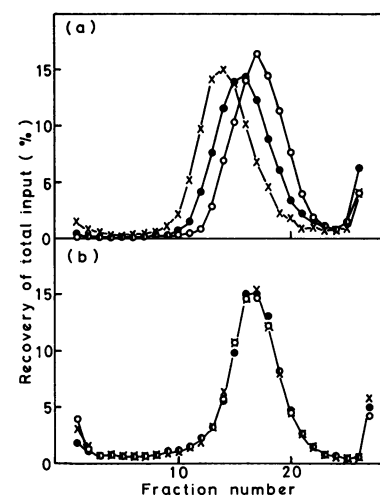

FIG. 3. Sedimentation patterns in neutral and alkaline sucrose of irradiated *E. coli* DNA after treatment with T4 endonuclease V. (*a*) Sucrose gradient centrifugation in 0.02 M K_3PO_4–0.05 M EDTA (pH 12.4) for 4 hr at 35,000 rpm. (*b*) Sucrose gradient centrifugation in 0.15 M NaCl–0.015 M sodium citrate (pH 7.0) for 3 hr at 34,000 rpm. ○, DNA irradiated (96 ergs/mm^2), treated with the enzyme; ●, DNA irradiated (32 ergs/mm^2), treated with the enzyme; ×, DNA irradiated (96 ergs/mm^2), without treatment.

Nature of the strand breaks: Formation of breaks in DNA can be detected by sedimenting DNA in an alkaline sucrose gradient. This technique revealed that the treatment of irradiated T4 DNA with the T4 enzyme results in an extensive decrease in sedimentation rate of the DNA, whereas the same treatment of nonirradiated DNA induces no decrease. In these experiments, it was also found that *E. coli* DNA that had been exposed to UV-irradiation is similarly attacked by the T4 enzyme.

In order to see whether the breaks formed in irradiated DNA by the T4 endonuclease are of single-strand or double-strand type, the sedimentation behavior of DNA was compared under neutral and alkaline conditions. As seen in Fig. 3, there was no detectable decrease in the size of DNA in neutral sucrose, whereas under alkaline conditions, where DNA is denatured, a considerable fragmentation of the DNA was observed. Therefore it is evident that the breaks formed in irradiated DNA are mostly of the single-strand type.

Site of the incision: If we assume that the enzyme introduces a break near a pyrimidine dimer, the question arises as to which side of a dimer the break is made. The enzyme may introduce a break either to the 5′- or to the 3′-side of a pyrimidine dimer.

[^{14}C]thymine-labeled T4 DNA, irradiated with UV, was treated with the T4 enzyme, denatured, and incubated with alkaline phosphatase to remove terminal phosphate. The DNA was then subjected to stepwise degradation by spleen or venom phosphodiesterase, and thymine and its dimers, released into the acid-soluble fraction, were determined. The results are shown in Fig. 4. Dimers are more rapidly released into acid-soluble material than is thymine upon treatment with the spleen enzyme. On the other hand, thymine and its dimers are released at almost equal rates by treatment with the venom enzyme. No such selectivity was observed when irradiated DNA, with or without treatment with pancreatic deoxyribonuclease, was subjected to stepwise degradation by the phosphodiesterases. Since it is known that spleen phosphodiesterase hydrolyzes a polynucleotide beginning from the 5′ end, whereas venom phosphodiesterase initiates cleavage at the 3′ end,[13,14] it is likely that more pyrimidine dimers are located in the region near the 5′ end of the DNA fragment produced by action of the T4 enzyme. This is consistent with the notion that the T4 endonuclease induces a break to the 5′-side of a pyrimidine dimer.

Discussion. It was suggested previously[7] that excision of pyrimidine dimers from UV-irradiated DNA in T4-infected cells consists of two different steps. First, a single-stranded break is induced at a point close to a pyrimidine dimer, and second a nucleotide fragment containing dimers is released from the DNA; the former step does not require Mg^{++}, whereas the latter does. Data presented in this paper clearly support this view. An enzyme, which induces single-stranded breaks specifically in UV-irradiated DNA in the absence of Mg^{++}, was isolated from T4-infected cells. The enzyme activity is not found in an extract of T4v_1-infected cells;[7] thus, it seems probable that the enzyme is responsible for the first step of excision repair.

The enzyme is unique in many properties. It introduces breaks into irradiated DNA but has no effect on native or denatured DNA. The enzyme re-

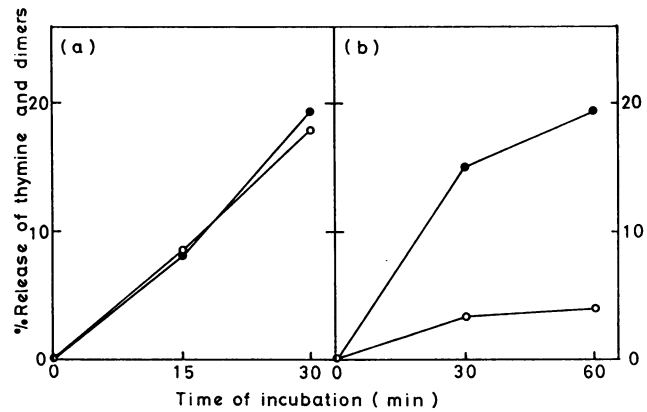

FIG. 4. The release of thymine and its dimers from endonuclease V-treated T4 DNA in the course of phosphodiesterase digestion. (*a*) Digestion with snake venom phosphodiesterase. (*b*) Digestion with bovine spleen phosphodiesterase. Endonuclease V treatment: The reaction mixture contained 86.4 nmol of irradiated [^{14}C]thymine-labeled T4 DNA, 120 μmol of Tris·HCl(pH 7.5), 30 μmol of $MgCl_2$, and 0.1 unit of T4 endonuclease V in 3 ml. After incubation at 37°C for 60 min, the reaction was stopped by the addition of trichloroacetic acid. Precipitated DNA was dissolved in 0.5 ml of 0.1 N NaOH and the pH was adjusted to 8.0 with 0.05 ml of 2 M Tris·HCl. Dephosphorylation: *E. coli* alkaline phosphatase (0.6 unit) was added to the endonuclease V-treated DNA and incubated at 45°C for 30 min. The DNA was precipitated with trichloroacetic acid, dissolved in 0.01 M NaOH, and then neutralized with 0.5 M Tris·HCl. Phosphodiesterase digestion: The reaction mixture contained; (*a*) 7.2 nmol of dephosphorylated DNA, 20 μmol of Tris·HCl (pH 8.9), and snake venom phosphodiesterase; or (*b*) 7.2 nmol of treated DNA, 20 μmol of Tris maleate (pH 6.5), and spleen phosphodiesterase. After incubation at 37°C for the periods indicated in the figure, perchloric acid was added to a final concentration of 0.6 N. The acid-soluble fractions were hydrolysed in 6 N perchloric acid at 100°C for 3 hr, neutralized, and applied to a Dowex-1 column to separate dimers from thymine. ●, thymine-containing dimers; ○, thymine.

action is a function of the UV-dose used for irradiation of the DNA, and the number of breaks formed almost corresponds to the number of dimers present in the DNA. Analysis of the reaction products suggests that the enzyme cleaves the phosphodiester bond to the 5′-side of a pyrimidine dimer. The inability of enzyme-treated DNA, even after dephosphorylation by phosphomonoesterase, to accept phosphate in the presence of polynucleotide kinase also supports this view (Shimizu, Yasuda, and Sekiguchi, unpublished results). It appears that this enzyme, which is induced in *E. coli* on infection with T4, is a new type of endonuclease. We propose that the enzyme be designated T4 endonuclease V. Endonucleases I–IV have already been described in normal or phage-infected *E. coli*.[15–18]

If endonuclease V has the biological role suggested here, then the enzyme that is responsible for the second step of dimer excision should be an exonuclease that hydrolyzes a polynucleotide in a 5′ → 3′ direction, or else a specific endonuclease that induces a break to the 3′-side of a dimer (Fig. 5). Recently, it was demonstrated that DNA polymerase from *E. coli* possesses a 5′ → 3′ exonuclease activity and can excise dimer-containing nucleotides from irradiated DNA *in vitro*.[19] Thus, there is a possibility that both excision and repair replication are catalyzed by a single enzyme, DNA polymerase. However, no direct evidence has yet

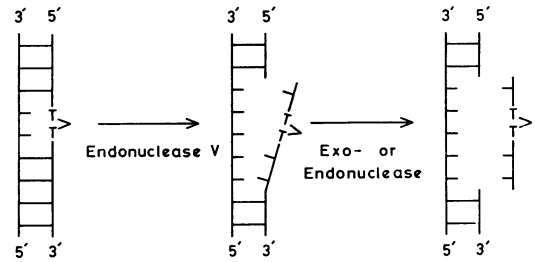

FIG. 5. A possible scheme for the initial steps in the repair of ultraviolet-irradiated DNA.

been obtained to indicate that DNA polymerase is involved in excision of dimers *in vivo*.

An endonuclease specific for irradiated DNA has been found in *Micrococcus luteus* (*M. lysodeikticus*).[5,20-22] The enzyme resembles T4 endonuclease V in many respects and may be concerned with dark repair of the bacterial DNA. It would be of interest to see whether similar enzymes function in mammalian cells to repair damaged DNA.

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