Substrate Range of the 40 000-Dalton DNA-Photoreactivating Enzyme from *Escherichia coli*†

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ABSTRACT: We determined the ability of the 40 000-dalton *Escherichia coli* photoreactivating enzyme to act on a variety of pyrimidine–pyrimidine photoproduct substrates in nucleic acids. The enzyme is at least as active on *cis-syn*-cyclobutylpyrimidine dimers in supercoiled DNA as in linear DNA, but inactive on dimers in RNA. Both the phosphodiester bond internal to the deoxyriboses of the pyrimidines of the dimer and the *N*-glycosyl bond joining the pyrimidine to deoxyribose must be intact for enzyme action. The enzyme has no activity toward (6-4) pyrimidine–cytosine products in DNA.

Photoreactivating enzymes (photolyase, EC 4.1.99.3) repair ultraviolet light (UV, 220–310 nm)[1] irradiated DNA by monomerizing *cis-syn*-cyclobutylpyrimidine dimers (PD) in a light (300–600 nm)-dependent reaction (Setlow, J. K., et al., 1965; Setlow, R. B., et al., 1965). The apparent specificity of these enzymes (Setlow & Setlow, 1963) has allowed their use as a marker of the existence and biological effects of the pyrimidine dimer (Sutherland, 1981).

A photoreactivating enzyme was isolated from *Escherichia coli* B and from an *E. coli* strain lysogenic for a λ phage carrying the *E. coli phr*A gene, mapped at 17.3 min (Sutherland et al., 1973; Snapka & Sutherland, 1980). The major protein in the purified preparation of Snapka & Sutherland (1980) was characterized as a moiety of slightly less than 40 000 Da by gel electrophoresis in the presence of sodium dodecyl sulfate, gel filtration in the presence of guanidinium chloride, and analytical ultracentrifugation. In addition, an *E. coli* photoreactivating enzyme from the *phr*B gene at 16.2 min has been characterized by gel electrophoresis in the presence of sodium dodecyl sulfate to have a molecular weight of 49 000 (Sancar et al., 1984a) and by calculation from the DNA sequence to have a molecular weight of 53 994 (Sancar et al., 1984). Since the 40 000-Da protein of Snapka and Sutherland was characterized under denaturing conditions and in the analytical ultracentrifuge where enzyme activity cannot be followed, Sancar et al. (1984a) suggested that the PRE activity of Snapka and Sutherland might be associated with the 50 000-Da protein. However, Ciarrocchi et al. (submitted for publication) have shown that the photoreactivating activity in the preparations of Snapka and Sutherland chromatographs on a gel filtration column as a moiety of about 40 000 Da. They also found that extracts of wild-type *E. coli* contain PRE activity corresponding to the 40 000-Da species, as well as one of approximately 50 000 Da, which may well correspond to the enzyme described by Sancar et al. (1984a). In addition to the difference in molecular weight, the two proteins differ in associated nonprotein moieties (Snapka & Sutherland, 1980; Sancar & Sancar, 1984; Jorns et al., 1984), amino acid composition (Snapka & Sutherland, 1980; Sancar et al., 1984), absorption spectra (Cimino & Sutherland, 1982; Sancar & Sancar, 1984; Jorns et al., 1984), and interactions with other DNA binding proteins (E. Seeberg, personal communication; Sancar et al., 1984b). These distinctive properties indicate that they are indeed two distinct proteins.

Tryptophan-containing oligopeptides and proteins can catalyze dimer destruction in a reaction catalyzed by light (Helene & Charlier, 1971; Helene et al., 1976) in the wavelength range absorbed by tryptophan ($\lambda < 300$ nm) (Sutherland & Griffin, 1980). However, because of side reactions, this reaction leads to photoinactivation rather than reactivation of dimer-containing polynucleotides (Chen et al., 1976). A true photoreactivating enzyme, on the other hand, not only

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[1] Abbreviations: UV, ultraviolet light; PRE, photoreactivating enzyme; PD, pyrimidine dimers; Da, dalton(s); bp, base pair(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol.

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should catalyze the light-dependent conversion of pyrimidine dimers to monomers but also should restore biological activity to UV-inactivated DNA. The 40 000-Da *E. coli* PRE monomerizes dimers in DNA (Sutherland & Chamberlin, 1973; Farland & Sutherland, 1979; Ahmed & Setlow, 1977) with an action spectrum which extends from 300 to 500 nm (Sutherland et al., 1973); its action restores the activity of UV-irradiated DNA (Yasbin et al., 1981; Wood, 1986). The 50 000-Da *E. coli* photolyase uses light from a BLB bulb (principal emission 365 nm) to catalyze the disappearance of sites for the uvr ABC endonuclease (Sancar et al., 1984a) and restores biological activity to pBR322 DNA (Sancar et al., 1984b). Smith et al. (1985) have shown that both *phr*A and *phr*B mutants show marked cellular photoreactivation; in the *phr*A mutant, the rate, but not the final extent, of photoreactivation was reduced, while in the *phr*B mutant, both the rate and final extent of photorecovery were reduced. These data indicate that both *phr*A and *phr*B gene products are active in photoreactivation in *E. coli*. The 40 000 and 50 000-Da *E. coli* photolyases both catalyze the light-dependent disappearance of pyrimidine dimers and restore biological activity to UV-irradiated DNA in vitro and in vivo; they both, then, are true photoreactivating enzymes.

We have examined the substrate range of the 40 000-Da *E. coli* photoreactivating enzyme. Our data indicate that the enzyme monomerizes *cis-syn*-cyclobutylpyrimidine dimers in linear and supercoiled DNAs but not in RNA. The enzyme does not photolyze dimers in DNA in which there is no phosphodiester bond internal to the two deoxyribose moieties nor does it act on dimers in which the *N*-glycosyl bond or the *N*-glycosyl and phosphodiester internal to the dimer were cleaved. The (6-4) pyrimidine–cytosine product was not a substrate for the enzyme. These data show that this *E. coli* photoreactivating enzyme requires for its activity a *cis-syn*-cyclobutylpyrimidine dimer in DNA with intact *N*-glycosyl bonds as well as a phosphodiester bond internal to the dimer.

Materials and Methods

Photoreactivating Enzyme. The 40 000-dalton *E. coli* photoreactivating enzyme was purified as previously described to fraction III (Snapka & Sutherland, 1980). For some experiments, small aliquots of fraction III enzyme were chromatographed on Bio Rex 70 to remove residual contaminating DNA.

Micrococcus luteus UV endonuclease was prepared by the method of Carrier & Setlow (1970) through fraction III. These preparations contained both the pyrimidine dimer *N*-glycosylase and apurinic endonuclease. UV-irradiated *E. coli* DNA cleaved only by the *N*-glycosylase was the kind gift of R. Grafstrom and L. Grossman, The Johns Hopkins University, Baltimore, MD.

Preparation and Measurements of Substrates. (A) Superhelical DNAs. Approximately 7 μg of naturally supercoiled pAT153 DNA, a plasmid of 3657 bp derived from pBR322 (Twigg & Sherratt, 1980), was UV-irradiated at 254 nm at an exposure rate of 2.6 J m^{-2} s^{-1} for 60 s, producing eight pyrimidine dimers (PD) per molecule. A portion of the irradiated DNA was then made linear by incubation in the presence of *Eco*RI (Bethesda Research Laboratories) which has only one recognition site per pAT153 molecule. The pyrimidine dimer content was determined by using the technique of Ciomei et al. (1984). In brief, all photoreactivated supercoiled DNA samples were incubated in the presence of *Eco*RI to linearize circular molecules, and in the presence of saturating amounts of *Micrococcus luteus* PD endonuclease to introduce a single-strand scission for every pyrimidine dimer not monomerized by incubation in light. Digestion products were then separated by electrophoresis on an alkaline agarose gel, and the number of UV endonuclease sensitive sites per molecule was calculated from the amount of linear single-stranded molecules not cleaved by the *M. luteus* endonuclease. With this technique, it is possible to measure up to eight enzyme-sensitive sites per molecule.

(B) Bacteriophage and Bacterial DNAs. ^{32}P-Labeled T7 bacteriophage were prepared by (a) infection of *E. coli* B grown in B-2 medium (Studier, 1975) containing 0.15 mCi of [^{32}P]orthophosphate/mL, (b) poly(ethylene glycol) precipitation, and (c) sedimentation at 40 000 rpm for 35 min in a CsCl gradient (three 1-mL steps of 21%, 31%, and 41% CsCl in 10 mM Tris, pH 8, and 1 mM EDTA and one 0.5-mL step of 62% CsCl) in the SW-50 rotor of the Beckman Model L ultracentrifuge. Phage were collected and dialyzed vs. 50 mM Tris, pH 8, 10 mM $MgCl_2$, and 0.5 M NaCl. DNA was extracted by treatment with distilled phenol and dialysis vs. 50 mM NaCl, 0.1 mM EDTA, and 10 mM Tris, pH 7, and was characterized by absorption spectroscopy. Specific activities of freshly prepared [^{32}P]DNAs ranged from 300 to 1000 cpm/pmol. [^{3}H]Thymidine-labeled *E. coli* AB 2500 DNA (22 000 cpm/μg) was the gift of W. H. Farland. DNAs were irradiated in a Johns' monochromator (Johns & Rauth, 1965) at 289 nm to a level of about 20% of the thymines as cyclobutylpyrimidine dimers.

Photoreactivation of pyrimidine dimers in [^{32}P]DNAs or [^{3}H]DNAs was measured by a nuclease digestion assay (Sutherland & Chamberlin, 1973; Farland & Sutherland, 1979); in some experiments, pyrimidine dimer monomerization was also followed by acid hydrolysis and thin-layer chromatography (Sutherland & Sutherland, 1969).

(C) RNA. PRE activity on UV-irradiated tRNA (Sigma) was tested in a competition assay; increasing amounts of UV-irradiated RNA were mixed with dimer containing T7 DNA; photoreactivating enzyme was added and exposed to photoreactivating light as usual. The rate of dimer disappearance was measured at 1:1 and 10:1 ratios of RNA dimers to DNA dimers. Dimer analysis was carried out basically by the method of Sutherland & Shih (1983).

(D) Photoligated $d(pT)_{10}$·Poly(dA). Double-strand d-$(pT)_{10}$·poly(dA) (P-L Biochemicals) in 50 mM sodium phosphate, pH 7.0, and 50 mM NaCl was labeled with ^{32}P by the method of Maxam & Gilbert (1980) and mixed with a large excess (>100:1) of unlabeled $d(pT)_{10}$·poly(dA). The mixture was irradiated with broad-band UV of wavelengths greater than 290 nm, and photoligation of adjacent oligothymidylate residues by pyrimidine dimer formation was monitored by gel electrophoresis and autoradiography (Lewis & Hanawalt, 1982). After irradiation, bands of oligo(dT) 10, 20, 30, and 40 bases long appeared. Production of pyrimidine dimers internal to dT_{10} was monitored by inclusion of a small amount of [2-^{14}C]$(dT)_{10}$·poly(dA)(P-L Biochemicals), irradiation as before, hydrolysis, and thin-layer chromatography (Reynolds et al., 1981).

E. coli PRE activity on these ligating dimers was determined by adding fraction III enzyme to 0.2 mL of 50 mM phosphate buffer, pH 7.0, 10 mM NaCl, and 1 mM EDTA containing 8 μg of irradiated $d(pT)_{10}$·poly(dA) plus 0.5 μg of irradiated ^{14}C-labeled *E. coli* DNA. Photoreactivation of ligating dimers would be observed as the disappearance of photoligated oligomers on gels; enzyme activity in the reaction mixture was checked by hydrolysis and chromatography of the [^{14}C]DNA. The ability of the enzyme to recognize internal dimers in the oligonucleotides was tested in a competition assay using 160

ng of irradiated ^{14}C-labeled *E. coli* DNA as test DNA and using irradiated or unirradiated DNAs as competitive inhibitors.

(*E*) *Defined-Sequence E. coli DNA Fragment.* A segment of the *E.* coli *lacI* gene extending from bp 561 to the sequence flanking the *lacI* gene was purified from the plasmid pMC1 (Calos et al., 1978) by digestion with restriction endonuclease *Bst*EII (New England Biolabs), ^{32}P labeled at the 3′ termini by using the Klenow fragment of pol I (New England Biolabs), and redigested with restriction endonuclease *Hinc*II (New England Biolabs). The fragment extending from bp 561 to bp 885 was separated on a 5% nondenaturing polyacrylamide gel and isolated by electroelution. After irradiation with 500 J/m^2 254-nm light from a germicidal bulb, the fragment either was incubated or was not incubated with 5 μL of fraction III *E. coli* photoreactivating enzyme. The 170-μL reaction mixture contained DNA, 20 mM phosphate buffer, pH 7.5, 0.1 mM dithiothreitol, and 2 mM EDTA in a siliconized glass tube. After 10 min of 37 °C dark preincubation to bind the enzyme, samples were photoreactivated in a 37 °C transparent water bath by illuminating from below with two cool-white fluorescent bulbs filtered through a 0.25-in. thickness of window glass. After phenol extraction, the fragment was treated with 1 M piperidine at 90 °C for 30 min to generate strand scissions at (6-4) lesion sites (Lippke et al., 1981) or with 10 μL of *M. luteus* pyrimidine dimer specific glycosylase plus apurinic endonuclease (Haseltine et al., 1980; gift of Dr. G. Chan) in 35 μL of 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, and 4 mM $MgCl_2$ for 60 min at 37 °C. Unirradiated samples were treated for guanine + adenine or cytosine + thymine Maxam–Gilbert DNA sequencing reactions. After lyophilization or precipitation, as appropriate, 2-μL samples containing 25 000 cpm were layered on a urea-containing 8% polyacrylamide gel and electrophoresed; the gel was then autoradiographed (Maxam & Gilbert, 1980; Brash & Haseltine, 1982).

Results

Photoreactivating enzymes can monomerize all types of cis-syn pyrimidine dimers in natural DNAs: the homodimers C[ ]C, T[ ]T, and U[ ]U, as well as the heterodimers C[ ]T, T[ ]C, U[ ]T, and T[ ]U (Setlow, 1966). Such determinations of substrate range employed linear DNAs with chromatographic identification of labeled dimers and resulting monomers (Setlow et al., 1964; Setlow, R. B., et al., 1965). We therefore determined the ability of the enzyme to act on dimers in supercoiled DNA. Superhelical pAT153 DNA was exposed to 156 J/m^2 of 254-nm radiation to produce eight dimers per molecule. After irradiation, part of the DNA was linearized with *Eco*RI, which has only one cleavage site in the pAT153 molecule. Fraction III PRE was then added to both DNAs, preincubated in the dark for 15 min, and exposed to photoreactivating light for 60 min. All samples were then treated with dimer-specific UV endonuclease and *Eco*RI. Figure 1 shows four alkaline agarose gels with results of such an experiment for PRE activity on linear (upper panels) and circular (lower panels) DNAs. Lanes f–t contain five quantities of PRE [2.26, 1.36, 0.79, 0.45, and 0.27 units (with activity in picomoles per hour)]; controls in lanes c–e contain 2.26 units (with unirradiated pAT153 DNA); each set of three (c–e, f–h, i–k, l–n, o–q, r–t) contains, first, no DNA (lanes c, f, i, l, o, and r), DNA with PRE kept in the dark (lanes d, g, j, m, p, and s), or DNA with PRE exposed to 60 min of photoreactivating light (lanes e, h, k, n, q, and t). Position and intensity markers were obtained by restricting pAT153 DNA with *Eco*RI plus *Bam*HI (BRL) (lanes a and v) and *Bgl*I (BRL)

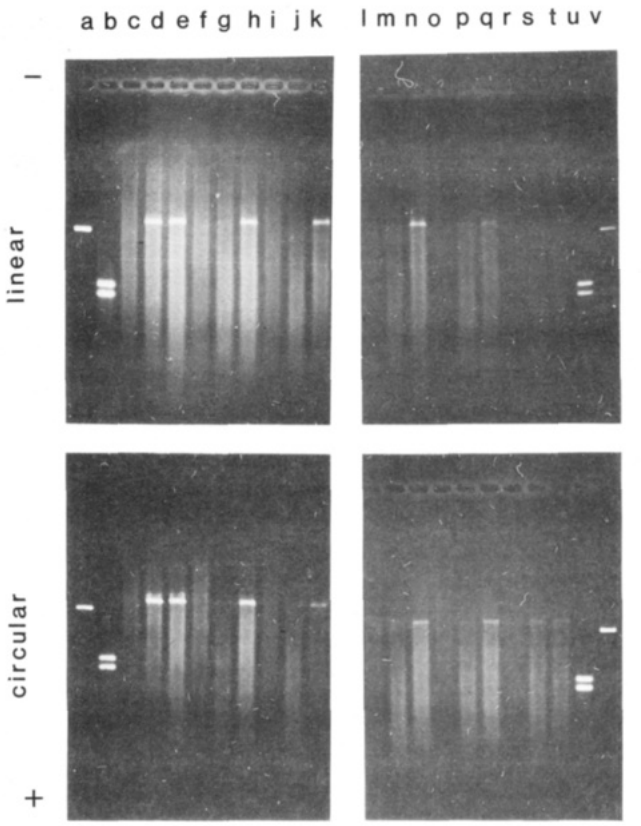

FIGURE 1: Determination of pyrimidine dimers in circular and linear pAT153 DNA by electrophoresis on 1% alkaline agarose gels after photoreactivation. Incubation mixtures (c–t) were incubated at 37 °C for 1 h and contained the following in a final volume of 30 μL: 28 mM potassium phosphate buffer, pH 7.1, 5.3 mM EDTA, 1.7 mM DTT, 40 mM Tris-HCl, pH 7.2, 43 mM NaCl, 4 mM $MgCl_2$, 6.7% (v/v) glycerol. The following activities of PRE (picomoles per hour) were present: 2.26 (c–e); 2.26 (f–h); 1.36 (i–k); 0.79 (l–n); 0.45 (o–q); 0.27 (r–t). Lanes d and e contained unirradiated control DNAs only. Irradiated circular or linear DNA samples (0.27 μg) were present in lanes g, h; j, k; m, n; p, q; and s, t only. The first sample in each set of three contains PRE at the stated amount and no added pAT153 DNA. Samples of lanes e, h, k, n, q, and t were incubated in the presence of PR light while all the others were incubated in the dark. The samples were then digested with saturating amounts of *Eco*RI (5 units) and *M. luteus* PD endonuclease (10 μg) for 30 min at 37 °C in a 60-μL reaction mixture containing 14 mM potassium phosphate buffer, pH 7.1, 25 mM Na-HEPES, pH 7.6, 52.5 mM Tris-HCl, pH 7.2, 50 mM NaCl, 7.75 mM $MgCl_2$, 5.8 mM EDTA, and 3.3% (v/v) glycerol. The samples were then dried under vacuum and resuspended in 20 μL of H_2O, and 5 μL of tracking dye containing 0.25 mg/mL bromophenol blue, 5 mg/mL sodium dodecyl sulfate, and 50% (v/v) glycerol was added. Position and intensity markers were also present during electrophoresis: lanes a and v, 0.30 μg of *Eco*RI- and *Bam*HI-restricted pAT153 DNA; lanes b and u, 0.38 μg of *Bgl*I-restricted pAT153 DNA. Electrophoresis buffer was 30 mM NaOH and 3 mM Na_2EDTA. Electrophoresis was at 25 V overnight at room temperature. Gels were neutralized by immersion in 0.1 M Tris-HCl, pH 7.0, for 10 min and then stained for 30 min with the same buffer containing ethidium bromide at 1 μg/mL. Gels were photographed on a Chromato-Vue transilluminator (UV Products Inc.) with a Polaroid MP4 camera on NP55 film.

(lanes b and u). Comparison of the upper and lower panels, and the quantitative analysis of such a gel (Figure 2), shows that the enzyme monomerizes pyrimidine dimer sites in supercoiled DNA at least as well as in linear DNA. In fact, the rate of photoreactivation of dimers in supercoiled DNA is actually greater than that on linear DNA, probably reflecting precession of the enzyme along the circular substrate (Ciarrocchi and Sutherland, submitted for publication).

Cyclobutyl dimers are also formed in RNA by UV radiation (Gordon et al., 1976). Can the 40-kDa *E. coli* DNA-photo-

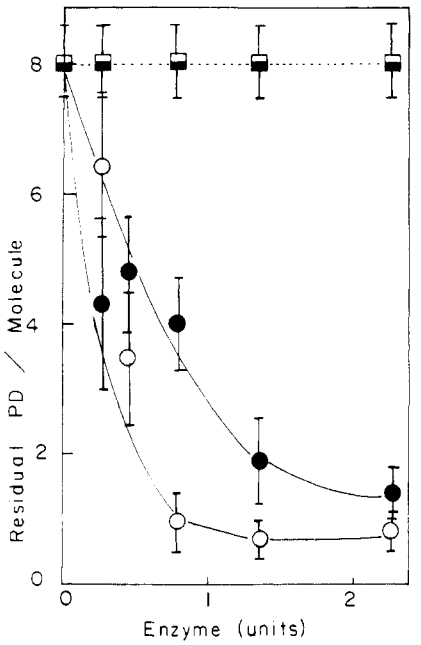

FIGURE 2: Photoreactivation of linear and supercoiled pAT153 DNAs. Pyrimidine dimer content was determined after incubation of linear (●) and circular (○) DNA samples with units of PRE activity (picomoles per hour) as shown in the presence of PR light and after incubation of linear (■) and circular (□) DNA PRE complexes in the dark for 1 h at 37 °C as in Figure 1. Data points are the average of two independent experiments.

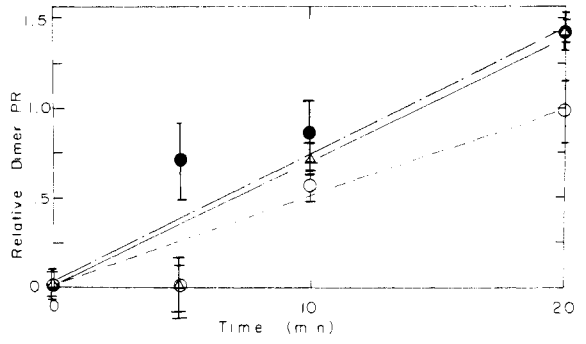

FIGURE 3: Photoreactivation of dimers of T7 DNA in the presence or absence of competing dimers in RNA. T7 DNA (122 pmol) exposed to 5 J/m^2 of 254-nm radiation was mixed with tRNA exposed to 5000 J/m^2 of 254-nm radiation to give approximate ratios of RNA dimers to DNA dimers of 1:1 (△) and 10:1 (●), or without RNA (○). After preincubation and photoreactivation for the times shown, samples were treated with 15 μg of *M. luteus* UV endonuclease, electrophoresed on an alkaline agarose gel, and analyzed by the method of Sutherland & Shih (1983).

reactivating enzyme monomerize pyrimidine dimers in RNA? We examined this possibility in a competition experiment: increasing quantities of RNA (exposed to 5000 J/m^2 from a 254-nm bulb) were added to T7 DNA containing 2.6 dimers per molecule. Fraction III enzyme was added, and the complexes were exposed to photoreactivating light as usual. Photoreactivation was analyzed by treatment with *M. luteus* PD endonuclease, gel electrophoresis, photography, and densitometry. Figure 3 shows that the UV-irradiated RNA did not compete with dimer-containing DNA for the enzyme. Since RNA was not a competitive inhibitor of the enzyme, it is unlikely that the enzyme either binds or photolyzes dimers in RNA.

The requirements for acceptance of a substrate by the enzyme, as delineated by the above experiments, include the presence of (1) a cis-syn pyrimidine dimer in (2) a deoxyribose polymer. We tested whether these were the only requirements

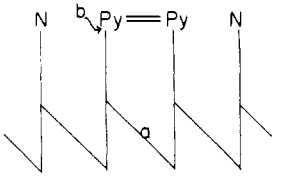

FIGURE 4: DNA segment showing individual bonds absent in tested substrates. Bond a was absent in photoligated dT_{10}·poly(dA), bond b cleaved in UV-irradiated DNA treated only with the *M. luteus* *N*-glycosylase, and bonds b and a cleaved in UV-irradiated DNA treated with *M. luteus* UV endonuclease.

Table I: Competitive Inhibition of Photoreactivating Enzyme Activity

competing DNA	quantity (μg)	dimer content (ng)	dimers split in test DNA (%)	competitive inhibn (%)
unirradiated d(pT)$_{10}$·poly(dA)	4	0	30.8	0[a]
irradiated *E. coli* DNA	0.64	20	6.8	78[b]
irradiated d(pT)$_{10}$·poly(dA)	4	[c]	20.3	34

[a] By definition. [b] Expected = 80%. [c] Four micrograms of irradiated d(pT)$_{10}$·poly(dA) containing 1.8 μg of unlinked oligonucleotides with 100 ng of internal dimers and 0.2 μg of photoligated oligonucleotide with 10–15 ng of internal dimers.

by constructing substrates containing *cis-syn*-cyclobutylpyrimidine dimers in a deoxyribose polymer, but with altered configuration resulting from the absence of particular bonds on the sugar–phosphate backbone or the lack of an *N*-glycosyl bond linking one moiety of the dimer and a deoxyribose. Figure 4 shows the bonds: (a) the phosphodiester linkage internal to the two deoxyribose moieties whose pyrimidines form the dimer, and (b) the *N*-glycosyl bond joining the 5′-pyrimidine to its deoxyribose.

The dimer substrate lacking bond a (phosphodiester internal to the dimer deoxyriboses) was constructed by photoligation of adjacent thymines in adjoining oligomers of (pT)$_{10}$·poly(dA) (Lewis & Hanawalt, 1982). Ligated oligomers appear as bands of 20, 30, and 40 bases on polyacrylamide gels; direct reversal by 254-nm radiation results in the disappearance of these multimer bands. [The corresponding increase in the oligomer band could not be detected because of the large amount of material already present in this band (Lewis & Hanawalt, 1982).] Fraction III enzyme was added to a mixture of ^{14}C-labeled *E. coli* DNA and ^{32}P-labeled photoligated (pT)$_{10}$·poly(dA) and exposed to photoreactivating light. Although 33% of the ^{14}C-labeled dimers were monomerized by the enzyme, no significant reduction of the multimer bands was observed.

It was possible that the enzyme failed to photoreactivate the photoligated dimers because it was unable to recognize dimers on short oligomers. The thymine oligomers should contain internal cis-syn thymine–thymine dimers as well as ligating thymine–thymine dimers. If the enzyme is able to recognize and bind to these dimers, then such a polymer should serve as a competitive inhibitor of enzyme activity on dimers in a natural DNA substrate. In Table I, row 2 shows that UV-irradiated *E. coli* DNA competes with irradiated ^{14}C-labeled *E. coli* DNA as substrate for the enzyme as expected; row 3 shows that UV-irradiated d(pT)$_{10}$·poly(dA) also competes, indicating binding by the photoreactivating enzyme. Since the enzyme does bind the irradiated d(pT)$_{10}$·poly(dA) polymer, it seems likely that its failure to photoreactivate the

ligating dimer results from an inappropriate configuration of the dimer lacking the phosphodiester bond internal to the deoxyribose moieties of the dinucleotide containing the dimer.

We next tested the ability of the photoreactivating enzyme to act on dimers when both bonds b and a of the DNA (Figure 4) are cleaved. This substrate was constructed by treatment of UV-irradiated ^{32}P-labeled T7 DNA with a partially purified preparation of *M. luteus* UV endonuclease, containing both *N*-glycosylase and apurinic endonuclease activities. UV endonuclease treatment of UV-irradiated T7 DNA completely prevented photoreactivation by the *E. coli* enzyme (10 and 6 cpm of dimers photoreactivated in 15 and 30 min of photoreactivation, respectively), although the same PRE preparation showed good activity on the same DNA not treated with the endonuclease (88 and 225 cpm of dimer photoreactivation at the same times).

Photoreactivation involves two major steps: (1) recognition and binding by the enzyme to the dimer site (which is independent of light absorption) and (2) light-dependent cleavage of the dimer to two parental monomers. Was the failure of the *E. coli* enzyme to photolyze the endonuclease-treated DNA theresult of successful binding but failure to photolyze the dimer, or of failure to bind the incised lesion? We tested the ability of the enzyme to bind ^{3}H-labeled DNA in a filter binding assay: the enzyme bound to UV-irradiated DNA to form a nonfilterable complex, while UV endonuclease treatment of the same DNA obliterated such complex formation. In contrast to untreated DNA, 70% of which was complexed by the PRE, only 6% of the DNA cleaved by the endonuclease was bound. Thus, the failure of the PRE to photoreactivate dimers after *M. luteus* UV endonuclease incision results from its inability to bind to DNA containing such sites. Partially purified endonuclease preparations contain both *N*-glycosylase (which cleaves at bond b of Figure 4) and apurinic endonuclease (cleaves at bond a) activities. We further investigated whether the prevention of photoreactivating enzyme binding to dimers required the cleavage of both bonds b and a, or whether cleavage of bond b alone would prevent complex formation. We found that glycosylase treatment of [^{3}H]DNA greatly reduced the ability of the PRE to bind the DNA in a filter binding assay. These results indicate that the *E. coli* enzyme requires for activity not only the presence of a *cis-syn*-cyclobutylpyrimidine dimer in a deoxyribose polymer but also a configuration in which the *N*-glycosyl bond and the internal phosphodiester bonds are intact.

Pyrimidine photoproducts other than *cis-syn*-cyclobutylpyrimidine dimers are also formed in DNA by UV radiation. One of these is the pyrimidine–cytosine adduct, identified as (6-4) pyrimidine–pyrimidone products on the basis of the equivalence of UV absorption spectra, fluorescence spectra, and HPLC mobility of alkali-labile dinucleotide and DNA products to those of authentic (6-4) photoproduct standards (Franklin et al., 1982). The presence of (6-4) products in cloned DNA fragments is detected by cleavage at the lesion site upon treatment with hot alkali. A cloned segment of the *E. coli lacI* gene was exposed to 500 J/m^2 of 254-nm radiation. Pyrimidine dimer sites were detected by cleavage with *Micrococcus luteus* UV endonuclease and electrophoresis on a urea-containing 8% polyacrylamide gel; (6-4) lesion sites were detected by treatment with 1 M piperidine at 90 °C for 30 min followed by electrophoresis. To test for photoreactivating enzyme activity on the two photoproducts, fraction III enzyme was added to the UV-irradiated DNA and exposed to photoreactivating light, and the DNA was analyzed for the two lesions after proteinase K digestion and phenol extraction.

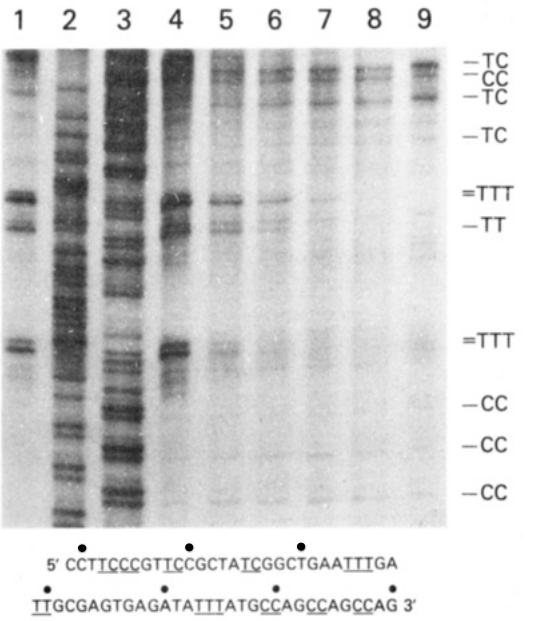

FIGURE 5: Photoreactivation of cyclobutane pyrimidine dimers but not (6-4) lesions by *E. coli* photoreactivating enzyme in vitro. Lane 1, scission of UV-irradiated DNA by *M. luteus* UV endonuclease. Lane 2, guanine plus adenine sequencing reaction (Maxam–Gilbert). Lane 3, cytosine plus thymine sequencing reaction. Lanes 4–8, UV endonuclease and piperidine plus heat cleavage of (6-4) lesion sites. Lanes 5–8, photoreactivating enzyme treatment subsequent to UV irradiation. Photoreactivating light present for 0, 5, 10, or 20 min. samples were exposed to only minimal room light when not being photoreactivated. Lane 9, piperidine plus heat cleavage only. The DNA sequence is indicated below; the 3′ base is bp 620 of the *lacI* gene. Pyrimidine sequences involved in prominent bands are indicated on the right: 622–623, 626–627, 630–631, and 675–676, piperidine-sensitive CC lesions; 664–665, 671–672, and 677–678, piperidine-sensitive TC lesions; and 635–636, 636–637, 650–651, 654–655, and 655–656, UV endonuclease sensitive sites.

Figure 5 shows the result of such an analysis. Lanes 1 and 9 show the cleavage patterns generated in the UV-irradiated fragment by *M. luteus* endonuclease and piperidine, respectively. Lane 4 shows the results of both these treatments: piperidine-sensitive lesions at CC in positions 622–623, 626–627, 630–631, and 675–676, and TC in positions 664–665, 671–672, and 677–678; endonuclease-sensitive sites at positions 635–636, 636–637, 650–651, 654–655, an 655–656. Lanes 5–8 show the result of addition of PRE to the fragment, exposure to 0 (lane 5), 5 (lane 6) 10 (lane 7), or 20 min (lane 8) of photoreactivating light. Figure 5 shows that after 20 min of photoreactivation treatment all the cis-syn pyrimidine dimer sites had disappeared, while the (6-4) photoproduct content of the DNA was unchanged. These data indicate that the *E. coli* photoreactivating enzyme did not act on the (6-4) pyrimidine–pyrimidine lesion in DNA, although it did monomerize cyclobutylpyrimidine dimers in the same DNA.

Discussion

The 40 000-Da *E. coli* photoreactivating enzyme quantitatively monomerizes cis-syn pyrimidine dimers in DNA (Ahmed & Setlow, 1977). We have now shown that the rate of dimer monomerization by the enzyme is as great or greater on dimers in superhelical DNA as in the same molecule in linear form. The enzyme does not act on dimers in RNA.

We have now defined further the substrate requirements of this enzyme: it does not act on a dipyrimidine photoproduct in DNA joined at the 6-4 position. In addition, P.-S. Song, W. H. Farland, and B. M. Sutherland (unpublished observations) have shown that neither the human nor this *E. coli* PRE could act on DNA adducts containing one pyrimidine

linked to 8-methoxypsoralen, or to 5,7-dimethoxycoumarin via a cyclobutyl ring. These results indicate that neither of these two conditions alone—the presence of a two-pyrimidine photoproduct in DNA or of a pyrimidine linked to another moiety by a cyclobutyl ring—is sufficient for recognition as substrate by the *E. coli* enzyme. We next constructed pyrimidine dimer substrates in DNA containing both the two pyrimidines and cyclobutyl rings characteristic of the cis-syn dimer, but without individual bonds in the immediate vicinity of the dimer. The first substrate (UV-irradiated DNA cleaved by partially purified *M. luteus* UV endonuclease) lacked the *N*-glycosyl bond linking one pyrimidine of the dimer to the deoxyribose moiety. The *E. coli* enzyme did not photoreactivate such dimers and bound the DNA containing them very poorly. We also determined the effect of cleavage of only the *N*-glycosyl bond; binding of the enzyme to DNA containing such dimers was very low. The enzyme also did not photoreactivate dimers in which the phosphodiester bond joining the deoxyribose moieties bonded to the dimer was not intact, but it could photoreactivate dimers in the same oligodeoxyribonucleotide in which the internal phosphodiester bond was intact. Photoreactivating enzyme activity from *Anacystis nidulans*, on the other hand, is reported to cleave pyrimidine dimers in DNA even if the internal phosphodiester was cleaved (Paterson et al., 1984).

The photoreactivating enzyme activity from commercial bakers' yeast was the first PRE whose substrate range was examined. Setlow, J. K., et al. (1965) showed that its substrate was the *cis-syn*-cyclobutylpyrimidine dimer in DNA. Rupert (1964) showed that it was inactive in photoreactivation of RNA. The trans-syn pyrimidine dimer (Rahn & Hosszu, 1968; Ben-Hur & Ben Ishai, 1968) and cytosine–thymine adduct (Patrick, 1970) were found not to be substrates for the enzyme activity from bakers' yeast. The presence of two distinct photoreactivating enzyme activities in extracts of commercial bakers' yeast (Boatwright et al., 1975; Madden, 1979) may be responsible for the apparently conflicting results on the activity of the yeast enzyme on UV-irradiated DNA treated with *M. luteus* UV endonuclease: Patrick & Harm (1973) found that yeast enzyme bound such DNA but could not photolyze the dimer, whereas Haseltine et al. (1980) observed that a yeast PRE preparation released free thymine from such DNA in a light-dependent reaction, indicating monomerization of the dimer. The 40 000-Da *E. coli* PRE is quite different from either of these photolyase activities, as it neither binds well nor photolyzes such UV endonuclease treated DNA.

The yeast and *E. coli* photoreactivating enzymes also differ in their affinity for dimers in DNA relative to the *M. luteus* endonuclease; Patrick & Harm (1973) found that the endonuclease could not nick at dimers to which yeast photoreactivating enzyme was bound, but Sutherland et al. (1980) showed that the endonuclease could displace the 40 000-Da *E. coli* PRE and thus nick at dimer sites. We found that *M. luteus* endonuclease pretreatment of DNA effectively prevents PRE–dimer complex formation and that the endonuclease can displace *E. coli* PRE already bound to dimer sites. The *E. coli* uvr ABC endonuclease, however, cannot displace this *E. coli* PRE from dimer sites (E. Seeberg, personal communication). [In contrast, the 50 000-Da *E. coli* PRE increases both the rate and extent of cleavage of UV-irradiated DNA by the *E. coli* uvr ABC endonuclease (Sancar et al., 1984b).] These results probably reflect the relative affinities of these repair enzymes for dimer in DNA: yeast PRE > *M. luteus* UV endonuclease > 40 000-Da *E. coli* PRE > *E. coli* uvr ABC endonuclease.

The 40 000-Da *E. coli* photoreactivating enzyme thus shows several requirements for composition and configuration of its substrate. These include (1) two pyrimidines joined by a cyclobutyl bond (2) in a deoxyribose polymer with (3) at least the following bonds intact: (a) the phosphodiester internal to the two deoxyribose moieties linked to the pyrimidines of the dimer and (b) the *N*-glycosyl bond joining pyrimidine to deoxyribose. The high specificity of this enzyme indicates its usefulness as a diagnostic tool for recognizing the presence and biological effects of pyrimidine dimers in DNA.

Registry No. DNA-photoreactivating enzyme, 37290-70-3.

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Fluorescence-Detected Circular Dichroism of Ethidium Bound to Poly(dG-dC) and Poly(dG-m^{5}dC) under B- and Z-Form Conditions†

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ABSTRACT: The equilibrium binding of ethidium to poly(dG-dC) and poly(dG-m^{5}dC) under conditions favoring B and Z forms was investigated with fluorescence-detected circular dichroism (FDCD) and optical titration methods. FDCD spectra indicate a similar geometry for the intercalated ethidium under both B- and Z-form conditions, even at low levels of bound ethidium. The magnitude of the 310–330-nm FDCD band as a function of the bound drug to base pair ratio (r) indicates ethidium binds to poly(dG-dC) in 4.4 M NaCl and to poly(dG-m^{5}dC) in 25 mM $MgCl_2$ by clustering. Under these conditions, circular dichroism spectra indicate the polymer is largely Z form. Thus, it appears ethidium clusters into regions it has induced into a right-handed form. For all conditions studied, the FDCD spectra provided no evidence for a left-handed binding site. Under B-form conditions, binding is random.

Pohl & Jovin (1972) discovered a salt-induced conformational transition in poly(dG-dC) that has been assigned to conversion to left-handed poly(dG-dC) (Wang et al., 1979, 1981; Patel et al., 1979; Drew & Dickerson, 1982; Wartell et al., 1983). Although ethidium binding to native and synthetic DNAs under B-form conditions has been well characterized (Lepecq & Paoletti, 1967; Dalgleish et al., 1971; Houssier et al., 1974; Aktipis et al., 1975; Krugh et al., 1975; Olmstead & Kearns, 1977; Bresloff & Crothers, 1981; Dahl et al., 1982), much less is known of the interaction of ethidium with left-handed DNA. Pohl et al. (1972) reported that ethidium binding to left-handed poly(dG-dC) in 4.4 M NaCl solution results in the reversal of the salt-induced conformational transition. Krugh & Walker (1984) and Walker et al. (1985) used circular dichroism and optical absorption spectroscopies to monitor the binding of ethidium to poly(dG-dC) and poly(dG-m^{5}dC) under both B- and Z-form conditions and concluded that ethidium intercalation results in the formation of a right-handed binding site. Conversely, Shafer et al. (1984) studied the binding of ethidium to left-handed poly(dG-dC) in 4.4 M NaCl and suggested that ethidium forms a left-handed intercalation site at low levels of bound drug. The plausibility of a left-handed intercalation site is supported by model building and theoretical studies (Gupta et al., 1983). Van de Sande & Jovin (1982) showed that Z* DNA [a condensed form of poly(dG-dC) in ethanol solution] supports the binding of several intercalating ligands, including ethidium.

A complicating factor in interpreting the CD[1] spectra of ethidium complexes with polymers is that the observed CD

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[1] Abbreviations: FDCD, fluorescence-detected circular dichroism; CD, circular dichroism; bp, base pair(s); EDTA, ethylenediaminetetraacetic acid.

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