Characterization of (6-4) Photoproduct DNA Photolyase*

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Pyrimidine (6-4) pyrimidone photoproduct is the second most abundant UV photoproduct in DNA. Recently, it was reported that Drosophila melanogaster cell-free extracts restored the biological activity of (6-4) photoproduct-containing DNA in a light-dependent reaction (Toda, T., Takemori, H., Ryo, H., Ihara, M., Matsunaga, T., Nikaido, O., Sato, K., and Nomura, T. (1993) Nature 361, 372-374) concomitant with the loss of (6-4) photoproduct antigenic sites and (6-4) photoproduct-cause alkali-labile sites. In the present study we show that the (6-4) photoproduct but not its Dewar isomer is the substrate for the enzyme, that the enzyme has an action spectrum peak at 400 nm, and that the efficiency of repair per incident photon is very low compared with cyclobutane pyrimidine dimer photolyases. Furthermore, we provide evidence that the (6-4) photoproduct photolyase converts the photoproduct to unmodified bases probably through an oxetane intermediate.

The ultraviolet (200-300 nm) component of sunlight is a major environmental mutagen and carcinogen. UV produces more than a dozen photoproducts in DNA; however, the most abundant are the cis,syn cyclobutane pyrimidine dimer, Pyr(c,s)Pyr, and the 6-4-pyrimidin-2'-one-photoproduc, or the (6-4) photoproduct, Pyr(6,4)Pyr (see Wang, 1976). These two lesions constitute 70-80% and 20-30% of total UV photoproducts, respectively. The (6-4) photoproduct is eliminated from both prokaryotes and eukaryotes. The cyclobutane pyrimidine dimer is a poorer substrate for excision repair; however, it can be reversed by DNA photolyases in organisms that contain the enzyme (Sancar, 1993; Kim and Sancar, 1993). DNA photolyase (deoxygeniboloabutadiyrimidine pyrimidine-lyase, EC 4.1.99.3) photoactivating enzyme is specific for cyclobutane type dimers; it does not repair (6-4) photoproducts (Brash et al., 1985). As a consequence, it has become common practice to expose UV-irradiated cells to photoreactivating light (350-450 nm) to study the effect of (6-4) photoproducts. Any residual mutagenic or cytotoxic effects remaining following photoreactivation are ascribed to (6-4) photoproducts (see Mitchell and Nair, 1989). This is a justifiable approach as it has been shown that in many organisms including man and Escherichia coli photoreactivating light does not eliminate (6-4) photoproducts from DNA, although at high doses of 300-350 nm wavelengths the photoproduct is converted to its Dewar isomer photochemically (Taylor and Cohrs, 1987; Taylor et al., 1988). This photoisomerization was suggested to explain the nonenzymatic loss of (6-4) photoproducts, that was correlated with type III photoreactivation (Ikenaga et al., 1918; 1971; Patrick, 1970), a process that is maximal at 313 nm and found only in certain bacteria (Ikenaga et al., 1970; Jagger et al., 1970).

However, two recent papers have provided strong evidence that, in some organisms at least, there might be a (6-4) photoreactivating enzyme (Toda and Ryo, 1992; Toda et al., 1993). It was found that cell-free extract of Drosophila melanogaster contained two factors with specific affinity for UV-irradiated oligomer, as determined by band shift assay. One of these (factor 2) was identified as the cyclobutane pyrimidine dimer DNA photolyase because the band was absent in a mutant lacking the enzyme (Toda and Ryo, 1992) when the experiment was conducted with UV-irradiated DNA photoreactivated with E. coli photolyase prior to mixing with the Drosophila cell-free extract. Thus it was concluded that factor 1 bound to (6-4) photoproduct. Surprisingly, however, exposure of the factor 1-DNA complex to high intensity light from a fluorescent lamp resulted in the disappearance of the retarded band. Similarly, when the DNA in factor 1 complex was exposed to light and then tested for (6-4) photoproducts by alkali hydrolysis and by disappearance of binding sites for Pyr(6,4)Pyr-specific monoclonal antibodies, all Pyr(6,4)Pyr were eliminated with the possible exception of C(6,4)C. Perhaps most significantly, this photoreactivation activity restored the biological function of a plasmid inactivated by Pyr(6,4)Pyr (Toda et al., 1993). It was proposed that Drosophila contained a photolyase specific for (6-4) photoproducts.

This finding was quite surprising because formation of the (6-4) photoproduct involves the transfer of the group at C4 (-NH, = OH) of the 3' base of the dinucleotide to the C-5 position of the 5' base concomitant with the formation of the sigma bond between the C-6 of the 5' base and the C-4 of the 3' base. Thus even if an enzyme breaks the 6-4 C-C bond the bases would not be restored to their original forms. To address this and many other related questions stemming from these exciting findings we decided to investigate this activity further. We used a defined substrate containing a single T(6,4)T in a 49-base pair duplex (Smith and Taylor, 1993) and nuclear extracts from a Drosophila cell line for these studies. Our work confirms the findings of Toda et al. (1993) and reveals that the enzyme may have a chromophore with λmax = 400 nm and that photoreactivation with this enzyme, most likely, restores the dinucleotides making up the (6-4) photoproduct to unmodified form.

EXPERIMENTAL PROCEDURES

Materials—MseI restriction endonuclease and polynucleotide kinase were purchased from New England Biolabs. DNA polymerase I was

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Enzyme and Substrate—Nuclear extract of the *D. melanogaster Kc* cell line was used as (6-4) photolyase. The extract was a kind gift of Dr. L. Searles (University of North Carolina). The extract was stored at -80 °C. Indeed, it was found that three or four freeze-thaw cycles did not affect the (6-4) photolyase activity. The substrate was a 49-base pair duplex containing either a (6-4) photoprotein or its *D. melanogaster* isomer at the center (the two Ts that make up the photodiester are in bold): 5′-GCATTGCCTGACGAAAATCCAGCTGAGCTGCTATAG-3′.

The preparation and characterization of the oligomer containing either the T6,4T or the T6,4D photoproduct have been described previously (Smith and Taylor, 1993; Svoboda et al., 1993). The oligomer was 5′-end labeled with [γ-32P]ATP (7,000 Ci/mmoll) and T4 polynucleotide kinase, annealed to the complementary strand, and the duplex was purified by gel electrophoresis as described previously (Smith and Taylor, 1993).

Photoreactivation Assay and Action Spectrum—The photodiester in the 49-base pair duplex is at an Msel site (which incises between the two Ts in the TTAA sequence) and thus is resistant to cleavage by Msel. The assay measures the restoration of the susceptibility of the duplex to Msel cleavage. The reaction mixture (100 μl) contained 50 μM Tris-Cl, pH 7.4, 5 mM MgCl2, 10 μM dithiothreitol, 1 mM ATP, 100 mM Tris-Cl, pH 7.4, 5 mM MgCl2, 2 μl of proteinase K (10 mg/ml) were added and incubated at 55 °C for 60 min. The DNA was then extracted with phenol/chloroform and precipitated with ethanol. The DNA was dissolved in 10 μl of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 50 mM glycerol, and 0.1 μl of cell-free extract, and about 0.1 μl of reference DNA (5′-end labeled with [γ-32P]ATP). The DNA was then mixed with 10 μl of Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol. Two units of Msel were added, and the mixture was incubated at 37 °C for 90 min. The digested DNA was separated on 12% polyacrylamide sequencing gels. The 49-mer band corresponding to unrepaired DNA and the 21-mer resulting from Msel incision of repaired DNA were excised and quantified by Cerenkov counting. The results shown in Fig. 2 indicate that the (6-4) photolyase is specific for the Kekule form and is essentially inactive on the Dewar isomer. The 1–2% activity observed with the Dewar isomer is probably caused by the low level of Kekule isomer contaminant in this T6,4D preparation.

**Photolytic Cross-section of (6-4) Photolyase**—Since the (6-4) photoprotein but not its *D. melanogaster* isomer absorbs in the near UV (310–330 nm) and since the photoreactivation activity appears to be specific for the Kekule form we considered the possibility that the photoreactivation resulted from direct excitation of the photoprotein to initiate a photochemical reaction facilitated by a chromophoreless (6-4) photoprotein-binding protein. This question is best answered by action spectrum measurements. Fig. 3 shows the absorption spectrum of T6,4D-T from the literature (Franklin et al., 1982) and the relative action spectrum of the (6-4) photolyase determined in this study. Clearly, the action spectrum is distinct from the T6,4D-T absorption spectrum. In fact, at the wavelengths (320–330 nm) corresponding to the T6,4D-T absorption peak the action spectrum has a valley, partly because at these wavelengths absorption of T6,4D-T leads to formation of nonsubstrate T6,4D-T. Thus, we tentatively conclude that the (6-4) photolyase has an intrinsic chromophore with an absorption peak at 400 nm.

**Photolytic Cross-section of (6-4) Photolyase**—Based on band shift assays (Todo and Ryo, 1992; Todo et al., 1993) the cyclobutane Tyr-c-Pyr photolyase and the Pyr (6-4) Pyr photolyase appear to be of comparable abundance. Yet, when we conducted photoreactivation experiments with T6,4D-T and T6,4D-T substrates the former was repaired more than 100-fold faster than the 6-4 photoprotein. To find out if this inefficient repair of the (6-4) photoprotein was because of inefficiency of the photoproduct on the primer-template mixture (10 μl) in 100 mM Tris-HCl, pH 7.4, 5 mM MgCl2, T4 polymerase (2 units), and one dNTP at 10 μM were added. The reaction was incubated at 37 °C for 30 min, and the reaction products were precipitated with ethanol and analyzed on 15% polyacrylamide sequencing gels.

**RESULTS**

**Fig. 1. Structures of T-T (6-4) photoproduct and its Dewar isomer.**

The reaction mixture was incubated at 37 °C for 90 min, and the reaction mixture was incubated at 37 °C for 90 min, and then the reaction products were analyzed on 12% polyacrylamide sequencing gels. For determining the base inserted across from the repaired lesion,
lanes respectively.

counting of the 49-mer and 21-mer bands.

tion coefficient (6) of the (6-4) photolyase.

chemical reaction we measured the photolytic cross-section of the (6-4) photolyase.

Photolytic cross-section is the product of the molar absorption of some other pyrimidine derivative not recognizable by endonuclease III and analyzed the reaction product on a sequencing gel. Fig. 5A shows that in this particular experiment about 30% of the (6-4) photoproduct has been eliminated as evidenced by sensitivity to MsoI restriction endonuclease (lane 2). However, when this DNA was treated with excess endonuclease III no cleavage occurred (lane 3) under conditions in which endonuclease III cleaved at all thymine glycols in a plasmid substrate (data not shown). Thus, it appears that photolyase does not generate a saturated pyrimidine ring as a product. To test for the formation of some other pyrimidine derivative not recognizable by endonuclease III we also tested the repaired DNA for template activity. Fig. 5B shows that T[6-4]T is a block for polymerase I (lane 1) but that after photoreactivation a fraction of the primer proportional to the fraction of T[6-4]T repaired is elongated past the photoproduct region (lane 2). Thus, we conclude that the photoreactivated DNA is no longer a block for DNA polymerase I. This is in agreement with the results of Todo et al. (1993), who...
showed restoration of transforming (replication) activity of a plasmid following photoreactivation, and provides further evidence that, most likely, (6-4) photolyase restores the pyrimidines to their normal form. A third line of evidence for this conclusion, of course, comes from the fact that the (6-4) photolyase renders the substrate susceptible to the \( \text{MseI} \) restriction endonuclease. It is well known that restriction endonucleases are quite sensitive to base modifications at the incision site, and \( \text{MseI} \) hydrolyzes the phosphodiester bond between the two thymines involved in \( \text{T}[6-4] \text{T} \); it is unlikely that the enzyme would recognize and incise at this sequence if following photoreactivation the bases were modified.

A final piece of evidence for restoration of the \( \text{T}[6-4] \text{T} \) to normal bases was provided by conducting chain elongation with single dNTPs using a primer terminating at the nucleotide preceding the lesion site. The results shown in Fig. 5C reveal that dA is incorporated very efficiently across the repaired lesion and that dG and dC are not incorporated at the detection limit of our assay. With dT as the sole nucleotide we do observe some (\(~1\%\)) chain elongation past the repaired lesion. However, we observe the same level of synthesis with undamaged DNA as well, and therefore we ascribe this synthesis to "slippage" of the template at this A-T-rich sequence. All of these data combined lead us to conclude that the (6-4) photolyase does restore \( \text{T}[6-4] \text{T} \) to canonical bases.

**DISCUSSION**

The work presented here confirms the findings of Todo et al. (1993) that \textit{Drosophila} contains a protein that mediates the light-dependent disappearance of the (6-4) photoproduct from DNA. We have shown further that the action spectrum peak for this activity is at 400 nm and that this activity is specific for the Kekule form of the photoproduct, being completely inactive on the Dewar form. We have obtained preliminary evidence that the photoreaction restores the bases to their canonical (unmodified) forms. Our findings raise two questions regarding the mechanism and significance of this activity.

**FIG. 4.** Ruppert plot for photoreactivation by (6-4) photolyase. Photoreaction at 360 nm was carried out under enzyme excess conditions such that during irradiation essentially all of the substrate was in enzyme-substrate complex. The level of repair was determined by digestion with \( \text{MseI} \).

**FIG. 5.** Restoration of canonical bases by (6-4) photolyase. Panel A, photoreactivated (PR) DNA was treated with \( \text{MseI} \) or endonuclease III as indicated. Panel B, photoreactivated DNA was used as a template for polymerase I. Elongation of the 10-mer primer to 49-mer (lane 2) indicates that the photoreversed bases do not block chain elongation by polymerase I. Panel C, nonphotoreactivated substrate, photoreactivated DNA and unmodified DNA (UM) were used as template for T4 DNA polymerase with 5'-labeled 27-mer primer in the presence of single dNTPs in each reaction as indicated. The degradation products seen in the absence of chain elongation are because of the 3' \( \rightarrow \) 5' exonuclease activity of T4 DNA polymerase. CFE, cell-free extract.
Regarding mechanism, it is most likely that reversal of the (6-4) product to 2 thymidines proceeds via the same oxetane intermediate that led to its formation. The presumed oxetane photoproduce of thymine has been shown to be unstable above -80 °C and thermally decompose to the (6-4) product (Rahn and Hosszu, 1969). It is not known, however, to what extent the oxetane intermediate also thermally decomposes back to the original dipyrimidine. The instability of the oxetane intermediate relative to the (6-4) product is probably caused by the inherent ring strain of the oxetane and the loss of aromatic stabilization of the pyrimidinone ring upon oxetane formation. The energy difference between the oxetane intermediate and the (6-4) product may not be that large, however, as the analogous sulfur intermediate (a thietane) has been shown to slowly interconvert with the (6-4) product in water at room temperature to give a 3:1 mixture at equilibrium (Clivio et al., 1991). It seems reasonable to expect that a protein could catalyze the interconversion of the oxetane intermediate and the (6-4) product, or the oxetane intermediate and 2 thymidines, and while bound to the protein, change the relative populations of these species. This suggests that the enzyme may be catalyzing the reversal of the (6-4) product by either of two general sequences of events (Fig. 6).

In one possible pathway, the enzyme may photochemically catalyze the formation of the oxetane from the (6-4) product and then thermally catalyze its conversion to 2 thymidines. Photochemical addition of the C-6 hydroxyl of the 5’ thymidine to the C-4 position of the pyrimidinone ring to form a dioxetane, or the C-6 amino group of (6-4) product of a Pyr site to form the corresponding azetidine intermediate, is preceded by the photochemical addition of diethylamine to either the C-4 of C-6 position of a 1,4,6-trisubstituted pyrimidinone (Nishio et al., 1981). For the photoenzymatic reaction to show a maximum at 400 nm rather than an absorption maximum of 330 nm for the pyrimidinone ring of (6-4) product suggests the presence of another chromophore. Cleavage by energy transfer from a chromophore with a $\lambda_{\text{max}} = 400$ nm to the pyrimidinone ring of the (6-4) product with a $\lambda_{\text{max}} = 330$ nm would be highly endergonic and would not occur with a detectable efficiency. It is conceivable, however, that upon binding to (6-4) product that the enzyme makes a charge transfer complex with it so that the absorption maximum is shifted to longer wavelengths. In such a complex, either direct excitation of the charge transfer complex (Mechanism I) or excitation transfer from a chromophore (Mechanism II) may lead to oxetane formation. Alternatively, it is possible that an excited state chromophore abstracts an electron from the pyrimidinone ring which is then attacked at C-4 by the hydroxyl group together with proton transfer and subsequent back electron transfer (Mechanism III).

In another possible pathway, the enzyme first thermally catalyzes the formation of the oxetane intermediate which then is converted to two thymines by a photochemical mechanism. The oxetane intermediate is expected to have a $\lambda_{\text{max}} < 250$ nm because of the disruption of the conjugation of the pyrimidinone ring, making it unlikely that cleavage of the oxetane intermediate could occur efficiently by direct excitation or excitation transfer from a chromophore (Mechanisms I and II). In a more plausible electron transfer mechanism, the excited state chromophore either abstracts (Mechanism IV) or donates (Mechanism V) an electron from/to the oxetane intermediate, and the resulting radicals collapse to the two canonical pyrimidines with concomitant back electron transfer.

The second question that arises from our results is the biological relevance of the (6-4) photolyase. The (6-4) photoproducts compared to Pyr[6-4]Pyr, are repaired very efficiently by nucleotide excision repair in all organisms tested (see Mitchell and Nairn, 1989; Svoboda et al., 1993). In contrast, the Pyr[6-4]Pyr photolyase has < 1% of the efficiency of Pyr[6-4]Pyr photolyase. Is the photolytic reaction observed a side reaction of a protein which binds (6-4) photoproduce with high affinity? Considering the fact that the $D.\ melanogaster\ mus-104$ mutant missing this activity is defective in postreplication repair (Tod and Ryo, 1982), this is a realistic possibility. With such a possibility in mind we reasoned that any protein that binds (6-4) photoproduce with high affinity might promote its photolysis with a low but detectable efficiency. Two such other proteins are known: the UvrB subunit of (A)BC excinuclease loaded onto DNA by the matchmaking activity of UvRA (Svoboda et al., 1993; Sancar and Hearst, 1993) and the human DDB/XPE protein (Hirschfeld et al., 1990; Reardon et al., 1993). We tested both proteins with the substrate used in this study, and even after extensive irradiation we failed to detect any reversal of the (6-4) photoproduce (data not shown). Although these experiments do not rule out the possibility that the primary function of the Drosophila (6-4) photolyase is something other than the extremely low efficiency photolysis, they do show that the (6-4)
Photolyase is probably not a homologue of either UvrB or DDB1-XPF protein and that not all proteins that bind to (6-4) photoproduct can promote its photolysis. Purification of the (6-4) photolyase to a high level of purity would help answer many of the questions regarding this extremely interesting protein.

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