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Invited Review

Repair of (6-4) Lesions in DNA by (6-4) Photolyase: 20 Years of Quest for the Photoreaction Mechanism[†]

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ABSTRACT

Exposure of DNA to ultraviolet (UV) light from the Sun or from other sources causes the formation of harmful and carcinogenic crosslinks between adjacent pyrimidine nucleobases, namely cyclobutane pyrimidine dimers and pyrimidine(6–4)pyrimidone photoproducts. Nature has developed unique flavoenzymes, called DNA photolyases, that utilize blue light, that is photons of lower energy than those of the damaging light, to repair these lesions. In this review, we focus on the chemically challenging repair of the (6–4) photoproducts by (6–4) photolyase and describe the major events along the quest for the reaction mechanisms, over the 20 years since the discovery of (6–4) photolyase.

INTRODUCTION

DNA absorbs photons of high energy (main absorption band centered at ~265 nm, *i.e.* in the UV-C range, with tail extending beyond 290 nm, *i.e.* in the UV-B). Photoexcited nucleobases undergo various chemical reactions, leading to modifications and to loss or alteration of the genetic information (1). A prominent example is photocycloaddition between two adjacent pyrimidines (see Fig. 1 for the case of thymine–thymine (TT) sites and Fig. 10 for thymine–cytosine (TC) sites), which gives rise to the formation of cyclobutane pyrimidine dimers (CPDs; specific lesions are noted 5' base$\searrow\swarrow$3' base) and pyrimidine(6–4)pyrimidone photoproducts, also called (6–4) photoproducts ((6–4) PPs; specific lesions noted 5' base(6–4)3' base) (2). It is noteworthy that (6–4) PPs are formed via an oxetane or an azetidine intermediate (Figs 1 and 10). The most frequent lesions induced by UV-C and UV-B light are T$\searrow\swarrow$T, T$\searrow\swarrow$C and T(6–4)C; C$\searrow\swarrow$T, C$\searrow\swarrow$C and T(6–4)T are less frequent; C(6–4)C and C(6–4)T are very rare (3,4). (6–4) PPs can be isomerized into their Dewar valence

isomers (Dewar PPs; specific lesions noted 5' base(Dew)3' base; see Figs 1 and 10) upon exposure to light in the 300–360 nm range, where the absorption band of (6–4) PPs is located (Fig. 1) (5). Significant amounts of Dewar PPs were observed upon irradiation of cells with simulated solar light (6).

These UV lesions inhibit DNA replication and transcription, thus leading to cell death or mutagenesis and carcinogenesis, if they are not repaired correctly (7). To maintain their genetic integrity, organisms have developed various DNA repair systems. Among the repair pathways, nucleotide excision repair (NER) is the most widespread (from bacteria to humans) and versatile machinery for repair of UV lesions and a number of other bulky lesions in DNA, and mutation of genes responsible for NER in humans causes an inherent disease, xeroderma pigmentosum (8). During NER, several proteins are associated to excise a short fragment of the DNA strand containing the UV lesion, and gap-filling DNA synthesis by DNA polymerase followed by ligation can achieve restoration of the intact DNA (9). The same mechanism was only recently established for plants (10). NER consumes energy-rich factors such as ATP and nucleotide triphosphates (11,12).

In 1949, a phenomenon named photoreactivation was independently discovered by Kelner (13) and by Dulbecco (14). Bacteria inactivated by UV irradiation could be reactivated when exposed to visible light (13); similarly, bacteriophages that had been UV-inactivated could be reactivated when infected bacteria were exposed to blue light (14). Rupert (15) demonstrated that photoreactivation is caused by an enzyme. It was later named DNA photolyase (PL) and was found in organisms from all kingdoms of life, with the remarkable exception of placental mammals (16). Contrary to NER, repair of UV lesions by PLs uses sunlight as the energy source and only involves one protein for recognition and repair of the lesion. The originally discovered PL selectively recognizes and repairs CPD lesions, but not (6–4) PP lesions (17). A (6–4) PP-specific PL was found in 1993, in the fly *Drosophila melanogaster* (18), followed by the frog *Xenopus laevis* (19), the plant *Arabidopsis thaliana* (20,21) and other higher organisms (22–24). After cloning of the PL genes (for the first time by Sancar and Ruppert (25) for the CPD-

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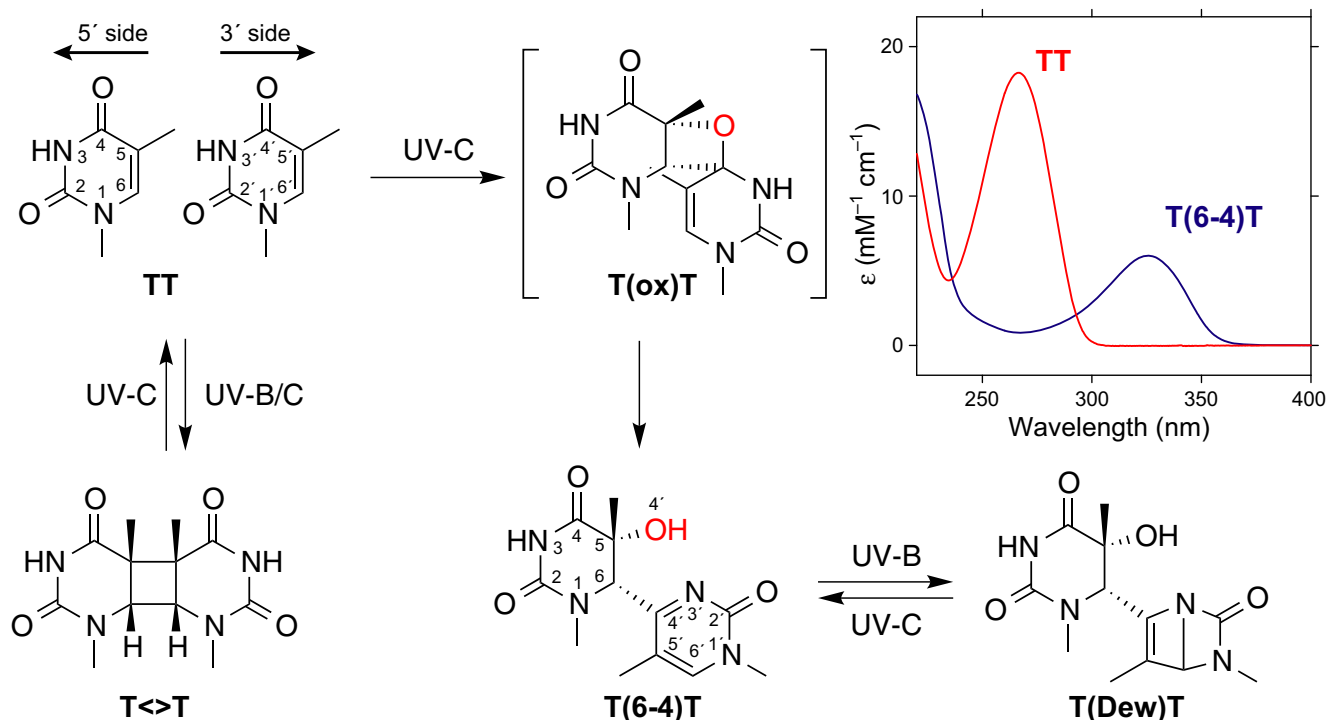


Figure 1. Formation of UV-induced DNA damages at a TT site (sketched in the upper left corner). The left column shows the formation of a T<>T lesion. The middle column illustrates the step-wise formation of a T(6-4)T lesion (bottom) through a T(ox)T oxetane intermediate (up). The photoconversion of the T(6-4)T lesion into its T(Dew)T Dewar isomer is represented in the lower right corner. The absorption spectra of the intact TT pair (red line) and of the T(6-4)T lesion (blue line) are given in the upper right corner.

specific PL from *Escherichia coli*), investigations using the purified enzymes have been performed, and their biochemical features, structures and reaction mechanisms have been analyzed.

CPD-specific PLs (CPD PLs) and (6-4) PP-specific PLs ((6-4) PLs) are rather homologous water-soluble globular proteins of ~55 kDa that harbor a non-covalently bound flavin adenine dinucleotide (FAD) chromophore. FAD in PLs can be found in three redox states, namely fully oxidized (FAD_{ox}), semi-reduced (FADH[•]) and fully reduced (FADH⁻) forms. Among them, the fully reduced FADH⁻, which absorbs 350–500 nm light, is the repair-active form. FAD_{ox} and FADH[•] can be converted to FADH⁻ by a separate photoreaction called photoactivation, which typically involves an intraprotein electron transfer (ET) through a chain of three tryptophan residues (26), exceptionally four in animal (6-4) PLs (27). A second chromophore (mostly a pterin or a deazaflavin) is present in many PLs and serves as a light-harvesting antenna that transfers excitation energy to the catalytic FAD cofactor, thereby increasing the effective absorption cross section of the enzyme. PLs are phylogenetically closely related to cryptochromes, flavoproteins that are involved in various blue light-regulated functions, but typically do not repair DNA (28). For detailed information and references on the protein organization of photolyases and cryptochromes, we refer to a comprehensive review by Sancar (29).

The mechanism of CPD repair by CPD PL is rather well established (see next section). In brief, the two intradimer C-C bonds split almost spontaneously upon ET from the photoexcited FADH⁻ to the CPD, leading to restoration of two intact pyrimidines. Repair of (6-4) PPs is chemically more challenging because cleavage of the C-C bond between the 5' and 3' components alone would yield two damaged nucleobases. In addition,

(6-4) PL has to accomplish the back transfer of a functional group (O4'H in the case of T(6-4)T (see Fig. 1)) from the 5' to the 3' base.

In the present review, we focus on major progress over the past 20 years in understanding the reaction mechanism of repair of (6-4) lesions by (6-4) photolyase and present our views (including unpublished results) on the ongoing controversies. A more detailed coverage of studies before 2003 can be found in Sancar's review (29). The reader may also consult Refs (2,30–32) for additional reviews and viewpoints including more recent works.

BACKGROUND: REPAIR OF CPD-TYPE LESIONS BY CPD PHOTOLYASE

We start with a brief summary (see also Fig. 2) of the well-established repair mechanism of CPD lesions by CPD photolyase, because the strong homology between (6-4) and CPD photolyases (see Introduction) suggests a similarity in the photorepair mechanisms for (6-4) and CPD lesions.

CPD PLs bind CPD-containing DNA substrates in a specific manner, with dissociation constant (K_d) of the order of 10^{-9} M and an association rate constant (k_{on}) of the order of 10^7 M⁻¹s⁻¹ (29). To ensure that the CPD comes close enough to the FAD cofactor for efficient ET, CPD PL flips the CPD lesion out of the DNA duplex (33,34) and accommodates it in the active site where FAD is buried (35). Upon photoexcitation of the fully reduced FADH⁻, an electron is transferred to the CPD in 30–250 ps, forming an anion radical of the CPD and the semi-reduced FADH[•] radical (36–39). The cyclobutane ring then splits almost spontaneously and rearranges into one intact base and the

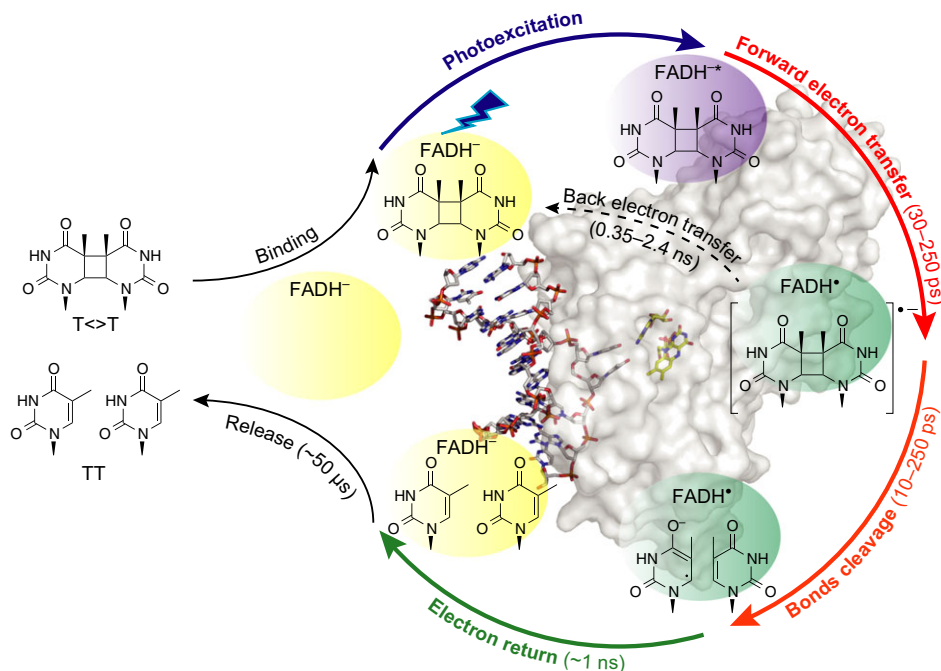


Figure 2. Sketch of the reaction cycle for photorepair of CPD lesions by CPD PL. The indicated ranges of time constant cover results obtained by different groups (see text). Inside the repair cycle, a structural overview is shown of CPD PL from *Anacystis nidulans* (surface representation in gray; the FAD cofactor is highlighted in sticks representation) bound to a double-stranded oligomer (in sticks representation on the left side) that initially contained a CPD lesion, which is flipped out of the double helix into the enzyme's binding pocket. In the X-ray crystal structure (1tez.pdb) (35), the two intradimer bonds are broken, presumably due to the synchrotron irradiation (35).

anion radical of the other base (38,39). The excess electron of the latter is finally returned to FADH• in ~ 1 ns (37–39), leading to the restoration of the second intact base and the catalytically active FADH⁻. The repaired DNA is released in ~ 50 μ s (40), and the enzyme is ready to bind and repair another CPD. CPD repair by photolyase is a single-photon process, and its reaction quantum yield is as high as 40–100% (29,41,42). Losses were attributed to (i) decay of the excited state FADH^{-*} to the ground state (intrinsic time constant ~ 1.3 ns (37,38)) in competition with ET to the CPD and to (ii) back ET from the reduced lesion to FADH• in competition with cleavage of the two intradimer bonds. The importance of loss channel (ii) (dashed arrow in Fig. 2) is still controversial (43,44), the back ET time constant having been estimated to 350 ps (38) or 2.4 ns (39).

REPAIR OF (6–4) PHOTOPRODUCTS

After their discovery, the binding and catalytic properties of (6–4) PLs were analyzed. In analogy with CPD PL, it was found that the repair-active redox state of the FAD cofactor was fully reduced FADH⁻ and that (6–4) PP-containing substrates were bound in a specific manner, with K_d of the order of 10^{-9} M (41,45). Repaired DNA was determined to be released in ~ 50 μ s (46), similar to the CPD case. However, the quantum yield (or efficiency) of photorepair of (6–4) PPs was found to be ~ 4 times (41) or even ~ 100 times (47) lower than for CPD repair. These findings suggested that the repair reaction of (6–4) PLs could share part of the reaction steps of CPD PLs, but that substantial differences remain.

It is worth recalling that, in the formation of the (6–4) PPs, a Paternò–Büchi reaction first yields an oxetane-bridged (or

azetidine-bridged for cytosine at 3') intermediate (48). This structure is thermodynamically unstable and rearranges (~ 2 ms for T (ox)T in aqueous solution (49)), to form a (6–4) PP (see Figs 1 and 10a). During this reaction, the O4' (or N4'H) in the 3' component is transferred to the 5' component and has thus to be returned to 3' during the repair reaction. Researchers have been very active addressing the challenging question of how (6–4) PLs achieve such return of the functional group.

A first working hypothesis: photoenzymatic splitting of a thermally formed oxetane intermediate

Based on the homology between photolyases, it was initially proposed that (6–4) PLs first catalyze the formation of a four-membered ring, similar to the cyclobutane for CPDs, in the dark. This intermediate was proposed to be the very oxetane- (or azetidine-)bridged compound found in the formation of the lesion. Photorepair would then follow just as for CPD PLs, by ET from the excited FADH⁻ to the transformed lesion (47). The idea of photoenzymatic splitting of a preformed oxetane intermediate was supported by studies of model compounds bearing an isoalloxazine and an oxetane attached to thymine derivatives. Illumination of such model compounds under reductive conditions resulted indeed in splitting of the oxetane ring (50–52).

In 2001, two histidines exclusively conserved among (6–4) PLs were identified (H354 and H358 in *Xenopus laevis* (6–4) PL (X164); generically noted His₁ and His₂ in this review), and mutation of either of these residues strongly impaired the photorepair activity (53). Structural simulation indicated that these residues were located in the putative active site of the (6–4) PL

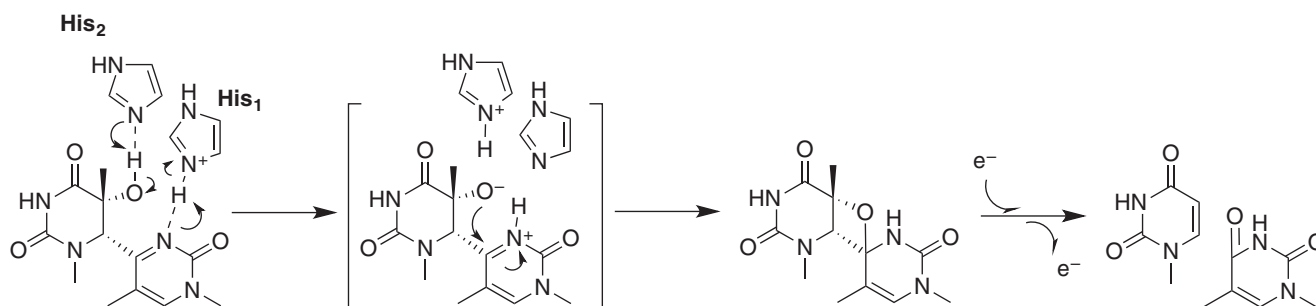


Figure 3. Early repair mechanism of (6-4)PP by (6-4) PL, proposed by Hitomi *et al.* (53). The reaction sequence first involves the formation of an oxetane intermediate in the dark, catalyzed by two conserved histidine residues (H354 and H358 in *Xenopus laevis* (6-4) PL). Photoinduced ET from the flavin to the oxetane then follows, with subsequent cleavage of the intradimer bonds and return of the excess electron to the flavin, as first suggested as one possible pathway by Kim *et al.* (47).

and were therefore considered to be responsible for an acid-base catalytic formation of the oxetane intermediate (Fig. 3). To function as an acid-base catalyst, the imidazole moiety of His₁ was supposed to be protonated and that of His₂ non-protonated (53). Such a protonation pattern was indeed supported for *XI64* at pH 9.5 by electron nuclear double-resonance measurements (54).

A crystal structure disproving thermal oxetane formation

In 2008, the crystal structure of *Drosophila melanogaster* (6-4) PL (*Dm64*) bound to a 15-mer DNA duplex containing a central T(6-4)T lesion was solved (55). The structure (see Fig. 4a) revealed that the lesion was flipped out of the double helix and accommodated in the active site of the enzyme, in a manner similar to the CPD case. Unexpectedly though, the lesion was seen to retain its original T(6-4)T structure; an oxetane was not formed in the dark, upon binding of the lesion to the enzyme. The conserved His₁ and His₂ residues (H365 and H369 in *Dm64*) were seen to form, together with a conserved tyrosine residue (Y423 in *Dm64*), a hydrogen bond network, proximal to the lesion. His₁ directly interacts with the hydroxy group (at the C5 position of the 5' component) that should be transferred during photorepair.

Remarkably, when the crystal of the enzyme-substrate complex was illuminated with white light in the presence of a reducing agent, the resulting structure showed that the (6-4) PP was perfectly repaired, with almost no change in the positions of the amino acid residues located in the close vicinity of the lesion.

Based on these observations, a first non-oxetane repair mechanism was proposed by the authors (55). Namely, upon formation of the anion radical of the (6-4) PP by ET from excited FADH⁻, the O4' located at the 5' component would be protonated by the initially protonated His₁, and a water molecule would transiently detach from C5 and subsequently attack C4' at the 3' component, thereby achieving the transfer of the O4'H functional group. Splitting of the C6-C4' bond would then follow, accompanied by back transfer of the excess electron (to FADH^{*}) and loss of a proton (Fig. 4b). Computational studies (see next section) did not retain this "transient water pathway" because of a too high activation barrier, but variants of this mechanism were devised and are still under consideration.

Computational search for a feasible reaction pathway

The structural data of Maul *et al.* (55) described above triggered a wealth of theoretical studies (30,56–67) aimed at finding a feasible repair mechanism for (6-4) PPs that does not imply the thermal formation of an oxetane intermediate prior to photoexcitation. A number of the explored pathways turned out to include steps with

unreasonably high activation barriers and were hence abandoned. This was in particular the case for the transient water pathway originally suggested by Maul *et al.* (55) (see Fig. 4b), for which Faraji *et al.* (62) calculated a barrier of nearly 50 kcal mol⁻¹ for the addition of the water molecule at the C4' position. In the following, we focus on those structure-based reaction mechanisms that were retained as feasible by their authors in their most recent publications. We tried to summarize them in Fig. 5 in a way that should facilitate comparisons. It should be noted that, as experimentalists, we avoided judging ourselves the appropriateness of the various computational methods used in the theoretical studies and focused on reporting main outcomes as presented by their authors.

Domratcheva and Schlichting (56) were the first to propose a variant of the transient water pathway based on a direct transfer of the hydroxyl group (O4'H), from the 5' to the 3' base. The mechanism was later refined by including the His₁ residue (H365 in *Dm64*) in the calculations (59) ("simple OH transfer" in Fig. 5, upper row). Forward electron transfer (FET) from photoexcited FADH⁻ to the (6-4) PP lesion would form the "5' base radical anion," in which the unpaired electron resides essentially on the 5' base (Fig. 5, intermediate II_a). The O4'H group would then move spontaneously (without barrier) from C5 to C4', followed by spontaneous cleavage of the C6-C4' bond and electron return to FADH^{*}.

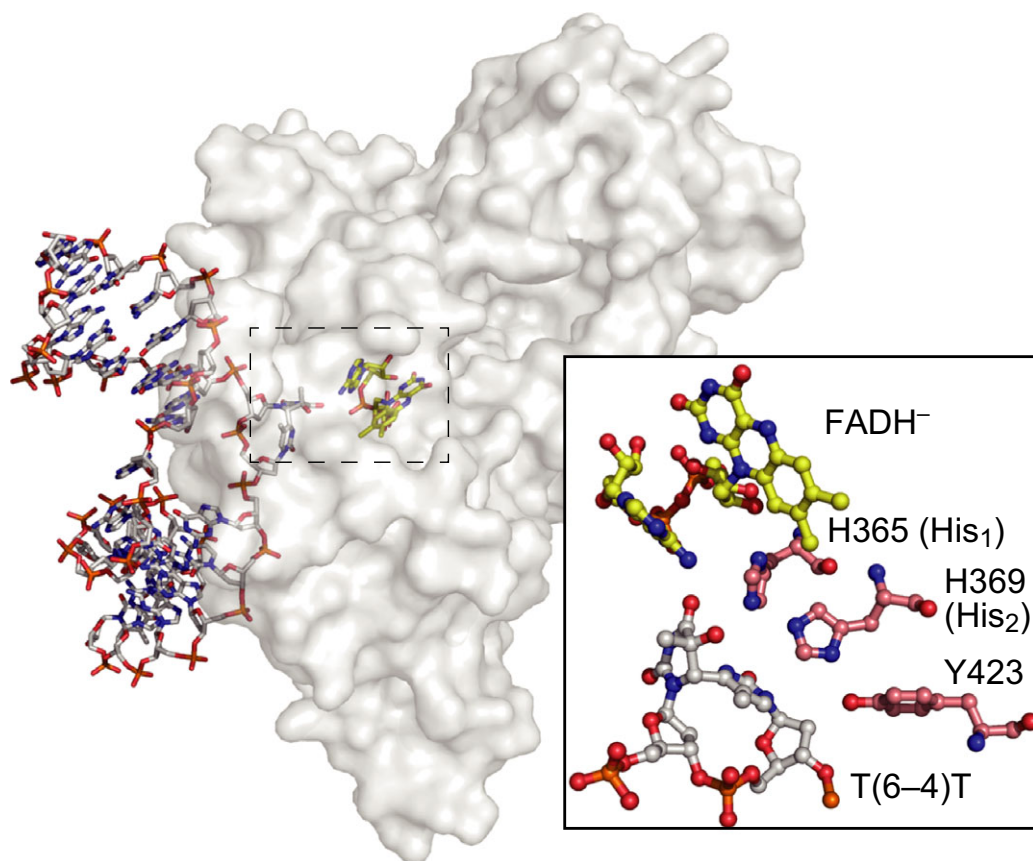
Remarkably, the 5' base radical anion was found to be an electronically excited state of the (6-4) PP radical anion, in the initial configuration (56). It is the high energy of this state that would allow transferring the OH group without barrier. It has been disputed whether the energy of the photon absorbed by FADH⁻ is sufficient to simultaneously transfer an electron to the lesion and excite electronically its radical anion (57,59).

It should be pointed out that, while several studies concluded that His₁ was protonated in the majority of the proteins (54,58,66,68,69), Domratcheva (59) retained that this residue should be found in its neutral (non-protonated) state^a. It is of note that Condic-Jurkic *et al.* (60) also concluded from their calculations that His₁ should be neutral at pH 7.

Several potential reaction pathways were explored and discussed by Faraji *et al.* (30,61–63,65,66). They finally retained a mechanism here termed "proton-transfer-steered OH transfer" (Fig. 5, middle row). In contrast to the "simple OH transfer" mechanism,

^aDomratcheva (59) calculated that if His₁ were initially protonated, the most stable state of the complex would be a biradical featuring the N3' protonated T(6-4)T neutral radical and FADH^{*}, coexisting with neutral His₁. This remarkable dark configuration would result from spontaneous (non-photoinduced) transfer of a proton (from His₁H⁺) and an electron (from FADH⁻) to the lesion. As the corresponding absorption spectrum was not experimentally detected in the dark, Domratcheva (59) anticipated that neutral His₁ was present in the active site.

a



b

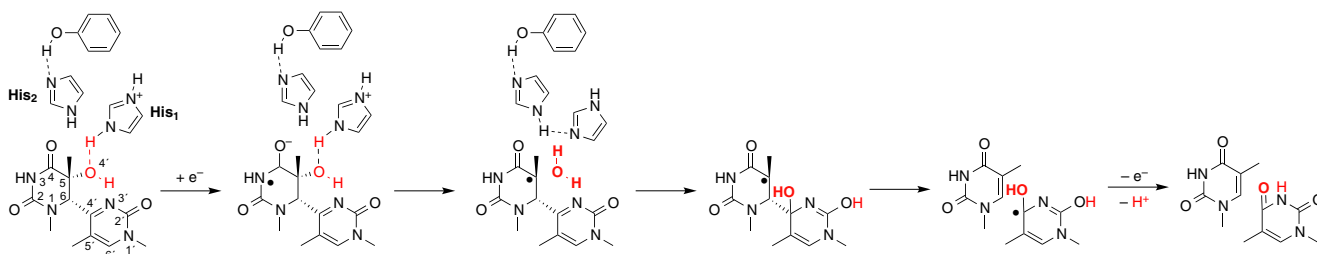


Figure 4. (a) Overall structure of a complex between the (6-4) PL of *Drosophila melanogaster* and a double-stranded DNA substrate containing a T(6-4) lesion, as determined by X-ray crystallography by Maul *et al.* (55). The inset shows in more detail the photocatalytic site, including the FADH⁻ cofactor, the T(6-4)T lesion and three conserved aromatic residues (H365, H369 and Y423). The lesion clearly retained its original T(6-4)T structure (with no sign of oxetane formation in the dark), but was successfully repaired upon irradiation of the crystal with white light (55). The presentation was prepared from 3cvu.pdb (55). (b) First non-oxetane repair mechanism of (6-4)PP by (6-4) PL, proposed by Maul *et al.* (55). This pathway involves the formation of a transient water molecule, indicated in red.

the histidine His₁ was assumed to be protonated in the dark (His₁H⁺)^b. Forward electron transfer from photoexcited FADH⁻ would yield the 5' base anion radical of the lesion (II_b, redrawn from their most recent publication (65)). Remarkably, the original calculations (61,62) indicated that the electron transferred from photoexcited FADH⁻ was first localized on His₁ (intermediate II_b[']), a possibility also considered by Moughal Shahi and Domratcheva (64). It was pointed out (61,62) that this contrasts with an experimental finding that the electron is initially transferred to the lesion (68). The discrepancy was suggested to be possibly due to

limitations in the calculation methods (61,62); it was also suggested that the electron on His₁ could be transferred immediately further to the lesion within small thermal fluctuations (66). The next step would be proton transfer from His₁H⁺ to the lesion anion radical, yielding III_b. The overall process of formation of III_b from photoexcited FADH⁻ was qualified as a proton-coupled electron transfer (61,62,66). Then, the O4'H group would be transferred from C5 to C4' (passing through an "oxetane-like" transition state (62,65) and yielding IV_b), followed by cleavage of the C6-C4' bond, return of the proton to His₁ and return of the excess electron to FADH[•]. The highest barrier in this pathway is ~20 kcal mol⁻¹ (for the OH transfer itself). It was argued that this value is likely to be overestimated due to limitations in the calculation methods and that the real value should be ~16 kcal mol⁻¹ or even lower (62).

^bDrew and Faraji (66) argued that proton transfer from His₁H⁺ to the lesion in the dark, as proposed by Domratcheva (59) if His₁ were initially protonated (see footnote a), would in fact be too slow to play any relevant role in the repair mechanism.

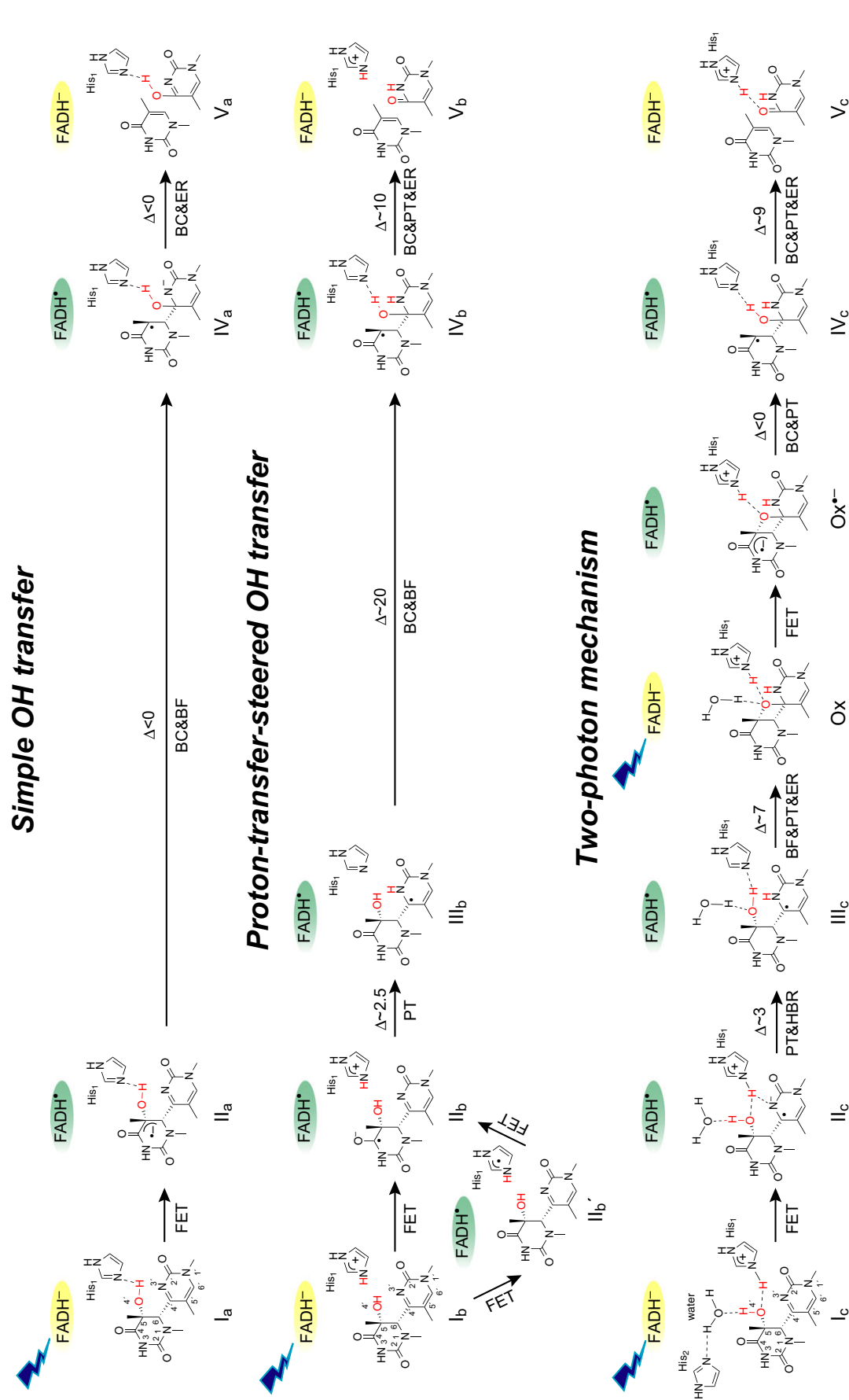


Figure 5. Three theoretically calculated reaction mechanisms for the repair of the T(6-4)T lesion by (6-4) PL, not involving the dark formation of an oxetane intermediate. The upper row (simple OH transfer) refers to the work of Domratcheva *et al.* (56,59); it involves the direct transfer of an OH group from the 3' base to the 5' base, assisted by the His₁ residue, supposed to be neutral (non-protonated). The middle row (proton-transfer-steered OH transfer) corresponds to the studies by Faraji *et al.* (30,61–63,65,66). OH transfer is here preceded by the protonation of the N3' atom of the 3' base by the protonated His₁. Two variants for the initial ET are sketched: either directly from the excited flavin to the lesion or through His₁. The lower row summarizes the two-photon mechanism proposed by Sadeghian *et al.* (58). The first photoexcitation yields the oxetane intermediate, which is repaired by the second photoexcitation. The layout of the figure is such that homologous intermediates (bearing same Latin numbering) may be found on a same column. This presentation in particular highlights that several reaction steps are shared nearly identically between the proton-transfer-steered OH transfer and the two-photon mechanisms (see text for details). FET = forward electron transfer; ER = electron return; PT = proton transfer; BC = bond cleavage; BF = bond formation; HBR = hydrogen bond reorganization; Δ = barrier in kcal mol⁻¹.

In contrast to the above proposals, Sadeghian *et al.* (58) claimed that (6-4) PPs could not be repaired by photolyase upon one single photoexcitation. Based on their calculations, they proposed a reaction mechanism that involves two successive photoexcitations of FADH⁻, each of them inducing ET to the lesion, bond rearrangements and electron return to FADH[•]. Their two-photon mechanism is summarized in Fig. 5, lower row. The first two reaction steps are very similar to the “proton-transfer-steered OH transfer” (except that the unpaired electron is mainly distributed in the 3' base for III_c (58) rather than in the 5' base as drawn for II_b in Ref. (65)). However, instead of a direct transfer of the O4'H group, O4' then forms a bond with C4' while keeping a bond with C5; this process occurs together with electron return to FADH[•] and proton return to His₁, yielding the oxetane-bridged dimer in its singlet ground state (Ox), the fully reduced flavin FADH⁻ and the protonated His₁H⁺. The calculated barrier for the overall III_c→Ox process is ~7 kcal mol⁻¹ (58).^c

Upon re-excitation of FADH⁻ by a second photon, an electron is injected into the oxetane intermediate, forming the oxetane radical anion Ox^{•-}. The C5-O4' bond splits spontaneously in concert with protonation of O4' by His₁H⁺, yielding intermediate IV_c where the O4'H group is completely transferred to the 3' base. The following steps are again very similar to the “proton-transfer-steered OH transfer” mechanism. The highest barrier in the second photoreaction would be ~9 kcal mol⁻¹ (for the cleavage of the C6-C4' bond), close to the ~10 kcal mol⁻¹ in the “proton-transfer-steered OH transfer.”

It is known that the T(ox)T oxetane converts to the T(6-4)T lesion in ~2 ms in aqueous solution (49). Such a short lifetime of the oxetane intermediate would make the sequential two-photon repair mechanism very inefficient under natural sunlight on Earth (estimated to excite FADH⁻ only about once every 3 s in average on a sunny day (72)). Sadeghian *et al.* (58), however, calculated that reversion to T(6-4)T of T(ox)T bound to (6-4) photolyase has an activation barrier as high as ~16 kcal mol^{-1d}. Taking this value as the activation free energy in transition state theory, the minimum lifetime (for a transmission coefficient of 1) of the enzyme-bound T(ox)T would be 0.14 s at 20 °C, that is much closer to the average time between two excitations of FADH⁻ under natural sunlight than the solution value. A much longer lifetime of T(ox)T in the binding pocket of (6-4) PL as compared to aqueous solution is consistent with

calculations showing a strong decrease in the activation barrier for the transition to T(6-4)T due to the presence of water molecules (73).

Sadeghian *et al.* (58) also considered other reverse reactions, which could potentially compete with the productive pathway and diminish the efficiency of photorepair. In the first photoreaction, two reversion channels leading to state I_c from precursors of state III_c^e were calculated to have low barriers (~2/3 kcal mol⁻¹). In the second photoreaction, the IV_c→Ox reversion was predicted to have a modest barrier (~7 kcal mol⁻¹). It should be stressed that these reversion reactions involve coupled proton and electron transfer and that theoretical prediction of the corresponding reaction rates is not straightforward (see footnote c). However, in view of the substantial barriers calculated for some of the potential forward reactions (see Fig. 5), we feel that it is desirable that potential reverse reactions are included as properly as possible in the computational quest for a feasible repair pathway.

The large number of controversial computational studies sketched above clearly called for experiments that could distinguish between the suggested mechanisms (or establish an alternative one).

Experimental investigation of primary processes

Remarkably, there is a strong predominance of computational over experimental studies of the (6-4) PP repair mechanism. This may be due to technical difficulties in monitoring a photoreaction that has a low quantum yield and is irreversible (so that improvement of the signal-to-noise ratio by signal averaging is particularly laborious). An additional difficulty is that proper test substrates must include several intact nucleobases (to ensure correct binding to the protein), which provide a strong background absorption around 265 nm, where restoration of intact nucleobases could be monitored directly.

Despite these difficulties, Li *et al.* (68) could monitor primary reactions related to the repair of a T(6-4)T lesion by the (6-4) PL of *Arabidopsis thaliana* (At64), by ultrafast fluorescence and transient absorption spectroscopy between 315 and 800 nm. Upon excitation of the fully reduced FADH⁻ cofactor, they observed electron transfer to the (6-4) PP in 225 ps, similar to the corresponding reaction for CPD repair by CPD photolyase. However, in contrast to the CPD case, ~90% of the FADH[•] radicals formed by this primary ET were re-reduced very quickly (in 50 ps) by back ET. The remaining ~10% of FADH[•] did not decay in the accessed time window (up to 3 ns), also in contrast to the CPD case where repair is completed by electron return to FADH[•] in ~1 ns (37–39). Transient absorption changes in the 315–370 nm range were attributed to a proton transfer from (protonated) His₁ (His364 in At64) to the reduced substrate, occurring in 425 ps. This assignment was supported by a significant kinetic isotope effect on this reaction (upon H₂O to D₂O exchange) and by a sensitivity to mutation of His₁ to asparagine. It was further shown that several point mutations of His₁ (H364N/M/Y/A/D/K) had no significant effect on the time constant of electron transfer from the excited FADH⁻ to the lesion, but completely suppressed long-lived (>1 ns) FADH[•]. DNA

^cFollowing discussions we had with theoreticians (F. Cailliez and A. de la Lande, personal communication), we would like to point out that using this barrier for an estimation of the reaction rate via transition state theory (TST) would be problematic. The methodology used in Ref. (58) is indeed based on the Born–Oppenheimer approximation. Yet, reaction III_c→Ox is likely to operate beyond this approximation, depending on the couplings between the electronic states that are involved. Evaluation of rate constants of bond breaking/formation (including proton transfer) in fact necessitates Marcus-like theories when they are coupled to ET (70,71). Only in the case of strong electronic coupling between charge transfer states can one reduce the rate expression to a TST-like equation. When the electronic coupling is weak, as is probably the case here in view of the spatial separation between the lesion and the flavin, the transmission coefficient should include an electron hopping probability, proportional to the square of the coupling. In this regime, estimating the kinetics of the reaction from the free-energy barrier solely is ill-advised as the prefactor equally matters and has the potential to severely diminish the reaction rate.

^dAccording to Ref. (58), this reversion would proceed in two steps: (i) concomitant reprotonation of O4' by His₁H⁺ and cleavage of the C4-O4' bond of T(ox)T (barrier ~16 kcal mol⁻¹) and (ii) back proton transfer from N3' of the lesion to His₁ (barrier ~2 kcal mol⁻¹). One may note that the product of step (i) is a closed-shell configuration where the protonated lesion coexists with FADH⁻. It is different from the biradical (open shell) ground state calculated by Domratcheva (59) if His₁ were initially protonated, where the reduced protonated lesion coexists with FADH[•] (see footnote a). Neither step (i) nor step (ii) is coupled to electron transfer, so that transition state theory might be applied to estimate the reaction rate (cf. footnote c).

^eIn these precursors (not shown in Fig. 5), N3' is already protonated, but the O4' proton is still hydrogen bonded to the water oxygen. These precursors could be reached from state III_c by overcoming a barrier of 8 kcal mol⁻¹ (58).

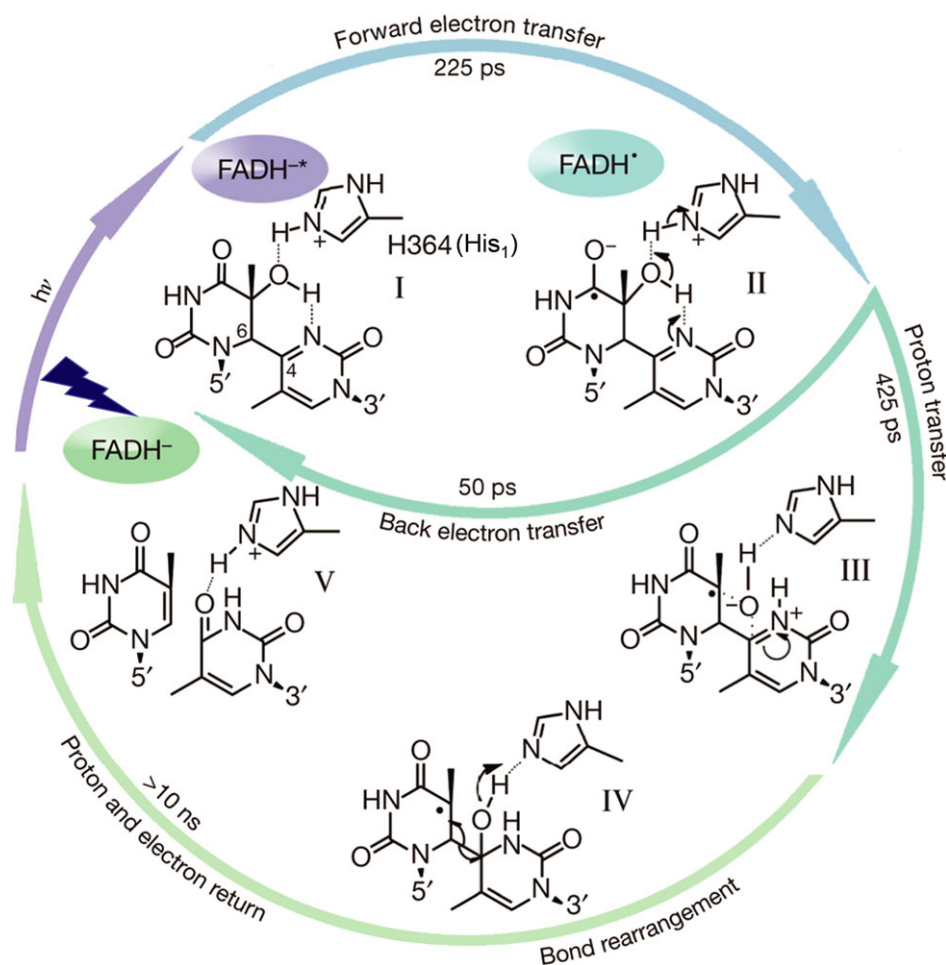


Figure 6. Reaction cycle for photorepair of a T(6-4)T lesion by the (6-4) PL of *Arabidopsis thaliana*, as proposed by Li *et al.* (68), on the basis of an experimental study of the reaction by ultrafast fluorescence and transient absorption spectroscopy. Adapted from Ref. (68) with permission from Macmillan Publishers Ltd: Nature, copyright 2010.

repair was found to be completely shut off as well in these mutant proteins, in line with a previous report for the corresponding H354A mutation in *XI64* (53).

Figure 6 shows the reaction scheme proposed by Li *et al.* (68). Importantly, the very unfavorable competition between productive protonation of the anion radical (structure II) (in 425 ps) and futile back ET (in 50 ps) would explain the relatively low quantum yield of (6-4) PP repair (as compared to CPD repair where the corresponding futile back ET is much slower; see above and Fig. 2).

Recent QM/MM calculations of the absorption spectra of different potential reaction intermediates assigned the spectral feature observed around 325 nm by Li *et al.* (68) to a (6-4) PP radical protonated at the N3' position by His₁ (65).

The essential role of His₁ in the repair reaction has recently been put into question by Yamada *et al.* (74). They concluded from an FTIR analysis of the repair function, that the His₁-to-alanine mutant of *XI64* (H354A) conserved its repair activity, although at much lower (not quantified) efficiency than the wild type. We have therefore tried to determine the absolute repair quantum yield of this very mutant.

Samples containing the photoreduced H354A mutant PL and an oligonucleotide carrying a central T(6-4)T were illuminated by strong continuous laser light (2 W cm^{-2}) at 408 nm for up to

65 min. Repair was quantified during the illumination period by monitoring the bleaching of the 325-nm absorption band of T(6-4)T (see Fig. 7a). The sample illuminated for 65 min was analyzed by reverse phase HPLC, confirming the restoration of intact thymine pairs within the substrate (Fig. 7b). The repair quantum yield was found to be 0.013%, that is ~500 times lower than that for the wild type (72). Yamada *et al.* (74) pointed out that “a decrease in volume by the H-to-A mutation is possibly compensated by the addition of water molecule(s).” We tentatively suggest that another protonated residue could protonate the (6-4) PP anion radical in the mutant protein, possibly via such water molecules. The associated proton transfer rate would, however, be much lower than with His₁ in the wild type and the efficiency of the proton transfer, in competition with back electron transfer to FADH*, much reduced.

Experimental evidence for a two-photon mechanism

As mentioned above, Sadeghian *et al.* (58) theoretically proposed a repair mechanism of (6-4) PP by (6-4) PL involving a sequence of two photoreactions. This possibility has never been experimentally tested until our recent work (72), which will be described below. It is in fact not so easy to distinguish between an ordinary one-photon mechanism and a sequential two-photon

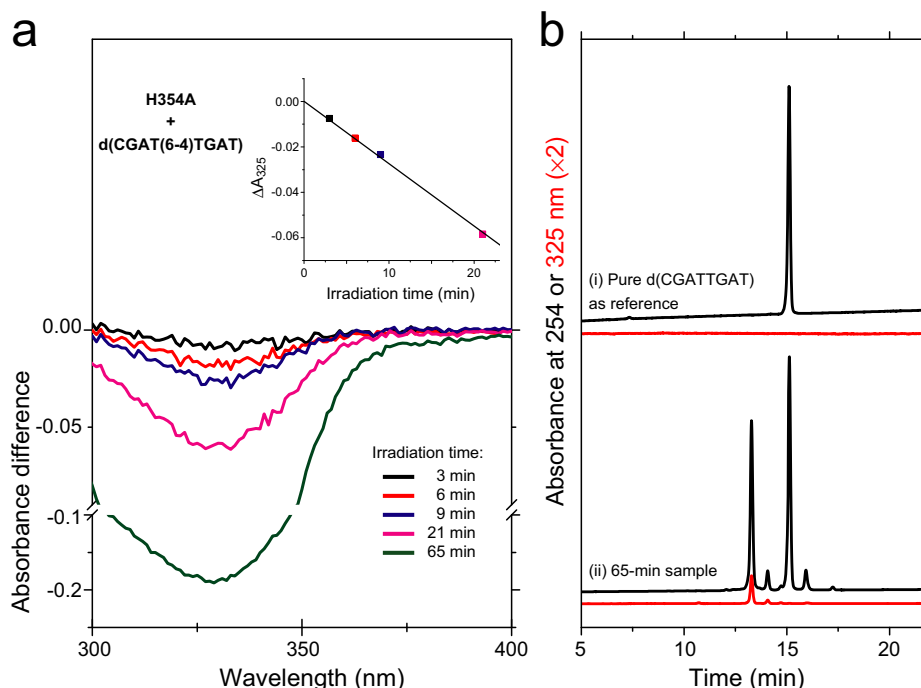
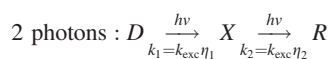
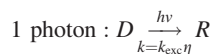


Figure 7. Photorepair of the (6-4)PP by *Xenopus laevis* (6-4) PL having His₁ (H354) mutated to alanine. (a) Samples of 100 μL containing 2.64 μM of the photoreduced H354A mutant PL and 40 μM of the 8-mer substrate, d(CGAT(6-4)TGAT), in a buffer composed of 10 mM phosphate (pH 7.0), 100 mM NaCl, 5% glycerol and 6 mM cysteine, were prepared anaerobically in a quartz cell with self-masking solid black walls and a sample chamber of $2 \times 2 \times 10$ mm (16.40-F/Q/GL14/Z15 from Starna) and kept at 10 $^{\circ}\text{C}$. The accessible volume of the samples (40 μL) was irradiated through the 2×2 mm window by continuous light of 2 W cm^{-2} at 408 nm from a laser diode (iBeam smart 405-S from Toptica). After given irradiation times, the samples were homogenized by shaking and absorption spectra were recorded in a spectrophotometer (Uvicon XS from Secoman). The light-minus-dark difference spectra are presented in panel a; the absorption changes at 325 nm for relatively short irradiations are plotted *versus* irradiation time in the inset. The repair quantum yield was calculated from the slope of a linear fit (solid line; forced to go through the origin), using a molar decadic absorption coefficient (ϵ) of $3600 \text{ M}^{-1} \text{ cm}^{-1}$ for FADH^- at 408 nm (72) and a differential absorption coefficient ($\Delta\epsilon$) of $-6000 \text{ M}^{-1} \text{ cm}^{-1}$ for the repair of T(6-4)T at 325 nm (72). (b) The sample irradiated for 65 min (ii) was analyzed by reversed-phase HPLC using a $\mu\text{Bondasphere C18}$ column (Waters), in a Gilson gradient-type analytical HPLC system equipped with a Waters 2996 photodiode-array detector. The flow rate was 1.0 mL min^{-1} , using a linear gradient of 5–13% acetonitrile in 0.1 M triethylammonium acetate (pH 7.0), generated over 20 min. The chromatogram of the pure 8-mer with intact TT bases (d(CGATTGAT)) is shown for reference (i). The black traces (254 nm) demonstrate the restoration of intact TT bases upon irradiation of H354A mutant PL mixed with the T(6-4)T-containing 8-mer. The peak in the red trace (325 nm) of sample (ii) marks the position of the (unrepaired) T(6-4)T-containing 8-mer.

mechanism. To illustrate this difficulty, let us consider a simple repair experiment under continuous illumination (with a constant excitation rate k_{exc}) and compare the following simplified models of both mechanisms:



Here, D represents the initial (6-4) PP lesion complexed to the (6-4) PL in the dark; X stands for the intermediate involved in the two-photon mechanism; R is the repaired lesion. The repair quantum yield for the one-photon mechanism is noted η , while the quantum yields of the consecutive reactions within the two-photon process are η_1 and η_2 . Let us additionally make the following basic assumptions: (1) The substrate binding and product release are much faster than the overall repair rate and are thus non-limiting, and (2) the substrate concentration is always in excess of the enzyme concentration and much higher than the dissociation constant of the complex. It is then straightforward to see that the repair ratio (*i.e.* lesions repaired per enzyme; noted x_r) increases linearly with irradiation time (t) in the one-photon mechanism; the slope is $k_{\text{exc}} \eta$. For the two-photon mechanism,

we derived an analytical expression of the repair ratio (see Ref. (72), Supporting Information, §4.2) that can be recast as follows within the present simplified framework:

$$x_r = \eta_1 \eta_2 \left[\frac{\exp(-(\eta_1 + \eta_2)k_{\text{exc}}t) - 1}{(\eta_1 + \eta_2)^2} + \frac{k_{\text{exc}}t}{\eta_1 + \eta_2} \right] \quad (1)$$

Equation 1 shows that the onset of photorepair is quadratic, as intuitively expected for a two-photon process, but that a linear regime rapidly follows (slope: $k_{\text{exc}} \eta_1 \eta_2 / (\eta_1 + \eta_2)$). This regime corresponds to a photostationary equilibrium, where the concentration of X remains constant. The steady state builds up exponentially with rate constant $k_{\text{exc}} (\eta_1 + \eta_2)$. Hence, unless both η_1 and η_2 are very small, the linear regime is reached when each enzyme has been excited just a few times. In any case, it is reached before one lesion has been repaired in average per enzyme. The quadratic onset may thus easily be missed in standard experiments. As an example, we compare in Fig. 8 the simulated dose-effect curves for a one-photon process with $\eta = 6\%$ (black line) and for the simplified two-photon process according to Eq. 1 with either $\eta_1 = 6.6\%$, $\eta_2 = 66\%$ (red line) or $\eta_1 = \eta_2 = 12\%$ (blue line), both yielding the same final slope as the one-photon process with $\eta = 6\%$.

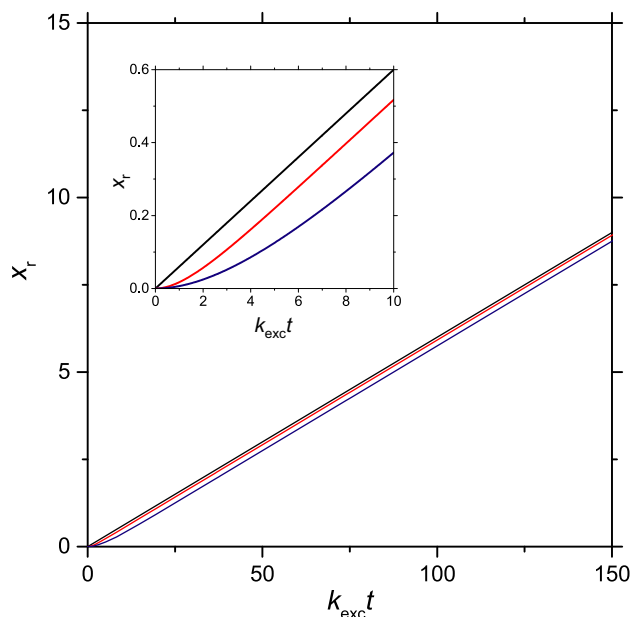


Figure 8. Simulated repair experiment under continuous illumination, illustrating the difficulty to distinguish between an ordinary one-photon mechanism and a sequential two-photon mechanism (see text for details). The buildup of the repair ratio (x_r) is plotted as a function of the irradiation dose ($k_{\text{exc}} t$). In black is represented the strictly linear repair curve corresponding to a one-photon process with quantum yield $\eta = 6\%$. The red and blue curves were calculated according to Eq. (1) for a simplified two-photon process with either $\eta_1 = 6.6\%$, $\eta_2 = 66\%$ (red) or $\eta_1 = \eta_2 = 12\%$ (blue). They both exhibit a brief quadratic onset, followed by a linear regime having the same final slope as the above one-photon process.

Revisiting previous experiments in the literature where repair curves were fitted linearly (41,47), we realized that in most cases the scattering of the data exceeded the deviation from linearity that could be expected for a two-photon process. Interestingly, in one of the experiments reported by Kim *et al.* (47) (Fig. 2B therein), the first data point is below the linear fit by more than the scattering of the other data, but this was not mentioned in the text and may hence not be considered as significant. Our own attempt to resolve the relevant onset of the repair curve under continuous illumination was unsuccessful because of an insufficient amplitude resolution (72).

To distinguish between a one- and a two-photon process, we devised a special experiment (72) where repair was triggered by non-saturating 100-ps laser flashes. The pulse duration was shorter than the reported lifetime of the excited flavin in the presence of substrate (225 ps (68)), which theoretically excludes double excitations of FADH^- within one flash. The repair reaction was monitored by recording absorption changes at 265 nm, where the recovery of the intact nucleobases is expected and, at 325 nm, where depletion of the T(6-4)T lesion should appear (see species spectra in Fig. 1). The observation at 265 nm was made possible by using a special substrate in which a single T(6-4)T was flanked by non-absorbing 5,6-dihydrothymines, instead of thymines. Using this approach, it was shown that the effect of the first flash was to deplete the T(6-4)T lesion without yielding substantial repair. Conversely, the effect of subsequent flashes, sent after a pre-irradiation burst, was to simultaneously deplete the T(6-4)T lesion and produce repair. An initial

quadratic behavior could be clearly observed at 265 nm with of series of 20 flashes spaced by 2 s (the so-called stairs experiment; see Fig. 9a). These results strongly suggested that photorepair by (6-4) PL actually requires two photoexcitations.

In a complementary experiment (72), we pre-irradiated a sample by a series of flashes (to accumulate the putative intermediate X) and then monitored the absorbance over minutes in the dark. We observed a slow recovery (time constant of ~ 100 s at 10°C) of the 325-nm band of the initial T(6-4)T lesion (Fig. 9b), thereby proving that intermediate X has a finite lifetime and spontaneously decays to the initial form of the lesion. We checked that a lifetime of 100 s is long enough to allow the absorption of a second photon under natural sunlight and thus to complete photorepair (72). Our spectral data are compatible with Sadeghian *et al.*'s proposal (58), according to which X would be an oxetane-bridged lesion (expected to absorb at lower wavelengths than the more conjugated T(6-4)T (49)), although a positive proof for this assignment is still lacking. As pointed out in Ref. (72), a lifetime of 100 s at 10°C would, according to transition state theory, correspond to an activation free energy of at most $19.2 \text{ kcal mol}^{-1}$ (for a transmission coefficient $\kappa = 1$; for instance, $17.9 \text{ kcal mol}^{-1}$ would be obtained with $\kappa = 0.1$). These values are rather close to the energy barrier of $\sim 16 \text{ kcal mol}^{-1}$ calculated by Sadeghian *et al.* (58) for the reversion of the enzyme-bound oxetane to the (6-4) PP. It thus appears that the observed lifetime of intermediate X is also consistent with the oxetane proposal, but, again, this does not constitute a proof. The two-photon repair cycle suggested by our results (72) is summarized in Fig. 9c.

The quantum yields of the individual photoreactions were estimated by fitting the stairs experiment with a two-photon repair model. The best fit (red line in Fig. 9a) yielded $\eta_1 = 6.7\%$ and $\eta_2 = 83\%$. Based on an error analysis of all data, confidence intervals of $5.8\% \leq \eta_1 \leq 8.8\%$ and $47\% \leq \eta_2 \leq 100\%$ were obtained (72).

REPAIR OF DEWAR PHOTOPRODUCTS

Dewar PPs are isomers of the (6-4) PPs featuring the 2-oxo-1,3-diazabicyclo[2.2.0]hex-5-ene structure in the 3' component (see Fig 1 for T(Dew)T and Fig. 10 for T(Dew)C). They are formed via an electrocyclic reaction of the 3' pyrimidine ring in (6-4) PPs upon photoexcitation in the 325-nm band of the (6-4) PP. No specific photolyase for Dewar PPs has been discovered so far (32), but (6-4) photolyase was found to repair the most frequent Dewar lesion, T(Dew)C, albeit with a very low quantum yield (one to two orders of magnitude lower than for the corresponding (6-4) PP, as estimated by ourselves from data by Glas *et al.* (75)). For the Dewar PP formed at TT sites, T(Dew)T, no significant repair by (6-4) PL was observed (47,75,76). The crystal structure of (6-4) PL complexed with DNA carrying an analogue of the T(Dew)C lesion revealed that this Dewar PP was accommodated in the active site in the same way as the corresponding (6-4) PP (77).

Concerning the reaction mechanism of repair of the T(Dew)C lesion by (6-4) PL, theoretical (78–80) and experimental studies (77,80) agree that the first step is reversion of the Dewar PP to a (6-4) PP upon electron injection by photoexcited FADH^- . The (6-4) PP would subsequently be repaired as discussed above. This implies that repair of the T(6-4)C lesion requires at least two subsequent photoreactions (or even three, if repair of the T

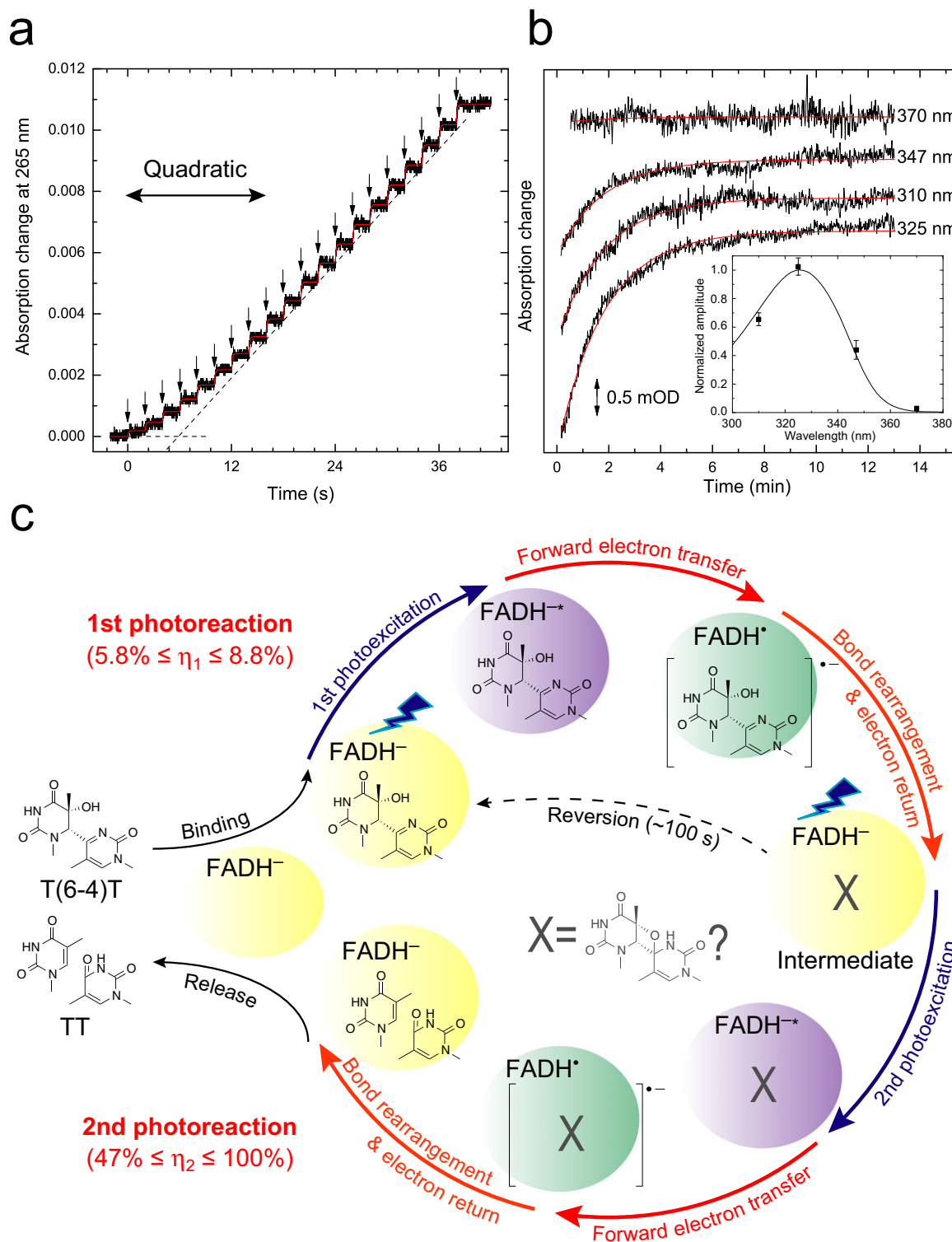


Figure 9. Experimental demonstration of the occurrence of a two-photon mechanism for the repair of a T(6-4)T lesion by the (6-4) PL of *Xenopus laevis*, according to Yamamoto *et al.* (72). (a) Absorption change at 265 nm induced by a series of twenty 100-ps laser flashes at 355 nm on a dark-adapted sample at 10 °C. The red trace represents the best fit of the data by a two-photon model (see Ref. (72), SI, Section 5.3, Eq. 19), yielding quantum yields of $\eta_1 = 6.7\%$ and $\eta_2 = 83\%$ for the first and the second photoreaction, respectively. (b) Long-term absorption changes at individual wavelengths of a dark-adapted sample at 10 °C exposed to 12 laser flashes just before time zero. The decays were globally fitted with a single exponential (red lines), yielding a time constant of ~ 100 s, attributed to the lifetime of the intermediate X formed by the first photoreaction. Inset: normalized amplitudes associated with the decay (squares, with corresponding error bars representing the standard amplitude errors of the fit), superimposed with the normalized absorption spectrum of T(6-4)T. (c) Proposed two-photon reaction cycle. The exact nature of intermediate X could not be determined by the experiments, but the spectroscopic data of panel b are compatible with the oxetane structure proposed by Sadeghian *et al.* (58). See text for further details. Panels (a) and (b) were reprinted with minor modifications from Ref. (72). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

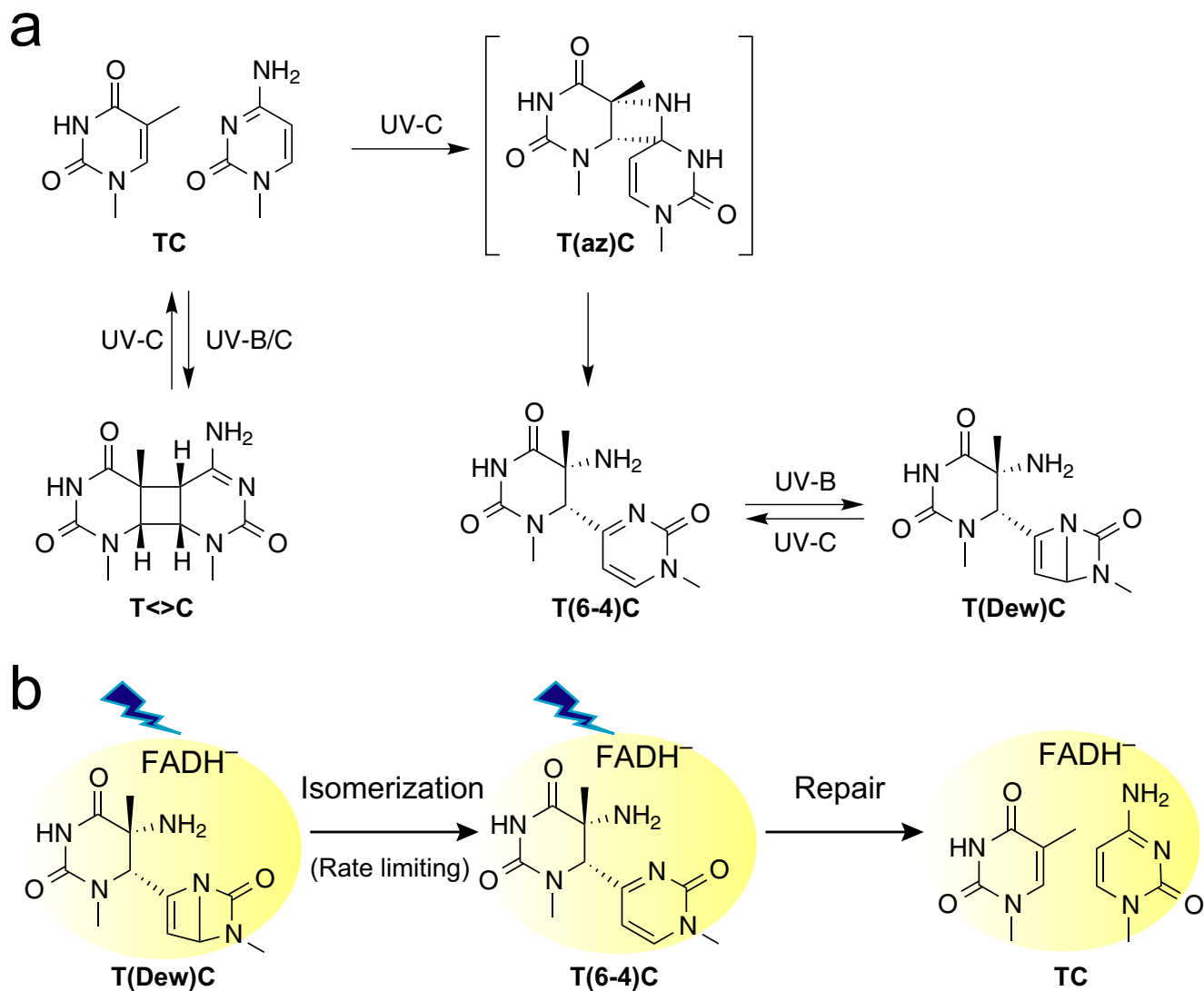


Figure 10. (a) Formation of UV-induced DNA damages at a TC site (upper left corner). The left column shows the formation of a T \rightleftharpoons C lesion. The middle column illustrates the step-wise formation of a T(6-4)C lesion (bottom) through a T(az)C azetidine intermediate (up). The photoconversion of the T(6-4)C lesion into its T(Dew)C Dewar isomer is represented in the lower right corner of the panel. (b) Photorepair of the T(Dew)C lesion by two sequential processes: photoreversion to the T(6-4)C isomer, assisted by the (6-4) PL, and photorepair of the T(6-4)C by the (6-4) PL. See text for further details.

(6-4)C requires two photoreactions, as in the T(6-4)T case). The reversion (isomerization) of the Dewar PP would be the rate-limiting step of overall repair (Fig. 10b). Experimentally, this reaction sequence was derived from the accumulation of the (6-4) PP when its repair in the second step was blocked (using a His₁-to-asparagine mutant photolyase) or slowed (using a TC-Dewar PP analog, consisting of thymidine and *N*⁴-methyl-2'-deoxycytidine) (77), while no accumulation of the (6-4) PP was observed in experiments with WT photolyase and the unmodified T(Dew)C lesion (80).

With respect to the inability of (6-4) PL to repair the T(Dew)T lesion (47,75,76), this cannot be attributed to poor substrate binding, as a high affinity for T(Dew)T-containing substrates has been demonstrated (41,45). Rather, reversion of the T(Dew)T to the T(6-4)T lesion appears to be inhibited, either by an unfavorable electron transfer from photoexcited FADH⁻ to T(Dew)T (2,77) or by a higher activation barrier for the splitting of the

N3'-C6' bond in the anion radical of the T(Dew)T lesion (compared to T(Dew)C) (79–81).

In view of the very low or insignificant quantum yields of Dewar PP repair by (6-4) PL, NER has been considered to be the most important repair mechanism for Dewar PPs even in cells expressing (6-4) PLs (32).

CONCLUDING REMARKS

More than 20 years after the discovery of (6-4) photolyase (18), the mechanism by which it repairs (6-4) photoproducts is still far from being established in detail. We have focused in this contribution on major experimental and theoretical advances in the field. At least for the T(6-4)T lesion, there is strong experimental evidence (72) that two consecutive photoreactions are required for the repair, in accordance with one singular previous theoretical study (58).

We feel that it should be a top priority of future research to establish experimentally the nature of the intermediate resulting from the first photoreaction (an oxetane-bridged dimer according to the theoretical prediction (58)). FTIR difference spectroscopy upon photoaccumulation of the intermediate could be a convenient technique. However, only small signals would be expected, because with quantum yields in the order of only 5–10% for the 1st photoreaction, but 50–100% for the 2nd one (72), only ~10% accumulation of the intermediate per photolyase could be achieved even under strong continuous light. Another obvious priority is to characterize each of the two photoreactions experimentally in as much detail as possible. Here, the quantum yields mentioned provide a serious challenge for monitoring the 2nd photoreaction. Ideally, one should study a sample in which 100% of the photolyases bind the intermediate, but at best ~10% would be accumulated by continuous light or repetitive flashes. Experiments on such a sample would monitor a mixture of ~90% photolyases bound to a (6-4) PP (and hence showing the 1st photoreaction) and of only ~10% photolyases bound to the intermediate (showing the 2nd photoreaction).

Primary steps of the repair reaction were studied by ultrafast spectroscopy (68) prior to the prediction of a two-photon process (58) and were hence discussed within the framework of a one-photon reaction. As the study used repetitive excitation, a fraction of the photolyases may have been bound to the intermediate, and the observed transient absorption changes may have represented a mixture of the 1st and 2nd photoreaction. For the reasons outlined above, however, at most ~10% should have been due to the 2nd photoreaction. As the sample was stirred during the experiment, the percentage was presumably even lower. We hence believe that the ultrafast data (68) essentially represent the 1st photoreaction. Indeed, the two primary forward reaction steps concluded from this study (electron transfer from FADH^{-*} to the (6-4) PP lesion and subsequent proton transfer from His_1 to the lesion anion radical; see Fig. 6) agree with the theoretical prediction for the 1st photoreaction (see Fig. 5, bottom row). Furthermore, the unfavorable competition between the productive proton transfer to the lesion anion radical (in 425 ps) and futile back electron transfer from the latter to FADH^{\bullet} (in 50 ps) concluded by the ultrafast study (68) would largely account for the low quantum yield of the 1st photoreaction concluded from our flash sequence experiments (72). The fraction of FADH^{\bullet} that escaped back electron transfer did not decay in the accessed time window (up to 3 ns), indicating that electron return from the lesion at the end of the photoreaction took longer than 10 ns (68).

We have undertaken preliminary transient absorption studies to estimate the electron return kinetics at 10 °C for both the 1st photoreaction (using single excitation flashes given to samples that were dark adapted for over 10 min) and the 2nd photoreaction (by using repetitive excitation and subtracting the contribution from the 1st photoreaction) and obtained time constants in the order of tens of microseconds and hundreds of nanoseconds, respectively. We mention these unpublished data here hoping that future computational quests for a repair pathway could already take them into account. More importantly, future experimental work should try to establish the missing steps of the 1st photoreaction (rearrangements of the lesion radical after its protonation) and to detail the 2nd photoreaction from its beginning.

So far, (6-4) photolyases from three different higher organisms were used for in-depth experimental and theoretical studies of (6-4) PP repair: the fly *D. melanogaster*, the frog *X. laevis* and the plant *A. thaliana*. These three proteins show strong

sequence homology, and it was implicitly assumed that results obtained on one protein apply to the other ones as well. Experimental verification of this assumption appears desirable. Recently, several members of the photolyase/cryptochrome family in microorganisms were found to repair (6-4) lesions, in addition to typical cryptochrome functions (82–85). Among these, the PhrB protein of the soil bacterium *Agrobacterium tumefaciens* (84,86) and the closely related CryB protein of the purple bacterium *Rhodobacter sphaeroides* (85) are of particular interest, because they are phylogenetically very distant from canonical (6-4) photolyases and feature specific structural elements and cofactors, like a [4Fe-4S] cluster. Homologous proteins exist in at least 350 bacterial organisms (86). It will be interesting to examine whether a (6-4) PP repair mechanism different from that of canonical (6-4) photolyases has evolved in prokaryotes.

Most of the experimental and theoretical investigations on the photorepair by (6-4) PL have been performed using T(6-4)T-containing substrates, but T(6-4)C lesions are formed in cells more efficiently than T(6-4)T (4,87), suggesting a more important role of the repair of T(6-4)C. It has early been reported that T(6-4)C and T(6-4)T are repaired with similar quantum yields (45), but it is not yet experimentally established whether or not the photorepair mechanism for T(6-4)T is applicable to T(6-4)C. A computational study (63) indicated that T(6-4)C could be repaired by a mechanism analogous (OH replaced by NH_2) to the “proton-transfer-steered OH transfer” shown in Fig. 5, middle row. It is worth mentioning that photorepair of T(Dew)C would require three photons, if photorepair of T(6-4)C proceeded via a two-photon mechanism. Detailed studies of the photorepair of the T(6-4)C lesion and of the first photoreaction in the repair of the T(Dew)C lesion should be included in future research.

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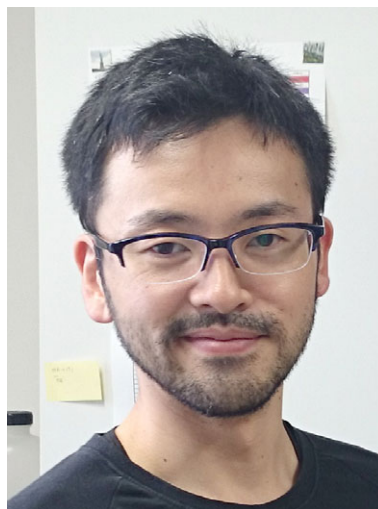
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