Title: Time-resolved crystallography captures light-driven DNA repair

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Abstract: Photolyase is an enzyme that employs light to catalyze DNA repair. To capture the reaction intermediates involved in photolyase's catalytic cycle, we conducted time-resolved crystallography measurements. We find the protein traps the excited state of the active cofactor, FAD, in a highly bent geometry. This excited state performs electron transfer to damaged DNA, inducing repair. We show the repair reaction, which involves the lysis of two covalent bonds, occurs *via* a single-bond intermediate. The resulting product does not have the same geometric complementarity for the active site as the substrate, explaining the change in affinity that drives product release.

One-Sentence Summary: Reaction intermediates determined by time-resolved crystallography reveal the excited state of FAD, a singly bonded thymine dimer, and mechanism of product release during DNA repair by photolyase.

Photolyases are enzymes that repair DNA lesions induced by solar radiation, elegantly employing light to do so (*1*). These enzyme's ancient origins suggest they were essential for early organisms to maintain genome integrity. They remain an important DNA repair mechanism in nearly all species today (*2–4*).

Distinct photolyases have evolved to repair the two most common DNA photolesions: cyclobutane pyrimidine dimers (CPD) and 6-4 adducts (*2–4*). CPDs account for ~80% of sunlight-induced DNA damage events. These lesions consist of two non-native carbon-carbon bonds between pyrimidine bases, most commonly sequence-adjacent thymines (*5–9*). CPD photolyases break these bonds, restoring the bases to their functional structure (Fig. 1). To do so, they consume a 350-450 nm photon as part of the catalytic cycle, making them one of the few known photoenzymes (*10*).

Repair by CPD photolyase begins with photoexcitation of a bound reduced flavin adenine dinucleotide cofactor (FADH-) (*6, 11*), either *via* direct photon absorption by FADH- or by resonant energy transfer from a second "antenna" cofactor that harvests radiation across a wider range of the visible spectrum (*12*). Within nanoseconds, the excited state (FADH-*) transfers an electron to the CPD lesion (*7, 13–16*). Facilitating electron transfer is a key function of the enzyme, as chemical models have shown that reduction of CPD is sufficient to break the pyrimidine dimer and produce repaired bases, even in the absence of the enzyme active site (*6, 17–19*).

Because the FADH-* excited state decays in tens of picoseconds in solution, an important question is how the enzyme stabilizes this excited state so that electron transfer occurs before de-excitation. The ratio of electron transfer to de-excitation events is a key factor in the overall quantum efficiency, which is very high in CPD photolyases with reported values from ~50% to >80% (*15, 20*). In contrast, chemical models of photolyase, lacking the enzyme's structure, achieve maximum quantum efficiencies of 1-5% (*6, 17–19*). To achieve these high quantum efficiencies, the enzyme must accommodate the transition from the ground to excited state, but then trap this state so that the de-excitation process is slower than the electron transfer timescale. The structure of this trapped intermediate, however, remains undetermined.

Of further interest is the role of the FAD binding mode, which is unique to the photolyase/cryptochrome family (*7–9, 21*). The enzyme bends FAD into a U-shaped conformation (Fig. 1A), such that the FAD adenine moiety sits between the electron-donating isoalloxazine ring and the electron-accepting CPD. This would presumably decrease the electron transfer rate and overall quantum efficiency as compared to a geometry where the isoalloxazine and the CPD were

immediately adjacent. While spectroscopic studies have suggested adenine mediates the electron transfer (*15*, *22*), a precise accounting of why this U binding mode was selected by evolution remains lacking. Structural characterization of adenine in the excited state is therefore of great interest.

After electron transfer to the CPD, the carbon-carbon bonds that form the nucleobase lesion are cleaved (*13*, *15*, *23*). Currently, the precise mechanism of carbon-carbon bond lysis is debated, especially whether the reaction proceeds *via* an intermediate with a single bond or not [see introduction of Ando *et al*., (2014) (*24*)].

Finally, following CPD lysis, rapid release of the cleaved bases is essential to enable the next turnover and maximize the total number of lesions repaired. Product release occurs in ~50 μs (*1*, *9*, *25*, *26*), significantly faster than the dissociation rate for the enzyme:substrate complex, which is on the order of seconds to minutes. This increased affinity for substrate over product is clearly advantageous; however, how the enzyme structure and dynamics that enable this discrimination have not yet been accounted for.

Time-resolved crystallography reveals the structural intermediates of photolyase catalysis

To determine the structure of the excited state, capture intermediates populated during CPD lysis, and characterize product release, we conducted time-resolved serial femtosecond crystallography (trSFX) experiments at the SwissFEL free-electron laser (*26*). We co-crystallized the CPD photolyase from the archaea *Methanosarcina mazei* (PL mmCPD), lacking the antenna cofactor, with a double-stranded DNA (dsDNA) substrate containing a synthetic *cis-syn* thymine dimer. Prior to crystallization, protein was photoreduced and maintained in this catalytically active state using an anaerobic environment. Our crystals form an orthorhombic lattice with two protein:DNA complexes in the asymmetric unit. All results here refer to one of these two complexes, which is better ordered than its counterpart (fig. S22). Employing a viscous media injector, a stream of microcrystals embedded in a cellulose matrix was delivered to the interaction region. DNA repair was initiated by a 1.1 ps pulse of 396 nm laser light and the resulting dynamics were probed by a pulse from the XFEL at time delays ranging from 3 ps to 100 μs.

To understand how photolyase interacts with the excited state FADH-*, we collected an ultrafast timepoint at 3 ps. Subsequently, to characterize CPD lysis, we collected five timepoints between 300 ps and 30 ns. Finally, to observe product release, we measured four timepoints between 1 μs and 100 μs (Fig. 1B, table S1). The resulting diffraction data enabled us to determine models of the dark state and, by refining against extrapolated structure factors (*26, 27*), 10 time-resolved structures. We observed quantitative improvement in the models by refining against data up to 2.1 Å to 2.4 Å depending on timepoint (table S1), however the resulting maps and models are comparable to structures determined from data with a highest resolution of 2.5-3.0 Å using standard methods (*26*).

Co-factor binding site facilitates dramatic butterfly bending of FADH- upon excitation

Repair chemistry begins with the photoexcitation of FADH-. To achieve high quantum efficiency, electron transfer to the substrate must be the fastest de-excitation pathway for the excited state. Transient absorption measurements show that FADH-* in solution exhibits three decay modes, with timescales of ~10 ps, ~50 ps, and ~2 ns (*28*) and branching ratios of 50-60%, 20-30%, and 10-15%, respectively. These same decay processes were observed in solutions of reduced flavin

mononucleotide (FNMH-), which lacks adenine, demonstrating quenching by adenine is not responsible for rapid decay (*28*). In contrast, similarly to other photolyases (*15*), PL mmCPD only shows a single ~2 ns decay mode in the visible spectrum (fig. S6). The enzyme channels all excited state population into this pathway, which is sufficiently long-lived to enable productive electron transfer.

We characterized this pathway by trSFX. Before laser illumination, the FADH- cofactor is in a bent geometry, characteristic of the reduced state, with the flanking benzene and pyrimidine rings forming a +13.5° "butterfly bending" angle around the N5-N10 axis (Fig. 2A). 3 ps after laser excitation, this bend inverts (Fig. 2B). The sp^3-hybridized N5 and N10 centers undergo pyramidal inversion, while the benzene and pyrimidine rings, distorted from their ground-state planar geometries, kink at -22.8°.

Spectroscopy performed by us in the absence of substrate (fig. S6) and others in both the presence and absence of substrate (*7*, *15*) suggest that FADH-* is the only populated species at 3 ps. Further, quantum mechanics calculations of reduced flavin excited states have suggested ring inversion upon excitation (*29*). Based on these observations, we assign the flavin geometry in our 3 ps structure to the FADH-* excited state.

The photolyase FADH- binding site enables 36.3° of butterfly-bending upon excitation, while simultaneously restricting FADH-* from reaching molecular geometries conducive to de-excitation. Butterfly bending disrupts only a single hydrogen bond between Arg378 and FAD N5 (2.9 → 4.0 Å, Fig. 2A). In contrast, all other stabilizing interactions between the protein and FAD, including hydrogen bonds with Asn403 and Asp409, are maintained upon excitation (Fig. 2A).

Further, ordered water nearby the isoalloxazine readily accommodates this dramatic butterfly bending, rearranging in response to excitation and forming a new water network within 3 ps (Figs. 2A, 2C). Specifically, upon excitation, a hydrogen bond between FAD carbonyl O2 and nearby highly coordinated water is broken as the water is displaced away from the isoalloxazine ring (2.7 Å → 3.9 Å). However, a second water strengthens its hydrogen bonding interaction with the same carbonyl (3.0 Å → 2.3 Å), simultaneously forming a new hydrogen bond with the sidechain of Ser268. These two waters are themselves hydrogen-bonded to one another both before and after their rearrangement. This water-toggle provides a second mechanism by which the FAD binding site is flexible enough to allow excitation, while simultaneously being able to form a hydrogen bond network in the excited state.

Electronic excitation disrupts adenine-associated water networks

To investigate why photolyase employs a U binding mode of FAD, inserting the adenine moiety in-between the electron-donating isoalloxazine ring and electron-accepting CPD, we interrogated our time resolved structures for dynamics that would reveal adenine's participation in the electron transfer reaction. At 3 ps, we observed disruption of two water networks in the active site that interact strongly with this adenine (Fig. 3).

The first network consists of a five-water cluster that fills a pocket in the active site of PL mmCPD near the 3' thymine (*9*). The second network contains two adenine-associated that waters fill a small void in the protein structure on the far side of the adenine ring (Fig. 3). One water from each group participates in a hydrogen bond with adenine atoms N7A and N1A in the dark state. At a 3 ps pump-probe delay, both these waters become disordered, while the adenine ring remains effectively stationary (Fig. 3, adenine atomic displacements ~0.2 Å). This rearrangement

propagates through the five-water cluster, disrupting a third water and inducing a 0.6 Å shift in the position of Arg256 (Fig. 3). At later timepoints, a shifting pattern of water density is observed (fig. S19), but by 10 ns, concurrent with the decay of the excited state FADH-*, both waters directly coordinating the adenine at N7A and N1A have regained order (fig. S19).

The water dynamics observed are notably specific, leading us to hypothesize that these water rearrangements may be caused by electronic coupling between the adenine and isoalloxazine systems in the excited state. We cannot exclude two alternatives; first, that a large difference dipole on the isoalloxazine system resulting from electronic excitation perturbs these waters through space without significant electronic coupling (see ref. (*30*)), or second that the adenine system is vibrationally hot and this thermal motion blurs the density of these coordinated waters. Indeed, the adenine ring B factors increase from 22 $Å^2$ to 37 $Å^2$ between dark and 3 ps, *vs.* 21 $Å^2$ to 29 $Å^2$ for the isoalloxazine system, suggestive of relatively increased disorder of the adenine (table S3). Insensitive to electronic structure, our crystallographic measurements cannot distinguish between these models, but provide a foundation upon which to design incisive future experiments and calculations that can.

Repair proceeds *via* stepwise bond-breaking of the cyclobutane pyrimidine

Between 300 ps and 3 ns we observe cleavage of the two carbon-carbon bonds that form the DNA lesion. Polder maps (*31*) omitting the thymine dimer region at 300 ps and 1 ns unambiguously show that the reaction proceeds *via* a transiently populated intermediate where the C5-C5' bond is broken (Fig. 4A, fig. S17, table S2). Whether or not the two cyclobutane bonds break concertedly or not has been extensively debated (*24*), but here we provide clear structural evidence for a single-bond mechanism.

At 3 ns, the second carbon-carbon bond is broken, and the planar, aromatic thymine systems are restored (Fig 4B). Between 300 ps and 3 ns, the structure of the FAD isoalloxazine ring flattens, reaching a bending angle of -4.1° by 3 ns (Fig. 2B), which we assign to the FADH• semiquinone state (*11*). Concomitantly, the hydrogen bond between the sidechain carbonyl of Asn403 and FAD N5 shortens (2.9 Å at 3 ps → 2.5 Å for 300 ps through 3 ns, table S3), confirming predictions from quantum calculations and IR spectroscopy measurements that show this interaction is essential to stabilize the semiquinone state (*32*).

Product release is governed by a geometric mismatch between product and active site

During repair, rearrangements of the product disrupt the geometric match between the bound thymines and the active site pocket, ultimately resulting in release of the repaired thymines. Immediately after lysis, the repaired nucleobases are forced apart, rotating from a thymine/thymine angle of 43° (dark) to nearly planar (15°, 10 ns) to enable π-stacking between the restored aromatic systems (Fig. 4B). As a result, the volume occupied by the thymine dimer increases significantly (462 $Å^3$, dark → 483 $Å^3$, 10 ns (*26*)), crowding the active site pocket and most notably displacing Met379 (Fig. 4B).

In this geometry, the 5' thymine π-stacks against Trp305 and maintains two strong hydrogen bonding interactions with Glu301 (Fig. 4B). In contrast, rotation of the 3' thymine disrupts the most mobile piece of the active site, a hinge consisting of Arg256, Asn257, and an ordered water, which holds the 3' thymine in place prior to repair. A hydrogen bond between 3' thymine N3 and the ordered water coordinated by Asn257 is broken by 300 ps (2.9 Å, dark → 3.9 Å, 300 ps),

causing the water to become mobile and move away from the nucleobase (Fig. 4B). The Arg256-Asn257 hinge, now uncoordinated, moves towards the 5' thymine, exposing the 3' thymine to solvent and providing a clear route for product release.

Between 10 ns and 1 μs, the enzyme:product complex undergoes only minor structural variations. The initial steps of product release are observed at 10 μs, with Met379, formerly pushed out of the active site, reversing direction and moving into the active site toward the 3' thymine (fig. S18). By 30 μs, this thymine flips out of the pocket completely (Fig. 5B, fig. S18, fig. S21), disrupting protein:DNA salt bridges between Arg411 and P_0 and Lys451 at P_{+1} (Fig. 5A). By 100 μs, the 5' thymine is mid-way through its withdrawal from the active site, and both repaired nucleobases exhibit significant disorder. In contrast, the phosphate backbone is still coordinated by salt bridges (Fig. 5C, fig. S18, fig. S21). We do not directly observe evidence of a restoration of Watson-Crick base pairing in our crystal structures, either because such rearrangements occur on longer timescales or are not compatible with the crystal lattice.

Our results indicate that prior to repair, the thymine dimer, with two ring systems strongly angled with respect to one another, is complementary with the active site geometry. In contrast, the repaired thymines, which adopt a planar π-stacked conformation, occupy a significantly larger volume in the active site and cannot form the same hydrogen bonding interactions at the 3' base, most notably with the Arg256-Asn257-water hinge system. Accordingly, thermal motion is sufficient to initiate release of the 3' base after tens of μs, followed by the 5' base hundreds of μs later (fig. S21). Geometric complementarity for substrate and mismatch for product, with respect to a largely static active site, underlies discrimination between substrate and product.

As with all enzymes, the structure of photolyase alters chemical rates to conduct biologically advantageous chemistry. Its catalyzed reaction begins with photoexcitation to a bent, trapped excited FADH-*. The long lifetime of this state facilitates electron transfer, resulting in DNA lysis via a one-bond mechanism. Finally, the repaired thymine dimer is not geometrically complementary to the active site and is therefore rapidly released. These processes together produce a powerful DNA repair machine, employed by nearly all lifeforms to survive under the sun.

References and Notes

1. A. Sancar, Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. *Chem Rev*. **103**, 2203–2237 (2003).
2. J. I. Lucas-Lledó, M. Lynch, Evolution of Mutation Rates: Phylogenomic Analysis of the Photolyase/Cryptochrome Family. *Mol Biol Evol*. **26**, 1143–1153 (2009).
3. Q. Mei, V. Dvornyk, Evolutionary History of the Photolyase/Cryptochrome Superfamily in Eukaryotes. *PLoS One*. **10** (2015), doi:10.1371/JOURNAL.PONE.0135940.
4. H. S. Black, F. R. DeGruijl, P. D. Forbes, J. E. Cleaver, H. N. Ananthaswamy, E. C. DeFabo, S. E. Ullrich, R. M. Tyrrell, Photocarcinogenesis: an overview. *J Photochem Photobiol B*. **40**, 29–47 (1997).
5. A. Sancar, Mechanisms of DNA Repair by Photolyase and Excision Nuclease (Nobel Lecture). *Angewandte Chemie International Edition*. **55**, 8502–8527 (2016).
6. T. Carell, L. T. Burgdorf, L. M. Kundu, M. Cichon, The mechanism of action of DNA photolyases. *Curr Opin Chem Biol*. **5**, 491–498 (2001).
7. M. Zhang, L. Wang, D. Zhong, Photolyase: Dynamics and Mechanisms of Repair of Sun-Induced DNA Damage. *Photochem Photobiol*. **93**, 78–92 (2017).
8. A. Mees, T. Klar, P. Gnau, U. Hennecke, A. P. M. Eker, T. Carell, L. O. Essen, Crystal structure of a photolyase bound to a CPD-like DNA lesion after in situ repair. *Science (1979)*. **306**, 1789–1793 (2004).

9. S. Kiontke, Y. Geisselbrecht, R. Pokorny, T. Carell, A. Batschauer, L. O. Essen, Crystal structures of an archaeal class II DNA photolyase and its complex with UV-damaged duplex DNA. *EMBO J*. **30**, 4437–4449 (2011).
10. L. O. Björn, Photoenzymes and Related Topics: An Update. *Photochem Photobiol*. **94**, 459–465 (2018).
11. M. Maestre-Reyna, C. H. Yang, E. Nango, W. C. Huang, E. P. G. Ngurah Putu, W. J. Wu, P. H. Wang, S. Franz-Badur, M. Saft, H. J. Emmerich, H. Y. Wu, C. C. Lee, K. F. Huang, Y. K. Chang, J. H. Liao, J. H. Weng, W. Gad, C. W. Chang, A. H. Pang, M. Sugahara, S. Owada, Y. Hosokawa, Y. Joti, A. Yamashita, R. Tanaka, T. Tanaka, F. Luo, K. Tono, K. C. Hsu, S. Kiontke, I. Schapiro, R. Spadaccini, A. Royant, J. Yamamoto, S. Iwata, L. O. Essen, Y. Bessho, M. D. Tsai, Serial crystallography captures dynamic control of sequential electron and proton transfer events in a flavoenzyme. *Nat Chem*. **14**, 677–685 (2022).
12. S. Kiontke, P. Gnau, R. Haselsberger, A. Batschauer, L. O. Essen, Structural and evolutionary aspects of antenna chromophore usage by class II photolyases. *Journal of Biological Chemistry*. **289**, 19659–19669 (2014).
13. A. W. MacFarlane IV, R. J. Stanley, Cis-Syn thymidine dimer repair by DNA photolyase in real time. *Biochemistry*. **42**, 8558–8568 (2003).
14. K. Brettel, M. Byrdin, Reaction mechanisms of DNA photolyase. *Curr Opin Struct Biol*. **20** (2010), pp. 693–701.
15. M. Zhang, L. Wang, S. Shu, A. Sancar, D. Zhong, Bifurcating electron-transfer pathways in DNA photolyases determine the repair quantum yield. *Science (1979)*. **354**, 209–213 (2016).
16. C. Tan, Z. Liu, J. Li, X. Guo, L. Wang, A. Sancar, D. Zhong, The molecular origin of high DNA-repair efficiency by photolyase. *Nat Commun*. **6**, 1–6 (2015).
17. Q. H. Song, W. J. Tang, X. B. Ji, H. B. Wang, Q. X. Guo, Do photolyases need to provide considerable activation energy for the splitting of cyclobutane pyrimidine dimer radical anions? *Chemistry - A European Journal*. **13**, 7762–7770 (2007).
18. J. Butenandt, A. P. M. Eker, T. Carell, Synthesis, crystal structure, and enzymatic evaluation of a DNA-photolesion isostere. *Chemistry - A European Journal*. **4**, 642–654 (1998).
19. R. Epple, E. U. Wallenborn, T. Carell, Investigation of flavin-containing DNA-repair model compounds. *J Am Chem Soc*. **119** (1997), doi:10.1021/ja964097u.
20. C. Tan, Z. Liu, J. Li, X. Guo, L. Wang, A. Sancar, D. Zhong, The molecular origin of high DNA-repair efficiency by photolyase. *Nature Communications 2015 6:1*. **6**, 1–6 (2015).
21. O. Dym, D. Eisenberg, Sequence-structure analysis of FAD-containing proteins. *Protein Science*. **10**, 1712–1728 (2001).
22. Z. Liu, X. Guo, C. Tan, J. Li, Y. T. Kao, L. Wang, A. Sancar, D. Zhong, Electron tunneling pathways and role of adenine in repair of cyclobutane pyrimidine dimer by DNA photolyase. *J Am Chem Soc*. **134**, 8104–8114 (2012).
23. V. Thiagarajan, M. Byrdin, A. P. M. Eker, P. Müller, K. Brettel, Kinetics of cyclobutane thymine dimer splitting by DNA photolyase directly monitored in the UV. **108** (2011), doi:10.1073/pnas.1101026108/-/DCSupplemental.
24. H. Ando, B. P. Fingerhut, K. E. Dorfman, J. D. Biggs, S. Mukamel, Femtosecond stimulated Raman spectroscopy of the cyclobutane thymine dimer repair mechanism: A computational study. *J Am Chem Soc*. **136**, 14801–14810 (2014).
25. A. Espagne, M. Burdin, A. P. M. Eker, K. Brettel, Very Fast Product Release and Catalytic Turnover of DNA Photolyase. *ChemBioChem*. **10**, 1777–1780 (2009).
26. Materials and methods are available as supplementary materials.
27. E. De Zitter, N. Coquelle, P. Oeser, T. R. M. Barends, J. P. Colletier, Xtrapol8 enables automatic elucidation of low-occupancy intermediate-states in crystallographic studies. *Communications Biology 2022 5:1*. **5**, 1–11 (2022).
28. Y. T. Kao, C. Saxena, T. F. He, L. Guo, L. Wang, A. Sancar, D. Zhong, Ultrafast dynamics of flavins in five redox states. *J Am Chem Soc*. **130**, 13132–13139 (2008).
29. Y. Ai, C. Zhao, J. Xing, Y. Liu, Z. Wang, J. Jin, S. Xia, G. Cui, X. Wang, Excited-State Decay Pathways of Flavin Molecules in Five Redox Forms: The Role of Conical Intersections. *Journal of Physical Chemistry A*. **122**, 7954–7961 (2018).
30. R. F. Pauszek, G. Kodali, M. S. U. Siddiqui, R. J. Stanley, Overlapping Electronic States with Nearly Parallel Transition Dipole Moments in Reduced Anionic Flavin Can Distort Photobiological Dynamics. *J Am Chem Soc*. **138**, 14880–14889 (2016).

31. D. Liebschner, P. V. Afonine, N. W. Moriarty, B. K. Poon, O. V. Sobolev, T. C. Terwilliger, P. D. Adams, Polder maps: Improving OMIT maps by excluding bulk solvent. *Acta Crystallogr D Struct Biol*. **73**, 148–157 (2017).
32. I. M. M. Wijaya, T. Domratcheva, T. Iwata, E. D. Getzoff, H. Kandori, Single Hydrogen Bond Donation from Flavin N5 to Proximal Asparagine Ensures FAD Reduction in DNA Photolyase. *J Am Chem Soc*. **138** (2016), doi:10.1021/jacs.5b10533.

Acknowledgments: The authors would like to thank J. Maracke, L. Gumprecht, H. Fleckenstein, D. Amores, A. Zhu, F. Jabbarpour and A. Peck for technical assistance, G. Consoli for advice concerning graphic design and presentation, and R. Stanley, D. Hekstra, M. Byrdin, K. Hadjidemetriou, G. Kurisu, and D. Zhong for stimulating discussions and feedback. TL would like to acknowledge S. Boutet for mentorship and support early in the project during his time at SLAC. NC would like to thank M. Weik for his mentorship and support. We acknowledge the Paul Scherrer Institute, Villigen, Switzerland for provision of free-electron laser beamtime at the Alvra instrument of the SwissFEL ARAMIS branch. Finally, the authors would like to acknowledge the support of the Helmholtz Association and DESY in the form of core funding and infrastructure.

Funding:

Helmholtz Association, Young Investigator Group (YIG) award (TJL)

Swiss National Science Foundation NCCR-MUST (CB)

Göran Gustafsson Foundation, Grant No. 1808 (JA)

Carl Trygger Foundation, CTS 21:1427 (JA)

Imperial College London President's PhD Scholarship (AF)

Cluster of Excellence ‘Advanced Imaging of Matter’, Deutsche Forschungsgemeinschaft (DFG), EXC 2056, project ID 390715994. (VA, SB, HNC)

National Science Foundation (USA) Graduate Research Fellowship (JH)

Department of Energy (USA), SCGSR program (JH)

Helmholtz Association, FISCOV/SFragX (PR)

Author contributions:

Conceptualization: TL

Methodology: TL, NC, DM, MR, NH

Investigation: TL, NC, DM, VA, AH, JS, DO, SG, AF, AP, AM, MG, OY, PR, VK, ES, EL, PM, JS, RS, CB, CC, EB, JHB, PJ, FD, DO, QB, MW, EDZ, DT, NH, MR, AK, JH, SR, DC, SW, SB, SB

Visualization: TL, NC

Funding acquisition: TL, HC

Project administration: TL, NC

Supervision: TL, HC

Writing: TL, NC

Competing interests: TJL is an employee and shareholder of CHARM Therapeutics.

Data and materials availability: All raw diffraction data (https://doi.org/10.16907/87a9f7a4-073e-430c-aa7f-6a194897074f) and processed CrystFEL stream files (https://doi.org/10.16907/49d16998-9faf-4ae9-b48a-17aea963be65) are available via SciCAT. Code used to perform data processing is available at https://github.com/virginia4/Swissfel_Scripts, code used to perform subsequent analysis is available at https://github.com/tjlane/mmcpd-scripts.

Supplementary Materials

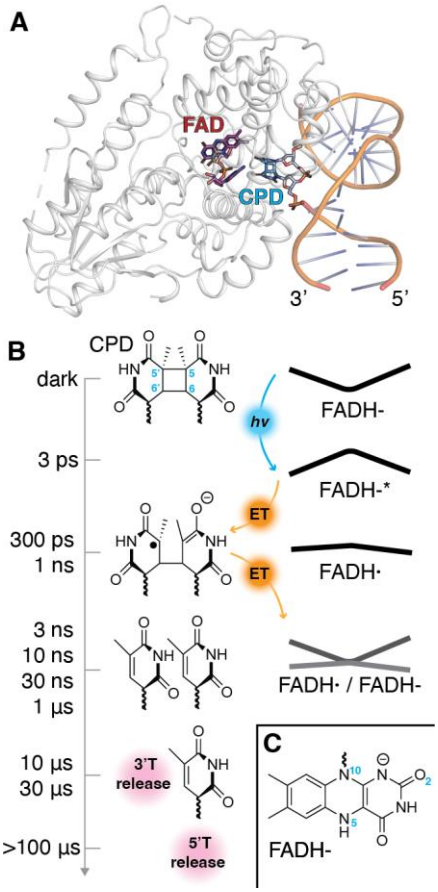

Fig. 1. The reaction mechanism of DNA photolyase captured by time-resolved crystallography. (**A**) Structure of photolyase co-crystallized with dsDNA 14-mer containing a cyclobutane pyrimidine dimer (CPD). CPD binds adjacent to the FAD cofactor. (**B**) Schematic of the reaction mechanism, with the pump-probe delays acquired in this study (left), the corresponding thymine dimer states (middle), and a schematic of the observed FAD bending angles (right). Blue line indicates photon absorption and orange line electron transfer. The final superimposed conformation of FADH-/FADH• reflects the ambiguity in redox state assignment from our crystal data alone (fig. S16, S21). At the longest timepoint studied (100 µs), the 5' thymine is only partially released, as indicated by the time axis. (**C**) Chemical structure of the isoalloxazine ring of FAD in the hydroquinone anion state. Abbreviations, *hv*: photon absorption, ET: electron transfer, blue text: atom numbering referred to in main text.

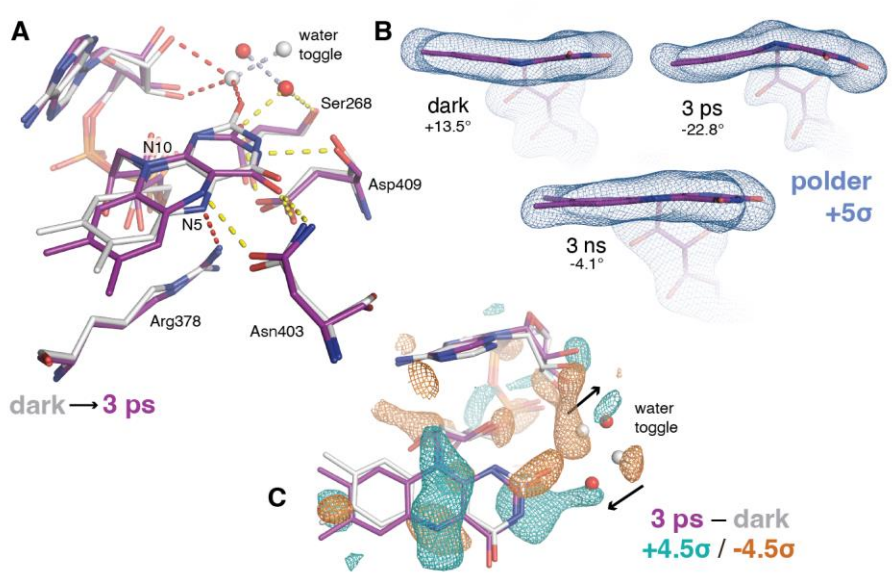

Fig. 2. FAD geometry and excited state stabilization. (**A**) Excited state of FAD (3 ps: purple, waters as red spheres) superimposed on the ground state (dark state: grey, waters as grey spheres). The FAD isoalloxazine ring participates in several hydrogen-bonding interactions in both the dark and 3 ps structures. Dashed lines show hydrogen bonding interactions (yellow: present at 3 ps, red: broken between dark state and 3 ps, grey: maintained water toggle hydrogen bond). (**B**) Excitation induces severe butterfly-like bending around the N5-N10 axis of FAD, with angles indicated. Maps: polder omit mF_{extr}-DF_c, 5 σ. Dark: fully reduced (FADH-), 3 ps: excited state (FADH-*), 3 ns: semiquinone (FADH•). (**C**) Same as (**A**), but rotated view with the isoalloxazine in the plane of the page and with $F_{o,3ps}$-$F_{o,dark}$ difference map superimposed (teal/orange, +/- 4.5 σ respectively).

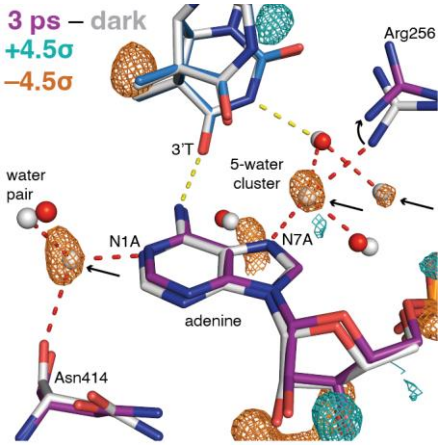

Fig. 3. Electronic excitation disrupts local water networks around adenine. Disrupted hydrogen bonds (red dashed lines) in the 5-water cluster and adenine-associated water pair at 3 ps following electronic excitation of FAD (3 ps: purple, red spheres/dark state: grey, grey spheres). Arrows highlight key waters that become disordered following electronic excitation of FADH-. Key retained hydrogen bonds shown as yellow dashed lines. Maps shown $F_{o,3ps}$-$F_{o,dark}$ (teal/orange, 4.5 σ) showing the loss of density at sites of water that is hydrogen-bonded to adenine in the dark state. In the dark model, N1A-water distance is 2.4 Å, N7A-water distance is 2.8 Å. See fig. S19 for $2mF_{extr}$-DF_c map.

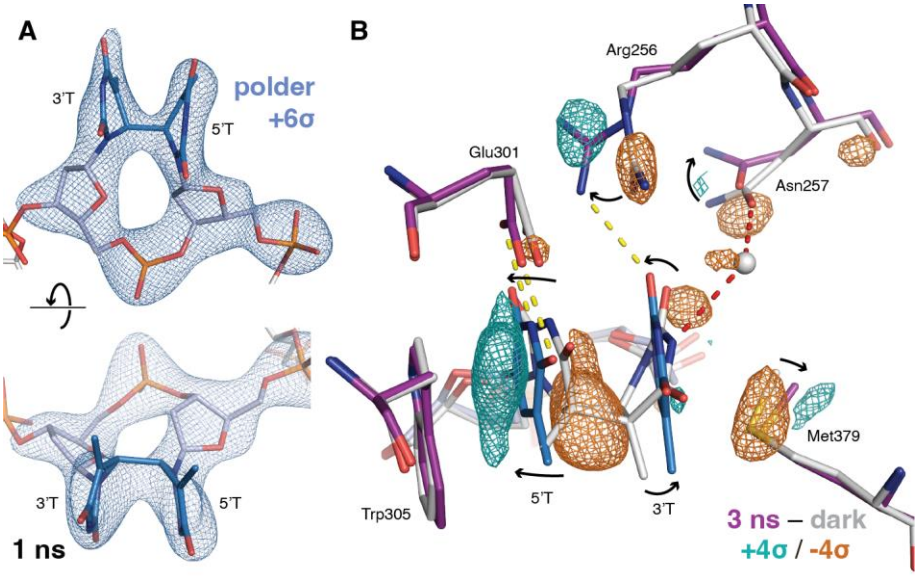

Fig. 4. DNA repair proceeds *via* a one-bond intermediate, resulting in geometric mismatch with active site. (**A**) Polder omit maps (blue, 6 σ) at 1 ns pump-probe delay provide evidence for a one-bond intermediate. (**B**) By 3 ns, repair is largely complete, with thymine bases forming a coplanar, π-stacked thymine geometry (3 ns: purple, dark state: grey, grey sphere, teal/orange $F_{o,3ns}$-$F_{o,dark}$ at 4 σ). This disrupts hydrogen bonding between the 3' thymine and an ordered water previously coordinated by Asn257 (red dashed lines, lost H-bonds). With this interaction disrupted, Arg256 forms a new interaction with the 5' thymine (yellow dashed lines, maintained H-bonds), weakening the interactions of the 3' thymine with the active site.

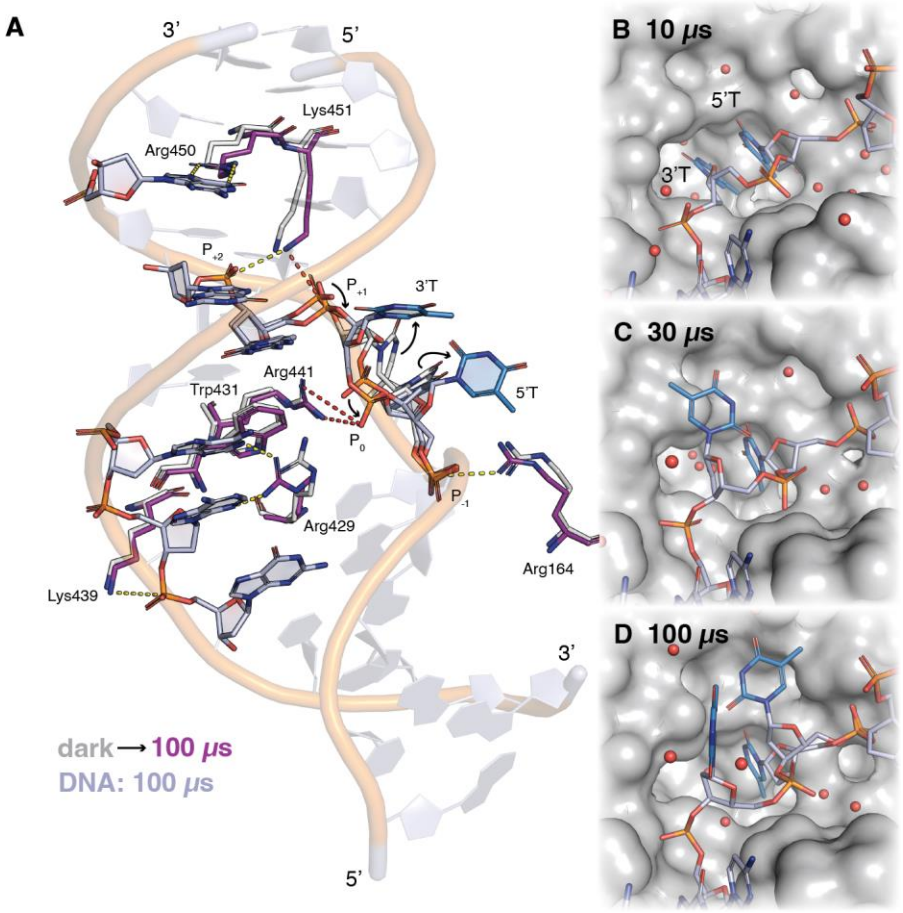

Fig. 5. Product release and disruption of DNA/photolyase interactions. (**A**) Overview of the DNA binding mode of photolyase before (dark state: grey) and after (100 µs: purple) repair. DNA backbone conformation shown is structure at 100 µs. Interactions that are retained or formed at 100 µs are shown in yellow dashed lines, while interactions that are lost, most notably at the thymine dimer site (P_0-Arg441 and P_{+1}-Lys451) are shown as red dashed lines. (**B**-**D**) Product release: (**B**, 10 µs) both thymines bound in the active site, (**C**, 30 µs) 3' release, (**D**, 100 µs) partial 5' release. After exiting the active site pocket, thymines are only partially ordered: conformation of 3' thymine at 30 µs and 5' thymine at 100 µs modeled outside of the pocket are partially occupied (fig. S18, fig. S21).