Binding and Catalytic Properties of *Xenopus* (6-4) Photolyase*

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The abbreviations used are: CPD, cis-syn-cyclobutane pyrimidine dimer; HPLC, high pressure liquid chromatography; GST, glutathione S-transferase; DTT, dithiothreitol; Pyr, pyrimidine; T[6-4]T, the (6-4) photoproduct of the corresponding dinucleotides.

*Xenopus* (6-4) photolyase binds with high affinity to DNA bearing a (6-4) photoproduct and repairs it in a light-dependent reaction. To clarify its repair mechanism of (6-4) photolyase, we determined its binding and catalytic properties using synthetic DNA substrate which carries a photoproduct at a single location. The (6-4) photolyase binds to T[6–4]T in double-stranded DNA with high affinity \( (K_D = 10^{-9}) \) and to T[6–4]T in single-stranded DNA and T[Dewar]T in double- and single-stranded DNA although with slightly lower affinity \( (K_D = 2 \times 10^{-8}) \). Majority of the T[6–4]T-(6-4) photolyase complex dissociates very slowly \( (k_{off} = 2.9 \times 10^{-5} \text{ s}^{-1}) \). Its absolute action spectrum without a second chromophore in the 350–600 nm region closely matches the absorption spectrum of the enzyme. The quantum yield \( (\phi) \) of repair is approximately 0.11. The fully reduced form \((E-FADH_2)\) of (6-4) photolyase is catalytically active. Direct analysis of the photoreactivated product showed that (6-4) photolyase restores the original pyrimidines. These findings demonstrate that *cis-syn*-cyclobutane pyrimidine dimer photolyase and (6-4) photolyase are quite similar, but they are different with regard to the binding properties.

The ultraviolet component of sunlight has mutagenic, carcinogenic, and lethal effects (1). The main products in DNA of UV light include the various oxidized nucleotides, cyclobutane pyrimidine dimers (CPD) (2), and (6-4) photoproducts (2, 3). Cells protect themselves against these lesions by eliminating the photoproducts from their genomes by excision repair or by photoreactivation. The phenomenon of photoreactivation (the reduction of lethal and mutagenic effects of UV radiation by simultaneous or subsequent irradiation with near UV or visible light) has been identified in a variety of organisms, and the enzyme responsible has been designated photoreactivating enzyme (DNA photolyase) (4). DNA photolyase binds to the photoproducts on DNA in a light-independent manner, then utilizes light energy to convert them to the original two pyrimidines, and dissociates from the repaired DNA. Two types of DNA photolyase are known, one specific for CPD (CPD photolyase) (5) and the other specific for (6-4) photoproduct (6-4) photolyase) (6).

Gene coding for CPD photolyase is widely distributed among species, being isolated from more than 16 organisms, and their enzymatic nature has been characterized in detail (4, 5, 7, 8). CPD photolyase has stoichiometric amounts of two noncovalent chromophores. One of these is FADH\(^2\) and the other is either methenyltetrahydrofolate or 5-hydroxy-5-deazaflavin (5). The proposed repair mechanism is as follows. The enzyme binds the DNA substrate, the second chromophore of the bound enzyme absorbs a UV-visible photon, and by dipole-dipole interaction transfers energy to FADH\(^2\) which, in turn, transfers an electron to the CPD in the DNA. The CPD splits and back electron transfer restores the dipyrimidine and the functional form of flavin ready for a new cycle of catalysis (5).

(6-4) photolyase activity has been found in *Drosophila melanogaster, Xenopus laevis, Crotalus atrox,* and *Arabidopsis thaliana* (6, 9, 10), the gene having been isolated from *Drosophila* (11) and *Xenopus* (12). Its enzymatic properties and reaction mechanism have yet to be well characterized. Isolation of the gene showed that (6-4) photolyase has an amino acid sequence similar to CPD photolyase and that the purified protein has two chromophores, one of which is an FADH\(^2\) similar to that of CPD photolyase. These findings suggest (6-4) photolyase repairs its substrate via a mechanism analogous to that of CPD photolyase, which involves electron transfer from the excited states of the enzyme to the damaged site resulting in restoration of the original components. The structure and chemical nature of the (6-4) photoproduct, however, suggest that direct application of the repair mechanism of the CPD to the (6-4) photoproduct is difficult. The (6-4) photoproduct has been proposed to be formed via an oxetane intermediate (13). The presumed oxetane photoproduct has been shown to be unstable above about 80 °C and to decompose thermally to the (6-4) photoproduct (14). The resulting (6-4) photoproduct involves the transfer of the group at C-4 of the 3′ base of the dinucleotide to the C-5 position of the 5′ base concomitant with formation of the sigma bond between the C-6 of the 5′ base and the C-4 of the 3′ base. Therefore, even if an enzyme breaks the 6–4 C–C bond, the bases would not be restored to their original forms. Furthermore, Kim et al. (15) rejected the possibility that electron transfer to the (6-4) photoproduct would result in the re-formation of the original DNA bases. Instead they proposed an ingenious repair mechanism in which the equilibrium between the (6-4) photoproduct and the oxetane shifts toward the latter upon enzyme binding. Electron transfer to the oxetane therefore could lead to oxetane splitting by a mechanism analogous to that proposed for cyclobutane splitting (oxetane intermediate model).
To test the probability of this oxetane intermediate model and to clarify the reaction mechanism, we investigated the binding and catalytic properties of Xenopus (6-4) photolyase using synthetic DNA substrates bearing one of the major UV photoproducts (T\(^\rightarrow\)A or T\(\rightarrow\)Dewar) at a single location. (6-4) photolyase binds to T\(\rightarrow\)A or T\(\rightarrow\)Dewar with a high affinity (\(K_d = 5 \times 10^{-9}\)) of the same order as the CPD photolyase but dissociates very slowly in comparison. Like the CPD photolyase, the fully reduced form (E-FADH\(^-\)) of (6-4) photolyase is catalytically active, but the quantum yield (\(\phi = 0.11\)) is lower than that of the Escherichia coli CPD photolyase (0.42). We also directly determined the structure of the repaired product by HPLC and showed that the (6-4) photoproduct is repaired to its original pyrimidines. These results and the accompanying paper by Sancar and colleagues (32) of D. melanogaster (6-4) photolyase show that the (6-4) photolyase binds to its substrate and repairs it by a mechanism similar to that by CPD photolyase.

**EXPERIMENTAL PROCEDURES**

**Substrates—**49-mers bearing the centrally located (6-4) photoproduct (T\(\rightarrow\)A or T\(\rightarrow\)Dewar) or its Dewar isomer (T\(\rightarrow\)Dewar) were used. The substrate sequences (the thymine dimer of the damaged strand is underlined) were TAATTATGGTGTACATGGCTGCTGCACGAATTAAGCAATTCGATTGCAGTTAATCATCGTCCGACATTGAACCTGAGCTACCATGCCTGCTGCACGAATTAAGCAATTCGATTG.

**Xenopus (6-4) Photolyase—**Sequence-specific binding activity, after which the fractions containing the (6-4) photoproducts were prepared as follows. The purified 49-mer band corresponding to unrepaired DNA and the 21-mer product of DNA was separated on 10% polyacrylamide sequencing gels. The 49-mer band was excised out and the DNA was purified.

**Determination of Dissociation Rate Coefficients (k\(\text{off}\))—**The reaction mixture (250 \(\mu\)l in binding buffer) contained 0.5 \(\mu\)M oligonucleotide substrate and 4–10 \(\mu\)M Xenopus (6-4) photolyase. The mixture was incubated on ice for 30 min, and then the pUC19 DNA irradiated with 25 kJ/m\(^2\) of 254 nm light was added to a concentration of 2.8 nM (\(~300 \text{ nM}\) (6-4) photoproduct), after which 20-\(\mu\)l aliquots taken at the indicated times were loaded on a running gel. After electrophoresis, the band fraction was quantified and plotted as a semi-logarithmic function of time to obtain the rate coefficient from the slope of the decay curve (19, 22).

**D\(\text{N}\)ase I Footprinting—**For footprinting Xenopus (6-4) photolyase, 50 \(\mu\)M DNA was mixed with 1 \(\mu\)M photolyase in 20 \(\mu\)l of binding buffer. After incubation on ice for 15 min, CaCl\(_2\) (8 mM), MgCl\(_2\) (10 mM), and 0.02 units of DNase I (Takara, Japan) were added, and the reaction was incubated at 22 °C for 3 min. D\(\text{N}\)ase I digestion was stopped by adding EDTA to 20 mM. DNA was precipitated with ethanol in the presence of 2 \(\mu\)g of RNA and then dissolved in formamide/dye and separated on 12% polyacrylamide sequencing gels.

**Photoreactivation Assay and Action Spectrum—**The photoreactivation assay was performed as described previously (26, 27) with some modifications. The photoactivated sample was placed in the irradiation chamber of a monochromator (Spectro Irradiator CRM-5, Japan Spectroscopic Co.) and exposed to 100 \(\mu\)M triethanolamine in the presence of 2 \(\mu\)g of RNA and then dissolved in formamide/dye and separated on 12% polyacrylamide sequencing gels.
unrepaired versus the light dose gave a line with a slope equal to \(-k_\text{off}\) (photolytic constant). The photolytic cross-section \((\phi\text{)}\) and quantum yield \((\psi\text{)}\) could be calculated from \(\phi \cdot \psi = (5.2 \times 10^6) k_\text{off} (\text{mm}^2 \text{ erg}^{-1} \text{cm}^{-2})\), where \(e\) is the molar extinction coefficient.

The absolute action spectrum of the \(E-\text{FADH}^+\) form of enzyme was generated by conducting the photoreactivation reactions at wavelengths ranging from 350 to 600 nm and by following procedures described previously (24). The relative action spectra of the \(E-\text{FADH}^+\) and \(E-\text{FADH}_\text{ox}\) forms of the enzyme were generated by following the procedures described (15) and then normalized to 370 nm, which showed the maximum activity.

Analysis of a Photoreactivation Product—The sequences of the oligonucleotides used are: TT 30-mer 5’ d(GCACGACCAACCGCCATTGG-GCAACCAGCACG) 3’; and (6-4) 30-mer 5’ d(GCACGACCAACCGCCATTGG-GACCAACCAGCA) 3’. The oligonucleotide containing the (6-4) photoproduct (6-4) 30-mer, 2.0 \(A_{260}\) units), synthesized as described previously (16), was mixed with the (6-4) photolyase (620 \(\mu\text{g}\) of Fraction I) in buffer (1.0 ml) containing 6.9 \(m\) \(M\) Tris-HCl, pH 7.5, and then alkaline phosphatase (from calf intestine, 1 \(\mu\text{l}, 1\) unit) was added. After incubation at 37 °C for 2 h, ethanol (300 \(m\)l) was added, and the mixtures were kept at \(-20\) °C for 2 h. The proteins were pelleted by centrifugation, and the supernatants were concentrated in vacuo and loading unto a running gel (Fig. 3). About 8% of the T\[6–4\]T and measuring the decay of the preexisting complexes as a function of time by taking samples from the reaction mixture and loading unto a running gel (Fig. 3). About 8% of the T\[6–4\]T-photolyase complexes dissociate with a rate constant \(k_\text{off} = 1.3 \times 10^3 \text{ s}^{-1}\), and the remaining complexes dissociate considerably slower \((k_\text{off} = 2.9 \times 10^2 \text{ s}^{-1})\). (6-4) photolyase dissociates from the T\[Dewar\]T substrate faster than do complexes with T\[6–4\]T. The rapidly dissociating class comprises approximately 48% of the T\[Dewar\]T-photolyase complexes present at time 0 and has the estimated \(k_\text{off} = 7.7 \times 10^4 \text{ s}^{-1}\), whereas the remaining complexes dissociate with an apparent rate constant \(k_\text{off} = 1.4 \times 10^4 \text{ s}^{-1}\) (Fig. 4). The reason why photolyase shows the biphasic dissociation rates is unclear. But in many reports DNA damage recognizing protein shows the biphasic dissociation rates (19, 22). The association rate constants \((k_\text{on})\) for both substrates were calculated from \(k_\text{on} = k_\text{off} / k_\text{off}\). For T\[6–4\]T, \(k_\text{on} = 2.6 \times 10^3 \text{ M}^{-1} \text{s}^{-1}\), and for T\[Dewar\]T it was 5.1 \times 10^3 \text{ M}^{-1} \text{s}^{-1}. These values are at least 1 order of magnitude slower than the on-rate from a diffusion-
controlled reaction.

Protection from DNase I Digestion by (6-4) Photolyase—To characterize further the interaction of (6-4) photolyase with DNA, we conducted DNase I footprinting experiments. DNase I footprinting gives a reasonable approximation of the contact site for DNA-binding proteins. Fig. 5 shows the DNase I footprints of (6-4) photolyase on the strands bearing the photoproduct (top, lanes 1–10) and complementary (bottom, lanes 11–16) strands. The enzyme protects 11 phosphodiester bonds around the (6-4) photoproduct from DNase I cleavage (Fig. 5, lane 6). In addition it inhibits hydrolysis of the 8th phosphodiester bond 5' to the photoproduct. Photoreactivation repaired almost all the (6-4) photoproduct, resulting in the loss of protection against DNase I digestion (lane 8). The bottom strand DNase I footprint extends over an 8 nucleotide-long region around the AA complementary to the photoproduct. In addition the 7th phosphodiester bond 5' to the AA sequence is partially protected (lane 14). The substrate bearing T[Dewar]T has a similar footprint (lane 10). The footprints of (6-4) photolyase are shown schematically in Fig. 6.

Substrate Specificity of (6-4) Photolyase—(6-4) photolyase binds to T[6–4]T and T[Dewar]T in single- and double-stranded DNA (Figs. 1 and 5). We tested the photoreactivating activity of (6-4) photolyase on these substrates using the photoreactivation assay (Fig. 7). This assay measures the restoration of restriction enzyme MseI susceptibility to the photoproduct with the TTAA site. (6-4) photolyase repair T[6–4]T in single as well as double-stranded DNA (Fig. 7, lanes 2 and 6), however, has no effect on the MseI site bearing T[Dewar]T (lane 3) or T[CPD]T (lane 4).

Absorption Spectra of Xenopus (6-4) Photolyase Carrying...
**Fig. 6. Diagram of the DNase I footprints of (6-4) photolyase.** This diagram is based on the data in Fig. 5. The two thymines that make up the photoproduct are underlined. The brackets and asterisks above and below the sequence, respectively, indicate the areas and bases of protection from DNase I digestion of the top and bottom strand.

**Fig. 7. Photoreactivation assay of various types of photoproducts.** The duplex oligomer (1 nM) carrying 5'-T6-4-T (lane 2), T[De-War]T (lane 3), or T[CPD'T]T (lane 4) or a single-stranded oligomer (1 nM) carrying T[6-4]T (lane 6) were mixed with 50 nM (6-4) photolyase and then were illuminated with fluorescent lamps for 30 min at room temperature and extracted with phenol/chloroform. Samples in lanes 2–4 were treated with MseI and then separated on 10% polyacrylamide gels. Samples in lane 6 were treated with MseI after annealing with a complementary strand (see “Experimental Procedures”). Samples in lanes 1 and 5 were illuminated without photolyase and treated the same as the samples in lanes 2 and 6, respectively.

**Only Flavin—** The semi-purified Xenopus (6-4) photolyase (Fraction I) has two chromophores; one has been identified as FAD (12), and the other chromophore has its absorption peak at 416 nm. Although the identity and structure of the second chromophore is not known, it has been proposed on the basis of spectroscopic changes associated with its chemical decomposition that (6-4) photolyase and its homolog utilize methenyltetrahydrofuran as a second chromophore (25). Further purification (Fraction II) results in the loss of the second chromophore and the oxidation of flavin. Findings from studies of CPD photolyase (5) show that flavin can be present in three different oxidation states with distinct absorption characteristics: fully reduced (FADH; $\lambda_{\text{max}} = 366$ nm), neutral radical (FADH; $\lambda_{\text{max}} = 580$ and 625 nm), and fully oxidized (FADH$_2$; $\lambda_{\text{max}} = 444$ nm). Fig. 8A shows the absorption spectrum of the purified enzyme without the second chromophore (Fraction II). The enzyme has prominent peaks at 450, 585, and 625 nm that are typical of the fully oxidized and neutral radical forms of flavin. Prolonged storage of the enzyme without a reducing reagent resulted in the further oxidation of the flavin to the fully oxidized form (FADH$_2$; Fig. 8B). Both the neutral radical and fully oxidized forms of the enzyme can be reduced to the fully reduced form ($\lambda_{\text{max}} = 374$ nm) by irradiation with UV/visible light under anaerobic conditions in the presence of DTT (Fig. 8C).

**Role of Flavin in the Catalytic Reaction—** We conducted a photoreactivation assay with Xenopus (6-4) photolyase containing only flavin to define the role of flavin in the catalytic reaction and compared our data with those for E. coli CPD photolyase. Fig. 9 shows the results of the light dose dependence of the repair of the (6-4) photoproducts by Xenopus (6-4) photolyase carrying fully reduced flavin (E-FADH$_2$). On the basis of these data and using the equation described under “Experimental Procedures,” we obtained $\varepsilon_{\text{max}} \phi = 630$ cm$^{-1}$ M$^{-1}$ for E. coli CPD photolyase (E-FADH$_2$). The values of $\varepsilon$ at 370 nm are almost identical for both the E. coli CPD photolyase and Xenopus (6-4) photolyase bearing FADH$_2$ ($\varepsilon_{\text{max}} = 5700$ cm$^{-1}$ M$^{-1}$). Xenopus (6-4) photolyase therefore reverses the (6-4) photoproducts in DNA with a quantum yield $\phi = 0.11$. This is comparable to the quantum yield $\phi = 0.42$ for the E. coli CPD photolyase under our experimental conditions. The efficiency of repair by (6-4) photolyase seems to be lower than that by the CPD photolyase.

To define further the role of flavin in the catalytic reaction, we determined the relative/absolute action spectra with enzyme bearing the various forms of flavin. Fig. 8 shows the relative/absolute action spectra in the near UV-visible region of the three forms superimposed on their respective absorption spectra. The action spectrum of E-FADH$_2$ closely matches its absorption spectrum in the near UV-visible region (Fig. 8C), whereas the relative action spectra of the purified photolyase that carries the neutral radical or fully oxidized flavin do not match their absorption spectra but are similar to the spectrum of fully reduced flavin (Fig. 8, A–C). In addition, the oxidation of flavin to the neutral radical or fully oxidized form results in a significant reduction in repair efficiency. These findings suggest that the fully reduced enzyme (E-FADH$_2$) is the catalytically active form and that the photoreactivating activity of the neutral radical or fully oxidized enzyme (E-FADH or E-FADH$_2$) is due to the reduced form of the oxidized enzyme to the catalytically active form (E-FADH$_2$).

**Direct Evidence for the Restoration of Normal Pyrimidines—** To determine directly the structure of the photoreacti-
vated product, we synthesized an oligonucleotide carrying only two thymidines, in which the (6-4) photoproduct is introduced, d(GCACGACCAACGCAT(6-4)TACGCAACCAGCACG) using the dinucleotide building block (16). After the photoreactivation with the Xenopus (6-4) photolyase (Fraction I), the reaction mixture was analyzed by reversed-phase HPLC. Results are shown in Fig. 10A. The largest peak, with the retention time of 14.0 min and UV absorption maxima at 255.6 and 325.0 nm, was the (6-4) 30-mer remaining after photoreactivation. The reaction product, eluted slightly later than the starting material, had no UV absorption in the long wavelength region (>300 nm). These two compounds could not be separated on an anion-exchange HPLC column. The peaks at the retention times of 3.7 and 8.8 min were assigned to an UV-absorbing contaminant in the reaction buffer and the flavin adenine dinucleotide chromophore, respectively. When the TT 30-mer, d(GCACGACCAACGCATTTACGCAACCAGCACG), was added to the reaction mixture prior to injection, this unmodified oligonucleotide was co-eluted with the photoreactivated product (Fig. 10B).

After purification by HPLC, this product was degraded to its nucleoside components with nuclease P1 and alkaline phosphatase. The TT 30-mer was treated similarly with the enzymes, and both degraded products were analyzed by HPLC. As shown in Fig. 11, there was no difference in the elution profiles of the photoreactivated (6-4) 30-mer and TT 30-mer reactions (Fig. 11, A and B). The chromatogram of a mixture of these two samples showed that all the peaks derived from the photoreactivated (6-4) 30-mer were identical with those of the TT 30-mer (Fig. 11C). The UV absorption spectrum, with a maximum at 263.6 nm, of the third peak in the (6-4) 30-mer sample was the same as that of the thymidine peak derived from the TT 30-mer. To confirm the identity, we calculated the nucleoside composition of each 30-mer from the peak areas (Table II).

**DISCUSSION**

Two types of photolyase are known, CPD photolyase and (6-4) photolyase. The repair reaction of CPD photolyase is well characterized. CPD photolyase absorbs light energy, and the photoexcited enzyme transfers an electron to CPD, generating a dimer radical anion. This anion is very unstable and undergoes spontaneous splitting to the original undamaged form. In contrast, the repair reaction of (6-4) photolyase is unclear. Since the formation of the (6-4) photoproduct involves the transfer of the group at C-4 of the 3’ base of the dinucleotide to the C-5 position of the 5’ base, restoration of the (6-4) photoproduct to the original undamaged dipyrimidine by single elec-
tron transfer is unlikely. On the other hand, the primary structure of both types of photolyase are similar and both have the same chromophores, indicative that the repair mechanism of (6-4) photolyase must be analogous to that of CPD photolyase but a more complex process. The repair reaction of photolyase can be divided into two steps, a light-independent binding reaction and a light-dependent catalytic reaction. To clarify the repair mechanism of (6-4) photolyase, we determined the enzymatic properties of (6-4) photolyase at each reaction step and compared them to those of CPD photolyase.

Xenopus (6-4) photolyase binds to T[6–4]T with the association constant \( K_A = 2.1 \times 10^7 \text{ M}^{-1} \). The value is in the same range as the E. coli CPD photolyase (2.6 \( \times 10^9 \text{ M}^{-1} \)) (5, 19).

Interestingly, (6-4) photolyase dissociates from its substrate-enzyme complexes much slower than does the CPD photolyase. More than 90% of the complexes dissociate very slowly with a half-life of \(-5 \text{ h} \), only 8% of the complexes dissociating rapidly with a half-life of \(-7 \text{ min} \). These values are markedly different from those for the CPD photolyase. The E. coli CPD photolyase dissociates much more rapidly, about 85% of the complexes dissociating with a half-life of 15–45 s (19, 26). (6-4) photolyase also binds to T[Dewar]T \( (K_A = 6.7 \times 10^7 \text{ M}^{-1}) \). This complex (photolyase-T[Dewar]T) dissociates at a rate of the same order as the CPD photolyase (55% of the complexes dissociate with a half-life of 45–60 s). Only the (6-4) photolyase-T[6–4]T complexes dissociate very slowly and, thus, have a lower association rate constant. The contact site on DNA for the (6-4) photolyase was determined by DNase I footprinting experiments. Despite the difference in the dissociating kinetics, the DNase I footprints made by (6-4) photolyase on DNA containing T[6–4]T and those on T[Dewar]T bear a remarkable resemblance. On both substrates, 11 phosphodiester bonds around the photoproducts are protected, and the 12th and 13th phosphodiester bonds of the photoproduct are hypersensitive to DNase I (Fig. 5), indicative that the (6-4) photolyase contact with DNA containing each type of photoproducts similarly and the difference in dissociation constants is due to the specific interaction between the damage recognition site of the enzyme and T[6–4]T. Slow association rate was also reported with UvrA protein (22). Such slow rates usually imply significant conformational change upon binding (27). Thus, the slow dissociation of the (6-4) photolyase-T[6–4]T complex might suggest that a change in the interaction between the enzyme and substrate occurs upon enzyme binding.

CPD photolyase has two chromophores. Its flavin chromophore is necessary and sufficient for the catalytic reaction (5). In case of the (6-4) photolyase, flavin chromophore also is sufficient for the catalytic reaction because the purified enzyme (Fraction II), which retains FAD but loses the second chromophore, is catalytically active as well as the semi-purified enzyme (Fraction I) with both FAD and the second chromophore. The purified (6-4) photolyase has three oxidative states of flavin, as is the case of the CPD photolyase: fully oxidized flavin FAD \(_{ox} \), neutral radical FADH \(_1 \), and fully reduced FADH \(_{-} \). The former two forms may be derived from oxidation during the purification step and can be reduced to the fully reduced form by illumination with near UV-visible light in the presence of DTT, as is the case for CPD photolyase (28), which suggests that catalysis may take place by electron dona-

| TABLE II |
|---------------|
| Nucleoside composition of TT 30-mer and the PR product |
| dA | dG | dC | T |
| Calculated for TT 30-mer | 10 | 6 | 12 | 2 |
| TT 30-mer | 9.13 | 5.53 | 13.16 | 2.18 |
| PR product | 9.31 | 5.47 | 13.10 | 2.12 |

Regarding the repair mechanism of (6-4) photolyase, the reverse reaction of (6-4) photoproduct formation has been proposed, which involves the restoration of the (6-4) photoproduct to 2 thymidines, proceeding via the same oxetane intermediate that led to its formation (oxetane intermediate model). In this model, the binding of (6-4) photolyase to DNA thermally converts the (6-4) photoproduct to its oxetane intermediate (15). If the (6-4) photolyase repairs its substrate to the original dinucleotide, the model most likely is valid. Recently, however, the free energy difference between the (6-4) photoproduct and oxetane species was reported as being too high to perturb the equilibrium between the two species by photolyase binding (29). This report is indicative that the oxetane intermediate model is an unlikely one and raises the possibility that the repaired product still has some degree of modification. The repair product has been analyzed using several enzymes (15). The photoreactivation product is recognized by restriction endonuclease but is not cleaved by E. coli endonuclease III. When photoreactivated DNA was used as the template for DNA polymerase I, elongation of the primer occurred, and the incorporated nucleotide was dA. In agreement with our previous findings (6), these results suggest that (6-4) photolyase restored the pyrimidines to their normal form. In the study reported here, we tried to directly determine the structure of the reaction product. We treated a large amount of a (6-4) photoproduct-bearing oligonucleotides with the Xenopus enzyme. Then, after illumination with fluorescent light, the reaction mixture was analyzed by HPLC. As shown in Fig. 10, the product separated from the (6-4) 30-mer was indistinguishable from the unmodified TT 30-mer in our HPLC system. To confirm that the product was identical with the unmodified oligonucleotide, both oligomers were degraded to their nucleoside components which were subjected to HPLC analysis. This method, used to detect modified nucleosides in synthetic oligonucleotides (30, 31), is very reliable for determining the nucleoside composition. As shown in Fig. 11, the elution profiles of the two degradation mixtures were identical. The UV absorption spectrum of the third peak in the photoreactivated mixture was identical with that of the thiymidine peak from the unmodified 30-mer (data not shown). It should be noted that the original (6-4) 30-mer had no thymidine in its sequence. Although the deoxy-cytidine content was greater in the nucleoside composition determined from the peak areas, a reasonable number was obtained for thyminidene (Table II). These results clearly demonstrate that the (6-4) photoproduct is repaired to its original pyrimidines by photoreactivation with the Xenopus (6-4) photolyase. Moreover, the normal structure of the reaction product strongly suggests that this photoreactivation proceeds via the oxetane/azetidine intermediate as proposed previously (15).

The accompanying paper (32) on Drosophila photolyase provides additional evidence for the similarities between the (6-4) photolyase and CPD photolyase. Although some minor differences exist between the Drosophila and Xenopus (6-4) photolyase with regard to substrate specificities and catalysis, these could reflect interspecies differences or experimental variabilities between laboratories.
REFERENCES

1. Freidberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, American Society for Microbiology, Washington, D.C.
2. Wang, S. Y. (1976) in Photochemistry and Photobiology of Nucleic Acids (Wang, S. Y., ed) Vol. 1, pp. 295–350, Academic Press, New York
3. Mitchell, D. L., and Nairn, R. S. (1989) Photochem. Photobiol. 49, 805–819
4. Sancar, G. B. (1990) Mutat. Res. 236, 147–160
5. Sancar, A. (1994) Biochemistry 33, 2–9
6. Todo, T., Takemori, H., Ryo, H., Ibbaru, M., Matsunaga, T., Nikaido, O., Sato, K., and Nomura, T. (1993) Nature 361, 371–374
7. Yasui, A., Eker, A. P. M., Yasuhira, S., Yajima, H., Kobayashi, T., Takao, M., and Okawara, A. (1996) EMBO J. 15, 6143–6151
8. Kato, T., Jr., Todo, T., Ayaki, H., Ishizaki, K., Motia, T., Mitra, S., and Ikemaga, M. (1994) Nucleic Acids Res. 22, 4119–4124
9. Kim, S.-T., Malhotra, K., Smith, C. A., Taylor, J. S., and Sancar, A. (1994) J. Biol. Chem. 269, 8535–8540
10. Fowler, K. W., Bui, C., and Essigmann, J. M. (1982) J. Biol. Chem. 257, 1050–1054
11. Kung, H. C., and Bolton, P. H. (1997) J. Biol. Chem. 272, 9227–9236
12. Zhao, X., Liu, J., Hsu, D. S., Zhao, S., Taylor, J. S., and Sancar, A. (1997) J. Biol. Chem. 272, 32580–32588