Similarities and Differences between Cyclobutane Pyrimidine Dimer (CPD) Photolyase and (6-4) Photolyase as Revealed by Resonance Raman Spectroscopy: Electron Transfer from FAD Cofactor to UV-damaged DNA*

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Running Title: Interactions between UV-Damaged DNA and FAD in Photolyase

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Cyclobutane pyrimidine dimer (CPD) and (6-4) photoproduct, two major types of DNA damage caused by ultraviolet (UV) light, are repaired under illumination with near-UV/visible light by CPD photolyase and (6-4) photolyase, respectively. To understand the mechanism of DNA repair, we examined the resonance Raman spectra of complexes between damaged DNA and the neutral semiquinoid and oxidized forms of (6-4) and CPD photolyases. The marker band for a neutral semiquinoid flavin and band I of the oxidized flavin, which are derived from the vibrations of the benzene ring of FAD, were shifted to lower frequencies upon binding of damaged DNA by CPD photolyase but not by (6-4) photolyase, indicating that CPD interacts with the benzene ring of FAD directly but that (6-4) photoproduct does not. Bands II and VII of the oxidized flavin and the 1398/1391 cm⁻¹ bands of the neutral semiquinoid flavin, which may reflect the bending of the U-shaped FAD, were altered upon substrate binding, suggesting that CPD and (6-4) photoproduct interact with the adenine ring of FAD. When substrate is bound, there is an upshifted 1528 cm⁻¹ band of the neutral semiquinoid flavin in CPD photolyase, indicating a weakened hydrogen bond at N5-H of FAD, and in (6-4) photolyase, band X seems to be downshifted, indicating a strengthened hydrogen bond at N3-H of FAD. These Raman spectra led us to conclude that the two photolyases have different electron transfer mechanisms as well as different hydrogen bonding environments, which account for the higher redox potential of CPD photolyase.

Irradiation of organisms with ultraviolet (UV) light causes damage to cellular DNA by inducing dimer formation between adjacent pyrimidine bases. This DNA damage causes mutation, cell death, and can lead to cancer (1). Cyclobutane pyrimidine dimer (CPD) and (6-4) photoproduct (Figure 1) account for most of this DNA damage, and these are repaired by CPD photolyase and (6-4) photolyase, respectively, under illumination by near-UV/visible light (2,3). Similar association constants of ca. 10⁻⁹ M were identified for CPD and (6-4) photoproduct in the complex of corresponding photolyase, whereas those for the undamaged DNA is more than four orders of magnitude lower or not detectable (4-6). Both photolyases contain flavin adenine dinucleotide (FAD) as an essential catalytic cofactor (7,8). The amino acid sequences of (6-4) and CPD photolyases, especially within the FAD binding region, are closely related, suggesting that these two enzymes share similar structure and reaction mechanisms (9); however, the quantum yield for photorecovery is significantly lower for (6-4) photolyase than for CPD photolyase (4,6,10).

The isoalloxazine ring of FAD in a photolyase can exist in one of three possible states: oxidized (FADox), neutral semiquinoid (FADH°),
and anionic fully reduced (FADH\(^-\)) forms (Figure 2). FADH\(^-\) is considered to be the active form of flavin in photolyase. The first step of the repair process is the specific recognition of CPD and (6-4) photoproduct by CPD photolyase (2) and (6-4) photolyase (9), respectively. After photoexcitation of the FADH\(^-\) by near-UV/visible light, an electron is transferred to the damaged DNA, leaving the flavin in the neutral semiquinoid form (FADH\(^0\)). Following bond cleavage of the DNA dimer, the electron is transferred back to FADH\(^0\) to restore the active state, FADH\(^-\) (4). In the case of the (6-4) photoproduct, it has been proposed that the bound substrate is converted to a four-membered oxetane ring intermediate (10) catalyzed by two conserved histidines in the active site (11).

Crystal structures of CPD photolyase have demonstrated that the FAD cofactor has an unusual U shape, with the isoalloxazine and adenine rings in close proximity (Figure 3) (12-15). In addition, the DNA dimer is flipped out of the DNA helix and approaches the FAD cofactor (5,16), but the distance between the DNA substrate and the FAD cofactor is not certain. Theoretical studies (17-19), with one exception (20), have predicted that the distance between them precludes van der Waals interactions. A long distance between FAD and the DNA substrate was also suggested by analysis of the electric dipole moment (21) and by EPR and ENDOR (22). The crystal structure of CPD photolyase complexed with a CPD-like DNA lesion (15), however, showed a direct van der Waals contact of 3 to 4 Å between the cofactor and the thymine dimer (Figure 3), a finding also supported by a femtosecond fluorescence and absorption spectroscopic study (23). Resonance Raman spectra have suggested that changes in the hydrogen bonding environment of the FAD cofactor in CPD photolyase after substrate binding (24) give rise to the increased redox potential of the FAD cofactor (25) and stabilize it in the catalytically active FADH\(^-\) state in the enzyme, but the precise alterations of the hydrogen bonding environments have not yet been determined. As for (6-4) photolyase, a crystal structure is not yet available. Although the high sequence homology between the two photolyases suggests that the interaction between (6-4) photoproduct and the FAD cofactor may be similar to that between CPD and FAD in CPD photolyase, the quantum yield for (6-4) photolyase is much lower (0.05–0.1) than that for CPD photolyase (0.7–0.98).

Resonance Raman spectroscopy is a sensitive tool for probing the interactions between the flavin cofactor and its surroundings in flavoproteins (26,27). To explore and compare the interactions between the damaged DNA and the FAD cofactor in (6-4) photolyase and CPD photolyase, we first constructed a His-tag expression system to obtain adequate amounts of purified, active enzymes. We then compared the resonance Raman spectra for complexes of the neutral semiquinoid and oxidized forms with damaged DNA. Our results suggest that CPD interacts with both the benzene ring (ring I) and adenine ring of the FAD cofactor, whereas (6-4) photoproduct interacts with only the adenine ring of the FAD cofactor. Such a structural difference may indicate different pathways of electron transfer for the two enzymes. In addition, the Raman spectra predict different substrate-induced changes in the hydrogen bonding environment for the two enzymes.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparation.** The gene of Arabidopsis thaliana (6-4) photolyase or Escherichia coli CPD photolyase was inserted at the Ndel and SacI sites of the pET-28a expression vector (Novagen). E. coli BL21(DE3) transformed with the vector was added to 0.5 L of LB medium in a 3-L flask and grown at 37°C to an OD\(_{600}\) of 1.5. The culture was then cooled to 26°C, adjusted to 0.2 mM IPTG, incubated for 24 h, and then harvested by centrifugation. The pellet was frozen at −80°C, thawed, resuspended in a lysis buffer (20 mM sodium phosphate, 0.5 M NaCl, 1 mM dithiothreitol [DTT], and 5% glycerol, pH 7.4), and sonicated. Cell debris was removed from the lysate by ultracentrifugation (40000 rpm, 1 h). The cell-free extract was loaded onto a HisTrap HP column (Amersham), and the fusion protein was eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, and 0.5 M imidazole, pH 7.4). Next, the sample was applied to a HiTrap Heparin HP column (Amersham) and eluted with
a linear gradient of 0.3 to 1 M NaCl. A 2-L culture of *E. coli* culture yielded approximately 12 mg of (6-4) photolyase and 10 mg of CPD photolyase. The N-terminal His-tag was not removed from the fusion protein.

To prepare the fully oxidized enzyme from the purified sample, (6-4) photolyase was exposed to air for more than 48 h and then applied to a HiLoad 16/60 Superdex 200 prep grade column (Amersham). Because the oxidization of CPD photolyase by air is very ineffective, the neutral semiquinoid FAD cofactor was removed by decreasing the pH, after which CPD photolyase was reconstituted with oxidized FAD (Sigma). All steps were performed according to the procedure described by Jorns et al. (28), except that the DTT was removed from the buffer and a HiTrap phenyl FF (high sub) column (Amersham) was used to remove the excess FAD.

The purified enzyme was stored in 20 mM sodium phosphate (pH 7.4) containing 0.5 M NaCl. Also, 10% glycerol was added to stabilize the reconstituted CPD photolyase. The purity of the protein after the Heparin column was determined by SDS-PAGE, and the monomeric form was identified by gel filtration chromatography on a HiLoad 16/60 Superdex 200 prep grade column. The concentration of the oxidized form of the enzyme was estimated on the basis of the FAD absorbance at 450 nm ($\varepsilon_{450} = 1.12 \times 10^4$ M$^{-1}$cm$^{-1}$) (29), and that of the neutral semiquinoid form was estimated from the absorbance at 580 nm ($\varepsilon_{580} = 4.8 \times 10^3$ M$^{-1}$cm$^{-1}$) (30).

**Substrate Preparation.** The substrate was prepared by irradiation of oligothymine (p(dT)$_8$ or p(dT)$_{10}$; Operon) for 30 min with 6.4 mW/cm$^2$ of 254 nm UV-light using a UV transilluminator (TFX-20-MC; Vilber Lourmat). The sample was loaded in a cylindrical spinning cell that was cooled during the UV irradiation by flushing with cold N$_2$ gas. The formation of dimers was monitored by the decrease of the monomer absorption band at 266 nm and the appearance of an absorption band of the (6-4) photoproduct at 325 nm (31).

**Enzyme Activity.** The enzyme activity of the His-tagged (6-4) and CPD photolyases was measured using a method based on the assay for CPD photolyase (32). Briefly, the complex of 2 μM photolyase and 50 μM UV-irradiated p(dT)$_8$ in 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM DTT was placed in a spinning cell and illuminated with a tungsten light (PHL-150; Sigma Koki) at room temperature. The UV-Vis absorption spectrum of the sample was measured as a function of time.

**Absorption and Resonance Raman Spectroscopy.** Optical absorption spectra of samples were recorded with a Hitachi UV-3310 UV/Vis spectrophotometer at room temperature. Resonance Raman spectra were obtained with a single monochromator (SPEX750M; Jobin Yvon) equipped with a liquid N$_2$-cooled CCD detector (Spec10:400B/LN; Roper Scientific). The excitation wavelengths were 568.2 and 488.0 nm from a krypton-argon mixed-gas ion laser (BeamLok 2060; Spectra Physics) for the semiquinoid and oxidized forms, respectively. The laser power at the sample point was 5 mW. Rayleigh scattering was removed with appropriate holographic notch filters (Kaiser Optical Systems). Raman shifts were calibrated with indene, and the accuracy of the peak positions of the well-defined Raman bands was ±1 cm$^{-1}$.

For the semiquinoid form, the measurements were made using an aliquot of 150 μM enzyme in 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. For the oxidized form, the buffer was the same but the enzyme concentration was 75 μM, and 10% glycerol was added for CPD photolyase. A two-fold excess of p(dT)$_{10}$ or UV-irradiated p(dT)$_{10}$ was added to the mixture. All measurements were performed with a spinning Raman cell containing 80 μL of sample solution, and all samples were cooled with cold N$_2$ gas. The structureless background in the raw spectrum was removed by a polynomial subtraction procedure using Igor Pro 5.03 (WaveMetrics). The resonance Raman spectra of oxidized CPD photolyase were normalized by the Raman band for glycerol in the buffer, so their differences both in frequency and intensity are reliable, whereas all the other Raman data were normalized by their most intense band presumably. Thus, only the differences in frequencies were considered.

**RESULTS**

**Enzyme Activity.** The time course curves for
dimer repair by 2 μM (6-4) photolyase and CPD photolyase in the presence of 50 μM UV-damaged p(dT)₈ are shown in Figures 4A and B, respectively. UV damage substantially reduced the 266-nm band of undamaged DNA and resulted in the appearance of a significant 325-nm band for the (6-4) photoproduct. Illumination of the damaged DNA complex with white light in the presence of (6-4) or CPD photolyases resulted in the gradual reappearance of the 266-nm band and, for the (6-4) photolyase, the disappearance of the 325-nm band. Therefore, although the enzymes used here have N-terminal His-tags, they are catalytically active.

Absorption Spectra of Photolyase Complexed with DNA Lesions. Immediately after purification, most of the (6-4) and CPD photolyase was in the neutral semiquinoid form with FADHº (Figures 5A and C, respectively). The absorption spectra for the fully oxidized (6-4) and CPD photolyases with FADox are shown in Figures 5B and D, respectively. Also shown are the spectra of the enzyme-substrate complexes with (dotted line) and without substrate (solid line). The absorbance maxima of FADHº (586 and 623 nm) and FADox (380 nm) in CPD photolyase were significantly shifted upon binding of the UV-damaged DNA. This blue shift is in agreement with previous findings (24,28). In contrast, substrate binding had little effect on the absorption spectra of (6-4) photolyase.

The absorption maxima around 590 and 630 nm in Figures 5A and C are due to the electronic transition of FADHº. These absorption maxima are absent from the spectra for FADox (Figures 5B and D). In accordance with these absorption spectra, we chose 568.2 and 488.0 nm as the Raman excitation wavelengths to selectively enhance the neutral semiquinoid and oxidized forms, respectively (arrows in Figure 5).

Resonance Raman Spectra of Neutral Semiquinoid Photolyase Complexed with DNA Lesions. The resonance Raman spectra of the neutral semiquinoid (6-4) photolyase and CPD photolyase are shown in Figures 6A and B, respectively. Figure 6 shows the spectra for enzyme alone (Figures 6A-a and B-a, respectively), with undamaged DNA (Figures 6A-b and B-b), and with UV-damaged DNA (Figures 6A-c and B-c). Some clear peaks are evident in the difference spectra between enzyme alone and enzyme-UV-irradiated DNA complex (Figures 6A-e and 6B-e) but not in the difference spectra between enzyme alone and enzyme-undamaged DNA complex (Figures 6A-d and 6B-d). These results were repeated in independent experiments, and they suggest that the structure and environment of the FAD cofactor change in both photolyases upon binding of a specific substrate and that undamaged DNA does not interact with the active site in either photolyase.

The band near 1606 cm⁻¹ in photolyases is a marker for the neutral semiquinoid flavin (33). Recent Raman studies suggested that there are two closely overlapped modes in the frequency region of this marker band and, in fact, splitting of this band can be observed for the D₂O substitution (24,34). The higher frequency counterpart arises from the stretching of Ring I, and the lower frequency counterpart comes from C-C/C-N5 stretching and N5-H bending of Ring II (Figure 2) (24,34,35). Upon substrate binding to CPD photolyase, there is a significant downshift in the frequency of this marker band. This shift, however, is not detected for the (6-4) photolyase. The 1528 cm⁻¹ band of CPD photolyase, which is assigned to the C-C/C-N5 stretching of Ring II and thought to be a sensitive indicator of hydrogen bonding to the N5-H of flavin (34), is upshifted, but this is not observed for (6-4) photolyase. The 1398 cm⁻¹ band of (6-4) photolyase and the corresponding band of CPD photolyase at 1391 cm⁻¹, which are tentatively assigned to the C-N10 stretching (34), are both upshifted. The 1338 cm⁻¹ band of (6-4) photolyase seems to be unaltered by substrate binding, whereas the bands of the both enzymes at 1331 cm⁻¹ seem to be less intensified in the substrate-bound form (Figure 6A). The sensitivity of this band to deuterium exchange indicates that it may be influenced by perturbations in the hydrogen bonding environment of the flavin ring (24). The 1298 and 1302 cm⁻¹ bands, which are assigned to C-N5 stretching (34) bands, are both upshifted only in CPD photolyase.

These substrate-induced frequency shifts of FADHº in (6-4) and CPD photolyases are summarized and quantitatively compared in Table 1. The frequency changes in CPD photolyase are much larger than those in (6-4) photolyase, and the results are compatible with the changes in the absorption spectra upon substrate binding (Figure
5). Notably, the shifts of the Raman bands at 1606 and 1607 cm\(^{-1}\) were the most different, indicating different interactions of UV-damaged DNA with FADH\(^{\circ}\) in the two photolyases.

**Resonance Raman Spectra of Oxidized Photolyase Complexed with DNA Lesions.** The vibrational assignments are much better established for the oxidized flavin than for the neutral semiquinoid form (35-38). Like the semiquinoid flavin, the oxidized flavin has a planar conformation (39) and should undergo a similar structural change upon substrate binding. Therefore, investigation of the oxidized enzymes could enhance the understanding of the structure and environment of the FAD cofactor in (6-4) and CPD photolyases.

The resonance Raman spectra of the oxidized (6-4) and CPD photolyases are shown in Figures 7A and B, respectively. Figure 7 shows the spectra for the enzyme alone (Figure 7A-a and 7B-a), with undamaged DNA (Figure 7A-b and 7B-b), and with UV-damaged DNA (Figure 7A-c and 7B-c). The customary numbering of the Raman bands (36) is indicated in the upper part of the figures. Comparison of the difference spectra between the enzyme alone and the enzyme-undamaged DNA complex (Figures 7A-d and 7B-d) and between the enzyme alone and the enzyme-damaged DNA complex (Figures 7A-e and 7B-e) reveals some peaks that are specifically caused by alterations in the structure and environment of the FAD cofactor upon binding of a damaged DNA substrate. Although the signal-to-noise ratio of the Raman spectra for the reconstituted oxidized CPD photolyase is not as high as that for the oxidized (6-4) photolyase, the frequency shifts observed upon substrate binding are clear and reproducible. The Raman band for glycerol (buffer component), which appears at 1456 cm\(^{-1}\), is indicated by a “G” in Figure 7B.

The band I at 1621 or 1622 cm\(^{-1}\) was assigned to the almost pure Ring I stretching vibration of the oxidized flavin (35-38). Upon substrate binding, a clear downshift of this band was observed only for CPD photolyase. Band II at 1577 or 1575 cm\(^{-1}\) was upshifted in both enzymes. Because this band is related to C4a=N5 and N1=C10a stretching, it has been used as a marker of the hydrogen bonding interactions at N1 and N5 of the oxidized flavin (38). In (6-4) photolyase, the frequency of band III is increased upon substrate binding, whereas those of bands IV and V are not. In contrast, the frequency changes of these weak bands in CPD photolyase cannot be reliably identified. A slight upshift at 1398 cm\(^{-1}\) and downshift at 1400 cm\(^{-1}\) were observed for band VI in (6-4) photolyase and CPD photolyase, respectively. C-N3 stretching and Ring I modes are believed to contribute to this band (35,38). Recent assignments have suggested that the strong band VII is related to the stretching of N10-C10a and N10-C1’ (ribityl) (35,38). Band VII at 1345 cm\(^{-1}\) in (6-4) photolyase was slightly upshifted, whereas in CPD photolyase, it changed from a split peak (1340 and 1350 cm\(^{-1}\)) to a single band. Band X at 1254 or 1250 cm\(^{-1}\) is another marker band for hydrogen bonding in a flavin moiety. It is thought to reflect the hydrogen bond at N3-H because of its assignment to C-N3 stretching coupled with N3-H bending (35,38). An increase in frequency was observed for band XI in both enzymes, whereas an increase for band XII was observed in only (6-4) photolyase.

The substrate-induced frequency shifts of FAD\(_{ox}\) in (6-4) and CPD photolyases are summarized and quantitatively compared in Table 2. The frequency changes in CPD photolyase are much larger than those in (6-4) photolyase and are similar to the results for the neutral semiquinoid enzymes. The substrate-induced downshift in band I and the merge of split band VII in CPD photolyase are the most significant differences with (6-4) photolyase, indicating that the substrate-flavin interactions in CPD photolyase are stronger than in (6-4) photolyase.

**DISCUSSION**

**Interactions between UV-damaged DNA and the Isoalloxazine Ring of FAD.** Figure 6 and Table 1 show that the marker band of the neutral semiquinoid flavin at 1607 cm\(^{-1}\) in CPD photolyase is significantly downshifted upon substrate binding, whereas the corresponding band in (6-4) photolyase is unaltered. This band is thought to be composed of two overlapping modes, namely, vibration of Ring I and the
C-C/C-N5/N5-H coupled mode of Ring II. Nishina et al. have reported that this marker band in a neutral semiquinoid riboflavin is insensitive to isotopic substitution of the C and N atoms in Rings II and III of flavin (40), indicating that the vibrations of Ring I are the major contributors to this band. This is also supported by our theoretical assignment of the neutral semiquinoid flavin. Furthermore, resonance Raman spectra in Figure 7 show that band I, which arises from the Ring I stretching of the oxidized flavin, is downshifted only in CPD photolyase upon substrate binding (Table 2). Therefore, the alterations of the Ring I vibrations of the isalloxazine ring cause the significant downshifts of the band at 1607 cm\(^{-1}\) of FADH\(^{\circ}\) and 1622 cm\(^{-1}\) of FADox in CPD photolyase. These Raman spectral features indicate that Ring I of the FAD cofactor interacts with the damaged DNA upon substrate binding in CPD photolyase but not in (6-4) photolyase.

The absorption spectrum of CPD photolyase shows a larger blue shift upon substrate binding than the spectrum for (6-4) photolyase (Figure 5). The apparent blue shift of the absorption band at 380 nm for FADox in CPD photolyase may indicate a decreased polarity around the isalloxazine ring because such a shift is also observed for flavin when the solvent is changed from polar to nonpolar (41). The difference Raman spectrum of FADox in CPD photolyase, which was normalized by the solvent band of glycerol, causes a downshift and decrease in intensity of band I as indicated by a negative band at 1625 cm\(^{-1}\) in the difference spectrum (Figure 7B-e). Such a change in band I in oxidized flavin has been observed when the polarity of its surroundings is reduced (42,43). Therefore, the downshifts of the Raman bands at 1607 cm\(^{-1}\) of FADH\(^{\circ}\) and 1622 cm\(^{-1}\) of FADox appear to reflect a decrease in the polarity around the isalloxazine ring when the pyrimidine bases of CPD approach. Specifically, there is a stronger hydrophobic interaction between CPD and Ring I of isalloxazine. The hydrophobic interaction has been identified to have the same range as the van der Waals-dispersion force (44). Therefore, the CPD and Ring I of isalloxazine in CPD photolyase may make a direct van der Waals contact (~4 Å), a possibility also suggested by the crystal structure of CPD photolyase containing a substrate analog (Figure 3) (15). MacFarlane et al. showed that the electric dipole moment of the CPD is responsible for the electrochromic shift of the electronic transition energy of FADox in CPD photolyase, and they estimated a distance of 5.5 to 8 Å between CPD and FAD (21). In a later report, they reduced this distance because they observed a very high rate of electron transfer (45). The absorption spectra upon substrate binding show less of a blue shift in the absorption bands for (6-4) photolyase than for CPD photolyase (Figure 5). This smaller change in electronic transition energy induced by damaged DNA indicates a greater distance between the (6-4) photoprodut and the FAD cofactor in (6-4) photolyase. Figure 8 shows a schematic illustration of the differences between the positions of DNA lesions and the isalloxazine ring in the FAD cofactor for the two photolyases.

**Interactions between UV-damaged DNA and the Adenine Ring of FAD.** Upon substrate binding, the band at 1391 cm\(^{-1}\) observed for FADH\(^{\circ}\) in CPD photolyase is upshifted (Figure 6). In our previous study, we proposed that this mode is derived mainly from the stretching of the N10-C10a bond in the isalloxazine ring.\(^{3}\) The X-ray crystal structure of CPD photolyase revealed that the FAD cofactor has a unique U shape, in which an adenine ring approaches to the isalloxazine ring near the N10-C10a moiety (12-15). Therefore, the upshift of the 1391 cm\(^{-1}\) band appears to arise from the alteration of the ring stacking conformation between adenine and isalloxazine rings. Furthermore, the pyrimidine bases of the CPD analog engage in hydrogen bonding interactions with the adenine ring of the FAD cofactor as shown in Figure 3. Accordingly, a change in ring stacking upon substrate binding is expected due to alterations of the hydrogen bonding interactions between the substrate and the adenine ring of the FAD cofactor, resulting in an upshift of the 1391 cm\(^{-1}\) band. The 1398 cm\(^{-1}\) band of (6-4) photolyase was also upshifted. Because the FAD binding domains of (6-4) and CPD photolyases are closely related (46), a similar conformational change is also expected to occur in (6-4) photolyase.

Band VII of oxidized flavin, which was assigned to the stretching of N10-C10a and N10-C1’ (ribityl), also reflects the vibrations of the N10 atom. Therefore, this mode should also be sensitive to the U-shaped bending and ring stacking conformation of FAD. For (6-4)
photolyase, this band is upshifted, and the split band VII in CPD photolyase merged into a single band upon substrate binding (Figure 7). These alterations also support the stronger interaction between the adenine ring and UV-damaged DNA (see Figure 8).

In (6-4) and CPD photolyases, band II (1577 and 1575 cm\(^{-1}\), respectively) is at a significantly lower frequency than for free FAD (1585 cm\(^{-1}\)) (47). The frequency of band II is known to be downshifted upon formation of a hydrogen bond at N1 of flavin (38). N1 and the 2'-OH of the ribityl chain are thought to form a strong hydrogen bond because of the U-shape of the FAD cofactor in CPD photolyase (24). Therefore, the lower frequency of band II in (6-4) and CPD photolyases should reflect a U-shaped conformation of the FAD cofactor. An apparent upshift of band II upon substrate binding is identified for both enzymes (Figure 7). This indicates the departure of the adenine ring from the isoalloxazine of FAD as a result of the hydrogen bonding interaction between adenine and the substrate.

Alterations in Hydrogen Bonding Interactions around FAD Induced by Substrate Binding. The crystal structure of CPD photolyase revealed that N5-H donates a hydrogen bond to an asparagine that is conserved in (6-4) photolyase. Figure 6 shows that the Raman band at 1528 cm\(^{-1}\) in CPD photolyase is upshifted upon substrate binding, whereas that at 1522 cm\(^{-1}\) in (6-4) photolyase is unaltered. This band is a marker for hydrogen bonding at N5-H in neutral semiquinoid flavin (34), and it has been empirically used in a Raman study of P-450 reductase (48). Therefore, the upshift of the 1522 cm\(^{-1}\) band indicates that the hydrogen bond at N5-H of FADH\(^{\circ}\) in CPD photolyase is weakened when the enzyme is bound to CPD. This Raman prediction is also supported by the more sensitive band at 1302 cm\(^{-1}\) in CPD photolyase, which involves C-N5 stretching (Figure 6, Table 1).

Another hydrogen bonding interaction may be formed at N3-H in both FADH\(^{\circ}\) and FAD\(_{ux}\). Band X of the oxidized flavin at 1254 cm\(^{-1}\), which reflects C-N3 stretching and N3-H bending, seems to be downshifted in (6-4) photolyase (Figure 7 and Table 2), indicating that the hydrogen bond would be strengthened by substrate binding. The slight frequency shifts of bands VI, XI, and XII in both enzymes (Figure 7, Table 2) may also reflect the alterations of the hydrogen bonding environment or vibrations of Ring I induced by substrate binding. On the other hand, the Raman bands of FADH\(^{\circ}\) at 1338 and 1331 cm\(^{-1}\) in (6-4) photolyase approach the profile of the single band at 1331 cm\(^{-1}\) in CPD photolyase. The lower frequency of band X suggests that the hydrogen bond at N3-H in CPD photolyase is stronger than that in (6-4) photolyase; therefore, the hydrogen bond at N3-H in (6-4) photolyase may be strengthened upon substrate binding.

Implication for Biological Functions. Although the crystal structure of CPD photolyase shows that Ring I is in the direction of the DNA lesion (15), previous studies revealed a lower density for unpaired electron and approximately for the whole electrons on the Ring I of semiquinoid flavin radical, suggesting that electron transfer between the isoalloxazine ring and CPD is not direct (49-51). It was assumed that the adenine ring of the U-shaped FAD fills the gap between the isoalloxazine ring and damaged DNA and provides effective coupling between them (20); however, a recent study using femtosecond absorption spectroscopy concluded that there is a direct electron transfer from FAD to CPD because intramolecular electron transfer could not be observed in the absence of substrate (23). Our Raman study also suggests that CPD makes a direct contact with Ring I of the isoalloxazine ring within a van der Waals distance and that it interacts with the adenine ring. Therefore, our results support a direct electron transfer from the flavin ring to CPD in CPD photolyase, although modulation of the electron transfer by the adenine ring cannot be ruled out. In contrast, in (6-4) photolyase, the (6-4) photoproduct is located slightly farther from the flavin ring and does not come into contact with it. This indicates that electron transfer in (6-4) photolyase from the flavin ring to the (6-4) photoproduct via the bridged adenine ring is more likely than a direct transfer. The putative electron transfer pathways in (6-4) and CPD photolyase are illustrated in Figure 8.

The hydrogen bonding environment is an important factor controlling the redox potential of flavin in flavoproteins (52). In a study on the control of the redox potential of flavodoxin, introduction of a \(\beta\)-branched threonine side chain
at position 57 was shown to change the protein environment of the isoalloxazine ring of FMN, and hydrogen bonding at N5-H of flavin was expected to be weaker than in the native enzyme based on the lower pH value of N5-H. This modification of the hydrogen bonding structure of the flavin ring is expected to lead to a significant increase in the redox potential for the semiquinone/hydroquinone couple (53). Furthermore, molecular orbital calculations showed that the weakened hydrogen bond at donor positions (N3-H and N5-H) would decrease the lowest unoccupied molecular orbital (LUMO) energy (54-56). This would enhance the electron acceptability, increasing the redox potential of flavin. In this study, we confirmed that the hydrogen bond at N5-H of FAD in CPD photolyase is weakened upon substrate binding. Therefore, weakening of the hydrogen bond at N5-H by the perturbation of the active site upon binding of CPD is expected to increase the redox potential of the CPD photolyase-substrate complex (25). In photolyases, FADH⁺ is generated during DNA repair and must be reduced to FADH⁻ before the next catalytic cycle. Such an increase in the redox potential upon CPD binding is in favor of the reverse electron transfer from the repaired DNA to FADH⁺ as well as the high quantum yield for CPD photolyase. In contrast, a strengthened hydrogen bond at N3-H in (6-4) photolyase may increase the LUMO energy and make it difficult for reverse electron transfer from the repaired DNA to reduce FADH⁺, which can lead to the relatively low quantum yield for this enzyme.

As known, an active photolyase is in an anionic fully reduced form. However, we have no data on the reduced enzyme, since its complex with the substrate is not so stable that the UV-damaged DNA is easily repaired by the probe light during resonance Raman measurements. Anionic reduced flavin as well as neutral semiquinoid and oxidized flavins are planar (39), and the FAD cofactors in the latter two redox states have been suggested to contact the substrate similarly according to our Raman spectra. In addition, the flipping of CPD out of the DNA helix into the active site cavity has been revealed to be independent of the redox states of the FAD cofactor by fluorescence study on oxidized and fully reduced CPD photolyase (16). Therefore, the interactions between substrate and FAD cofactor observed in this work were supposed to occur in the fully reduced enzyme, too.

Conclusion: In the current study, we reported UV-Vis absorption and resonance Raman spectra of the neutral semiquinoid and oxidized (6-4) and CPD photolyases as well as their changes upon substrate binding. We demonstrated that a direct hydrophobic interaction may occur between CPD and Ring I of the FAD in CPD photolyase but that this does not occur for (6-4) photolyase, indicating that the electron transfer mechanisms in these two enzymes may be different. Another hydrogen bond may be formed between UV-damaged DNA and the adenine ring of FAD in both enzymes upon substrate binding. The substrate may also weaken the hydrogen bond at N5-H in CPD photolyase and strengthen the hydrogen bond at N3-H in (6-4) photolyase. The former change could explain the increased redox potential of FAD recently observed in CPD photolyase. These findings should help establish the structure of the DNA-FAD complex and the mechanism by which substrate binding modulates both enzymes.
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FOOTNOTES

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1The abbreviations used are: UV, ultraviolet; FAD, flavin adenine dinucleotide; FADH+, neutral semiquinoid FAD; FADH-, anionic fully reduced FAD; FADox, oxidized FAD; FMN, flavin mononucleotide; CPD, cyclobutane pyrimidine dimer; EPR, electron paramagnetic resonance; ENDOR, electron-nuclear double resonance; LUMO, lowest unoccupied molecular orbital.

2The prominent near-UV absorption maxima around 340 nm shown as a dotted line arises from the UV-damaged DNA of the (6-4) photoproduct (31). The presence of this band as well as the invariable frequencies of all absorption bands before and after Raman measurement (data not shown) indicate the presence of an adequate amount of UV-damaged DNA substrate in this study.

FIGURE LEGENDS

Figure 1. Structures of the UV-induced DNA lesions. R, H or CH₃.

Figure 2. Redox states and numbering scheme for the isoalloxazine ring of FAD in photolyase.

Figure 3. Structure of the FAD cofactor and a CPD-like DNA lesion in CPD photolyase (PDB ID: 1TEZ). The distances of possible interactions are given in Å.

Figure 4. Time course of absorption change of UV-damaged p(dT)₈ upon near-UV/visible light irradiation in the presence of (6-4) photolyase (A) and CPD photolyase (B). The absorbance at 266 nm (circles) is due to undamaged DNA, and that at 325 nm (triangles) is due to the (6-4) photoproduct.

Figure 5. Absorption spectra of (6-4) and CPD photolyases in the absence (solid line) and presence (dotted line) of UV-irradiated p(dT)₁₀. (A) Neutral semiquinoid (6-4) photolyase, (B) oxidized (6-4) photolyase, (C) neutral semiquinoid CPD photolyase, (D) oxidized CPD photolyase. The arrows indicate the Raman excitation wavelengths.

Figure 6. Resonance Raman spectra of 150 μM neutral semiquinoid (6-4) photolyase (A) and CPD photolyase (B). In both (A) and (B), subpanel (a) shows the spectrum for enzyme alone, (b) shows the spectrum in the presence of 300 μM p(dT)₁₀, (c) the spectrum in presence of 300 μM UV-irradiated p(dT)₁₀, (d) the difference spectrum [(b) − (a)]×3, and (e) the difference spectrum [(c) − (a)]×3. The Raman excitation wavelength was 568.2 nm.

Figure 7. Resonance Raman spectra of 75 μM oxidized (6-4) photolyase (A) and CPD photolyase (B). In both (A) and (B), subpanel (a) shows the spectrum for enzyme alone, (b) shows the spectrum in the presence of 150 μM p(dT)₁₀, (c) shows the presence of 150 μM UV-irradiated p(dT)₁₀, (d) the difference spectrum [(b) − (a)]×3, and (e) the difference spectrum [(c) − (a)]×3. The Raman excitation wavelength was 488.0 nm. Band numbering is according to (36). G, Raman band of glycerol. Insert: Band X of (6-4) photolyase in the absence (solid line) and presence (dotted line) of UV-irradiated p(dT)₁₀.

Figure 8. Schematic illustration for the positions of a substrate and the putative electron transfer pathways in (6-4) photolyase (A) and CPD photolyase (B). (6-4)PP represents (6-4) photoproduct.
Table 1: Raman frequency shifts (cm$^{-1}$) of FADH$^\circ$ in (6-4) photolyase and CPD photolyase upon substrate binding.

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Table 2: Raman frequency shifts (cm\(^{-1}\)) of FADox in (6-4) photolyase and CPD photolyase upon substrate binding.

<table>
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Figure 1: Diagram showing the relationship between dipyrimidines, UV-induced CPD, and the (6-4) photoproduction.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

(A) (6-4) photolyase

(B) CPD photolyase

(a) photolyase (b) +DNA (c) +UV-damaged DNA (d) [(b)-(a)]x3 (e) [(c)-(a)]x3

Raman Shift (cm$^{-1}$)

FADox $\lambda$ = 488.0 nm

\( \gamma = 488.0 \, \text{nm} \)
Figure 8
Similarities and differences between cyclobutane pyrimidine dimer (CPD) photolyase and (6-4) photolyase as revealed by resonance Raman spectroscopy: Electron transfer mechanism from FAD cofactor to UV-damaged DNA

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