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UV-endonuclease from calf thymus with specificity toward pyrimidine dimers in DNA

(DNA-Sepharose/photoreactivation/PM2 DNA)

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ABSTRACT **We describe the partial purification of an endonuclease from calf thymus that nicks phage PM2 DNA irradiated with UV doses producing only a few pyrimidine dimers per molecule. It has much less activity on DNA that has been subjected to enzymatic photoreactivation after UV irradiation. The calf thymus endonuclease is different from other mammalian UV-endonucleases so far described in that it seems to be dimer specific. The enzyme is stimulated by Mg^{2+} and is inactive in the presence of EDTA. It binds to UV-irradiated DNA-Sepharose from which it is released by low concentrations of KCl. Gel filtration data indicate that the endonuclease may belong to a high molecular weight protein or protein complex. The enzyme is very labile and freezing increases its lability.**

The excision of dimers from UV-irradiated DNA is started by an endonuclease that nicks the dimer-containing strand in the vicinity of the dimer. Endonucleases that specifically recognize dimers have been purified from phage T4 infected *Escherichia coli* and from several bacterial species (for review see ref. 1). Nevertheless attempts to isolate dimer-specific endonuclease from mammalian cells have not been successful. The UV-dependent endonucleolytic activities isolated from rat liver (2, 3), calf thymus (4), several types of cultured human cells (5–8), and mouse cells (9) apparently recognize photoproducts other than dimers. Most of these endonucleases were tested on DNA molecules exposed to doses of UV that introduced many dimers per molecule, and the numbers of endonucleolytic nicks observed were 10- to 100-fold less than the numbers of dimers. In one case tested, enzymatic photoreactivation did not affect the substrate properties of the UV-irradiated DNA (4). The heavily irradiated DNA molecules contain alkali stable photoproducts other than dimers (10) that seem to be recognized by these UV-dependent endonucleases. Bovine cells have been shown to possess an efficient dimer excision system (11). To ensure that we were isolating a dimer-specific endonulcease, all assays were performed on phage PM2 DNA containing not more than 2 or 3 dimers per molecule. At the doses used, the probability of the presence of other products was negligible. We describe the isolation of a dimer-specific endonuclease from calf thymus.

MATERIALS AND METHODS

PM2 DNA. ^{3}H-Labeled phage PM2 DNA was prepared by a slight modification of the procedure of Espejo and Canelo (12) and Masamune *et al.* (13). A concentrated labeled phage suspension from 100 ml of lysate was diluted 1:4 with 100 mM NaCl/1 mM EDTA/10 mM Tris·HCl, pH 8.0, and lysed by the addition of Sarkosyl to 0.5%, and the DNA was isolated by two extractions with isoamylalcohol/chloroform (1:24). Two volumes of ethanol and 0.1 vol of 3 M NaOAc were added and the DNA was precipitated overnight at −20°C. The DNA was collected, dissolved in and dialyzed against the above buffer, and stored up to 5 weeks in small portions at −20°C. This PM2 DNA contained 85–95% covalently closed duplexes with a specific activity of about 4×10^4 cpm/μg.

Enzyme Assay. Because a single nick converts covalently closed circular duplex DNA into a form that can be bound to nitrocellulose filters (14), endonucleolytic activity was estimated by the conversion of PM2 DNA from a nonbinding to a binding form. The 100-μl assay mixture contained 10 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM NaCl, and about 0.1 μg of ^{3}H-labeled PM2 DNA (about 4000 cpm per assay). After 5–15 min of incubation at 37°C with enzyme, the reaction was stopped by addition of 2 ml of 0.1 M Na_2HPO_4/0.3 M NaCl/25 mM EDTA/0.1 M NaOH solution titrated to pH 11.9. The unbroken DNA was renatured by addition of 0.5 ml of 2 M Tris·HCl, pH 6.0, bringing the mixture to a pH of 7.6–7.8. After addition of 3.5 ml of 0.3 M NaCl/30 mM Na citrate, the mixture was filtered through nitrocellulose B6 filters, pore size of 0.45 μm (Schleicher & Schuell), and washed with 3–5 ml of the same solution. The radioactivity of the nicked DNA bound to the filter (r) was determined in a toluene-based scintillation fluid in a Packard scintillation counter. The total radioactivity per assay mixture (T) and the amount bound to the filter from assay mixtures incubated without enzyme (r_0) were also determined in order to correct the assay results from the initially nicked molecules by the formula: % nicked by endonuclease = $[(r - r_0)/(T - r_0) \times 100]$.

All enzyme fractions were tested on UV-irradiated (15 J/m^2) and on nonirradiated DNA. One unit of enzyme is defined as the amount of enzyme that, in 5 min at 37°C, induces one break per PM2 molecule irradiated with 15 J/m^2 of UV light. Because all enzyme preparations contained some nonspecific endonuclease, the units of UV-endonuclease were defined as the total endonuclease units minus the nonspecific units.

UV-Irradiation. Assay mixture (2 ml containing about 0.5–1.0 μg of DNA per ml) in a 48-mm-diameter glass petri dish was irradiated with primarily 254-nm radiation at a dose rate of 0.6 W/m^2. The light source was a single 15-W Phillips germicidal lamp placed 60 cm from the sample.

Dimer-Specific Endonuclease from *Micrococcus luteus*. The enzyme was prepared according to Carrier and Setlow (15). Incubation for 5 min at 37°C in 10 mM Tris·HCl, pH 8.0/20 mM NaCl/5 mM EDTA with 5 μl of *M. luteus* extract had no effect on unirradiated DNA and nicked 95% or more of DNA irradiated with 20 J/m^2 as detected by the filter assay or by sedimentation in alkali. *M. luteus* UV-endonuclease used in some experiments was a generous gift from F. Ahmed, Brookhaven National Laboratory.

DNA-Sepharose. The agarose was precycled according to

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Abbreviation: UV-endonuclease, endonuclease acting preferentially on UV-irradiated DNA compared to nonirradiated DNA.

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March *et al.* (16), and UV-irradiated calf thymus DNA was linked to the activated agarose according to Arndt-Jovin *et al.* (17). Twenty milliliters of a washed Sepharose 4B (Pharmacia) slurry suspended in 20 ml of 2 M K_2CO_3 was treated at ice temperature with constant stirring with 7 ml of acetonitrile and 3 ml of cyanogen bromide. The reaction was continued for 2 min and the reagents were rapidly removed on a fritted glass funnel by washing with 10 vol of cold water and 10 vol of cold 10 mM potassium phosphate buffer, pH 8.0. The activated agarose was immediately mixed with 20 ml of a 2 mg/ml calf thymus DNA solution in the buffer containing 20 mM NaCl. The DNA had previously been exposed to UV light at 2.4×10^4 J/m^2. The mixture was agitated for 16–18 hr at room temperature, and the unbound DNA was removed by successive washings with buffer, 1 M KCl, water, and 10 mM Tris·HCl at pH 8.0.

Enzymatic Photoreactivation. [^{3}H]PM2-DNA (50 μg/ml; 8×10^4 cpm/ml) in 50 mM buffer, pH 7.0 containing 1 mM EDTA was irradiated with increasing doses of UV light. Half of each sample (0.5 ml) was incubated with 25 μl of *Escherichia coli* photolyase (a generous gift from B. M. Sutherland, Brookhaven National Laboratory) at 37°C for 1 hr in the dark and the other half was incubated under illumination from two black light fluorescent lamps at 10 cm from the light source. During illumination the samples were covered with an 0.8-cm-thick glass plate to eliminate wavelengths shorter than 320 nm. After photoreactivation the samples were deproteinized with phenol and dialyzed extensively against 10 mM Tris·HCl, pH 8.0/10 mM NaCl.

Miscellaneous. The concentration of proteins in column fractions was recorded by an LKB UVicord at 280 nm. In crude extracts and enzyme preparations the protein content was assayed according to Lowry *et al.* (18) with bovine serum albumin as a standard. KCl concentration was determined by a Radiometer Conductivity Meter.

RESULTS

Enzyme Purification. Fresh thymus from 3- to 5-month-old animals was cooled with ice and used less than 1.5 hr after slaughter. All subsequent purification steps were carried out at 0–5°C. Clean tissue (20–26 g) was cut into small pieces and suspended in 7–40 ml of 50 mM Tris·HCl, pH 8.0, containing 10% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM diisopropylfluorophosphate. The tissue was homogenized in a Sorvall Omnimixer and the homogenate was centrifuged at $12,000 \times g$ for 10 min. The supernatant fluid was centrifuged for an additional 30 min at $140,000 \times g$ in a type 60 rotor in a Beckman ultracentrifuge. The clear supernatant (crude extract) containing about 40 mg of protein per ml was used for enzyme testing and further purification steps.

Crude extract (15–18 ml) was applied to a DEAE-Sephadex A-50 column (2.5 × 35 cm) equilibrated with 50 mM Tris·HCl, pH 8.0/20% glycerol/5 mM 2-mercaptoethanol. The column was eluted with the same buffer at 1.2–1.4 ml/min, and 5- to 7-ml fractions were collected. The UV-specific activity eluted together with the nonspecific activity immediately after the void volume of the column (Fig. 1). Both activities were associated with the first peak of proteins and continued to elute until a second peak of proteins started to come off the column. The endonuclease-containing material of the first peak (fraction I, column fractions 6–11) and the region containing relatively low amounts of protein (fraction II, column fractions 12–16) were pooled separately for further use. Application of a linear salt gradient (0–0.5 M NaCl) released no further UV-specific activity.

In some experiments further purification was achieved by rechromatography of fraction I on a second DEAE-Sephadex A-50 column. Both endonucleases appeared again in the first fractions after the void volume of the column. The pooled active material of the second DEAE-Sephadex column showed UV-specific activity on a significantly lowered background of nonspecific activity towards untreated DNA (fraction III).

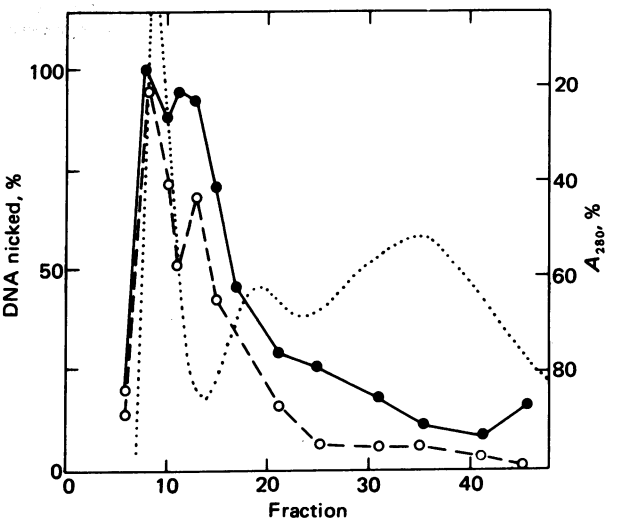

FIG. 1. Separation of endonucleases by DEAE-Sephadex (A-50) chromatography. Crude extract (85 ml at 45 mg of protein per ml) was applied to 2.5 × 12 cm column preequilibrated and eluted with 50 mM Tris·HCl, pH 8.0/20% glycerol/5 mM 2-mercaptoethanol. Five-microliter fractions were incubated for 5 min at 37°C under standard assay conditions on unirradiated (○) and on UV-irradiated PM2 DNA (●). The protein profile is indicated by A_{280} (·····).

The UV-endonuclease was further separated from the nonspecific endonucleolytic activity by affinity chromatography on DNA-Sepharose. Two milliliters of fraction I or 20 ml of fraction III was dialyzed against two changes of 25 vol of 5 mM Tris·HCl, pH 8.0, for about 1 hr. The dialyzed material was mixed for 30 min with 15–20 ml of DNA-Sepharose, the mixture was packed into a column (1.8 × 10.0 cm), and the unbound protein was washed free with 10 mM Tris·HCl, pH 8.0/20% glycerol/2 mM 2-mercaptoethanol. The column-bound endonucleolytic activities were eluted with 50 ml of a 0–0.5 M KCl gradient in the buffer. As seen in Fig. 2 low concentrations of salt (15–25 mM KCl) eluted the UV-specific endonucleolytic activity whereas higher salt concentrations released the nonspecific activity. The UV-endonuclease obtained after DNA-Sepharose chromatography (fraction IV) contained only a low amount of nonspecific endonucleolytic activity.

The results of a representative preparation are summarized in Table 1. Two successive purification steps decreased the amount of protein about 400-fold. The specific activities achieved were low and there was a very high loss of total UV-endonuclease activity, mainly because of the instability of the nuclease. The enzyme becomes labile during purification and, in most preparations, the enzyme fractions were inactive on the second day of isolation. Only in some exceptional cases did fractions I and II retain activity on the second and third day after isolation. All enzyme fractions were kept at +4°C and contained 20% glycerol, which considerably enhanced the stability of the enzyme. Freezing increased the instability of the UV-endonuclease. The enzyme could be recovered in crude extracts prepared from thymuses kept frozen at −20°C or in liquid nitrogen and was detectable in crude extracts containing 50% glycerol stored at −20°C or frozen in liquid nitrogen. However the UV-activity disappeared rapidly if such frozen

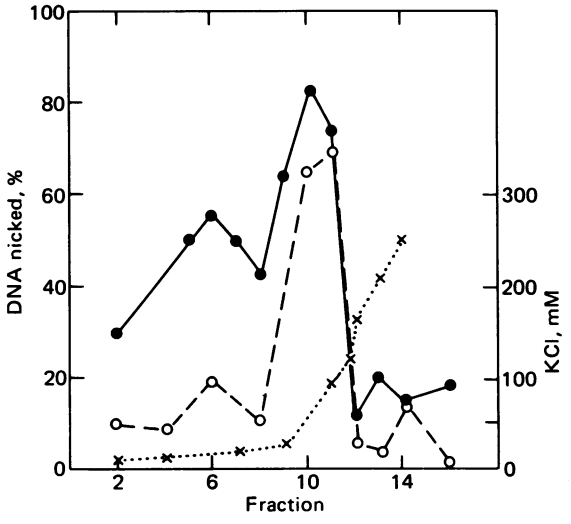

FIG. 2. Separation of endonuclease on DNA-agarose. Twenty milliliters of fraction III was mixed with 20 ml of DNA-Sepharose for 30 min, and the mixture was packed into a column and washed with 5 mM Tris·HCl/20% glycerol. The column was eluted with 50 ml of a 0–0.5 M KCl gradient in the same buffer. Portions (15 μl) of column fractions were incubated for 5 min at 37°C with unirradiated (○) or with UV-irradiated PM2 DNA (●). ×, Concentration of KCl.

material was used for further isolation steps. Thus, for every new set of experiments crude extracts were prepared from fresh thymuses and all purification steps were done on the first day of isolation. Crude extracts from some thymuses treated as usual showed no UV-specific activity. We have no explanation for this observation.

Properties of UV-Endonuclease. Maximum activity of the UV-endonuclease was observed between pH 7.5 and 8.0, and 5–15 mM $MgCl_2$ was optimal for its activity. It was inactive in the presence of 10 mM EDTA. When assayed in the presence of 10 mM $MgCl_2$ small quantities of NaCl (5–15 mM) stimulated its activity. 2-Mercaptoethanol (5 mM) had no effect on the activity. The number of nicks the enzyme makes increased with the amount of enzyme and time of incubation (Fig. 3).

We have no exact data about the molecular weight of the UV-specific enzyme. It seems to belong to a high molecular weight protein or protein complex. It elutes from Sephacryl S-200 and Sepharose 6B columns close to the void volume (Fig. 4). Attempts to measure the sedimentation coefficient of the enzyme were unsuccessful because no UV-specific activity was recovered from sucrose gradients after 12 hr of centrifugation at 26,000 rpm in an SW 50.1 rotor.

Specificity for Dimer-Containing DNA. In routine experiments the enzyme activity was tested on PM2 DNA irradiated

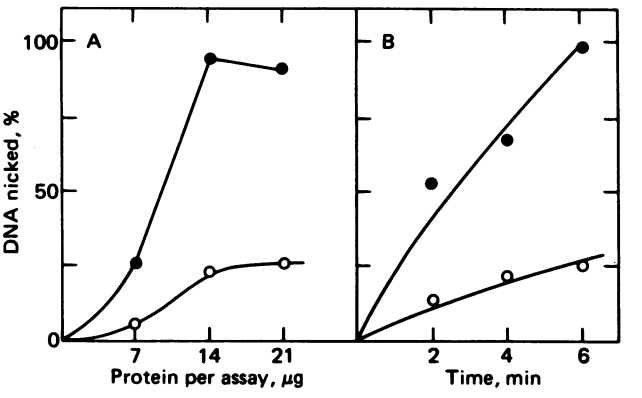

FIG. 3. Production of nicks in unirradiated (○) and UV-irradiated (15 J/m²) (●) PM2 DNA by any enzyme preparation purified through DEAE-Sephadex and DNA-Sepharose columns. The DNA was incubated in a standard reaction mixture for 5 min with increasing amounts of protein (*A*), or for various times with 14 μg of protein (*B*).

with a UV dose (15 J/m²) producing not more than two to three dimers per DNA molecule. Such a substrate is free from other photoproducts, which appear only after significantly higher doses of radiation (10). To be sure that the UV-endonuclease from calf thymus cells recognizes dimers in the UV-irradiated DNA, the dose dependence of break induction was studied. As seen in Fig. 5, the fraction of DNA molecules nicked by the enzyme increased with dose, and the fraction nicked by the calf enzyme was similar to that nicked by the dimer-specific endonuclease from *M. luteus*. The latter enzyme makes one break per pyrimidine dimer in PM2 DNA (19).

Enzymatic photoreactivation experiments confirm that dimers are the sites recognized by the calf thymus UV-endonuclease. As shown in Fig. 6, treatment with photolyase significantly reduced the number of sites in the UV-irradiated DNA that were sensitive to the calf thymus and the *M. luteus* enzymes. Because the pyrimidine dimer is the only known enzymatically photoreversible damage produced in DNA by UV (20), these data imply that the pyrimidine dimer is the lesion recognized by the enzymes.

DISCUSSION

The UV-specific endonucleolytic activity found in crude extracts of calf thymus was separated by affinity chromatography on DNA-Sepharose from an endonucleolytic activity that nicks unirradiated DNA. Both enzymes bind to UV-irradiated DNA-Sepharose but elute at different salt concentrations. Our final preparation contained a rather low background of nonspecific activity but was pure enough to investigate some properties of the enzyme.

Table 1. Purification of calf thymus UV-endonuclease

Fraction	Volume, ml	DNA substrate, μl/unit: Unirradiated	DNA substrate, μl/unit: Irradiated	Protein, mg/ml	UV-endonuclease, units/ml	Specific activity, units/mg	Total units
Crude extract	8	1.75	0.4	39.1	1900	50	16,000
DEAE-Sephadex (fraction I)	15	0.9	0.2	4.1	3900	950	58,000
DNA-Sepharose (fraction IV)	6.5	*	30	0.12	33	280	220

Fresh calf thymus (30 g) was homogenized with 12 ml of buffer and assayed on PM2 DNA irradiated at 15 J/m² at 254 nm.

* Activity was too low to define enzyme units.

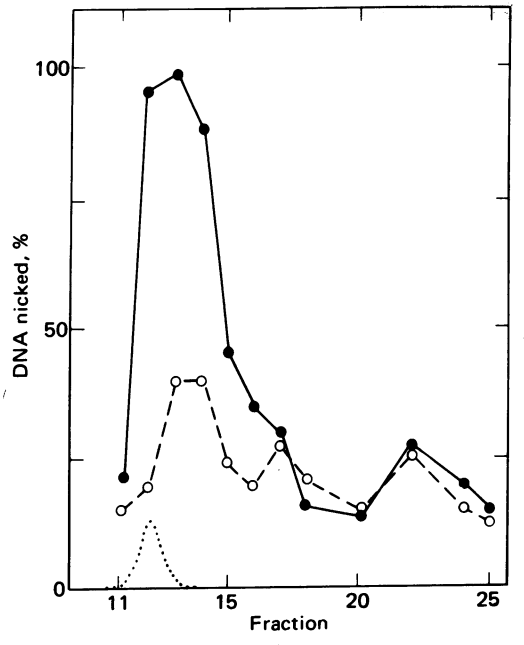

FIG. 4. Chromatography on Sepharose 6B. Fraction I (1 ml) was applied to a 10 × 1.5 cm column preequilibrated with the buffer used in Fig. 1, and the column was eluted with the same buffer. Portions (15 μl) were incubated for 5 min at 37°C with unirradiated (○) and UV-irradiated (●) DNA. ·····, Elution pattern of Dextran blue.

The calf thymus UV-specific endonuclease is different from mammalian endonucleases so far described (2–8). Unlike others that act on heavily irradiated DNA, the calf enzyme is active on DNA irradiated with doses that introduce not more than 2 or 3 dimers per molecule. The number of nicks made by the enzyme is comparable to the number of dimers. It is close to the number of nicks made by the dimer-specific UV-endonuclease of *M. luteus* used in parallel experiments on the same substrate. Even more convincing are the results obtained by enzymatic photoreactivation of the UV-irradiated substrate, which significantly reduces the number of nicks introduced by the calf enzyme and by the dimer-specific *M. luteus* endonuclease.

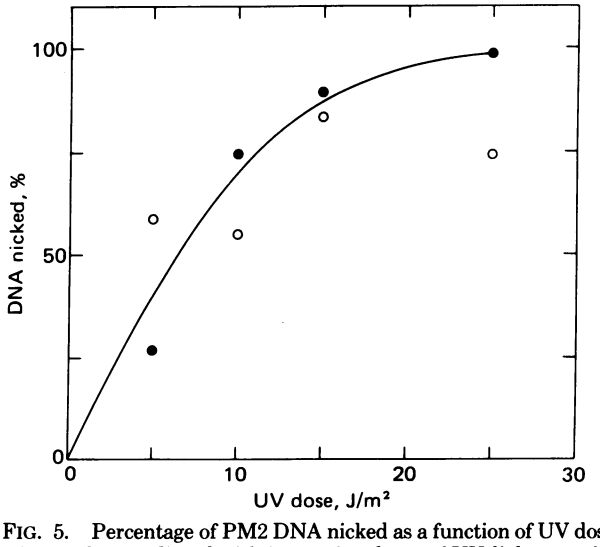

FIG. 5. Percentage of PM2 DNA nicked as a function of UV dose. DNA samples irradiated with increasing doses of UV-light were incubated for 5 min at 37°C with 5 μl of calf enzyme in the standard reaction mixture (○) or with 5 μl of *M. luteus* UV-endonuclease in 10 mM Tris·HCl, pH 8.0/20 mM NaCl/5 mM EDTA (●).

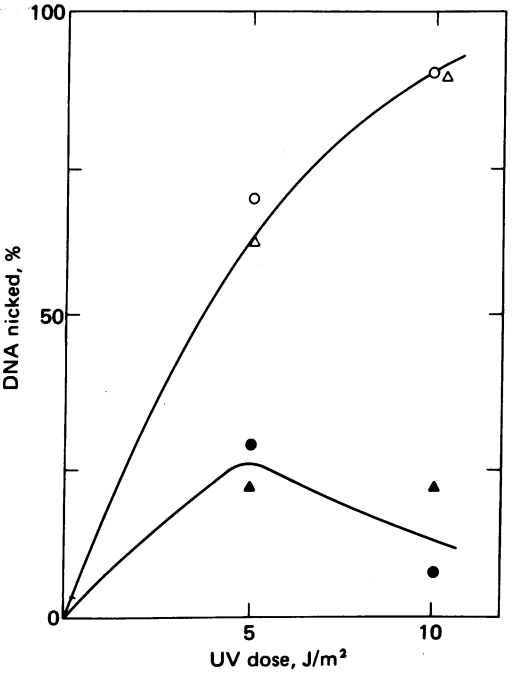

FIG. 6. Effect of enzymatic photoreactivation of UV-irradiated PM2 DNA on the ability of the DNA to be nicked by the UV-dependent endonuclease from calf thymus (○, ●) or by the *M. luteus* enzyme (△, ▲). Irradiated DNAs were incubated with photoreactivating enzyme in the dark (open symbols) or in the light (filled symbols) before use in endonuclease assays.

These data suggest that the calf enzyme is an endonuclease that recognizes pyrimidine dimers in DNA. All UV-endonucleases isolated previously from animal or human cells seem to recognize damages other than dimers (see introduction).

The dimer-specific endonuclease from calf thymus is labile after isolation. Only in exceptional cases did enzyme preparations contain activity on the second or third day after isolation. This lability could be one of the reasons why previous attempts to isolate such an activity from mammalian sources were unsuccessful. The enzymes can be isolated from frozen tissue but freezing increases its lability. The UV-specific activity in column fractions prepared from frozen material (tissue or crude extract) is lost after 1 or 2 hr. A freeze-sensitive dimer excision activity has been observed in human cells (21) and in crude extracts prepared from frozen cells of human origin (22). The lability of these activities suggests that a human dimer-specific endonuclease might have features in common with the enzyme from calf thymus.

The dimer-specific calf endonuclease is inactive in the presence of EDTA and is stimulated by Mg^{2+}. On gel filtration columns it behaves like a high molecular weight protein or protein complex. These features are different from those described for the dimer-specific endonucleases isolated from *M. luteus* and phage T4 infected cells, and from the ATP-independent UV-specific *E. coli* enzyme. Judging from the small number of nicks per dimer (10, 23), the latter enzyme is not dimer specific. These three enzymes are low molecular weight proteins (11,000–20,000) active in the presence of EDTA (for review see refs. 1 and 24). The calf enzyme seems to be more related to the ATP-dependent endonucleolytic activity observed in toluenized *E. coli* (25), because the latter is also inactive in the presence of EDTA. Moreover, gene products from the *uvrA*, *uvrB*, and *uvrC* genes take part in the activity of the *E. coli* ATP-dependent, UV-endonuclease that is dimer specific (26). The molecular weight of the *uvrA*-gene product is 100,000 and

that of the *uvrB* and *uvrC* is close to 70,000 (27). These data suggest that a complex type of dimer-specific UV-endonuclease exists, which is either composed of several polypeptide chains (the *E. coli* enzyme) or may be a high molecular weight protein or part of an enzyme complex (the calf thymus enzyme). The integrity of this polymer of complex enzyme seems to be essential for maintaining its dimer-specific activity.

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