BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Flavin Adenine Dinucleotide Chromophore Charge Controls the Conformation of Cyclobutane Pyrimidine Dimer Photolyase α-Helices

I M. Mahaputra Wijaya,[†] Tatsuya Iwata,[†,‡] Junpei Yamamoto,[∥] Kenichi Hitomi,[⊥,@] Shigenori Iwai,[∥] Elizabeth D. Getzoff,[⊥] John T. M. Kennis,[§] Tilo Mathes,[§] and Hideki Kandori*[,†,‡]

[†]Department of Frontier Materials and [‡]OptoBio Technology Research Center, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan

[§]Department of Physics and Astronomy, VU University Amsterdam, Amsterdam, The Netherlands

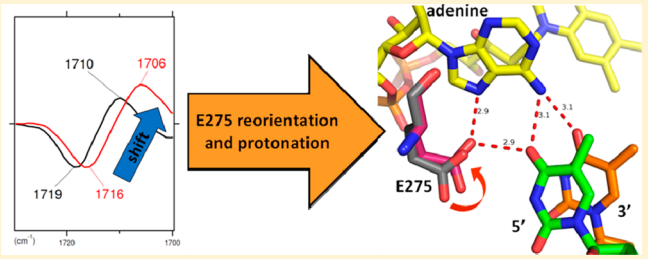
[∥]Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka 560-8531, Japan

[⊥]Department of Molecular Biology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California 92037, United States

[@]Section of Laboratory Equipment, National Institute of Biomedical Innovation, 7-6-8, Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

Supporting Information

ABSTRACT: Observations of light-receptive enzyme complexes are usually complicated by simultaneous overlapping signals from the chromophore, apoprotein, and substrate, so that only the initial, ultrafast, photon−chromophore reaction and the final, slow, protein conformational change provide separate, nonoverlapping signals. Each provides its own advantages, whereas sometimes the overlapping signals from the intervening time scales still cannot be fully deconvoluted. We overcome the problem by using a novel method to selectively isotope-label the apoprotein but not the flavin adenine dinucleotide (FAD) cofactor. This allowed the Fourier transform infrared (FTIR) signals to be separated from the apoprotein, FAD cofactor, and DNA substrate. Consequently, a comprehensive structure−function study by FTIR spectroscopy of the *Escherichia coli* cyclobutane pyrimidine dimer photolyase (CPD-PHR) DNA repair enzyme was possible. FTIR signals could be identified and assigned upon FAD photoactivation and DNA repair, which revealed protein dynamics for both processes beyond simple one-electron reduction and ejection, respectively. The FTIR data suggest that the synergistic cofactor−protein partnership in CPD-PHR linked to changes in the shape of FAD upon one-electron reduction may be coordinated with conformational changes in the apoprotein, allowing it to fit the DNA substrate. Activation of the CPD-PHR chromophore primes the apoprotein for subsequent DNA repair, suggesting that CPD-PHR is not simply an electron-ejecting structure. When FAD is activated, changes in its structure may trigger coordinated conformational changes in the apoprotein and thymine carbonyl of the substrate, highlighting the role of Glu275. In contrast, during DNA repair and release processes, primary conformational changes occur in the enzyme and DNA substrate, with little contribution from the FAD cofactor and surrounding amino acid residues.



DNA photolyase (PHR) is a flavoprotein that specifically repairs ultraviolet (UV) light-induced DNA damage by utilizing near-UV−blue light. There are two types of PHRs, cyclobutane pyrimidine dimer PHR (CPD-PHR) and (6−4) PHR, which specifically repair CPD and (6−4) photoproducts, respectively.[1,2] In addition, homologous photolyase proteins that do not have DNA repair ability, at least not for double-stranded DNA, are called cryptochromes (CRYs). The function of CRYs is manifold, such as in the circadian clock,[3] regulation of protein expression,[3,4] and magnetic sensing.[5] All CRY/PHR family proteins bind flavin adenine dinucleotide (FAD) noncovalently as their main catalytic cofactor.[6−9] Some also bind an additional second chromophore, either 5,10-methylenetetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin (HDF), as a light-harvesting antenna to assist in FAD activation by energy transfer.[10,11]

CPD-PHR from *Escherichia coli* is a 55 kDa protein that binds FAD and MTHF as primary and second chromophores, respectively.[10] Although CPD-PHR from *E. coli* is catalytically active in the reduced form of FAD ($FADH^-$) *in vivo*,[12] CPD-PHR is oxidized upon protein purification. Most *E. coli* CPD-PHR enzymes form stable resting states in the form of a neutral semiquinone ($FADH^•$) during *in vitro* experiments.[10] In the presence of external electron donors and upon illumination

Received: May 25, 2014
Revised: July 28, 2014
Published: August 25, 2014

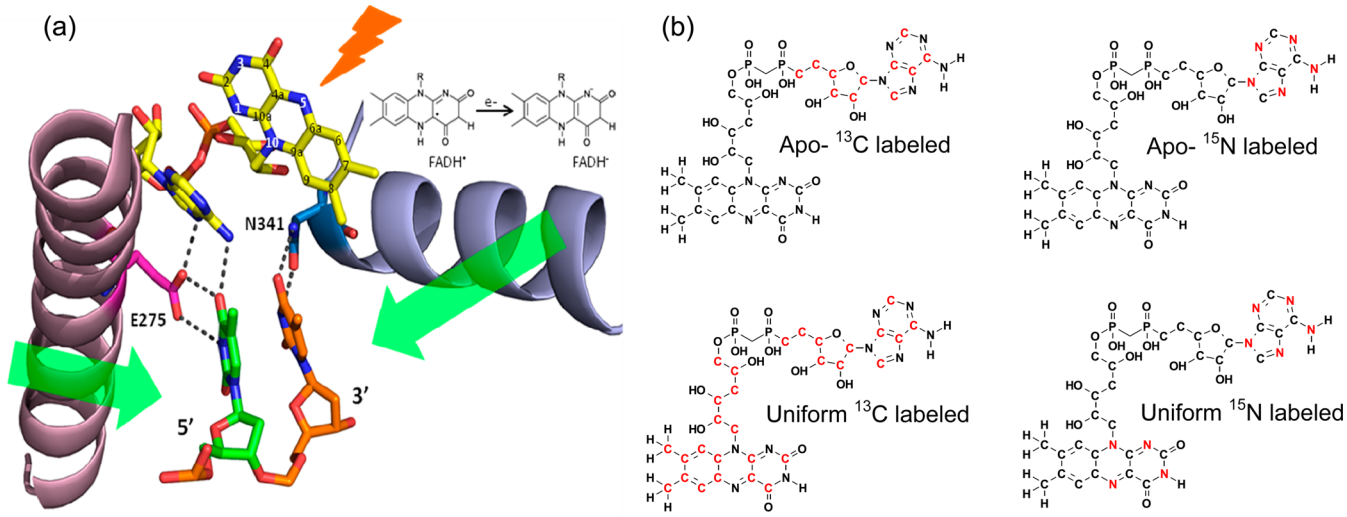


Figure 1. (a) Illustration of conformational changes in α-helices indicated by green arrows. Hydrogen bonds between the C_4=O group of DNA 5′ and 3′ and its pairing residues Glu275 and Asn341 may have changed as a result of geometric changes to FAD translated through light pink- and light purple-colored α-helices upon photoactivation of *Anacystis nidulans* (PDB entry 1TEZ) CPD-PHR. (b) Schematic figures of FAD showing isotope-labeled sites of FAD upon apo-^{15}N, apo-^{13}C, uniform ^{15}N, and uniform ^{13}C isotope labeling.

with >550 nm light, which is absorbed by the FADH$^{\bullet}$ form of PHR, the enzyme is reduced to the FADH^{-} form, the functional state of CPD-PHR for DNA repair.[13]

The FAD chromophore of PHR is noncovalently bound and buried inside the protein.[14] Upon light-induced excitation, FAD is reduced by a nearby tryptophan through a conserved tryptophan triad that wires FAD to the protein surface.[15,16] For DNA repair, CPD-PHR recognizes CPD lesions in the DNA strand and flips out the CPD lesion from the DNA strand.[17] The CPD lesion is bound in the binding cavity in the proximity of the FAD cofactor, which is situated at the bottom of the cavity.[18] When excited by near-UV−blue light, the excited FADH^{-} ejects an electron to the CPD substrate to break the dimer and thereby oxidizes FADH^{-} to the FADH$^{\bullet}$ form. Finally, the electron returns to FADH$^{\bullet}$ after the CPD is broken to re-form FADH^{-}; the repaired thymines flip back to base pair with their corresponding adenines, and the repaired DNA strand is released in a very efficient manner.[19]

Some details of the transfer of an electron from the excited FADH^{-} to the DNA substrate and its return to FAD after DNA repair have been proposed.[20] However, the wider context of the CPD-PHR molecular mechanism, conformational changes to the enzyme and DNA, and specifically the catalytic role of important amino acid residues, especially in the DNA binding pocket, during repair have yet to be elucidated (Figure 1a). Information from UV−visible (UV−vis) spectroscopy together with ultrafast spectroscopy is limited to changes in the FAD moiety and some aromatic residues.[20,21] However, these methods have not been used to understand local structural changes in amino acids at the enzyme's active site, the interaction of amino acids near the FAD cofactor, or the alteration of related global enzyme−substrate conformations, and how these could facilitate electron transfer and substrate interaction.

Previously, use of Fourier transform infrared (FTIR) spectroscopy provided new insight into the α-helical diversity of CPD-PHR and (6−4) PHR upon their specific interaction with their respective DNA substrates.[13] Each PHR enzyme may have evolved according to its substrate, subsequently resulting in high binding constants, at least *in vitro*. In this study, the aim was to elucidate the FTIR signals of CPD-PHR and identify specific amino acids known to possess FTIR signature signals,[22] especially those in the DNA binding pocket as shown in Figure 1a. To achieve this, uniformly ^{13}C- and ^{15}N-labeled proteins as well as selectively labeled apoprotein samples in which the FAD cofactor remained unlabeled (i.e., apo-labeled) were employed. Light-induced FTIR spectroscopy has been used to elucidate the molecular mechanisms of various proteins. Using this advantage, the light-induced activation of the enzyme's FAD cofactor (photoactivation) was systematically measured in the presence and absence of DNA substrate, and light-induced DNA repair (photorepair) by CPD-PHR. Both photoactivation and photorepair were selectively conducted through illumination of different light wavelengths (see Materials and Methods), utilizing the difference absorbance of unphotolyzed state (FADH$^{\bullet}$) and catalytically active fully reduced state (FADH^{-}).[13] Differences in the enzyme's conformational responses upon measurement of light-induced FAD activation were expected in the presence and absence of DNA substrate; meanwhile, changes in DNA substrate structure and enzyme conformation upon DNA release were expected upon light-induced DNA repair (see Results).

The riboflavin auxotrophic *E. coli* strain CpXribF, which cannot synthesize riboflavin itself and utilizes external sources of riboflavin, was used to make apo-labeled proteins.[23] Accordingly, proteins produced in this strain correspond to apo-labeled enzymes that bind FAD whose riboflavin part is unlabeled while the adenosine moiety and the protein is labeled with isotope, as shown in Figure 1b (red-colored atom). In combination with FTIR spectroscopy, these selectively labeled proteins allow for discrete assignments of vibrational signatures to structural changes in the apoprotein, identification of some specific amino acids, FAD cofactor, and DNA substrate upon chromophore activation, and repair by CPD-PHR.

To the best of our knowledge, the FTIR spectral assignment of light-induced FAD activation and DNA repair is very limited because only uniform ^{15}N isotope-labeled enzymes have been studied.[24] The comparison of uniform and apo-labeled enzyme with unlabeled enzymes in this study, through the observation of selective isotope-induced shifts in FTIR spectra and

dx.doi.org/10.1021/bi500638b | *Biochemistry* 2014, 53, 5864−5875

therefore unambiguous assignment of FTIR spectra, will provide a better understanding of the structural changes of CPD-PHR during FAD photoactivation and DNA repair. Furthermore, these results will be of general interest for vibrational spectroscopic studies of the CRY/PHR family.

MATERIALS AND METHODS

Protein Expression for Unlabeled, Uniform Isotope-labeled, and Apo-Labeled Enzymes. Unlabeled *E. coli* CPD-PHR was prepared as N-terminally His_6-tagged protein using an expression system in *E. coli* strain BL21(DE3). In this report, we used the E109A mutant, which does not carry MTHF, as the WT enzyme.[13] *E. coli* was grown in M9 minimal medium. [^{13}C]Glucose and $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, 99.0% purity) were used to prepare uniform ^{13}C and ^{15}N labels, respectively. Isotope-labeled enzyme with unlabeled FAD (riboflavin part), i.e., apo-labeled enzyme, was expressed in *E. coli* strain CpXribF, which is unable to synthesize riboflavin.[23] Riboflavin was added at a final concentration of 30 μM to M9 minimal medium in which strain CpXribF was grown. The expressed CPD-PHR enzyme consists of isotope-labeled apoenzyme and the adenosine moiety of the FAD cofactor, but the riboflavin moiety remains unlabeled (see Figure 1b). Isotope labeling efficiency was believed to be >90% from the FTIR spectra (see Results).

Preparation of Double-Stranded CPD Substrate for FTIR Measurement. The double-stranded DNA (dsDNA) used in this work has the same sequence as previously described,[13] and the dsDNA substrate was prepared according to the method of Murata et al.[25]

FTIR Spectroscopy and FTIR Sample Preparations. FTIR spectroscopy was conducted with an FTS-7000 instrument (DIGILAB) equipped with a cryostat (Optistat DN-Oxford Instrument) in which all samples were kept at 277 K. Illumination light was supplied by a 300 W xenon lamp (MAX-302, Asahi Spectra) with >550 and >390 nm filters for FAD activation and DNA repair, respectively.[13,26] Samples for FTIR measurement of unlabeled, uniformly isotope-labeled, and apo-labeled enzymes and DNA substrate were prepared at a concentration of 2 mM. This concentration minimized water absorbance while allowing good enzyme and DNA substrate turnover, resulting in a 1:1 stoichiometry between the enzyme and dsDNA substrate.

For light-induced FTIR measurements of CPD-PHR FAD activation, samples were illuminated with >550 nm light in the absence or presence of DNA substrate for 2 min. Following photoactivation, samples were further illuminated with >390 nm light to induce DNA repair. A baselinelike signal was obtained in the absence of DNA substrate;[13] meanwhile, photorepair signals appeared in the presence of DNA substrate.

On the FTIR spectra, the isotope-labeled enzyme would shift the signals that originated from a specific selected isotope-labeled moiety. The expected shifted signals from unlabeled to isotope-labeled enzymes are represented by s-shaped arrows, while straight arrows represent unshifted signals that confirm the origin is from the same moiety.

RESULTS

Light-Induced FAD Activation of Uniformly Labeled and Apo-Labeled CPD-PHR. Upon light-induced activation, the FAD cofactor of the CPD-PHR enzyme was reduced by one electron from $FADH^{\bullet}$ to $FADH^{-}$. Although the isoalloxazine ring of FAD is a conjugated system with delocalized π electrons, theoretical calculations indicate strong localization of electron density on the FAD pyrimidine ring on the fully reduced form ($FADH^{-}$),[27] which is possibly accompanied by structural changes in the apoprotein. Information about the FAD cofactor and apoprotein could not be disentangled in a previous study that recorded light-induced difference FTIR spectra of FAD activation.[13] To assign the FTIR signals in the absence and presence of substrate, photoactivation of uniformly labeled and apo-labeled enzymes was compared. Light-induced difference FTIR spectra of FAD activation ($FADH^{-}$ minus $FADH^{\bullet}$) for the unlabeled, uniformly ^{13}C-labeled, and apo-^{13}C-labeled samples are shown in Figures 2 and 3 in the regions of 1730−1560 and 1560−

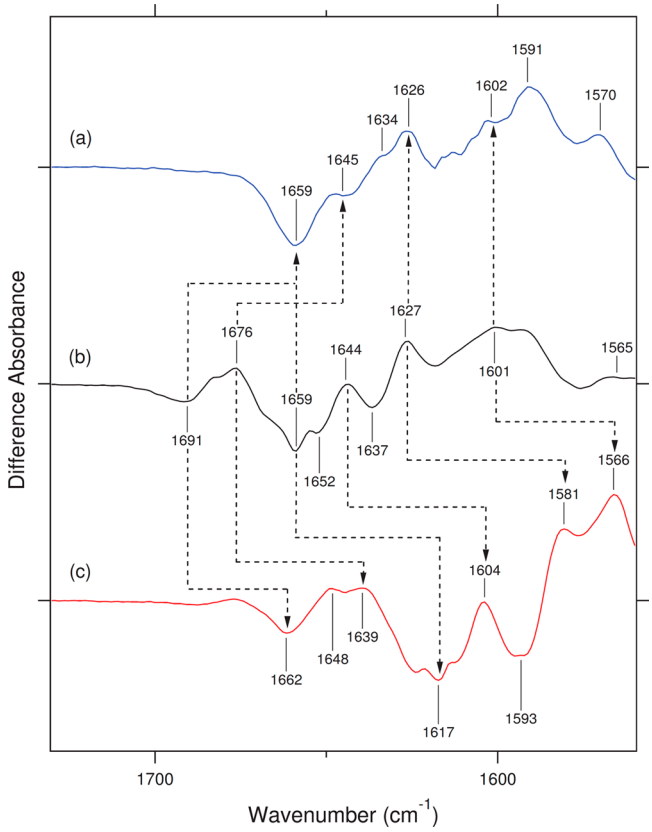


Figure 2. Light-induced difference FTIR spectra of CPD-PHR at 277 K showing photoactivation of apo-^{13}C-labeled (blue), unlabeled (black), and uniformly ^{13}C-labeled (red) samples in the absence of CPD substrate in the region of 1800−1550 cm^{-1}. One division of the *y*-axis corresponds to 0.008 absorbance unit.

1000 cm^{-1}, respectively. In Figures S1 and S2 of the Supporting Information, difference FTIR spectra for uniformly ^{15}N-labeled and apo-^{15}N-labeled samples are shown. In the C=O stretching vibrational region (1760−1600 cm^{-1}), the isotope-induced spectral downshift of C=O stretches is expected to be ~35−40 cm^{-1} following the substitution of ^{12}C with ^{13}C. In detail, the uniformly ^{13}C-labeled enzyme is expected to show downshifts of all C=O stretches. Concurrently, in the apo-^{13}C-labeled enzyme, all C=O stretches from the apoprotein are expected to show downshifts, while the C_2=O and C_4=O stretches in the isoalloxazine ring are not. It is noteworthy that the adenosine moiety of FAD is ^{13}C-labeled under these conditions but does not have any C=O groups. On the other

dx.doi.org/10.1021/bi500638b | *Biochemistry* 2014, 53, 5864−5875

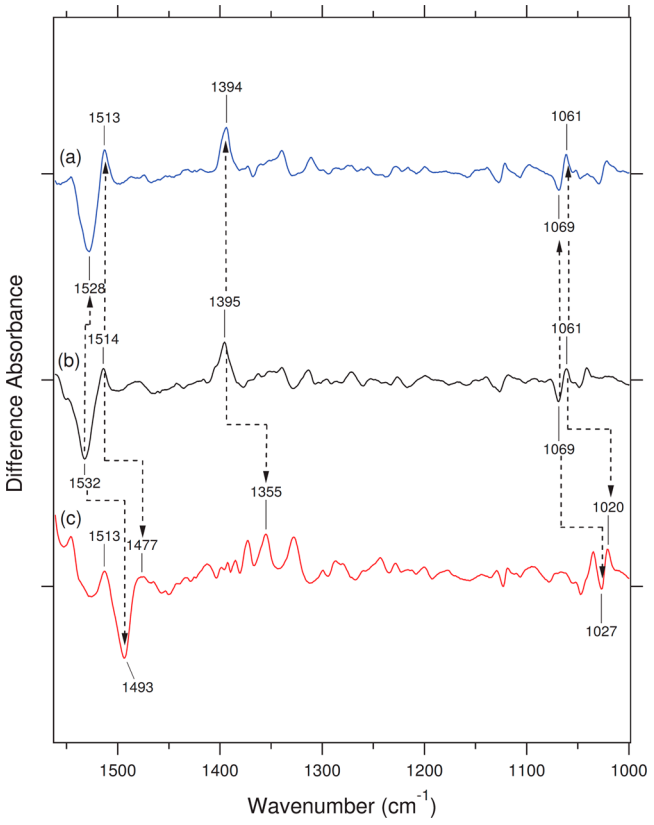


Figure 3. Light-induced difference FTIR spectra of CPD-PHR at 277 K showing photoactivation of apo-^{13}C-labeled (blue), unlabeled (black), and uniformly ^{13}C-labeled (red) samples in the absence of CPD substrate in the region of 1550−1000 cm^{-1}. One division of the *y*-axis corresponds to 0.008 absorbance unit.

hand, further spectral assignment is possible with the apo-labeled and uniformly ^{15}N-labeled enzyme, and a downshift for the signals corresponding to the C−N and C═N vibrations of FAD isoalloxazine ring are expected, depending on the interaction with the surrounding enzyme environment.

Because the origin of the signals from FAD or apoprotein can be discriminated in this way, the uniform and apo-labeled spectra both yield spectral shifts, which can be used as a reference for spectral assignment in the presence of DNA substrate. Shifted signals in both uniformly labeled and apo-labeled enzymes originate from apoprotein, while the shifted signals on uniformly labeled enzyme, but unshifted signals in apo-labeled enzyme, originate from the FAD (riboflavin) moiety.

The peaks at 1691 (−)/ 1676 (+) cm^{-1} (Figure 2b) downshift to 1662 (−)/1639 (+) cm^{-1} in the spectrum of uniformly ^{13}C-labeled enzyme (Figure 2c) and are also shifted to 1659 (−)/1645 (+) cm^{-1} in the activation of the apo-^{13}C-labeled enzyme spectrum (Figure 2a). These results indicate that both signals at 1691 (−) and 1676 (+) cm^{-1} originate from C═O stretches of apoprotein. The band at 1691 (−) and 1676 (+) cm^{-1} may represent C═O stretching vibrations of asparagine, with the most likely candidate being Asn378 near N_5 of FAD. This assignment was further supported by the unshifted 1691 (−) cm^{-1} peak upon H−D exchange,[13] confirming the compact FAD binding environment made the H−D exchange processes was likely not possible. The carbonyl moiety of Asn378 may form or strengthen the hydrogen bond interaction with H5 of FADH$^-$ and thereby stabilize the protonation state of N_5 to stabilize both reduced and semiquinoid forms to prevent further oxidation. On the other hand, the 1676 (+) cm^{-1} signal may also be attributed to amide I. However, the appearance of a slight shoulder over 1676 (+) cm^{-1} in the absence of CPD substrate, which becomes more prominent in the presence of CPD substrate, suggests that the 1691 (−) and 1676 (+) cm^{-1} signals consist of two overlapping pairs of peaks. In the future, site-specific isotope labeling is needed to clearly assign the origin of these signals.

Another peak at 1659 (−) cm^{-1} is shifted to 1617 (−) cm^{-1} in uniformly ^{13}C-labeled enzyme, but not in apo-^{13}C-labeled enzyme (Figure 2a,c). This indicates that the 1659 (−) cm^{-1} peak originates from FAD, most likely the C_2═O group of FADH$^{\bullet}$. This peak was also not shifted during the previous H−D exchange experiment,[13] further supporting the assignment to the C_2═O group of FAD. The small negative peak at 1652 cm^{-1} presumably originates from amide I of α-helices, which cannot be clearly assigned in apo-^{13}C-labeled enzyme, possibly because of spectral overlap with other peaks. However, a clear shift and an increase in intensity were observed for the uniformly ^{13}C-labeled enzyme in the presence of CPD substrate (see Figure 4c). Assignment of this negative peak to α-helices indicates that the rearrangement of α-helices is ubiquitous to the changed state of FAD charge and enzyme interaction with DNA substrate, in agreement with our previous finding.[13] The positive peak at 1644 cm^{-1}, which shifts to 1604 cm^{-1} in the uniformly ^{13}C-labeled enzyme (red spectra), could not be observed in the apo-^{13}C-labeled enzyme (blue spectra) and is

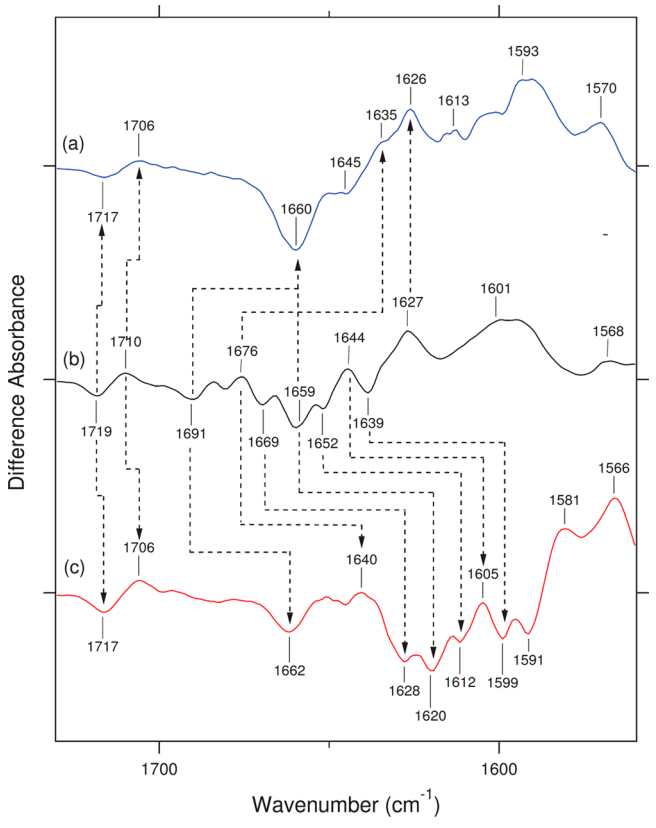


Figure 4. Light-induced difference FTIR spectra of CPD-PHR at 277 K showing photoactivation of apo-^{13}C-labeled (blue), unlabeled (black), and uniformly ^{13}C-labeled (red) samples in the presence of CPD substrate in the region of 1800−1550 cm^{-1}. One division of the *y*-axis corresponds to 0.008 absorbance unit.

dx.doi.org/10.1021/bi500638b | *Biochemistry* 2014, 53, 5864−5875

possibly overlapped at either 1645 or 1602 cm^{-1} in apo-^{13}C-labeled enzyme. However, this positive peak most likely originates from a change in the C_4=O vibration of $FADH^-$, whose intensity is expected to increase upon formation of a new C_{4a}=C_{10a} bond. Moreover, the formation of a new C_{4a}=C_{10a} bond of $FADH^-$ from the C_{4a}–C_{10a} bond of $FADH^•$ can be explained by the signal observed at 1627 (+) cm^{-1}. These assignments are supported by the fact that these signals in the H–D exchange experiment conducted previously are unshifted[13] and also were not shifted in the apo-^{13}C- or apo-^{15}N-labeled enzyme, in contrast to the clear shift observed for uniformly ^{13}C- and ^{15}N-labeled enzyme.

With regard to the signal assignment for the lower-frequency region of ^{13}C- and ^{15}N-labeled enzymes (Figure 3 and Figure S2 of the Supporting Information) which us due to the lower signal-to-noise ratio in this region, only major peaks were carefully assigned. The negative peak at 1532 cm^{-1} in Figure 3b would correspond to the C_{10a}=N_1 group of $FADH^•$ because this peak is shifted to 1493 (−) and 1523 (−) cm^{-1} for the uniformly ^{13}C- and ^{15}N-labeled samples, respectively (Figure 3c and Figure S2c of the Supporting Information), and the assignment was further supported by the absence of a shift in the previous H–D exchange experiment.[13] This change in the C_{10a}=N_1 vibration implies the formation of a new single bond (C_{10a}–N_1) of $FADH^-$. The negative peak at 1532 cm^{-1} also shifted slightly to 1528 (−) cm^{-1} upon apo-^{13}C and apo-^{15}N labeling (Figure 3a and Figure S2a of the Supporting Information). This observation and assignment is not necessarily inconsistent with the shift obtained by uniform ^{13}C and ^{15}N isotope labeling, which indicates that possibly not only the C=N group from the FAD moiety but also other parts of the protein (amide II) contribute around this frequency. The peak at 1514 (+) cm^{-1} (Figure 3b and Figure S2b of the Supporting Information) is assigned to FAD because this peak was shifted only upon uniform ^{13}C or ^{15}N labeling, not in apo-labeled enzymes. The peak at 1395 (+) cm^{-1} was downshifted to 1355 (+) cm^{-1} in the uniformly ^{13}C-labeled enzyme (Figure 3c) but was not downshifted in apo-^{13}C- or apo-^{15}N-labeled enzyme (Figure 3a and Figure S2a of the Supporting Information). Thus, we conclude that this band also originates from FAD and indicates a newly formed C_{10a}–N_1 stretching vibration. Note that the signal is not detected in apo-^{15}N-labeled enzyme (Figure S2c of the Supporting Information), probably because of an overlap with another peak. This peak was not shifted upon H–D exchange,[13] thus supporting the assignment of this peak to the C_{10a}–N_1 group of FAD. The 1395 cm^{-1} peak (Figure 3b) could be the marker band for determining the efficiency of isotope labeling, because >90% of this peak intensity was shifted to 1355 cm^{-1}, leaving a barely noticeable signal at 1395 cm^{-1} for the uniformly ^{13}C-labeled enzyme (Figure 3c); thus, we concluded the labeling efficiency reached >90%. Meanwhile, the peaks at 1069 (−) and 1061(+) cm^{-1} were also assigned to FAD because they remain unshifted in apo-^{13}C- and apo-^{15}N-labeled enzymes, respectively (Figure 3a and Figure S2a of the Supporting Information), but shifted upon uniform ^{13}C and ^{15}N labeling to 1066 (−)/1057 (+) and 1027 (−)/1020 (+) cm^{-1}, respectively (Figure 3c and Figure S2c of the Supporting Information), where these peaks were also not shifted upon H–D exchange, as shown previously.[13]

FAD Activation of Uniformly Labeled and Apo-Labeled CPD-PHRs in the Presence of DNA Substrate. Figures 4 and 5 show the light-induced difference FTIR spectra

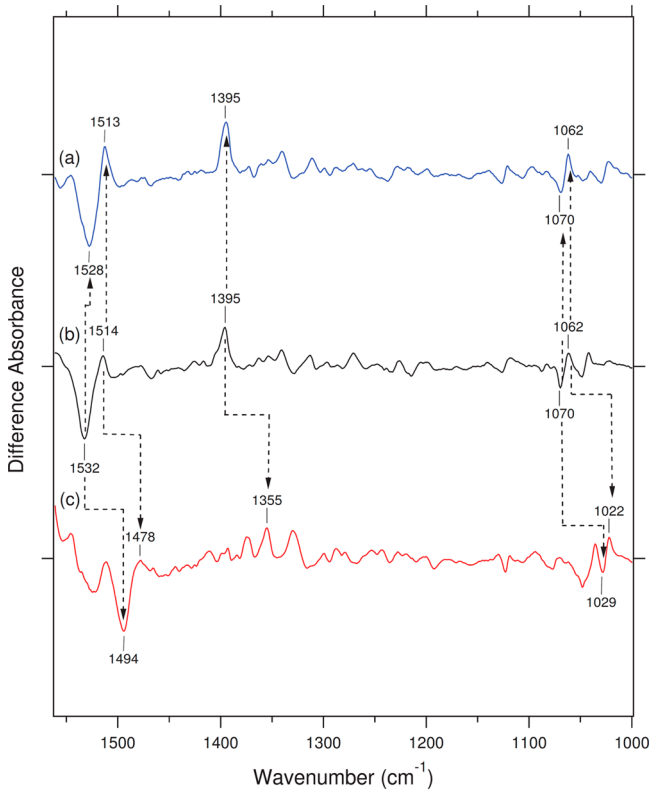


Figure 5. Light-induced difference FTIR spectra of CPD-PHR at 277 K showing photoactivation of apo-^{13}C-labeled (blue), unlabeled (black), and uniformly ^{13}C-labeled (red) samples in the presence of CPD substrate in the region of 1550–1000 cm^{-1}. One division of the *y*-axis corresponds to 0.008 absorbance unit.

of FAD activation for the unlabeled and uniformly ^{13}C- and apo-^{13}C-labeled samples in the presence of the unlabeled CPD substrate, and Figures S3 and S4 of the Supporting Information show the spectra of the uniformly ^{15}N- and apo-^{15}N-labeled enzymes, respectively. Under our measurement conditions, CPD is already bound to the enzymes prior to FAD activation. In the presence of CPD substrate, the light-induced FAD activation of CPD-PHR results in spectral features that are more complex than those in the absence of substrate.

In the presence of CPD substrate, the peaks at 1719 (−)/1710 (+) cm^{-1} newly appeared in the photoactivation spectra. These peaks shifted slightly to 1717 (−)/1706 (+) cm^{-1} in both uniformly ^{13}C- and apo-^{13}C-labeled enzymes (Figure 4a,c). If the peaks originated from CPD-PHR, a downshift of ∼35–40 cm^{-1} is expected upon ^{13}C labeling. Thus, the bands originate from the CPD substrate. The most likely candidates are C_4=O stretches of the CPD substrate because the C_2=O stretches of the CPD substrate are expected to appear at a much lower frequency, as previously observed for the C_2=O stretching vibrations of the thymine analogue.[28] However, the subtle shift of these peaks by a few wavenumbers may be due to a mixture of other subtle signals from the apoprotein, probably C=O stretching vibrations of protonated carboxylic acid residues. Previously, the 2–4 cm^{-1} shift of these peaks was observed upon H–D exchange,[13] further supporting the possibility that the presence of exchangeable proton(s) was involved in this shift. A possible candidate of the origin of the signal will be discussed below.

In the presence of CPD substrate, some peaks newly appeared while other peaks became sharper and showed higher

dx.doi.org/10.1021/bi500638b | *Biochemistry* 2014, 53, 5864–5875

Table 1. FTIR Spectral Assignment for FAD Activation

wavenumber (cm^{-1})	assignment	isotope shift (cm^{-1}) ^{13}C apo	^{13}C uniform	^{15}N apo	^{15}N uniform	description
1719 (−)	C_4=O	−2	−4	0	0	C_4=O group of DNA is downshifted upon hydrogen bond formation with E275 and N_7 of FAD's adenine (see Discussion)
1710 (+)	C_4=O	−2	−4	0	0	
1691 (−)	C=O	−32	−29	−1	−1	Asn or amide I, protein moiety or amide I[24]
1676 (+)	protein	−31	−37	+1	+2	in apo-labeled and uniformly ^{15}N-labeled, this peak overlapped with the shoulder at 1684 cm^{-1} (see Results)
1669 (−)	protein	*a*	−42	−1	−2	
1659 (−)	C_2=O	0	−42	0	−1	1724 cm^{-1}, hydrated free FAD[ox33]
						1660 cm^{-1}, *E. coli* CPD-PHR by Raman spectroscopy[34]
1652 (−)	protein	*a*	−40	−2	−2	α-helices, more prominent in the presence of CPD substrate, shift only −2 cm^{-1} in both apo-labeled and uniformly ^{15}N-labeled samples
1644 (+)	C_4=O	*a*	−40	0	0	FAD ($FADH^-$)
						1702 cm^{-1}, hydrated free FAD[ox33]
1627 (+)	C_{4a}=C_{10a}	0	−46	−1	−4	FAD ($FADH^-$)
						$\nu(C-C)_{ring}$ of FAD[ox35]
1601 (+)		*a*	−35	0	*a*	FAD ($FADH^-$), C_{10a}=N_1 group of oxidized lumiflavin[33]
1568 (+)		*a*	*a*	+1	−2	FAD ($FADH^-$)
1532 (−)	C_{10a}=N_1	−4	−39	−4	−9	FAD ($FADH^•$), ν(ring I) and $\delta(N_5-H)$[34]
1514 (+)		−1	−37	−1	−4	FAD ($FADH^-$)
1395 (+)	C_{10a}–N_1	−1	−40	0	*a*	FAD ($FADH^-$), 1501 cm^{-1} was observed in oxidized lumiflavin[34]
1070 (−)	$C_{7\alpha}$–H or $C_{8\alpha}$–H	0	−42	0	−3	FAD ($FADH^•$), 1069 cm^{-1} in the absence of CPD substrate
1063 (+)	$C_{7\alpha}$–H or $C_{8\alpha}$–H	0	−41	0	−4	FAD ($FADH^-$), 1061 cm^{-1} in the absence of CPD substrate

[a]A shifted signal upon particular isotope labeling could not be observed.

intensities. These phenomena are attributed to the formation of a complex structure upon binding to CPD substrate and may reflect structural changes or alterations to the CPD binding regions. The appearance of a slightly sharper peak at 1669 (−) cm^{-1} upon activation in the presence of CPD substrate (Figure 4b) suggests that this peak originates from CPD-PHR enzyme because it also shifts upon uniform ^{13}C labeling. Another negative peak at 1659 cm^{-1} (Figure 4b), which was previously assigned to the C_2=O stretching vibration of $FADH^•$, is further supported by these spectra, because the shift appears only in uniformly ^{13}C-labeled enzyme (Figure 4c). In this spectrum, the peak at 1652 (−) cm^{-1}, which is attributed to structural changes in α-helices, becomes sharper than in the absence of CPD substrate (Figure 2). This indicates that the structural change of CPD-PHR α-helices is different upon binding of CPD substrate. Interestingly, the formation of this signal is triggered by a FAD reduction. Most likely, this phenomenon is attributed to subtle changes in FAD's shape upon reduction, which subsequently induces structural changes to the protein environment.

The positive peak at 1644 cm^{-1}, which was assigned to the C_4=O stretch of $FADH^-$ (Figure 4b), is also supported here because it shifted in uniformly ^{13}C-labeled enzyme, even in the presence of CPD. This signal was not affected by CPD binding because the C_4=O group of FAD is located distal to CPD substrate. It should be noted that this peak in apo-^{13}C-labeled enzyme could not be observed probably because of an overlap with other peaks around 1645 cm^{-1} (Figure 4a). The peak at 1601 cm^{-1} could not be properly assigned in this experiment because of a complicated shift with other possibly overlapping peaks.

In the lower-frequency region, as shown in Figure 5 and Figure S4 of the Supporting Information, for ^{13}C and ^{15}N labeling, respectively, the negative peak at 1532 cm^{-1} is assigned to the C_{10a}=N_1 stretch of $FADH^•$, as it showed a downshift to 1494 cm^{-1} in the ^{13}C-labeled enzyme (Figure 5c, red spectra). In the presence or absence of CPD substrate, this peak appeared at the same frequency (Figure 3). This indicates that the presence of CPD substrate does not affect this bond, possibly because of the fairly long distance between both CPD substrate and this bond, as observed in the CPD-PHR crystal structure.[18] The slight downshift to 1528 cm^{-1} observed for apo-^{13}C- and apo-^{15}N-labeled enzyme (Figure 5a, blue spectra, and Figure 4a of the Supporting Information) may indicate the presence of C=N stretching from other moieties or an overlapping amide II vibration at this frequency.

Another positive peak at 1514 cm^{-1} was further confirmed to originate from FAD because it shifted only in uniformly ^{13}C- and ^{15}N-labeled enzymes [Figure 5c and Figure S4c of the Supporting Information (red spectra)], but not in apo-^{13}C- or apo-^{15}N-labeled enzymes (blue spectra) (Figure 5 and Figure S4 of the Supporting Information, respectively), despite the presence of CPD. It is difficult to assign a particular signal to a specific part of FAD. The next positive peak at 1395 cm^{-1} most likely originated from the C_{10a}–N_1 stretch of $FADH^-$. Further support for this assignment to the C_{10a}–N_1 stretch is the fact that the signal at 1395 cm^{-1}, like the C_{10a}=N_1 stretch, was not affected by the presence of CPD substrate. Additionally, the peak did not shift in apo-^{13}C-labeled spectra in Figure 5a (blue line) but shifted to 1355 cm^{-1} in uniformly ^{13}C-labeled enzyme (red line), as shown in Figure 5c. Similarly, this peak also did not shift upon apo-^{15}N labeling (Figure S4 of the Supporting Information) but could not be properly assigned in uniformly ^{15}N-labeled samples because of overlapping peaks around 1390 cm^{-1}, where the resulting shifted peak should be situated, as in the absence of CPD substrate.

The last signal that was identified is a pair at 1069 (−)/1061 (+) cm^{-1}, which shifted slightly to become 1070 (−)/1062 (+)

dx.doi.org/10.1021/bi500638b | *Biochemistry* 2014, 53, 5864−5875

cm^{-1} in the presence of CPD substrate. Similar to previous assignments for FAD activation in the absence of CPD substrate, this pair of signals is attributed to the isoalloxazine moiety of FAD because it was not shifted in apo-^{13}C- or apo-^{15}N-labeled enzymes but was shifted in uniformly labeled enzymes. The observed shift indicates that FAD was slightly perturbed by the presence of CPD, which is consistent with previous findings.[13] Signals from this frequency possibly originate from the $C_{7\alpha}$–H or the $C_{8\alpha}$–H bending vibrations of FAD because both groups are located near CPD substrate,[18] providing the possibility for steric interaction between CPD substrate and FAD. The assignment of FTIR spectra of CPD-PHR photoactivation is summarized in Table 1.

DNA Repair of Light-Induced Uniformly Labeled and Apo-Labeled CPD-PHRs. Light-induced FAD activation of CPD-PHR produces the functionally active $FADH^-$ state required for DNA repair. The $FADH^-$ state remains stable in the presence of an electron donor during the time frame of the FTIR measurements that were conducted. The CPD-PHR enzyme in the absence of CPD substrate was illuminated with >390 nm light, which is absorbed by $FADH^-$. Although the oxidized (FAD^{ox}) enzyme also absorbs light in this region, it is not reduced under these conditions as previously described.[13] In the presence of DNA substrate, new signals appeared as a result of CPD cleavage by the CPD-PHR enzyme. Because the light-induced DNA repair signal includes not only the cleavage of CPD but also the release of thymines from the enzyme, the new peaks that appeared on the positive side correspond to the repaired DNA together with free enzyme. On the other hand, the negative peaks correspond to unrepaired CPD substrate bound to the enzyme and the enzyme bound to CPD substrate.

The photoreaction by >390 nm light resulted in new peaks on the difference FTIR spectra as shown in Figures 6 and 7 and Figures S5 and S6 of the Supporting Information for ^{13}C- and ^{15}N-labeled samples, respectively. The newest and most prominent peak pair at 1721 (+)/1705 (−) cm^{-1} and the peak pair downshift only by 1–2 cm^{-1} in both uniformly ^{13}C-labeled and apo-^{13}C-labeled enzymes, respectively (Figure 6a,c). This is in agreement with a previous assignment to the C_4=O stretch of repaired thymines.[13] The spectral difference at 1710–1700 cm^{-1} between spectrum b of Figure 6 and spectra a and c shows that the contribution of the C=O stretch from protonated carboxylic acid may be involved (see below).

The broad negative band at 1705 cm^{-1}, which shows a slight shift to 1696 cm^{-1} for the ^{15}N-labeled enzyme (Figure S5 of the Supporting Information), may originate from two C_4=O groups in CPD that bind to different amino acid residues of CPD-PHR enzyme. The possibility of overlap with amide C=O stretching vibrations from glutamine or asparagines cannot be completely dismissed because this negative peak shifts in only uniformly labeled and apo-^{13}C-labeled enzyme, not in uniformly labeled or apo-^{15}N-labeled samples, as shown in Figure S5 of the Supporting Information. The 1705 (−) cm^{-1} peak was also shifted to 1692 (−) cm^{-1} upon H–D exchange;[13] the shift supports the idea of the involvement of replaceable proton(s) upon bound C_4=O groups of CPD substrate with its nearby residue pictured in Figure 1a, the most likely candidate for the proton being that from protonated carboxylic acid. The shifted peak of the C_4=O stretch from 1705 (−) to 1721 (+) cm^{-1} upon photorepair also may indicate differences in the binding conditions of the C_4=O group of CPD substrate bound to the enzyme complex as in Figure 1a, and the C_4=O group of the repaired thymines bound to adenine in the

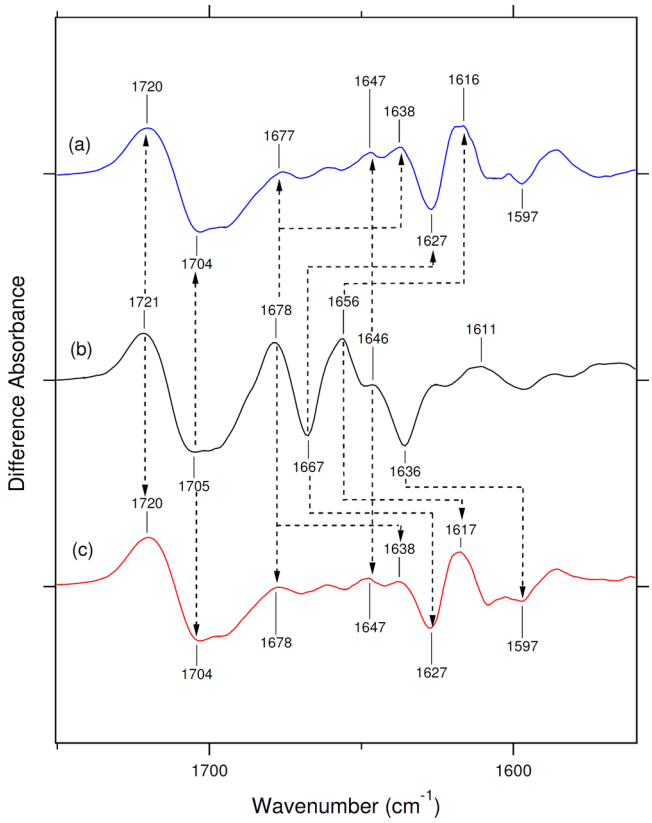


Figure 6. Light-induced difference FTIR spectra of CPD-PHR at 277 K showing photorepair of previously photoactivated apo-^{13}C-labeled (blue), unlabeled (black), and uniformly ^{13}C-labeled (red) samples in the presence of CPD substrate in the region of 1800–1560 cm^{-1}. One division of the *y*-axis corresponds to 0.007 absorbance unit.

DNA strand. The C=O moieties of CPD, which might form hydrogen bonds with water and/or the enzyme moiety, may be required for binding or for stabilization of the thymine anion radical during the cleavage of CPD. Both uniform and apo-^{15}N labeling also revealed subtle contributions of apoenzyme to the 1705 cm^{-1} peak as this broad peak shifted slightly to 1696 cm^{-1} in both labeling patterns (Figure S5a,c of the Supporting Information).

The second positive peak at 1678 cm^{-1} is assigned to a recovered C_2=O group of DNA with a major contribution from the protein. This peak shifted partially to 1638 cm^{-1}, but a small but observable signal at 1678 cm^{-1} in both uniformly labeled and apo-^{13}C-labeled enzyme remained and thus indicated the contribution of protein (spectra c and a of Figure 6, respectively). As reported previously, a small contribution of C_2=O stretching vibrations also appears at this frequency.[28] A slight shift observed previously in H–D exchange might have originated only from protein;[13] meanwhile, the contribution of the C_2=O group of DNA can be excluded, which may not involve a proton bound to the enzyme. A new sharp negative peak at 1667 cm^{-1} may originate from amide I of the enzyme, which shifted to 1627 cm^{-1} in both uniformly labeled and apo-^{13}C-labeled enzymes, which is further support from the previous H–D exchange experiment, as this peak was not shifted upon H–D exchange.[13] The positive peak at 1656 cm^{-1} probably originates from amide I, attributed mainly to α-helices, because of the shift to 1617 and 1616 cm^{-1} in uniformly labeled and apo-labeled enzymes, respectively (spectra c and a of Figure 6, respectively), which previously was not shifted as a

dx.doi.org/10.1021/bi500638b | *Biochemistry* 2014, 53, 5864–5875

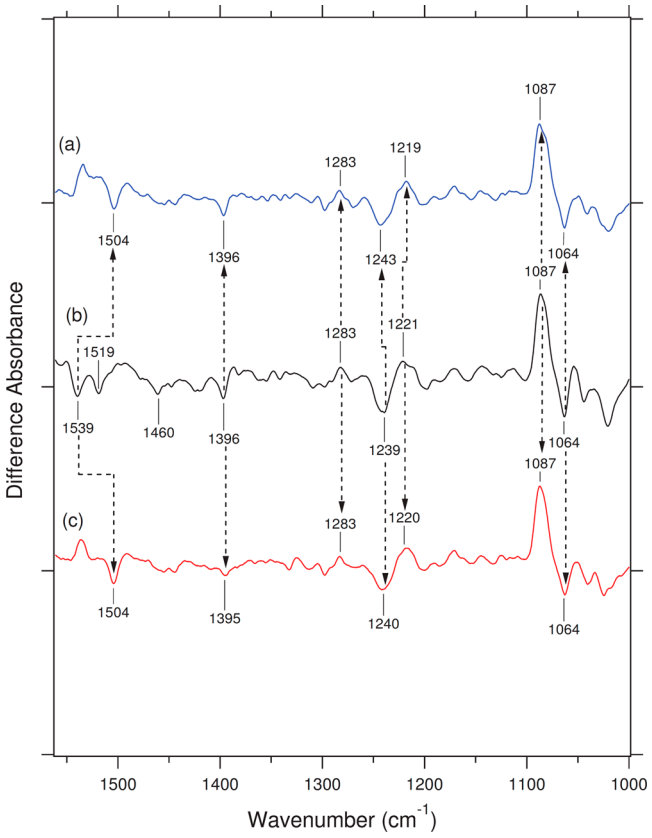


Figure 7. Light-induced difference FTIR spectra of CPD-PHR at 277 K showing photorepair of previously photoactivated apo-^{13}C-labeled (blue), unlabeled (black), and uniformly ^{13}C-labeled (red) samples in the presence of CPD substrate in the region of 1560−1000 cm^{-1}. One division of the *y*-axis corresponds to 0.007 absorbance unit.

result of the H−D exchange experiment that further supported this assignment.[13] Interestingly, a new, small but consistently positive peak at 1646 cm^{-1} can be assigned to the repaired DNA or normal intact thymine because the peak did not shift in all ^{13}C- and ^{15}N-labeled samples. A possible contribution may be by the newly formed C_5=C_6 stretch of the repaired thymine after the cleavage of cyclobutane. A prominent peak was observed at 1636 (−) cm^{-1} that is attributed to the protein, because it shifts in both uniformly labeled and apo-^{13}C-labeled enzymes. The same holds true for the similar shifted and less prominent peaks at 1611 (+), 1551 (+), 1539 (−), and 1519 (−) cm^{-1}.

In the lower-frequency region (Figure 7 and Figure S6 of the Supporting Information), the negative peak at 1396 cm^{-1} did not shift in the ^{13}C- or ^{15}N-labeled enzyme, or after H−D exchange,[13] thereby confirming their origin as the CPD substrate, as previously reported.[24] On the other hand, the negative peak at 1460 cm^{-1} did not shift for the ^{15}N-labeled enzyme, but the shift was unclear for the ^{13}C-labeled enzyme. The three unshifted peaks at 1283 (−), 1239 (−), and 1221 (+) cm^{-1} may originate from asymmetric stretches of PO_2^- in the DNA phosphate backbone, while the three unshifted peaks at 1087 (+), 1063 (−), and 1054 (+) cm^{-1} likely originate from symmetric stretches of PO_2^- in the DNA phosphate backbone. Peaks around 1200 and 1000 cm^{-1} were previously reported to originate from the DNA phosphate backbone.[29] The candidate signals that were assigned as originating from PO_2^- in the DNA phosphate backbone were also supported by a previous H−D exchange experiment in which no shift in these signals was observed.[13] The assignment of FTIR spectra for CPD repair by CPD-PHR enzyme is summarized in Table 2.

Origin of a Protonated Carboxylic Acid Residue. We found that the C=O stretch from a protonated carboxylic acid residue may be involved in FAD photoactivation in the presence of CPD substrate. Upon FAD activation and DNA repair, in unlabeled spectra, the >1700 cm^{-1} region is a mixture of C=O stretches from both apoprotein and DNA, while both apo-labeled and uniformly ^{13}C-labeled enzyme originated only

Table 2. FTIR Spectral Assignments for DNA Repair

		isotope shift (cm^{-1})				
		^{13}C		^{15}N		
wavenumber (cm^{-1})	assignment	apo	uniform	apo	uniform	description
1721 (+)	C_4=O	0	0	0	0	recovered and increment of DNA C_4=O stretches due to cyclobutane cleavage
1705 (−)	C_4=O	−1	−1	−9	−9	C_4=O group of CPD bound with enzyme, shift in signal due to the presence of enzyme contribution
1678 (+)	C_2=O and protein	−1 −40	0 −40	−1	−2	small contribution from the C_2=O group,[28] majority contribution from enzyme (amide I) that shifts upon isotope ^{13}C labeling
1667 (−)	protein	−40	−40	+1	+1	amide I
1656 (+)	protein	−40	−39	+2	0	α-helices
1646 (+)	C_5=C_6	+1	+1	−1	−2	formation of new C_5=C_6 bonds of repaired thymines
1636 (−)	protein	*a*	−37	−3	−2	shifts of the signals originating from isotopically labeled enzyme
1611 (+)	protein	*a*	*a*	+1	+6	
1539 (−)	protein	−35	−35	*a*	*a*	
1460 (−)	CPD	*a*	*a*	0	0	CPD substrate,[24] 1640 cm^{-1} signal possibly overlapped in both apo-labeled and uniformly ^{13}C-labeled enzymes
1396 (−)	CPD	0	0	0	0	
1283 (+)	PO_2^-	0	0	0	+1	DNA phosphate backbone (asymmetric)[29]
1239 (−)	PO_2^-	+4	+1	0	−1	DNA phosphate backbone (asymmetric)[29]
1221 (+)	PO_2^-	−2	−1	0	−1	DNA phosphate backbone (asymmetric)[29]
1087 (+)	PO_2^-	0	0	0	0	DNA phosphate backbone (symmetric)[29]
1064 (−)	PO_2^-	0	0	0	0	DNA phosphate backbone (symmetric)[29]

[a]A shifted signal upon particular isotope labeling could not be observed.

dx.doi.org/10.1021/bi500638b | *Biochemistry* 2014, 53, 5864−5875

from DNA (Figure 8a). As such, the signal from double difference spectra (unlabeled minus uniformly ^{13}C-labeled and

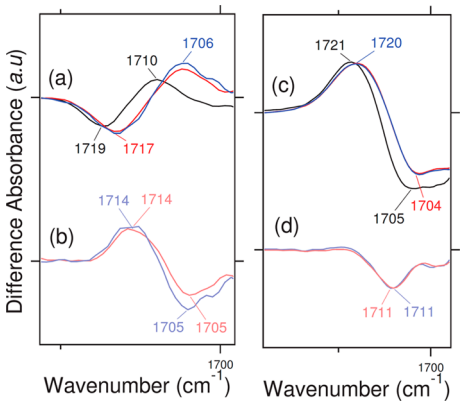


Figure 8. Light-induced difference FTIR spectra of CPD-PHR at 277 K showing (a) photoactivation of apo-^{13}C-labeled (blue), unlabeled (black), and uniformly ^{13}C-labeled (red) samples in the presence of CPD substrate, (b) double-difference spectra upon FAD activation of black minus blue/light blue and black minus red/light red, (c) photorepair of a previously photoactivated sample of apo-^{13}C-labeled (blue), unlabeled (black), and uniformly ^{13}C-labeled (red) samples, and (d) double-difference spectra upon DNA repair of black minus blue/light blue and black minus red/light red in the >1690 cm^{-1} region. *a.u* indicates arbitrary units.

unlabeled minus apo-^{13}C-labeled) would then result only from apoprotein (Figure 8b). Double-difference signals from apo-labeled and uniformly ^{13}C-labeled enzyme yielded similar double-difference spectra upon FAD activation, which is most likely from the carboxylate protonation signal.[22] During photorepair processes in the presence of CPD substrate, which can be observed from the double difference of the spectra in Figure 8c, only an apoprotein signal was observed (Figure 8d), which indicates carboxylic acid deprotonation. Because the positive signal of apoprotein upon photoactivation (1714 cm^{-1}) and the negative signal upon photorepair (1711 cm^{-1}) appear in a similar frequency region, as shown in spectra b and d of Figure 8, respectively, this suggests that the origin is identical. No positive signal in Figure 8d can be interpreted to mean that carboxylic acid was deprotonated after CPD repair followed by dissociation from CPD-PHR. Deprotonation of glutamic acid would yield symmetric and asymmetric vibrations of deprotonated glutamate (COO^-). However, the double-difference spectra of Figure 6 (spectrum b minus spectrum c) around 1553−1575 and 1397−1424 cm^{-1} for $\nu_{symmetric}(COO^-)$ and $\nu_{asymmetric}(COO^-)$, respectively, are difficult to assign because the strong signals overlap, and a strong candidate for both symmetric and asymmetric stretches for deprotonated carboxylate could not be found (data not shown). This is possibly due to the small contribution from glutamate symmetric and asymmetric stretch vibration signals that were disabled by the presence of those strong overlapping signals. Another possibility is that carboxylic acid remains protonated and shifted to the ∼1700 cm^{-1} region, overlapping with signals from other origins.

The most likely candidate of a carboxylic acid that is involved in FAD activation and DNA repair would be Glu275, which is present in the CPD binding pocket, because it alters its C═O stretching vibration upon FAD activation, as proposed previously.[13] The accompanying changes of the C_4═O stretch of CPD substrate with carboxylic acid C═O stretch only in the presence of CPD substrate suggest that some interactions occurred between these moieties, although no change in the protonation state of specific interacting residues was observed in the absence of CPD, as can be seen by the flat >1700 cm^{-1} region in Figure 2b. Another alternative that cannot be ruled out is the possibility that the C_4═O group of CPD may be indirectly affected by ^{13}C labeling through coupled vibrations via hydrogen bonds. The downshift of C_4═O stretches of CPD substrate [from 1719 (−) to 1710 (+) cm^{-1}] upon reduction of FAD is intriguing because the formation of a new or stronger hydrogen bond of this DNA moiety with the enzyme moiety is caused only by the reduction of a single electron of the FAD cofactor.

DISCUSSION

In this paper, structural information about CPD-PHR was extracted from the FTIR spectra of FAD activation and DNA repair using uniform labeling with ^{13}C and ^{15}N isotopes and selective unlabeling of the riboflavin moiety of the FAD cofactor. The signals from apoprotein, FAD cofactor, and DNA upon FAD activation and DNA repair were clearly obtained and distinctly identified. On the basis of the isotope shift of the FTIR signals, the observed data deepen our understanding of the detailed molecular steps of the complete CPD-PHR function, from light-induced, one-electron reduction of FAD and subsequent environmental changes in the apoprotein moiety to subsequent cyclobutane cleavage by FAD excitation to finally the release of the DNA substrate.

Light-Induced Structural Changes in FAD Activation. In this experiment, the light-induced FAD activation of CPD-PHR consists of one-electron reduction of FAD, from $FADH^•$ to $FADH^-$. The repair spectra yield fully activated $FADH^-$ in complex with the DNA substrate and its subsequent release after repair. In this process, the FAD redox state remains unchanged and the resulting repair spectra reflect only the molecular changes of CPD cleavage, together with DNA substrate and enzyme conformation.

Photoactivation of uniformly ^{13}C- and ^{15}N-labeled enzymes in the absence and presence of CPD substrate showed that light-induced FAD reduction causes not only structural changes in FAD itself but also structural changes in the apoprotein (see Figures 2 and 4 and Figures S1 and S3 of the Supporting Information). Kao et al. proposed the dynamics of free FAD upon light excitation probed by ultrafast spectroscopy.[30] FAD was proposed to go through a bending motion from FAD^{ox} to $FADH^-$ forms, which was analogous to a butterfly bending movement.[30] Despite the difference in the unphotolyzed states between the calculation (FAD^{ox})[30] and FTIR experiments ($FADH^•$) described herein, the FAD shape should have gone through a partial butterfly bending motion upon activation ($FADH^•$ to $FADH^-$) if the latter was continuous from an oxidized state to a fully reduced state. The FAD isoalloxazine ring planarity is expected to change upon reduction of $FADH^•$, even though the degree of FAD bending in this experiment could not be determined. However, despite subtle changes in the degree of FAD planarity, this would obviously affect protein conformation, although it is difficult to know which part of the protein changes and the extent to which the shape of the protein changes as the FAD redox state changes. The FAD activation spectra observed here should have recorded FTIR signals that were correlated to the bending of FAD. However, the appearance of several major signals that can be attributed to

dx.doi.org/10.1021/bi500638b | *Biochemistry* 2014, 53, 5864−5875

apoprotein such as 1691 (−), 1676 (+), and 1669 (−) cm^{-1} in Figures 2b and 4b, which can be generally assigned to simultaneous amide I vibrations of the enzyme, might have masked the FAD bending signal. The 1652 (−) cm^{-1} peak (Figures 2b and 4b) in particular could be a clue for determining which part of the enzyme changed, driven by FAD bending, because this peak is assigned to structural changes involving α-helices. This observation then queries the role of these conformational changes to the protein upon FAD reduction.

To address the purpose of these protein conformational changes, the discrete changes in α-helices upon FAD reduction were closely examined. In partial agreement with a previous report,[21] as illustrated in Figure 1a, it is possible that FAD reduction not only provides the catalytically active state but also alters the shape of the apoprotein. From assigned changes that originated from α-helices, we thus propose that the signal of α-helices originated from α-helices near FAD, especially those α-helices that are expected to form the active site and the DNA binding pocket, colored light pink and light purple, respectively, in Figure 1a.[18] These α-helices may alter its position, as illustrated by a green arrow in Figure 1a, to become optimized for binding with DNA substrate, even though the extent and direction of the altered condition triggered by the FAD bending movement could not be determined from this experiment. These results demonstrate how CPD-PHR not only utilizes photons to change the redox state of its FAD cofactor but also utilizes the subsequent change to FAD's shape to structurally preconfigure its conformation to optimally facilitate DNA repair in general.

Roles of the Glutamic Acid Residue in the DNA Binding Pocket for DNA Repair. An interesting feature observed in the FTIR spectra is the slight shift of the 1719 (−)/1710 (+) cm^{-1} peaks that was mainly assigned to a downshift of the C_4=O group of the DNA substrate upon activation (Figure 4b). How and why did the C_4=O group of DNA become downshifted upon FAD reduction? As discussed previously, FTIR spectra indicate that α-helices changed upon FAD activation (Figures 2 and 4 and Table 1). The next logical step was to confirm which α-helices had "moved" the FAD bending motion. Evidence of the α-helices that form the DNA binding pocket lies in the slight shift of pairs of peaks from 1719 (−)/1710 (+) to 1717 (−)/1706 (+) cm^{-1} in both uniformly ^{13}C-labeled and apo-^{13}C-labeled spectra (Figure 4, red and blue lines), but not in ^{15}N-labeled and apo-^{15}N-labeled spectra (Figure S3 of the Supporting Information, red and blue lines). The latter signal shifts, which originated from the glutamate moiety, were previously masked. Interestingly, an α-helix that contributes to the DNA binding pocket and is proposed to have "moved" upon FAD activation contains a glutamate at position 275 (PDB entry 1TEZ)[18] and is situated within contact range of the adenine moiety of FAD, as can be seen in Figures 1b and 9a.

Superimposition of glutamate at position 275 of CPD-PHR with (PDB entry 1TEZ)[18] and without (PDB entry 1QNF) CPD substrate (Figure 9a, red and gray, respectively) reveals slight differences in the orientation of Glu275, suggesting subtle differences in enzyme conformation induced by the presence of CPD substrate (red arrow). The carboxyl group of Glu275 is within hydrogen bonding distance of the C_4=O group of the displayed 5′ side of CPD substrate (see Figure 9a), and this study implies that FAD activation accompanies hydrogen bonding alteration of the protonated Glu275 in the presence of DNA. The addition of E275's hydrogen bonds with the C_4=O group of the 5′ end of CPD substrate only upon FAD activation is functionally intriguing, because both the C_4=O groups of the 5′ and 3′ ends of DNA should have already bound with the $N_{6\alpha}$−H group, as can be seen in Figure 9a.

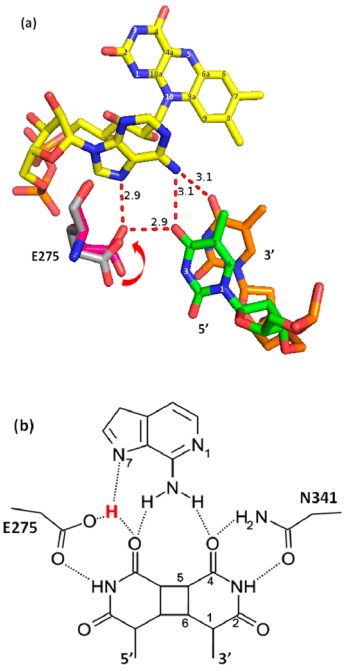


Figure 9. (a) Glu275 side chain that shows different orientations in the absence (gray, PDB entry 1QNF) and presence (pink, PDB entry 1TEZ) of CPD substrate. The interactions of 5′-thymine and Glu275 carboxyl with the adenine moiety of FAD upon reduction resemble Hoogsteen base pairing. (b) A proton (red), which dangles between Glu27 and N_7 of adenine and the C_4=O group of the CPD substrate, retracts to be in the vicinity of the Glu275 carboxyl and C_4=O group of the CPD substrate.

Interestingly, N_7 of adenine, which is also within hydrogen bond distance of the carboxyl moiety of Glu275, is a strong candidate that could be involved in these shifts. In the absence of CPD substrate, the carboxyl of Glu275 may be deprotonated and receive a proton from another moiety upon CPD substrate binding (Figure 9b, red), even though Glu275 may be already protonated and share a single hydrogen with another moiety, a possibility that cannot be fully excluded. This possibility is supported by the presence of a C=N shift signal upon FAD reduction, as already described in Results, as observed in Figure 5, which indicates that other C=N stretches were also involved in the spectra. The shifts may have formed when a proton was dangled between the carboxyl of Glu275 and N_7 of adenine. This proton of protonated Glu275 may have also been retracted by N_7 of the adenine moiety and can act as a proton acceptor. This is evidenced by N_7 of adenine, which is situated within hydrogen bonding distance of Glu275 (Figure 9b).

In the presence of CPD substrate, Glu275 is protonated near the substrate. The straightforward interpretation of Figure 8d is that Glu275 is deprotonated after DNA release. However, we cannot fully exclude the possibility that Glu275 is still protonated in the absence of the substrate. The peaks at 1719 (−)/1710 (+) cm^{-1}, which are attributed to the downshift of the C_4=O group of CPD substrate due to formation of a new hydrogen bond, coincide with a change in the hydrogen bonding network of Glu275. These signals also coincide with a

dx.doi.org/10.1021/bi500638b | Biochemistry 2014, 53, 5864−5875

signal of C═N shifts upon FAD reduction that originate from N_7 of the adenine moiety.

Upon FAD reduction in the presence of CPD substrate, the shifted C_4═O stretches of CPD, which were accompanied by hydrogen bonding alteration of protonated Glu275, presumably with a retracted proton from N_7 of the adenine moiety, were all located in the same vicinity as the DNA binding pocket. These clues suggest the involvement of a single hydrogen atom that is the origin of the signals for all three moieties. In the absence of CPD substrate, Glu275 might become deprotonated and a proton is situated near N_7 of adenine. Only in the presence of the C_4═O group of CPD substrate in the vicinity of the Glu275 carboxyl moiety would the proton be retracted. Glu275 would thus be protonated and dangle with the C_4═O group of the 5′ end of DNA that had previously bound with the $N_{6\alpha}$–H group of adenine, as illustrated in Figure 9b.

Another plausible explanation that cannot be ruled out is illustrated in Figure 1a: instead of the 5′ end of DNA, it is also possible that the C_4═O group from the 3′ end forms a hydrogen bond with the amine moiety of asparagine at position 341 (Asn341) due to FAD bending. This would also result in similar frequency shifts upon activation but is less likely to happen because the C═O stretch from the asparagine moiety is expected to appear at a lower frequency. To clearly elucidate the role of these amino acid residues, a future mutational study of Glu275 and Asn341 needs to be performed.

The exact role of the formation of a new Glu275 hydrogen bonding network with the C_4═O group of CPD substrate together with N_7 of adenine upon $FADH^{\bullet}$ reduction still needs to be determined. The intriguing formation of a hydrogen bond among N_7 of adenine, the carboxyl moiety of Glu275, and the C_4═O group of the 5′ CPD substrate might function in CPD repair. These issues need to be addressed by theoretical calculations in the future. The idea of the importance of the E275 upon CPD repair was partially supported by a mutational study through mutation of glutamate to alanine by Sancar et al., which reduced the quantum yield to 40%.[31] Mutant enzyme E275Q was made in an attempt to upshift the carboxylate protonation–deprotonation signal; however, the mutant enzyme lost its ability to bind CPD substrate, which further confirmed the importance of glutamate for the CPD binding process (data not shown). In addition, the hydrogen bonding network, as illustrated in Figure 9a, among these three moieties resembles the existing Hoogsteen base pairing, in addition to utilizing a single hydrogen that is dangled for these three moieties (Figure 9b).

In conclusion, this study suggests a possible new role for the FAD adenine moiety in the CPD binding pocket: N_7 of adenine pairs with the carboxyl of Glu275 and the C_4═O group of 5′ thymine. This resembles an existing Hoogstein base pair. Furthermore, no changes in the FAD signal upon substrate release were observed, unlike previously reported shifts in the FAD signal upon binding to CPD substrate.[32] This may have been caused by subtle changes in signal intensity caused by changes in the interaction of FAD with its surrounding residues upon CPD binding, causing a considerable overlap with other strong signals from amide I. In summary, this indicates that the main changes that occurred upon CPD substrate binding and release with CPD-PHR were due to the changes in the conformation of the apoenzyme and the repaired CPD.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

FTIR spectra of CPD-PHR at 277 K showing photoactivation of apo-^{15}N-labeled (blue), unlabeled (black), and uniformly ^{15}N-labeled (red) samples in the absence of CPD substrate in the regions of 1800–1550 cm^{-1} (Figure S1) and 1550–1000 cm^{-1} (Figure S2); light-induced difference FTIR spectra of CPD-PHR at 277 K showing photoactivation of apo-^{15}N-labeled (blue), unlabeled (black), and uniformly ^{15}N-labeled (red) samples in the presence of CPD substrate in regions of 1800–1550 cm^{-1} (Figure S3) and 1550–1000 cm^{-1} (Figure S4); and FTIR spectra of CPD-PHR at 277 K showing photorepair of the previously photoactivated apo-^{15}N-labeled (blue), unlabeled (black), and uniformly ^{15}N-labeled (red) samples in the presence of CPD substrate in the regions of 1800–1560 cm^{-1} (Figure S5) and 1560–1000 cm^{-1} (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Frontier Materials, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan. Phone and fax: 81-52-735-5207. E-mail: kandori@nitech.ac.jp.

Funding

This work was supported by grants from the Japan Society for the Promotion of Science to J.Y. (25870400) and the Japanese Ministry of Education, Culture, Sports, Science and Technology to H.K. (25104009). T.M. was supported by the Chemical Sciences council of The Netherlands Organization for Scientific Research (NWO-CW) through an ECHO grant to J.T.M.K. J.T.M.K. was supported by a NWO-CW VICI grant.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PHR, photolyase; CPD, cyclobutane pyrimidine dimer; FAD, flavin adenine dinucleotide; $FADH^-$, reduced form of FAD; $FADH^{\bullet}$, semiquinoid form of FAD; FAD^{ox}, oxidized form of FAD; MTHF, 5,10-methylenetetrahydrofolate; PP, photoproduct; FTIR, Fourier transform infrared; UV–vis, ultraviolet–visible; PDB, Protein Data Bank.

■ REFERENCES

(1) Sinha, R. P., and Hader, D. P. (2002) UV-induced DNA damage and repair: A review. *Photochem. Photobiol. Sci. 1*, 225–236.

(2) Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A., and Ellenberger, T. (2006) *DNA repair and mutagenesis*, 2nd ed., American Society for Microbiology, Washington, DC.

(3) Cashmore, A. R. (2003) Cryptochrome: Enabling plants and animal to determine circadian time. *Cell 114*, 537–543.

(4) Vitaterna, M. H., Selby, C. P., Todo, T., Niwa, H., Thompson, C., Fruechte, E. M., Hitomi, K., Thresher, R. J., Ishikawa, T., Miyazaki, J., Takahashi, J. S., and Sancar, A. (1999) Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc. Natl. Acad. Sci. U.S.A. 96*, 12114–12119.

(5) Gegear, R. J., Casselman, A., Waddell, S., and Reppert, S. M. (2008) Cryptochrome mediates light-dependent magnetosensitivity in *Drosophila*. *Nature 454*, 1014–1018.

(6) Lin, C., Robertson, D. E., Ahmad, M., Raibekas, A. A., Jorns, M. S., Dutton, P. L., and Cashmore, A. R. (1995) Association of flavin adenine dinucleotide with the *Arabidopsis* blue light receptor CRY1. *Science 269*, 968–970.

(7) Todo, T., Kim, S. T., Hitomi, K., Otoshi, E., Inui, T., Morioka, H., Kobayashi, H., Ohtsuka, E., Toh, H., and Ikenaga, M. (1997) Flavin adenine dinucleotide as a chromophore of the *Xenopus* (6–4) photolyase. *Nucleic Acids Res. 25*, 764–768.
(8) Sancar, A., and Sancar, G. B. (1984) *Escherichia coli* DNA photolyase is a flavoprotein. *J. Mol. Biol. 172*, 223–227.
(9) Malhotra, K., Kim, S. T., Batschauer, A., Dawut, L., and Sancar, A. (1995) Putative blue-light photoreceptors from *Arabidopsis thaliana* and *Sinapis alba* with a high degree of sequence homology to DNA photolyase contain the two photolyase cofactors but lack DNA repair activity. *Biochemistry 34*, 6892–6899.
(10) Jorns, M. S., Sancar, G. B., and Sancar, A. (1984) Identification of a neutral flavin radical and characterization of a second chromophore in *Escherichia coli* DNA photolyase. *Biochemistry 23*, 2673–2679.
(11) Eker, A. P. M., Hessels, J. K. C., and van de Velde, J. (1988) Photoreactivating enzyme from the green alga *Scenedesmus acutus*. Evidence for the presence of two different flavin chromophores. *Biochemistry 27*, 1758–1765.
(12) Payne, G., Heelis, P. F., Rohrs, B. R., and Sancar, A. (1987) The active form of *Escherichia coli* DNA photolyase contains a fully reduced flavin and not a flavin radical, both *in vivo* and *in vitro*. *Biochemistry 26*, 7121–7127.
(13) Wijaya, I M. M., Zhang, Y., Iwata, T., Yamamoto, J., Hitomi, K., Iwai, S., Getzoff, E. D., and Kandori, H. (2013) Detection of distinct α-helical rearrangements of cyclobutane pyrimidine dimer photolyase upon substrate binding by Fourier transform infrared spectroscopy. *Biochemistry 52*, 1019–1027.
(14) Tamada, T., Kitadokoro, K., Higuchi, Y., Inaka, K., Yasui, A., de Ruiter, P. E., Eker, A. P., and Miki, K. (1997) Crystal structure of DNA photolyase from *Anacystis nidulans*. *Nat. Struct. Biol. 4*, 887–891.
(15) Byrdin, M., Eker, A. P., Vos, M. H., and Brettel, K. (2003) Dissection of the triple tryptophan electron transfer chain in *Escherichia coli* DNA photolyase: Trp382 is the primary donor in photoactivation. *Proc. Natl. Acad. Sci. U.S.A. 100*, 8676–8681.
(16) Park, H. W., Kim, S. T., Sancar, A., and Deisenhofer, J. (1995) Crystal structure of DNA photolyase from *Escherichia coli*. *Science 268*, 1866–1872.
(17) Berg, B. J. V., and Sancar, G. B. (1998) Evidence for dinucleotide flipping by DNA photolyase. *J. Biol. Chem. 32*, 20276–20284.
(18) Mees, A., Klar, T., Gnau, P., Hennekke, U., Eker, A. P., Carell, T., and Essen, L. O. (2004) Crystal structure of a photolyase bound to a CPD-like DNA lesion after in situ repair. *Science 306*, 1789–1793.
(19) Kim, S. T., and Sancar, A. (1993) Photochemistry, photophysics, and mechanism of pyrimidine dimer repair by DNA photolyase. *Photochem. Photobiol. 57*, 895–904.
(20) Heelis, P. F., and Sancar, A. (1986) Photochemical properties of *Escherichia coli* DNA photolyase: A flash photolysis study. *Biochemistry 25*, 8163–8166.
(21) Liu, Z., Tan, C., Guo, X., Kao, Y. T., Li, J., Wang, L., Sancar, A., and Zhong, D. (2011) Dynamics and mechanism of cyclobutane pyrimidine dimer repair by DNA photolyase. *Proc. Natl. Acad. Sci. U.S.A. 108*, 14831–14836.
(22) Barth, A. (2000) The infrared absorption of amino acid side chains. *Prog. Biophys. Mol. Biol. 74*, 141–173.
(23) Mehlhorn, J., Steinocher, H., Beck, S., Kennis, J. T., Hegemann, P., and Mathes, T. (2013) A set of engineered *Escherichia coli* expression strains for selective isotope and reactivity labeling of amino acid side chains and flavin cofactors. *PLoS One 8*, 79006.
(24) Schleicher, E., Hessling, B., Illarionova, V., Bacher, A., Weber, S., Richter, G., and Gerwert, K. (2005) Light-induced reactions of *Escherichia coli* DNA photolyase monitored by Fourier transform infrared spectroscopy. *FEBS J. 272*, 1855–1866.
(25) Murata, T., Iwai, S., and Ohtsuka, E. (1990) Synthesis and characterization of a substrate for T4 endonuclease V containing a phosphorodithioate linkage at the thymine dimer site. *Nucleic Acids Res. 18*, 7279–7286.
(26) Yamada, D., and Kandori, H. (2014) FTIR spectroscopy of flavin-binding photoreceptors. *Methods Mol. Biol. 1146*, 361–376.
(27) Prytkova, T. R., Beratan, D. N., and Skourtiss, S. S. (2007) Photoselected electron transfer pathways in DNA photolyase. *Proc. Natl. Acad. Sci. U.S.A. 104*, 802–807.
(28) Hernández, B., Navarro, R., Vergoten, G., and Hernanz, A. (2001) *Ab initio* vibrational calculations on ara-T molecule: Application to analysis of IR and Raman spectra. *Biopolymers 62*, 193–207.
(29) Taillandier, E., and Liquier, J. (1992) Infrared spectroscopy of DNA. *Methods Enzymol. 211*, 307–335.
(30) Kao, Y. T., Saxena, C., He, T. F., Guo, L., Wang, L., Sancar, A., and Zhong, D. (2008) Ultrafast dynamics of flavin in five redox states. *J. Am. Chem. Soc. 130*, 13132–13139.
(31) Vande Berg, B. J., and Sancar, G. B. (1998) Evidence for dinucleotide flipping by DNA photolyase. *J. Biol. Chem. 273*, 20276–20284.
(32) Li, J., Uchida, T., Todo, T., and Kitagawa, T. (2006) 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. *J. Biol. Chem. 281*, 25551–25559.
(33) Wille, G., Ritter, M., Friedemann, R., Mantele, W., and Hubner, G. (2003) Redox-triggered FTIR difference spectra of FAD in aqueous solution and bound to flvoprotein. *Biochemistry 42*, 14814–14821.
(34) Murgida, D. H., Schleicher, E., Bacher, A., Richter, G., and Hildebrandt, P. (2001) Resonance raman spectroscopic study of the neutral flavin radical complex of DNA photolyase from *Escherichia coli*. *J. Raman Spectrosc. 32*, 551–556.
(35) Lively, C. R., and McFarland, J. T. (1990) Assignment and the effect of hydrogen bonding on the vibrational normal modes of flavins and flavoproteins. *J. Phys. Chem. 94*, 3980–3994.