modynamics alone would have been extremely difficult.

Supplementary Material Available

One figure showing melting curves of poly(A) in 5 mol % propanol and 10 mol % ethanol; three figures showing Eyring plots of poly(A) in 5 mol % ethanol, 5 mol % urea, and 10 mol % ethanol for both the two-state and kinetic Ising models; and eight figures of absorption and circular dichroism spectra at three temperatures for poly(A) in water, 5 mol % urea, 10 mol % ethanol, and 10 mol % glycerol (12 pages). Ordering information is given on any current masthead page.

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Binding of T4 Endonuclease V to Deoxyribonucleic Acid Irradiated with Ultraviolet Light†

Patricia C. Seawell, Thomas J. Simon,‡ and Ann K. Ganesan*

ABSTRACT: Endonuclease V of bacteriophage T4 binds to UV-irradiated deoxyribonucleic acid (DNA) but not to unirradiated DNA. We have developed an assay to detect this binding, based on the retention of enzyme–DNA complexes on nitrocellulose filters. The amount of complex retained, ascertained by using radioactive DNA, is a measure of T4 endonuclease V activity. The assay is simple, rapid, and specific, which makes it useful for detecting T4 endonuclease V activity both in crude lysates and in purified preparations. We have used it to monitor enzyme activity during purification and to study binding of the enzyme to DNA under conditions that minimize the ability of the enzyme to nick DNA. From our data we conclude that (1) T4 endonuclease V binds to UV-irradiated DNA but not to DNA that has been previously incised by the endonuclease, (2) equilibrium between the free and complexed form of the enzyme is attained under our reaction conditions, (3) dissociation of enzyme–DNA complexes is retarded by sodium cyanide, and (4) retention of enzyme–DNA complexes on nitrocellulose filters is enhanced by high concentrations of saline–citrate.

Endonuclease V of bacteriophage T4 is an important biochemical probe for identifying and quantitating pyrimidine dimers in DNA. It produces nicks in DNA strands containing dimers, making approximately one incision for each dimer (Friedberg & King, 1969, 1971; Yasuda & Sekiguchi, 1970; Simon et al., 1975). To complement studies of the incising properties of T4 endonuclease V (Minton et al., 1975; Yasuda & Sekiguchi, 1976), we have developed an assay to measure the binding of the enzyme to DNA. The assay, a modification of previously described procedures (Jones & Berg, 1966; Riggs et al., 1970; Hinkle & Chamberlin, 1972; Madden et al., 1973; Braun & Grossman, 1974), involves a binding reaction in which enzyme and radioactive, UV-irradiated DNA are mixed under conditions that inhibit incision but permit the endonuclease to bind to UV-irradiated DNA. After dilution to prevent formation of new enzyme–DNA complexes the mixture is filtered through nitrocellulose filters that retain enzyme–DNA complexes but not free DNA. The amount of

† From the Department of Biological Sciences, Stanford University, Stanford, California 94305. *Received October 26, 1979*. This work was supported by a grant from the National Institutes of Health (GM 19010).

‡ Present address: Department of Medicine, University of California Medical Center, San Diego, CA 92103.

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radioactive DNA retained provides a measure of T4 endonuclease V binding activity. For clarity we use the term *binding* to indicate the formation of complexes between T4 endonuclease V and UV-irradiated DNA and the term *retention* to indicate trapping of the complexes on filters. For retention to occur, the enzyme must bind to DNA; however, under certain conditions not all enzyme–DNA complexes are retained. In this report we describe the assay and discuss some of the factors that affect binding and retention.

Experimental Procedure

(*I*) *Crude Lysates of T4-Infected Escherichia coli.* Phage stocks were prepared (Little, 1973) with *E. coli* CR63 as the host for the amber mutant T4amN82-rPT8-nd28x6 (Snustad et al., 1976) and *E. coli* B41 for the wild-type (T4D) and the endonuclease V deficient mutant (T4*denV1*) (Harm, 1963; Wood & Revel, 1976).

Lysates were prepared from *E. coli* B41 grown at 37 °C to a density of 5×10^8 cells/mL in 1% tryptone broth (Difco) containing 0.5% NaCl and 1 μg/mL thiamine hydrochloride. The culture was divided into four 200-mL portions. A control portion was left uninfected, while the other three were infected with T4D, T4*denV1*, and T4amN82-rPT8-nd28x6, respectively, at a multiplicity of infection of 5. After 15 min of incubation, chloramphenicol was added to a final concentration of 150 μg/mL. Five minutes later the cells were chilled on ice, harvested by centrifugation, washed and resuspended at a density of 10^{10} cells/mL in 50 mM Tris-HCl (pH 7.5) containing 10% sucrose, quick frozen in a dry ice–acetone bath, and stored overnight at −20 °C. Cells of *E. coli* B62 (*tonA*) infected with T4amN82 (New England BioLabs, Beverly, MA) were resuspended (0.78 g/10 mL) in the Tris–sucrose buffer and processed along with the other cells. The cells were lysed with lysozyme (Wickner et al., 1972) and the supernatants, clarified by centrifugation, were stored at 4 °C.

(*II*) *T4 Endonuclease V.* The enzyme used in these studies was obtained from cells of *E. coli* B62 infected with T4amN82, lysed with lysozyme in 50 mM Tris-HCl (pH 7.5) containing 10% sucrose. Further purification (~300-fold) of the lysate was achieved by chromatography through DEAE-cellulose[1] and phosphocellulose (Seawell et al., 1981). In the presence of 10 mM EDTA, the enzyme preparation contained no detectable nuclease activity against undamaged DNA as determined by nicking of superhelical DNA (Seawell & Ganesan, 1981), and it was also free of activity against uracil-containing DNA from bacteriophage PBS-2 (a gift of Dr. Stuart Linn, University of California, Berkeley, CA) as determined by the release of acid-soluble [^{3}H]uracil. However, the preparation did incise apurinic DNA and DNA treated with methyl methanesulfonate (Nishida et al., 1976). The protein concentration of the enzyme preparations, quantified by the Bio-Rad protein assay (Bio-Rad Laboratories Technical Bulletin 1051) with bovine serum albumin as the standard, ranged from 0.011 mg/mL to 0.8 mg/mL.

(*III*) *Isolation of DNA.* ColE1 plasmid DNA was isolated from AG118 (kindly provided by Dr. Ray Rodriguez, University of California, Davis, CA). This strain is a ColE1 transformant of AB2487, F⁻ *recA13 thyA16 drm-1 thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 supE44* (Bachmann, 1972). Cultures were grown at 37 °C in Difco Bacto Minimal Broth Davis containing 0.4% glucose, 0.05% vitamin-free casein hydrolysate (Nutritional Biochemical Co.), 10^{-3} M L-threonine, L-leucine, L-arginine, L-proline, and L-histidine, respectively, 0.5 μg/mL thiamine hydrochloride, and 8×10^{-5} M thymine (10 μg/mL) to obtain nonradioactive DNA. To obtain radioactive DNA, we used a concentration of 3.6×10^{-5} M (4.6 μg/mL) 2-[^{14}C]thymine (New England Nuclear, 54.6 Ci/mol). ColE1 DNA was amplified by the addition of chloramphenicol (Clewell, 1972) (150 μg/mL) to exponentially growing cultures at a density of 5×10^8 cells/mL. After 16–20 h of incubation, cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl (pH 8) containing 25% (w/v) sucrose, and lysed (Clewell & Helinski, 1969). Equilibrium sedimentation of the cleared lysate in CsCl gradients containing ethidium bromide separated the superhelical plasmid DNA from host DNA and allowed the DNA to be visualized by long-wave UV (Mineralite, UV Products, San Jose, CA). The superhelical DNA was collected and the dye removed by shaking the DNA solution gently with 5–8 equal volume changes of 2-propanol which had been previously equilibrated with buffer [10 mM Tris-HCl (pH 8), 100 mM NaCl] saturated with CsCl. The DNA was then dialyzed against the Tris–NaCl buffer. Exposure to light of wavelengths below 500 nm was minimized from the time of addition of ethidium bromide to the DNA until after dialysis. Without this precaution, a significant fraction of the unirradiated DNA was sensitive to our T4 endonuclease V preparation. The specific activity of ColE1 DNA varied between 4.8×10^4 and 5.5×10^4 cpm/μg.

(*IV*) *Sonication of DNA.* ColE1 DNA was fragmented by sonication with the microprobe of a Branson sonifier cell disrupter, Model 200. One milliliter of DNA solution (6 μg/mL) in a 12 × 75 mm polystyrene tube in an ice bath was exposed to half-second pulses (intensity setting 7) alternating with half-second pauses for 5 min. The average molecular weight of the fragments was determined by electrophoresis through a 6% polyacrylamide slab gel (Bolivar et al., 1977a) calibrated with HpaII restriction fragments of pBR322 DNA (Bolivar et al., 1977b; Sutcliffe, 1978).

(*V*) *Cleavage of DNA by Restriction Endonucleases.* Superhelical ColE1 DNA was converted to the linear form by incubation with EcoRI (New England BioLabs) in 50 mM Tris-HCl (pH 7.5) containing 50 mM NaCl and 5 mM $MgCl_2$. The reaction, containing 5 μg of DNA and 50 units of EcoRI, was incubated at 37 °C for 30 min and then terminated by the addition of EDTA (50 mM final concentration). Total conversion to the linear form was verified by agarose gel electrophoresis (Seawell & Ganesan, 1981).

(*VI*) *Equilibrium Sedimentation in CsCl.* When it was necessary to reisolate ColE1 DNA after treatment with T4 endonuclease V, the DNA was sedimented to equilibrium in neutral CsCl. To recover radioactive DNA we added the DNA to 2 mL of NET and adjusted the density to 1.707 g/mL with CsCl. The mixture was centrifuged for 24 h at 35 000 rpm, 20 °C, in a Beckman SW50.1 rotor. Fractions were collected and sampled for radioactivity. The peak fractions were pooled and dialyzed twice against 1 L of NET. To recover nonradioactive DNA we dissolved it in 3 mL of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, containing 400 μg/mL propidium diiodide. The density was adjusted to 1.55 g/mL with CsCl, and the mixture was centrifuged for 24 h at 38 000 rpm, 20 °C, in a Beckman SW50.1 rotor. The superhelical or nicked circular DNA was visualized and collected, and the dye was removed as described under section III above.

(*VII*) *Ultraviolet Irradiations.* The incident dose rate of the source, an unfiltered 15-W germicidal lamp, was measured

[1] Abbreviations used: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; NET, 0.1 M NaCl–0.01 M EDTA–0.01 M Tris (pH 8); SSC, 0.15 M NaCl–0.015 M trisodium citrate; Tris, tris(hydroxymethyl)aminomethane.

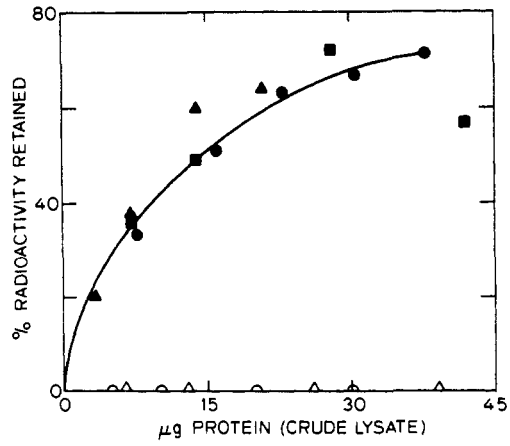

FIGURE 1: Retention of UV-irradiated DNA depends upon T4 endonuclease V. Standard binding reactions containing 105 ng of [^{14}C]ColE1 DNA (4990 cpm) irradiated with 200 J/m^2 were incubated with increasing amounts of crude lysates of uninfected *E. coli* cells (○) or cells infected with T4D (▲), T4amN82 (●), T4amN82-rPT8-nd28x6 (■), or T4*denV1* (△). The data have been corrected for retention of unirradiated DNA (≤100 cpm) and expressed as percent of input radioactivity retained.

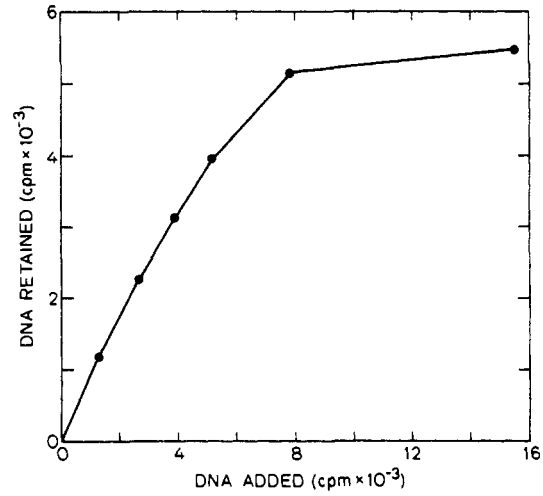

FIGURE 2: Efficiency of retention of irradiated DNA. Standard binding reactions containing various amounts of [^{14}C]ColE1 DNA (55 cpm/ng) irradiated with 200 J/m^2 (18 dimers per molecule) were incubated with a constant amount of T4 endonuclease V. Each point is the average of duplicate samples.

by an IL254 germicidal photometer (International Light, Newburyport, MA) and adjusted with perforated grilles to 0.07 or 0.9 J/(m^2 s) (the lower rate being used for doses ≤50 J/m^2). Solutions of DNA (3.8–7.5 μg/mL) 1–2 mm deep were irradiated in depressions of a glass palette on a rotating platform shaker. Under these conditions the fraction of thymine residues converted to pyrimidine dimers was 4.2×10^{-5} per J/m^2, corresponding to approximately 0.09 pyrimidine dimers per ColE1 molecule per J/m^2 (P. C. Seawell, J. C. Hunt, and A. K. Ganesan, unpublished experiments).

(VIII) Binding Assay. The standard binding assay consists of three stages: (1) incubation of T4 endonuclease V with irradiated DNA in reaction buffer, (2) dilution of the reaction mixture, and (3) filtration of the mixture through nitrocellulose filters. Before each experiment, filters (Millipore, HAWP, 25-mm diameter) were floated on the surface of distilled water, and those that did not wet rapidly and uniformly were discarded; the rest were submerged in water until used. Stainless steel filter holders and chimneys (Tracer Labs) were chilled on ice and rinsed with cold distilled water between samples. The standard reaction contained approximately 100 ng of [^{14}C]ColE1 DNA in 0.3 mL of reaction buffer [9 mM Tris-HCl (pH 8), 90 mM NaCl, 9 mM EDTA, and 10% (w/v) ethylene glycol]. After enzyme addition the mixture was shaken briefly and then incubated for 3 min in an ice water bath. The reaction was diluted with 5 mL of ice-cold 14× SSC (2.1 M NaCl and 0.21 M trisodium citrate) (Marmur, 1961) and filtered at a flow rate of 2–3 mL/min. The filter was then rinsed with an additional 5 mL of 14× SSC and dried. The amount of radioactivity retained on the filter was determined by liquid scintillation spectrometry. Under these conditions, T4 endonuclease V produced less than one incision for every 10 pyrimidine dimers in the DNA as determined by nicking of superhelical DNA (Seawell & Ganesan, 1981).

Results

(I) Binding Is Specific for T4 Endonuclease V. Several lines of evidence indicate that the retention we measure depends upon the formation of complexes between T4 endonuclease V and UV-irradiated DNA. (A) In the presence of 9 mM EDTA we observed retention of UV-irradiated DNA incubated with crude lysates of *E. coli* infected with any of three derivatives of bacteriophage T4 that produce endonuclease V: T4D, T4amN82, and T4amN82-rPT8-nd28x6 (Figure 1). In contrast, no retention was detected after incubation with lysates of uninfected cells or cells infected with T4*denV1* (Figure 1), a mutant that does not produce T4 endonuclease V (Harm, 1963; Friedberg & King, 1969). Less than 5% of unirradiated DNA was retained after incubation with any of the crude lysates of Figure 1 or with purified T4 endonuclease V (data not shown). However, retention of unirradiated DNA was detected after incubation with each of the crude lysates when Mg^{2+} replaced EDTA in the binding reaction (data not shown). (B) During purification of T4 endonuclease V through DEAE-cellulose and phosphocellulose the binding assay revealed only one peak of activity (Seawell et al., 1981). This peak coincided with the peak of incising activity of T4 endonuclease V as determined by nicking of irradiated superhelical DNA in the presence of EDTA (Seawell & Ganesan, 1981). (C) The amount of DNA retained depended upon the concentration of T4 endonuclease V when crude extracts were assayed (Figure 1) and when purified preparations were studied.

(II) Retention Depends upon the Concentration of Substrate DNA. The amount of DNA retained on filters increased with the concentration of irradiated DNA in reactions containing excess T4 endonuclease V. From the initial slope of the curve in Figure 2 the efficiency of retention of ColE1 DNA irradiated with 200 J/m^2 (18 dimers per molecule) was calculated to be 90%. In other experiments the value varied between 70 and 100%, depending upon the batch of filters used. A similar phenomenon has been observed with other binding reactions (Jones & Berg, 1966; Riggs et al., 1970; Yarus & Berg, 1970; Hinkle & Chamberlin, 1972) and may reflect different efficiencies of retention of protein–DNA complexes by different batches of filters. For this reason we usually designed each experiment to require no more than one box of filters.

(III) Retention Depends upon the Average Number of Pyrimidine Dimers per Substrate DNA Molecule. The amount of DNA retained on filters after incubation with T4 endonuclease V increased with the dose of UV to which the DNA had been exposed (Figure 3). These data indicate that the probability of a DNA molecule being retained by a nitrocellulose filter depends upon the number of pyrimidine dimers the molecule contains and thus the number of sites to

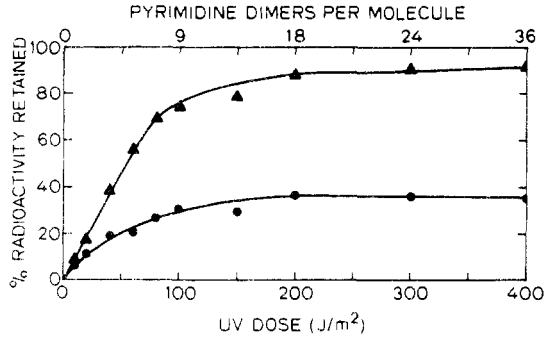

FIGURE 3: Retention of DNA depends upon the average number of pyrimidine dimers per molecule. Standard binding reactions containing 105 ng of [^{14}C]ColE1 DNA (4990 cpm) irradiated with various doses of UV were incubated with T4 endonuclease V activity present in 55 ng (●) or 200 ng (▲) of protein.

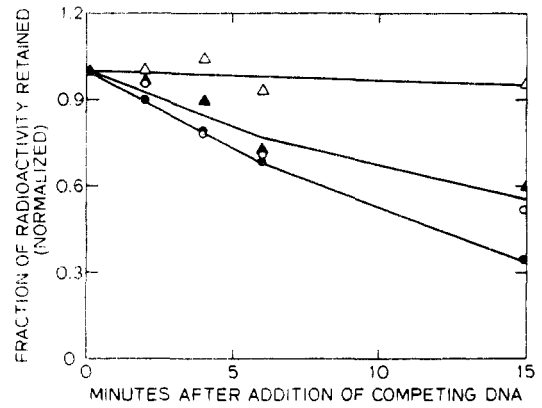

FIGURE 4: Effect of competing nonradioactive DNA on retention of radioactive DNA. Standard binding reactions containing 66 ng of [^{14}C]ColE1 DNA (2700 cpm) irradiated with 200 J/m^2 were incubated with a limiting amount of T4 endonuclease V. After 3 min nonradioactive ColE1 DNA was added and incubation continued for various periods of time before dilution with 14× SSC [containing 0.02 M NaCN to retard dissociation of the enzyme from DNA (see Results, section VII)]. Competing DNA: 126 ng, irradiated with 20 J/m^2 (△); 1260 ng, irradiated with 20 J/m^2 (○); 126 ng, irradiated with 200 J/m^2 (▲); 1260 ng, irradiated with 200 J/m^2 (●). Each point is the average of duplicate samples and has been normalized to the retention of [^{14}C]DNA in the absence of competing DNA (1350 cpm).

which endonuclease can bind. If one enzyme molecule bound to a DNA molecule were sufficient to cause retention of that DNA molecule, according to the Poisson distribution 63% of a DNA population containing an average of one dimer per molecule should be retained by saturating amounts of endonuclease. However, after a dose of 10 J/m^2, which produces approximately 0.9 pyrimidine dimers per molecule of ColE1, only 7% of the DNA was retained (Figure 3, ▲). Because the relationship between the fraction of DNA retained and the dimer content of the DNA was linear from the origin, we concluded that each dimer or, by inference, each molecule of bound T4 endonuclease V contributes equally to the probability that a DNA molecule is retained. From the data in Figure 3 we calculated a probability of approximately 0.1 per enzyme molecule bound.

Although DNA molecules containing only a few pyrimidine dimers were not efficiently retained, competition experiments indicated that T4 endonuclease V does bind to them (Figure 4). Nonradioactive DNA containing an average of 1.8 pyrimidine dimers per molecule (irradiated with 20 J/m^2) competed for limiting amounts of endonuclease as effectively as one-tenth the amount of DNA containing an average of 18 dimers per molecule (irradiated with 200 J/m^2) (Figure 4, compare ○ and ▲).

Table I: Effect of DNA Conformation and Size upon Retention[a]

DNA	UV dose (J/m^2)	pyrimidine dimers per molecule (or fragment)	radioact retained (cpm)
superhelical	0	0	42
(4.2 × 10^6)	20	1.8	992
	40	3.6	2086
	60	5.4	2382
linear	0	0	57
(4.2 × 10^6)	20	1.8	848
	40	3.6	1872
	60	5.4	2772
sonicated	0	0	741
(2.4 × 10^5)	350	1.8	1339
	700	3.6	1706
	1051	5.4	2192

[a] Standard binding reactions containing 63 ng of [^{14}C]ColE1 DNA (3260 cpm) irradiated with various UV doses were incubated with a constant amount of T4 endonuclease V sufficient to saturate the DNA irradiated with the highest UV dose. The data are averages of duplicate samples.

The effect of the UV dose upon retention appears to be independent of the DNA conformation. Similar retention was observed with linear and superhelical DNA containing the same average number of pyrimidine dimers per molecule (Table I). Linear DNA reduced by sonication to an average molecular weight of 2.4 × 10^5 required approximately the same number of dimers per fragment for retention as did full-length molecules with a molecular weight of 4.2 × 10^6 (Table I).

The slope of the UV dose–response curve and the pyrimidine dimer content required for maximum retention varied with different batches of filters. However, DNA containing an average of 18 pyrimidine dimers per molecule was maximally retained by all filters tested.

(IV) Dynamic Equilibrium Is Attained between Free and Complexed Enzyme. Binding of the purified preparation of enzyme to irradiated DNA at 0 °C reached its maximum within 3 min, after which time a gradual decrease was observed (data not shown). To determine whether a dynamic equilibrium was reached between free enzyme and enzyme bound to irradiated DNA, we performed a competition experiment (Figure 4). A limiting amount of T4 endonuclease V was used in this experiment to ensure that all the enzyme present in the reaction mixture was bound to radioactive substrate DNA when the competing DNA was added. When the total number of pyrimidine dimers contributed by nonradioactive DNA was 20 times greater than the number present in the radioactive DNA, we observed a significant loss of retained radioactivity (Figure 4, ●). In contrast, when the total number of dimers present in the nonradioactive DNA was relatively small, little loss of retained radioactivity occurred (Figure 4, △). These results indicate that enzyme–DNA complexes are formed and dissociated reversibly at 0 °C.

(V) Irradiated DNA Incised by T4 Endonuclease V Is No Longer a Substrate to Which the Enzyme Can Bind. No retention was observed when irradiated DNA incised by T4 endonuclease V was subsequently used as the substrate in the standard binding assay (Table II), nor did incised DNA compete effectively with unincised, irradiated DNA for limiting amounts of the enzyme (Table III). Thus, T4 endonuclease V does not appear to bind to pyrimidine dimers at or near incision sites in DNA.

(VI) Retention Depends upon the Composition of the Dilution Buffer. Increasing the concentration of NaCl and trisodium citrate in the dilution buffer increased the amount

Table II: Effect of Incision by T4 Endonuclease V upon Retention of UV-Irradiated DNA[a]

UV dose (J/m²)	incision	radioact retained (% of total)
0	−	0
50	−	46
50	+	0
100	−	59
100	+	1

[a] [^{14}C]ColE1 DNA was irradiated, incised by T4 endonuclease V, and reisolated by equilibrium sedimentation in CsCl. The reisolated DNA was tested as a substrate for T4 endonuclease V in the standard binding assay. Unirradiated DNA treated with T4 endonuclease V was also reisolated and tested as a substrate with or without irradiation. The data are averages of duplicate samples and have been corrected for retention of unirradiated DNA in the presence of enzyme (<2% of input DNA). Input DNA varied between 4900 and 5710 cpm.

Table III: Effect of Incision of UV-Irradiated DNA by T4 Endonuclease V upon the Subsequent Ability of the DNA to Compete for Limited Endonuclease[a]

competing DNA		substrate DNA retained	
first UV dose	second UV dose	cpm	% of max
no competing DNA		996	58
−	−	916	53
+	−	1165	68
−	+	573	33

[a] Unirradiated and irradiated (1.7 J/m²) nonradioactive competing DNA was treated with T4 endonuclease V and centrifuged in CsCl-propidium diiodide gradients; superhelical DNA was isolated from the former and nicked circles were isolated from the latter. One portion of the superhelical DNA was then irradiated with 1.7 J/m². The substrate DNA, 57 ng of [^{14}C]ColE1 (5360 cpm) irradiated with 50 J/m², was mixed with 1200 ng of competing DNA before addition of a limiting amount of T4 endonuclease V. Under the conditions used, saturating amounts of the enzyme (with no competing DNA) resulted in the retention of 1720 cpm.

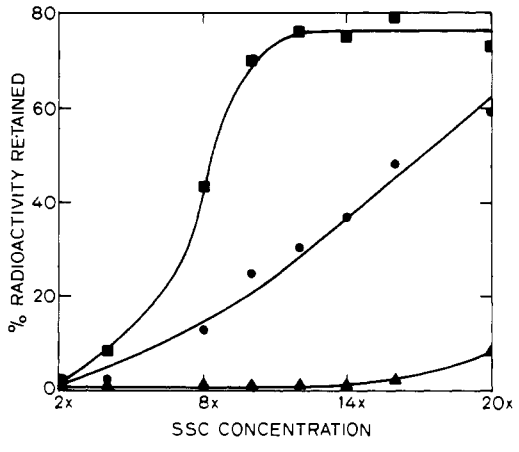

FIGURE 5: Effect of the concentration of dilution buffer on retention. Standard binding reactions containing 101 ng of [^{14}C]ColE1 DNA (4990 cpm) irradiated with 200 J/m² were incubated with T4 endonuclease V activity in 0 (▲), 50 (●), or 200 (■) ng of protein. Reactions were diluted by the addition of 5 mL of various concentrations of buffer (expressed as multiples of SSC). Filters were rinsed with an additional 5 mL of buffer.

of retention observed (Figure 5). With saturating levels of T4 endonuclease V, maximum retention was observed at a concentration of buffer 12-fold higher than that of SSC (12× SSC). With lower enzyme levels higher concentrations of

Table IV: Effect of Composition of Dilution Buffer upon Retention[a]

dilution buffer	radioact retained (% of total)
1.5 M NaCl	21.4
1.65 M NaCl	34.0
0.15 M trisodium citrate	9.2
1.5 M NaCl + 0.15 M trisodium citrate (10× SSC)	58.5
0.15 M Na_2EDTA	13.1
1.5 M NaCl + 0.15 M Na_2EDTA	54.2

[a] Standard binding reactions containing 101 ng of [^{14}C]ColE1 DNA (4990 cpm) irradiated with 200 J/m², and saturating amounts of T4 endonuclease V, were diluted with 5 mL of the indicated buffer, filtered immediately, and rinsed with an additional 5 mL of buffer.

Table V: Effect of Buffer Concentration upon Retention of DNA-Enzyme Complexes by Nitrocellulose Filters[a]

dilution buffer	filtration buffer	min in dilution buffer	radioact retained (% of total)	
			expt 1	expt 2
7× SSC	7× SSC	0	2	
		3	2	
		6	2	
14× SSC	7× SSC	0	2	
		3	2	
		6	2	
7× SSC	14× SSC	0	30	
		3	21	
		6	13	
14× SSC	14× SSC	0	31	15
		3	21	11
		6	14	9
NET	14× SSC	0		18
		3		14
		6		13

[a] Standard reactions containing 93 ng of [^{14}C]ColE1 DNA (5120 cpm) irradiated with 200 J/m², and limiting amounts of T4 endonuclease V, were diluted with 3 mL of dilution buffer. After 0, 3, or 6 min additional buffer was added to adjust the concentration to that of the filtration buffer. The mixture was immediately filtered and the filters were rinsed with an additional 5 mL of filtration buffer: 7× SSC, 1.05 M NaCl-0.105 M trisodium citrate; 14× SSC, 2.1 M NaCl-0.21 M trisodium citrate; NET, 0.1 M NaCl-0.01 M EDTA-0.01 M Tris-HCl (pH 8).

dilution buffer were needed to maximize the retention observed (Figure 5). Retention of irradiated DNA in the absence of enzyme began to increase at 16× SSC. Unirradiated DNA, with or without enzyme, was not retained in the presence of SSC concentrations up to 18×. Thus, in order to maximize enzyme-specific retention, we chose 14× SSC for standard use.

NaCl and trisodium citrate act synergistically in the dilution buffer; the retention observed when both were present was greater than the sum of the retention observed with the two separately (Table IV). Na_2EDTA can substitute for trisodium citrate (Table IV). No retention was observed when 10× or 14× SSC was used as the reaction buffer (data not shown).

The concentration of NaCl and trisodium citrate in the dilution buffer appears to affect retention primarily by altering the efficiency with which DNA–enzyme complexes are trapped on nitrocellulose filters rather than by influencing the rate of dissociation of the complexes. Dilution of binding reactions with NET, 7× SSC, or 14× SSC resulted in the same amount of DNA being retained provided that the diluted reactions were filtered at the same time after dilution and were adjusted to

Table VI: Effect of NaCN on the Binding of T4 Endonuclease V to Irradiated DNA[a]

	NaCN in standard reaction	additions after 3 min		incubation after additions (min)	radioact retained (cpm)
		NaCN	competing DNA		
A	–	–	–	0	1600
	–	–	–	10	1430
B	+	–	–	0	1440
	+	–	–	10	1520
C	–	–	+	0	1410
	–	–	+	10	400
D	–	+	+	0	1350
	–	+	+	10	1330

[a] Standard reaction mixtures containing [^{14}C]ColE1 DNA (50 ng, 3040 cpm) irradiated with 200 J/m^2 were incubated with limiting amounts of T4 endonuclease V (80 ng of protein) either with (B) or without (A, C, and D) 0.02 M NaCN. After 3 min of incubation the following additions were made: none (A and B); nonradioactive competing DNA (500 ng; 200 J/m^2) (C); NaCN (0.02 M final concentration), followed by nonradioactive competing DNA (500 ng; 200 J/m^2) (D). Reactions were diluted immediately or after 10 min with 5 mL of 14× SSC containing 0.02 M NaCN, filtered, and rinsed with an additional 5 mL of 14× SSC + 0.02 M NaCN. The data are averages of duplicate samples.

14× SSC immediately prior to filtration (Table V). The amount of radioactivity retained decreased when diluted reactions were not filtered immediately (Table V), indicating that dissociation of enzyme–DNA complexes occurred. The rate of dissociation appeared to be the same in NET, 7× SSC, and 14× SSC. In contrast, when samples were adjusted to 7× SSC prior to filtering, no significant retention was observed whether reactions had been diluted originally with 7× SSC or 14× SSC (Table V).

(VII) Cyanide Inhibits the Dissociation of Enzyme–DNA Complexes. Although cyanide inhibits incision of UV-irradiated DNA by T4 endonuclease V (P. Seawell, unpublished experiments), it does not cause a commensurate reduction in binding of the enzyme to DNA. However, it appears to inhibit the dissociation of enzyme–DNA complexes. The addition of 0.02 M NaCN to standard binding reactions did not alter the amount of radioactive DNA retained on the filters (Table VI, compare A and B). On the other hand, when nonradioactive, competing DNA was added to standard binding reactions, a decrease in the retention of irradiated, radioactive DNA was observed after 10 min of incubation in the absence, but not in the presence, of 0.02 M NaCN (Table VI, compare C and D). Furthermore, when standard binding reactions were diluted with 14× SSC, the amount of radioactivity retained on the filters decreased as the time between dilution and filtration increased. The decrease occurred more slowly when the 14× SSC contained 0.02 M NaCN (Figure 6).

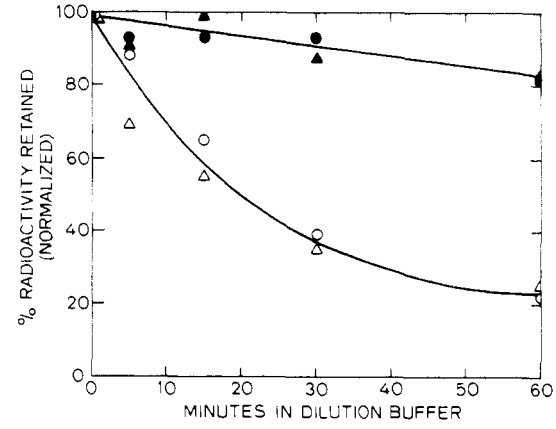

FIGURE 6: Effect of NaCN upon the dissociation of enzyme–DNA complexes in dilution buffer. Standard binding reactions containing 96 ng of [^{14}C]ColE1 DNA (4780 cpm) irradiated with 200 J/m^2 and limiting amounts of T4 endonuclease V (triangles, 70 ng of protein; circles, 140 ng of protein) were diluted with 5 mL of 14× SSC (open symbols) or 14× SSC containing 0.02 M NaCN (closed symbols) and held for various times at 0 °C before filtration. The data have been normalized to the radioactivity retained after 0 min of holding (△, 860 cpm; ▲, 1004 cpm; ○, 1577 cpm; ●, 1864 cpm).

Discussion

To study the binding of T4 endonuclease V to DNA we have developed an assay that depends upon selectively trapping DNA–enzyme complexes on nitrocellulose filters. The amount of DNA retained on the filters under various conditions provides information about the interaction between the enzyme and DNA. Binding is sufficiently specific under our conditions that it can be used to measure T4 endonuclease V activity in crude extracts (Figure 1) as well as in purified preparations. The specificity results in part from including EDTA in the reaction buffer. EDTA prevents binding of DNA by proteins that require divalent cations but has only a slight effect upon the activity of T4 endonuclease V (Yasuda & Sekiguchi, 1970; Friedberg & King, 1971). The enzyme incises irradiated DNA in the reaction buffer at 37 °C, but the DNA is not retained on the filters, probably due to dissociation of DNA–enzyme complexes after incision. The endonuclease does not bind detectably to irradiated DNA that it has incised (Tables II and III). At 0 °C incision and dissociation are inhibited, and enzyme–DNA complexes can be trapped on the filters.

The amount of DNA retained on filters depends upon several factors in addition to the amount of endonuclease (Figures 1, 3, and 5) and DNA (Figure 2) in the reaction mixture. The composition of the filtration buffer affects retention. A high concentration of NaCl containing trisodium citrate or EDTA favors retention (Figure 5, Tables IV and V) without apparently altering the rate of dissociation of enzyme–DNA complexes (Table V). Furthermore, increasing the pyrimidine dimer content of the substrate DNA increases retention (Table I, Figure 3). Although competition experiments indicated that T4 endonuclease V binds to DNA containing only a few dimers per molecule (Figure 4, ○), such DNA was not efficiently retained on filters (Table I, Figure 3). At saturating enzyme levels retention increased as a linear function of dimer content from an average of 0.9 to approximately 10 pyrimidine dimers per DNA molecule (Figure 3, ▲). If each dimer was complexed with endonuclease, it appears that each molecule of T4 endonuclease V bound to a DNA molecule increases the probability that the DNA molecule is retained by a filter. Our data are not consistent with the hypothesis that all DNA molecules bound to a certain number (n) of enzyme molecules have a high probability of retention while DNA molecules bound to fewer ($n - 1$) enzyme molecules have a negligible probability. We calculated the fraction of DNA molecules expected to contain a minimum of 1, 2, or 3 pyrimidine dimers, respectively, for various UV doses, using the Poisson expression and assuming that dimers were randomly distributed among DNA molecules. None of the curves generated by plotting these values as a function of the UV dose were similar to the curve obtained by plotting the fraction of DNA retained as a function of the UV dose it had received. For the experiment shown in Figure 3 (▲) each enzyme molecule bound contributed 0.1 to the probability that a DNA molecule would be retained. However, the value observed in other experiments varied between 0.1 and 0.2,

depending upon the batch of filters used. This made it difficult to determine the precise number of enzyme molecules bound to DNA from the amount of DNA retained on filters.

Under our standard assay conditions T4 endonuclease V binds to irradiated DNA relatively rapidly, as indicated by the observation that the amount of DNA retained on filters reached a plateau within 3 min after the addition of enzyme to the reaction. Results of experiments in which the endonuclease was allowed to bind to radioactive substrate DNA before the addition of nonradioactive competing DNA (Figure 4) indicated that the plateau reflects a dynamic equilibrium between free enzyme and enzyme bound to DNA. Based upon the decrease observed in the amount of radioactive DNA retained on filters, we inferred that approximately 50% of the enzyme–DNA complexes dissociated within 10 min after the addition of competing DNA (Figure 4). Dilution of reaction mixtures into filtration buffer (14× SSC) did not greatly alter the rate of dissociation of enzyme–DNA complexes (Table V, Figure 6), although it prevented the formation of new complexes (data not shown). However, dissociation can be inhibited by NaCN. At a concentration of 0.02 M, NaCN had no detectable effect on the binding of T4 endonuclease V to DNA (Table VI) nor on the retention of DNA on filters (data not shown), but it did retard dissociation of enzyme from DNA when present in the reaction mixture (Table VI) or in the filtration buffer (Figure 6).

Because of its specificity and simplicity the binding assay has proven valuable for monitoring the activity of T4 endonuclease V during purification (Seawell et al., 1981). Because it can provide information about events involved in the interaction of the endonuclease with DNA prior to incision, the assay should be helpful in elucidating the mechanism of action of the enzyme. In addition, results of experiments with radioactive substrate DNA and nonradioactive competing DNA (Figure 4) indicate that the assay should prove useful for determining the pyrimidine dimer content of nonradioactive DNA.

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