Supporting Information

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SI Methods

Kinetic Data Analysis. The scheme of cyclobutane pyrimidine dimer (CPD) repair by photolyase is shown in Fig. 1. Assuming that the enzyme-substrate complex concentration involved in reaction is n_0 upon excitation at time-zero, the dynamics of each step involved in the entire reaction will be solved by fitting the following equations according to the reaction scheme:

$$[\mathrm{FADH}^{-*}]_{t=0} = n_0 \quad \textbf{[S1]}$$

$$\frac{d[\mathrm{FADH}^{-*}(t)]}{dt} = -(k_{\mathrm{FET}} + k_{\mathrm{LT}})[\mathrm{FADH}^{-*}(t)] \quad \textbf{[S2]}$$

$$\frac{d[T\langle\rangle T^{-}(t)]}{dt} = k_{\mathrm{FET}}[\mathrm{FADH}^{-*}(t)] - k_{\mathrm{sp1}}[T\langle\rangle T^{-}(t)] - k_{\mathrm{BET}}[T\langle\rangle T^{-}(t)] \quad \textbf{[S3]}$$

$$\frac{d[T\text{-}T^{-}(t)]}{dt} = k_{\mathrm{sp1}}[T\langle\rangle T^{-}(t)] - k_{\mathrm{sp2}}[T\text{-}T^{-}(t)] - k_{\mathrm{BET}}[T\text{-}T^{-}(t)] \quad \textbf{[S4]}$$

$$\frac{d[T^{-}(t)]}{dt} = k_{\mathrm{sp2}}[T\text{-}T^{-}(t)] - k_{\mathrm{ER}}[T^{-}(t)] \quad \textbf{[S5]}$$

$$\frac{d[T(t)]}{dt} = k_{\mathrm{sp2}}[T\text{-}T^{-}(t)] + k_{\mathrm{ER}}[T^{-}(t)] \quad \textbf{[S6]}$$

$$[\mathrm{FADH}^{\bullet}(t)] = [T\langle\rangle T^{-}(t)] + [T\text{-}T^{-}(t)] + [T^{-}(t)] \quad \textbf{[S7]}$$

$$[\mathrm{FADH}^{-}(t)] = n_0 - [\mathrm{FADH}^{-*}(t)] - [\mathrm{FADH}^{\bullet}(t)] \quad \textbf{[S8]}$$

Before pump excitation, the $[\mathrm{FADH}^{-}]$ is n_0 and the concentrations of FADH^{-*}, $\mathrm{FADH}^{\bullet}$, $T\langle\rangle T^{-}$, $T\text{-}T^{-}$, T^{-}, and T are zero. According to the Beer-Lambert law, the signal of femtosecond transient absorption at any wavelength can be acquired as:

$$\begin{aligned}\Delta A(t) \propto{}& \varepsilon_{\mathrm{FADH}^{-*}}([\mathrm{FADH}^{-*}(t)] - [\mathrm{FADH}^{-*}]_{t<0}) \\ &+ \varepsilon_{\mathrm{FADH}^{\bullet}}([\mathrm{FADH}^{\bullet}(t)] - [\mathrm{FADH}^{\bullet}]_{t<0}) \\ &+ \varepsilon_{\mathrm{FADH}^{-}}([\mathrm{FADH}^{-}(t)] - [\mathrm{FADH}^{-}]_{t<0}) \\ &+ \varepsilon_{T\langle\rangle T^{-}}([T\langle\rangle T^{-}(t)] - [T\langle\rangle T^{-}]_{t<0}) \\ &+ \varepsilon_{T\text{-}T^{-}}([T\text{-}T^{-}(t)] - [T\text{-}T^{-}]_{t<0}) + \varepsilon_{T^{-}}([T^{-}(t)] - [T^{-}]_{t<0}) \\ &+ \varepsilon_{T}([T(t)] - [T]_{t<0}) \propto \varepsilon_{\mathrm{FADH}^{-*}}[\mathrm{FADH}^{-*}(t)] \\ &+ \varepsilon_{\mathrm{FADH}^{\bullet}}[\mathrm{FADH}^{\bullet}(t)] + \varepsilon_{\mathrm{FADH}^{-}}([\mathrm{FADH}^{-}(t)] - n_0) \\ &+ \varepsilon_{T\langle\rangle T^{-}}[T\langle\rangle T^{-}(t)] + \varepsilon_{T\text{-}T^{-}}[T\text{-}T^{-}(t)] \\ &+ \varepsilon_{T^{-}}[T^{-}(t)] + \varepsilon_{T}[T(t)]. \quad \textbf{[S9]}\end{aligned}$$

We observed FADH^{-*} at all wavelengths, while $\mathrm{FADH}^{\bullet}$ signal was probed from wavelengths of shorter than 620 nm. The FADH^{-} signal is captured from 500 to 260 nm. All thymine-related intermediates, which have absorption in UV region, were detected below 360 nm. The thymine product was detected clearly below 300 nm in absorption transients. There is no $T\langle\rangle T$ absorption, which is mainly shorter than 250 nm, at all wavelengths we probed, and thus no signal contains the $T\langle\rangle T$ contribution.

In addition, the decay of FADH^{-*} is controlled by both lifetime emission (k_{LT}) and forward ET, thus we observed a total rate of excited-state decay as $k = k_{\mathrm{LT}} + k_{\mathrm{FET}}$ at all wavelengths. The forward ET rate was then deconvoluted from the fitting results. Also, according to Eq. **S7**, the concentration of $\mathrm{FADH}^{\bullet}$ is equal to the sum of three thymine-related intermediates. Because the $T\langle\rangle T^{-}$ has trace amount accumulation due to the ultrafast k_{sp1}, the sum of signal of $T\text{-}T^{-}$ and T^{-} will be almost identical to that of $\mathrm{FADH}^{\bullet}$. Due to the fact that we do not have any absorption spectra of $T\text{-}T^{-}$ and T^{-} in protein environment, it is not easy to differentiate the total signal of $T\text{-}T^{-}$ and T^{-} intermediates from that of $\mathrm{FADH}^{\bullet}$. Instead, only the species with the stronger absorption, $T\text{-}T^{-}$ or T^{-}, can be usually obtained as shown in the inset of Fig. 2*A*, which is also the reason that only one intermediate, either $T\text{-}T^{-}$ or T^{-}, shows the absorption spectrum in the UV region from 260 to 360 nm. Thus, the results of $T\text{-}T^{-}$ and T^{-} are not the true absorption spectra and only represent the relative absorption between two intermediates. The magnitudes of $T\text{-}T^{-}$ and T^{-} were calibrated by comparison with the known flavin ground-state absorption.

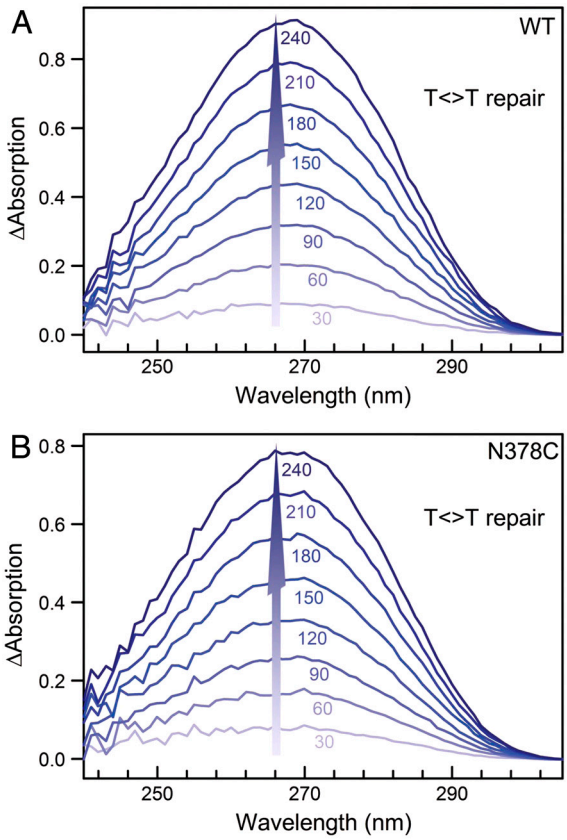

Fig. S1. (*A* and *B*) Steady-state measurements of repair efficiency of $T\langle\rangle T$ by mutant photolyase. Under the same conditions, a reference repair of $T\langle\rangle T$ by wild-type photolyase was conducted. Difference absorption spectra of $T\langle\rangle T$ repair by the wild-type and N378C mutant relative to their time-zero absorption spectra, respectively, were taken from 30 to 240 min illumination under white light and anaerobic conditions. Multiple independent experiments were taken to obtain an average value of absorption at 266 nm with visible-light illumination time. Given the quantum yield of 0.82 for $T\langle\rangle T$ repair by the wild-type, the repair efficiency of N378C mutant is calibrated by the change of distinctive 266-nm absorption.