Electron Fate and Mutational Robustness in the Mechanism of (6-4)Photolyase-Mediated DNA Repair

Hisham M. Dokainish, ‡ Daichi Yamada, ‡ Tatsuya Iwata, § Hideki Kandori, ‡,§ and Akio Kitao ‡,‡

‡ Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
§ Department of Frontier Materials, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan

Research indicates that the formation of (6-4)PP lesions is strongly associated with the development of skin cancer. Therefore, it is important to understand the mechanism of (6-4)PP repair. Here, we describe the mechanism of the photorepair cycle, the roles of the active site His365 and His369 residues, and the robustness against mutation of these residues. Using molecular dynamics simulations, quantum mechanical/molecular mechanical (QM/MM) calculations, large-QM/MM which includes 982 atoms in the QM layer, Marcus theory, and Fourier transform infrared (FTIR) spectroscopic measurements, we found that an electron is transferred to the 2'-base of (6-4)PP to form a radical; subsequently a proton transfer from His365 to (6-4)PP causes water formation, which induces further OH transfer upon water activation and ultimately DNA repair. A H365A mutant repairs (6-4)PP similarly, with His369 compensating for His365 through a 2'-base radical. Our findings also provide an explanation of the observed low quantum yield in the wild-type and mutant proteins.

KEYWORDS: enzyme catalysis, large-QM/MM, photolyase (PHR), DNA repair, Marcus theory, FTIR, DNA photolesion, pyrimidine(6-4)pyrimidine

INTRODUCTION

Harmful covalent modifications of pyrimidine in DNA are generally triggered by UV irradiation, creating either cyclobutane pyrimidine dimers (CPDs) or pyrimidine(6-4)pyrimidone photoproducts ((6-4)PPs). Both lesions are cytotoxic, and their constant formation hinders vital cellular processes, potentially leading to mutation and cell death. Historically, the mechanism was proposed via the formation of a four-membered-ring oxetane intermediate either before or after photoexcitation. However, this mechanism was later excluded on the basis of an X-ray structure of the (6-4)PHR/DNA complex as well as an unfeasible calculated barrier. In addition, such a long-lived intermediate has not been detected experimentally. Later, Maul et al., solely on the basis of their X-ray structure of the complex, proposed repair via proton transfer from the active site to O4', forming a water molecule. However, afterward, Li et al., on the basis of their ultrafast spectroscopy experiment, proposed a modified mechanism starting by His proton transfer to O4' without water formation and alternatively O4'H transfer of its proton to N3'. Furthermore, other mechanisms have been proposed on the basis of experimental and theoretical investigations. In summary, three primary repair pathways have been proposed (Figure 1b): (1) concerted OH transfer (oxetane-like transition state) via one-photon excitation, (2) formation of a four-membered-ring oxetane intermediate either before or after one- or two-electron transfer, and (3) repair mediated by...
Although other mechanisms have been proposed, including conical intersection of the electronic excited state, these repair processes are generally thought to occur in the ground state. Most of the proposed mechanisms also assume that the first step in the reaction is proton transfer from an active site histidine (His) to the pyrimidine N3′, on the basis of observations that the (6-4)PHR reaction rate in D2O (deuterated His364 in Arabidopsis thaliana, equivalence of His365 in this work) is reduced by 50%.

The (6-4)PHR active site encloses two vital His residues (His365 and His369 in Drosophila melanogaster). Mutation of one or both of these His residues reportedly leads to termination of the repair process. Recent elegant Fourier transform infrared (FTIR) spectroscopy studies, however, demonstrated that, despite a drastic reduction in the catalytic rate, mutation of either His to Ala does not abolish the repair process, indicating mutational robustness of the enzyme. In a recent study aimed at further elucidating the role of vital His residues, we found that the HIP365/HID369 (HIP, both Nδ and Nε are protonated; HID, Nδ is protonated) is the most probable combination in the active site. This result supports the proposed role of HIP365 as a proton donor.

![Figure 1. Proposed mechanism of (6-4)PP repair by (6-4)PHR: (a) overall photorepair and electron dynamic cycle in (6-4)PHR including the main intermediates; (b) schematic representations of pyrimidine(6-4)pyrimidone ((6-4)PP repair) pathways proposed previously. The (6-4)PP structure includes atom numbering. All of the mechanisms consider OH transfer to C4′ followed by C4′–C6 bond breakage: (1) concerted direct OH transfer pathways in which an oxetane-like transition state occurs after the protonation of N3′, in the presence of anionic radical substrate (one photon mechanism), (2) OH transfer occurring through the formation of a four-membered-ring oxetane in the mechanism either before or after forward electron transfer (FET) or after back electron transfer (BET) which is followed by another photon excitation (two-photon mechanism), and (3) OH transfer through the formation of a water molecule as the first step followed by OH transfer.](image-url)
4)PHR is unknown. Likewise, the role of His369 in both the wild type (6-4)PHR (WT) and mutant has yet to be determined. Furthermore, although the electronic state of the reactive complex (RC) has not been identified, most of experimental studies suggest that a 3′-base radical RC (3′RC) is formed in the active site, whereas the results of theoretical investigations suggest that a 5′-base radical RC (5′RC) is formed. Therefore, we focus primarily on determining whether RC is 3′RC or 5′RC, as this information would significantly affect the current understanding of the reaction mechanism. For this purpose, we employed a combined synergistic approach involving quantum chemical/molecular mechanical (QM/MM), QM/QM/MM, and exceptionally large QM/MM calculations, molecular dynamics (MD) simulations, FTIR spectroscopic analyses, and Marcus theory. On the basis of the results of these analyses, we propose a complete and reasonable mechanism of repair of (6-4)PP DNA lesions by both WT and H365A (6-4)PHR.

**METHODS**

**MD Simulation.** A representative structure from our previous MD study on the X-ray crystallographic structure of the *D. melanogaster* (6-4)PHR/DNA complex including a T(6-4)T lesion and FADH− was used as the starting structure for the QM/MM approach. Similarly, the same X-ray structure was used to run a 100 ns MD simulation of the H365A mutant enzyme. MD preparation steps, force field, photoproduct parameters, MD procedure, and the clustering algorithm for the mutant complex were similar to those in our previous study of the WT protein. In the mutant complex, His369 was

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**Figure 2.** 3′-base reactive complex radical (3′RC) versus 5′-base reactive complex radical (5′RC) formation. (a) Structural changes in the reactive complex (RC) upon electron transfer to the prereactive complex (PRC) and then to post-BET. Schematic representation of the structural changes in the (6-4)PHR active site before electron transfer in PRC and after the forward electron transfer (FET) to either the 3′- or 5′-base rings. In addition, structural changes after backward electron transfer (BET) are shown. (b) Potential energy surfaces of key electronic states in (6-4)PHR upon formation of 3′RC or 5′RC. Green, red, and blue surfaces represent the excited state FADH−*, ground state biradical 3′RC/5′RC, and ground state FADH− post-back electron transfer (post-BET), respectively. (c) Calculated energy values representing the reorganization energy for BET (λ1), energy difference between PRC and RC (ΔE1), and the energy difference between RC and post-BET (ΔE2), reaction barrier (ΔE*) in eV and the ratio of the catalytic rates of BET and FET (K_BET/K_FET) obtained using the QM/QM/MM approach.
considered to be protonated after mutating His365. The programs VMD, AMBER, and CPPTRAJ were used for trajectory visualization, running simulations, and analyses/clustering, respectively.25–27

**QM Calculation.** All QM calculations were performed using the Gaussian 09 (G09) suite of programs.28 QM/MM calculations, within the ONIOM formalism,29 were performed using the unrestricted hybrid-meta-GGA density functional method UM06-2X for the high layer and the AMBER parm96 force field for the MM layer.30,31 DFT optimization was performed using G09d01 default criteria. The choice of UM06-2X was based on our previous study of the His protonation states. In addition, this functional is suitable for the large-QM layer at the UM06-2X/6-31G(d) level of theory, the transition state 2 (TS2; see the Results and Discussion) barrier was found to be reduced from 28.1 to 27.5 kcal mol⁻¹. In contrast, the QM/QM/MM energies show an increase of TS2 barrier from 25.7 to 29.0 kcal mol⁻¹. All transition states were confirmed using frequency and intrinsic reaction coordinate calculations. IR calculations were performed on the model III QM layer upon truncating the MM layer at the UM06-2X/6-31G(d) level of theory using the first QM/MM optimization step.

**FET/BET Reaction Rate Calculation.** To examine two electron transfer steps, forward electron transfer (FET) and backward electron transfer (BET) in the repair photocycle, we employed Marcus theory.33 We considered three potential energy surfaces (PES) of the donor and acceptor to gain insights into the driving forces for ET (ΔE) and the ET reaction barriers (ΔE*). Subsequently we calculated the FET/BET reaction rates. To construct the required PESs for Marcus theory calculations, several energies were calculated, including energy values representing the reorganization energy for FET (Δλ₂), the reorganization energy for BET (Δλ₁), the energy difference between PRC and RC (ΔE₈), and the energy difference between the RC and post-BET (ΔE₆) structures, shown in Figure 2a. The previously described QM/QM/MM approach was used to determine the energies of PRC (triplet FADH⁺), biradical 3'-base and 5'-base reactive complex, and singlet 3'-base and 5'-base RC post-BET (structures shown in Figure 2a) and reorganization energies for FET and BET. Note that, for the FET reaction, we assumed ΔE₁= −Δλ₁, which indicates a barrierless step for forward electron transfer. Subsequently the denominator in eq 1 is equal to 1.

\[
K_{FET} = \frac{e^{(\frac{\lambda_2 + \Delta E_8}{4k_B T})}}{\frac{e^{(\frac{\lambda_1 + \Delta E_6}{4k_B T})}}{4k_B T}}
\]

Finally, the \(K_{FET}/K_{BET}\) catalytic rate ratios were calculated using eq 1.

**H365A (6-4)PHR Mutant/DNA Complex Calculation.** The H365A (6-4)PHR mutant/DNA complex was also investigated in a fashion similar to that for WT, in which relative energies were determined using the large-QM/MM approach after two cycles of QM/MM and QM/QM/MM optimization. The QM layer included (6-4)PP, His369, Lys246, Asn406, Tyr306, FADH⁺, and three water molecules (Figure S5 in the Supporting Information). The protonation state of His369 was decided to be both protonated (HIP) on the basis of the following analyses. (1) The main interatomic distances in the active site of the H365A mutant with HIP369 agreed with those in the X-ray structure of the H365N mutant (PDB: 3CVW). This analysis was conducted similarly to our previous study on the wild type.22 (2) PROPKA calculations of the wild-type and mutant (6-4)PHR showed an increase in His369 pKₐ by 0.78. (3) There are no other residues in the active that can act as proton donors.
FTIR Spectroscopy. The WT and His354Ala mutant of *Xenopus laevis* (6-4)PHR were expressed in *Escherichia coli* as fusion proteins with a His<sub>6</sub> tag at the N terminus. Double-stranded DNA including (6-4)PP lesion was synthesized as previously described. The damaged DNA substrate had the following sequences (TT represents the (6-4)PP lesion):

\[
\begin{align*}
5'\text{-CGCGAA}T\text{-GTGC}G\text{-CC}-3' \\
3'\text{-GC}G\text{CTTAACGC}G\text{G}-5'
\end{align*}
\]

Light-induced difference FTIR spectroscopy is a powerful, sensitive, and informative method for studying the structural and chemical characteristics of the activation and repair processes of (6-4)PHR. We used redissolved samples, and FTIR spectra were measured using an FTS-7000 (DIGILAB) spectrophotometer, as reported previously. The source of illumination was a high-power xenon lamp (MAX-303, ASAHI SPECTRA). Low-temperature spectra were measured using a cryostat (Optistat DN, Oxford, U.K.) and a temperature controller (ITC 4, Oxford, U.K.) with liquid nitrogen as the coolant. FTIR spectra were constructed from 128 interferograms with a spectral resolution of 2 cm<sup>-1</sup>. The observed shift is in agreement with that observed experimentally at low temperature (77 K) to 1712 cm<sup>-1</sup> (Figure 4). The spectra were calculated using only the QM layer of the optimized QM/MM model at the UM06-2X/6-31G(d) level of theory. A scaling factor of 0.94 was used to match experimental results. The C<sub>4</sub>···O<sub>4</sub> bond by 0.09 Å, from 1.21 to 1.30 Å. Consequently, O<sub>4</sub> becomes strongly hydrogen bonded to Lys246 at a distance of 1.86 Å. Even though O<sub>4</sub>···His365 hydrogen bond is maintained in 5′RC, formation of 3′RC replaces this bond with an N3<sub>′</sub>···His365 hydrogen bond. In 3′RC, the C<sub>4</sub>···N3<sub>′</sub> double bond becomes a single bond, C<sub>4</sub>···N3<sub>′</sub>, with a distance of 1.39 Å (Figure 2a). Both RCs were also optimized after back electron transfer (BET), and their IR spectra were calculated. The structure of 3′RC after BET was found to maintain a strong hydrogen bond, O<sub>4</sub>···Lys246, and the IR frequency of C<sub>4</sub>···N3<sub>′</sub> stretching was downshifted from 1738 to 1712 cm<sup>-1</sup> (Figure 3). In contrast, no significant change in the 3′RC structure was observed after BET.

To determine whether the 3′RC or 5′RC scenario is more plausible, we considered the experimental results demonstrating that (6-4)PHR exhibits very rapid BET (50 ps),<sup>9</sup> which leads to a very low quantum yield (~0.11) in comparison to its sister enzyme, CPD PHR (quantum yield ~1).<sup>20,24</sup> For this purpose, we calculated the ratio of the BET and forward electron transfer (FET) catalytic rates (K<sub>BET/K<sub>FET</sub></sub>) on the basis of Marcus theory,<sup>33</sup> using calculated energy differences ΔE<sub>BET</sub>, ΔE<sub>FET</sub>, λ<sub>1</sub>, and λ<sub>2</sub> (Figure 2b). FET to both RCs was found to occur as a barrierless step accompanied by a large driving force (Figure 2b,c), in agreement with previous experimental results that showed a drastic reduction of the lifetime of FADH<sup>−</sup> from 3 ns to 225 ps in the presence of substrate.<sup>9</sup> K<sub>BET/K<sub>FET</sub></sub> for 3′RC was 45%, which reflects a small driving force for BET (ΔE<sub>BET</sub> = −0.23 eV). Therefore, the formation of 3′RC is particularly favored over BET, but this contradicts the experimentally observed dominance of BET.<sup>9</sup> In addition, previous theoretical studies showed that N3<sub>′</sub> protonation occurs as a barrierless step.<sup>16,42</sup> Altogether, these results exclude the 3′RC scenario, because the repair process should be the dominant next step after FET combined with minor BET reactions.
**Figure 4.** Assignment of substrates in the (6-4)PHR reaction by FTIR spectroscopy at 77 K. (a–c) Light-induced difference FTIR spectra of (6-4)PHR without (gray line) and with (black line) unlabeled, $^{15}$N3-labeled (blue line), or $^{18}$O4-labeled (red line) (6-4)PP measured at 77 K. (d, e) Double difference FTIR spectra of unlabeled minus $^{15}$N3-labeled (blue line) and $^{18}$O4-labeled (red line) (6-4)PP, respectively. The black spectra show the baseline, which indicate the noise level of each spectrum (see Figure S6 in the Supporting Information for details). In (a)–(e), one division of the y axis corresponds to 0.0005 absorbance unit. (f) Interpretation of the observed $^{18}$O4-labeled isotope shifts in (c) and (e). In the light-induced difference spectrum (top panel), positive and negative signals originate from the post-FET/BET and PRC states, respectively, where black and red bands show the spectra of the unlabeled and $^{18}$O4-labeled (6-4)PP, respectively. In the $^{16}$O–$^{18}$O double difference spectrum (bottom panel), the signals of PRC are downshifted, whereas those for the post-BET state are upshifted. (g) Tentative assignment of the observed vibrational signals at 77 K. The N3' nitrogen and O4' oxygen are indicated in blue and red, respectively.

In contrast, the driving force for BET in S'RC is stronger, as $\Delta E_2$ was found to be $-1.69$ eV, which leads to a $K_{2,BET}/K_{1,FET}$ ratio of 99%. This result indicates that the formation of S'RC in (6-4)PHR causes significant BET and a subsequent low-quantum-yield reaction. Therefore, the S'RC scenario shows good agreement with previous experimental results.

In addition, the larger driving force in the S'RC BET in comparison with FET aligns with the experimental data, indicating that BET occurs over a longer time frame (225 ps) than BET (50 ps). Collectively, the theoretical analyses strongly support the S'RC scenario.

**Intermediates Identified by Low-Temperature FTIR Spectroscopy Supporting the S'RC Scenario.** The results of FTIR spectroscopy experiments also support the S'RC scenario. FTIR spectroscopy was used to assign structural changes in RCs after FET and BET at 77 K, in which the repair reaction is prevented by low temperature (Figure 4). The spectrum obtained in the absence of DNA was identical with the baseline spectrum (gray line in Figure 4a); in contrast, entirely different spectral features were obtained for a light-dependent reaction involving damaged DNA carrying a (6-4)PP lesion (black line). On the basis of analogies with the previous (6-4)PHR report, a negative peak at 1738 cm$^{-1}$ was identified as C4\text{=O} stretching vibrations of the (6-4)PP, and the corresponding positive band could be the 1718 cm$^{-1}$ peak. These results indicate that this oxygen forms a stronger hydrogen bond network (Figure 4g). It is worth noting that a band at 1535 cm$^{-1}$, which is a characteristic of FADH*, was not observed. Therefore, we concluded that the difference FTIR spectra detected the PRC and post-BET states.

Next, in order to identify the origin of the bands in the difference FTIR spectra, unlabeled (6-4)PHR with $^{15}$N3- and $^{18}$O4-labeled (6-4)PP were analyzed (Figure 4b,c). The double difference analysis successfully isolated the vibrations involving the O4' motion of (6-4)PP; however, no signal originating from N3' motion was observed (Figure 4d,e and Figure S6 in the Supporting Information). Figure 4f depicts our interpretation of the origin of the shifts. The FTIR analysis using $^{18}$O4-labeled (6-4)PP revealed that the vibration at 1714 cm$^{-1}$ originated from the PRC state and one vibration at 1698 cm$^{-1}$ originated from the state after BET. Generically, C=O stretches appear in the frequency region of 1780–1660 cm$^{-1}$ and show a downshift by 30 cm$^{-1}$ after $^{18}$O labeling; however, the observed bands actually shifted by only 5–10 cm$^{-1}$, suggesting that the bands were not C=O vibrations. Thus, it is reasonable to conclude that these bands originated from an O4'-H bending vibration (Figure 4g). These results suggest that the structural change takes place only in the S'-base during FET and BET, which is consistent with the computational results suggesting that the electron transfers from FADH to the S'-base rather than the 3'-base.

**Complete Mechanism of (6-4)PP Repair through Activated Water Formation.** The (6-4)PP repair mechanism was also investigated using QM/MM calculations. To better account for electrostatic interactions between the active site and highly charged surrounding residues, energies were obtained using a large-QM/MM approach in which the QM layer...
includes 982 atoms. O4′ of 5′RC (simply referred to as RC hereafter) is strongly hydrogen bonded to His365 (Figure 5). In fact, electron transfer to the 5′-base elongates the C5—O4′ bond distance by 0.03 Å, from 1.40 Å in PRC to 1.43 Å in RC. Accordingly, the negative charge (Mulliken charges) of O4′ is increased in RC by −0.05. Moreover, the distance between the His365 proton and N3′ and their orientations indicate that no interaction occurs between them, which excludes an N3′ protonation reaction. Collectively, these data suggest that a proton is transferred from His365 to O4′.

This proton transfer occurs against a free energy barrier of 8.9 kcal mol$^{-1}$ (Figure 6a). The same step was previously reported using a smaller QM layer to have a barrier of 16 kcal mol$^{-1}$. In transition state 1 (TS1), the C5—O4′ distance increases to 1.71 Å, which is followed by proton transfer to O4′. TS1 induces the formation of the first intermediate (I1), in which O4′ of a newly formed water (W) is situated in close proximity to C4′, allowing OH transfer in the following step (Figure 5 and 6). The structure of I1 also shows a hydrogen-bonding network that includes His365, His369 and two water molecules in the active site, which might play a role in maintaining W in a position suitable for the subsequent reaction. Notably I1 is more stable than RC, by −20.5 kcal mol$^{-1}$, and the barrier for the reverse reaction is 29.4 kcal mol$^{-1}$. This is in agreement with the previous study suggesting that proton transfer in the (6-4)PHR reduces/blocks BET.9
We next elucidated the subsequent step in the repair process that is the nucleophilic attack of W on C4' (Figures 5 and 6). Although the water formation mechanism was originally thought to occur, subsequent theoretical studies were unable to optimize a feasible mechanism for the formation of the O4'−C4' bond. Indeed, water is a poor nucleophile and its activation is essential for oxygen to act as a nucleophile; however, Faraji et al. modeled this step without activating the water molecule, leading to a very high barrier TS. On the basis of the structure of I1, we found that W can be activated through His365, as they are strongly hydrogen bonded. In TS2, the O4'−C4' distance is shortened to 1.68 Å and a proton is located approximately halfway between O4' and NHis365. This step occurs against a feasible barrier of 13.4 kcal mol$^{-1}$, which is the rate-limiting step of the entire reaction. Remarkably, in comparison with all previous studies of the (6-4)PHR repair mechanism, this is the lowest reported barrier.

The subsequent intermediate I2 not only shows the formation of a C4'−O4' bond but also accompanies proton transfer to N3' (Figures 5 and 6). Indeed, the formation of the C4'−O4' breaks the C4'−N3' double bond, forming a single bond, and subsequently, N3' has only two bonds, which would form an unstable structure. Sadeghian et al. reported that the formation of the O4'−C4' bond via water formation leads to an unstable structure, but they did not consider the protonation of N3' in their study. I2 is more stable than RC by −15.5 kcal mol$^{-1}$.

The final step in the repair is breakage of the C6−C4' bond (Figures 5 and 6). This step occurs via TS3, in which this bond is elongated to 2.13 Å. The energy barrier for this step is 10.9 kcal mol$^{-1}$. The structure of I3 shows that (6-4)PP is now repaired, forming two nonbonded pyrimidines. I3 is more stable than I2 and RC by −17.7 and −33.2 kcal mol$^{-1}$, respectively. However, the 3'-base pyrimidine ring is still a cationic radical. Therefore, the subsequent step involves BET to FADH*, producing FADH$^+$. To restore the 3'-base pyrimidine protonation state, the next step is proton transfer to the active site His (Figures 5 and 6b). The structure of I3$'$ shows that O4'−H is strongly hydrogen bonded to His369 through a water molecule. TS4 was found to involve two proton transfers from O4' to the water molecule and subsequently from the water to His369. This step occurs.

Figure 6. (6-4)PP repair mechanism through activated water formation. (a) Free energy surface of the (6-4)PHR repair mechanism via water formation: overall free energy surface obtained using the large-QM/MM approach in which 982 atoms were included in the QM layer at the UM06-2X/6-311G(d,p) level of theory. Significant structural changes to (6-4)PP are also shown for all intermediates. (b) Schematic representations of the (6-4)PHR repair mechanism through activated water formation. The proposed repair mechanism involves water formation as the first step of the reaction, followed by water activation and OH transfer to C4'. Finally, C4'−C6 bond breakage occurs, leading to the formation of nonbonded pyrimidine. Afterward, BET to FADH* occurs, which leads to restoration of the pyrimidine protonation state and full repair.
changes are also shown for all of the intermediates. Using the large-QM/MM approach, in which 927 atoms were included in the QM layer at the UM06-2X/6-311G(d,p) level of theory. Significant structural changes are also shown for all of the intermediates.

Figure 7. Structural changes and mechanism of the repair by H365A (6-4)PHR. (a) Superposition of representative structures of the wild-type and H365A (6-4)PHRs. The mutation of His365 to Ala induces a positional change in His369, which comes into close contact with O4′, allowing catalysis to occur. (b) Distances from His369 nitrogen (NE2) to (6-4)PP N3′ and O4′ atoms throughout a 100 ns MD simulation of the H365A mutant (6-4)PHR. (c) Free energy surface of H365A (6-4)PHR repair through water formation. The free energy surface was obtained using the large-QM/MM approach, in which 927 atoms were included in the QM layer at the UM06-2X/6-311G(dp) level of theory. Significant structural changes are also shown for all of the intermediates.

via a barrierless transition state, which induces the formation of the product complex (PC), indicating that the (6-4)PP lesion is fully repaired. Finally, the initial protonation state of the active site His can be easily restored after DNA release from the active site, in which His369 is now protonated and can transfer its proton to His365 mediated by water.

The current mechanism (Figure 6b) is in good agreement with that proposed by Maul et al. on the basis of their X-ray structure. The proposed mechanism also provides an explanation for the previous experimental observations demonstrating a low quantum yield of (6-4)PHR, the role of His369 in the catalytic mechanism (Figure 6a) being a dominant factor in the reaction.

Repair Mechanism Involving H365A Mutant. Understanding how the H365A mutant retains repair activity, as recently reported, is challenging, because His365 is essential for repair activity in WT. MD simulation of the H365A mutant/DNA complex shows that the position of His369 changes significantly, coming into close contact with O4′(6-4)PP (Figure 7a), in agreement with the X-ray structure of the H365N mutant (PDB: 3CVW). Furthermore, the distance between His369 and N3′ is too large for proton transfer (Figure 7b), and no water molecule reached a position suitable for mediating proton transfer from His369 to N3′ throughout the simulation. The only stabilized RC optimized from the MD structure was a 3′RC; no stable 5′RC was obtained. In contrast to WT, the structure of RC in the mutant was similar to that of PRC, except for some changes in the hydrogen bond network involving water (Figure S7 in the Supporting Information). Although significant positional changes were observed in WT before and after electron transfer, there were no changes in the mutant. The formation of 3′RC in the mutant complex was also supported by FTIR spectroscopic analysis, which showed no changes in the spectrum before and after electron transfer at 77 K, unlike the case with WT (Figure S8 in the Supporting Information).

In RC (Figure S7 in the Supporting Information), more water molecules are located in the active site and His369 becomes strongly hydrogen bonded to O4′, at a distance of 1.86 Å, which also suggests a water formation mechanism similar to that of WT. In addition, no direct/indirect interactions were observed between His369 and N3′, thus excluding the possibility that N3′ protonation is the first step in the reaction.

The formation of a water molecule (W) in the mutant complex is energetically feasible (against a barrier of 27.6 kcal mol\(^{-1}\)) through TS1 (Figure 7c). In TS1, the C5′−O4′ distance increases to 1.67 Å, and this increase is accompanied by a proton transfer from His369 to O4′. The resulting intermediate (I1) is slightly more stable than RC, with an energy difference of −8.2 kcal mol\(^{-1}\). Notably, the experimentally observed low repair activity of H369A (6-4)PHR\(^{21}\) can be explained by the TS1 energy difference between the mutant and WT; the relative energy of TS1 is 18.7 kcal mol\(^{-1}\) higher than that in WT. In fact, the obtained result indicates 134-fold reduction in the catalytic rate upon the mutation, which agrees with the 150-fold reduction observed experimentally.\(^{21}\) Furthermore, the mutant I1 relative to RC is 8.1 mol\(^{-1}\) less stable than the WT I1. Similar to the case for WT, the post-BET RC in the mutant is more stable than RC by −98.4 kcal mol\(^{-1}\) (−1 eV), which is indicative of a large driving force for BET. Notably, the formation of TS1 is the rate-limiting step of the mechanism.

In I1, the oxygen atom of W is situated at 3.06 Å from C4′. W is stabilized by a hydrogen-bonding network with three other water molecules: His369, Asn406, and Lys246 (Figure S7 in the Supporting Information). Subsequently, W is activated by His369 and then attacks C4′ through TS2, in which the C4′−O4′ hydrogen bond distance is shortened to 1.73 Å and a proton is almost transferred from W to His369. The free energy barrier for this step is also high in comparison with WT (27.9 kcal mol\(^{-1}\)). Although the formation of the C4′−O4′ bond and breaking of the C4′−C6 bond occur in two consecutive steps in WT, both reactions are concomitant in the mutant, and the
optimized intermediate from TS2 includes two detached pyrimidines. The energy of the PC in this case is close to that of RC, with a small energy difference of $-3.7$ kcal mol$^{-1}$. The PC includes an isomer of one of the pyrimidine rings, the protonation state of which is readily restored once it is released from the protein.

**CONCLUSION**

Our results show that (6-4)PHR repairs (6-4)PP lesions through the formation of a 5′RC, which is followed by water formation and the activation reaction (see the Supporting Information for a summary). The water formation/activation mechanism was also observed with the H365A mutant complex, with the exception that 3′RC rather than 5′RC is formed.

In this study, the roles of two His residues in the active site of (6-4)PHR were elucidated. In WT, His365 plays a major role in the repair mechanism. This residue is strongly hydrogen bonded to O4′ of 5′RC, transfers its proton to O4′, induces water formation, and activates this water in TS2. Conversely, the role of His369 is secondary. In the His369Ala mutant, His369 compensates for the role of His365. His369 is strongly hydrogen bonded to O4′ in RC and causes a proton transfer from His369 to O4′. His369 also activates this water, which attacks C4′ and causes the proton to be transferred back to His369. Therefore, the two His residues in the active site appear to contribute to the robust nature of the (6-4)PHR lesion repair process.

**ASSOCIATED CONTENT**

1. Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.7b00751.

Figure S1–S9 and Table S1 as described in the paper and supporting text (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**
*A.K.: tel and fax, +81-3-5841-2297; e-mail, kitao@i.u-tokyo.ac.jp.

**ORCID**
Hisham M. Dokainish: 0000-0002-4387-4790
Hideki Kandori: 0000-0002-4922-1344
Akio Kitao: 0000-0002-5221-0806

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