Escherichia coli DNA photolase stimulates uvrABC excision nuclease in vitro
(pyrimidine dimers/DNA repair/DNA–protein interactions)

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ABSTRACT Pyrimidine dimers are the major photoproducts produced in cellular DNA upon UV irradiation. In Escherichia coli there are dark and photorepair mechanisms that eliminate the dimers from DNA and prevent their lethal and mutagenic effects. To determine whether these repair mechanisms act cooperatively or competitively in repairing DNA, we investigated the effects upon one another of DNA photolase, which mediates photorepair, and uvrABC excision nuclease, an enzyme complex of the uvrABC gene products, which catalyzes nucleotide excision repair. We found that photolase stimulates the removal of pyrimidine dimers but not other DNA adducts by uvrABC excision nuclease. The two subunits of uvrABC excision nuclease, the uvrA and uvrB proteins which together bind to the dimer region of DNA, had no effect on the activity of photolase. T4 endonuclease V, which like photolase is specific for pyrimidine dimers, was inhibited by photolase, suggesting that these two proteins recognize the same or similar chemical structures in UV-irradiated DNA that are different from those recognized by uvrABC excision nuclease.

In Escherichia coli UV (200–300 nm)-induced DNA damage is repaired by three molecular mechanisms: photoreactivation (1), nucleotide excision repair (2, 3), and postreplication-recombination repair (4). Of these repair mechanisms, the postreplication-recombination pathway does not involve an enzymatic machinery that recognizes the photoproducts and removes them; rather, it operates on the gaps generated by the replicative mechanism opposite dimers to initiate a recombinational process that ultimately leads to “diluting out” of the photoproducts. On the other hand, photoreactivation and nucleotide excision repair involve enzymes that specifically recognize the DNA damage and directly eliminate the photoproducts from DNA. Photoreactivation is mediated by DNA photolase, a flavoprotein (5) that acts specifically on the major UV photoproduct, the cis–syn pyrimidine dimer, and catalyzes its photolysis back to pyrimidines by 300–500-nm light (6, 7). Nucleotide excision repair is initiated by uvrABC excision nuclease (an enzyme complex of the uvrABC gene products), which cuts the eighth phosphodiester bond 5′ and the fourth or fifth phosphodiester bond 3′ to a pyrimidine dimer to generate a 12- to 13-nucleotide-long single-stranded DNA carrying the pyrimidine dimer. The oligonucleotide is removed, and the resulting gap is filled by DNA polymerases and sealed by ligase (8). While photolysis is specific for pyrimidine dimers, uvrABC excision nuclease removes, in addition to pyrimidine dimers, the second major UV photoproduct, 6-4′-(pyrimidine-2′-one)-thymine [Thy(6-4)Pyo] (8, 9) as well as other nucleotide adducts generated by such diverse chemicals as psoralens, cis-diaminedichloroplatinum(II), nitrous acid, and mitomycin C. Thus, the fact that photolysate and uvrABC excision nuclease have different action mechanisms and substrate specificities suggests that they interact with pyrimidine dimers differently. However, it is conceivable that binding of one enzyme to the dimer substrate may interfere with binding of the other. Such a situation becomes of physiological significance when photolysate binds to pyrimidine dimers but cannot repair them for lack of photoreactivating light. Under such conditions, does the enzyme impede DNA repair by uvrABC excision nuclease? While in vivo data (10, 11) have suggested that photolysate in fact aids dark repair, it has been reported (12) that in vitro the enzyme inhibits uvrABC excision nuclease. Having recently purified both enzymes to homogeneity (8, 13), we decided to study their effects on one another.

MATERIALS AND METHODS

Bacterial Strains. E. coli K-12 derivatives CSR603 (recA1 uvrA6 phr-1) and AB2487 (recA1 thyA) were obtained from B. J. Bachmann and P. Howard-Flanders, respectively.

Enzymes. The subunits of uvrABC excision nuclease were prepared essentially as described by Sancar and Rupp (8) with the exception of uvrB protein, for which a strain carrying the tac-uvrB plasmid pUNC211 (in which uvrB protein constitutes about 15% of total cellular proteins upon induction) was used as the starting material. E. coli DNA photolysate was purified as described (13). T4 endonuclease V was a generous gift from E. C. Friedberg and R. A. Schultz.

DNA Purification. Nonradioactive pBR322 plasmid was purified from AB2487/pBR322 by Sarkosyl lysis, followed by centrifugation in CsCl/ethidium bromide density gradients. [3H]pBR322 was prepared as follows: AB2487/pBR322 was grown in K medium containing thymidine (2.5 μg/ml) to A600 = 0.6, at which time chloramphenicol was added to 200 μg/ml and growth was continued for another hour. At this point [3H]thymidine (78.1 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) was added to a final concentration of 2 μCi/ml, and incubation was continued for 14 hr. The cells were collected, and the plasmid DNA was purified as described above. The specific activity of the plasmid was 3.98 × 10^6 cpm/μg. The plasmid concentration was determined by using an extinction coefficient of 20 M^-1 cm^-1 at 260 nm and a M, of 2.88 × 10^6.

DNA Damaging Treatments. UV-damaged pBR322 was obtained by irradiating the DNA at 10 μg/ml with a General Electric lamp at a rate of 10 W/cm^2 for 100 sec in 10 mM Tris chloride, pH 7.4/10 mM NaCl/1 mM EDTA (buffer A). This treatment resulted in 1.7–1.8 lethal hits per molecule as measured by transformation (14). Of the lethal lesions, 95% were photoreactivatable and, therefore, were assumed to be pyrimidine dimers. cis-Pt(NH3)2Cl2-damaged DNA was pre-

Abbreviation: Thy(6-4)Pyo, 6-4′-(pyrimidine-2′-one)-thymine.

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pared by incubating pBR322 at 100 μg/ml with cis-
Pt(NH3)2Cl2 (0.1 mM) at 23°C in buffer A for 35 min and then
dialyzing the mixture for 24 hr against the same buffer. This
treatment produced on the average 1.43 lethal hits per mole-
cule as measured by transformation.

Enzyme Assays. The uvrABC excision nuclease buffer
(175 μl) contained 50 mM Tris chloride (pH 7.5), 100 mM
KCl, 10 mM MgCl2, 10 mM dithiothreitol, 2 mM ATP, 0.57
pmol of [3H]pBR322, and uvrA, -B, and -C subunits at the
amounts indicated in Results. The reaction mixture was
incubated at 23°C; 10-μl samples were taken at time intervals
and mixed with an equal volume of a solution containing 50
mM Tris chloride (8.0), 20 mM EDTA, 1% NaDodSO4, 10%
glycerol, and 0.05% bromophenol blue. The samples were
loaded onto a 1% agarose gel, which was run and stained
with ethidium bromide by standard procedures. The bands
corresponding to covalently closed circular and open circu-
lar DNA were cut out, dissolved in 1 ml of 1 M HClO4, and
neutralized with NaOH; then 10 ml of scintillant was added,
and the radioactivity was quantitated in an LKB liquid scinti-
illation counter. From the fraction of covalently closed cir-
cular DNA remaining at each time point, the number of cuts
made by uvrABC excision nuclease was calculated by using
the Poisson distribution.

Photolyase was assayed by the E. coli transformation as-
say as described (13, 15) with the following modifications.
The reaction was conducted in 115 μl of uvrABC excision nuclease
buffer containing 0.9 pmol of pBR322 and 0.05
pmol of photolyase. The mixture was illuminated with a
General Electric black light at a rate of 2 mW/cm2; 5-μl sam-
ple were taken at time intervals and mixed with an equal
volume of 20 mM EDTA and then used to transform compe-
tent CSR603 cells.

T4 endonuclease V assay was conducted at 37°C in 20 μl
doctor buffer A containing 0.07 pmol of pBR322 and the amount
of enzyme indicated in Results.

RESULTS

Effect of Photolyase on uvrABC Excision Nuclease. To
determine the effect of photolyase on excision of pyrimidine
dimers by the excision nuclease, we wanted to use experi-
mental conditions such that, at the time of addition of the
nuclease, most or all of the pyrimidine dimers were bound
to photolyase. This was achieved by preincubating UV-irradi-
ated pBR322 DNA with about a 16-fold molar excess of pho-
tolyase over pyrimidine dimers. To find out what fraction of
pyrimidine dimers was bound to photolyase under these con-
tions, the mixture was exposed to a single photoreactivat-

Fig. 1. Cutting of UV-irradiated pBR322 DNA by uvrABC excision nuclease (ABC) in the presence (Lower) or absence (Upper) of photolyase (PL) as assayed by agarose gel electrophoresis. The reaction mixture (175 μl) contained 0.57 pmol of [3H]DNA 0.52, 1.2, and 2.7 pmol of uvrA, uvrB, and uvrC proteins, respectively, and 14.8 pmol of PL when indicated. The irradiated DNA contained 1.7 photoproducts per mole-
cule, and 1.6 of these were photoreactivable and presumed to be pyrimidine dimers. In the reactions with photolyase, the DNA was
preincubated with the enzyme for 10 min at 23°C before the addition of uvrABC excision nuclease. (Left) Unirradiated DNA. (Right) Irradiated
DNA. Lanes 1 through 16 contain samples (10 μl each) taken at 0, 0.5, 1, 1.5, 2, 2.5, and 3 through 12 min after addition of uvrABC excision
nuclease.

Table 1. Determination of the number of photolyase–pyrimidine
dimer complexes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Surviving fraction</th>
<th>Lethal hits per molecule</th>
<th>Dimers per molecule</th>
<th>Fraction of dimers repaired</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>UV(50 J/m²)</td>
<td>0.170</td>
<td>1.77</td>
<td>1.71</td>
<td>—</td>
</tr>
<tr>
<td>UV + flash</td>
<td>0.35</td>
<td>0.59</td>
<td>0.57</td>
<td>0.66</td>
</tr>
<tr>
<td>UV + black light</td>
<td>0.90</td>
<td>0.10</td>
<td>—</td>
<td>1.0</td>
</tr>
</tbody>
</table>

pBR322 DNA was irradiated at 254 nm with a fluence of 50 J/m²
at a concentration of 10 μg/ml in buffer A and then mixed with
photolyase in uvrABC excision nuclease buffer. The reaction mix-
ture (555 μl) contained 0.9 pmol of DNA and 27 pmol of enzyme.
After a 10-min incubation in the dark at 23°C, the mixture was
exposed to a single flash and a sample was taken. The rest of the
mixture was photoreactivated for 30 min with black light at a rate of
2 W/cm². The number of lethal hits per molecule at various stages
of the experiment were determined by the method of Sancar and
Rupert (14), and the number of dimers per molecule was calculated
by assuming that the lesions remaining after 30 min of photo-
reactivation were nondimer photoproducts. Recent experiments
(unpublished data) show that photolyase does not act on Thy(6-
4)Pyo, which probably accounts for most of the "nonphotoreactiva-
ting light flash of about 1-msec duration (16). The results (Ta-
ble 1) show that this treatment resulted in photolysis of 66% of
the dimers. Because in this photolyase preparation only
70% of the molecules contain the flavin chromophore (5, 17)
necessary for photolysis, we conclude that the fraction of
dimers bound by photolyase was 66/70 = 0.94. This calcula-
tion assumes that photolyase molecules with or without the
chromophore are equally efficient in binding to dimers. This
assumption was confirmed by nitrocellulose filter-binding
experiments in which it was found that ~100% of the dimers
were complexed with the enzyme under similar experimental
conditions (unpublished data).

Having established the conditions such that, at any given
moment, most of the dimers are bound to photolyase, we
then treated the UV-irradiated DNA with uvrABC excision
nuclease in the presence or absence of photolyase and fol-
lowed the cutting kinetics by this enzyme (Figs. 1 and 2).
The excision nuclease had no effect on unirradiated DNA in
the presence or absence of photolyase. With irradiated DNA
the enzyme converted covalently closed circles to open cir-
cles by incising on both sides of the photoproducts (8), and
this conversion was stimulated by photolyase, as can be seen
qualitatively in Fig. 1. When the data from Fig. 1 and two
other experiments conducted under identical conditions were analyzed quantitatively, the results shown in Fig. 2 were obtained. Photolyase stimulated both the initial rate and the extent of the cutting reaction by a factor of $\approx 2$. Since about 95% of the UV lesions in the DNA used in these experiments were pyrimidine dimers, we conclude that photolyase stimulates the removal of pyrimidine dimers by uvr-ABC excision nuclease.

**Stimulation of uvrABC Excision Nuclease by Photolyase Is Specific for Pyrimidine Dimers.** To understand the mechanism of stimulation by photolyase, it is important to know whether the enzyme stimulates the removal of other DNA adducts that are substrates for uvrABC excision nuclease but not for photolyase. For this purpose we used DNA containing cis-Pt(NH$_3$)$_2$Cl$_2$ or psoralen adducts and conducted the cutting reaction in the presence or absence of photolyase. The results obtained with cis-Pt(NH$_3$)$_2$Cl$_2$-damaged DNA are shown in Fig. 3. The rate and extent of cutting by uvrABC excision nuclease were the same regardless of whether photolyase was present or absent in the reaction mixture. Similarly no stimulation of cutting of psoralen-damaged DNA was observed (data not shown), indicating that the effect of photolyase on the activity of uvrABC excision nuclease is specific for pyrimidine dimers.

**Effect of uvrA and uvrB Proteins on Photolyase.** Having established that photolyase stimulates the removal of pyrimidine dimers by uvrABC excision nuclease, we wanted to find out if the effect was reciprocal—i.e., does the excision nuclease stimulate the photolysis of pyrimidine dimers by photolyase? Since preincubation of UV-irradiated DNA with saturating amounts of uvrABC excision nuclease will result in excision of all pyrimidine dimers before the addition of photolyase, we conducted the experiment with only two subunits of the excision nuclease. Data from several laboratories (18-21) suggest that uvrA and uvrB proteins are the subunits involved in specific recognition of and binding to the dimer-containing region of DNA in a reaction driven by ATP hydrolysis by the uvrA subunit. Therefore, we addressed the question of whether the binding of these subunits has any effect on photolyase. UV-irradiated DNA was preincubated with a molar excess of uvrA and uvrB proteins and then mixed with limiting amounts of photolyase and photoreactivated (Fig. 4). The same rate and extent of photoreactivation were observed, whether uvrA and uvrB proteins were present or absent in the reaction mixture, suggesting that the binding of these subunits to DNA does not affect the binding of photolyase.

**DNA Photolyase Inhibits T4 Endonuclease V.** The results presented above suggest that the contact sites of photolyase and uvrABC excision nuclease on UV-irradiated DNA do not overlap. Another enzyme that acts on UV-damaged DNA is T4 endonuclease V. This enzyme like photolyase, but unlike uvrABC excision nuclease, repairs only pyrimidine dimers; its action mechanism strongly suggests that, when it binds to DNA, it is in close contact with the dimer: the enzyme cleaves the N-glycosyl bond of the 5' pyrimidine of the dimer and then the phosphodiester bond 3' to the apyrimidinic deoxyribose that is generated by the first reac-

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**Fig. 3.** Kinetics of cutting of cis-Pt(NH$_3$)$_2$Cl$_2$-damaged DNA by uvrABC excision nuclease in the presence ($\triangle$) or absence ($\square$) of photolyase. The experimental conditions were the same as in Fig. 1 except that the DNA contained 1.45 lethal cis-Pt(NH$_3$)$_2$Cl$_2$ adducts per pBR322 instead of UV photoproducts.

**Fig. 4.** Photoreactivation of UV-irradiated DNA in the presence ($\triangle$) or absence ($\square$) of uvrA and uvrB proteins. The reaction mixtures (115 $\mu$L) contained 0.9 pmol of pBR322 (1.7 dimers per molecule) and 0.05 pmol of photolyase in uvrABC excision nuclease buffer; where indicated, the mixture was preincubated for 10 min with 14 pmol of uvrA and 12.8 pmol of uvrB proteins before the addition of photolyase. In both cases the samples were kept in the dark for 5 min after the addition of photolyase and then exposed to photoreactivating light (2 W/cm$^2$). Samples (5 $\mu$L) were taken at the indicated times, mixed with an equal volume of 20 mM EDTA, and then used for transforming CSR603. The amount of dimers repaired was calculated by the increase in transformation frequency as described (13, 15).
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Fig. 5. Inhibition of T4 endonuclease V by photolyase. The reaction mixtures (20 µl) contained 0.07 pmol of pBR322 (irradiated with 150 J/m², which produced 4.4 dimers per molecule) in T4 endonuclease V buffer and 7.4 pmol of photolyase and 22 pmol of endonuclease where indicated. The reaction mixtures were incubated at 37°C for 30 min and then analyzed on an agarose gel. Lanes: 1, irradiated DNA; 2, DNA treated with endonuclease; 3, irradiated DNA preincubated with photolyase at 23°C for 10 min before addition of the endonuclease. Quantitative analysis of the gel shows that T4 endonuclease V produced 1.96 and 0.71 nicks per molecule in the absence (lane 2) and presence (lane 3) of photolyase, respectively. The linear DNA in lanes 2 and 3 is probably due to a single-strand-specific endonuclease contaminant in the T4 endonuclease V preparation used. Such an endonuclease would cut across the nicks produced by T4 endonuclease V and generate linear molecules.

DISCUSSION

The significance of the results presented in this paper are 2-fold. From the physiological point of view, our findings suggest that two enzymes that repair pyrimidine dimers in E. coli act in concert to help the survival of the organism. The demonstration in vitro that photolyase stimulates uvrABC excision nuclease is in agreement with the in vivo results of Harm and Hillebrandt (10), who found that E. coli phr- mutants were more UV sensitive than phr+ cells even in the dark, and with the results of Yamamoto et al. (11), who found that photolyase-overproducing cells were more UV resistant than wild-type cells and that this increased resistance was observed only in Uvr+ cells. On the other hand, our results are in contradiction with an earlier report (12) that photolyase inhibits uvrABC excision nuclease in vitro. However, those studies were conducted with the so-called photolyase R protein, whose identity as a true photolyase and its physiological significance in photoreactivation is questionable (13). Our finding of inhibition of T4 endonuclease V by E. coli photolyase is in accordance with those of Patrick and Harm (27) that yeast photolyase inhibits Micrococcus luteus UV endonuclease, an enzyme that has a size similar to that of T4 endonuclease V and the same action mechanism (28). The inhibition of T4 endonuclease V by photolyase is probably of little physiological significance in E. coli, as the amount of T4 endonuclease V produced upon infection by T4 phage is in vast excess over photolyase (26) and, under such conditions, no significant inhibition is to be expected. However, our finding that photolyase inhibits an enzyme that initiates dimer removal by a glycosylase–apurinic acid endonuclease type of mechanism suggests that such an inhibition might be detrimental should both enzymes be present in the same organism. It is interesting to note that M. luteus lacks photolyase (29), and it is tempting to speculate that other bacteria that lack photolyase (Haemophilus influenzae, Streptococcus pneumoniae, Bacillus subtilis) initiate dimer excision by a glycosylase–apyrimidinic acid endonuclease type of mechanism rather than by an excision nuclease.

From the physicochemical point of view, our results reveal certain aspects of the mechanism of binding of uvrABC excision nuclease and photolyase to dimer-containing DNA. The excision nuclease hydrolyzes the eighth phosphodiester bond 5’ and the fourth or fifth phosphodiester bond 3’ to the dimer (8). Thus, the incision sites are apart by about one turn of the double helix, suggesting that the enzyme binds to DNA on one face of the helix that does not include the two pyrimidines making the dimer; therefore, it does not interfere with photolyase, which recognizes the dimer, as opposed to uvrABC excision nuclease, which recognizes the helical distortion caused by the dimer (and by other “bulky” base adducts). Recent experiments (unpublished data) show that E. coli photolyase has the same turnover number for dimers in oligo(dT)₃ containing more than 4 thymidine residues as for native DNA and that the enzyme repairs dimers in oligo(dT)₃ at 43% of maximum rate. That the dimer is the important structure recognized by photolyase, and it is reasonable to assume that the enzyme interacts with a four-to-six nucleotide region containing the dimer and that these nucleotides do not interact with uvrABC excision nuclease. Binding of uvrA protein to UV-damaged DNA partially unwinds the helix (unpublished data), thus, one can speculate that the net effect of the binding of UvrA/B to DNA is to increase the single-strandedness created by the dimer. Since photolyase is equally efficient on single- and double-stranded DNA, this increase in single-strandedness is not expected to affect photoreactivation, in agreement with our observation that uvrA and uvrB proteins did not affect the activity of photolyase. (The effect of uvrC protein on photolyase was not investigated. It is conceivable that, upon formation of the uvrA/B/DNA complex, uvrC may have an effect on the binding of photolyase.) On the other hand, since the pyrimidine dimer per se is not the structure recognized by the excision nuclease, it is possible that photolyase, by binding to the dimer and changing the DNA helix parameters, makes the DNA a better substrate for the excision nuclease. Alternatively, photolyase by binding to the dimer may facilitate the dissociation of uvrABC excision nuclease from DNA after the incision events; as it has been reported (21) that the nuclease remains complexed with DNA for a long period after the incision reaction, such an effect might promote the turnover of this enzyme, thus increasing the rate and extent of the cutting. Whatever the mechanism is, it is clear from our results that the excision nuclease is stimulated by photolyase specifically in its action on pyrimidine dimers. Indeed, it is ironic that uvrABC excision nuclease is named as such since the major UV photoproduct, the pyrimidine dimer, is one of the poorest substrates for the enzyme, presumably because the distortion caused by the dimer is minimal (30) as compared to that caused by other DNA adducts. In our limited survey of various substrates, we found (unpublished data) that the psoralen-pyrimidine mono- and di-adducts to be the best substrates followed by Thy(6-4)Pyo, certain cis-Pt(NH₃)₂Cl₂ adducts, and then pyrimidine dimers.

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