Purification and Characterization of Normal and Mutant Forms of T4 Endonuclease V*

Yusaku Nakabeppe, Katsumi Yamashita, and Mutsuo Sekiguchi

From the Department of Biology, Faculty of Science, Kyushu University 33, Fukuoka 812, Japan

Endonuclease V of bacteriophage T4 has been purified to physical homogeneity from T4D-infected *Escherichia coli* 1100. The enzyme, whose molecular weight is 16,000, possesses two distinct catalytic activities, a pyrimidine dimer-DNA glycosylase and an apurinic/apyrimidinic endonuclease. They acted on UV-irradiated poly(dA)-poly(dT) in a sequential manner; the glycosylase cleaved the N-glycosyl bond between the 5'-pyrimidine of a dimer and the corresponding sugar and then the endonuclease hydrolyzed a phosphodiester bond on the 3'-side of the apyrimidinic site. The 5'-termini thus generated were phosphorylated by T4 polynucleotide kinase only after they had been subjected to direct photoreversal and then treated with alkaline phosphatase. By using two phage mutants, *uvs*-5 and *uvs*-13, it was shown that occurrence of an amber mutation in the *denV* gene caused a simultaneous loss of the two activities. Suppression of the mutation of *uvs*-5 rendered both activities partially active. When the mutation of *uvs*-13 was suppressed, a mutant form of enzyme that possessed only a glycosylase activity was produced. This suggests that there are two distinct domains in a single enzyme, each of which corresponds to one of the activities.

UV irradiation results in formation of pyrimidine dimers in DNA, which are potentially lethal and mutagenic damages for organisms. T4 endonuclease V appears to be involved in excision of dimers from the DNA (1, 2). The enzyme is specifically active on UV-irradiated DNA, and mutant phages which are unable to produce the enzyme exhibit increased sensitivity to UV (3, 4).

It was originally thought that T4 endonuclease V induces a strand break by a single catalytic reaction. However, a two-step mechanism for the incision of DNA containing dimer has been proposed by Grossman and co-workers (5, 6) and evidence to support this view has been presented by others (7–11). We have recently demonstrated that T4 endonuclease V possesses two catalytic activities, a pyrimidine dimer-DNA glycosylase and an apurinic/apyrimidinic DNA endonuclease (12).

To characterize these unique enzyme activities, we have purified T4 endonuclease V to an apparent physical homogeneity. This paper describes the purification procedure and properties of the two enzyme activities associated with the purified enzyme preparations. We also analyzed mutant forms of the enzyme, partially purified from mutant phage-infected cells. The results obtained here support the view that T4 endonuclease V possesses two distinct active sites or domains, each of which corresponds to the glycosylase specific for pyrimidine dimer and the AP DNA endonuclease.

**MATERIALS AND METHODS**

**Bacteria and Bacteriophages**

*Escherichia coli* strains B (Su' ), 1100 (End l-, Su' ), and CR63 (Su' ) were used in these experiments. Bacteriophage T4D and T4uvs were provided by Dr. W. Harm (3). T4uvs-5 and T4uvs-13, having an amber mutation in the *denV* gene, were obtained from Dr. L. van Minderhout (13).

**Chemicals and Enzymes**

Poly(dA)-poly(dT) was purchased from Boehringer Mannheim. [methyl-*H]*TTP and [*-32P]*ATP (carrier-free) were obtained from New England Nuclear. [*-32P]*ATP was obtained from Amersham. NaBH₄ was purchased from Merck-Schuchardt. *E. coli* DNA polymerase I and pancreatic DNase I were purchased from Bethesda Research Laboratories and Sigma, respectively. Bacterial alkaline phosphatase and T4 polynucleotide kinase were purchased from Worthington.

**DNA and Polynucleotides**

T4 [*-32P]*DNA and [*H]*thymine-labeled poly(dA)-poly(dT) were prepared as described previously (1, 12). Single-stranded poly(dT) was obtained by centrifuging [*H]*thymine-labeled poly(dA)-poly(dT) in alkaline CsCl (14). To prepare an intermediate polynucleotide that has AP sites in the vicinity of pyrimidine dimers, UV-irradiated poly(dA)-poly(dT) (640 nmol) was treated with 6.2 units of T4 endonuclease V (Fraction VI) in 2.7 ml of 9.6 mM EDTA/56 mM NaCl/32 mM Tris-HCl (pH 8.5) at 37°C for 30 min. The mixture was shaken with phenol and dialyzed against four 0.5-liter changes of 1 mM EDTA/0.1 mM NaCl/10 mM Tris-HCl (pH 7.5).

**Assay of Enzyme Activities**

Procedure 1—T4 endonuclease V activity was determined by measuring degradation of UV-irradiated T4 [*-32P]*DNA (840 J/m²) in the presence of an extract of T4-infected *E. coli* 1100 (15). One unit of T4 endonuclease V activity was defined as the activity that releases 24 nmol of nucleotide as acid-soluble materials. This assay was used throughout purification of the enzyme.

Procedure 2—The assay of pyrimidine dimer-DNA glycosylase activity depends on formation of alkali-labile sites in UV-irradiated synthetic polymer. The reaction mixture contained 1.0 nmol of UV-irradiated (4200 J/m²) [*H]*polynucleotide-poly(dT) (3.6 × 10⁸ dpm/nmol), 3.2 nmol of Tris-HCl (pH 7.5), 0.96 µmol of EDTA, 1 µmol of NaCl, and an enzyme in 100 µl. Incubation was at 37°C for 20 min, unless otherwise indicated. At the end of incubation, 20 µl of 1 N NaOH was added (final pH 13) and the mixture was further incubated at 37°C for 80 min. After alkali treatment, the mixture was chilled and precipitated with the addition of 30 µl of 50% trichloroacetic acid. After standing for 30 min at 0°C, the sample was centrifuged and the supernatant solution was taken as the acid-soluble fraction. The radioactivity was determined in a liquid scintillation counter.

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The abbreviations used are: AP, apurinic/apyrimidinic; SDS, sodium dodecyl sulfate.
Normal and Mutant Forms of T4 Endonuclease V

Procedure 3—Nicking activity toward UV-irradiated poly(dA)-poly(dT) was assayed under the conditions essentially the same as those for the assay of glycosylase except that alkali treatment was omitted.

Procedure 4—For the assay of AP DNA endonuclease activity, an intermediate polymer that has AP sites near pyrimidine dimers was used as substrate. The conditions and procedures were the same as procedure 3.

Determination of Pyrimidine Dimer

The pyrimidine dimer content of UV-irradiated poly(dA)-poly(dT) was determined by Dowex I column chromatography as described by Sekiguchi et al. (16).

Photoreversal Irradiation

Solution of polymer was irradiated at 0 °C for a given time at a distance of 10 cm from a germicidal lamp (approximate dose, 420 J/m²/min). To 50 μl of the sample, 50 μl of carrier DNA (partially digested salmon sperm DNA, 1 mg/ml) and 250 μl of 95% ethanol were added. After standing at 0 °C for 30 min, the mixture was centrifuged and the ethanol-soluble fraction was taken. To identify the material, the fraction was evaporated by aspiration at 30 °C and dissolved in 10 μl of 50 mM potassium phosphate buffer (pH 7.0). 5 μl of the solution was applied to a cellulose thin layer sheet (Kodak) together with 12 μg each of thymine and deoxythymidine. The sheet was developed in 50 mM potassium phosphate buffer (pH 7.0) for 1 h at room temperature.

Sodium Borohydride Treatment

To a chilled solution of polymer, 0.25 volume of 0.5 M sodium borate and 0.1 volume of NaBH₄ (2 M in 50 mM NaOH) were added. After 2 h in ice, the reaction was terminated by addition of 0.1 volume of H₃PO₄ (17). The polymer was dialyzed against 1 mM EDTA/0.1 M NaCl/10 mM Tris-HCl (pH 7.5) at 0 °C.

Protein Determination

Protein was determined by the Bio-Rad protein assay method (Bio-Rad Technical Bulletin 1051).

Polyacrylamide gel (15%) electrophoresis in the presence of SDS was essentially as described by Laemmli (18).

Phosphorylation of Oligonucleotides

The reaction mixture (100 μl) contained 5 μmol of Tris-HCl (pH 7.5), 1 μmol of MgCl₂, 0.5 μmol of 2-mercaptoethanol, and 1 nmol of [γ-³²P]ATP (1 × 10⁶ cpm/nmol), 0.5 nmol of H-labeled fragments, and various amounts of T4 polynucleotide kinase. After incubation at 37 °C for 30 min, the reaction was terminated by addition of 100 μl of 0.2 M sodium pyrophosphate. The mixture was dialyzed against four 1-liter changes of 200 mM NaCl/10 mM Tris-HCl (pH 7.5) at 0 °C.

Incorporation of dTMP into Incised Polymer

The reaction mixture (450 μl) contained 6.7 μmol of potassium phosphate buffer (pH 7.4), 0.1 μmol of 2-mercaptoethanol, 0.67 μmol of MgCl₂, 0.6 μmol of NaCl, 3.3 nmol of [γ-³²P]ATP (50 μCi/nmol), 3 nmol of incised polymer, and 0.3 unit of E. coli DNA polymerase I. After incubation at 37 °C for various times, 100-μl aliquots were removed and mixed with 100 μl of 1 N HCl in 0.1 M sodium pyrophosphate and dried. The radioactivity was measured in a liquid scintillation counter.

RESULTS

Purification of T4 Endonuclease V

T4 endonuclease V was purified to Fraction III by a modification of the method of Yasuda and Sekiguchi (15) and further purified by a procedure newly developed. Table I summarizes the result of a typical purification from T4D-infected E. coli 1100. Unless otherwise indicated, all operations were carried out at 0–4 °C.

Preparation of Extract—E. coli 1100 cells were grown in M9-casamino acid medium at 37 °C with aeration to a concentration of 1 × 10⁹ cells/ml and infected with T4D at a multiplicity of 5. Fifteen min after infection, the culture was chilled rapidly by pouring onto crushed ice. Cells were collected in a refrigerated centrifuge and the cell paste was stored at −20 °C. Frozen cells (50 g, approximately 2 × 10¹⁵ cells) were suspended in 200 ml of 10 mM Tris-HCl (pH 7.5) containing 1 mM glutathione (reduced form), 10% (v/v) ethylene glycol, 2 mM EDTA and disrupted in an ultrasonic disintegrator. The lysate was centrifuged at 10,000 × g for 30 min and the supernatant fluid (205 ml) was taken as the extract (Fraction I).

Fractional Phase Partition—To 205 ml of Fraction I, 3.35 ml of 20% (w/v) dextran T500, 66 ml of 30% (w/v) polyethylene glycol 6000, and 34.4 g of NaCl were added with continuous stirring. After additional stirring for 60 min, the mixture was centrifuged at 10,000 × g for 30 min and the upper layer was taken and dialyzed against three 2-liter changes of 10 mM potassium phosphate (pH 6.5) containing 10% (v/v) ethylene glycol, 10 mM 2-mercaptoethanol, 2 mM EDTA overnight. The dialyzed solution was centrifuged at 10,000 × g for 30 min and the clear supernatant fluid (494 ml) was taken (Fraction II).

CM-Sephadex Chromatography—Fraction II was applied to a column of CM-Sephadex C-25 (1.5 × 10 cm) equilibrated with PEMS (0.01 M potassium phosphate, pH 6.5/10% (v/v) ethylene glycol/0.01 M 2-mercaptoethanol/2 mM EDTA). After washing the column with 100 ml of PEMS containing 0.15 M KCl, 500 ml of PEMS containing a linear gradient (0.15–0.5 M) of KCl were applied. The flow rate was 15 ml/h and 10 ml of fractions were collected. The enzyme activity appeared after passage of 150 ml of gradient solution. The active fractions (100 ml) were pooled (Fraction III).

Bio-Gel P-10 Gel Filtration—Fraction III was condensed to 8.7 ml with countercurrent against Sephadex G-100 powder and applied to a column of Bio-Gel P-10 (2.0 × 140 cm) that had been equilibrated with 3 liters of PEMS containing 0.5 M KCl. The column was eluted with the same buffer. The flow rate was 10 ml/h and 2 ml of fractions were collected. The enzyme activity was eluted after passage of 170 ml of buffer. Fractions with specific activity greater than 100 units/mg of protein were pooled (Fraction IV, 16 ml).

Phosphocellulose Chromatography—Fraction IV (15 ml) was diluted with PEMS to 0.2 M of KCl and applied to a column of phosphocellulose P11 (0.9 × 15 cm) equilibrated with PEMS containing 0.2 M KCl. The column was washed with 20 ml of the same buffer, followed by a linear gradient of 200 ml of the buffer with limits of 0.2 and 0.6 M of KCl. The flow rate was 8 ml/h and 3 ml of fractions were collected. The enzyme activity appeared at 0.43 M of KCl. Fractions (12 ml) with specific activity greater than 1000 units/mg of protein were pooled (Fraction V).

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Volume</th>
<th>Specific activity</th>
<th>Purification fold</th>
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<td>4449</td>
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<td>1.0</td>
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<td>434</td>
<td>1.38</td>
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<tr>
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<td>586</td>
</tr>
<tr>
<td>V Phosphocellulose</td>
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<td>11</td>
<td>1577</td>
<td>2464</td>
</tr>
<tr>
<td>VI Irradiated DNA</td>
<td>234</td>
<td>8</td>
<td>2630</td>
<td>4109</td>
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</tbody>
</table>

Purification of T4 endonuclease V from T4-infected E. coli 1100
Irradiated DNA-cellulose Chromatography—Fraction V (11 ml) was diluted with PEMS to 0.2 M KCl and applied to a column (0.5 cm X 11 cm) of UV-irradiated DNA-cellulose pre-equilibrated with PEMS containing 0.2 M KCl. After washing the column with 10 ml of the same buffer, 80 ml of PEMS containing a linear gradient of 0.2 to 1.0 M KCl were applied. The enzyme activity appeared after passage of 26 ml of gradient solution. The active fractions were pooled (Fraction IV, 8 ml) and after addition of an equal volume of glycerol, stored at -20°C.

The enzyme was purified about 4,100-fold at the final step of purification (Fraction VI). SDS-polyacylamide gel electrophoresis revealed that Fraction VI contains only a single polypeptide whose molecular weight is approximately 16,000. Some additional bands were detected in less purified preparations (Fig. 1).

The specific activity of the homogeneous preparation of T4 endonuclease V was 2630 units/mg of protein; that is, 1 unit of the enzyme corresponds to about 1.4 x 10^16 molecules of the polypeptide. Since 4450 units of the enzyme were obtained from 2 x 10^10 cells, we estimate that about 3000 molecules of T4 endonuclease V are present in a single T4D-infected cell.

Two Activities Associated with T4 Endonuclease V

Assay Conditions—When poly(dA)-poly(dT) irradiated with UV was incubated with purified preparation (Fraction VI) of T4 endonuclease V, alkali-labile sites were first produced, which were converted to nicks by the secondary reaction. We have shown in a previous paper (12) that the two activities possess different pH optima; the activity to produce alkali-labile sites was optimal in a relatively broad pH range (pH 6.0 to 6.5) whereas the activity to form nicks had a narrow optimum, pH 6.5.

We found, furthermore, that optimum salt concentrations for the two activities were different. As shown in Fig. 2, the activity to produce alkali-labile sites was stimulated when 25 to 100 mM NaCl were present in the reaction mixture while the nicking activity was rather inhibited by NaCl. A similar observation was recently made by other investigators. Thus, by performing a limited reaction with T4 endonuclease V at pH 8.5 in the presence of 50 mM NaCl, irradiated polymer was converted to an intermediate form that carried a large number of alkali-labile sites but only a few nicks. Characterization of the intermediate will be described in a latter section.

Co-purification—Availability of such an intermediate makes it possible to assay the activity responsible for the second reaction, namely AP endonuclease, independent of the activity for the first reaction, pyrimidine dimer-DNA glycosylase. Taking advantage of this, we have determined the two activities in various enzyme fractions. As shown in Table II, the ratio of the endonuclease activity to the glycosylase activity was nearly constant throughout the last three steps of purification. This confirms the notion that the two activities are the integral parts of a single enzyme.

Characterization of an Intermediate

Reduction with Sodium Borohydride—The intermediate produced by a limited reaction with T4 endonuclease V contains many alkali-labile sites and becomes acid-soluble when exposed to alkali (Fig. 3). When, however, the intermediate was treated with sodium borohydride, it became resistant to alkali. The reduced form remained intact even after a prolonged treatment with alkali (Table III).

It has been shown that β-elimination is promoted at alkaline pH when aldehyde radicals, but not reduced alcohol forms, are present in polydeoxyribo nucleotide. It seems, therefore, that there are aldehyde radicals at apyrimidinic sites in the intermediate.

Photoreversal—Radany and Friedberg (7) demonstrated that free thymine was released when irradiated DNA incu...
with NaBH₄. The reduced or nonreduced intermediate was incubated with T4 endonuclease V at pH 8.5 and a portion of the isolated intermediate was reduced with alkali. and then the reaction mixture was acidified.

We examined the effect of photoreversal on the phosphorylation. It was found that an active phosphorylation took place when the fragment had been subjected to photoreversing light and then treated with alkaline phosphatase. As shown in Fig. 5b, extents of phosphorylation increased with increasing doses of UV. No increase in phosphorylation was observed when treatment with phosphatase was omitted. This indicates that the 5'-termini of the products are phosphorylated and that the enzymatic phosphorylation is interfered with by the presence of a thymine dimer at the 5'-terminus.

The 3'-Termini—Generation of nicks in irradiated polymer by T4 endonuclease V did not enhance the priming activity for polydeoxyribonucleotide synthesis by E. coli DNA polymerase I (8). Treatment of the nicked polymer with either phosphatase or alkali alone had no effect for the priming activity. When, however, the incised polymer was treated with alkali and then with phosphatase, a considerable increase in priming activity was observed (Table IV). This result is consistent with the idea that deoxyribose is linked to the 3'-terminus through a phosphodiester bond (see Fig. 3), since the deoxyribose moiety can be removed by alkali-promoted β-elimination.

Characterization of an End Product

An extended incubation of UV-irradiated poly(dA)-poly(dT) with T4 endonuclease V yielded acid-soluble oligonucleotides, whose length is less than 15 nucleotides. We analyzed the termini of such oligonucleotides by using enzymes with known specificity.

The 5'-Termini—T4 polynucleotide kinase catalyzes the transfer of phosphate group from ATP to 5'-hydroxyl termini of oligo- or polynucleotides (19). However, when the oligonucleotide fragments generated by T4 endonuclease V were subjected to the kinase reaction, no phosphorylation took place whether they had been treated with alkaline phosphatase or not (Fig. 5a). Under the conditions used, DNase I-generated fragments which had been treated with phosphatase were rapidly phosphorylated.

Since it was suspected that inaccessibility of polynucleotide kinase to the endonuclease V-generated fragment might be due to the presence of a thymine dimer at the 5'-terminus, we have examined the effect of photoreversal on the phosphorylation. It was found that an active phosphorylation took place when the fragment had been subjected to photoreversing light and then treated with alkaline phosphatase. As shown in Fig. 5b, extents of phosphorylation increased with increasing doses of UV. No increase in phosphorylation was observed when treatment with phosphatase was omitted. This indicates that the 5'-termini of the products are phosphorylated and that the enzymatic phosphorylation is interfered with by the presence of a thymine dimer at the 5'-terminus.

### Table III

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NaBH₄</th>
<th>Alkali</th>
<th>Acid-soluble fraction (%)</th>
<th>Alkali-labile fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>+</td>
<td>12.4</td>
<td>52.6</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>65.0</td>
<td>4.4</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>−</td>
<td>15.1</td>
<td>4.4</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>19.5</td>
<td>4.4</td>
</tr>
</tbody>
</table>

An extended incubation of UV-irradiated poly(dA)-poly(dT) with T4 endonuclease V yielded acid-soluble oligonucleotides, whose length is less than 15 nucleotides. We analyzed the termini of such oligonucleotides by using enzymes with known specificity.

Fig. 6 shows UV sensitivity levels of T4 strains in two types of hosts, E. coli strain B and CR63. Strain CR63 carried a supD mutation and thus is able to suppress an amber mutation(s) in either the host or phage genome whereas strain B

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**Fig. 3. Diagrammatic presentation of enzymic and chemical cleavages of dimer-containing poly(dT).**

**Fig. 4. Release of free thymine from an intermediate on photoreversal irradiation.** a, effect of UV dose. Irradiation with a germicidal lamp was carried out on ice at a distance of 10 cm from the lamp for various times (approximate dose, 420 J/m²/min). ○, UV-irradiated poly(dA)-poly(dT), □, intermediate (UV-irradiated poly(dA)-poly(dT) that had been subjected to a limited reaction with T4 endonuclease V). b, chromatographic identification of thymine. 5 nmol of the intermediate were irradiated for 30 min under the conditions described above and the ethanolic fraction (430 µl) was obtained. The sample was applied to thin layer chromatography, and the sheet was monitored at 254 nm with a densitometer (top). Sections corresponding to 0.05 Rf were cut and the radioactivity in each fraction was determined (bottom). No radioactivity was found in fractions with Rf < 0.5 in the chromatogram (data not shown). Arrows indicate the positions of thymine (left) and thymidine (right).
FIG. 5. Phosphorylation of the 3'-ends created by T4 endonuclease V. a, susceptibility of various DNA fragments. To prepare DNase I-generated fragments, [H]thymine-labeled poly(dT) (50 nmol) was incubated with 180 µg of pancreatic DNase I in 1 ml of 50 mM Tris-HCl (pH 8.3)/10 mM MgCl₂/20 mM CaCl₂ at 37 °C for 1 h. The acid-soluble fraction was collected. Under these conditions, 66% of poly(dT) was converted to acid-soluble forms. After dialysis against two 2-liter changes of 1 mM EDTA/10 mM Tris-HCl (pH 7.5), a half-portion (15 nmol) was treated with 3.85 units of T4 endonuclease V (Fraction VI) at 37 °C for 2 h. The acid-soluble fraction was then taken and the reaction was at 37 °C for 30 min and then shaken with phenol, and the aqueous layer was directly shaken with phenol, and the other was shaken after alkali treatment (pH 13, 1 h at 37 °C). The aqueous layer was dialyzed against two 2-liter changes of 1 mM EDTA/10 mM Tris-HCl (pH 7.5) at 0 °C overnight. One-half of the dialyzed sample (about 20 nmol of polymer) was incubated with 0.5 units of T4 endonuclease V (Fraction VI) in 1.5 ml of 9.6 mM EDTA/32 mM Tris-HCl (pH 7.5)/10 mM NaCl at 37 °C for 2 h. The acid-soluble fraction (59%) was then applied to a small column of CM-Sephadex C-25 (bed volume, 1 ml), followed by washing with 1 ml of 0.15 M KCl. The enzyme was then eluted with 3 ml of PEMS containing 0.5 M KCl. The enzyme was then eluted with 3 ml of PEMS containing 0.5 M KCl (Fraction III).

Partial Purification—To remove interfering enzyme activities, crude extracts of phage-infected cells (5 x 10⁸ cells for each preparation) were processed to a step of phase partition according to the procedure described in a preceding section. The enzyme fraction (Fraction II) was then applied to a small column of CM-Sephadex C-25 (bed volume, 1 ml), and the column was washed with 10 ml of PEMS containing 0.15 M KCl, followed by washing with 1 ml of PEMS containing 0.5 M KCl. The enzyme was then eluted with 3 ml of PEMS containing 0.5 M KCl (Fraction III).

Phosphorylation—To interfere with enzyme activities, crude extracts of phage-infected cells (5 x 10⁸ cells for each preparation) were processed to a step of phase partition according to the procedure described in a preceding section. The enzyme fraction (Fraction II) was then applied to a small column of CM-Sephadex C-25 (bed volume, 1 ml), and the column was washed with 10 ml of PEMS containing 0.15 M KCl, followed by washing with 1 ml of PEMS containing 0.5 M KCl. The enzyme was then eluted with 3 ml of PEMS containing 0.5 M KCl (Fraction III).

Crude extracts were prepared from uninfected cells and from cells infected with various phage strains, and the two enzyme activities associated with T4 endonuclease V were determined. Levels of both glycosylase and AP endonuclease activities in E. coli B cells infected with v, uvs-5, or uvs-13 and CR63 cells infected with v were essentially the same as those of uninfected cells. When the amber mutation of uvs-5 was suppressed by the supD mutation, significant increases in both activities were observed. There was some increase in glycosylase activity but no detectable increase in AP endonuclease in uvs-13-infected CR63 cells. However, an accurate quantification of phage-induced AP endonuclease activity was difficult with crude extracts because such extracts contained substantial amounts of host AP endonuclease activity, which is indistinguishable from the phage enzyme under the present assay conditions.

Enzyme Activities—Fig. 8 shows pyrimidine dimer-DNA glycosylase and AP endonuclease activities in these enzyme fractions. Both activities were almost completely absent from the samples of E. coli B cells infected with T4v, uvs-5, or uvs-

Crude extracts were prepared from uninfected cells and from cells infected with various phage strains, and the two enzyme activities associated with T4 endonuclease V were determined. Levels of both glycosylase and AP endonuclease activities in E. coli B cells infected with v, uvs-5, or uvs-13 and CR63 cells infected with v were essentially the same as those of uninfected cells. When the amber mutation of uvs-5 was suppressed by the supD mutation, significant increases in both activities were observed. There was some increase in glycosylase activity but no detectable increase in AP endonuclease in uvs-13-infected CR63 cells. However, an accurate quantification of phage-induced AP endonuclease activity was difficult with crude extracts because such extracts contained substantial amounts of host AP endonuclease activity, which is indistinguishable from the phage enzyme under the present assay conditions.

Partial Purification—To remove interfering enzyme activities, crude extracts of phage-infected cells (~5 x 10¹¹ cells for each preparation) were processed to a step of phase partition according to the procedure described in a preceding section. The enzyme fraction (Fraction II) was then applied to a small column of CM-Sephadex C-25 (bed volume, 1 ml), and the column was washed with 10 ml of PEMS containing 0.15 M KCl, followed by washing with 1 ml of PEMS containing 0.5 M KCl. The enzyme was then eluted with 3 ml of PEMS containing 0.5 M KCl (Fraction III).

Possesses no suppressor. T4v, which is defective in the denV gene (3), was more sensitive to UV than T4D wild type in either type of hosts. T4uvs-5 and T4uvs-13, each of which has an amber mutation in the denV gene (13), were as sensitive as T4v, when plated on E. coli strain B (Su) but exhibited normal or intermediate sensitivity on strain CR63 (Su).
Normal and Mutant Forms of T4 Endonuclease V

Two distinct catalytic activities, a pyrimidine dimer-DNA glycosylase and an AP endonuclease. It would therefore appear that both glycosylase and AP endonuclease activities are associated with a single polypeptide chain.

Genetic evidence in support of this idea was also presented in this paper. A partially purified enzyme fraction from cells infected with T4us, a mutant that is defective in the denV gene, was deficient in both activities. Moreover, occurrence of an amber mutation in the denV gene caused a simultaneous loss of the two activities. When the amber mutation in T4us 5 was suppressed by the supD mutation of host bacteria, both activities were recovered.

The two activities associated with T4 endonuclease V act on UV-irradiated DNA in a sequential manner. First, the glycosylase cleaves the glycosyl bond of one of the pyrimidine residues of a dimer to yield an AP site in the DNA, and then the AP endonuclease hydrolyzes the phosphodiester bond near the AP site. The presence of AP sites in the intermediate was evidenced by the fact that reduction with sodium borohydride decreased greatly the sensitivity of the intermediate to alkaline. In addition, it was shown that free thymine was released from the intermediate on direct photo-reversal, indicating that one of the thymine residues of a dimer is free from the corresponding deoxyribose. The end product of T4 endonuclease V reactions was characterized by using appropriate enzymes, and the results obtained were consistent with the idea that the enzyme cleaves the phosphodiester bond between the nucleotides of a pyrimidine dimer to yield 3'-hydroxyl and 5'-phosphoryl termini on the 3'-side of the AP site (see Fig. 3). Such a model for the mechanism of incision has been proposed for Micrococcus luteus UV endonuclease by Grossman and co-workers (5, 6), who analyzed incised DNA fragments by gel electrophoresis. Recent studies with T4 endonuclease V also supported this view (7-11). In view of the remarkable similarity in the properties of T4 endonuclease V and M. luteus UV endonuclease (1, 20-22), there is a possibility that the M. luteus enzyme also carries two activities in a single molecule although definite evidence to show this has not been available.

It has been shown that the AP endonuclease activity associated with T4 endonuclease V is more thermolabile than is the glycosylase activity associated with the same enzyme (12). It is also noted that the optimum pH and ionic strength for the two activities are different; the glycosylase is active in a relatively broad pH range, from 6.0 to 8.5, and stimulated by the addition of 50 mm NaCl whereas the AP endonuclease has a narrow pH optimum, near pH 6.5, and is rather inhibited by NaCl. These results imply that there are two distinct domains in a single enzyme, one for the glycosylase and the other for the endonuclease.

A partially purified preparation of endonuclease V derived from T4us-infected E. coli CR63 contained a glycosylase but not an AP endonuclease activity. Despite this defective-ness, the size of the mutant enzyme was similar to that of the wild type enzyme. A reasonable interpretation of these findings is that the two activities are handled by different regions of a polypeptide chain. Suppression of an amber mutation sometimes causes substitution of a single amino acid at the site of mutation. If such substitution occurred within a domain, one of the activities might be lost. On the other hand, when a right amino acid or one that exerts an analogous function has been inserted by suppression, both activities may be recovered. The latter might be the case for T4us-infected E. coli CR63.

It was noted that uvs-13 is only partly suppressed in terms of UV sensitivity. Since E. coli possesses a large amount of AP endonucleases but no equivalent glycosylase activity, it is

*Fig. 7. SDS-polyacrylamide gel analysis of partially purified preparations (Fraction III) from cells infected with various T4 strains.* Gel electrophoresis was carried out as described in Fig. 1, and proteins were quantified by densitometric tracing of Coomassie brilliant blue-stained gels. Dotted lines indicate the position of the 16,000-dalton polypeptide (T4 endonuclease V). Numbers below indicate molecular weight standards: 25,000, chymotrypsinogen; 17,000, myoglobin; 13,000, cytochrome c.

*Fig. 8. Enzyme activities in partially purified preparations (Fraction III) from various infected cells.* a, T4D-infected E. coli B b, T4D-infected E. coli CR63 c, T4us-infected E. coli B, d, T4us-infected E. coli CR63, e, T4us-5-infected E. coli B, f, T4us-5-infected E. coli CR63, g, T4us-13-infected E. coli B, h, T4us-13-infected E. coli CR63. o, pyrimidine dimer-DNA glycosylase; 0, AP endonuclease.
likely that the host endonucleases can substitute the missing phage endonuclease. The intermediate UV sensitivity of *uus-13* in CR63 cells might be due to a decreased level of glycosylase activity in the mutant enzyme.

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Purification and characterization of normal and mutant forms of T4 endonuclease V.

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