Similarities and Differences between Cyclobutane Pyrimidine Dimer Photolyase and (6-4) Photolyase as Revealed by Resonance Raman Spectroscopy

ELECTRON TRANSFER FROM THE FAD COFACTOR TO ULTRAVIOLET-DAMAGED DNA *

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The cyclobutane pyrimidine dimer (CPD) and (6-4) photoproduct, two major types of DNA damage caused by UV light, are repaired under illumination with near UV-visible light by CPD and (6-4) photolyases, 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 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 the (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 U-shaped FAD, were altered upon substrate binding, suggesting that CPD and the (6-4) photoproduct interact with the adenine ring of FAD. When substrate was bound, there was an upshifted 1528 cm⁻¹ band of the neutral semiquinoid flavin in CPD photolyase, indicating a weakened hydrogen bond at N(5)–H of FAD, and band X seemed to be downshifted in (6-4) photolyase, indicating a weakened hydrogen bond at N(3)–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 light causes damage to cellular DNA by inducing dimer formation between adjacent pyrimidine bases. This DNA damage causes mutation and cell death and can lead to cancer (1). The cyclobutane pyrimidine dimer (CPD) and (6-4) photoproduct (Fig. 1) account for most of this DNA damage, and these are repaired by CPD and (6-4) photolyases, respectively, under illumination by near UV-visible light (2, 3). Similar association constants of ~10⁻⁹ M were identified for CPD and the (6-4) photoproduct in the complex of the corresponding photolyase, respectively, whereas those for the undamaged DNA were >4 orders of magnitude lower or not detectable (4–6). Both photolyases contain 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 (Fig. 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 the (6-4) photoproduct by CPD photolyase (2) and (6-4) photolyase (9), respectively. After photoexcitation of FADH⁻ by near UV-visible light, an electron is transferred to the damaged DNA, leaving the flavin in the neutral semiquinoid form (FADH⁻). Following bond cleavage of the DNA dimer, the electron is transferred back to FADH⁺ 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-member 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 (Fig. 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 FAD cofactor is not certain. Theoretical studies (17–19), with one exception (20), have predicted that the distance between them precludes van der Waals...
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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 electron nuclear double resonance (22). However, the crystal structure of CPD photolyase complexed with a CPD-like DNA lesion (15) showed a direct van der Waals contact of 3–4 Å between the cofactor and the thymine dimer (Fig. 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 photolyase after substrate binding (24). Resonance Raman spectra have suggested that 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 liters of LB medium in a 3-liter flask and grown at 37 °C to A600 = 1.5. The culture was then cooled to 26 °C, adjusted to 0.2 mM isopropyl β-D-thiogalactopyranoside, incubated for 24 h, and then harvested by centrifugation. The pellet was frozen at −80 °C, thawed, resuspended in lysis buffer (20 mM sodium phosphate, 0.5 M NaCl, 1 mM dithiothreitol, and 5% glycerol, pH 7.4), and sonicated. Cell debris was removed by ultracentrifugation at 40,000 rpm for 1 h. The cell-free extract was loaded onto a HiTrap HP column (Amersham Biosciences), 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 Biosciences) and eluted with a linear gradient of 0.3–1 M NaCl. A 2-liter culture of E. coli culture yielded −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 >48 h and then applied to a HiLoad 16/60 Superdex 200 prep grade column (Amersham Biosciences). Because the oxidation 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 dithiothreitol was removed from the buffer, and a HiTrap phenyl FF (high sub) column (Amersham Biosciences) was used to remove excess FAD.

The purified enzyme was stored in 20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl, 10% glycerol was also added to stabilize the reconstituted CPD photolyase. The purity of the protein after application to the heparin column was determined by SDS-PAGE, and the monomeric form was identified by gel filtration chromatography on the HiLoad 16/60 Superdex 200 prep grade column. The concentration of the oxidized form of the enzyme was estimated based on the FAD absorbance at 450 nm (ε450 = 1.12 × 10^4 M⁻¹ cm⁻¹) (29), and that
of the neutral semiquinoid form was estimated from the absorbance at 580 nm ($\varepsilon_{580} = 4.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (30).

Substrate Preparation—The substrate was prepared by irradiation of oligothymine (oligo(dT)$_8$ or oligo(dT)$_{10}$, Operon Biotechnologies) for 30 min with 254 nm UV light (6.4 milliwatt/cm$^2$) 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 passed through liquid N$_2$. Formation of dimers was monitored by the decrease in 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, a complex of 2 $\mu\text{M}$ photolyase and 50 $\mu\text{M}$ UV-irradiated oligo(dT)$_8$ in 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1 mM dithiothreitol was placed in a spinning cell and illuminated with a tungsten light (PHL-150, Sigma Koki Co., Ltd.) at room temperature. The UV-visible 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-visible 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, Inc.). 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 milliwatts. 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 $\pm 1 \text{ cm}^{-1}$.

For the semiquinoid form, the measurements were made using an aliquot of 150 $\mu\text{M}$ 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 $\mu\text{M}$, and 10% glycerol was added for CPD photolyase. A 2-fold excess of oligo(dT)$_{10}$ or UV-irradiated oligo(dT)$_{10}$ was added to the mixture. All measurements were performed with a spinning Raman cell containing 80 $\mu\text{l}$ 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 $\mu\text{M}$ (6-4) and CPD photolyases in the presence of 50 $\mu\text{M}$ UV-damaged oligo(dT)$_8$ are shown in Fig. 4 (A 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 photolyase 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) photolyase resulted in the gradual reappearance of the 266 nm band and, for the (6-4) or CPD photolyase, the disappearance of the 325 nm band. Therefore, although the enzymes used here have N-terminal His tags, they are catalytically active.
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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 Fig. 5 (A and C) are due to the electronic transition of FADH+. These absorption maxima are absent from the spectra for FADox (Fig. 5, B 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 (Fig. 5, arrows).

Resonance Raman Spectra of Neutral Semiquinoid Photolyase Complexed with DNA Lesions—The resonance Raman spectra of the neutral semiquinoid (6-4) and CPD photolyases are shown in Fig. 6 (A and B, respectively). Fig. 6 shows the spectra for the enzyme alone (traces a), with undamaged DNA (traces b), and with UV-damaged DNA (traces c). Some clear peaks are evident in the difference spectra between the enzyme alone and the enzyme-UV-irradiated DNA complex (traces e) but not in the difference spectra between the enzyme alone and the enzyme-undamaged DNA complex (traces 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 overlapping modes in the frequency region of this marker band and that, 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 the C–C/C–N(5) stretching and N(5)–H bending of Ring II (Fig. 2) (24, 34, 35). Upon substrate binding to CPD photolyase, there was a significant downshift in the frequency of this marker band. However, this shift was not detected for the (6-4) photolyase. The 1528 cm⁻¹ band of CPD photolyase, which is assigned to the C–C/C–N(5) stretching of Ring II and thought to be a sensitive indicator of hydrogen bonding to N(5)–H of flavin (34), was upshifted, but this was 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–N(10) stretching (34), were both upshifted. The 1338 cm⁻¹ band of (6-4) photolyase seemed to be unaltered by substrate binding, whereas the bands of both enzymes at 1331 cm⁻¹ seemed to be less intensified in the substrate-bound form (Fig. 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–N(5) stretching (34), were upshifted only in CPD photolyase.

These substrate-induced frequency shifts of FADH+ in (6-4) and CPD photolyases are summarized and quantitatively compared in Table 1. The frequency changes in CPD photolyase were much larger than those in (6-4) photolyase, and the results are compatible with the changes in the absorption spectra upon substrate binding (Fig. 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+ 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

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5 The prominent near-UV absorption maximum 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), indicates the presence of an adequate amount of UV-damaged DNA substrate in this study.
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The resonance Raman spectra of the oxidized (6-4) and CPD photolyases are shown in Fig. 7 (A and B, respectively). Fig. 7 shows the spectra for the enzyme alone (traces a), with undamaged DNA (traces b), and with UV-damaged DNA (traces c). The customary numbering of the Raman bands (36) is indicated. Comparison of the difference spectra between the enzyme alone and the enzyme-undamaged DNA complex (traces d) and between the enzyme alone and the enzyme-damaged DNA complex (traces e) reveals some peaks that were 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 was not as high as that for the oxidized (6-4) photolyase, the understanding of the structure and environment of the FAD cofactor is clear and reproducible. The Raman band for glyceral (buffer component), which appears at 1465 cm⁻¹, is indicated by G in Fig. 7B.

Band I at 1621 or 1622 cm⁻¹ is 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 C(4a)=N(5) and N(1)=C(10a) stretching, it has been used as a marker of the hydrogen bonding interactions at N-1 and N-5 of the oxidized flavin (38). In (6-4) photolyase, the frequency of band III was increased upon substrate binding, whereas those of bands IV and V were not. In contrast, the frequency changes of these weak bands in CPD photolyase could not be reliably identified. A slight upshift at 1398 cm⁻¹ and downshift at 1400 cm⁻¹ were observed for band VI in (6-4) and CPD photolyases, respectively. C–N(3) 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 N(10)–C(10a) and N(10)–C(1') (ribityl) (35, 38). Band VII at 1345 cm⁻¹ in (6-4) photolyase was slightly upshifted, whereas it changed from a split peak (1340 and 1350 cm⁻¹) to a single band in CPD photolyase. 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 N(3)–H because of its assignment to C–N(3) stretching coupled with N(3)–H bending (35, 38). This band seemed to be downshifted only in (6-4) photolyase. Bands XI (1228 and 1222 cm⁻¹) and XII (1180 and 1178 cm⁻¹) may be related to stretching of C–N(3) and Ring I as well as N(3)–H bending (35, 38). An increase in frequency was observed for band XI in both enzymes, whereas an increase for band XII was observed only in (6-4) photolyase.

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

### DISCUSSION

Interactions between UV-damaged DNA and the Isoalloxazine Ring of FAD—Fig. 6 and Table 1 show that the marker band of the neutral semiquinoid flavin at 1607 cm⁻¹ in CPD

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**TABLE 1**

| Mode       | Frequency shift (cm⁻¹) |
|------------|-----------------------|
| **FADH° in (6-4) photolyase** |                          |
| 1606       | 0                     |
| 1522       | 0                     |
| 1398       | +1                    |
| 1338       | 0                     |
| 1298       | 0                     |
| **FADH° in CPD photolyase** |                          |
| 1607       | −2                    |
| 1528       | +1                    |
| 1391       | +2                    |
| 1331       | 0                     |
| 1302       | +1                    |
photolyase was significantly downshifted upon substrate binding, whereas the corresponding band in (6-4) photolyase was unaltered. This band is thought to be composed of two overlapping modes, viz. vibration of Ring I and the C=O/C=N(5)–H coupled mode of Ring II. Nishina et al. (40) have reported that this marker band in a neutral semiquinoid riboflavin is insensitive to isotopic substitution of the carbon and nitrogen atoms in Rings II and III of flavin, 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, the resonance Raman spectra in Fig. 7 show that band I, which arises from the Ring I stretching of the oxidized flavin, was downshifted only in CPD photolyase upon substrate binding (Table 2). Therefore, the alterations of the Ring I vibrations of the isoalloxazine ring—Upon substrate binding, the band at 1391 cm\(^{-1}\) observed for FADH\(^{+}\) in CPD photolyase was upshifted (Fig. 6)—caused the significant downshifts of the bands at 1607 cm\(^{-1}\) of FADH\(^{+}\) and 1622 cm\(^{-1}\) of FAD\(_{ox}\) 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 compared with the spectrum of (6-4) photolyase (Fig. 5). The apparent blue shift of the absorption band at 380 nm for FAD\(_{ox}\) in CPD photolyase may indicate a decreased polarity around the isoalloxazine ring because such a shift is also observed for flavin when the solvent is changed from polar to non-polar (41). The difference Raman spectrum of FAD\(_{ox}\) in CPD photolyase, which was normalized by the solvent band of glycerol, shows a downshift and decrease in intensity of band I as indicated by a negative band at 1625 cm\(^{-1}\) in the difference spectrum (Fig. 7B, trace e). Such a change in band I in the oxidized flavin is observed when the polarity of its surroundings is reduced (42, 43). Therefore, the downshifts of the Raman bands at 1607 cm\(^{-1}\) of FADH\(^{+}\) and 1622 cm\(^{-1}\) of FAD\(_{ox}\) appear to reflect a decrease in the polarity around the isoalloxazine ring when the pyrimidine bases of CPD approach. Specifically, there is a stronger hydrophobic interaction between CPD and Ring I of isoalloxazine. The hydrophobic interaction has been identified to have the same range as the van der Waals dispersion force (44). Therefore, CPD and Ring I of isoalloxazine 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 (Fig. 3) (15). MacFarlane and Stanley (21) showed that the electric dipole moment of CPD is responsible for the electrochromic shift of the electronic transition energy of FAD\(_{ox}\) in CPD photolyase, and they estimated a distance of 5.5–8 Å between CPD and FAD. In a later report (45), they reduced this distance because they observed a very high rate of electron transfer. The absorption spectra upon substrate binding show less of a blue shift in the absorption bands for (6-4) photolyase than for CPD photolyase (Fig. 5). This smaller change in electronic transition energy induced by damaged DNA indicates a greater distance between the (6-4) photoproduct and the FAD cofactor in (6-4) photolyase. Fig. 8 shows a schematic illustration of the differences between the positions of DNA lesions and the isoalloxazine ring in the FAD cofactor for the two photolyases.

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**TABLE 2**

| Band | FAD\(_{ox}\) in (6-4) photolyase | FAD\(_{ox}\) in CPD photolyase |
|------|-------------------------------|-------------------------------|
|      | Mode Frequency shift cm\(^{-1}\) | Mode Frequency shift cm\(^{-1}\) |
| I    | 1621 0                        | 1622 -2                       |
| II   | 1577 +1                       | 1575 +2                       |
| VI   | 1398 <+1                      | 1380 <+1                      |
| VII  | 1345 <+1                      | 1330, 1334 <+3, +7           |
| X    | 1254 <+1                      | 1250 0                        |
| XII  | 1180 <+1                      | 1178                           |

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\(^{6}\) Li, J., Uchida, T., Ohta, T., Todo, T., and Kitagawa, T. (June 28, 2006) *J. Phys. Chem. B* 10.1021/jp062998b, submitted for publication.
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We have proposed that this mode is derived mainly from the stretching of the N(10)–C(10a) bond in the isoalloxazine ring. 6 The x-ray crystal structure of CPD photolyase revealed that the FAD cofactor has a unique U shape, in which an adenine ring approaches the isoalloxazine ring near the N(10)–C(10a) 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 isoalloxazine 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 Fig. 3. Accordingly, a change in ring stacking upon substrate binding is expected because of 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 the oxidized flavin, which is assigned to the stretching of N(10)–C(10a) and N(10)–C(1’) (ribityl), also reflects the vibrations of the N-10 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 was upshifted, and the split band VII in CPD photolyase merged into a single band upon substrate binding (Fig. 7). These alterations also supported the stronger interaction between the adenine ring and UV-damaged DNA (Fig. 8).

In (6-4) and CPD photolyases, band II (1577 and 1575 cm⁻¹, respectively) is at a significantly lower frequency compared with free FAD (1585 cm⁻¹) (47). The frequency of band II is known to be downshifted upon formation of a hydrogen bond at N-1 of flavin (38). N-1 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 was identified for both enzymes (Fig. 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.

Alters in Hydrogen Bonding Interactions around FAD Induced by Substrate Binding—The crystal structure of CPD photolyase revealed that N(5)–H donates a hydrogen bond to an asparagine that is conserved in (6-4) photolyase. Fig. 6 shows that the Raman band at 1528 cm⁻¹ in CPD photolyase was upshifted upon substrate binding, whereas that at 1522 cm⁻¹ in (6-4) photolyase was unaltered. This band is a marker for hydrogen bonding at N(5)–H in the neutral semiquinoid flavin (34), and it has been empirically used in a Raman study of cytochrome P-450 reductase (48). Therefore, the upshift of the 1522 cm⁻¹ band indicates that the hydrogen bond at N(5)–H of FADH+ 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–N(5) stretching (Fig. 6 and Table 1).

Another hydrogen bonding interaction may be formed at N(3)–H in both FADH+ and FADox. Band X of the oxidized flavin at 1254 cm⁻¹, which reflects C–N(3) stretching and N(3)–H bending, seemed to be downshifted in (6-4) photolyase (Fig. 7 and Table 2), indicating that the hydrogen bond would be weakened by substrate binding. The slight frequency shifts of bands VI, XI, and XII in both enzymes (Fig. 7 and 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+ 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 N(3)–H in CPD photol- yase is weaker than that in (6-4) photolyase; therefore, the hydrogen bond at N(3)–H in (6-4) photolyase may be weakened 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 electrons and approximately for the whole electrons on Ring I of the 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 photolyases are illustrated in Fig. 8.
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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 β-branched threonine side chain at position 57 was shown to change the protein environment of the isoalloxazine ring of FMN, and hydrogen bonding at N(5)–H of flavin was expected to be weaker than in the native enzyme based on the lower pK_a value of N(5)–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 (N(3)–H and N(5)–H) would decrease the lowest unoccupied molecular orbital 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 N(5)–H of FAD in CPD photolyase is weakened upon substrate binding. Therefore, weakening of the hydrogen bond at N(5)–H by 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. Similarly, a weakened hydrogen bond at N(3)-H in (6-4) photolyase may also decrease the lowest unoccupied molecular orbital energy and facilitate the reduction of FADH^-.

An active photolyase is in an anionic fully reduced form. However, we have no data on the reduced enzyme because 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 and 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 photolyases (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 this study, we reported UV-visible 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 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 N(5)–H in CPD photolyase and at N(3)–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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