Supplementary Figure 1. Transient-absorption signals of three mutants E274A, N378C and R342A with a pump wavelength at 400 nm and a wide range of probe wavelengths from visible to UV region. a, Absorption transients probed at 800 nm for detection of the excited-state flavin (FADH$^{•*}$) and at 620 nm mainly for the intermediate-state flavin (FADH$^{•}$). Shown in the inset is the deconvolution of the FADH$^{•*}$ and FADH$^{•}$ contributions for R342A and the latter is from two contributions of the branched back ET and the repair channels. b-d, Absorption transients probed at b, 300 nm, c, 270 nm and d, 266 nm with distinct dynamic patterns for each mutant. Shown in the insets is the deconvolution of the transient signals with detection of initial reactants, subsequent intermediates and final products for b, R342A, c, N378C and d, E274A.
Supplementary Figure 2. The deactivation lifetimes of the wild type and mutant photolyases. The absorption transients were probed at 800 nm with excitation of 400 nm in the absence of the thymine dimer substrate, showing the deactivation dynamics of the excited state.
Supplementary Figure 3. Determination of dissociation constants. The binding percentages of enzyme-substrate complex were measured for the wild-type and mutant photolyases (PL) with different T<>T-containing oligo(dT)$_{15}$ substrate concentrations. The dissociation constants were obtained by fitting the titration curve with the equation $[ES]/[E]=[S]/([S]+K_d)$. 
Supplementary Table 1. Results of various reaction times, efficiencies of the elementary steps and overall repair quantum yields of the wild-type and mutant photolyases. 

<table>
<thead>
<tr>
<th></th>
<th>$\Phi_T$</th>
<th>$\beta$</th>
<th>$\tau_{LT}$</th>
<th>$&lt;\tau_{FET}&gt;^b$</th>
<th>$\Phi_{FET}^c$</th>
<th>$&lt;\tau_{SP2}&gt;$</th>
<th>$&lt;\tau_{BET}&gt;$</th>
<th>$\Phi_{SP2}^c$</th>
<th>$&lt;\tau_{ER}&gt;$</th>
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<tr>
<td>WT</td>
<td>0.82</td>
<td>0.71</td>
<td>1300</td>
<td>236</td>
<td>0.85</td>
<td>88</td>
<td>2400</td>
<td>0.96</td>
<td>625</td>
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<tr>
<td>M345A</td>
<td>0.72</td>
<td>0.71</td>
<td>1169</td>
<td>140</td>
<td>0.89</td>
<td>88</td>
<td>368</td>
<td>0.81</td>
<td>200</td>
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<tr>
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<td>0.71</td>
<td>3374</td>
<td>1181</td>
<td>0.74</td>
<td>88</td>
<td>839</td>
<td>0.91</td>
<td>500</td>
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<td>0.71</td>
<td>1351</td>
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<td>1242</td>
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<tr>
<td>R226A</td>
<td>0.53</td>
<td>0.71</td>
<td>1309</td>
<td>480</td>
<td>0.73</td>
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<td>126</td>
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<tr>
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<td>0.64</td>
<td>1600</td>
<td>595</td>
<td>0.73</td>
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<td>166</td>
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<tr>
<td>E274A</td>
<td>0.38</td>
<td>0.71</td>
<td>1044</td>
<td>615</td>
<td>0.62</td>
<td>31</td>
<td>52</td>
<td>0.62</td>
<td>75</td>
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</table>

All times are in unit of picosecond. Here, $\beta$ is the stretched parameter and $\Phi_T$ is the overall repair quantum yield of the wild-type and mutant photolyases. $<\tau_{FET}>$ is the forward ET time calculated from $[(\Gamma\beta\beta^{-1})^{-1} - \frac{1}{\tau_{LT}}]^{-1}$. $\tau$ is the observed time constant of FADH$^+$ decay with substrate probed at 800 nm, while $\tau_{LT}$ is that in the absence of substrate. $\Phi_{FET}$ and $\Phi_{SP2}$ are the branching quantum yields of the forward ET and the ring splitting and calculated by $\Phi_{FET} = <\tau_{FET}>^{-1}/(<\tau_{FET}>^{-1} + \tau_{LT}^{-1})$ and $\Phi_{SP2} = <\tau_{SP2}>^{-1}/(<\tau_{SP2}>^{-1} + <\tau_{BET}>^{-1})$, respectively. Thus, $\Phi_T = \Phi_{FET} \times \Phi_{SP2}$. 

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Supplementary Table 2. The various energies obtained in electron transfer reactions.\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>$\Delta G_{\text{FET}}^0$</th>
<th>$\lambda_{\text{FET}}$</th>
<th>$\Delta G_{\text{BET}}^0$</th>
<th>$\lambda_{\text{BET}}$</th>
<th>$\Delta G_{\text{ER}}^0$</th>
<th>$\lambda_{\text{ER}}$</th>
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<td>-0.22</td>
<td>1.20</td>
<td>-2.26</td>
<td>1.37</td>
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<tr>
<td>M345A</td>
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<td>1.24</td>
<td>-2.17</td>
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<td>N378C</td>
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<td>-2.39</td>
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<tr>
<td>R342A</td>
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<td>1.27</td>
<td>-0.68</td>
<td>1.27</td>
<td>-2.13</td>
<td>1.49</td>
</tr>
</tbody>
</table>

\textsuperscript{a}All energies are in unit of eV. The 2.48 eV for the $S_1 \leftrightarrow S_0$ at 500 nm is used in calculation of $\Delta G_{\text{FET}}^0$. 

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\textsuperscript{a}All energies are in unit of eV. The 2.48 eV for the $S_1 \leftrightarrow S_0$ at 500 nm is used in calculation of $\Delta G_{\text{FET}}^0$.
Supplementary Note 1. Supplementary kinetic data analyses

The scheme of the dimer repair by photolyase is shown in Fig. 1a. Assuming that the enzyme-substrate complex concentration involved in the reaction is $n_0$ upon excitation at time zero, the kinetics of each elementary step involved in the entire reaction can be solved by fitting the following equations according to the reaction scheme:

$$[\text{FADH}^-]_{t=0} = n_0$$  \hspace{1cm} (1)

$$\frac{d[\text{FADH}^-](t)}{dt} = - (k_{\text{FET}} + k_{\text{LT}})[\text{FADH}^-](t)$$  \hspace{1cm} (2)

$$\frac{d[\text{T}<>\text{T}^-](t)}{dt} = k_{\text{FET}}[\text{FADH}^-](t) - k_{\text{SP1}}[\text{T}<>\text{T}^-](t) - k_{\text{BEY}}[\text{T}<>\text{T}^-](t)$$  \hspace{1cm} (3)

$$\frac{d[\text{T}-\text{T}^-](t)}{dt} = k_{\text{SP1}}[\text{T}<>\text{T}^-](t) - k_{\text{SP2}}[\text{T}-\text{T}^-](t) - k_{\text{BEY}}[\text{T}-\text{T}^-](t)$$  \hspace{1cm} (4)

$$\frac{d[\text{T}^-](t)}{dt} = k_{\text{SP2}}[\text{T}-\text{T}^-](t) - k_{\text{ER}}[\text{T}^-](t)$$  \hspace{1cm} (5)

$$\frac{d[\text{T}](t)}{dt} = k_{\text{SP2}}[\text{T}^-](t) + k_{\text{ER}}[\text{T}^-](t)$$  \hspace{1cm} (6)

$$[\text{FADH}^+](t) = [\text{T}<>\text{T}^-](t) + [\text{T}-\text{T}^-](t) + [\text{T}^-](t)$$  \hspace{1cm} (7)

$$[\text{FADH}^-](t) = n_0 - [\text{FADH}^+](t) - [\text{FADH}^\Delta](t)$$  \hspace{1cm} (8)

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

$$\Delta A(t) \propto \varepsilon_{\text{FADH}^-}([\text{FADH}^-](t) - [\text{FADH}^-]_{t=0}) + \varepsilon_{\text{FADH}^-}([\text{FADH}^+](t) - [\text{FADH}^+]_{t=0})$$

$$+ \varepsilon_{\text{T}<>\text{T}^-}([\text{T}<>\text{T}^-](t) - [\text{T}<>\text{T}^-]_{t=0}) + \varepsilon_{\text{T}-\text{T}^-}([\text{T}-\text{T}^-](t) - [\text{T}-\text{T}^-]_{t=0}) + \varepsilon_{\text{T}}([\text{T}^-](t) - [\text{T}^-]_{t=0})$$

$$\propto \varepsilon_{\text{FADH}^-}([\text{FADH}^-](t) + \varepsilon_{\text{FADH}^-}([\text{FADH}^+](t) + \varepsilon_{\text{FADH}^-}([\text{FADH}^-](t) - n_0)$$

$$+ \varepsilon_{\text{T}<>\text{T}^-}([\text{T}<>\text{T}^-](t) + \varepsilon_{\text{T}-\text{T}^-}([\text{T}-\text{T}^-](t) + \varepsilon_{\text{T}}([\text{T}^-](t) + \varepsilon_{\text{T}}[\text{T}](t))$$  \hspace{1cm} (9)

We observed $\text{FADH}^-\Delta^-$ at all wavelengths, while $\text{FADH}^-$ signal was probed from the wavelengths of shorter than 620 nm. The $\text{FADH}^+$ 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. The absorption of $\text{T}<>\text{T}$ is mainly at the wavelengths shorter than 250 nm, which is out of our probing range. Thus, none of transients contains the $\text{T}<>\text{T}$ signal.

In addition, the decay of $\text{FADH}^-\Delta^-$ results from both the lifetime deactivation ($k_{\text{LT}}$) and forward ET, thus we observed a total rate of excited-state decay as $k = k_{\text{LT}} + k_{\text{FET}}$ at all wavelengths. The forward ET rate was then deconvoluted from the fitting results. Also, according to equation (7), the concentration of $\text{FADH}^-$ is equal to the sum of three thymine-related intermediates. Since the trace amount accumulation of $\text{T}<>\text{T}$ can be neglected due to the ultrafast $k_{\text{sp1}}^{13}$, the sum of concentrations of $\text{T}-\text{T}^-$ and $\text{T}^-$ will be almost identical to that of $\text{FADH}^\Delta$. 

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All the time scales of each elementary step for the wild type and mutant photolyases are listed in supplementary table 1.

Supplementary Note 2. Analyses of electron-transfer processes

The repair of the dimer by photolyase involves a series of critical electron transfer reactions, which plays a vital role in the repair efficiency. The electron transfer in proteins is strongly influenced by the active-site environment\textsuperscript{19, 36, 37}. To get more insight into how the active-site residues modulate the ETs in repair, we analyzed the forward ET, futile back ET and electron return (ER) using the empirical ET formula below\textsuperscript{24, 38}.

\[
k_{ET} = \frac{4\pi^3}{h^2 B k_b T^2} J^2 \times 10^{-3.1(\Delta G^0 + \lambda)^2/\lambda}
\]  

(10)

where \(k_{ET}\) is the ET rate in s\(^{-1}\), \(h\) and \(B\) are Planck and Boltzmann constants, respectively, and \(T\) is temperature in Kelvin, \(J\) is the electron coupling matrix element, \(\Delta G^0\) is the standard free energy difference between the reactants and the products in eV, and \(\lambda\) is the reorganization energy in eV. For simplicity, equation (10) can be rewritten as follows\textsuperscript{24, 39}.

\[
\log k_{ET} = 13 - A - 3.1 \frac{(\Delta G^0 + \lambda)^2}{\lambda}
\]  

(11)

where \(A\) is related to the electron coupling term.

Given that the reduction potentials of T<>T/ T<>T\(^-\) and FADH’/FADH\^-\) for the wild type are -1.96 V and +0.08 V vs. NHE\textsuperscript{39-42}, respectively, and assuming the S\(_1\)←S\(_0\) transition of FADH\^-\) at 500 nm (2.48 eV), the free energy \(\Delta G^0\) for the forward ET of the wild type is calculated to be -0.440 eV. Our recent study elucidated the electron tunneling pathway of forward ET from FADH\(^-\) to substrate T<>T through mediation of the intervening adenine, and resolved the electron coupling \(J\) as 3.0 meV and the term \(A\) as 1.8825. Thus, we determined the reorganization energy of 1.2 eV for FET in the WT. For the electron return, the free energy \(\Delta G^0_{ER}\) can be derived by subtracting \(\Delta G^0_{FET}\) from -2.70 eV, based on the energy of the S\(_1\)←S\(_0\) transition of FADH\(^-\) (2.48 eV) and the reduction potential difference (0.22 eV) between T<>T/ T<>T\(^-\) and T/ T\(^-\\)\textsuperscript{39, 43}. Given the electron coupling term of 2.6 meV and the term A as 2.02 from our recent study\textsuperscript{25}, we obtain the reorganization energy of 1.373 eV for ER in WT.

Since the single-position mutations do not affect the folding and local structure of the enzyme (the circular dichroism spectra of mutants do not show any obvious changes from that of the wild type and the mutant enzymes show similar repair), we can assume that the mutants have the same electron-coupling constants as the wild type for the FET and ER, respectively. The \(\Delta G^0_{FET}\) and \(\Delta G^0_{ER}\) have the certain relationship, thus we have two equations of (11) for the FET and ER reactions with three unknowns of one free energy, FET and ER reorganization energies of \(\lambda_{FET}\) and \(\lambda_{ER}\), respectively, for the mutants. With the following three constraints of (1) \(\lambda_{ER}\) is larger than \(\lambda_{FET}\), (2) The larger \(\Delta G^0_{FET}\) and the faster \(\tau_{FET}\), and the larger \(|\Delta G^0_{ER}|\), the faster \(\tau_{ER}\), and (3) Both reorganization energies are in the certain range according to the wild-type values\textsuperscript{44, 45}, we can determine the three values for all mutants shown in supplementary table 2.

Assuming that futile back ET has the similar \(J\) and \(\lambda\) values as for the forward ET, the
free energies $\Delta G_{\text{BET}}^0$ of the back ET can be obtained. The assumption is reasonable given by the ultrafast breaking of the C5-C5’ bond and the electron still localized near the 5’ site.

The energy parameters involved in forward ET, futile back ET and electron return reactions for the wild type and all mutants are listed in supplementary table 2 and plotted in Fig. 3.

**Supplementary Note 3. Influence of the mutations on the electron transfer reactions and the dimer splitting**

(a) Modulation of the forward ET by the active-site residues

The driving forces ($-\Delta G^0$) for the forward ET change by mutations (Fig. 3 and Supplementary Table 2), suggesting the modulation by the active-site residues. After purification, the flavin cofactor in N378C and N378S is in oxidized form FAD$_{\text{ox}}$, in contrast to the radical FADH$^-$ in the wild type and other mutants. The FAD$_{\text{ox}}$ can be photoreduced to the fully reduced form FADH$^-$ in presence of external electron donors. However, no accumulation of radical FADH$^-$ was observed during the photoreduction, indicating that the formed FADH$^-$ is unstable and readily goes further to form FADH$^-$. Additionally, FADH$^-$ in N378C or N378S is more difficult to be reoxidized compared to the wild type under aerobic conditions. These observations suggest that the mutation of N378 would increase the reduction potential of FADH$^-/FADH^-$, leading to a less negative $\Delta G_{\text{FET}}^0$ compared with the wild type. M345 is located between the flavin and the 3'-thymine and the sulfur atom in its side chain is in van der Waals contact with the thymine ring$^{17}$. The sulfur-aromatic interactions have been extensively studied$^{46,47}$, and sulfur-containing amino acids were found to be weak electron donors$^{48,49}$. The mutation of M345 could make the dimer anion radical formed during repair reaction more stable after the forward ET, and result in the increase in reduction potential of T<>T/ T<>T$^-$. Therefore, the free energy $\Delta G_{\text{FET}}^0$ is more negative and the forward ET becomes more favorable.

(b) Regulation of cyclobutane ring splitting and futile back ET

After the forward ET from the excited state FADH$^-$, the excess negative charge makes the anionic radical T<>T$^-$ unstable and the C5-C5’ bond splits instantaneously. The resulting T-T$^-$ evolves along two pathways of either the C6-C6’ bond splitting or the back ET without repair. For the N5 position mutants (N378C and N378S), we observed the same second-bond splitting time in 88 ps as the wild type, in agreement with the fact that the mutation only affects the flavin cofactor. At the binding site, the mutation of the charged/polar residues E274 and R226 at the 5’ side diminishes stabilization of the anionic radical, resulting in much faster second bond-breaking time of 30 and 50 ps, respectively. Interestingly, R342A and M345A have similar time scales as the wild type, suggesting that the excess electron is mainly localized at the 5’ side after the C5-C5’ bond breakage, consistent with the observation in our previous study$^{25}$. We also observed the shorter time scales of the back ET for all six mutants compared to the wild type (2.4 ns). For N378C or N378S, the faster back ET process is mainly caused by the modulation of the redox potential of the flavin cofactor. Our recent theoretical studies have shown that free energy of larger than 1.7 eV is required to lengthen and break the C5-C5’ bond$^{21}$. Thus, the free energy for back ET after the C5-C5’ breakage must be around -0.34 eV (-1.96+1.7-0.08 eV) (Fig. 4). Assuming the reorganization energy to be similar as the forward ET ($\approx 1.2$ eV), the back ET should be in the Marcus normal region. The increased free energies $\Delta G_{\text{BET}}^0$ ($\Delta G_{\text{BET}}^0 = \varphi_{\text{T-T-T}} - \varphi_{\text{FADH}^-/\text{FADH}^+}$) of N378C < N378S < WT determine the back ET rates in a decreased order of N378C > N378S > WT. At the binding site, with the strong
hydrophilic interactions with T-T", the excess negative charge can be delocalized and the anionic radical can be stabilized by residues E274, R226 and R342. Mutations of these residues reduce the stabilization and dramatically accelerate the back ET rates, even more than one order of magnitude.
Supplementary References


