Selective Inhibition by Methoxyamine of the Apurinic/Apyrimidinic Endonuclease Activity Associated with Pyrimidine Dimer–DNA Glycosylases from *Micrococcus luteus* and Bacteriophage T4†

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ABSTRACT: The UV endonucleases [endodeoxyribonuclease (pyrimidine dimer), EC 3.1.25.1] from *Micrococcus luteus* and bacteriophage T4 possess two catalytic activities specific for the site of cyclobutane pyrimidine dimers in UV-irradiated DNA: a DNA glycosylase that cleaves the 5′-glycosyl bond of the dimerized pyrimidines and an apurinic/apyrimidinic (AP) endonuclease that thereupon incises the phosphodiester bond 3′ to the resulting apyrimidinic site. We have explored the potential use of methoxyamine, a chemical that reacts at neutral pH with AP sites in DNA, as a selective inhibitor of the AP endonuclease activities residing in the *M. luteus* and T4 enzymes. The presence of 50 mM methoxyamine during incubation of UV- (4 kJ/m^2, 254 nm) treated, [^{3}H]thymine-labeled poly(dA)·poly(dT) with either enzyme preparation was found to protect completely the irradiated copolymer from endonucleolytic attack at dimer sites, as assayed by yield of acid-soluble radioactivity. In contrast, the dimer–DNA glycosylase activity of each enzyme remained fully functional, as monitored retrospectively by release of free thymine after either photochemical- (5 kJ/m^2, 254 nm) or photoenzymic- (*Escherichia coli* photolyase plus visible light) induced reversal of pyrimidine dimers in the UV-damaged substrate. Our data demonstrate that the inhibition of the strand-incision reaction arises because of chemical modification of the AP sites and is not due to inactivation of the enzyme by methoxyamine. Our results, combined with earlier findings for 5′-acting AP endonucleases, strongly suggest that methoxyamine is a highly specific inhibitor of virtually all AP endonucleases, irrespective of their modes of action, and may therefore prove useful in a wide variety of DNA repair studies.

Living organisms possess a battery of enzymatic systems to remove potentially deleterious lesions in their cellular DNA [reviewed in Lindahl (1982), Paterson et al. (1984), and Friedberg (1985)]. In one of these processes, termed excision repair, error correction is accomplished by a series of reactions leading to the release of the defective material from the genome, followed by insertion of a normal nucleotide sequence into the resulting single-strand gap by repair synthesis and ligation of newly synthesized and preexisting material so as to restore the damaged site to its correct structure.

The nature of the initial steps in this generalized mechanism is primarily dependent upon the type of DNA damage. For example, a limited number of frequently occurring single base defects, such as uracil and 3-methyladenine, are released as free bases by a panel of damage-recognizing enzymes termed DNA glycosylases (Lindahl, 1982). Each glycosylase catalyzes the hydrolysis of the *N*-glycosyl bond linking a specific inappropriate or damaged base with its backbone sugar. The adjacent phosphodiester bond 5′ to the resulting "denuded" sugar may then be acted upon by a separate AP[1] endonuclease, generating a 3′-hydroxy nucleotide terminus and a deoxyribose 5′-phosphate at the 5′ terminus. The deoxyribose phosphate moiety can then be removed by either a 5′ → 3′-directed exonuclease or a 3′-acting AP endonuclease.

The repair of solar UV-induced DNA cyclobutane pyrimidine dimers in *Micrococcus luteus* and T4 phage-infected *Escherichia coli* is accomplished in a somewhat similar fashion (see Figure 1). A pyrimidine dimer–DNA glycosylase cleaves the *N*-glycosyl bond between the 5′ member of the dimerized pyrimidines and its corresponding deoxyribose. The AP site, so formed, is then attacked by a 3′-acting AP endonuclease, creating a deoxyribose at the 3′ terminus and a pyrimidine dimer, linked by a single *N*-glycosyl bond, at the 5′ terminus (Haseltine et al., 1980; Demple & Linn, 1980; Radany & Friedberg, 1980). Several lines of independent evidence strongly suggest that the pyrimidine dimer–DNA glycosylase and its functionally associated AP endonuclease from T4 phage are companion activities that reside in a single 16-kDa polypeptide encoded by the *den*V gene (McMillan et al., 1981; Nakabeppu & Sekiguchi, 1981; Warner et al., 1980; Valerie et al., 1984). Similarly, the same two catalytic activities from *M. luteus* are thought to be carried by a single small protein (Grafstrom et al., 1982). The 3′-acting AP endonucleases (with their attendant dimer–DNA glycosylases) have been arbitrarily designated as class I AP endonucleases to distinguish them from the 5′-acting AP endonucleases that are denoted as class II enzymes (Linn, 1982).

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[1] Abbreviations: AP, apurinic/apyrimidinic; UV, ultraviolet; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TTP, thymidine triphosphate; TCA, trichloroacetic acid; PCR, photochemical reversal; PER, photoenzymatic reversal; PR, photoreversal; TLC, thin-layer chromatography; Thy, thymine; kDa, kilodalton.

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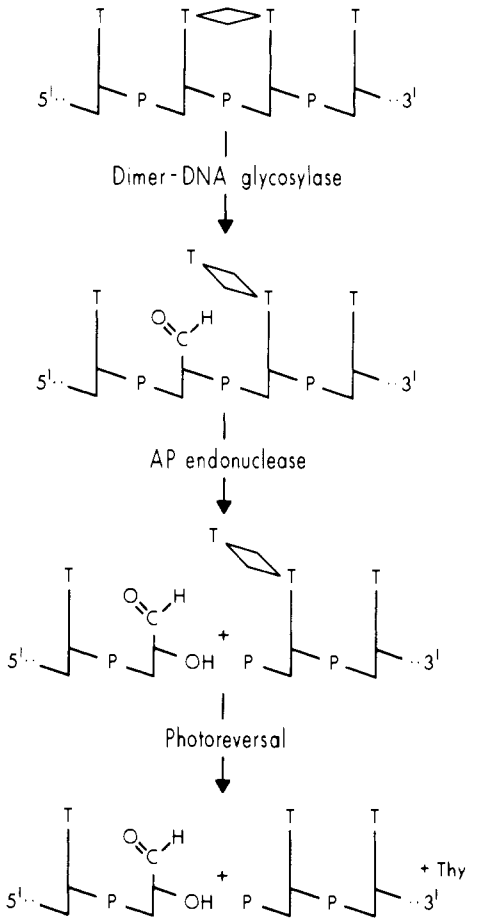

FIGURE 1: Two-step model for incision of UV-irradiated poly(dA)·poly(dT) by *M. luteus* UV endonuclease and phage T4 endonuclease V at sites of cyclobutane pyrimidine dimers. A dimer–DNA glycosylase initially hydrolyzes the *N*-glycosyl bond between the 5′-pyrimidine of the dimer and the corresponding deoxyribose, creating an AP site. Subsequently, an AP endonuclease cleaves the phosphodiester bond on the 3′ side of the baseless site. The glycosylase activity may be monitored by the measurement of free thymine released from short oligonucleotides after photoreversal of the cyclobutane pyrimidine dimers by either UV light or purified *E. coli* DNA photolyase in the presence of fluorescent light.

Since AP sites would appear to be common intermediate structures in the repair of numerous lesions routinely occurring in cellular DNA (Loeb, 1985), a chemical reagent capable of recognizing such structures should prove to be useful in a variety of DNA repair studies. A reagent that seems to possess this very property and may therefore be considered a promising tool for examining AP sites is methoxyamine. The chemical reacts with the aldehyde groups present at AP sites (Coombs & Livingston, 1969; Talpaert-Borlé & Liuzzi, 1983); this reaction has been shown to render the phosphodiester bond immediately upstream from each site refractory to the incising action of class II AP endonucleases isolated from rat liver and calf thymus (Liuzzi & Talpaert-Borlé, 1985). The same report also provided evidence that methoxyamine does not affect calf thymus uracil–DNA glycosylase activity under conditions in which the reagent totally inhibits the action of the class II AP endonuclease from the same source.

The purpose of this study was to explore further the potential of methoxyamine to serve as a selective AP site reagent by investigating its inhibitory effects on class I AP endonucleases that coreside with dimer–DNA glycosylases in *M. luteus* and T4 phage UV endonucleases. Our data clearly demonstrate that methoxyamine does indeed selectively inhibit 3′-acting AP endonucleases; it would thus appear that the reagent can recognize virtually all AP sites, irrespective of whether such sites are operated on by class I or class II AP endonucleases. [A preliminary account of our findings has been reported elsewhere (Weinfeld et al., 1986).]

Materials and Methods

Enzymes. A crude protein extract from wild-type *M. luteus* cells was prepared according to the procedure of Paterson and co-workers (1981). This procedure was similar to the initial steps followed by Carrier and Setlow (1970) in the purification of a UV endonuclease activity from *M. luteus*. In brief, spray-dried cell powder (6 g) of ATCC strain 4698 (Miles Chemical Co., Elkhart, IN) was washed in 10 mM Tris-HCl buffer (pH 8.0), resuspended in the same buffer with 0.2 M sucrose and 0.2 g/mL lysozyme (Calbiochem, San Diego, CA), and subjected to osmotic shock by the addition of water. The resulting lysate was sonicated and then centrifuged to remove cell debris, after which ammonium sulfate was added slowly to the collected supernatant to a final concentration of 65% saturation and the precipitated proteins were pelleted by centrifugation and stored at −80 °C. At this stage, further purification by phosphocellulose chromatography was required to remove nonspecific endonucleolytic activities. All manipulations associated with the additional phosphocellulose purification step were performed at 4 °C. Frozen ammonium sulfate precipitate was dissolved in 15 mL of 20 mM potassium phosphate (pH 7.5) and 2 mM 2-mercaptoethanol (buffer A) and dialyzed 5 times for 1 h each time against 35 volumes of the same buffer to remove trace amounts of ammonium sulfate. This crude UV endonuclease preparation [15 mL of ~50 mg of protein/mL; equivalent to fraction II of Carrier and Setlow (1970)] was loaded onto a Whatman P-11 phosphocellulose column [(1.6 × 30 cm) preequilibrated with buffer A] at the rate of 0.5 mL/min. After the column was washed with 120 mL of buffer A over a 4-h period, the UV endonuclease was eluted with a linear gradient (500 mL) of 20–500 mM potassium phosphate (pH 7.5) and 2 mM 2-mercaptoethanol at the rate of ~25 mL/h. Eighty-four, 6-mL fractions were collected. The major activity peak eluted from the column at about 250 mM potassium phosphate. Six peak activity fractions [detected by the AP endonuclease assay (see below)] were pooled, after which glycerol was added to a final concentration of 30% (v/v). This preparation of *M. luteus* UV endonuclease (56 mL of ~130 μg of protein/mL) was stored at −20 °C for use throughout the study. Its specific activity was estimated to be 425 units/mL according to the AP endonuclease assay. Enzyme maintained under these conditions retained essentially full activity for at least 2 months, the maximum period during which the preparation was stored for use in the work described here.

Phage T4 endonuclease V, a second pyrimidine dimer specific endodeoxyribonuclease, was kindly supplied by Dr. A. K. Ganesan (Stanford University, Palo Alto, CA). Under our standard AP endonuclease assay conditions, the activity of this preparation was 660 units/mL.

E. coli photoreactivating enzyme (photolyase, EC 4.1.99.3), the product of the *phr*B gene that catalyzes monomerization of *cis-syn*-cyclobutane pyrimidine dimers in a light- (300–600 nm) dependent reaction (Setlow, 1966), was a generous gift of Dr. A. Sancar (University of North Carolina, Durham, NC). This enzyme preparation (8 mg/mL, fraction VII; Sancar et al., 1984) was supplied in a 50 mM Tris-HCl (pH 7.4) buffer containing 50 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 50% glycerol (v/v).

Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Preparation of [^{3}H]Thymine-Labeled Poly(dA)·Poly(dT). $(dA)_{600}$·$(dT)_{20}$ (Pharmacia Canada, Dorval, Quebec) served as a template-primer substrate for the synthesis of tritiated poly(dA)·poly(dT) catalyzed by the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim Canada, Dorval, Quebec). The radioactive polymer was prepared as described (Talpaert-Borlé et al., 1979) in a 1-mL reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5 mM $(dA)_{600}$·$(dT)_{20}$, 0.5 mM TTP, 2 nmol of [^{3}H]TTP (68.8 Ci/mmol; New England Nuclear Canada, Montreal, Quebec) and 15 units of the Klenow fragment. The reaction was carried out at 37 °C for 24 h. The synthesized copolymer was isolated from the reaction mixture in the following manner. After addition of 250 μL of 1 M sodium acetate (pH 5.5), the polymeric material was precipitated with two volumes of ice-chilled ethanol; the pellet was then dissolved in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 10 mM NaCl (buffer B) and dialyzed at 4 °C overnight against 2 L of the same buffer. The resulting preparation of ^{3}H-labeled poly(dA)·poly(dT) (360 μM phosphate; specific activity ~200 cpm/pmol of thymine) had an acid-soluble labeled component, measured in 7.5% TCA, of ≤1% and hence was essentially free of contaminating nucleic acid material of low molecular weight.

UV Irradiation of [^{3}H]Thymine-Labeled Poly(dA)·Poly(dT). The radioactive copolymer, at a concentration of 36 μM phosphate in buffer B, was exposed at ambient temperature to 4 kJ/m^2 of far-UV radiation delivered by two 15-W (low-pressure mercury vapor) germicidal lamps (Model GE 15T8; General Electric, Toronto, Ontario), emitting 97% of their radiant energy at 254-nm wavelength. The incident fluence rate from the UV source was 2.5 W/m^2, as measured by potassium ferrioxalate actinometry (Jagger, 1967).

AP Endonuclease Assay. The AP endonuclease activities of the *M. luteus* and T4 UV endonuclease preparations were determined by measuring the degradation of [^{3}H]thymine-labeled poly(dA)·poly(dT) according to the procedure of Nakabeppu and Sekiguchi (1981). The standard (20-μL) reaction mixture contained UV- or sham-irradiated radioactive copolymer (10 μM phosphate, ~2 × 10^4 cpm) and one of the two UV endonuclease preparations (4 μL) in buffer C [50 mM Tris-HCl (pH 7.5), 25 mM EDTA, and 5 mM dithiothreitol]. Incubation was performed at 37 °C for 30 min, unless indicated otherwise. At the completion of the incubation period, 100 μL of bovine serum albumin (1 mg/mL) and 72 μL of 20% TCA (final concentration, 7.5%) were added, and the reaction mixture was vortexed. The sample was then placed on ice for 10 min, after which time the acid-insoluble material was pelleted by centrifugation (10000*g* for 15 min at 4 °C). A 150-μL portion of the supernatant was carefully withdrawn, mixed with 5 mL of HP cocktail (Beckman Instruments, Palo Alto, CA), and counted in a liquid scintillation system to compute the radioactivity in the TCA-soluble fraction. This value, when expressed as a percentage of total radioactivity, became a measure of the amount of [^{3}H]thymine-labeled material released from the copolymer and, in turn, of the AP endonuclease activity of a given UV endonuclease preparation. One unit of AP endonuclease activity was defined as that amount of purified enzyme which converted 1 pmol of the radioactive poly(dA)·poly(dT) preparation to acid-soluble oligonucleotides in 1 min under our standard reaction conditions.

When the action of methoxyamine was investigated, a 1-μL aliquot of a 1 M aqueous solution (neutralized to pH 7.5 with NaOH) of methoxyamine hydrochloride (Sigma Chemical Co., St. Louis, MO) was added to the standard reaction mixture to give a final concentration of 50 mM.

Dimer–DNA Glycosylase Assay. The assay of the dimer–DNA glycosylase activity of the two procaryotic UV endonucleases exploited a unique property of the thymine–thymidylate product of the glycosylase-mediated reaction, namely, liberation of thymine as a free base upon photoreversal of the cyclobutane ring joining the dimerized pyrimidines (Demple & Linn, 1980; Radany & Friedberg, 1980). The assay conditions were the same as those described above for the AP endonuclease assay except that, on completion of the 30-min incubation period, the standard reaction mixture was subjected to dimer-photoreversal treatment and then analyzed for the presence of free thymine by thin-layer chromatography. Photoreversal treatment was administered in one of two ways: (i) exposure to a second, dimer-monomerizing fluence (5 kJ/m^2) of far-UV (germicidal) radiation or (ii) incubation with *E. coli* DNA photolyase in the presence of fluorescent light; the former treatment is henceforth referred to as PCR and the latter as PER. For PER, 1 μL of the photolyase preparation was added, and the reaction mixture was then incubated at 23 °C for 1 h under fluorescent light (two General Electric F15-T8D lamps, incident fluence ~8 W/m^2). When the reaction mixture was subsequently assayed, for photoreleased thymine, by TLC on silica gel (60 F 254 precoated aluminum-backed, 0.2 mm thick sheets; Merck, Darmstadt, West Germany), a chloroform/methanol mixture (3:1 v/v) was used as the eluent and nonradioactive thymine served as an internal standard (Talpaert-Borlé & Liuzzi, 1982). The poly(dA)·poly(dT) substrate remained at the origin of the chromatogram while thymine migrated with an R_f value of 0.67. On completion of the run, silica gel in the areas of the origin and of the thymine standard were scraped off the TLC sheet and placed in scintillation vials containing 0.5 mL of H_2O. Finally, each sample was counted for radioactivity in a liquid scintillation spectrometer with 5 mL of HP cocktail (Beckman Instruments, Palo Alto, CA). The results were expressed as a percentage of total radioactivity eluting with thymine.

Preincubation of M. luteus UV Endonuclease with Methoxyamine. To test for possible inactivation of the bacterial UV endonuclease by the chemical, a 400-μL solution of the purified enzyme was incubated with 25 μL of 1 M methoxyamine hydrochloride (pH 7.5; 50 mM final concentration) in 500 μL of buffer C under the conditions described for the AP endonuclease assay; 25 μL of water was added to a second 400-μL sample, which served as a control. After incubation at 37 °C for 30 min, the two enzyme samples were dialyzed at 4 °C overnight against 2 L of buffer C. Lastly, 4 μL of each dialyzed enzyme was added to 16 μL of the standard reaction mixture containing either UV- or sham-irradiated radioactive copolymer, and the AP endonuclease activity of each enzyme sample was measured as described earlier. The results, expressed as a percentage of total radioactivity, were corrected for the dilution of the enzyme preparations.

RESULTS

Inhibition of M. luteus UV Endonuclease by Methoxyamine. We first explored the inhibitory action of methoxyamine toward the two well-defined catalytic functions of the bacterial UV endonuclease.

(*i*) *AP Endonuclease Activity.* The DNA-backbone-incising activity of the UV endonuclease was determined by measuring the release of TCA-soluble radioactivity from UV- or sham-treated, [^{3}H]thymine-labeled poly(dA)·poly(dT) following incubation of the substrate with the partially purified enzyme. The enzyme preparation purified on phosphocellulose con-

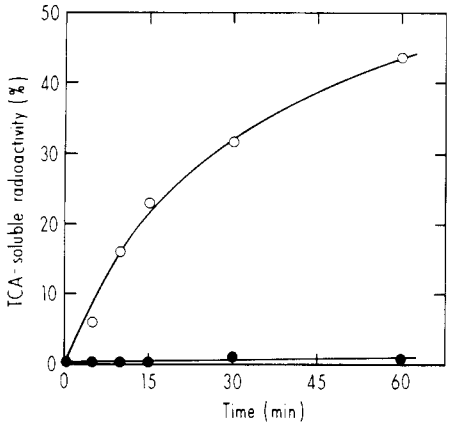

FIGURE 2: Specificity of *M. luteus* UV endonuclease preparation for poly(dA)·poly(dT) containing UV-induced cyclobutane pyrimidine dimers. [^{3}H]Thymine-labeled poly(dA)·poly(dT), UV irradiated (4 kJ/m^2, 254 nm) (○) or not (●), was incubated with 4 μL of purified *M. luteus* UV endonuclease. Incubations were conducted for the indicated times under the conditions described for the AP endonuclease assay under Materials and Methods. At the end of the incubations, the TCA-soluble fraction was measured. The results, corrected for background, are expressed as a percentage of total counts.

Table I: Effect of Methoxyamine on the AP Endonuclease Activity of *M. luteus* UV Endonuclease and Bacteriophage T4 Endonuclease V[a]

sample	UV light	methoxyamine	UV endonuclease		% TCA soluble
			M. luteus	phage T4	
a	−	−	−		0.9
b	−	−	+		1.3
c	+	−	−		1.1
d	−	+	+		1.1
e	+	−	+		30.1
f	+	+	+		1.3
g	−	−		+	3.0
h	−	+		+	1.2
i	+	−		+	47.0
j	+	+		+	1.8

[a] [^{3}H]Thymine-labeled poly(dA)·poly(dT), UV-irradiated (4 kJ/m^2, 254 nm) or not, was incubated with 4 μL of *M. luteus* UV endonuclease or phage T4 endonuclease V in the presence or absence of 50 mM methoxyamine (pH 7.5). The reaction conditions were those described for the AP endonuclease assay under Materials and Methods. The TCA-soluble fraction is expressed as a percentage of the total radioactivity.

tained no measurable endonucleolytic activity toward nonirradiated copolymer, even when the reaction was carried out for 60 min (cf. lines a and b in Table I; also see Figure 2). Similarly, exposure of the copolymer to UV radiation did not by itself lead to the release of acid-soluble radioactivity (cf. lines a and c, Table I). In keeping with the well-documented ability of the *M. luteus* enzyme to degrade UV-irradiated DNA, incubation of the bacterial preparation with 254-nm-damaged substrate yielded substantial amounts of TCA-soluble material (cf. lines a and e, Table I). Figure 2 shows the kinetics of the enzymatic degradation of the UV-treated poly(dA)·poly(dT). After the standard 30-min incubation period, ~30% of the copolymer was rendered acid soluble; the level increased to about 45% by 60 min. In contrast, the introduction of 50 mM methoxyamine into the reaction mixture abolished the degradative action of the enzyme, reducing the level of radioactivity released from the UV-damaged substrate to background (cf. lines a and f, Table I). These data demonstrated that the chemical was indeed capable of preventing *M. luteus* UV endonuclease from introducing scissions in the sugar–phosphate backbone at the site of pyrimidine dimers in UV-irradiated poly(dA)·poly(dT).

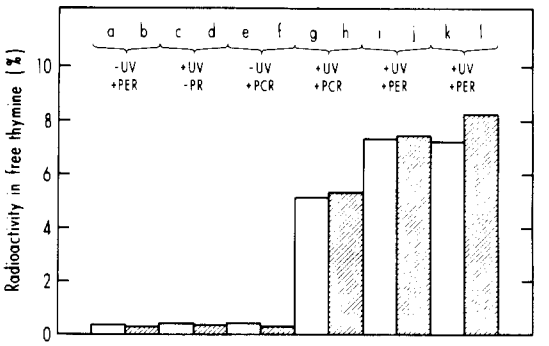

FIGURE 3: Effect of methoxyamine on the dimer–DNA glycosylase activity of *M. luteus* UV endonuclease and phage T4 endonuclease V. [^{3}H]Thymine-labeled poly(dA)·poly(dT) was incubated with 4 μL of *M. luteus* UV endonuclease (lanes a–j) or phage T4 endonuclease V (lanes k and l) in the presence (hatched bars) or absence (open bars) of 50 mM methoxyamine (pH 7.5) under the conditions described for the dimer–DNA glycosylase assay under Materials and Methods. −UV, unirradiated substrate; +UV, irradiated (4 kJ/m^2, 254-nm light) copolymer; +PER, enzymatic photoreversal by *E. coli* DNA photolyase plus fluorescent light; +PCR, photochemical reversal by 5 kJ/m^2 of 254-nm UV light; −PR, no photoreversal of either form. The free thymine released after photoreversal was isolated by silica gel TLC. The results are given as a percentage of total counts in thymine.

(ii) Dimer–DNA Glycosylase Activity. As described in the introduction, the UV endonuclease from *M. luteus* incises DNA at the site of pyrimidine dimers by a two-step mechanism in which backbone cleavage at an AP site constitutes the second reaction. One explanation for the observed inhibition of the UV endonuclease activity by methoxyamine could therefore have been that the chemical inhibited the first reaction (i.e., attack on the 5′-glycosyl bond of the dimerized pyrimidines by a dimer–DNA glycosylase activity), thus precluding the formation of AP sites. This possibility was tested directly by measuring the liberation of free thymine on photoreversal of UV-treated copolymer previously incubated with the bacterial enzyme. Our findings are presented in Figure 3. As anticipated, omission of either 254-nm irradiation, that is, the initial dimer-inducing exposure (Figure 3a,b,e,f) or the secondary dimer-photoreversing treatment (Figure 3c,d), resulted in negligible generation of free thymine. In contrast, ~5.5% of the total radioactivity was released in thymine when both UV treatments were administered; moreover, the extent of the photoliberation was independent of the inclusion of methoxyamine in the reaction mixture, signifying that the dimer–DNA glycosylase activity of *M. luteus* UV endonuclease was unaffected by the presence of the chemical reagent.

As an alternative to photochemical reversal, we also investigated the use of photoenzymatic reversal, mediated by *E. coli* DNA photolyase, as a means of generating free thymine at a thymine–thymidylate structure attached to the sugar–phosphate backbone by a single *N*-glycosyl bond. As is evident in Figure 3g–j, not only was the catalytic function of the photoreactivating enzyme unimpaired by methoxyamine but in fact the PER treatment proved to be more proficient at monomerizing pyrimidine dimers than the PCR treatment.

Inhibition of the AP Endonuclease Activity of Phage T4 Endonuclease V by Methoxyamine. The product of the *den*V gene of bacteriophage T4 has been shown to possess properties similar to those of *M. luteus* UV endonuclease (Nakabeppu & Sekiguchi, 1981; Demple & Linn, 1980; Gordon & Haseltine, 1980). The AP endonuclease and dimer–DNA glycosylase assays were therefore performed on the T4 UV endonuclease preparation to determine whether the same selective

Table II: Effect of Preincubation of *M. luteus* UV Endonuclease with Methoxyamine on the Associated AP Endonuclease Activity[a]

sample	UV light	preincubated enzyme[b]	% TCA soluble
a	−	−	1.3
b	−	−MA	1.4
c	−	+MA	1.5
d	+	−	1.4
e	+	−MA	11.5
f	+	+MA	11.1

[a] *M. luteus* UV endonuclease was preincubated in the presence or absence of 50 mM methoxyamine as detailed under Materials and Methods. After removal of methoxyamine by dialysis, aliquots of the enzyme were then incubated with [^{3}H]thymine-labeled copolymer under the conditions described for the AP endonuclease assay under Materials and Methods. The results, expressed as percent of total counts soluble in 7.5% TCA, were corrected for the dilution effect observed as a result of enzyme dialysis. [b] (−), no preincubated enzyme in the reaction mixture; −MA, enzyme preincubated in the absence of methoxyamine; +MA, enzyme preincubated in the presence of methoxyamine.

inhibition by methoxyamine could be obtained with the phage enzyme. The addition of the chemical to the reaction mixture containing T4 endonuclease V and UV-irradiated copolymer was indeed found to reduce the AP endonuclease activity of the phage enzyme to a background level (Table I, lines g–j) without compromising its glycosylase activity (Figure 3k,l). It would thus seem likely that the chemical exerts its inhibitory action on the two procaryotic UV endonucleases by the same mechanism.

Mode of Action of Methoxyamine. The experimentation described above clearly demonstrated that methoxyamine specifically blocks the second (backbone-incision) step in the nicking action of *M. luteus* or T4 UV endonuclease on UV-irradiated DNA. In attempting to elucidate the mechanism of action of the chemical, two possibilities were entertained, namely, that the reagent may chemically modify either the enzyme (presumably in the vicinity of the active site of its AP endonuclease function) or the substrate, UV-damaged DNA (probably at an AP site). The first hypothesis was tested by incubating aliquots of purified *M. luteus* UV endonuclease with or without methoxyamine, dialyzing the reaction mixtures exhaustively (to remove all traces of the chemical), and finally exposing UV-irradiated copolymer to the enzyme samples to assay for AP endonuclease activity. The results, summarized in Table II, showed that preincubation of the bacterial enzyme with the chemical caused no reduction in AP endonuclease activity compared to that exhibited by enzyme preincubated alone. The AP endonuclease activities in both preincubated samples were, however, only ~32% of that routinely found in a nonpreincubated sample of the same UV endonuclease preparation (Table I, line e). In all likelihood, this substantial loss of AP endonuclease activity occurred when the two preincubated samples underwent dialysis overnight.

We previously reported that the covalent reaction of methoxyamine with the aldehyde group at AP sites, generated in polynucleotides by uracil–DNA glycosylase, prevents alkaline degradation (i.e., strand backbone rupture) mediated by a β-elimination mechanism (Liuzzi & Talpaert-Borlé, 1985). To ascertain whether AP sites formed by a dimer–DNA glycosylase (present in *M. luteus* UV endonuclease) were liable to similar modification by methoxyamine, despite the close proximity of thymine–thymidylate dimerized structures (and possibly UV endonuclease protein as well), UV-irradiated copolymer was first incubated with a saturating amount (10 μL) of the purified bacterial enzyme in the presence or absence of the chemical and was then subjected to alkali before TCA-soluble radioactivity was measured. Incubation of the substrate with only the *M. luteus* enzyme converted some 50–60% of the [^{3}H]thymine label into an acid-soluble form. This occurred irrespective of subsequent alkaline treatment, indicating that the dimer–DNA glycosylase and the AP endonuclease activities of the UV endonuclease were both fully functional (Table III). In sharp contrast, inclusion of methoxyamine in the reaction mixture fully protected the substrate from degradation; this held true even when the substrate was subsequently alkali-treated, implying that the AP sites had been effectively rendered alkali stable and thus refractory to modification by a β-elimination reaction.

Table III: Methoxyamine-Mediated Inhibition of Strand Cleavage Induced by Alkali or *M. luteus* UV Endonuclease[a]

UV endonuclease	methoxyamine	alkali	% TCA soluble
+	−	+	60.7
+	+	+	1.8
+	−	−	53.4
+	+	−	1.6

[a] UV-irradiated (4 kJ/m^2, 254 nm), [^{3}H]thymine-labeled poly(dA)·poly(dT) was incubated for 2 h with 10 μL of *M. luteus* UV endonuclease preparation under the conditions described for the AP endonuclease assay in order to obtain maximal modification of the polymeric substrate. After reaction, an equal volume of 0.1 M NaOH was added, and the mixture was incubated at 65 °C for an additional 8 min. This treatment quantitatively cleaved all the AP sites. The solutions were then neutralized with 0.1 M HCl, after which the acid-soluble fraction was determined as outlined under Materials and Methods.

Discussion

As noted earlier, methoxyamine has been shown previously to be an effective inhibitor of calf thymus and rat liver AP endonucleases, two 5′-acting (class II) AP endonucleases, but not of calf thymus uracil–DNA glycosylase (Liuzzi & Talpaert-Borlé, 1985). Accordingly, it was of interest to determine if this same reagent could likewise selectively block the 3′-acting (class I) AP endonuclease activities associated with the UV endonucleases from *M. luteus* and bacteriophage T4. The results presented here demonstrate that in the presence of 50 mM methoxyamine (pH 7.5) the AP endonuclease activities of both procaryotic enzymes are indeed unable to degrade UV-irradiated poly(dA)·poly(dT), whereas the companion dimer–DNA glycosylase activities remain fully functional (Table I and Figures 3 and 4). In combination with earlier findings of other investigators [e.g., Haseltine et al. (1980), Demple and Linn (1980), and Radany and Friedberg (1980)], these observations provide overwhelming evidence that the *M. luteus* and T4 enzymes act on UV-irradiated polynucleotides in two distinct sequential steps, namely, hydrolysis of the 5′-glycosyl bond of the dimer followed by cleavage of a phosphodiester bond on the 3′ side of the resulting AP site, rather than via one single concerted reaction. Earlier studies in several other laboratories yielded results that, although less direct, are entirely consistent with this interpretation (Warner et al., 1980; Nakabeppu & Sekiguchi, 1981; Seawell et al., 1980).

More specifically, the data summarized in Tables II and III reveal that the inhibitory action of methoxyamine results from chemical modification of the substrate, AP sites, rather than from poisoning of the enzyme, AP endonuclease. On a priori grounds, this modification of the substrate may block the action of the enzyme in one of two ways: either the chemical structurally alters the AP site (i.e., by forming a covalent linkage with its aldehyde moiety) so that the enzyme can no longer recognize (and hence bind to) the base-free site or, alternatively, the methoxyamine-induced modification may

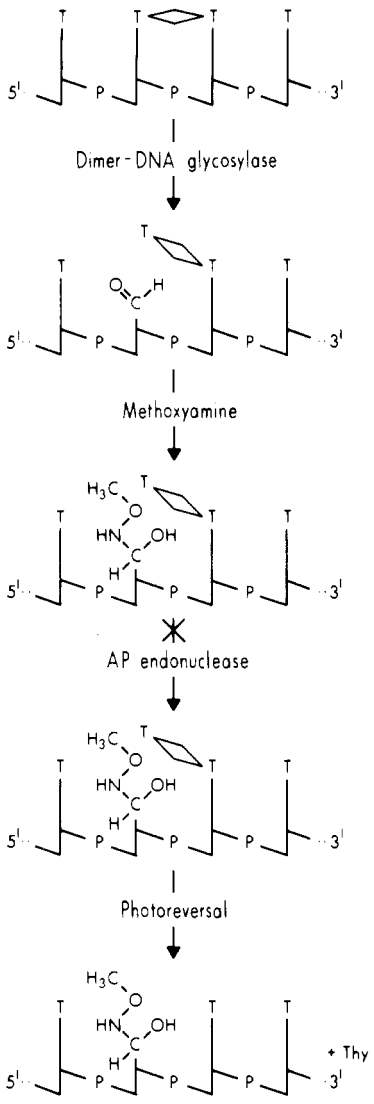

FIGURE 4: Presumed reaction of methoxyamine with the aldehyde group present at the AP site, resulting in selective inhibition of the AP endonuclease activity of *M. luteus* UV endonuclease and phage T4 endonuclease V. Inhibition is believed to result because methoxyamine-reacted AP sites are no longer a substrate for the AP endonuclease activity. This model implies that the reaction of methoxyamine with AP sites may be considerably faster than the incision of the phosphodiester backbone by the enzyme. The dimer–DNA glycosylase activity of both UV endonucleases can still be demonstrated by the release of thymine from the intact polymeric substrate after photoreversal of the cyclobutane pyrimidine dimers by either UV light or purified *E. coli* DNA photolyase plus fluorescent light.

render the phosphodiester bonds adjacent to the AP site refractory to the catalytic activity of the AP endonuclease even though the enzyme can still bind to the site. For those AP endonucleases that appear to function by direct hydrolytic cleavage of an adjacent phosphodiester bond, as is believed to be the case for class II AP endonucleases, the former possibility is the more likely explanation for the observed inhibition. The answer is far less certain for class I AP endonucleases, however, simply because the precise mechanism by which these enzymes introduce backbone scissions at AP sites remains unclear. Several lines of independent evidence (Bailly & Verly, 1984; Valerie et al., 1984; Toulmé & Saison-Behmoaras, 1985) suggest that the attack by class I enzymes on the phosphodiester bond 3′ to an AP site does not typify the action of hydrolases, and hence these enzymes may not function as true endonucleases. In T4 UV endonuclease at least, the carboxy-terminal sequence of the protein forms a crucial part of the endonuclease's catalytic domain. Since this region has been shown to be rich in basic and aromatic amino acid residues, it is conceivable that the AP endonuclease activity of the T4 enzyme may produce single-strand breaks via a base-catalyzed β-elimination reaction similar to that proposed for DNA-binding oligopeptides containing lysine and tryptophan residues [for a review, see Toulmé and Saison-Behmoaras (1985)]. Methoxyamine addition, which is known to block this kind of reaction by masking the aldehyde function of the AP site (Coombs & Livingston, 1969; Liuzzi & Talpaert-Borlé, 1985), would thus inhibit the incising action of this class I AP endonuclease even if the enzyme were still able to bind to the chemically modified site [e.g., by using aromatic amino acids to recognize AP sites (Behmoaras et al., 1981)].

This study may provide additional insight into the two-step mechanism by which the *M. luteus* and T4 UV endonucleases incise UV-irradiated DNA at the site of pyrimidine dimers. It is an intriguing observation that in the presence of methoxyamine these two enzymes retain their full glycosylic activity but, despite each having a molecular weight of ~16 000 (Carrier & Setlow, 1970; Nakabeppu & Sekiguchi, 1981), are unable to confer on the resulting AP sites protection from attack by the chemical (Figures 2–4). Several other research groups have independently shown that in a T4 enzyme limited reaction, UV-irradiated substrate is converted to an intermediate structure that harbors many alkali-labile sites but few strand breaks (Warner et al., 1980; Seawell et al., 1980; Nakabeppu & Sekiguchi, 1981; Evans et al., 1984). It would thus appear that the initial dimer–DNA glycosylase step may well proceed considerably faster than the subsequent AP endonuclease reaction. One of the simplest interpretations of these and other findings is that the mode of action of the T4 enzyme involves two separate enzyme–substrate encounters in which the enzyme does not remain bound to its substrate on completion of the glycosylase reaction but must detach and reorient itself before performing its AP endonuclease function. Such a mechanism would readily explain the apparent ease with which methoxyamine can react with AP sites generated by the two procaryotic UV endonucleases.

The work reported here involving the photolyase encoded by the *phr*B gene of *E. coli* has served to define further the substrate requirements of this 49-kDa photoreactivating enzyme recently described by Sancar and co-workers (Sancar et al., 1984). Interestingly, we found that methoxyamine does not interfere with the enzyme's ability to catalyze the photolytic monomerization of pyrimidine dimers in UV-damaged substrate (see Figure 3). Moreover, our data indicate that this photolyase preserves its full activity toward DNA glycosylase modified cyclobutane pyrimidine dimers situated either within the intact, methoxyamine-treated copolymer or even at the end of short oligonucleotides released by the AP endonuclease activities of the *M. luteus* and T4 UV endonucleases (Figures 1 and 4). Our laboratory has also observed that this same *E. coli* enzyme, as well as photolyases purified from two other microbial sources (*Streptomyces griseus* and *Anacystis nidulans*), can likewise photoreverse pyrimidine dimers in which the phosphodiester bond linking the two sugar moieties attached to the dimer is broken (Paterson et al., 1984; unpublished data). It is noteworthy that the substrate requisites for the photolyase utilized here contrast sharply with those reported recently by Sutherland et al. (1986) for a second photoreactivating enzyme purified from *E. coli*, a 40-kDa protein that is the product of the *phr*A gene (Snapka & Sutherland, 1980). The latter enzyme is inactive toward sites containing a pyrimidine dimer when an interruption is intro-

duced in certain bonds in the immediate vicinity of the dimer, namely, (i) the *N*-glycosyl bond joining the 5′ member of the dimer to its deoxyribose and (ii) the phosphodiester linkage between the dimer-forming pyrimidines. These collective results lead us to speculate that the photolyases characterized thus far can be divided into two major classes on the basis of their substrate requirements: those that recognize the dimer themselves and those that recognize some local topological deformation of the bihelical macromolecule caused by the presence of the dimer. The first class would be exemplified by the 49-kDa *E. coli* protein and the photolyases purified from *S. griseus and A. nidulans*, whereas the 40-kDa *E. coli* protein would be representative of the second class.

None of the enzymes employed thus far in studies with methoxyamine, that is, two mammalian class II AP endonucleases, two procaryotic UV endonucleases, a calf thymus uracil–DNA glycosylase, and the *E. coli* 49-kDa photolyase, appear to react adversely with this chemical reagent (Liuzzi & Talpaert-Borlé, 1985; this paper). It remains to be determined whether this is a general property of the reagent. The cytotoxicity of methoxyamine toward rodent cells has been measured (Reznik & Shapiro, 1975) but, unfortunately, this analysis was carried out at pH 6.0—conditions under which the chemical is less specific, reacting with cytosine residues in addition to AP sites (Budovskii et al., 1968; Talpaert-Borlé & Liuzzi, 1983). If it is found that, under physiological conditions, methoxyamine does not induce gross pathological reactions, such as rampant degradation of cellular genomic DNA, then the reagent could be gainfully exploited in assays designed to monitor the formation of AP sites in vivo and thus to identify enzymatic repair pathways involving DNA glycosylases and AP endonucleases. Experiments of this nature are currently in progress in our laboratory.

Registry No. AP endonuclease, 65742-70-3; UV endonuclease, 81611-73-6; methoxyamine, 67-62-9.

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