DNA Repair

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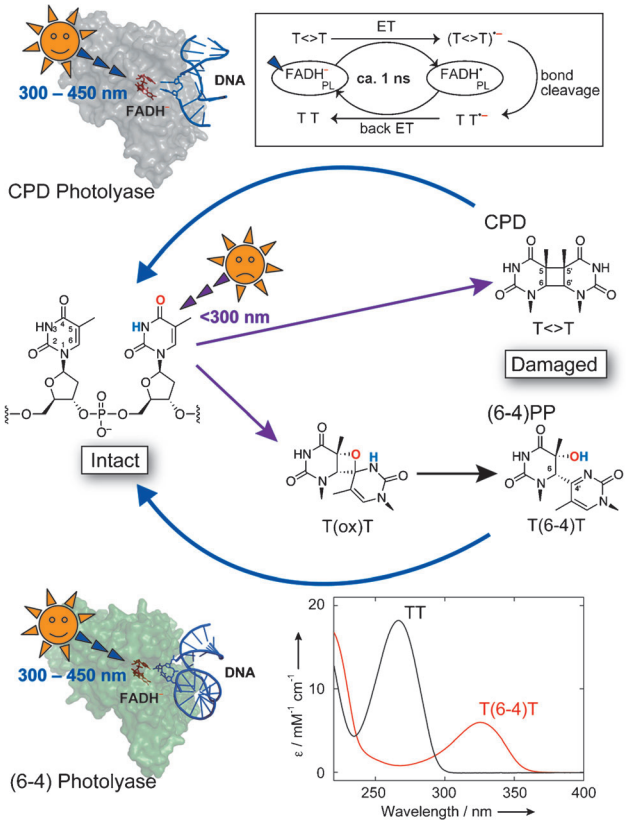
Repair of the (6–4) Photoproduct by DNA Photolyase Requires Two Photons**

Junpei Yamamoto, Ryan Martin, Shigenori Iwai, Pascal Plaza,* and Klaus Brettel**

Ultraviolet (UV) irradiation of DNA induces two major types of harmful cross-links between adjacent pyrimidine bases (see Scheme 1): cyclobutane pyrimidine dimers (CPD) and pyrimidine(6–4)pyrimidone photoproducts ((6–4)PPs; denoted T(6–4)T in the case of two thymines). While CPDs are formed directly by [2+2] cycloaddition, formation of the (6–4)PP proceeds through an oxetane intermediate (or azetidine in the case of cytosine as the 3′ base).[1,2] In many organisms, these lesions are repaired by DNA photolyases (PL)—flavoenzymes using blue or near UV light for their catalytic action.[2,3] For CPD repair, it has been established that the transfer of an electron from the photo-excited fully reduced flavin cofactor ($FADH^-$) to the lesion induces the cleavage of the intradimer bonds and the restoration of the intact pyrimidines in approximately one nanosecond, including return of the excess electron to the flavin cofactor (Scheme 1, top inset).[4,5] Repair of the (6–4)PP by (6–4) photolyase is far less understood. It is chemically more challenging than repair of the CPD because of the requirement to transfer a functional group (OH in the case T(6–4)T) from the 5′ to the 3′ base, in addition to intradimer bond cleavage. Remarkably, the quantum yield of repair of the (6–4)PP (approximately 3–11 %)[6] is much lower than that of the CPD (approximately 50–100 %).[2,7] According to a recent study using repetitive femtosecond flash excitation, excited $FADH^-$ transfers an electron to the (6–4)PP in 225 ps; the excess electron is either returned to the flavin in 50 ps without repair of the (6–4)PP, or repair takes place and the excess electron supposedly returns to the flavin in more than 10 ns.[8]



Scheme 1. Center) UV-induced damage of DNA, yielding CPD or (6–4)PP lesions, for the case of two thymines. Top) photorepair of CPD by CPD photolyase (structure from PDB: 1tez).[13] Top inset: CPD repair mechanism. Bottom) photorepair of (6–4)PP by (6–4) photolyase (structure from PDB: 3cvu).[11] Bottom inset: Absorption spectra of intact TT pair and T(6–4)T lesion.

This and other experimental or theoretical studies generally assumed that the (6–4)PPs, like CPDs, are repaired upon absorption of a single photon by the enzyme.[8–11] The possibility of a two-photon process has been ignored, except for one computational study by Sadeghian et al.[12] They suggested that a first photo-induced electron transfer (ET) from $FADH^-$ converts T(6–4)T to the oxetane-linked dimer, denoted T(ox)T, also involved in photodamage (see Scheme 1); after return of the excess electron to the flavin, a second photo-induced ET from $FADH^-$ splits the oxetane ring with restoration of two intact thymines and return of the electron. Of note, it was long believed[2,6,10] that the (6–4)PP is converted to the oxetane (or azetidine for the T(6–4)C lesion) upon binding to the enzyme in the dark. However, this idea was disproven by a crystal structure showing an unmodified (6–4)PP in the binding pocket of the enzyme.[11]

[*] Dr. J. Yamamoto, Prof. S. Iwai
Division of Chemistry, Graduate School of Engineering Science, Osaka University
1-3 Machikaneyama, Toyonaka, Osaka 560-8531 (Japan)
E-mail: yamamoto@chem.es.osaka-u.ac.jp
R. Martin, Dr. P. Plaza
Département de Chimie, UMR 8640 CNRS-ENS-UPMC, Ecole Normale Supérieure
24 rue Lhomond, 75005 Paris (France)
E-mail: pascal.plaza@ens.fr
Dr. K. Brettel
UMR 8221, CEA-Institut de Biologie et de Technologie de Saclay, CNRS, Université Paris Sud
91191 Gif-sur-Yvette (France)
E-mail: klaus.brettel@cea.fr

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Herein, we studied repair of a T(6–4)T containing substrate by *Xenopus laevis* (6–4) photolyase, under photon-regulated conditions, to distinguish between a one- and a two-photon process. Repair was monitored spectroscopically using the recovery of the 265 nm absorption band of intact thymines and depletion of the 325 nm band of the T(6–4)T (see spectra in Scheme 1, bottom inset). To facilitate detection at 265 nm, as in previous works on CPD repair,[4,7] we used a substrate with greatly decreased background absorption at 265 nm: the 10-mer oligonucleotide d(HHHH-T(6–4)TTHHH), where H represents non-absorbing dihydrothymine. This special substrate (referred to as DHT (6–4)PP 10-mer herein) was repaired as well as the corresponding conventional substrate (Supporting Information, Figure S1).

Upon excitation of $FADH^-$ by continuous light at 384 nm (excitation rate, $k_{exc} = 0.13\ s^{-1}$; Figure S2), an apparently linear absorption increase at 265 nm and bleaching at 325 nm were observed (Figure 1). The slopes of the linear

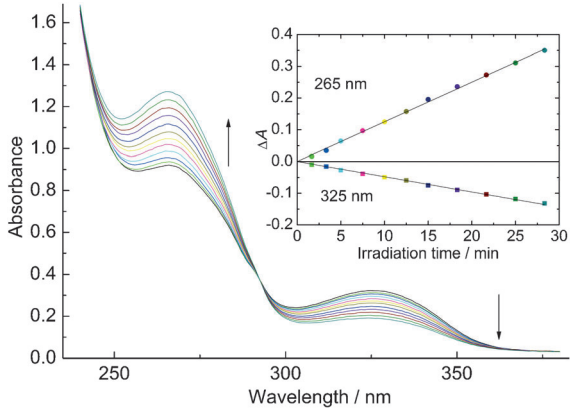

Figure 1. Photorepair of DHT (6–4)PP 10-mer by *Xenopus laevis* (6–4) photolyase under continuous excitation at 384 nm at 10 °C. The solution contained 60 μM DHT (6–4)PP 10-mer, 1.7 μM of photoreduced (Supporting Information, Section 1 and Figure S7b) *Xl* (6–4) photolyase, 10 mM phosphate buffer at pH 6.8, 100 mM NaCl, 6 mM cysteine, and 5% glycerol in an anaerobic cell. Inset: absorption changes at 265 nm and 325 nm as a function of irradiation time, corresponding to TT restoration and T(6–4)T depletion, respectively. The colors of the symbols correspond to their spectra.

regression lines correspond to repair quantum yields of 5.9% and 6.1%, respectively (see Supporting Information, Section 2). These data apparently support a one-photon process, because a two-photon reaction would yield an initial quadratic shape of the repair curve. However, as detailed in Section 4.2 of the Supporting Information, the initial quadratic behavior was virtually impossible to detect with this setup because of insufficient amplitude resolution.

We therefore devised a much more sensitive method, using excitation by single-turnover flashes (355 nm, 100 ps duration; approximately 0.15 photons absorbed per $FADH^-$; see Supporting Information, Section 4.3 for details) and quantification of repair using the flash-induced absorption changes (ΔA) at 265 nm and 325 nm (see Supporting Information). As a reference, we applied excitation in successive

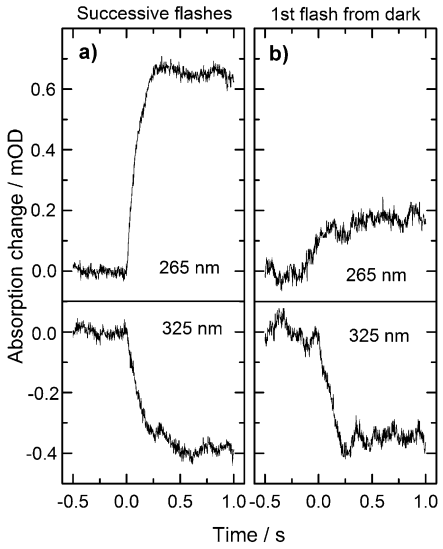

Figure 2. a) Averaged absorption changes (top: at 265 nm; bottom: at 325 nm) induced by eight successive laser flashes (355 nm, 100 ps duration, 4.78 $mJ\,cm^{-2}$, spaced by 4 s), observed on a sample having been exposed to eight such flashes immediately before. Average of three experiments. b) Absorption changes induced by a single such flash on a sample that had been incubated in the dark for at least 20 min. Average of seven experiments. The samples contained 4.9 μM photoreduced *Xl* (6–4) photolyase and 20 μM DHT (6–4)PP 10-mer. Other sample conditions were as in Figure 1.

flashes (interval = 4 s); the sample was exposed to prior irradiation by eight flashes and the absorption changes induced by the eight following flashes were averaged (Figure 2a).

As expected, we observed an absorption increase at 265 nm and a bleaching at 325 nm with a similar amplitude ratio as in the continuous wave (CW) light experiments (Figure 1). The absolute amplitude at 265 nm corresponds to a repair quantum yield of 5.7% (see Supporting Information for absorption coefficients used). Next, we recorded the signals induced by the first excitation flash applied to a dark-adapted sample (Figure 2b). Strikingly, ΔA_{265} turned out to be much smaller on the first flash than in the successive flashes regime, while ΔA_{325} was essentially unchanged. This observation clearly indicates that absorption of the first photon induced depletion of the T(6–4)T but not formation of the intact TT. Thus, repair of the (6–4)PP requires more than one photoexcitation. Our data suggest that a first photoreaction converts T(6–4)T into an intermediate X (that absorbs much less than T(6–4)T at 325 nm and much less than intact TT at 265 nm); a second photoreaction may convert X to the final repaired product (according to Equation (1)):

$$PL - T(6-4)T \xrightarrow{light} PL - X \xrightarrow{light} PL - TT \qquad (1)$$

The small, but non-negligible ΔA_{265} on the first flash was presumably caused by the second photoreaction occurring in a small fraction of the enzymes (approximately 1.5%; see below) that contained X before the first flash owing to excitation by the monitoring light (see Supporting Information). If the absorption of X at 265 nm were not negligible, it

would contribute to the first flash ΔA_{265} as well. Control experiments in the absence of either substrate or enzyme (Figure S4) revealed small but non-negligible signals for the substrate-only sample at 325 nm and for the enzyme-only sample at 325 nm and 265 nm. The latter presumably reflect some spurious long-lived photooxidation of $FADH^-$, which may be more prominent in the absence than in the presence of substrate because the excited state of $FADH^-$ is longer lived when it is not quenched by ET to the substrate.[8] The substrate-only sample showed a significant (approximately 15% of the enzyme-plus-substrate sample) flash-induced bleaching at 325 nm that can be attributed to some formation of the non-absorbing Dewar isomer[14] owing to direct excitation by the 355 nm flash of T(6–4)T in the red-most tail of its 325 nm band (Scheme 1 and Supporting Information). The signals observed in these control experiments were taken into account for the quantitative data evaluation below.

The evolution from low repair on the first flash to normal repair in the successive flashes regime was studied in a separate experiment. A series of 20 flashes spaced by two seconds was given to a dark-adapted sample while the formation of repaired TT was continuously monitored at 265 nm. The resulting trace looks like stairs with increasing step height at the beginning and virtually constant step height at the end. This behavior reflects an increasing repair yield until a quasi steady state with constant repair yield is reached. It can be well described (red fit line in Figure 3) by a two-photon repair model, as outlined below. The overall shape is close to the expected quadratic behavior that we were not able to resolve in the continuous light experiments (Figure 1; Figure S3). Interestingly, stairs with the same shape were observed repeatedly in the same sample when it was kept in darkness for one hour between experiments, while stairs with constant high steps (even from the beginning) appeared when the second experiment started shortly after the first one. We hypothesized that the intermediate X formed by the first photoreaction would decay to the initial T(6–4)T in less than one hour.

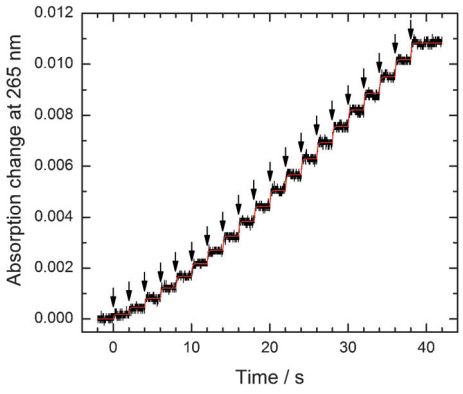

Figure 3. Absorption change at 265 nm induced by a series of 20 laser flashes (6.5 mJ cm^{-2}, spaced by 2 s), on a dark-adapted sample (so-called stairs measurement). Average of two experiments. The red line represents the best fit of the data by our two-photon model (Supporting Information, Section 5.3, Equation (19)). The sample contained 3.9 μM of photoreduced *Xl* (6–4) photolyase and 20 μM DHT (6–4)PP 10-mer. Other conditions were as in Figure 2.

To estimate the decay time of X more precisely, stairs measurements were performed at different times of dark adaptation after a preceding stairs measurement (Figure 4a). We defined the "repair lag" (see Figure 4a) as a measure of

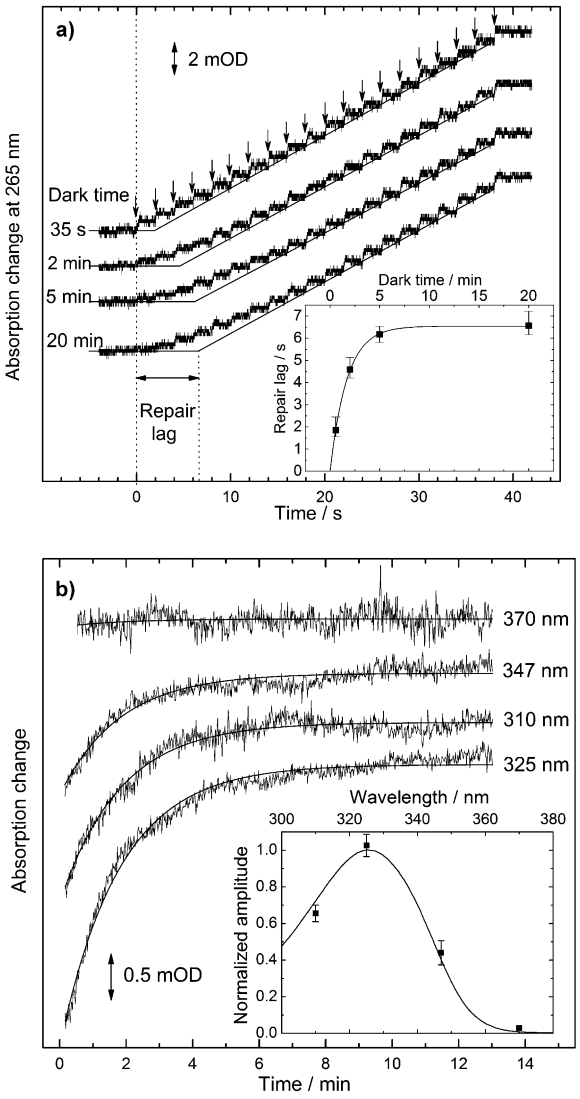

Figure 4. a) Stairs measurements (as in Figure 3) performed at different dark times after a preceding series of 20 flashes. Other conditions were as in Figure 3. The "repair lag" is a measure of the time necessary to achieve a constant step height within a flash sequence. It was obtained as indicated by the straight lines (see Supporting Information, Section 3, for details). Inset: Repair lag as a function of dark time (error bars obtained as outlined in Supporting Information, Section 3). A fit with a single-exponential decay function through the origin yielded a time constant of 1.7 min (solid line). b) Long-term absorption changes at individual wavelengths of a dark-adapted sample (8.8 μM photoreduced *Xl* (6–4) photolyase and 33 μM DHT (6–4)PP 10-mer) exposed to 12 laser flashes (5.0 mJ cm^{-2}) within approximately 16 s just before time zero. Other conditions of sample and laser flashes were as in Figure 2. Two, four, and three experiments were averaged at 310, 325 and 347 nm, respectively. About 10 s were required to transfer the sample from the laser flash set-up to the spectrophotometer. The decays were globally fitted with a single exponential, yielding a time constant of 1.8 min (smooth lines). Inset: normalized amplitudes associated with the decay (error bars representing the standard amplitude errors of the fit), superimposed with a normalized absorption spectrum of T(6–4)T.

the number of excitations required to achieve a constant step height. The repair lag increased systematically with dark incubation time (Figure 4a inset); the increase was well fitted by a single exponential curve with a time constant of 1.7 minutes, which we assigned to the spontaneous decay of X back to the original T(6–4)T lesion.

Next we searched for spectral changes that correlated with this time constant and hence to characterize the intermediate, X. A dark-adapted sample was exposed to 12 excitation flashes (to accumulate a significant amount of X), then its absorbance at a fixed wavelength was monitored over 14 minutes in a spectrophotometer. Significant absorption increases on a timescale of minutes were observed at three wavelengths within the 325 nm absorption band of the T(6–4)T (Figure 4b). Global analysis with a single exponential yielded a best-fit time constant of 1.8 minutes, very close to the 1.7 minute recovery time of the repair lag. The relative amplitudes of the fit curves overlaid well with the 325 nm absorption band of the T(6–4)T (Figure 4b inset). Taken together, these results strongly suggest that intermediate X is characterized by a complete bleaching of the 325 nm band of the T(6–4)T and reverts to the T(6–4)T with a rate constant $k_x \approx 0.01\ s^{-1}$. Our spectral data also suggest that, with formation of X, the excess electron on the lesion was returned to the flavin cofactor, because a contribution from the $FADH^{\bullet}$-minus-$FADH^{-}$ difference spectrum[4] should otherwise have been observed.

We quantitatively analyzed our experimental data within the framework of Equation (2):

$$\begin{aligned} &PL + T(6-4)T \xrightarrow[\text{binding}]{\text{Substrate}} PL - T(6-4)T \underset{k_x}{\overset{\text{light}(\eta_1)}{\rightleftharpoons}} \\ &PL - X \xrightarrow{\text{light}(\eta_2)} PL - TT \xrightarrow[\text{release}]{\text{Product}} PL + TT \end{aligned} \qquad (2)$$

where η_1 and η_2 are the quantum yields of the first and second photoreaction, respectively. Substrate binding and product release are expected[15] to be much faster than the overall repair rate (less than $0.01\ s^{-1}$ in all our experiments) and are hence considered to be non-limiting (Supporting Information, Section 4).

Yield η_1 can be estimated from ΔA_{325} for the first flash (Figure 2b, bottom). After correcting for Dewar isomer formation and spurious photo-oxidation of $FADH^{-}$, a conservative estimate yields $5.4\% \le \eta_1 \le 11.8\%$ (Supporting Information, Sections 5.2, 6.2). Upon subsequent flashes, X builds up and reaches a quasi steady-state level. From the observation that ΔA_{325} in the successive flash regime was similar to the first flash (Figure 2, bottom), we conclude that the fraction of complexes present in the state PL-X in our successive flash regime was rather low, in fact less than 20% according to a conservative estimation (Supporting Information). Knowing this fraction, η_2 can be estimated from ΔA_{265} in the successive flash regime (Figure 2a, top), giving $\eta_2 > 27\%$ (Supporting Information). Hence, we can safely conclude that the quantum yield of the first photoreaction is well below that of the second one and may in fact be the main cause of the low overall repair quantum yield.

To obtain more accurate estimates of the two quantum yields, we fitted the stairs in Figure 3 according to Equation (2), with k_x fixed at $0.01\ s^{-1}$ and allowing for the presence of PL-X before the first flash in a small fraction of the complexes owing to the actinic effect of the monitoring light (Supporting Information, Sections 4.4 and 5.3 for the calculations). The best fit (red line in Figure 3) gave $\eta_1 = 6.7\%$, $\eta_2 = 83\%$, and 1.88% for the fraction in state PL-X prior to the first flash. Based on an error analysis of the fit and taking into account the data presented in Figure 1 and 2, we concluded that only quantum yields lying in the ranges $5.8\% \le \eta_1 \le 8.8\%$ and $47\% \le \eta_2 \le 100\%$ (imposed physical limit) would be consistent with our data (Supporting Information, Section 6).

For the steady-state (SS) repair quantum yield under continuous light at an excitation rate k_{exc} the proposed reaction scheme gives Equation (3) (Supporting Information, Section 4):

$$\eta_{SS} = \eta_1\eta_2/(\eta_1 + \eta_2 + k_x/k_{exc}) \qquad (3)$$

At $k_{exc} = 0.13\ s^{-1}$ as used in Figure 2, the quantum yields $\eta_1 = 6.7\%$ and $\eta_2 = 83\%$ obtained from the best fit of the stairs measurement (Figure 3) give $\eta_{SS} = 5.7\%$, rather close to the values of 5.9% and 6.1% derived from the data in Figure 1 at 265 nm and 325 nm, respectively.

With respect to the chemical nature of intermediate X, our experimental data strongly suggest that X is non-absorbing in the 325 nm band of T(6–4)T (Figure 4b) and non- or only weakly absorbing at 265 nm (Figure 2b). These spectral features are consistent[16] with X being the oxetane-bridged dimer T(ox)T, as theoretically proposed by Sadeghian et al.[12]

According to our results, PL-X reverts to PL-T(6–4)T in approximately 100 s, that is, much slower than the approximately 2 ms reported by Marguet and Markovitsi[16] for the T(ox)T→T(6–4)T transition in an enzyme-free solution, apparently contradicting the assignment of X as the oxetane. However, this difference could reflect a stabilization of the oxetane by the enzyme. The computational study[12] indeed estimated an activation energy of around 16 kcal mol^{-1} for the transition from the oxetane in the active site to the T(6–4)T. From our observed lifetime of the intermediate X at 10 °C of approximately 100 s, the free-energy barrier estimated by transition-state theory would be 19.2(17.9) kcal mol^{-1} with an assumed transmission coefficient of 1(0.1). This reasonable agreement between the activation barrier predicted by computation and the one derived from our experimental data strengthens the proposal that intermediate X is the oxetane, stabilized by interaction with the enzyme.

This strong stabilization may be required to achieve a reasonable overall repair yield in a two-photon process under solar irradiation on Earth (η_{SS} indeed decreases hyperbolically with increasing k_x/k_{exc}; see Equation (3)). An estimation (Supporting Information, Section 7) based on the solar irradiance spectrum for clear sky and the absorption spectrum of $FADH^{-}$ yields an excitation rate k_{exc} on the order of $0.4\ s^{-1}$. This is much higher than the decay rate of X determined in our study ($0.01\ s^{-1}$), indicating that a close to optimal repair yield can be achieved in cells directly exposed to sunlight.

Remarkably, the quantum yield we obtained for the conversion of X to intact thymines (47–100%) is comparable to the repair quantum yield of CPD photolyase (around 50–100%).[2,7] If X were indeed the oxetane-bridged dimer (a proposal compatible with all our data but not actually proven by the present study), this similarity could be explained by the four-membered ring-opening reaction, conserved between CPD and (6–4) photolyases.

During the two decades following the discovery of (6–4) photolyase, its reaction mechanism has been believed to be shared with that of the structurally homologous CPD photolyase that can repair its substrate upon absorption of a single photon. Our experimental results provide clear evidence that, for the chemically much more challenging repair of the (6–4)PP, nature resorts to a sequential two-photon mechanism with a long-lived intermediate. This new data should allow for the design of adequate experiments to detail the intricate mechanism of (6–4)PP repair by photolyase.

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