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FTIR Study of Light-Dependent Activation and DNA Repair Processes of (6−4) Photolyase

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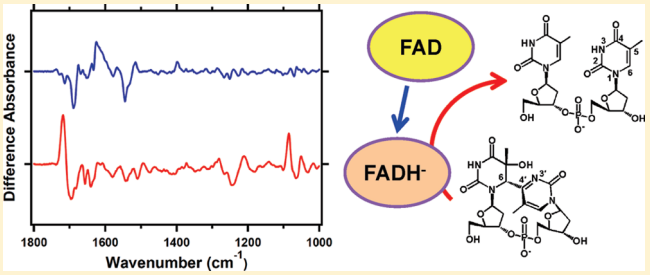
Supporting Information

ABSTRACT: The UV component of sunlight threatens all life on the earth by damaging DNA. The photolyase (PHR) DNA repair proteins maintain genetic integrity by harnessing blue light to restore intact bases from the major UV-induced photoproducts, cyclobutane pyrimidine dimers (CPD), and (6−4) photoproducts ((6−4) PPs). The (6−4) PHR must catalyze not only covalent bond cleavage between two pyrmidine bases but also hydroxyl or amino group transfer from the 5′- to 3′-pyrimidine base, requiring a more complex mechanism than that postulated for CPD PHR. In this paper, we apply Fourier transform infrared (FTIR) spectroscopy to (6−4) PHR and report difference FTIR spectra that correspond to its photoactivation, substrate binding, and light-dependent DNA repair processes. The presence of DNA carrying a single (6−4) PP uniquely influences vibrations of the protein backbone and a protonated carboxylic acid, whereas photoactivation produces IR spectral changes for the FAD cofactor and the surrounding protein. Difference FTIR spectra for the light-dependent DNA damage repair reaction directly show significant DNA structural changes in the (6−4) lesion and the neighboring phosphate group. Time-dependent illumination of samples with different enzyme:substrate stoichiometries successfully distinguished signals characteristic of structural changes in the protein and the DNA resulting from binding and catalysis.



UV components of sunlight are harmful to life by triggering various chemical reactions inside cells. Organisms have developed diverse defense systems. Photolyases (PHRs) are unique DNA repair enzymes that maintain genetic integrity by reverting UV-induced photoproducts on DNA strands into normal bases with blue light.[1,2] Most prokaryotes have a single PHR that specifically repairs cyclobutane pyrimidine dimer (CPD, right in Figure 1), whereas some higher eukaryotes possess an additional PHR that restores pyrimidine−pyrimidone (6−4) photoproduct ((6−4) PP, left in Figure 1) to parental bases. CPDs and (6−4) PPs both arise from UV-induced [2 + 2] cycloaddition reactions between adjacent pyrimidine bases in DNA strands (middle in Figure 1). Yet the two new covalent bonds between adjacent pyrimidines formed by rearrangement of the two C_5−C_6 double bonds are stabilized in CPD, whereas the heterologous four membered-ring derived from a reaction between the C_5−C_6 double bond of the 5′-base and the functional group, carbonyl (thymine) or amino (cytosine) group, at the C_4 position of the 3′-base is considered to be extremely unstable and rapidly rearranges into (6−4) PP. The two PHR enzymes show some similarities in reaction mechanism; however, to restore the undamaged original bases, (6−4) PHR[3] must not only cleave a covalent bond between two bases like CPD PHR does but also catalyze a hydroxyl (thymine) or an amino group (cytosine) transfer from the 5-position of the 5′-base to the 4-position of the 3′-base. Thus, a more complex mechanism has been postulated for (6−4) PHR than for CPD PHR.[4] The discovery of (6−4) PHR occurred 40 years after the first isolation of a CPD PHR gene. Because of the greater structural complexity of (6−4) PP compared to CPD, the synthesis of DNA oligonucleotide substrates carrying a single (6−4) PP is more difficult. However, structural determination of (6−4) PHR provides insights into product recognition by the enzyme.[5,6]

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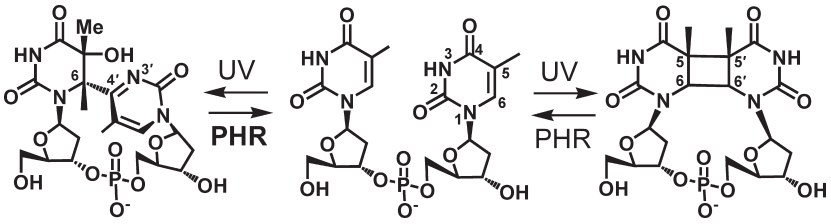

Figure 1. Molecular structures of two thymines (middle) and two major UV-induced photoproducts, cyclobutane pyrimidine dimers (CPD) (right) and (6−4) photoproducts (left).

The structures of (6−4) PHRs show an overall fold similar to that of CPD PHRs, consisting of α/β and α barrel domains connected with a unique long loop.[5,6] Flavin adenine dinucleotide (FAD) is the common chromophore for both PHRs. The chromophore is buried in the α barrel domain with the redox-active isoalloxazine rings sequestered from solvent. Reduced FAD is the active form for PHR catalysis. It has been proposed that PHRs provide an electron to the substrate from reduced $FADH^-$ by light excitation, split the covalent bonds, and withdraw the redundant electron back from DNA, while the cofactor returns from the radical semiquinone form to the reduced form. The overall reaction is a photocycle between the enzyme and DNA. The oxidized FAD is a resting state in purified enzymes for both CPD and (6−4) PHRs.[7] PHRs purified from organisms, however, are artificially subject to oxidation, becoming inactive. Remarkably, PHRs have a system to regain activity. In the presence of reducing agent, buried FADs inside PHRs can be light-dependently reduced. PHRs have the tryptophan triad chain, considered important for this photoreduction, that links FAD to the protein surface. In *E. coli* CPD PHR, substitution of the outside tryptophan of the triad chain disturbs this process.[8] (6−4) PHRs structurally conserve the tryptophan triad chain with some modification. Two photons are needed for this photactivation: the oxidized form of FAD is first converted to the semiquinone by light-induced one electron and one proton transfers and then to the reduced form $FADH^-$ by light-induced one electron transfer. When the reduced enzyme absorbs another photon in the presence of the (6−4) PP, electron transfer from $FADH^-$ to the photoproduct initiates the repair process.

Light-induced difference Fourier-transform infrared (FTIR) spectroscopy is a powerful, sensitive, and informative method to study structure−function relationships in photoreceptive proteins, as shown for rhodopsins.[9] In fact, changes in the hydrogen bonding of even a single water molecule can be detected, leading to elucidation of its functional importance.[10,11] To date, this spectroscopic method has been applied to various flavoproteins utilizing blue light.[12−15] Most of these studies, however, simply detected subtle interaction changes between polypeptides and flavins in a homogeneous system. It is very challenging to capture the entire biological process from reaction light-triggered through a cofactor to signaling to other macromolecules.

We have tackled the complicated DNA repair process of (6−4) PHR with FTIR spectroscopy. In this paper, we applied FTIR spectroscopy to full-length *Xenopus laevis* (6−4) PHR and authentic DNA carrying a single (6−4) PP to gain insights into the reaction mechanisms. Consequently, here, we have successfully observed the photoactivation, defined cofactor redox states, and obtained the repair signals. We strongly believe our information and system would give a clue to understanding the molecular mechanism of (6−4) PHR.

MATERIALS AND METHODS

Sample Preparation. *Xenopus* (6−4) PHR was prepared as described previously.[7] The protein expressed in *E. coli* does not bind second chromophore, such as MTHF.[7] The (6−4) PP was synthesized and incorporated into double-stranded DNA according to the method described previously.[16] The damaged DNA substrate had the following sequence:

5′-CGCGAATTGCGCCC-3′
3′-GCGCTTAACGCGGG-5′

In FTIR measurements, strong IR absorption by water in the protein sample can be problematic and is often reduced by using hydrated films.[10,11] For hydrated films, a water drop placed next to, but not onto, the dry film allows rehydration of the sample with minimal water. In the present study, however, we wanted a sample with higher water content to facilitate enzyme turnover, so we prepared the (6−4) PHR sample with or without the (6−4) PP as follows. First, we put 2 μL of the sample solution containing 1 mM (6−4) PHR, estimated from the absorbance at 450 nm (ε_{450} = 11 200 M^{-1} cm^{-1})[17] before concentration, in 50 mM Tris-HCl buffer (pH 8.0) and 200 mM NaCl on an IR window (BaF_2), and dried. For measurements of (6−4) PHR in complex with the (6−4) PP, 2 or 1 μL of 2 mM (6−4) PP in aqueous solution was gently mixed with the sample solution of (6−4) PHR on the IR window and then dried. We then put 0.4 μL of the 50 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl directly onto the dried film and sandwiched by another IR window. We used such redissolved samples for FTIR spectroscopy. (6−4) PHR in D_2O was prepared by diluting the (6−4) PHR with the same buffer prepared in D_2O and concentrating by Amicon YM-30 device (Millipore) for three times. (6−4) PP was dissolved in D_2O. The same procedure was carried out except for using D_2O buffer for the preparation of redissolved samples. It is noted that the oxidized form of (6−4) PHR can bind (6−4) PP,[6] where >99% of (6−4) PHR binds (6−4) PP from the values $K_a = 2.1 \times 10^8$ M^{-1},[7] 5 mM (6−4) PHR, and 10 (or 5) mM (6−4) PP.

In the IR spectrum of the resulting redissolved sample in H_2O (Figure S1A), the high absorbance of the water O−H bending frequency (∼1650 cm^{-1}) remains <0.6, which allows accurate detection of light-induced difference spectra even in this frequency region. Samples of the undamaged DNA and the (6−4) PP were similarly prepared. Typical absorption at 450 nm was 0.034 (Figure S1B), where the optical path length was estimated to be ∼6 μm using Abs_{450} = 0.034, ε_{450} = 11 200 M^{-1} cm^{-1}, and 5 mM (6−4) PHR in 0.4 μL of buffer.

UV−vis and FTIR Spectroscopy. UV−vis and IR spectra of the redissolved sample were measured using V-550DS (JASCO) and FTS-7000 (DIGILAB) spectrophotometers, respectively.[12,18] Samples were placed in an Oxford Optistat-DN cryostat mounted

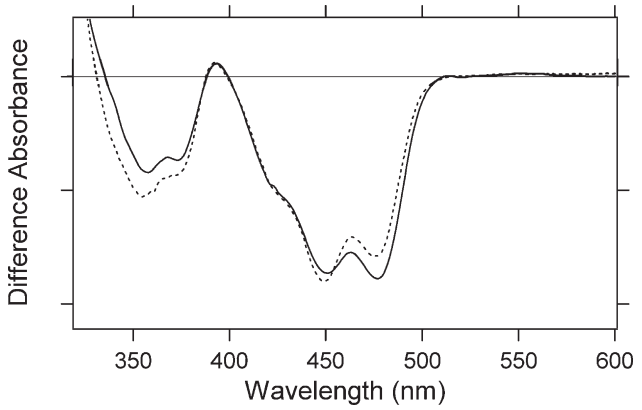

Figure 2. Light-induced difference UV−vis spectra of *Xenopus* (6−4) PHR at 277 K, showing photoactivation (FAD photoreduction) by illumination at >390 nm. The reduced−oxidized difference spectrum in the redissolved sample conditions used for our FTIR analyses (solid line) closely resembles that in solution (dotted line). One division of the *y*-axis corresponds to 0.02 absorbance units.

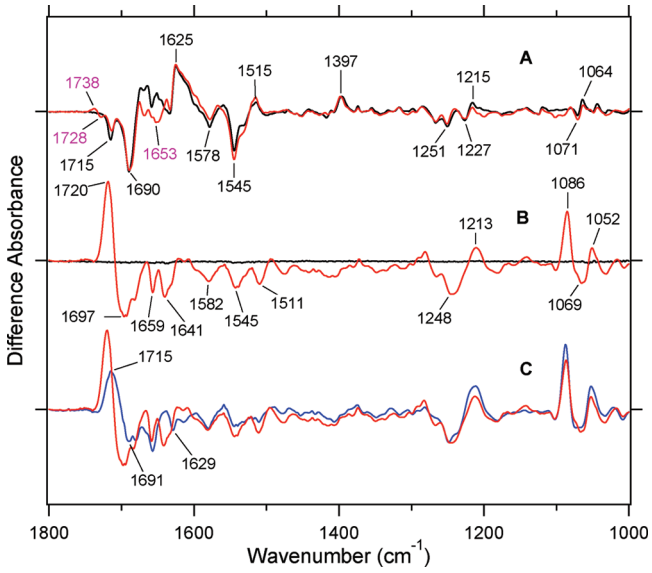

Figure 3. Light-induced difference FTIR spectra of *Xenopus* (6−4) PHR at 277 K showing the photoactivation (A) and photorepair (B, C) processes. (A) The inactive oxidized enzyme with (red line) and without (black line) the (6−4) PP was photoactivated (FAD photoreduced) by illumination at >450 nm for 6 min. Since the resultant reduced enzyme does not absorb light at these wavelengths, light-induced photorepair did not occur even in the presence of (6−4) PP. (B) Active reduced enzyme with (red line) and without (black line) the (6−4) PP was light-induced for photorepair by illumination at >390 nm for 20 min. (C) Photorepair is measured in H_2O (red line reproduced from B) vs D_2O (blue line). The molar ratio of enzyme to substrate was 1:2. One division of the *y*-axis corresponds to 0.008 absorbance units.

in the spectrophotometer, which was also equipped with a temperature controller (ITC-4, Oxford). The illumination source was a 1 kW halogen−tungsten lamp, and illuminations at >390 or >450 nm represent without or with a yellow filter (VY-45, Toshiba), respectively. The FTIR spectra were constructed from 128 interferograms with spectral resolution of 2 cm^{-1}. The difference spectrum was calculated by subtracting the spectrum recorded before illumination from the spectrum recorded after illumination. Six to eight difference spectra obtained in this way were averaged for each difference spectrum.

RESULTS AND DISCUSSION

Application of FTIR Spectroscopy to (6−4) Photolyase and Assignments of Photoactivation by Difference Spectra. In PHRs, the reduced form of FAD has been proposed to be the active form for DNA repair, whereas isolated PHRs in the "resting" state have undergone FAD oxidation.[7] PHRs inactivated by FAD oxidation can be reactivated by light-dependent reduction, also termed "photoactivation".[19] We monitored this photoactivation process in (6−4) PHR with FTIR spectroscopy. To better mimic solution conditions, we measured FTIR spectra of the redissolved sample sandwiched between BaF_2 windows, rather than in the more widely used hydrated films, which contain much less water (see details in Materials and Methods).[a] Under the redissolved sample conditions that we used for FTIR analyses, UV−vis spectra showed that the oxidized FAD of resting state (6−4) PHR (Figure S1) was reduced by continuous illumination with light above 390 nm. Under these photoactivation conditions, we did not detect significant absorption near 600 nm, characteristic of the semiquinone form of FAD. Figure 2 shows reduced-minus-oxidized UV−vis difference spectra by the illumination of the redissolved sample and in dilute solution, enabling us to identify characteristic peaks for the oxidized (before illumination) and reduced (after illumination) forms of (6−4) PHR. It should be noted that the redissolved sample of *Synechocystis* sp. Cryptochrome (CRY)-DASH, a PHR homologue with a similar fold and the same cofactor, exhibited different photoreaction from those in diluted solution, suggesting possible unfolding of protein by the drying process, and we thereby applied FTIR spectroscopy to the concentrated solution without drying.[20] In contrast, (6−4) PHR shows similar spectra between redissolved and solution samples (Figure 2), implying that (6−4) PHR is more tolerant of drying than is CRY-DASH.

To elucidate the mechanism of light-dependent DNA repair by PHRs, it is critical to discriminate between the photoactivation (photoreduction of FAD) and photorepair (conversion of damaged DNA to intact DNA) processes, which may occur simultaneously. Thus, for difference FTIR spectroscopy, we isolated light-induced photoactivation of (6−4) PHR (Figure 3A) from photorepair by use of a yellow filter (>450 nm). It should be noted that the illumination at >450 nm possibly causes photorepair as well, but the entirely different FTIR spectra for photoactivation and photorepair (see below) indicate that photorepair is negligible under the present conditions. The black line in Figure 3A shows the reduced-minus-oxidized spectrum for (6−4) PHR in the absence of substrate. Based on analogies with CPD PHR and other flavoproteins, major negative peaks at 1715, 1578, and 1545 cm^{-1} can be respectively identified as $C_4{=}O$, $C_{4a}{=}N_5$, and $C_{10a}{=}N_1$ stretching vibrations of the oxidized FAD.[21] A strong negative peak at 1690 cm^{-1} possibly contains the $C_2{=}O$ stretching vibration of oxidized FAD. Similarly, the positive peak at 1397 cm^{-1} was proposed to be the in-plane rocking mode for $N_5{-}H$ as observed at 1396 cm^{-1} in the light-induced reduction of CPD PHR.[15] Reduction of the buried FAD cofactor requires protonation at FAD N_5 from a neighboring proton donor in the protein. For photoreduction of plant CRY, a negative FTIR band observed at 1735 cm^{-1} suggested that a carboxylic acid is the FAD proton donor.[14] The reduced-minus-oxidized spectrum for (6−4) PHR in the absence of substrate

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(Figure 3A, black line) exhibits no significant signals in this frequency region characteristic of protonated carboxylic acids. Consistent with the FTIR results, plant CRY anchors the redox-active FAD N_5 position with a hydrogen bond to the carboxylic acid Asp,[22] whereas PHRs conserve this position as Asn.[6] Despite this difference in anchoring amino acid, the PHR/CRY proteins all show light-dependent reduction of FAD. By analogy with *E. coli* CPD PHR, this reduction occurs via a conserved Trp triad forming an electron transfer pathway between FAD and the solvent-exposed protein surface, and this Trp triad is considered to be important for the light-dependent reduction of PHR/CRY family proteins, at least *in vitro*.[23] Unlike plant CRYs, PHRs may directly utilize the Trp cascade.

Observation of (6−4) Photolyase Dynamics by FTIR Spectroscopy. We were able to discriminate between the two different redox states of (6−4) PHR by FTIR spectroscopy. Hence, we subjected the enzyme with an authentic DNA substrate to FTIR analyses. For the analyses, we synthesized a 14 base-pair DNA oligonucleotide carrying a single thymine−thymine (6−4) PP at the center and compared this with intact damage-free DNA. Regardless of the presence of substrate, (6−4) PP, overall light-induced difference FTIR spectra of (6−4) PHR with damaged DNA (Figure 3A, red line) at the initial stage are quite similar to that of (6−4) PHR without DNA (Figure 3A, black line). This overall similarity indicates that the (6−4) PHR predominantly changes the interactions between the polypeptide and FAD by the light excitation. The presence of (6−4) PP produces substantial spectral perturbations arising from the recognition process (Figure 3A). The amide-I vibrations at 1670−1630 cm^{-1} are clearly impacted by the presence of (6−4) PP. A negative band at 1653 cm^{-1} suggests α-helical perturbation.[24] Secondary structural perturbation may occur in the (6−4) PHR upon the photoproduct binding. Another possibility is that the signal originates from the structural change of (6−4) PP. (6−4) PP in a KBr matrix has a band at 1695 cm^{-1},[25] and the band may be shifted by aggregation in the DNA and/or binding to the protein (see also Figure 3B, red line). In addition, paired peaks are newly observed at 1738 (+)/1728 (−) cm^{-1} (Figure 3A, red line). Because these peaks are sensitive to D_2O (data not shown), we consider that alteration of hydrogen-bonding network involving a protonated carboxylic acid may occur during the light-dependent DNA repair. The crystallographic structure of (6−4) PHR reveals that the DNA repair enzyme has the positively charged groove to fit DNA,[6] while DNA is negatively charged due to the phosphate backbone. Association and dissociation between two macromolecules with opposite charge could impact protonation and deprotonation of a carboxylate, which subsequently undergoes hydrogen-bonding changes to stabilize the complex during the repair and to enable the dissociation after the restoration.

Detection of (6−4) Photoproduct Repair by (6−4) Photolyase. To focus on the light-dependent DNA repair process, after we confirmed that the enzymatic formation of reduced state was completed with 6 min illumination at wavelength above 450 nm, we further illuminated the reduced (6−4) PHR with DNA with light including shorter wavelengths (>390 nm) that can be absorbed by the reduced enzyme. In the absence of DNA, the observed spectrum was identical to the baseline (Figure 3B, black line), confirming that the previous 6 min illumination at >450 nm was sufficient for complete FAD reduction. In contrast, entirely different spectral features were obtained for light-dependent enzyme-catalyzed repair of damaged DNA carrying the (6−4) PP (Figure 3B, red line). The difference FTIR spectrum for the light-dependent repair reaction exhibits strong peaks at 1720 (+), 1697 (−), 1248 (−), 1213 (+), 1086 (+), and 1052 cm^{-1}. These spectral features most likely originate from the DNA containing (6−4) PP, during its conversion to normal bases by (6−4) PHR. To support this idea, we measured simple IR spectra on the DNA oligonucleotides alone. The IR spectra indicate that the intact and the damaged DNA have characteristic absorption bands at 1086 and 1052 cm^{-1} and at 1077 cm^{-1}, respectively (Figure S2). Therefore, we concluded that the peaks at 1086 and 1052 cm^{-1} in the difference FTIR spectrum (Figure 3B) are likely derived from the restored intact DNA. IR bands at ∼1230 and ∼1090 cm^{-1} are reported to arise from asymmetric and symmetric PO_2^- stretching vibrations, respectively,[15,26,27] and this assignment is consistent with unshifted frequencies for the bands at 1250−1210 and 1090−1050 cm^{-1} in D_2O (Figure 3C, blue line). To access a (6−4) PP in double-stranded DNA and catalyze its restoration, the (6−4) PHR flips the damaged moiety out of the duplex.[7] Although the crystallographic structure of the (6−4) PHR in complex with DNA carrying the (6−4) PP[5] demonstrate the base-flipping mode for damage recognition, the present FTIR study reveals that the DNA distortion is accompanied by severe structural perturbation of its phosphate moiety.[15]

The light-dependent DNA repair spectrum also exhibits numerous negative bands at 1800−1300 cm^{-1} (Figure 3B, red line). Possibly, these peaks are due to the physical interactions between the (6−4) PHR and the substrate. In the difference FTIR spectrum of repair, two strongest bands at 1720 (+) and 1697 (−) cm^{-1} exhibit spectral downshifts in a D_2O buffer (Figure 3C). Likewise, a negative band at 1641 cm^{-1} also downshifts to 1629 cm^{-1}. These D_2O-induced shifts indicate that the peaks did not arise from the amide-I vibration. The thymine−thymine (6−4) PP has two H/D exchangeable groups, an N−H and an O−H group in the base moiety. When the (6−4) PP is restored to normal bases, two parental thymines, a new peak for the C═O group at C_4 position of 3′-thymine should appear. The positive band at 1720 cm^{-1} likely corresponds to the recovered C_4═O stretch. The D_2O-sensitive nature of this peak supports this assignment.[28] Peaks at 1248 (−)/1213 (+) cm^{-1} are also observed in the photorepair of CPD with *E. coli* CPD PHR, which are assigned as asymmetric PO_2^- stretch of DNA backbone.[15] Therefore, we concluded that the light-dependent DNA repair process could be directly monitored in our FTIR system.

Extraction of Signals from the DNA Interface with (6−4) Photolyase during Repair. To observe the photoactivation and light-dependent DNA repair processes (Figure 3), we performed the above experiments with excess substrate, at a 1:2 molar ratio of enzyme to (6−4) PP. Under these conditions, ideally, the enzyme should be all in a 1:1 complex with DNA carrying a (6−4) PP. During light illumination, however, the reaction mixture actually consists of proteins and oligonucleotides at various stages of the DNA repair process, so important information may be masked. To overcome this problem, we made time-dependent FTIR measurements on samples containing different enzyme−substrate ratios.

To extract more data on the light-dependent DNA repair process, we tuned for different intermediates by varying the molar ratio of enzyme to substrate. At 1:1 stoichiometry, most of the damaged DNA substrate is initially enzyme-bound (Figure 4A). At later time points, the difference FTIR spectrum

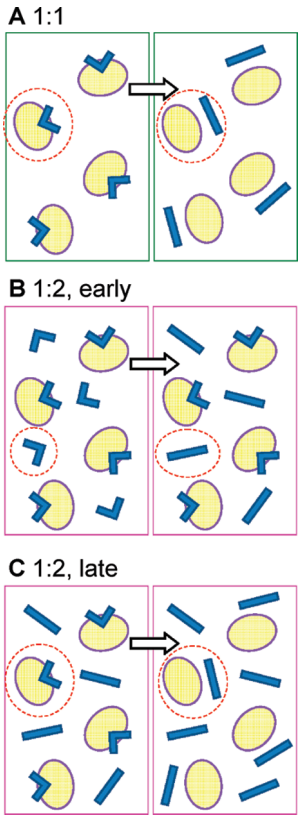

Figure 4. Schematic drawing illustrating the distinct information obtained by the time-dependent illumination measurements at different enzyme−substrate molar ratios. (A) For the 1:1 molar ratio of PHR (yellow ellipse) to (6−4) PP (bent blue line), all (6−4) PPs are bound to PHR in the dark. Upon illumination, the difference FTIR spectra always reflect differences between bound and unbound PHR and between damaged and repaired DNA. The spectral shape is invariant with illumination time, although the overall intensity varies. (B, C) For the 1:2 molar ratio of PHR to (6−4) PP, half of the (6−4) PPs are initially bound to PHR, but the remaining half are unbound. (B) At the early stage of illumination, as repaired DNA (straight blue line) is released from the enzyme into solution, a new (6−4) PP binds to the empty PHR. The difference FTIR spectra correspond to the structural differences between unbound damaged and repaired DNA only because the enzyme−substrate complex remains in steady state. (C) At the late stage of illumination, after (6−4) PP is depleted, repaired DNA released from the enzyme can no longer be replaced by new (6−4) PP, so empty PHR accumulates. The difference FTIR spectra correspond to the structural changes of PHR as well as those of DNA.

changes as the damaged DNA is repaired and released. Finally, we expect that the difference FTIR spectrum will become invariant with time, when the resultant mixture simply contains free enzyme and restored intact DNA. With excess substrate at the 1:2 molar ratio, some damaged DNA remains unbound at the early stage of illumination (Figure 4B,C). Subsequently, as restored DNA is released from the enzyme into solution, additional (6−4) PP substrate binds (6−4) PHR. During this time period, the difference FTIR spectra mainly indicate structural changes between DNA substrate and product because the enzyme−DNA complex maintains a steady-state concentration (Figure 4B). At the late stage of illumination, however, there is less damaged DNA substrate than (6−4) PHR. Once the damaged DNA substrate is used up, repaired DNA product released from the enzyme−DNA complex cannot be replaced with damaged DNA substrate, and free (6−4) PHR enzyme accumulates. The difference FTIR spectra would indicate structural changes in both the protein and DNA (Figure 4C). Eventually, the FTIR spectra of 1:2 ratio mixture should resemble the final spectrum of the 1:1 mixture.

Time-Dependent FTIR Spectroscopic Analyses of (6−4) Photolyase with Damaged DNA. In Figure 5, we show comparisons of actual time-dependent FTIR spectra corresponding to different enzyme−substrate ratios. At 1:1 stoichiometry, the initial 4 min illumination produced the strongest intensities, and then overall spectral intensities decreased following further illumination. Only negligible changes were observed in the fifth (16−20 min), sixth (20−24 min), and seventh (24−28 min) illumination intervals (Figure 5A). Damaged double-stranded DNA containing (6−4) PP was completely repaired in 30 min. For the mixture with excess substrate, we illuminated for a longer time to allow all (6−4) PPs to be eliminated from the reaction mixture (Figure 5B). The overall spectral shapes from individual time intervals all look similar, showing sharp positive peaks at 1086 and 1720 cm^{-1}, which arise from the C═O group at the C_4 position of the restored 3′-thymine. Hence, we plotted integrated peak intensity of the positive 1720 cm^{-1} bands from both conditions, against accumulated illumination time (Figure 5C). For both molar ratios, the integrated peak intensity linearly increases and then plateaus. After 28 min illumination, the peak intensity for the sample with excess substrate (1:2 stoichiometry; pink circles) was twice that of the 1:1 stoichiometry mixture (green circles). Such quantitative correlations confirm the assignments and interpretations of FTIR signals observed in the present study.

Then, how much do the FTIR spectra differ between the early and late stages of illumination? In samples with 1:1 enzyme−substrate stoichiometry, the FTIR spectra remain unchanged in shape between the early (Figure 6A; 0−4 min, black line) and late (Figure 6A; 16−20 min, red line) stages of illumination. The identity of the scaled spectra is shown by the flat double difference spectrum (Figure 6C green line), which lies along the baseline. In contrast, for samples with excess substrate (1:2 enzyme−substrate stoichiometry), the early (Figure 6B; 0−4 min, black line) and late (16−20 min, red line) spectra differ significantly. The late stage spectrum gains new positive peaks at 1667 and 1650 cm^{-1} and more intense peaks at 1244 (−) and 1086 (+) cm^{-1}. These signals imply that the species they represent is increasing significantly from the early to the late stage of the reaction.

As illustrated in Figure 4, the early stage spectrum of the 1:2 stoichiometry sample would have less information on the (6−4) PHR alone, while the late stage mixture should have more free enzyme. The double difference FTIR spectrum that we obtained from the time-dependent experiments with excess substrate (Figure 6C, pink line) corresponds to the initial recognition process of (6−4) PHR to (6−4) PP (i.e., [free protein + free substrate] minus [protein−substrate complex]). Thus, we infer that the signals at 1244 (−) and 1086 (+) cm^{-1} originate from changes in phosphate vibrations due to DNA distortions induced by (6−4) PHR to recognize the photoproduct within the duplex. Other peaks in the 1800−1500 cm^{-1} range likely arise from conformational changes in the enzyme (amide I and amide II) and ring moiety of the (6−4) PP upon substrate binding (Figure 6C).

Among the peaks in the double difference spectrum at 1800−1500 cm^{-1}, the spectral feature at 1750−1720 cm^{-1}, characteristic frequencies for protonated carboxylic acids, are

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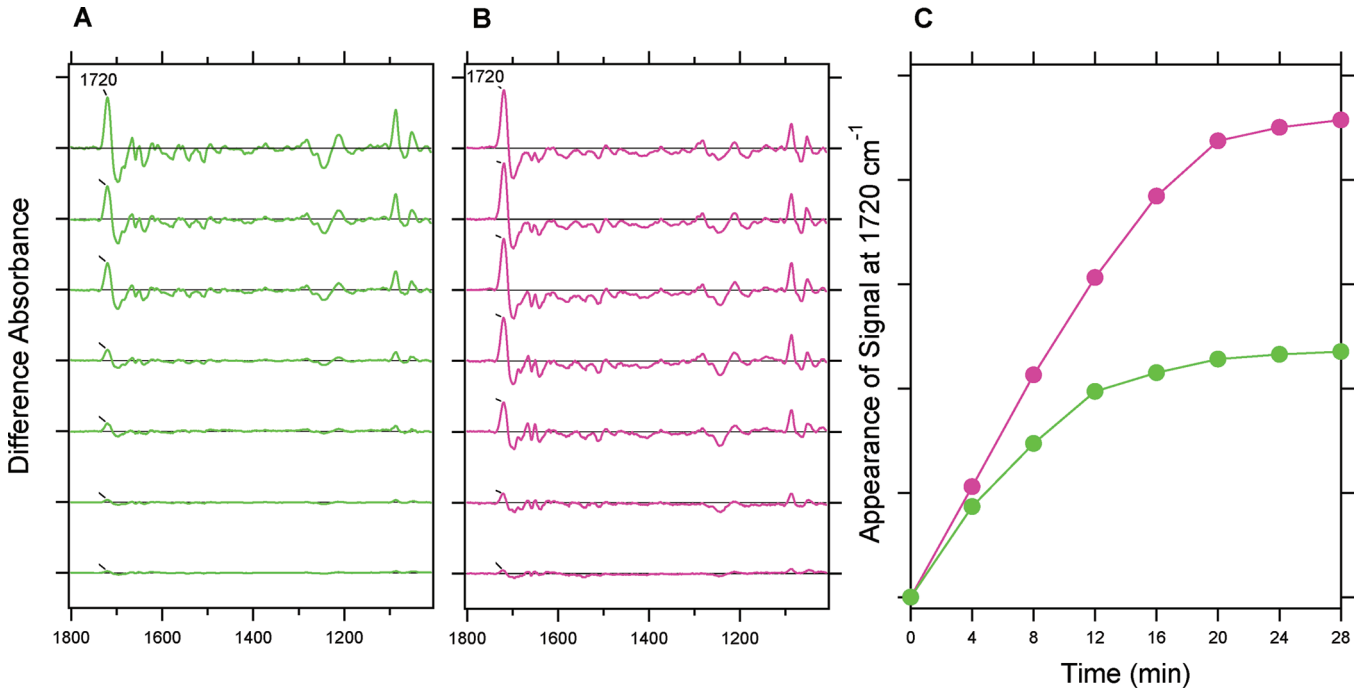

Figure 5. Light-induced difference FTIR spectra of *Xenopus* (6–4) PHR illuminated at >390 nm at 277 K, in the redissolved samples containing (6–4) PHR and the (6–4) PP substrate in molar ratios of 1:1 (A) and 1:2 (B). Each FTIR difference spectrum was collected during a 4 min illumination period, starting with the 1–4 min time interval at the top, and ending with the 24–28 min time interval at the bottom. The sum of the first five spectra in (B) (20 min cumulative illumination) is also shown in Figure 3B. (C) Illumination time-dependent DNA repair. The cumulative peak intensity of the positive band at 1720 cm^{-1} in the difference FTIR spectra is plotted versus total illumination time at >390 nm. The molar ratio of PHR and (6–4) PP is 1:1 (green circles) and 1:2 (pink circles). One division of the *y*-axis corresponds to 0.0013 absorbance units in (A) and (B) and 0.001 absorbance units in (C).

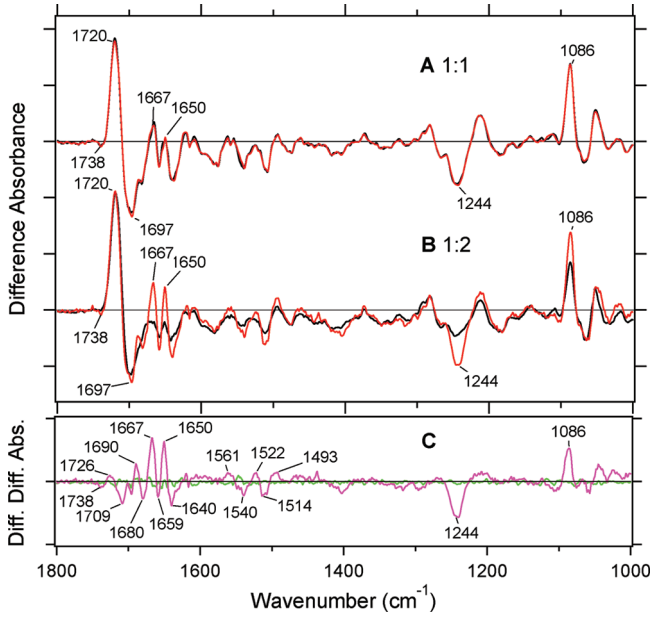

Figure 6. FTIR spectral comparison of the early (0–4 min; black line) and late (16–20 min; red line) stages of illumination for the 1:1 (A) and 1:2 (B) enzyme–substrate mixtures. In (A), the fifth spectrum in Figure 5A is scaled up 7-fold for comparison. In (B), the fifth spectrum in Figure 5B is scaled up 2-fold for comparison. In (C), double difference spectra (red-minus-black) from (A) and (B) are shown by green and pink line, respectively. One division of the *y*-axis corresponds to 0.0015 (A) and (B) and 0.0005 (C) absorbance units.

particularly noted for the (6–4) PHR reactions with the damaged DNA. A negative peak at 1738 cm^{-1} is present in the spectra of the 1:1 stoichiometry mixture (Figure S3A and Figure 6A) and at the late stage of the 1:2 excess mixture (Figure S3B and Figure 6B, red line), whereas a corresponding band is absent in the spectrum at the early stage of the 1:2 excess mixture (Figure S3B and Figure 6B, black line). Consequently, the respective double difference spectra indicate the bands at 1738 (−)/1726 (+) cm^{-1} for the 1:2 excess mixture, but not for the 1:1 stoichiometry mixture (Figure 6C and also see Figure S3C in more detail). The photoactivation spectrum of (6–4) PHR was obtained by subtraction of spectral intensities for oxidized (6–4) PHR from those of the reduced enzyme. Remarkably, the spectral features in the double difference spectrum of the 1:2 excess mixture (Figure 6C, pink line) are inverted to with respect to those present in the photoactivation spectrum in the presence of (6–4) PP (Figure 3A, red line). It is thus likely that a protonated carboxylic acid alters its hydrogen bond dependent on the redox and substrate binding, which exhibits the frequency at 1738 cm^{-1} for the reduced form binding a (6–4) PP and at 1728 (1726) cm^{-1} for the free reduced form and the oxidized form binding a (6–4) PP.

REMARKS

In this study, we report difference FTIR spectra that correspond to the photoactivation (light-induced FAD reduction) process and light-dependent DNA repair reactions of (6–4) PHR. Here we accomplished a monitoring system to decipher

the intricate enzymatic reactions from recognition through dissociation. With this monitoring, we discriminated between the photoactivation and repair processes, identified some significant conformational changes in the enzyme and substrate, and profiled the overall procedure. Together with mutagenesiss,[29] isotopic labeling,[30] structural studies,[6] ultrafast reaction dynamics,[31] and theoretical calculations,[32,33] this work opens a new understanding of the molecular mechanisms of (6−4) PHR in atomic detail.

■ ASSOCIATED CONTENT

Supporting Information. IR and UV−vis absorption spectra of (6−4) PHR (Figure S1), IR absorption spectra of intact and damaged DNA (Figure S2), spectral comparison of the early and late stage of illumination for the 1:1 and 1:2 mixtures in the 1800−1720 cm^{-1} region (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

CRY, cryptochrome; PHR, photolyase; FAD, flavin adenine dinucleotide; $FADH^-$, reduced form of FAD; FTIR, Fourier transform infrared; CPD, cyclobutane pyrimidine dimer; (6−4) PPs, (6−4) photoproducts; UV−vis, UV−visible.

■ ADDITIONAL NOTE

[a] In the previous study for *Synechocystis* sp. Cry-DASH, we used concentrated solutions.[20] It should be noted that control of hydration is easier for redissolved samples than for concentrated solutions, where the former contains less water than the latter. Here we found that the light-induced FTIR spectra of *Xenopus* (6−4) PHR are identical between redissolved samples and concentrated solutions. In contrast, light-induced FTIR spectra of Cry-DASH differ between them, and it seems that the sample is denatured by drying. This indicates that *Xenopus* (6−4) PHR is more tolerant of drying than Cry-DASH.

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