Photolesions

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Characterization of the Intermediate in and Identification of the Repair Mechanism of (6-4) Photolesions by Photolyases

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Abstract: *Quantum mechanics/molecular mechanics calculations are employed to assign previously recorded experimental spectroscopic signatures of the intermediates occurring during the photo-induced repair of (6-4) photolesions by photolyases to specific molecular structures. Based on this close comparison of experiment and theory it is demonstrated that the acting repair mechanism involves proton transfer from the protonated His365 to the N3′ nitrogen of the lesion, which proceeds simultaneously with intramolecular OH transfer along an oxetane-like transition state.*

UV radiation causes two of the most abundant mutagenic and cytotoxic DNA photolesions: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs).[1–4] Persistence of these lesions can interfere with essential processes such as transcription and DNA replication leading to mutation, nucleotide misincorporation and eventually to cell death. In fact, (6-4) photoproducts are supposed to be the major players in the formation of skin cancer.[5–9] To maintain genetic integrity, many organisms have developed an elegant mechanism to repair these lesions using photo-activated enzymes called DNA photolyases.[10–12] These are highly efficient enzymes utilizing themselves with UV/blue light to eliminate these UV-derived photoproducts. After DNA binding through the dinucleotide flipping mechanism,[13] the enzymatic repair occurs in three sequential steps:[5–16] 1) photo-induced electron transfer from the catalytic cofactor, reduced flavine adenine dinucleotide ($FADH^-$) to the lesion;[13–17] 2) electron-induced splitting of the lesion; and 3) back electron transfer to $FADH^\bullet$ (Figure 1). Experimental[1–35] and theoretical[36–47] studies have been performed intensively over the past years, to trace all steps of the repair. While the functional mechanism of CPD photolyases has been identified,[7,8,18,19,23,26,36,37] the details of the repair mechanism operating in (6-4) photolyases is still a matter of ongoing debate. The initial steps of the repair mechanism, that is, light absorption, energy transfer[13–15] and generation of the catalytic electron[16,17] are now well understood, however,

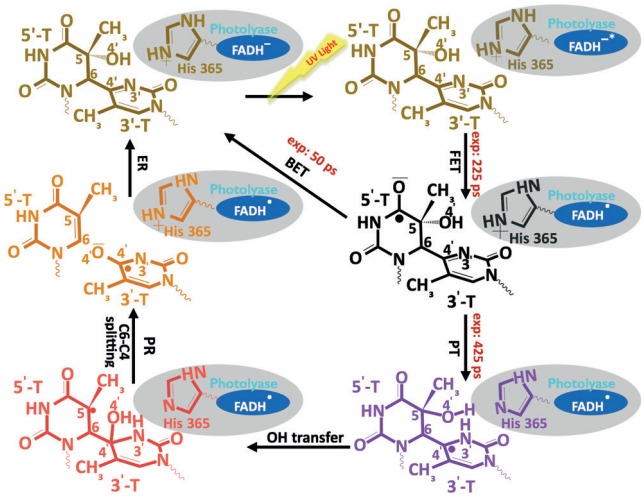

Figure 1. Suggested repair photocycle of (6-4) photoproduct by (6-4) photolyases. FET: forward electron transfer, BET: back electron transfer, PT: proton transfer, PR: proton return, ER: electron return.

the terminal repair of the 6-4PP after the transfer of the electron is not yet finally determined.[40,41,44,47] Unlike in CPD repair, a single-electron transfer to the 6-4PP is not sufficient[28] for their repair and it was thus suggested that additional factors are required to restore the original bases, which left the field open to hypotheses and speculations. A final proof of the mechanism is hence still missing.

The current status of experimental physicochemical and theoretical research on the repair mechanism of 6-4PP by (6-4)-photolyases has been reviewed by several authors[5,7,9] and it has become apparent that many aspects of the mechanism have been clarified. We refer to Ref. [9] and references therein for a more complete coverage of the vast amount of literature on the system. In summary, on the basis of most recent experimental findings[25–27] along with the geometrical arrangement of the 6-4PP in the resolved X-ray structure,[21,22,24] the majority of researchers seem to agree that the repair of the 6-4PP occurs not via formation of a stable oxetane ring *prior* to the electron transfer. Also, it is now widely accepted that the terminal repair occurs in the electronic ground state[25,42] of the generated photolesion radical anion. Therefore, we confine ourselves in the following to the steps involved *after* electron transfer, that is, the electron-induced repair mechanism, since this catalytic electron triggers the terminal repair.

Until today, various mechanisms of 6-4PP repair initiated by the catalytic electron have been proposed, out of which the following five are considered to be the most promising

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candidates: I) The oxetane mechanism,[29–33] which represents one of the earliest proposed mechanism in the literature. Here, 6-4PP is assumed to be converted to an oxetane ring, which requires the attack of the O4′ oxygen at the C4′ carbon involving intermediate **I** (Figure 2). II) The transient water mechanism,[24,43,46] in which the nearby His365 residue plays a crucial role, since proton transfer from this protonated His365 to O4′ is postulated to precede the cleavage of H_2O4' from the 5′ thymine involving intermediate **IIb** (Figure 2). III) The so-called proton transfer steered mechanism,[25,43–46] in

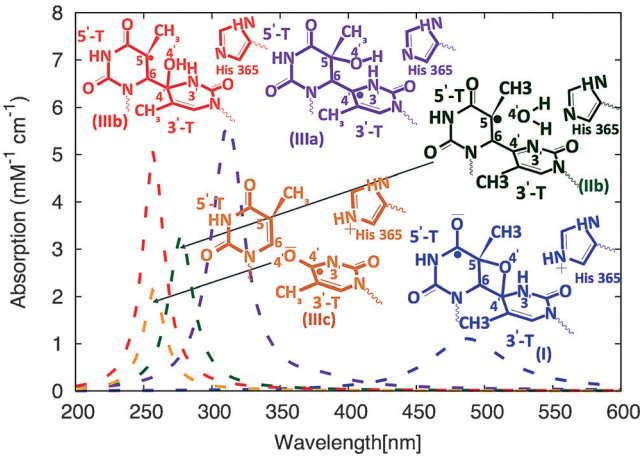

Figure 2. Molecular structure and theoretical absorption spectra of intermediates (**I**, blue; **IIb**, green; **IIIa**, purple, **IIIb**, red) involved in the repair mechanisms I–III as well as the repaired base pair (IIIc, yellow; see text).

which proton transfer from the protonated His365 to the N3′ nitrogen of the 3′ thymine occurs simultaneously with an oxetane-like transition state involving intermediates **IIIa** and **IIIb** (Figure 2). IV) Neutral histidine mechanism[47,48] in which OH transfer from 5′ thymine to 3′ thymine is assisted by a neutral His365 residue (see the Supporting Information (SI)). V) The two sequentially absorbed photons mechanism, in which upon absorption of the first photon, the proton is transferred from the protonated His365 to the N3′ nitrogen of the 3′ thymine leading to intermediate **Va** (identical to **IIIa**). Intermediate **Va** ultimately converts to a short-lived oxetane-like intermediate **Vb** that is stabilized by the enzyme, allowing repair to be completed by the second photon (see SI). The mechanisms (I)–(III), which are the main focus of the present work, all have in common that, in accord with the experiment discussed here, they require the absorption of one single photon only, that His365 is protonated, and that the repair proceeds in the electronic ground state of the generated radical anion. However, to draw reliable conclusions, we have also included the mechanism featuring the neural His365 (IV) as well as the proposed two-photon mechanism (V) within our study.

To identify the involved reaction intermediates and thereby to test the different proposed repair mechanisms, ultrafast transient absorption spectroscopy had been performed and presented in Ref. [25]. A proton transfer between a histidine residue in the active site and 6-4PP has been identified, which is induced by the initial photo-induced electron transfer from $FADH^-$ to 6-4PP, occurs in 425 ps and leads to 6-4PP repair in tens of ns (see Figure 1). To further corroborate the initial proton transfer during the repair reaction, the experiment was also carried out in D_2O instead of H_2O. The repair through D^+ transfer slows down by an isotope effect of 2.6 ($\kappa_H/\kappa_D = 2.6$) in accordance with the mass difference between hydrogen and deuterium. Such a ratio indicates also that there are no significant quantum-tunneling effects for proton transfer in photolyases. To check the pH dependence of the repair mechanism, the steady-state enzyme activity has been studied over a pH range from 7 to 9 and no changes were observed.[25] It has been shown that His365 remains protonated over even a wide basic pH range. All these results are consistent with proton transfer from His365 to $6\text{-}4PP^{\bullet-}$, as one of the key steps in the repair photocycle.

The repair process has been further examined by detection of the 6-4PP repair intermediates and the recovery of $FADH^-$ in the UV region. Knowing the absorption spectra of 6-4PP and of flavins in various forms ($FADH^-$, $FADH^-$*, and $FADH^{\bullet}$) as well as their related dynamics, obtained by visible light probing and shown in Ref. [25], we measured the transient dynamics of the 6-4PP intermediate, formed in about 425 ps with protonated, and in about 1100 ps with deuterated His365. We determined its absorption spectrum with a peak around 325 nm (see Figure 3). Thus, in this system, we are able to detect the repair reaction intermediates of both the cofactor ($FADH^{\bullet}$) and the substrate ($6\text{-}4PPH^{\bullet}$). After protonation, the observed protonated intermediate radical decays in >10 ns, corresponding to a series of atom rearrangements including bond breaking and formation, to complete the 6-4PP repair on a timescale of at least tens of ns. In the SI an illustration of how the thymine restoration was detected by the increase in absorption at 260 nm and by the decrease in absorption of the 6-4PP intermediate at 325 nm is provided. Although the experimentally observed mechanism seems to be, at first glance, simple, its molecular details however are not at all clear due to the successive structurally and chemically challenging reactions.

A primary question of interest here is the molecular structure of the protonated 6-4PP intermediate observed at around 325 nm. Being able to relate this signal to one of the possible intermediates occurring in the suggested repair mechanisms (I–V) discussed above would immediately allow us to identify the acting molecular mechanism. For that objective, we have extended previous theoretical analyses towards a more elaborate theoretical treatment, that is, the calculation of UV/Vis spectra of all possibly involved intermediates using a quantum mechanics/molecular mechanics (QM/MM) framework. For the details of the calculations please see the Supporting Information.

The primary mechanistic question to address is to which site the proton is transferred from the protonated His365, since this initial proton transfer distinguishes the intermediates occurring in the different mechanisms involving protonated His365, I–III and V. QM/MM calculations of the absorption spectra of the different intermediates and comparison with the experimental signatures help to identify the

molecular structure of the protonated intermediate, and ultimately to determine which the operating repair mechanism in (6-4) photolyases is. Therefore, electronic spectra of $FADH^-$, $FADH^\bullet$, 6-4PP, as well as intermediates involved in the five different proposed repair mechanisms (I–V) have been computed at the theoretical level of scaled-opposite-spin configuration interaction singles with perturbative doubles (SOS-CIS(D)[49]) (see SI for further information). The accuracy of this method has been shown to be about 0.2 eV for typical ππ* and nπ* excitations, which is here sufficient to assign the intermediates in the applied QM/MM framework. In fact, the excitation energies obtained for selected structures in gas phase at SOS-CIS(D) agree with the ones obtained at higher level of theory[16,42,45] such as ADC(2)[50] and CC2.[51] It must be noted that the electronic spectra reported here refer to vertical excitation to the corresponding lowest excited states (S_1). The only exception is $FADH^-$ for which the first three excited states are included in order to account for the unbound nature of the close lying excited states embedded into the continuum.[16]

As is illustrated in Figure 2, among all possible theoretically proposed intermediates of the (6-4)PP repair, **I–III**, only one falls into the range of the signal observed experimentally at 325 nm. This is the intermediate **IIIa** that corresponds to the direct protonation of N3′ (protonated 6-4PP) leading to an oxetane-like transition state and further to intermediate **IIIb**. Interestingly, the precursor of the oxetane-like intermediate, **Va**, suggested within the two-photon mechanism in Ref. [34] and Ref. [40], is indeed the protonated 6-4PP intermediate (**IIIa**) occurring in the mechanism III. In other words, our proton transfer steered mechanism (III) and the two-photon mechanism share an identical intermediate. This allows us to speculate that photolyases function with one photon under natural low-light conditions, however, they can utilize also two photons, if available, to facilitate the repair. Clearly, if one photon provides just enough energy to perform repair, two photons provide twice as much energy. Although the intermediates involved in the mechanism IV, considering neutral His365, can not be directly compared with the present experimental spectra due to the fact that His365 stays protonated over a wide basic pH range here, we have calculated the spectra of all the intermediates involved in the neutral His365 mechanism, as it was proposed in Ref. [47]. None of the intermediates therein appear in the energy window in which the experimental 6-4PP intermediate is detected. To avoid an extensive number of drawings in Figure 2, we confine ourselves to presenting only the 6-4PP related intermediates in which the His365 is protonated and refer to the SI for more data.

In Figure 3 we show the simulated absorption spectra of all species involved in the (6-4)-photolesion repair cycle according to the identified mechanism (III), and compare them with the experimental recordings (see SI for more data). The calculated line spectrum is convoluted with Lorentzians of suitable full width at half maximum (FWHM) of the corresponding experimental spectrum, to account for instrumental resolution and other broadening effects. The comparison between theory and experiment shows a very satisfactory

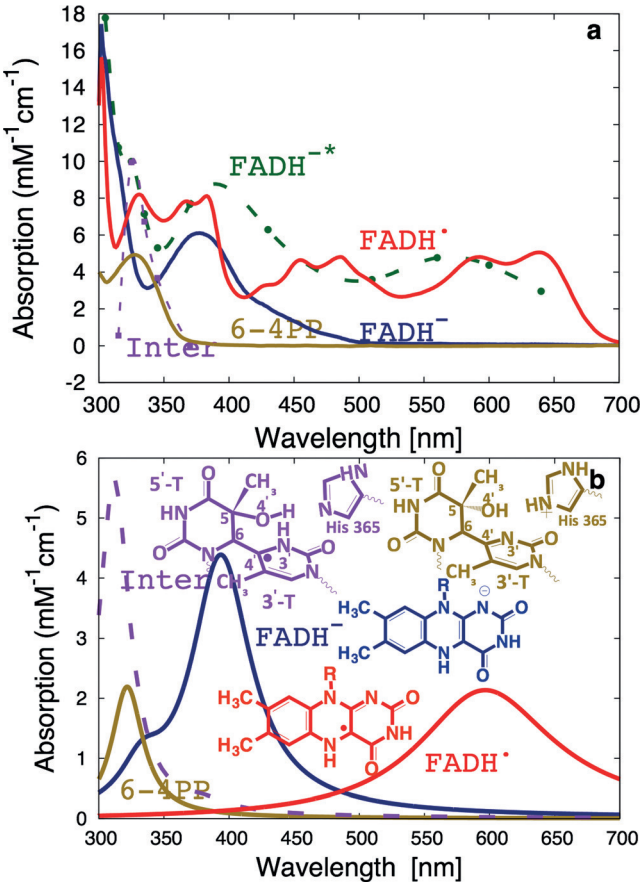

Figure 3. a) Experimental and b) theoretical absorption spectra and coefficients of purified protein with $FADH^\bullet$ (red), converted active form $FADH^-$ (dark blue), damaged 6-4PP (yellow) and 6-4PP related reaction intermediate **IIIa** (Inter., purple).

qualitative, partly quantitative, agreement. The finding that the experimentally observed intermediate corresponds to the intermediate IIIa of the proposed mechanism (III), that is, proton-transfer to N3′ and formation of the so-called protonated 6-4PP (Figure 2), is in agreement with previous studies of the energetics of these three different repair mechanisms. For it has been shown[40,43,46] that mechanism III requires the lowest activation energy. Hence it is clear that the (6-4) photolesion repair proceeds via the electron-transfer coupled proton transfer from protonated His365 to N3′ of the lesion making the concerted transfer of the OH group from C5 to C4′ via an oxetane-like transition state feasible.

In order to simulate the influence of the protein as a polar environment versus nonpolar environment, the point charge field of the protein is screened to simulate situations between weakly to strongly polar media. Over this large range of variation, the general trend observed for the calculated excitation energies and their relation to the respective structures remains the same for different levels of electronic structure employed (see SI).

This is the first time that the experimental spectroscopic signature of the detected 6-4PP intermediate[52,53] is assigned theoretically to a specific molecular structure determining the operating molecular mechanism of the electron-induced

restoration of (6-4) photolesions. Thereby, all pieces of the electron-induced (6-4) photolesion repair puzzle are finally put together. In the future, we aim at providing even more details on the molecular structure of the 6-4PP related intermediates by means of time-resolved infrared spectroscopy to further corroborate our structural assignment of the experimentally observed intermediate.

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