Inhibition of Enzymic Incision of Thymine Dimers by Covalently Bound 2-[*N*-[(Deoxyguanosin-8-yl)acetyl]amino]fluorene in Deoxyribonucleic Acid†

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ABSTRACT: The effects of DNA adducts of the carcinogen 2-[*N*-(acetoxyacetyl)amino]fluorene on enzymic incision of thymine dimers was investigated. *Escherichia coli* DNA labeled with [^{3}H]thymidine was reacted with the carcinogen. Thymine dimers were then introduced into the modified DNA by irradiation with monochromatic 254-nm light in the presence of the photosensitizer silver nitrate. This DNA containing both types of damages, mainly 2-[*N*-[(deoxyguanosin-8-yl)acetyl]amino]fluorene and thymine dimers, was then used as substrate for pyrimidine dimer-DNA glycosylase, purified from *E. coli* infected by bacteriophage T4. Activity was assayed by measuring release of free labeled thymine after photoreversal of the enzyme-reacted DNA by 254-nm light. The V_{max} of the enzyme was decreased when it was reacted with the extensively arylamidated substrate. This inhibition of incision of pyrimidine dimers was increased with the number of carcinogen–DNA adducts, although no enzymic activity against modified guanines was present. Therefore, carcinogen-modified purine moieties can interfere with initiation of excision repair of ultraviolet-induced pyrimidine dimers. This suggests an indirect pathway by which modified DNA bases can be mutagenic.

The excision repair pathway removes damaged sites from DNA. It is initiated by DNA glycosylases that incise glycosylic bonds linking the modified bases to sugars. A variety of such glycosylases have been purified and characterized from diverse sources, each with its own particular substrate specificity (Lindahl, 1982). Because complex mixtures of genotoxic agents may be present, an organism may be exposed to several types of DNA damages simultaneously. If one type of damaged moiety can interact with a DNA glycosylase that initiates excision repair of another, persistence of that glycosylase substrate may result, leading to subsequent mutagenesis.

The effects of other types of DNA damages on uracil-DNA glycosylases from a variety of sources have been studied. Excision of uracil by these purified enzymes is reduced by a number of other types of modifications in the substrate DNAs. These include uracil dimers (Duker et al., 1981; Leblanc et al., 1982), apurinic sites (Duker et al., 1982), apyrimidinic sites (Talpaert-Borle et al., 1982), and guanines modified at the C-8 position (Duker et al., 1982; Duker & Hart, 1984). These alterations introduce either bulky distortions of the DNA structure or single-stranded regions. Because it is also a substrate for the glycosylase, 5-fluorouracil in DNA can reduce enzymic uracil excision (Caradonna & Cheng, 1980; Ingraham et al., 1980; Warner & Rockstroh, 1980). 7-Methylguanine or 5-azacytosine, which cause few deformities or local DNA denaturations, have little effect on uracil-DNA glycosylase activity (Duker et al., 1982; Chao & Duker, 1984). It is important to determine if interference with glycosylase action by unrelated types of DNA damages is unique to uracil-DNA glycosylase, or might be a general mechanism, impeding initiation of excision repair of other damaged DNA moieties.

Pyrimidine dimer-DNA glycosylase (PD glycosylase)[1] initiates excision repair of ultraviolet- (UV) induced pyrimidine dimers by cleaving the glycosylic bond between the 5′-pyrimidine and the corresponding deoxyribose (Haseltine et al., 1980; Gordon & Haseltine, 1980). The enzyme has been well characterized, and studies indicate a strict specificity of the activity for DNA cyclobutyldipyrimidines (Friedberg et al., 1981). Therefore, the effects of guanine adducts resulting from reaction of DNA with the carcinogen 2-[*N*-(acetoxyacetyl)-amino]fluorene (AAAF) on PD glycosylase activity were examined. The presence of the adducts 2-[*N*-[(deoxyguanosin-8-yl)acetyl]amino]fluorene (dGuo-AAF) in the substrate resulted in reduced incision of thymine photodimers by the glycosylase. This indicates that reduction of glycosylase-initiated excision repair may be a general pathway of indirect mutagenesis by damaged DNA moieties.

Experimental Procedures

DNA Preparation and Modification. *Escherichia coli* W3110 (thy$^-$) J. Cairns strain (DeLucia & Cairns, 1969) was obtained from the *E. coli* Genetic Stock Center at Yale University School of Medicine, New Haven, CT, and grown according to Frenkel et al. (1981). The DNA was labeled to a specific activity of 10000–15000 dpm/μg by addition of 20 μCi/mL [*methyl*-^{3}H]thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) and purified as previously described (Frenkel et al., 1981). The DNA was reacted with AAAF (obtained from the National Cancer Institute Carcinogen Repository, Bethesda, MD) under the conditions of Santella et al. (1981) and the carcinogen extracted according to Duker & Hart (1984). Substrate DNA was then irradiated with monochromatic 254-nm light for 3 min (total fluence = 5000

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[1] Abbreviations: UV, ultraviolet; AAAF, 2-[*N*-(acetoxyacetyl)-amino]fluorene; PD glycosylase, pyrimidine dimer-DNA glycosylase; dGuo-AAF, 2-[*N*-[(deoxyguanosin-8-yl)acetyl]amino]fluorene; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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J/M^2) in the presence of the photosensitizer $AgNO_3$ according to Rahn & Landry (1973) with the apparatus previously described (Duker et al., 1981). The irradiated DNA was separated from silver ions by sequential dialysis into 10 mM Tris-HCl/10 mM EDTA, pH 7.8, containing 1.0, 0.5, 0.2, and 0.1 M NaCl, respectively. Poly(dA)·poly(dT) labeled in thymine was synthesized and irradiated according to Nakabeppu & Sekiguchi (1981).

Analysis of DNA Damage. The degree of guanine modification in the AAAF-reacted DNA was estimated by the A_{305}/A_{260} of the modified labeled DNA (Fuchs & Daune, 1972). Unlabeled *E. Coli* DNA was reacted with [^{3}H]AAAF (424 mCi/mmol, National Cancer Institute Carcinogen Repository, Bethesda, MD) and extracted as above. After denaturation and enzymic digestion to deoxyribonucleosides by S1 nuclease and acid phosphatase (Poirier, 1981), the hydrolysate was applied to a 4-mL Sephadex LH-20 column. Analysis of dGuo-AAF was performed by column elution and thin-layer chromatography according to Amacher et al. (1977), with marker dGuo-AAF synthesized and purified by the method of Poirier (1981). This assay separates another reaction product, probably 3-[[(deoxyguanosin-N^2-yl)acetyl]-amino]fluorene (Amacher et al., 1977). The quantity of reacted DNA was measured according to Burton (1968). Thymine dimers were assayed by the method of Carrier & Setlow (1971).

Enzymology. PD glycosylase was purified from T4-infected *E. coli* (purchased as a frozen paste from the New England Enzyme Center, Boston, MA) by the method of Friedberg et al. (1980). Measurement of alkali-mediated enzymic release of radioactive material from UV-irradiated poly(dA)·poly(dT) (Nakabeppu & Sekiguchi, 1981) was used as the assay during the purification procedure. Enzyme fraction IV was used for all enzymological studies. The reaction conditions were those of Radany & Friedberg (1980). Enzyme activity was assayed by quantitation of release of free thymine into the acid-soluble fraction following direct photoreversal of substrate DNA according to Radany & Friedberg (1980). The released material was identified as thymine by paper chromatography (Carrier & Setlow, 1971). The Lineweaver–Burk plot was analyzed by the program of Cleland (1967). DNA challenge experiments were performed by allowing the reaction to proceed for 5 min against the labeled UV-irradiated substrate followed by addition of an equal quantity of unlabeled unmodified or AAAF-reacted *E. coli* DNA. In a separate experiment, the enzyme was incubated in ice for 1 min was unlabeled AAAF-modified or control DNA. An equal quantity of labeled UV-irradiated substrate was added, the temperature raised to 37 °C, and enzyme activity assayed.

Results

The introduction of dGuo-AAF into *E. coli* DNA by reaction with [^{3}H]AAAF is shown in Figure 1. Modification of the DNA was rapid, with substitution of about 5% of the DNA guanines after 5 min. Thereafter, the reation was slower, with about 8 and 16% modifications at 15 min and 3 h, respectively. About 90% of the radioactivity bound to DNA was recovered as a single peak coincident with marker dGuo-AAF at an R_f of 0.75 on the thin-layer chromatogram. The remaining 10% was recovered at an R_f of 0.40–0.45. The increase in labeled DGuo-AAF content approximated the rise of A_{305}/A_{260} after 5 min of reaction time. Therefore, at high levels of guanine modification, the A_{305}/A_{260} permits a valid estimate of dGuo-AAF content.

Even with large quantities of dGuo-AAF, the introduction of thymine photodimers in photosensitized DNA by UV irradiation at 254 nm in unaffected. This is shown in Figure 2. *E. coli* DNA was reacted with AAAF for the times indicated. Silver nitrate was then added, and the DNA was irradiated. No substantial reduction of thymine dimer content was noted, even in DNA reacted with AAAF for 3 h. This demonstrates that irradiated DNA is a valid substrate for PD glycosylase irrespective of dGuo-AAF content. Therefore, the glycosylase can be assayed with this denaturing lesion present in the substrate DNA.

The reactions of PD glycosylase with AAAF-modified substrates were assayed. The DNAs were first reacted with AAAF, then photosensitized, and irradiated. After removal of the photosensitizer $AgNO_3$ by dialysis, the DNA was reacted with PD glycosylase. Release of free thymine upon

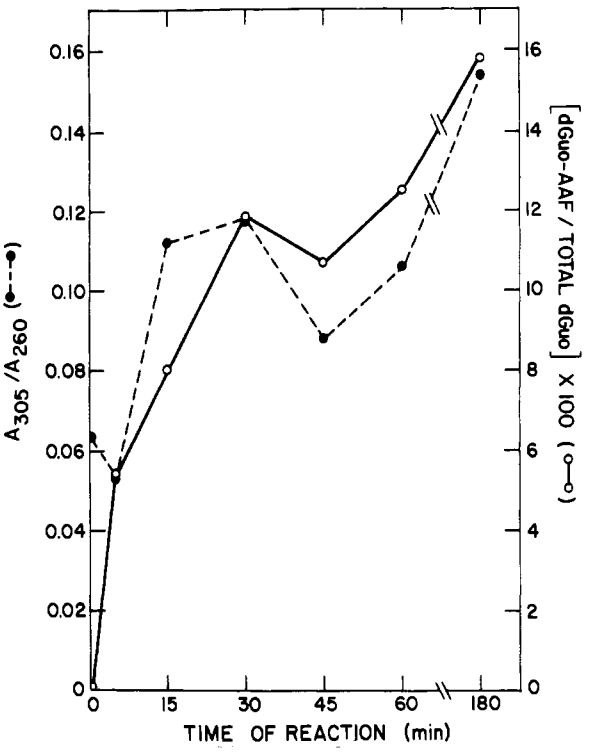

FIGURE 1: Formation of dGuo-AAF in *E. coli* DNA. The DNA was reacted for the times indicated and the extent of DNA modification assayed by spectrophotometry (●) and by chromatography (○). Each point represents the average of two determinations.

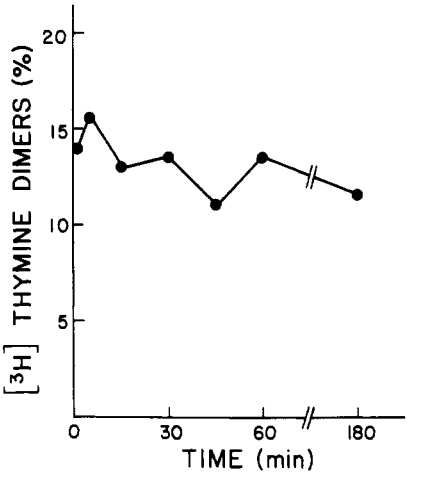

FIGURE 2: Formation of thymine dimers in AAAF-modified DNA. *E. coli* DNA was reacted with AAAF for the times indicated. $AgNO_3$ was added and the DNA irradiated at a total fluence of 5000 J/M^2. Thymine dimer content was then assayed by acid hydrolysis and paper chromatography. Each point represents the average of two determinations.

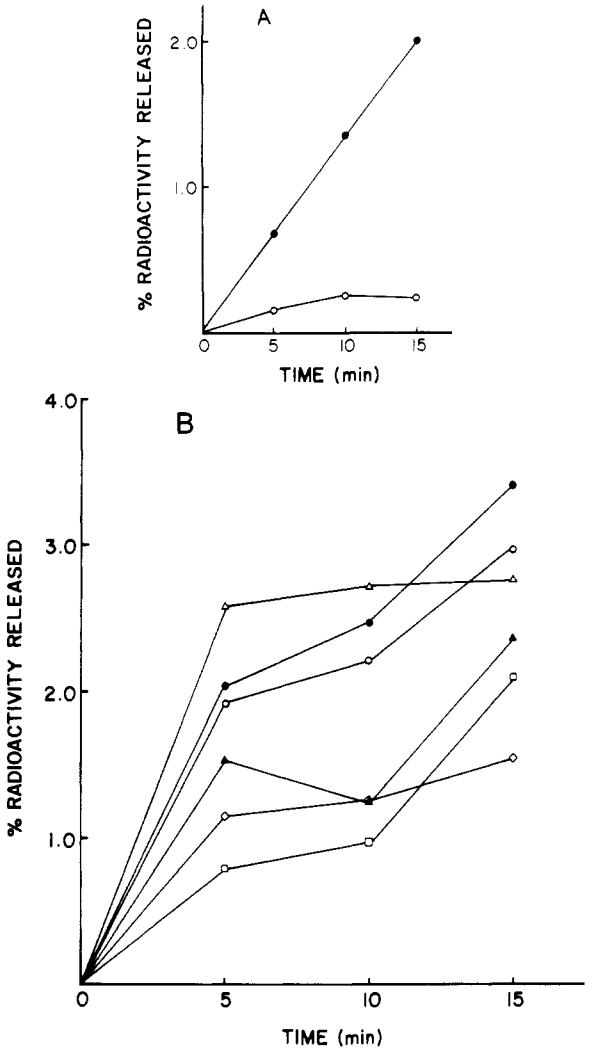

FIGURE 3: Pyrimidine dimer-DNA glycosylase activity against DNA modified by AAAF. *E. coli* DNA, labeled with [^{3}H]thymidine, was reacted with AAAF, photosensitized, irradiated and dialyzed, and 2.0 μg was used as substrate for 5.0 μL of pyrimidine dimer-DNA glycosylase. After the enzyme reacted for the times indicated, the DNA was photoreversed and precipitated by 5% trichloroacetic acid, and the acid-soluble radioactivity was identified as thymine by paper chromatography. (A) DNA reacted with AAAF for 3 h (○) and control DNA (●); (b) DNA reacted with AAAF for 5 (△), 15 (○), 30 (◇), 45 (▲), and 60 min (□) and control DNA (●). Each point represents the average of two determinations.

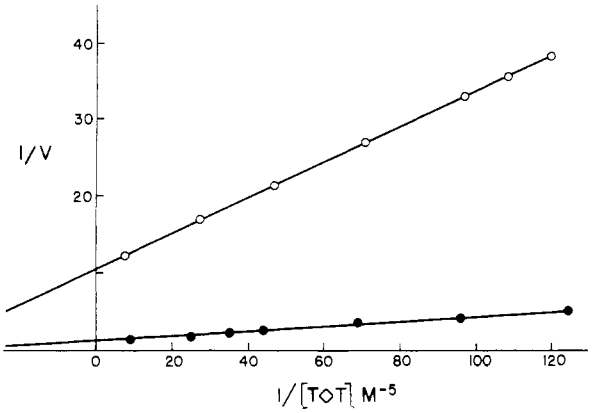

FIGURE 4: Double-reciprocal plot of bacteriophage T4-infected *E. coli* pyrimidine dimer-DNA glycosylase with DNA reacted with AAAF for 3 h (○) and control UV-irradiated *E. coli* DNA (●). The enzyme reaction time was 5 min, with 5 μL of enzyme per reaction mixture. Each point represents the average of four determinations.

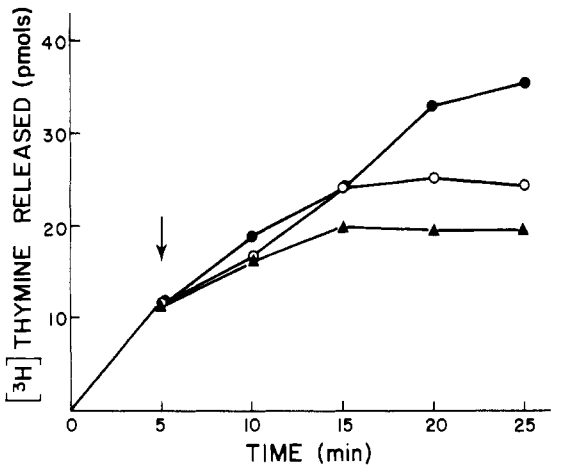

FIGURE 5: Challenge of UV-irradiated DNA substrate by AAAF-modified DNA. A total of 2.4 μg of photosensitized, UV-irradiated *E. coli* DNA was incubated with 2 μL of enzyme at 37 °C for 5 min. At that point, an equal quantity of DNA that had been reacted with AAAF (▲), control unmodified DNA (○), or no DNA (●) was added. Each point represents the average of two determinations.

photoreversal was compared between the control and arylamidated substrates. The first result is shown in Figure 3A. A substantial difference is present between DNA reacted for 3 h with AAAF and the control. Reduction of PD glycosylase activity with increasing dGuo-AAF present in the substrate is demonstrated in Figure 3B. Two families of curves were obtained. While reduction of dimer incision by PD glycosylase was minimal in DNAs reacted with AAAF for 5 and 15 min, significant inhibition was present with substrate DNAs having a higher dGuo-AAF content.

The alteration of PD glycosylase by dGuo-AAF in DNA is further demonstrated in Figure 4. The doubly damaged DNA had been reacted for 3 h with AAAF before irradiation. The V_{max} was reduced from 97.4 ± 0.7 pmol min^{-1} (mL of enzyme)$^{-1}$ of dimers incised for the control substrate to 9.5 ± 2.4 pmol min^{-1} mL^{-1} for the arylamidated DNA. The K_m was unaffected, being 0.31 ± 0.05 μM for thymine dimers in DNA for the control and 0.22 ± 0.14 for the modified substrate. Control experiments using DNA reacted with labeled carcinogen showed no enzymic release of modified purines.

The basis for the observed reduction of the V_{max} was investigated by challenging enzymic incision of UV-irradiated DNA by addition of an equal quantity of unlabeled AAAF-reacted or unmodified control DNA to the reaction mixture. The result is shown in Figure 5. Release of labeled thymine was reduced after addition of the unlabeled DNAs. This indicates that the enzyme binds well to DNA even when no photodimers are present. Reversal of this experiment gave a similar result, as shown in Figure 6. PD glycosylase was incubated in ice for 1 min with unlabeled control or AAAF-reacted DNA. Photosensitized substrate DNA was then added, the temperature raised to 37 °C, and the glycosylase assayed. Enzyme activity commenced more slowly in the presence of prior binding with the other DNAs than in their absence. This shows that the inhibition described here is not of the irreversible type.

Discussion

The standard method of determining if the simultaneous repair of two different forms of DNA damage is by a common mechanism or by independent pathways is by exposure of cells concomitantly to a pair of DNA-modifying agents. Should the repair pathways for the different agents overlap, exposure to both agents would yield less repair synthesis than the two

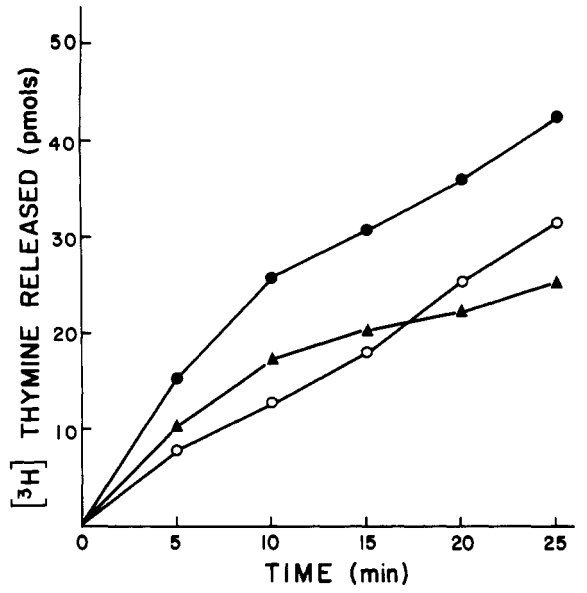

FIGURE 6: Challenge of AAAF-modified DNA by UV-irradiated substrate. A total of 2 μL of enzyme was incubated in ice for 1 min with 4.1 μg of *E. coli* DNA that had been reacted with AAAF for 3 h (▲), unreacted control DNA (○), or no DNA (●). An equal quantity of UV-irradiated substrate DNA was then added, the temperature raised to 37 °C, and enzymic activity assayed. Each point represents the average of two determinations.

types of damages inflicted separately would elict. If the pathways are totally independent, the sum of DNA repair synthesis should be additive of that elicited by both agents. In the case of studies of repair of AAAF and UV damages, contradictory results have been obtained. Exposure of human cells to both forms of damage resulted in additive repair synthesis, indicating separate rate-limiting steps for the different forms of damage (Ahmed & Setlow, 1977; Ahmed & Setlow, 1978). Measurement of direct excision of damaged DNA adducts yielded a similar result (Amacher et al., 1977). Another group, however, found no additivity in repair synthesis elicited by both damaging agents, indicating a common repair pathway (Brown et al., 1979). The multiplicity of variables in these systems make interpretation difficult. Therefore, we used a well-characterized enzyme that initiates excision repair of pyrimidine dimers and investigated how it is affected by dGuo-AAF in the DNA substrate.

The enzyme studied is the *denV* gene product of bacteriophage T4. This enzyme has two activities on the same molecule, the PD glcyosylase that cleaves the glycosylic bond between the 5′-pyrimidine of the dimer and the adjacent sugar and an endonuclease for DNA apurinic and apyrimidinic sites (Nakabeppu & Sekiguchi, 1981; Nakabeppu et al., 1982). However, under standard conditions the two activities do not act in a concerted manner and intact apyrimidinic sites persist under conditions of substrate excess (Nakabeppu & Sekiguchi, 1980; Seawell et al., 1980). Therefore, commonly used endonuclease assays that measure nicking of superhelical DNA, degradation of linear DNA after coupled exonuclease digestion, or DNA filter binding assays (Friedberg et al., 1980) will underestimate the amount of glycosylic bonds incised. The assay used here was release of thymine from enzyme-reacted DNA after photoreversal, since it alone is specific for the PD glycosylase activity (Radany & Friedberg, 1980; Warner et al., 1980; Nakabeppu et al., 1982). Because PD glycosylase hydrolyzes only one of the glycosylic bonds linking the pyrimidine photodimer to the two sugars, the 5′-thymine remains linked to the DNA only by the cyclobutane ring. Monomerization of the dimer by photoreversal at 254 nm breaks the cyclobutane ring, resulting in release of the 5′-thymine from the DNA. The linear quantitative relationship between recovered radioactivity in free thymine and photoreversal of thymine dimers ensures that measurement of released thymine provides a specific assay for the PD glycosylase activity without need for consideration of subsequent DNA breakage (Radany et al., 1981).

Since extensive modification of DNA by AAAF does not significantly reduce the thymine dimer yield, the effects of dGuo-AAF on PD dimer glycosylase activity can be examined directly in the absence of complicating factors. The levels of damage were high; 3 hours of reaction with AAAF yielded about 40 modified purines per 1000 nucleotides, twice the thymine dimer yield in photosensitized DNA. While the performance of kinetic studies here dictated higher levels of damage to the DNA substrates than could be tolerated by living cells, the quantities of dGuo-AAFs and pyrimidine dimers were within 1 order of magnitude of each other. This is similar to the ratios obtained in the DNA of cultured cells (Amacher et al., 1977). The 10% yield of putative 3-[[(deoxyguanosin-N^2-yl)acetyl]amino]fluorene here was similar to the 15% found in the cell cultures (Amacher et al., 1977).

The observed reaction of PD dimer glycosylase against the arylamidated substrate indicates that initiation of excision repair of pyrimidine dimers is directly affected by the presence of other, unrelated types of DNA alterations. The *denV* gene product has been well characterized, and no damaged purines have been found to be substrates for its activity (Friedberg et al., 1981). Our finding here that no AAAF adducts of *E. coli* DNA were released by the enzyme preparation is in keeping with these reports. It is unlikely that dGuo-AAFs and pyrimidine dimers are in proximity to each other, since AAAF adducts are preferentially formed in alternating dG-dC sequences (Harvan et al., 1977). While pyrimidine dimers in either denatured or duplex DNA are incised by PD glycosylase, there is no evidence of strong preference of the enzyme for single-stranded regions (Friedberg et al., 1981). This makes the possibility of enzyme binding at such regions unlikely. These are induced by reaction of DNA with AAAF (Yamasaki et al., 1977). The challenge experiments performed do not indicate a strong preference of the *devV* gene product for binding to arylamidated DNA. PD glycosylase is not processive under the conditions used here (Hanawalt et al. 1982). This eliminates the possibility of blockage of movement of the enzyme along the DNA substrate by dGuo-AAF.

It is possible that apurinic sites could be present in the arylamidated DNA. While the major product of AAAF reaction with DNA is dGuo-AAF, other minor N-7-substituted products that rapidly depurinate are formed (Tarpley et al., 1982). These might account for some of the alkali-labile sites detected in a study of AAAF damage to a defined DNA sequence (Bases et al., 1983). There is virtual absence of activity of the *denV* gene product against base loss sites under these conditions (Nakabeppu et al., 1982). It is nevertheless possible that the glycosylase could be inhibited due to binding of the endonuclease activity to intact apurinic sites, which are quite stable in these circumstances (Lindahl & Andersson, 1972). It has not yet been determined if the endonuclease activity of the *devV* gene product prefers substrate apyrimidinic sites over apurinic sites, as has been reported for a similar enzyme purified from *Micrococcus luteus* (Grafstrom et al., 1982).

These studies show that the presence of dGuo-AAF in DNA reduces initiation of excision repair of pyrimidine dimers. The AAAF-modified guanines are not substrates for the PD gly-

cosylase. Therefore, an extraneous type of damage can induce repair of pyrimidine dimers even though it is excised by a completely different pathway. These results should not be extrapolated to other systems. PD glycosylase initiation of repair of pyrimidine dimers may be restricted to *M. luteus* and T4-infected *E. coli*. It is not the mechanism of pyrimidine dimer excision in human cells (La Belle & Linn, 1982) or uninfected *E. coli* (Seeberg, 1978). This is the second glycosylase found to be inhibited by dGuo-AAF in DNA. Therefore, this phenomenon, found both in PD glycosylase and in uracil-DNA glycosylase (Duker & Hart, 1984), may be a general manifestation of carcinogen modifications of DNA. A wide variety of persistent damaged DNA moieties may perturb glycosylase-initiated excision repair of other altered bases. Should this be the case, carcinogens may exert mutagenic effects by the hindrance of excision repair of chemically and etiogenically unrelated types of DNA base modifications.

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Registry No. dGuo-AAF, 37819-60-6; PD-glycosylase, 75302-33-9; AAAF, 75871-36-2; thymine dimer, 28806-14-6.

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