Role of Two Histidines in the (6-4) Photolyase Reaction*

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The reaction mechanism of Xenopus (6-4) photolyase was investigated using several mutant enzymes. In the active site, which is homologous between the cis,syn-cyclobutane pyrimidine dimer and (6-4) photolyases, four amino acid residues that are specific to (6-4) photolyase, Glu254, His354, Leu355, and His358, and two conserved tryptophans, Trp291 and Trp398, were substituted with alanine. Only the L355A mutant had a lower affinity for the substrate, which suggested a hydrophobic interaction with the (6-4) photoprodut. Both the H354A and H358A mutations resulted in an almost complete loss of the repair activity, although the Trp291 and Trp398 mutants retained some activity. Taking the pH profile of the (6-4) photolyase reaction into consideration with this observation, we propose a mechanism in which these histidines catalyze the formation of the four-membered ring intermediate in the repair process of this enzyme. When deuterium oxide was used as a solvent, the repair activity was decreased. The proton transfer shown by this isotope effect supports the proposed mechanism. The substrate binding and the reaction mechanism are discussed in detail using a molecular model.

Excitation of bases in DNA strands, induced by the ultraviolet component of sunlight, triggers various chemical reactions and causes genetic mutations (1). Since the pyrimidine bases absorb in the UV region (200–300 nm), UV irradiation causes a [2 + 2] reaction at adjacent pyrimidine sites, resulting in the formation of two major photoproducts, the cis,syn-cyclobutane pyrimidine dimer (CPD) and the pyrimidine-pyrimidone (6-4) photoprodut (6-4) photoprodut. Unlike the CPD, the primary photoprodut of the [2 + 2] addition between the C-5-C-6 double bond and the carbonyl or iminyl group, oxetane for TT or azetidine for TC, is not actually observed but undergoes rapid rearrangement at temperatures above –80 °C, leading to the formation of the (6-4) photoprodut, as shown in Fig. 1 (2, 3). Consequently, the hydroxyl or amino group at the C-4 position of the 3’-base is transferred to the 5’-pyrimidine ring in the (6-4) photoprodut. Although both the CPD and the (6-4) photoprodut are formed by UV at adjacent pyrimidine sites in DNA strands, the structural features of the (6-4) photoprodut are quite different from those of the CPD. Therefore, the influence of these two photolesions on replication also differs (4).

Photoproducts must be repaired in cells to maintain genetic integrity. Photolyase is a unique DNA repair enzyme that eliminates UV-derived photoproducts by electron transfer from the catalytic cofactor, FAD, using light in the near UV/blue region (5, 6). Thus far, two types of photolases have been isolated. One is a DNA photolyase specific for the CPD (referred to as CPD photolyase in this report) and the other is a (6-4) photolyase specific for the (6-4) photoprodut (7, 8). Photoreactivation of the CPD has been found in a wide range of organisms. The Escherichia coli and Anacystis nidulans enzymes were characterized in detail, and their structural information is available (9, 10). The homologous genes have been isolated from many sources (5, 7, 11, 12). In contrast, the (6-4) photolyase activity has been detected in some higher eukaryotes. The CDNs of (6-4) photolyase have been cloned from Drosophila melanogaster (13), Xenopus laevis (14), Danio rerio (15), Arabidopsis thaliana (16). Interestingly, the (6-4) photolases are quite similar to the CPD photolases, although (6-4) photolyase does not bind or repair the CPD (13). The (6-4) photolyase has been considered to have evolutionarily diverted from the CPD photolyase (17). In fact, we have shown that the (6-4) photolases possess features quite similar with those of the CPD photolases (14, 18). On the other hand, unlike the CPD, Kim et al. (19) rejected the possibility that a single electron transfer to the (6-4) photoprodut could result in the restoration of the original DNA bases. It was suggested that a function additional to the common photolase activities is required for the (6-4) photolyase to restore the original bases.

It was proposed that the (6-4) photolyase repairs the (6-4) photoprodut first by converting it to a four-membered ring and then by splitting the two pyrimidines, as does the CPD photolyase (oxetane intermediate model, see Ref. 19). In previous reports, we showed by high pressure liquid chromatography analyses that both the T6-4/T and T6-4/C photoproduts are restored completely to the original bases, despite the difference in the functional group at the C-4 of the 3’-base (18, 20). These results strongly suggested that the transfer of the –NH2 or –OH group of the (6-4) photoprodut is an intramolecular reaction within the substrate. Additionally, Zhao et al. (21) supported the oxetane intermediate model by using chemically synthesized analogues of the (6-4) photoproduct as the substrate. However, the (6-4) photoproducts neither form this intermediate nor turn into the original bases spontaneously. Theoretical calculations on the base portion of T6-4/T and its oxetane isomer estimated the gap to be about 14.5–16.5 kcal/mol.

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‡ The abbreviations used are: CPD, cis,syn-cyclobutane pyrimidine dimer; T6-4/T, the (6-4) photoprodut of the corresponding dinucleotides; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; (6-4) photoprodut, pyrimidine-pyrimidone (6-4) photoprodut.
mol, suggesting that perturbation of the (6-4)/oxetane equilibrium is unlikely to be a feature of the photoenzymic repair mechanism as the computed value exceeds the likely difference in binding energy between the two species (22). Although the cycloreversion process by electron transfer has been studied intensively (18, 19, 21, 23, 24), the mechanism of this intermediate formation by the (6-4) photolyase is still unclear.

In this paper, we identified the residues crucial for the photoactivation by the (6-4) photolyase. Due to the high similarity in the primary structures at the active sites, the reason for the marked difference in the substrate specificity of each type of photolyase has been unclear. A structural model of the (6-4) photolyase in complex with the (6-4) photoproduct was constructed to discuss the substrate recognition and the reaction mechanism. We propose that the two conserved histidine residues, which are not present in the CPD photolyases, act as an acid and a base to catalyze the formation of the four-membered ring intermediate.

**EXPERIMENTAL PROCEDURES**

**Binding and Repair Assays—Xenopus (6-4) photolyase and its substrate, the 49-mer oligonucleotide containing a single (6-4) photoproduct, were prepared for in vitro assays, as described previously (14, 18, 25). The sequence of the oligonucleotide was 5'-d(ACTTCTGAGGCGTTGCTGG-3'). The (6-4) photoproduct derived from two thymidines was incorporated into the TTAA sequence context, which is the recognition site of the MseI restriction endonuclease. The oligonucleotide was labeled isotopically at the 5' terminus, annealed with the complementary strand, and then used as the substrate.

To detect the photolyase activity in vitro, restriction site restoration assays were performed, as described previously (18). The substrate (1 nM) was treated with the photolyases in 30 μl of a 50 mM buffered solution containing 1 mM dithiothreitol under daylight irradiation by a fluorescent lamp. The mixture was diluted to 150 μl with sterile water, treated with the same volume of phenol/chloroform (50:50, v/v), and precipitated with ethanol. The pellet was dissolved in 10 μl of sterile water, digested with MseI (New England Biolabs, Inc.) overnight, subjected to electrophoresis on a 10% polyacrylamide gel containing 7 M urea, and visualized by autoradiography. The cleaved and uncleaved fragments were quantified with a Fuji bioimaging analyzer (BAS-2000, Fuji Photo Film). For the isotope effect, D2O, KOD, urea, and visualized by autoradiography. The cleaved and uncleaved fragments were quantified with a Fuji bioimaging analyzer (BAS-2000, Fuji Photo Film). The amino acid sequences of the (6-4) photolyase were aligned by using the commercial available software, GENETYX-MAC Version 8.0 (Software Development Co., Ltd.). A tertiary model of Xenopus (6-4) photolyase from Gnu22 to Pro250 was constructed by comparative modeling based on the structure of E. coli CPD photolyase (Protein Data Bank code, 1dpn) (9), using our original programs as follows: a loop search method for the backbone structure (26), a dead-end elimination method for the side chains (27), and an energy minimization method for structure refinement (28), using the AMBER force field (29). The coordinates of the free (6-4) photoproduct of thymidyl(3') → 5')-thymidine, derived from an NMR study (30), were kindly provided by Dr. Byung-Seok Choi. The (6-4) photoproduct was manually docked to the active site of Xenopus (6-4) photolyase, using the molecular graphics program Insight II (Molecular Simulation Inc.), and the energy of the complex conformation was further minimized (28).
The 49-mer oligonucleotide duplex (0.5 nM) was incubated on ice with C residues are conserved (Fig. 3). Particularly, Trp291 and residues are not very well conserved, most of the aromatic and (6-4) photolyases. Although the proposed DNA binding servation indicates the structural similarity between the CPD (6-4) photolyase. The flavin chromophore is deeply buried in (6-4) photolyase. The sequences were reported in Refs. 9 and 10. The conservation of the FAD-binding sites and the substrate (31, 32, 34) and to mediate the electron transfer (31, 34). The conservation of the FAD-binding sites and the (6-4) photolyase reaction. The substrate specificity and the apparent reaction of the (6-4) photolyases are quite different from those of the CPD photolyases, although both photolyases repair the damage by photoinduced electron transfer from reduced FAD to the substrate (18). The pH profile of Xenopus (6-4) photolyase. A single T(6-4)T photoprodact in a 49-mer oligonucleotide was photoreactivated with Xenopus (6-4) photolyase at various pH values. The activity was detected with the described coupled enzyme assay (18), which is based on the restoration of the restriction enzyme sensitivity of a recognition sequence containing the photoprodact by photoreactivation. Repair by Xenopus (6-4) photolyase was most efficient at high pH (pH 8.5–9.0), although flavin is most efficiently excited at a lower pH. The maximal activity occurred at pH 8.5 and was 2.4-fold higher than that at pH 6.5, whereas a remarkable decrease of catalysis was observed upon approaching pH 6.0.

A1α Substitution—To determine the catalytic residues essential for the formation of the proposed four-membered ring intermmediate, a site-specific mutation analysis was carried out. Although the crystal structures of the CPD photolyases did not contain the substrate (9, 10), the mutation analysis and other experiments located the interaction sites with DNA in the C-terminal helical domain (31–36). The C-terminal halves of the (6-4) and CPD photolyases are highly conserved. The homology of Xenopus (6-4) photolyase to E. coli CPD photolyase increases to 35.8% in this region, whereas the (6-4) photolyases share 26.5–28.5% homology with E. coli CPD photolyase in the full-length comparison (Fig. 3A). This region of E. coli CPD photolyase also contains most of the amino acids involved in the binding of the flavin cofactor, and the FAD-binding sites are well conserved in the (6-4) photolyase (Fig. 3B). Eleven of 14 amino acids involved in FAD binding in Xenopus (6-4) photolyase are homologous, and 8 of them are identical in Xenopus (6-4) photolyase. The flavin chromophore is deeply buried in the center of the C-terminal helical domain (9, 10). This conservation indicates the structural similarity between the CPD and (6-4) photolyases. Although the proposed DNA binding residues are not very well conserved, most of the aromatic residues are conserved (Fig. 3C). Particularly, Trp277 and Trp384 of the Xenopus enzyme, equivalent to Trp277 and Trp384 of E. coli photolyase, respectively, are conserved in all of the (6-4) photolyases. Trp277 and Trp384 of E. coli CPD photolyase are supposed to be located between the cofactor and the sub-
and were irradiated with UV light at either 0.3 or 0.6 J/m². At a series of mutant vectors containing the (6-4) photolyase gene, wild type E. coli the reactivating light (14). By using this feature, we investigated increased resistance to UV light in the presence of the photolyases were used for the repair assays.

The (6-4) photolyase binds the substrate independent of light and initiates the repair only upon absorbing a near UV-visible photon, as CPD photolyase does. To characterize the substrate binding of the (6-4) photolyase mutants, EMSA was performed in the dark by using the 49-mer oligonucleotide containing a single T(6-4)/T photoproduct as a substrate. All of the obtained photolyases displayed an affinity for the substrate. We generated the binding isotherms of the mutants under the conditions of constant substrate and various enzyme concentrations (Fig. 4). All mutants except Leu355 resulted in a large decrease in the affinity to the substrate.

Analysis of DNA Repair by Ala-substituted Mutants—To study the catalysis by the mutants, we used two assays. First, we tested the photoreactivating activity of the photolyases in vitro. Fig. 5A shows the results of the restriction site susceptibility assays. The repair activity was not reduced by the mutation at either Gln291 or Leu355, whereas H358A showed a decrease in the cleaved fraction (lane 7). In addition, as revealed in the figure, the H354A mutant lost the catalytic activity (lane 5). The ratio of DNA repaired by the bulky residue mutants, histidines and tryptophans, was quantified at different times (Fig. 5B). Removal of the α-system of Trp291 or Trp398 resulted in a reduction of the activity to 2(6-4)% of the wild type, whereas the mutation of the histidine residues had a particularly large influence on the catalysis. The H354A mutant lost the repair activity completely, and the rate of repair by the H358A mutant was only 1.5% that by the wild type enzyme.

Since E. coli does not photoreverse the (6-4) photoproduct, the production of the (6-4) photolyase in cells would result in an increased resistance to UV light in the presence of the photoreactivating light (14). By using this feature, we investigated the in vivo activity of the photolyases (Fig. 5C). E. coli SY2 (uvrA−, recA−, phr−) cells containing pRT2, which carries the wild type E. coli CPD photolyase gene, were transformed with a series of mutant vectors containing the (6-4) photolyase gene and were irradiated with UV light at either 0.3 or 0.6 J/m². At these UV doses, the main photoproduct is the CPD (70–80%), whereas the (6-4) photoproduct forms 20–30% of the total photoproducts. Although irradiation at 0.3 J/m² did not cause any apparent difference between the mutants (data not shown), the results at 0.6 J/m² reflected those of the in vitro photoreactivation experiments, as shown in Fig. 5C. At 0.6 J/m², the survival rate of control cells transformed with the pGEX-4T-2 construct was 2.1 × 10⁻² at this UV dose.
contrast, the survival rates of the cells transformed with pGEX-H354A and pGEX-H358A were 0.9–3.5 \times 10^{-2} and 1.8–3.3 \times 10^{-2}, respectively. The H354A and H358A mutants showed no increase in the UV resistance by photoreactivation (1.1 \pm 0.6- and 1.2 \pm 0.4-fold against the control cells transformed with pGEX-4T-2). Apparently, the phenotype of the mutants that lack the histidine residue in the active site was different from that of W291A or W398A. From these results, we concluded that both His^{354} and His^{358} are essential for the catalytic activity of (6-4) photolyase.

**Isotope Effect**—The results of the repair assays revealed an unusual pH dependence of the (6-4) photolyase reaction (Fig. 3). Unlike other amino acids, the pK_a value of histidine is variable due to the properties of the imidazole. Hence, we reasoned that these properties of the histidine could be used for the formation of the oxetane or azetidine intermediate, and we assumed that either His^{354} or His^{358} could act as a general acid, and the other as a general base, to facilitate the formation of the proposed four-membered ring. To obtain data supporting this proposition, the isotope effect was tested. We photoreactivated the T(6-4)T-containing 49-mer with the photolyase in heavy water. If proton transfer is involved in the (6-4) photolyase reaction, then the isotope effect by deuterium would cause a difference in the rate of the photoreactivation. As shown in Fig. 6, the deuterated reaction resulted in a 44% decrease in the rate of repair.

**DISCUSSION**

To gain insight into the interactions between the (6-4) photolyase and its substrate, we constructed a structural model of *Xenopus* (6-4) photolyase over the region comprising amino acids 221–420 by using the crystal structure of *E. coli* photolyase (9) as a starting point (Fig. 7). Since the C-terminal halves of the proteins, which interact with the FAD and contain the putative substrate-binding site, are highly conserved between the (6-4) and CPD photolyases, as shown in Fig. 3A, this model can be used reliably to discuss the substrate recognition and the catalytic reaction. The cavity leading to the chromophore in the *E. coli* CPD photolyase is conserved in the (6-4) photolyase (Fig. 7). Compared with the cavity in the CPD photolyase, the path to the chromophore in the (6-4) photolyase is somewhat narrow due to the bulky residues of His^{354} and His^{358}, as shown in Fig. 7A. All of the residues shown in Fig. 3C are located on...
Two Histidines in the (6-4) Photolyase Reaction

The surface of this cavity. The proposed catalytic residues, His\textsuperscript{354} and His\textsuperscript{358}, are found on the opposite rim of the cavity to the \(\pi\)-systems of Trp\textsuperscript{291} and Trp\textsuperscript{398}, and the side chains of these histidines and tryptophans are spatially close to each other.

From the crystal structure of E. coli CPD photolyase, Park et al. (9) predicted that the interaction for its substrate recognition occurs near the rim of the cavity. At this site, the residues are hydrophobic on one side and polar on the other, and this asymmetry fits well with the hydrophobic cyclobutane ring and the polar opposite edges. This substrate-binding site has been verified by site-directed mutagenesis (31, 32), docking simulations (33–35), and atomic force microscopy (36). We put the substrate, i.e. a dinucleoside monophosphate containing the (6-4) photoproduct, at the corresponding site in the (6-4) photolyase with the same orientation of the 5' and 3' components. The internucleoside phosphate was located near the conserved basic residue (Lys\textsuperscript{430}). In the final model after energy minimization, His\textsuperscript{354}, Leu\textsuperscript{355}, and His\textsuperscript{358} are close to the photoproduct, as shown in Fig. 7A. When the photoprotein is incorporated into a DNA strand, both the 5' and 3' extensions can interact with the enzyme along the trace of the positive electrostatic potential shown in Fig. 7B. In this study, we showed that Leu\textsuperscript{355} is crucial for the substrate binding (Fig. 4). From these results and the docking model, it can be concluded that the hydrophobic interaction between the 3'-pyrimidone ring and Leu\textsuperscript{355} plays an important role in substrate binding. It is noteworthy that Arg\textsuperscript{242} of E. coli CPD photolyase, which is the counterpart of Leu\textsuperscript{355} of Xenopus (6-4) photolyase, is also required for the substrate binding (32), although leucine is quite different from arginine in terms of polarity.

Since the FAD is buried deeply in the center of the C-terminal helical domain, the conservation of the FAD-binding site suggests a structural similarity between the CPD and (6-4) photolyases. In addition, not only the \(\pi\)-systems of Trp\textsuperscript{277} and Trp\textsuperscript{384} at the proposed active site of E. coli CPD photolyase (equivalent to Trp\textsuperscript{291} and Trp\textsuperscript{398} in Xenopus (6-4) photolyase) but also most of the aromatic residues are well conserved at the region surrounding the FAD, as shown in Fig. 3A. Considering the similarity in the electron transfer between the CPD and (6-4) photolyases (18), the aromatic residues in the (6-4) photolyase probably play a role similar to that of the corresponding residues in the CPD photolyase. Like the CPD photolyase, which exhibits high activity over the pH range from 5.2 to 7.9 (37), the FAD in the (6-4) photolyase is surrounded by these aromatic residues and lies in the hydrophobic core (Fig. 7), which enables efficient electron transfer free from environmental influences. However, the (6-4) photolyase exhibited a remarkable pH dependence with maximal activity at pH 8.5, as shown in Fig. 2. The inhibitory effect at neutral pH observed for the (6-4) photolyase should result from a catalytic function of this enzyme, which is absent for CPD photolyases.

We noticed that protonation of histidine would agree well with the inhibitory effect observed for the activity of (6-4) photolyase. Since the function of histidine is uniquely influenced by pH, as compared with other amino acids, it is possible that the histidine(s) at the active site caused the unusual pH dependence of the (6-4) photolyase. Indeed, the mutants in this study showed that the two histidines at the active site are required for the catalysis to repair the (6-4) photoprotein, in contrast to the CPD photolyases (Fig. 5). We proposed previously that the (6-4) photolyase donates an electron in a manner similar to that of the CPD photolyase (18), and the oxetane intermediate model proposed by Kim et al. (19) is most likely. According to their model, the (6-4) photolyase repairs the (6-4) photoprotein first by converting it to an intermediate with the four-membered ring, oxetane for TT or azetidine for TC, which is also formed in the photochemical reaction to the (6-4) photoproduct, as shown in Fig. 1 (3). Zhao et al. (21) supported this model by using synthetic analogs of the (6-4) photoprotein as substrates. Laser flash photolysis, fluorescence quenching, and product analysis experiments also supported the oxetane reversal mechanism (24). On the other hand, Heelis and Liu (22) suggested that perturbation of the T(6-4)/oxetane equilibrium is unlikely to be a feature of the photoenzymic repair mechanism as the value estimated for the free energy difference between the hydrated T(6-4)T and oxetane species (\(-14.5\) to \(-16.5\) kcal/mol) exceeds the likely difference in binding energy between the two species. To repair (6-4) photoproducts efficiently, the (6-4) photolyases should enhance the rate of formation of the four-membered ring intermediates. Zhao et al. (21) suggested Gln\textsuperscript{304}, Asp\textsuperscript{397}, and Asp\textsuperscript{399} at the active site of Drosophila (6-4) photolyase (corresponding to Gln\textsuperscript{298}, Asp\textsuperscript{386}, and...
Asp\textsuperscript{388}, respectively, in Xenopus (6-4) photolyase) as candidates for the catalytic residues. However, two Asps are also conserved in the CPD photolyase, and in our model, these Asps are located on the opposite side to Gln\textsuperscript{288} in Xenopus (6-4) photolyase. In addition, the alanine substitution at Gln\textsuperscript{288} had no effect on the (6-4) photolyase activity, as shown in Fig. 5, A and C. Therefore, we propose that His\textsuperscript{354} and His\textsuperscript{358}, which are located at the active site and are crucial for the activity, catalyze the intermediate formation. Fig. 8 shows a possible mechanism for the formation of the four-membered ring catalyzed by two histidines. In the docking model, the locations of His\textsuperscript{354} and His\textsuperscript{358} would allow hydrogen bonding to the N-3 of the 3'-pyrimidone and the hydroxyl group on the 5'-pyrimidine, respectively. Therefore, His\textsuperscript{358} abstracts a proton from the hydroxyl group or the protonated amino group at C-5 of the 5'-base, and at the same time, His\textsuperscript{354} protonates the N-3 of the 3'-base to generate a highly electrophilic iminium ion. A nucleophilic attack to the cationic 3'-C-4 by the oxygen anion or the nitrogen lone pair results in the formation of the oxetane or azetidine intermediate. The inhibition at neutral pH and the isotope effect observed in this study can be explained by this mechanism. At neutral pH, His\textsuperscript{358}, which triggers the repair process of the (6-4) photolyase, is protonated and fails to ab-}

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