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1 **Watching the entire DNA repair process by a photolyase at atomic resolution in real time**

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48

49 **Abstract**

50 Photolyases, a ubiquitous class of flavoproteins, are light-dependent enzymes that employ blue
51 light to repair DNA photolesions. Here, we determined the structural mechanism of photolyase-
52 catalyzed repair of a cyclobutane pyrimidine dimer (CPD) lesion at atomic and picosecond
53 resolutions via time-resolved serial femtosecond X-ray crystallography (TR-SFX). We obtained
54 18 snapshots at different time points, whose predominantly accumulated structures were solved by
55 structure factor extrapolation followed by structural refinement. As this light-triggered process
56 involving electron transfer to the CPD lesion required a high laser power for achieving reasonable
57 repair efficiencies, we carefully addressed and discussed potential multiphoton effects. Each
58 structural snapshot is assignable to elementary steps of the catalytic cycle given time-dependent
59 changes in four reaction loci and corroborating data from complementary methods. The resulting
60 3D movie depicts first the repair of CPD lesions in the pico- to nanosecond range and how it is
61 orchestrated by conformational changes of the catalytic flavin and other active site moieties.
62 During the first nanoseconds, intermediate structures show the steps of forward electron transfer
63 between the coenzyme FADH⁻ and the CPD lesion, bond breaking, ring opening, and back electron
64 transfer. These chemical steps are followed by recovery of the enzymatic moieties involved in
65 catalysis, forming the fully reduced enzyme-product complex at 500 nanoseconds. Finally, during
66 product release, back-flip intermediates of the thymine bases to reanneal the dsDNA were captured
67 at 25-200 microseconds. Our data cover now the complete molecular mechanism of one of the
68 most widespread DNA repair mechanisms, and importantly, its chemistry and enzymatic catalysis
69 at work in real time and at atomic resolution.

70 The last 5-10 years have seen major breakthroughs in the determination and analysis of
71 macromolecular structures, contributed by two powerful techniques, cryo-electron microscopy
72 (cryo-EM) and time-resolved serial femtosecond crystallography (TR-SFX) using X-ray free
73 electron lasers (XFEL). While the major advantage of cryo-EM is its capability to solve large
74 complexes (1, 2), TR-SFX enabled the monitoring of light-induced structural changes of
75 macromolecules in real time (3–5). So far, the majority of TR-SFX studies addressed *trans-cis*
76 isomerization and resulting conformational changes in photoreceptors (6–10) or ion pumps (11,
77 12), and the redox mechanism of photosystems (13–17). Only recently several TR-SFX reports
78 have started to analyze the structural mechanisms of enzymatic catalysis (16–21).

79 Photolyases catalyze the repair of damaged DNA containing UV-induced lesions, including
80 cyclobutane pyrimidine dimers (CPD) and the 6-4 photoproduct (6-4PP) (22, 23). CPD lesions are
81 the most abundant in nature upon UVB exposure and the major cause for skin cancer in humans
82 (24). Catalysis by CPD photolyases involves multiple redox reactions and a multi-step splitting of
83 the cyclobutane ring (25). The enzyme is first activated via photoreduction of its coenzyme flavin
84 adenine dinucleotide (FAD) from the oxidized (FAD_{ox}) to the reduced state (FADH^-) (26). This
85 process requires two single-electron transfer steps mediated by an electron transfer chain involving
86 three tryptophan residues, yielding respectively the radical semiquinone intermediate ($\text{FAD}^{\bullet-}$ or its
87 protonated form FADH^\bullet) and the reduced, anionic hydroquinone state (FADH^-) (27). Only the
88 latter is capable of catalyzing light-induced DNA repair. The mechanisms of photoreduction and
89 DNA repair have been elucidated by extensive spectroscopic and theoretical studies over the past
90 three decades (22, 26, 28–32). However, relatively limited information from high-resolution
91 crystal structures is available, and, except from apo-forms (33–35), all such structures were
92 obtained using synchrotron X-ray radiation that induced both photoreduction (36–38) and DNA
93 repair reactions (39).

94 Recently, we employed TR-SFX to elucidate the structural mechanism of the photoreduction
95 processes in the *Methanosarcina mazei* class II CPD photolyase (*MmCPDII*) (33). We identified
96 an Asn/Arg-Asp redox sensor triad that regulates FAD re-hybridization and protonation, and
97 observed buckling and twisting of the isoalloxazine ring of the coenzyme FAD, which occurred in
98 the submicrosecond regime after the light-triggered electron transfer step. The TR-SFX study of
99 the photoreduction process has set the stage to study the main function of the *MmCPDII* photolyase,
100 the now presented repair of CPD lesions.

101 On the basis of extensive spectroscopic and computational analyses (22, 23, 25, 26, 40, 41),
102 the repair of the cyclobutane-bridged TT dimer ($T\langle\rangle T$, **1** in **Fig. 1A**) by photolyases was suggested
103 to go through three intermediates. First, a forward electron transfer (forward ET or FET) from the
104 reduced coenzyme $FADH^-$ produces $(T\langle\rangle T)^{\bullet-}$ (**2**), where the excess electron may be delocalized
105 between the two pyrimidines. The resulting CPD radical anion undergoes then cleavage of its C5-
106 C5' bond to produce $(T_T)^{\bullet-}$ (**3**) and further cleavage of the C6-C6' bond to give $(T+T)^{\bullet-}$ (**4**). The
107 latter then transfers an electron back to the flavin coenzyme to produce the repaired thymines (T+T,
108 product, designated as **5** in **Fig. 1A**). While such a repair mechanism is chemically feasible and
109 well supported by spectroscopic data, structures of its reaction intermediates await elucidation in
110 the enzyme bound form. Furthermore, given the moderate quantum yields for DNA repair, at least
111 in class II photolyases, the underlying interactions between reaction intermediates, the
112 isoalloxazine ring of the coenzyme and active site residues of the photolyase, remain to be probed
113 by high-resolution data. The first goal of this TR-SFX study was hence to identify and characterize
114 five reaction intermediates corresponding to the chemical intermediates of **Fig. 1A** in the DNA-
115 enzyme complex, **Int1 to Int5**. Their assignment was based on observed changes of the
116 coenzyme's geometry, conformations of active site residues and the chemical state of the CPD
117 during its repair.

118 When complexed to photolyases, the TT dimer in UV-damaged DNA, which retains base
119 pairing with adenine counterbases in the unbound state, though somewhat twisted (42), flips out
120 of double-stranded DNA (dsDNA) to interact with the FAD coenzyme and active site residues. This
121 double-flip causes a strong kink in the canonical B-DNA structure, and creates an unpaired bubble
122 that is stabilized by the photolyase's bubble-intruding region (BIR) (43). Accordingly, a second
123 goal of this TR-SFX study was to identify the conformational intermediates (**Con**), which
124 correspond to the inverse conformational changes required for dsDNA reannealing and dissolution
125 of the DNA-photolyase complex after completion of the chemical CPD repair. As photolyases bind
126 regular, i.e. already repaired, dsDNA >4 orders of magnitude weaker than UV-damaged dsDNA
127 (44), this analysis may be particularly rewarding given that spectroscopic signatures of thymine
128 bases are essentially identical before and after flip-back so that very little dynamic information is
129 available for the late part of the reaction cycle.

130
131

132

133 **Results**

134 **Tailoring TR-SFX for uncovering the photolyase mechanism**

135 Our DNA repair study depended on co-crystals of fully reduced *Mm*CPDII (32) and CPD-
136 containing DNA (**Fig. 1B**) (43), which were grown within 12 h prior to use directly at XFEL sites
137 using anaerobic tents and safety light conditions. Two sets of TR-SFX data were collected: One
138 covers the CPD repair reaction itself from 100 ps to 10 ns (the ps-ns series) as performed at
139 SwissFEL, where crystals were excited via a 0.98 ps long, 10 μ J, 400 nm pump laser pulse with a
140 50 μ m diameter focal spot. For the second time series that covers relaxation of the photolyase-
141 product complex and DNA release (10 ns - 200 μ s, the ns- μ s series, SACLA), samples were
142 excited via a 3 ns long, 150 μ J, 408 nm pump laser pulse with a 100 μ m focal spot diameter.
143 Although illumination at \sim 400 nm likely generates a mixture of S1 and S2 excited FADH^{*} species,
144 the S2 \rightarrow S1 decay has been noted to occur within a few ps, considerably faster than FET (45).

145 For photolyase-mediated DNA repair TR-SFX conditions were optimized for achieving
146 anticipated DNA repair yields, as detailed in **Supplementary Text S1** and **figs. S1, S2**. A brief
147 summary is provided here: (i) We confirmed that the oxidized form of *Mm*CPDII, was unable to
148 initiate FET towards CPD and failed to cause any effects including repair of the CPD under very
149 high laser powers, both under ps-ns and ns- μ s conditions (**figs. S1A, B**). (ii) Prior to TR-SFX
150 experiments addressing CPD repair itself, we validated that difference density signatures of the 10
151 ns TR-SFX structure (**fig. S1E**) coincided with that initially reported from X-ray radiation-
152 triggered repair (**fig. S1C**) (38, 39) and low-dosage LED-induced repair (**fig. S1D**). (iii) Reduction
153 of laser power from 10 μ J to 5 μ J in the ps-ns series caused diminished signature signals (**fig. S2B**).
154 (iv) Control experiments for light contamination assured clean dark datasets (**figs. S2A, B**).

155 In TR-SFX experiments the power and energies of laser excitation pulses generally exceed
156 one absorbed photon per chromophore thus implying potential multiphoton effects in light-
157 triggered systems, which may affect the photochemical reaction course and dynamics (46–51). We
158 found that not more than \sim 68 % of incident light is accessible for microcrystals embedded in grease
159 due to light scattering (**Supplementary Text S2** and **fig. S3**). Accordingly, calculation of photon
160 dosages as absorbed by the enzyme have to take this and other factors like the quantum yields into
161 account. Nevertheless, our results indicated that up to 10 and 30 photons are potentially absorbed

162 per FAD chromophore per pulse for the ps-ns and the ns- μ s series, respectively (**Supplementary**
163 **Text S3**), which are high enough to cause activation of the FADH⁻ coenzyme to levels higher than
164 the S1 state. However, electronic relaxation of FADH^{-*} to S1 from higher states than S2 is
165 expected to proceed even faster than the S2 \rightarrow S1 transition by internal conversion (45), i.e. in the
166 sub-ps range. Given the time-resolution of our TR-SFX experiments (**Table 1**), multiply excited
167 FADH⁻ species should have been decayed prior to the first catalytic event, i.e. electron-transfer to
168 the CPD lesion.

169

170 **Analyzing TR-SFX data for photolyase-mediated CPD-DNA repair**

171 Given the complex set of TR-SFX data a brief explanation of methods for structural analysis and
172 nomenclature is given first. For structure factor extrapolation and refinement, we followed
173 previously described guidelines (5, 9, 52, 53). For both time-series, the basic conditions and main
174 structural features are summarized in **Table 1**, while the detailed structural parameters are listed
175 in **tables S1 and S2**. Each structure is given a name in column 1 of **Table 1**, e.g. F10ns and N10ns
176 for the 10 ns snapshots, where the one letter code indicates the type of pumping laser (F for
177 femtosecond laser pump in the ps-ns series, and N for nanosecond pump in the ns- μ s series),
178 followed by the delay time after illumination (with “dark” designating a non-illuminated time-
179 resolved control structure). Exceptions are the steady-state structures in the oxidized (ox/ss) and
180 fully reduced states (Fdark, Ndark). Isomorphous difference electron density maps (ΔF_o) (54) were
181 calculated to decipher structural differences, which are designated as difference between two states,
182 $\Delta F_o(Y-X)$.

183 Major properties of each structural snapshot are depicted in columns 3-11 of **Table 1**,
184 which include features from the four main reaction loci (**Fig. 2**). These properties are considered
185 in the interpretation of each structural snapshot. For the ps-ns series the focus was on CPD repair.
186 The structural snapshots were hence assigned to match the TT status of the proposed intermediates
187 **1-5 (Fig. 1A)**, and designated as **Int1-Int5** of the photolyase-DNA complexes (**Table 1**). For the
188 ns- μ s series with its already completed CPD repair reaction, structural snapshots reflecting post-
189 repair reactions are designated as conformational intermediates (**Con1-Con4**), with close variants
190 being further subdivided by a, b, ...

191 Structural analysis of the TR-SFX data was conducted in three rounds: First, on the basis
192 of mainly structural changes at CPD and FADH⁻ (two of the four reaction loci, **Fig. 2**), each time-

193 point was assigned intermediates playing a role in the catalytic cycle. Next, structural changes in
194 the other two reaction loci were included to fine-tune the interpretation of each time point. Finally,
195 all datasets and interpretations were considered together along with additional supporting evidence
196 derived from electron density analyses, singular value decomposition (SVD), and quantum
197 mechanical (QM) calculations.

198

199 **The four main reaction loci of CPD-DNA repair by *Mm*CPDII**

200 Global structures of the *Mm*CPDII-DNA complex as determined via SFX in both the oxidized and
201 fully reduced states (ox/ss, Fdark., **Fig. 2A**, and Ndark) agree well with prior synchrotron
202 structures of the DNA complex in the oxidized states (37, 43) and *Mm*CPDII SFX structures
203 without bound DNA in all redox states (33). However, difference density peaks are found at four
204 main loci: the CPD structure (**Fig. 2B**), the FAD coenzyme (**Fig. 2C**), the active site moieties R256
205 and 5WC (**Fig. 2D**), and the class II photolyase-defining BIR (**Fig. 2E**). These loci show also all
206 prominent difference density map features after light-triggered DNA repair, though the general
207 protein fold itself is not affected (**fig. S4**). In this section, we survey the four main reaction loci
208 and explain how the relevant data in **Table 1** were obtained.

209 (1) CPD locus: To track CPD repair itself, we used two key indicators based on the
210 characteristic CPD difference density features. First, we determined the C5-C5' and C6-C6' peak-
211 to-peak distances (designated as C-C peak distances) between the maxima of the difference map
212 peaks along the axes of the C5-C5' and C6-C6' bonds (**Table 1**, column 8 and footnote c,
213 **Materials and methods summary, fig. S5**). Secondly, we calculated the average integrated
214 negative electron densities (designated as negative densities) from difference maps around C5 and
215 C6 atoms as a function of time (**Table 1**, column 9 and footnote e, **Supplementary Text S4**, and
216 **fig. S13B**), which is a good indicator of the accumulation of repair intermediates where the
217 corresponding bond had been broken.

218 (2) FAD locus: Subtle redox-dependent changes around the flavin site, including increased
219 buckling of the FAD upon reduction (**Fig. 2C**), is consistent with our recent studies of *Mm*CPDII
220 photoreduction (33). There, we showed by monitoring ρ_C and ρ_N dihedral angles (**Fig. 1C**) that the
221 isoalloxazine ring of the FAD coenzyme undergoes a sequence of buckling and twisting motions
222 during the process of photoreduction and protonation. In the oxidized state FAD_{ox}, the
223 isoalloxazine moiety was only very mildly buckled (ρ_C , ρ_N : 2.0°, 2.0°), whereas in the catalytic

224 FADH⁻ state, the isoalloxazine moiety was strongly buckled (ρ_C , ρ_N : 14.3°, 14.5°). In the
225 semiquinone FADH[•] state, it moved closer to planarity (ρ_C , ρ_N : 4.6°, 4.8°) (33). Since FADH⁻ is
226 transiently oxidized into FADH[•] upon forward ET toward CPD, then recovered upon reaction
227 completion, the ρ_C and ρ_N dihedral angles may hence well reflect the electron flow during CPD
228 repair (**Table 1**, columns 4 and 5).

229 (3) 5WC/R256 locus. In the active site, the substrate interacts with several key features of
230 the enzyme (**Fig. 2D**), including the adenine moiety of FAD, the highly conserved residue R256,
231 and the structurally conserved five-water cluster. In the fully reduced state, i.e. Fdark and Ndark,
232 the 5WC bridges the bisphosphate backbone of FAD and R256 so that it contributes to the
233 electrostatic stabilization of the anionic FADH⁻. Upon FET, R256 moves to stabilize the CPD
234 radical anion and the 5WC becomes disordered or dynamic. Changes in 5WC/R256 during repair
235 are monitored in **Table 1** (column 6 and footnote f).

236 (4) BIR locus: In the bubble-intruding region (**Fig. 2E**), the D428/W431/R441 triad acts as
237 a lock, both stabilizing the unpaired bubble, and firmly maintaining the protein-DNA complex
238 (43), while R429 is more flexible, and capable of dynamically interacting with both CPD
239 complementary bases, dA7' and dA8'. Here, post-repair base flip-back induced opening of the
240 BIR lock, as shown in **Table 1** (column 7 and footnote g).

241
242 Given our structural snapshots the entire process of photolyase-mediated repair of dsDNA
243 can be assigned to three periods, i.e. early, middle, and late events, which correspond to CPD repair,
244 active site recovery, and thymine back flip-DNA release, respectively. The loci of CPD structure
245 and FAD geometry will be considered first to assess the primary repair process. The remaining
246 two reaction loci will be then separately addressed. Taken together the results are then used to
247 assemble a molecular movie covering DNA repair and ordered product release by photolyases.

248

249 **Early events: Electron transfer-driven repair of the CPD lesion**

250 The refined structures and difference maps from the ps-ns series monitor both the ring-opening
251 steps of the CPD repair reaction and the concomitant FAD isoalloxazine moiety dynamics (**Fig.**
252 **3A**). The C5-C5' and C6-C6' peak distances as well as the integrated negative electron densities
253 (**Fig. 3B**) and the flavin dihedral angles ρ_C , ρ_N (**Fig. 3C**) are plotted over time. The structure of
254 each time point in Figure 3A represents the predominantly accumulated species of a snapshot

255 during CPD repair. Although the structural changes shown here are subtle, the $\Delta F_c(Y\text{-dark})$ of
256 each individual structure is always maximally correlated to its corresponding $\Delta F_o(Y\text{-dark})$ (**figs.**
257 **S5A, S5B**), supporting the reliability and significance of the refined structural changes, and
258 yielding a clear view of the process of DNA repair.

259 Accordingly, we have observed all proposed CPD repair intermediates **1** to **5** (**Fig. 1A**) in
260 the enzyme bound form as **Int1/Int1*** to **Int5** (**Table 1**), where the F_{dark} structure represents **Int1**
261 ($T\langle\rangle T/FADH^\bullet$). At 650 ps, the magnitude of the negative density feature around the C5-C5' bond
262 is much higher than around the C6-C6' bond. In good agreement, the C5-C5' peak distance is 1.9
263 Å (**Figs. 3A, B** and **Table 1**) implying a broken C5-C5' and an intact C6-C6' bond, suggesting
264 that the CPD ring-opening intermediate (T_T) $^\bullet$, **Int3**, is dominant in the F650ps snapshot. At
265 F1ns, with the negative density around C5-C5' being strong and that around C6-C6' growing, both
266 C5-C5' and C6-C6' difference density peak distances reach 1.9 Å. Here, the dominant species
267 corresponds to the repaired CPD intermediate ($T+T$) $^\bullet$, **Int4**. Interestingly, these trends continue
268 to the F2ns snapshot, suggesting a continuing build-up of **Int4**. This in turn suggests that F1ns may
269 still contains a contribution by **Int3**.

270 The first structure after photon absorption corresponds to the activated coenzyme, $FADH^\bullet$
271 * . The F100ps snapshot (**Fig. 3A**) might represent this state since its CPD structure is unaltered
272 while its isoalloxazine ring has greatly changed. Nevertheless, the R256 side chain has moved
273 toward CPD, suggesting that FET from $FADH^\bullet$ has already onset at this point. In the F250ps
274 snapshot, the isoalloxazine ring continued to swing, as shown by sign-changes of its ρ_C and ρ_N
275 angles (**Fig. 3C**). Although the CPD structure remains still unchanged, the nearby active site
276 moieties R256/5WC have already onset to move as described later. These changes suggest that
277 FET is in progress during both F100ps and F250ps snapshots, which represent a mixture of species,
278 $T\langle\rangle T/FADH^\bullet$ (**Int1***) and ($T\langle\rangle T$) $^\bullet/FADH^\bullet$ (**Int2**). This ET-dependent reorganisation is in good
279 agreement with such ultrafast redox transitions being stabilized via rapid isoalloxazine fluttering
280 (55), and harmonic motions of other light-gathering cofactors after absorption (49).

281 In the F450ps snapshot, a C5-C5' peak distance could be measured for the first time as C5-
282 C5' negative density has accumulated. Nevertheless, in the refined structure both the C5-C5' and
283 C6-C6' bonds remained intact. This snapshot can be hence assigned to an **Int2/3** mixture, where
284 **Int2** is the major species. Interestingly, from F450ps and up to F1ns, structures showed relatively

285 planar FAD isoalloxazine geometries compared to the dark, fully reduced state (**Fig. 3C**), which
286 is expected for the semiquinoid FADH[•] form (33). This indicates that FET is mostly finished in
287 the F450ps snapshot, and that the radical anionic CPD species dominate these snapshots. However,
288 immediately after the 1 ns delay (at 2 ns and 3.35 ns), there are remarkable oscillatory motions of
289 the FAD's isoalloxazine ring as indicated by ρ_C and ρ_N dihedral angles, which correlate well with
290 fluttering that is associated with rapid relaxation after sudden redox changes (22). This fluttering
291 suggests back electron transfer (BET) from the thymine radical anion to the coenzyme and is in
292 good agreement with a BET time constant of ~700 ps according to time-resolved spectroscopic
293 studies of CPD repair by photolyases (56, 57). Accordingly, we assign the F2ns and F3.35ns
294 snapshots as representatives for the BET reaction, which should contain varying mixtures of
295 (T+T)^{•-}/FADH[•] (**Int4**) and (T+T)/FADH[•] (**Int5**). In the F6ns snapshot, the isoalloxazine
296 oscillatory motion appears to have receded, and therefore we propose that F6ns consists
297 predominantly of the product after completion of the CPD repair, **Int5**. Since the structural
298 properties of the next snapshot F10ns are very similar to F6ns (**Fig. 3A** and **Table 1**), F10ns
299 represents further accumulation of the **Int5** product.

300

301

302 **Middle events: base stacking and FAD site recovery**

303 The ns- μ s series of TR-SFX data addresses conformational changes of the repaired thymines and
304 enzymatic moieties during product release. Throughout this series, the status of the intact thymines
305 (T+T) and the coenzyme FADH[•] corresponds chemically to **Int5**. Nevertheless, given that the T+T
306 and FADH[•] conformations and active site arrangements also differ in detail besides later
307 conformational changes of the photolyase-product complex, we describe them as distinct
308 conformational intermediates. Notably, the N10ns snapshot (**Con1**, first structure in **Fig. 4A**) and
309 the F6ns/F10ns snapshots (**Int5**, **Fig. 3A**) coincide closely thus providing further evidence for the
310 adequacy of experimental parameters used in both series. To our surprise, before onset of product
311 release, the intact thymines stay fully stacked in the active site for up to 500 ns (**Table 1**, **Fig. 4A**).
312 Apparently, the active site undergoes relaxation before the two repaired thymine bases start to flip
313 back out of the active site. In the N100ns and N500ns snapshots, the isoalloxazine geometry of the
314 FADH[•] coenzyme returns to the initial fully reduced state (**Fig. 4A**, **4C**). These two structures,
315 assigned as **Con2a** and **Con2b** intermediates, respectively, differ only slightly for the 5WC/R256

316 locus (see below). Interestingly during photoreduction of *Mm*CPDII without bound DNA, the
317 isoalloxazine ring adopts similarly its full buckling at about 300 ns after its light-driven FADH^\bullet
318 $\rightarrow \text{FADH}^-$ transition (33).

319 An interesting observation from these data is the rapid decrease of isoalloxazine dihedral
320 angles during FET, but slow increase and recovery after BET (**Fig. 3C, Table 1**). A possible
321 explanation is that FET occurs between CPD and the light-excited $\text{FADH}^{*\bullet}$, with a singlet S_1 shell
322 occupying electron being transferred (58). The oxidation of $\text{FADH}^{*\bullet}$ into FADH^\bullet is a downhill
323 transition between an unstable, high-energy state to a stable low-energy state, whereas reduction
324 of FADH^\bullet to FADH^- represents a transition between two ground states (D_0 to S_0). Although BET
325 transiently perturbs the FADH structure, as evidenced by strong torsion and fluttering between 1
326 and 3 ns (**Fig. 3C**), the accumulated energy within the isoalloxazine moiety is only slowly
327 dispersed over hundreds of nanoseconds, as seen both here and previously for the *Mm*CPDII
328 photoreduction (33).

329
330

331 **Late events: base back-flip and onset of reannealing of double-stranded DNA**

332 The isoalloxazine geometry no longer changes in the 25 μs and 200 μs snapshots (**Fig. 4D**). Here,
333 the repaired thymine bases finally move out of the active site and flip back towards the dsDNA.
334 In continuation from prior time-points, where post-repair movement concentrated around the 5'-
335 thymine (**Figs. 3A, 4A**), it is the 5'-thymine (dT7) that first starts to exit the active site during the
336 N25 μs snapshot. However, this back-flipping base is too disordered to be refined as only the
337 pentose is well resolved (**Fig. 4D**). Since the two thymine bases are no longer stacked and 5'-T
338 flips back, this structure can be considered as a “thymine back-flipping intermediate” (**Con3a**, TT
339 status: T/T). Meanwhile, the 3'-thymine dT8 is tilted in such a way that it occupies the full space
340 within the active site (**Fig. 4D**). The N200 μs snapshot is particularly interesting, since the two
341 thymine bases continued the back-flipping process, but were captured at different stages in the two
342 complexes in the asymmetric unit. In complex A, the dT7 has further moved and its density has
343 now become well defined (intermediate **Con3b**, TT status: T/T). In complex B, both thymine bases
344 have flipped back and undergo base-pairing with their respective complementary bases. This
345 complex can be designated as the “product-reannealing complex” (**Con4**), and its TT status (-T-
346 T-) corresponds to regular double-stranded DNA. Notably, there are extra difference signals in

347 complex A, which are fittable to **Con4** (30 to 50%), and likewise in complex B that correspond to
348 **Con3b** (30 to 40%) (**fig. S7**). This population shift between complexes A and B in the N200 μ s
349 snapshot likely derives from crucial crystal contacts made by complex A, but not complex B (**fig.**
350 **S8**). In terms of thymine back-flipping, complex B is hence less constrained by the crystal lattice
351 and can restore the integrity of the duplex DNA in a shorter timeframe. Considering that repaired
352 dsDNA is known to dissociate from the photolyase (36, 43), it is reasonable to consider **Con4** as a
353 transient intermediate captured just prior to final complex dissociation. Unlike all structures up to
354 500 ns that show only few difference density peaks in the DNA region (**Fig. 4E**, left), the N200 μ s
355 complex B reveals extensive difference density features along the entire dsDNA (**Fig. 4E** right).

356 Overall, the presented timeline outlines all chemical and structural changes undergone by
357 the substrate CPD-DNA and the coenzyme FAD during the *Mm*CPDII reaction cycle. Next, the
358 crucial contribution of the enzyme itself will be analyzed, providing a detailed mechanistic
359 description of *Mm*CPDII reactivity, including additional support for our assignment of structural
360 intermediates.

361

362 **Active site dynamics during CPD repair: The 5WC/R256 locus**

363 Concomitant to the reaction course of CPD repair as outlined before, further ΔF_o peaks nearby
364 hint at conformational changes in two highly conserved features of the class II photolyase active
365 site, the 5WC and the side chain of R256 (**Fig. 2D**). Only in the catalytically active fully reduced
366 (**Int1**) but not the oxidized state (ox/ss), the 5WC interacts with one of the N η atoms of R256 to
367 form a square bipyramidal structure that connects the FAD phosphate backbone, the CPD 3'-
368 thymine and R256 (**Fig. 2D**). Upon reaction initiation, i.e. at 100 ps, R256 quickly retracts from
369 the 5WC towards the CPD, with the side chain being still mobile and showing two alternative
370 conformations (**Fig. 5A**). During the further reaction, the 5WC loses its order while the R256-CPD
371 interaction persists, as shown for **Int3** of the F650ps snapshot as an example (**Fig. 5B**). Based on
372 these structures and the corresponding structures of all time points (**fig. S9**), we suggest that the
373 5WC/R256 locus allows fast reorganization during the electron transfer reaction. In the resting, i.e.
374 fully reduced state, 5WC/R256 electrostatically stabilizes the negatively charged FADH $^-$. Upon
375 FET, R256 preferably stabilizes electrostatically the CPD $^{\bullet-}$ anion radical rather than the FADH $^{\bullet}$
376 radical, and destabilizes the ordered 5WC. In support of this hypothesis, the 5WC could not be
377 found in either the ox/ss (**Fig. 2D**) or in any of the room temperature apo-structures of *Mm*CPDII

378 **(fig. S10)** (33), indicating that for the formation of a square bipyramidal structure of 5WC, anionic
379 FAD and a bound CPD substrate are necessary. In *Mm*CPDII-CPD-DNA complexes obtained
380 under cryogenic conditions at the synchrotron, a highly similar six-water cluster (6WC), in which
381 the sixth water replaces R256, can be observed (37, 43) **(fig. S10)**. Given that such a 6WC
382 transiently appears during the BET, i.e. in the F3.35ns snapshot **(fig. S9)**, we propose that, for the
383 synchrotron structures, the 6WC mimics an intermediate derived either from cryo-trapping or X-
384 ray induced ET.

385 Our results on the electrostatic stabilization of the CPD radical anion by R256 are
386 corroborated by previous mutational analysis (56, 59). These studies showed that the
387 corresponding R342A mutation in *Ec*CPDI caused impaired binding for photodamaged DNA and
388 a diminished quantum yield for CPD repair (56). Accordingly, the 5WC/R256 locus of class II
389 photolyases may act as a fail-safe device for FADH⁻, priming it for FET activity only in the
390 presence of a bound CPD lesion.

391 The 5WC is reordered at 500 ns **(Fig. 5C)**, when the isoalloxazine ring of FADH⁻ has
392 returned to its relaxed fully reduced state. Accordingly, the **Con2b** intermediate can be understood
393 as the “enzyme-recovered product complex” prior to initiation of product release. In the N100ns
394 snapshot (**Con2a**) the 5WC adopts a slightly different geometry when compared to both the dark
395 and N500ns states, which apparently represents a structural snapshot of the process towards the
396 enzyme-recovered product complex. The concept of enzyme recycling is well known, but it is
397 notable that the enzyme does not rush to release the product before resetting the conformations of
398 its active site moieties that have been altered during catalysis. In this way, the enzyme may drive
399 product release, while being primed for cognate substrate recognition and the next reaction cycle.

400

401 **Role of the BIR during product release by photolyases**

402 While the 5WC/R256 locus is involved in fine-steering the active site’s affinity for cognate CPD
403 substrates and repaired products, the BIR acts in stabilizing the unpaired bubble of the bound
404 dsDNA, i.e. the DNA region vacated by CPD flip-out upon binding to *Mm*CPDII **(Fig. 2E)** (43).
405 Unlike the 5WC/R256 locus, the BIR locus remains essentially unchanged during the first 500 ns
406 **(Fig. 5D, fig. S11)**, but shows first, only slight changes for the N25μs snapshot (**Con3a**) and, as
407 expected, large-scale changes in both complexes of the N200μs snapshot, where the two thymines
408 have partially entered the unpaired bubble (**Con3b** and **Con4, Figs. 5E, 5F, fig. S7, fig. S11**). As

409 the 5'-thymine first flips back towards the unpaired bubble (**Fig. 5E**), the resulting rotation of the
410 intra-CPD phosphate causes BIR residue R441 to swivel away from the phosphate by changing its
411 center of mass distance from 4.4 Å to 7.2 Å (**Fig. 5E**). This opens the BIR “lock” and allows both
412 thymines to return sequentially to the unpaired bubble. Once filling the unpaired bubble, the two
413 thymine bases stack well with the preceding dC6 base and interact with their complementary bases
414 dA7' and dA8', essentially displacing D428 and R429, while R441 moves back closer to the dT8
415 phosphate, stabilizing it in its new orientation (**Fig. 5F**). However, the remaining BIR element
416 W431 remains interacting with dC9 and the thymine bases, which rationalizes why the repaired
417 DNA, after losing so many interactions at the active site, still sticks to the enzyme before full
418 release, and why the bound and still kinked DNA at this point is highly dynamic as shown by the
419 large number of diffuse difference density peaks around the DNA backbone (**Fig. 4E**, right
420 structure). Indeed, clustering analysis of steered molecular dynamics simulations restrained by the
421 $\Delta F_o(200\mu s\text{-dark})$ electron density map revealed that dsDNA in complex A adopts a single major
422 conformation similar to the starting structure (**fig. S12**). In contrast, the dsDNA of complex B
423 showcases two major conformations with different degrees of deformation in the unpaired bubble
424 region, supporting that DNA release starts at the unpaired bubble, and extends from it towards the
425 upstream and downstream regions of the DNA (**fig. S12**).

426 Upon full DNA release, even this last protein-DNA interaction will be broken, and dC9
427 and -T-T- co-stack again as in canonical dsDNA. It is feasible that CPD-DNA binding by
428 *Mm*CPDII follows the reverse sequence of events: First, W431 intercalates between the 3'-thymine
429 of the CPD lesion and its downstream base enforcing suboptimal base stacking on both sides of
430 the CPD lesion. Then, an onset of interactions between R429 and the counterbases dA7' and dA8'
431 (37) supplies further stabilization. Finally, the R441-D428 lock facilitates CPD phosphate rotation,
432 thus causing the CPD flip into the enzyme's active site.

433 Overall, our results not only characterized the intermediates of TT-dimer repair, but also
434 demonstrated the associated dynamic processes of key active site moieties. Furthermore, we have
435 identified and characterized intermediates in the entire catalytic process in addition to chemical
436 steps, and rationalized how damaged dsDNA binds to the active site, gets repaired, how the enzyme
437 recovers, and then how the repaired bases return to dsDNA during product release.

438
439

440 **Complementary approaches to validate the TR-SFX derived photolyase mechanism**

441 To provide further support, we performed three additional analyses on the structural TR-SFX data
442 as a whole, with details provided in **Supplementary Texts S4-S6**. First, we developed a kinetic
443 model (**Supplementary Text S4** and **fig. S13A**) based on the accumulation of negative densities
444 around the two affected bonds, C5-C5' and C6-C6' during the first 3.35 ns of the reaction (**Fig.**
445 **3B, fig. S13B**). By numerical integration of this model, we could determine that indeed **Int3**, i.e.
446 (T_T)^{•-}, was the dominant species between 450 to 650 ps, while a mixture of **Int4** and **Int5** became
447 dominant after 1 ns (**fig. S13C**), as described by our molecular structures (**Fig. 3A**). These results
448 show that the kinetic data derived from the negative density accumulation correlate well with the
449 derived intermediate structures and thus produce a valid reaction mechanism (**fig. S13D**).

450 Second, we performed singular value decomposition (SVD) analysis of both the ps-ns and
451 ns- μ s time-series as described in **Supplementary Text S5, fig. S14** and **S15**. For the ns-ps series,
452 the first main component (ISV1) acted by symmetrically separating the CPD bases, while the
453 second one (ISV2) dampened the effect of the first one in the vicinity of C6-C6', effectively
454 delaying their separation (**fig. S14A and B**). Meanwhile, SVD results for the ns- μ s series agreed
455 very well with our molecular structures (**Fig. 4**), and supported a three-state mechanism for DNA
456 release. Here, the first component effectively represented local CPD changes prevalent at 100 to
457 500 ns (ISV1), the second global DNA changes during complex release at 200 μ s (ISV2), and the
458 final one base flip-back between 25 and 200 μ s (ISV3) (**fig. S15**). Since SVD is performed over
459 the entire asymmetric unit including complexes A and B, we performed further structural analyses
460 to show that complexes A and B of 200 μ s involve shifts of populations (**fig. S7**).

461 Third, we sought to validate our interpretations of time-dependent structural changes via
462 QM computational analysis by using the density functional theory (DFT) method to calculate the
463 highest occupied molecular orbitals (HOMO) of the TR-SFX derived atomic coordinates of CPD
464 repair intermediates (**Supplementary Text S6** and **fig. S16A**). Our data showed overlapping
465 orbitals up to 650 ps (**Int3**) and formation of a node at 1 ns (**Int4, fig. S16B**), supporting complete
466 rupture of the cyclobutane ring between 650 ps and 1 ns. Furthermore, when the calculation was
467 repeated with addition of a negative charge to CPD, node formation then occurred at 3.35 ns, i.e.
468 BET involving **Int4** and **Int5** (**fig. S16C**), reflecting the extra time needed for back electron
469 transfer.

470

471 Discussion

472

473 Comparing TR-SFX data with kinetic data from spectroscopic studies of photolyases

474 Even though this work focussed on structural details of the intermediates and processes during
475 catalysis of DNA repair by a class II photolyase, the kinetics and order of occurrence of
476 intermediate species should resemble that from spectroscopic studies in solution, though detailed
477 data can differ due to different experimental conditions and sample sources. Overall, our results
478 are in close agreement with results of previous ultrafast time-resolved spectroscopic studies,
479 wherever a comparison can be made, such as the chemical structures of the intermediates of
480 cyclobutane repair (22, 25, 57), and the roles of active site residues such as R256 and hydration
481 dynamics (56). The role of primary hydration dynamics in stabilizing and mediating the charge
482 transfer reactions, which had been previously hypothesized based on ultrafast spectroscopic
483 analysis of the DNA repair reaction (25), has been now given a molecular and structural basis.
484 Here, the R256-5WC locus plays a key role in stabilizing the negative charge of the electron that
485 travels back and forth between the FADH⁻ coenzyme and the CPD lesion. In addition, while the
486 exact timing of each step may differ between our TR-SFX study and previous spectroscopic studies
487 due to different conditions and/or model systems used, e.g. double-stranded CPD-DNA vs. UV-
488 damaged oligo-dT, the relative timing of different steps is in very good agreement.

489 Furthermore, we can also address some of the differences in the kinetics and quantum
490 yields reported previously for different photolyases. For example, our data clearly indicate that the
491 occupancy of activated complexes during CPD repair stays constant from 100 ps to 10 ns (**table**
492 **S1**). This signifies that, once FET has occurred, the reaction proceeds with almost 100% efficiency
493 and, conversely, that the observed quantum yields for repair depend on non-productive de-
494 excitation of FADH^{-*}. Another possible culprit for lowered quantum yields is the proposed
495 intramolecular ET (iET) between the FADH⁻ isoalloxazine and adenine moieties (41, 60). We
496 believe that the latter is a plausible explanation, although our data showed no significant time-
497 dependent conformational changes around the adenine moiety. iET is part of the FET pathway in
498 class II photolyase mediated DNA repair, but not predominant in class I photolyases (41). This
499 hypothesis, which also implies that iET is completed within the overall FET time-frame of about
500 100-250 ps, fits well with the comparatively low quantum yield of *Mm*CPDII (~25%, **fig. S18**)
501 compared to yields of 45–100 % reported for class I photolyases (40, 61). These results suggest

502 that different photolyases, despite high structural homology, differ in their kinetic schemes, as also
503 reflected by the variations of kinetic constants and quantum yields depending on class, species,
504 substrate, and reaction conditions (41, 57, 60, 62). Since our TR-SFX analysis of *Mm*CPDII
505 simultaneously examined multiple reaction loci, as well as chemical and conformational properties,
506 the order of events found in this study should reflect the real time events, though it was not our
507 intention to determine detailed kinetic constants. Nevertheless, our finding that BET occurs 1-2 ns
508 after photon absorption and FET (**Fig. 3B**) is comparable to previous reports about class II
509 photolyases, where (T+T)^{•-} appeared ~600 ps after photon absorption, and BET was completed
510 850 ps later (21).

511 Prior spectroscopic data had suggested that bond breaking occurred sequentially, with C5-
512 C5' breaking first, followed by C6-C6' (**Fig. 1A**) (22, 23, 25, 28, 59). Our results have validated
513 this mechanism and characterized the chemical structure of the ring opening intermediate in the
514 enzyme bound form.

515

516 **The issue of multiphoton effects on DNA repair by photolyases**

517 While it is conceivable that FAD dynamics, particularly the resulting FET kinetics, is affected by
518 multiphoton excitation, the focus of this work is the repair of CPD-lesion comprising DNA, which
519 should be minimally affected, if at all, by multiphoton effects based on the following mechanistic
520 and experimental considerations.

521 First, the structural moiety being repaired, CPD, does not interact at 400 nm with the
522 excitation light. Initiation of its repair only requires an electron via FET from the excited FADH^{-*}
523 *. Since our results show that FET is completed in ~250 ps, whereas CPD repair takes place mainly
524 during 0.45-1.0 ns, the repair mechanism itself should be unaffected by the mechanism and kinetics
525 of FET. Previous spectroscopic studies showed that electronic relaxation of free FADH^{-*} proceeds
526 within 5-10 ps, but takes about 1.8 ns when bound to free photolyases (27), indicating sufficient
527 time for generating higher states than S1 by excitation of FADH^{-*}. However, relaxation of FADH^{-*}
528 * from higher states, i.e. S2 and higher, occurs within a few ps (45) or even faster by internal
529 conversion. Even if multiple photon absorption by FADH^{-*} may cause high-energy states, which
530 eject a free electron by reaching the ionization continuum, this process might be biologically
531 feasible. In the presence of the 8-hydroxy-deazaflavin antenna chromophore, light energy absorbed
532 by the antenna is predicted to cause free electron ejection from FADH^{-*} by a process called

533 intermolecular coulombic decay, ICD (63). Although the effects on quantum yield cannot be
534 discounted, those on the FET speed appear rather unlikely according to our TR-SFX data, as
535 previously published FET time constants from time-resolved spectroscopy range between 200 to
536 600 ps, e.g. 209 ps for a class I and 565 ps for a class II photolyase (41). Thermal effects caused
537 by vibrational cooling upon back-conversion of high-energy states of FADH^{-*} are also unlikely to
538 affect catalysis as we did not observe any kind of conformational changes for the photolyase-DNA
539 complex in its oxidized state under multiphoton conditions (**fig. S1A, B**).

540 Second, the interpretation of our results is based on multiple active site moieties (**Fig. 2A-**
541 **E**) that all display different time-dependent changes. As mentioned above, CPD repair is by orders
542 of magnitude slower than multiphoton relaxation. Based on **Fig. 6A**, the recovery of active site
543 residue conformations (500 ns) and the thymine back-flipping and product return (microsecond
544 range) are even slower, thus unlikely to be affected by any multiphoton effect.

545 Third, it is interesting to note the high degree of correlation between the CPD ΔF_0 features
546 in the F10ns and N10ns snapshots (**Materials and methods summary**). Since simultaneous
547 multiphoton excitation may occur in the ps-ns series (pulse duration: 0.98 ps), but not in the ns- μ s
548 series due to long pulse duration (3 ns) as addressed in **Supplementary Text S3**, we would expect
549 that the F10ns and N10ns snapshots and their difference maps differ significantly, if multiphoton
550 excitation affected repair kinetics in the ps-ns series.

551 Finally, as described in the beginning of Results, power titration results suggested that the
552 power used in our experiments are just enough, in contrast to the calculated values suggesting
553 excessive photons. This disparity has also been reported recently (64), suggesting that far fewer
554 photons than the nominal values are absorbed for reasons requiring further investigation. An
555 alternative scenario, especially for proteins optimized for light-driven electron transfer, is that
556 “proteins may have evolved to direct all deposited energy toward functional outcomes” (46, 50).

557

558 **The issue of identifiable reaction intermediates from TR-SFX experiments**

559 While many TR-SFX reports focused on dynamic features of a specific chemical or physical step
560 (6, 10, 49, 65), and some others used kinetic models from spectroscopic studies to guide their
561 search for reaction intermediates (20), our work dealt with an entire catalytic cycle with many
562 elementary steps not assigned before, like ordered product release. In the following, we address
563 some of the general issues encountered in our work. Intermediates of chemical or enzymatic

564 reactions are commonly considered stable enough when being detectable by kinetic or biophysical
565 methods. These methods are usually limited by their capacity to detect characteristic signals for
566 each intermediate moiety, and thus provide mostly local information about the reactants
567 themselves. Notably, the IUPAC Gold Book (66) defines an intermediate as “a molecular entity
568 with a lifetime appreciably longer than a molecular vibration that is formed from the reactants and
569 reacts further to give the products of a chemical reaction”. Since TR-SFX captures global
570 information about all atoms within the enzyme-substrate complex, in our case a photolyase bound
571 to the biopolymeric CPD-DNA, it should be able to unravel multiple intermediates, or molecular
572 entities, in a chemical step or a process (e.g. conformational change, product release) particularly
573 since they can be stabilized by the enzyme. This is what we have accomplished in this work, as
574 summarized in **Table 1**.

575 On the other hand, since each time point may consist of multiple intermediates with varying
576 populations, each snapshot listed in **Table 1** represents either the predominant species of that
577 particular snapshot or a mixture of species. Importantly, we based our assignments not only on the
578 refined structural models, but also on the features of the ΔF_o maps, such as C-C peak distances
579 and accumulation of negative densities along the relevant bonds (**Fig. 3B**). These assignments
580 were then further validated by other relevant structural changes at the active site, such as the status
581 of the dihedral angles of the FAD and the structural movements of the 5WC/R256 locus (**Table**
582 **1**), and by different experimental approaches (**Supplementary Texts S4-S6**).

583 Furthermore, the reliability and significance of the refined structural changes is supported
584 by correlation coefficient analysis (**fig. S6**), which quantifies the similarity between the observed
585 and calculated difference electron density maps. Here, and especially within the initial 2 ns, where
586 prior solution spectroscopic studies predicted that most chemical changes would occur (40, 58),
587 individual structures strongly correlated with their corresponding density maps, but not with those
588 of their neighbouring time-points, suggesting that each snapshot harboured mostly a distinct
589 conformation.

590 A conformational change is commonly considered as a simple two-state process, where a
591 time-dependent structural change corresponds to a shift of population between two states. In our
592 view, a conformational change could be a continuous or multi-step process, as demonstrated
593 recently by a temperature-resolved cryo-EM study of a ligand-induced conformational change of
594 another enzyme (67). In the present work, we found that the active site of the enzyme goes through

595 a multi-step process (10 ns – 500 ns) to recover from catalysis, and we identified two variants,
596 **Con2a** and **Con2b** in the process. For the product release, we also identified two variants (**Con3a**
597 and **Con3b**) in the process of back-flipping of the repaired thymines, and characterized the
598 reannealed product **Con4**. Interestingly, increased conformational freedom around the DNA leads
599 to the shift of population between **Con3b** and **Con4** (**fig. S7**).

600 In summary, for the CPD repair in the ps-ns series, the structure from a snapshot often
601 consists of multiple intermediates. Those with distinct structures in a predominant state can be well
602 characterized, such as the ring opening intermediates in the F650ps (C5-C5' cleaved) and F1ns
603 (C6-C6' also cleaved) snapshots. Those of the electron transfer processes (FET, BET) are not
604 directly resolvable by TR-SFX before occurrence of the next step. However, the ET processes can
605 be indirectly identified from changes in the coenzyme and active site residues.

606

607 **A new era in enzymology: watching multi-step catalysis as a whole in real time**

608 Our results described in this work are summarized in an overall time line (**Fig. 6A**) by a molecular
609 movie (**Fig. 6B**, or **Video S1**). Real-time recording of molecular movies such as this signals a new
610 era in describing the processes and structural basis of (photo)enzymatic catalysis, particularly for
611 the multi-step reactions catalyzed by photolyases. Here, FET reaction leads to accumulation of
612 bond-cleavage intermediates, followed by ring opening, BET, post-repair recovery of enzyme and
613 coenzyme, and thymine back-flip and DNA release. The events of CPD repair are mediated by
614 structural fluctuations of the FAD coenzyme and the surrounding active site moieties including
615 the 5WC/R256 locus, whereas thymine back-flip and the onset of DNA release proceed along the
616 BIR region of the unpaired bubble. Interestingly, we also observed the directionality of DNA repair,
617 i.e. the 5'-T moves away from the 3'-T after opening of the CPD ring so that the 5'-T leaves the
618 active site first.

619

620

621 **Materials and methods summary**

622

623 **Sample preparation**

624 *Mm*CPDII-DNA co-crystals were grown according to previously published conditions (37, 43)
625 after applying further optimizations for enzyme activity and large-scale microcrystal production.

626 Here, the *Mm*CPDII protein solution was directly activated prior to crystallization via
627 photoreduction in the presence of DTT, white light and under anaerobic conditions, followed by
628 mixing with CPD-containing DNA. After an incubation period of ~2 h at 23 °C, crystals were
629 harvested, mixed with grease and loaded into injectors according to previously established
630 protocols (68). To preserve enzyme activity, all steps were performed under anaerobic conditions
631 in a COY Labs vinyl anaerobic chamber. Further, to prevent accidental DNA repair, all
632 experimental steps after enzyme photoreduction were performed under safety light (640 nm). To
633 confirm enzyme status, in-solution reoxidation was followed by UV-Vis absorption spectroscopy
634 (**fig. S17A**), which showed that *Mm*CPDII remained in the fully reduced state under experimental
635 conditions for at least 24 hours after photoreduction. Although the presence of photodamaged
636 DNA is known to further stabilize FADH⁻ in photolyases, we further confirmed *Mm*CPDII *in*
637 *crystallo* status via *in crystallo* UV-Vis absorption spectroscopy of 20-hour old crystals (**fig. S17B**).
638 No crystals older than 20 hours were used for any of the experiments presented in this work.
639 Further details for sample preparation can be consulted in Supplementary methods.

640

641 **Data collection**

642 At SACLA (69), data was collected at the BL2 beamline using a 30 Hz pulse frequency and 10
643 keV X-ray with a pulse duration of <10 fs and focused to a focal spot with a diameter of 1.5 μm.
644 For time-resolved experiments, a 15 Hz, 150 μJ, 408 nm OPO pump laser with a 3 ns pulse length
645 with a 100 μm diameter focal spot was used. During all experiments, the DAPHNIS (70) chamber
646 was flooded with a 98% helium atmosphere to increase signal to noise ratio, and to prevent
647 oxidation of the sample. Samples were extruded at 2.6 μL/min through a 75 μm nozzle (71).
648 Images were collected on a short-working distance octal multi-port detector with 50 mm sample
649 to detector distance (72). Initial light and dark splitting as well as initial processing was performed
650 by the Cheetah pipeline (73).

651 At SwissFEL (74), the XFEL was also 1.5 μm in diameter with a ~20 fs pulse duration, but
652 run at 100 Hz. For time-resolved data collection, the in-house laser was set for a pulse duration of
653 0.98 ps, with a pulse energy of 10 μJ and a focal spot diameter of 50 μm running in a 3:1 setup.
654 Accordingly, for every three light images, one dark image was collected. Sample was extruded at
655 5 μL/min through a 75 μm nozzle, while the chamber was maintained at 200 mbar after flooding
656 and evacuating three times with helium.

657 The average crystal size was $50 \times 50 \times 50 \text{ }\mu\text{m}^3$ with an $1/e$ penetration depth of $103 \text{ }\mu\text{m}$ at
658 400 nm . Further details and control experiments for light contamination and laser power can be
659 found in **Supplementary methods, Supplementary Texts 1-3** and **figs. S1-S3**.

660

661 **Processing, refinement and analysis**

662 Data processing with CrystFEL (75, 76) and refinement procedures are described in detail in
663 Supplementary methods. Briefly, we followed previously described guidelines (5, 9, 52, 53) to
664 perform structure factor extrapolation followed by structural refinement (**tables S1-S2** for
665 refinement statistics). Additionally, two features were given particular attention, namely the exact
666 structure of the CPD cyclobutane ring (**Fig. 1A**) and the dihedral angles ρ_C and ρ_N of the FAD
667 isoalloxazine moiety (**Fig. 1C**). To accurately determine the crucial CPD and FAD geometries, we
668 performed real-space correlation-coefficient (CC) based refinement of both features. Here, we
669 searched for maximum correlation between a $\Delta F_o(Y-X)$ map and an equivalent calculated
670 difference map ($\Delta F_c(Y-X)$) based on a fully refined static structure (X) and the time-resolved
671 structure to be refined (Y). This approach produces a quantitative quality parameter, CC, which
672 can be compared for a given structure versus all experimental data to quantify how well small
673 structural changes, such as bond elongation during CPD repair, can be monitored in our time
674 courses (**fig. S6**). Our occupancies achieved by light-triggering (**tables S1-S2**) are close to the
675 experimental quantum yield of $24 \pm 1\%$ for DNA repair by *Mm*CPDII (**fig. S18, table S3**) for the
676 ns- μs series (20-22%) and about half of that for ps-ns series (11-13%).

677 The asymmetric unit contains two complexes. Unless otherwise stated, the maps shown in
678 the figures are always from complex A, since those of complex B are much noisier than complex
679 A (I/σ values of 3 or below).

680

681 **Consistency between F series and N series**

682 In order to control for consistency between the ps-ns and ns- μs series, we collected the 10 ns time-
683 point both at SwissFEL and SACLA. Two important factors need to be considered when
684 comparing these two 10 ns snapshots. The first one is resolution. Given the lower resolution of
685 N10ns (2.7 \AA), atomic positions are less well defined than in the high-resolution counterpart F10ns
686 (2.15 \AA). Secondly, and more importantly, is pulse duration. For the F10ns snapshot, each crystal
687 is illuminated for 0.98 ps , while for N10ns, for 3 ns . Accordingly, reaction coherence is higher in

688 F10ns than in N10ns. For example, the variance for the ps-ns series is expected to be ± 0.001 ns,
689 i.e. the pump pulse duration. Meanwhile, in the ns- μ s series, the variance could amount up to ± 3
690 ns, i.e. an error of 30% relative to N10ns, which decreases for longer delay times. Accordingly,
691 the N10ns snapshot corresponds more to a mixture of the F6ns and F10ns snapshots, implying that
692 their correlation coefficients around the CPD are all the same (N10ns/F6ns: 0.62; N10ns/F10ns:
693 0.61, F6ns/F10ns: 0.67).

694

695 **Calculation of C-C peak distances**

696 Bias-free monitoring of distances between peaks from difference maps is more reliable than atom-
697 atom distances from refined structural models in tracking structural changes by TR-SFX. In our
698 study, only the 5'-T, not the 3'-T appears to move upon opening of the cyclobutane, leading to
699 mainly negative peaks for the cyclobutane and positive peaks at the 5'-T side. To determine the
700 distance between the negative cyclobutane and the positive 5'-T difference density peaks along
701 the C5-C5' and C6-C6' bonds, ΔF_0 maps were generated with the phenix isomorphous difference
702 tool as described in **Supplementary Materials** and shown in **Figures 3A and 4**. The CCP4 tool
703 fft was then used to extract electron densities at a 2.5σ contour level. To ensure an identical
704 number of voxels existed in each map even at different resolutions, the grid was set to $1/4 \times$
705 maximum resolution. Next, a Python script was used first to position a dummy atom on the C5 and
706 C6 positions, and then to move them along the C5-C5' and the C6-C6' bond axes in steps of
707 0.385 \AA (half the atomic radius of carbon) for 11 steps (4.235 \AA) towards, and beyond, the 5'-T.
708 In both cases, the dummy atom was then used as a mask to extract the integrated electron density
709 at each position and plotted versus distance (**fig. S5**). The distance between minimum (cyclobutane
710 peak) vs. maximum (5'-T peak) along each axis was then extracted from the plots. Importantly, if
711 either a minimum or a maximum could not be determined, no peak distance could be calculated,
712 and therefore no value was assigned (noted as a dash "-" on **Table 1**), or plotted (**Figs. 3B and**
713 **4B**).

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Author Contributions

1016 MMR, AR, JY, SI, JS, YB, LOE, and MDT conceived the research and designed experiments.
1017 MMR, PHW, EN, YH, MS, AF, CHY, EPGNP, WJW, HJE, SF-B, CY, SE, NC, MW, HLG, TW,
1018 HYW, CCL, WCH, KFH, YKC, JHL, JHW, WG, CWC, AHP, KCY, WTL, YCC, DG, EB, GK,
1019 CC, CM, CB, MS, YJ, AY, RT, TT, FL, KT, MAA, FB, VF, PG, SK, LK, VR, CJR, EMS, MW,
1020 LW, RS, AR, JY, YB, and LOE performed experiments. MMR, DO, KN, RS, AR, and LOE were
1021 responsible for on-site data processing at SwissFEL, MMR, CHY, YJ, RS, AR, and LOE fulfilled
1022 the same role at SACLA. PJMJ was responsible for pump laser setup at SwissFEL, while SO at
1023 SACLA. MMR, PHW, EN, CHY, SK, RS, AR, JY, YB, LOE and MDT analyzed the data. MMR,
1024 PHW, EPGNP, LOE, and AR performed *in crystallo* spectroscopy. PHW performed in-solution
1025 spectroscopy. WZ and PM determined quantum yield spectroscopically. NC and AR performed
1026 SVD analyses. CHY performed QM calculation. MMR, PHW and CHY established and analyzed
1027 the refinement protocol. DZ provided critical insight on the early events of dimer repair. MMR,
1028 CHY, LOE, and MDT wrote the manuscript.

1029 **Competing Interests**

1030 The authors declare no conflict of financial interest.

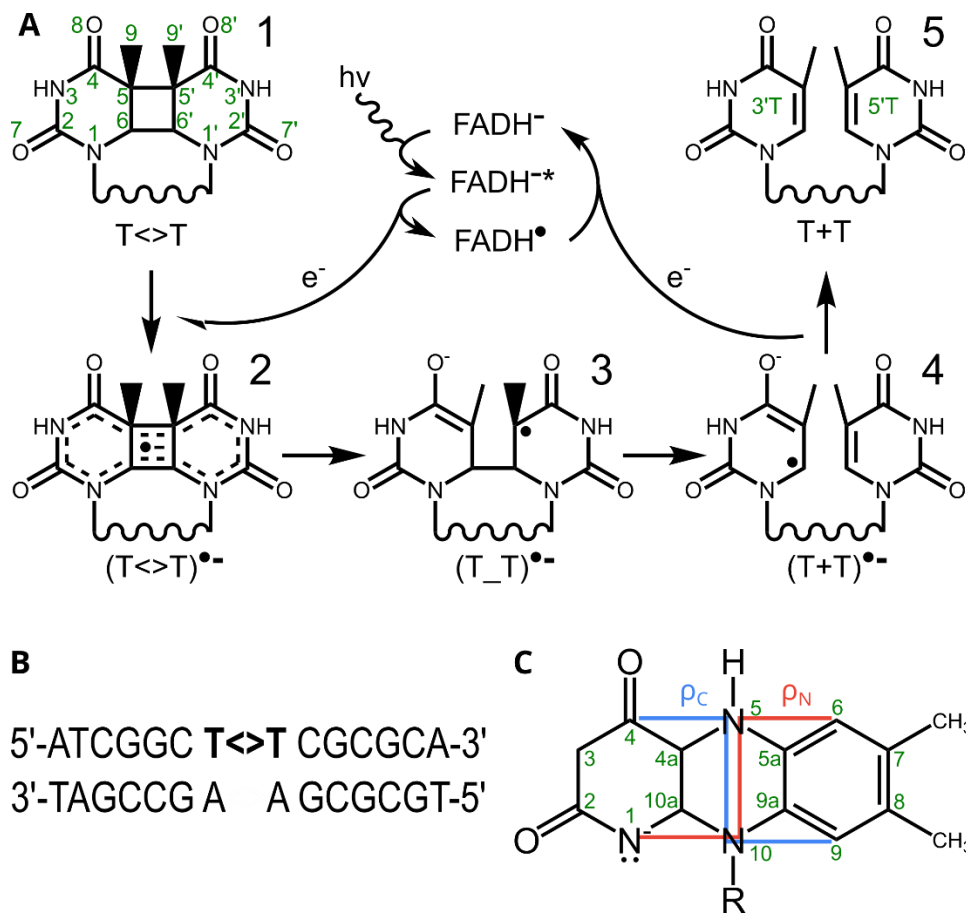
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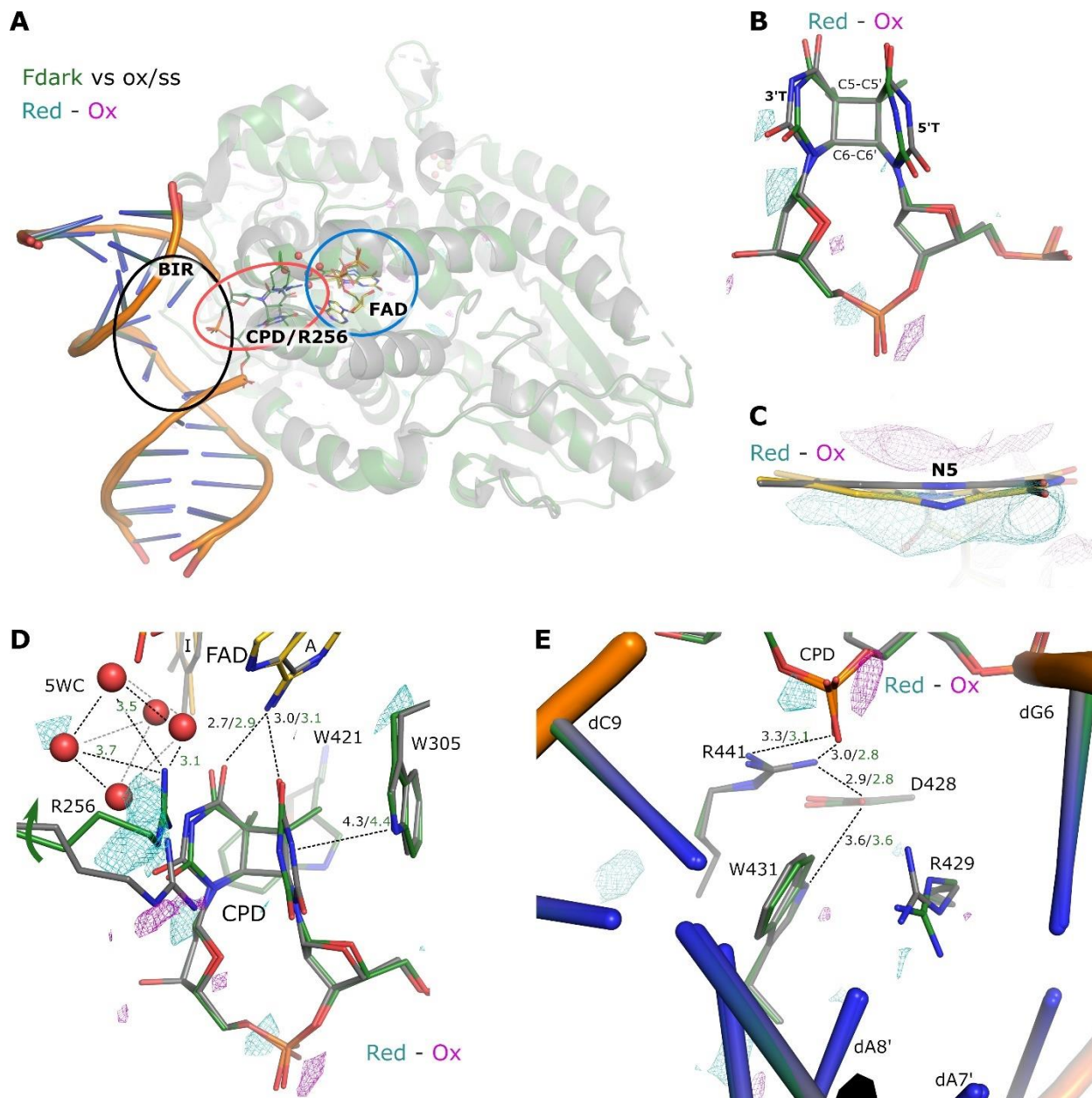
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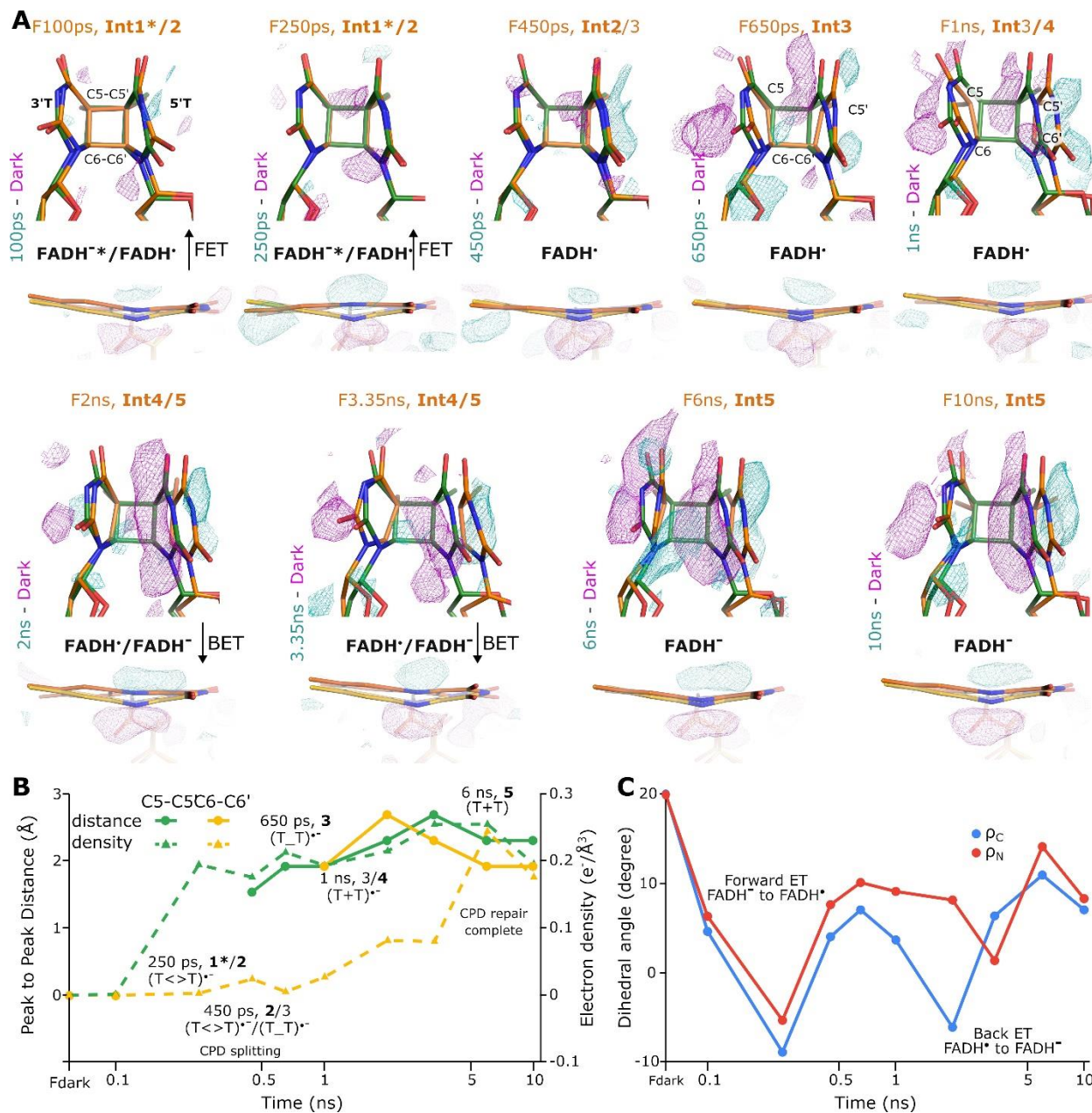
Fig. 1. Proposed intermediates during repair of T<>T lesions as catalyzed by DNA photolyases. (A) Canonical scheme of DNA repair by CPD-photolyases. **(B)** The CPD-lesion comprising dsDNA used in this work, which contained the *cis-syn* CPD with its phosphodiester linkage (43). **(C)** Illustration of the ρ_C and ρ_N dihedral angles of the FADH⁻ isoalloxazine moiety. As shown by TR-SFX of photoreduction, the isoalloxazine moiety of FAD in *Mm*CPDII undergoes symmetrical buckling or bending ($\rho_C = \rho_N$) or asymmetrical twisting ($\rho_C \neq \rho_N$) upon redox change (33). Most importantly, the transition from one geometry to another is orders of magnitude slower than electron transfer itself.



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1080 **Fig. 2. Static structures of *Mm*CPDII complexes with damaged DNA and illustration of the four main**
 1081 **reaction loci.** Structure of Fdark (reduced, green) superposed with ox/ss (steady state oxidized, gray) are
 1082 shown along with 3σ -contoured difference maps between the dark and oxidized states, $\Delta F_o(\text{dark-ox/ss})$
 1083 (positive peaks in cyan, negative peaks in magenta). (A) Global structures showing the overall fold, with
 1084 the bound DNA backbone in orange. (B) Structures of the bound CPD. (C) Structures of the isoalloxazine
 1085 ring in the oxidized (FAD_{ox}, gray) and fully reduced form (FADH⁻, gold), showing increased buckling of
 1086 the FAD in the reduced state. (D) The 5WC/R256 locus at the active site. The interactions between CPD,
 1087 FAD and active site residues R256, W305, and W421, and the five-water cluster (5WC) that only appears
 1088 in the Fdark state are also shown. In the presence of the 5WC, R256 undergoes a conformational change,
 1089 with its guanidinium moiety becoming the final vertex in the bipyramidal 5WC. Some interatomic distances
 1090 are highlighted by dashed lines with values in Ångstrom nearby. (E) The bubble-intruding region (BIR)

1091 with its D428/W431/R441 locking triad, as well as R429, which mostly interacts with the unpaired bases
1092 dA7' and dA8' complementary to the thymine dimer. The lack of significant difference electron density
1093 here suggests little conformational change related to the flavin's redox state for this region.
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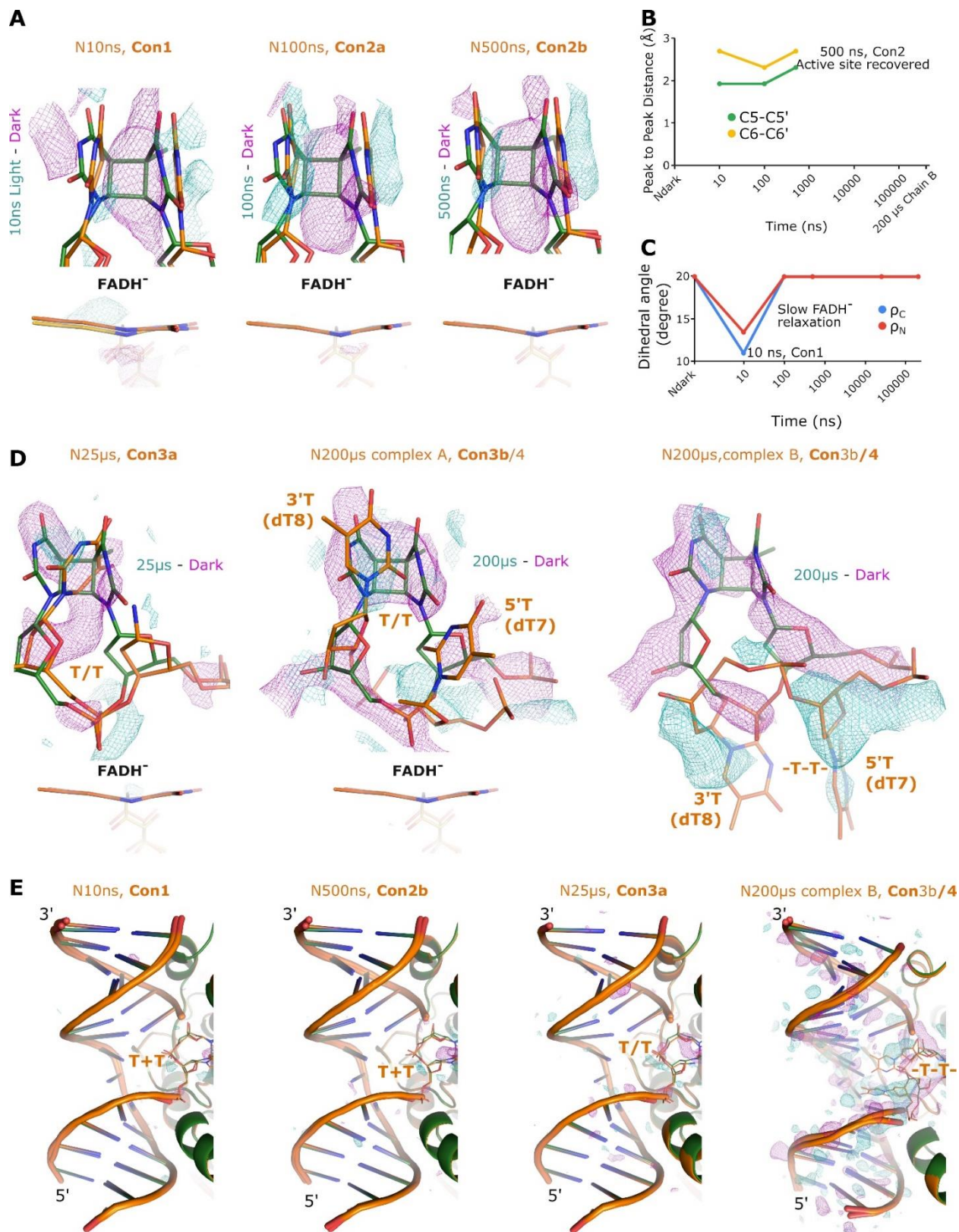
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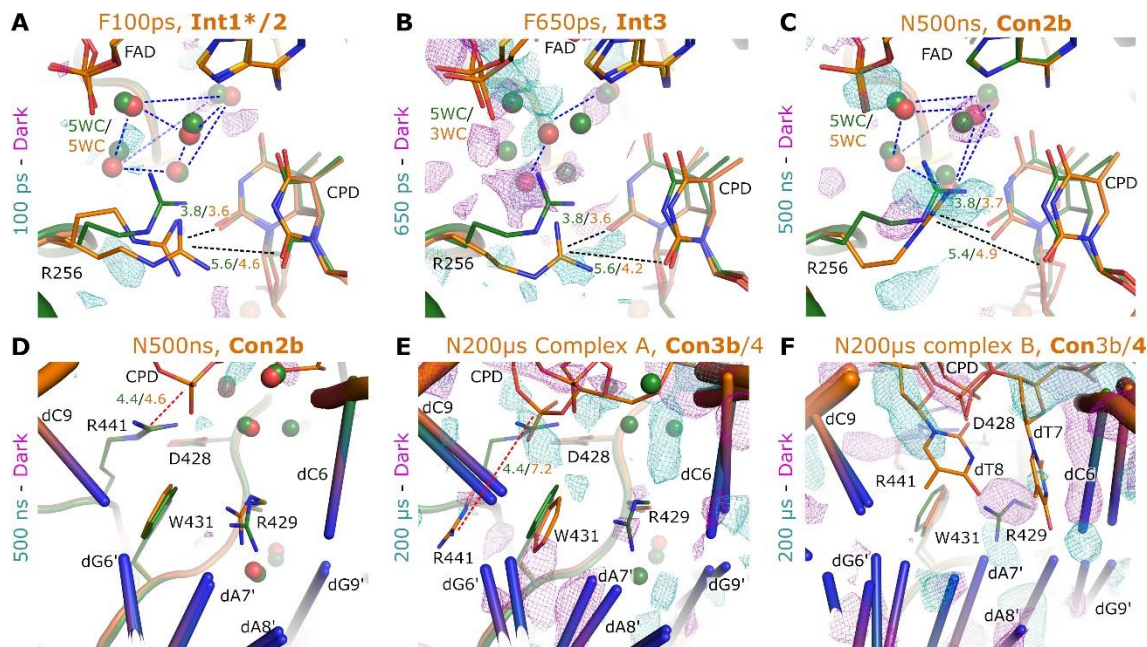
Fig. 3. Bond breaking and ring opening during cleavage of the cyclobutane ring. (A) Structures of the CPD moiety (T \leftrightarrow T) of the bound DNA as well as the FAD isoalloxazine moiety from the first TR-SFX series (orange), from F100ps to F10ns, each superposed with the reduced dark structure (Fdark in green and FADH⁻ in gold). Each panel also shows 3 σ -contoured Δ Fo(Y-dark) maps, with positive peaks in cyan and negative ones in magenta. (B) Plots of C5-C5' (green) and C6-C6' (orange) peak distances (full lines) and average integrated negative electron densities (dashed lines) over time. Since both the negative cyclobutane and the positive 5'-T peaks are necessary to calculate peak distances, when either is missing, the distance could not be calculated and no value is plotted (100 to 250 ps for C5-C5' and 100 ps to 650 ps for C6-C6'). (C) Plots of ρ_C (blue) and ρ_N (red) dihedral angles over time.



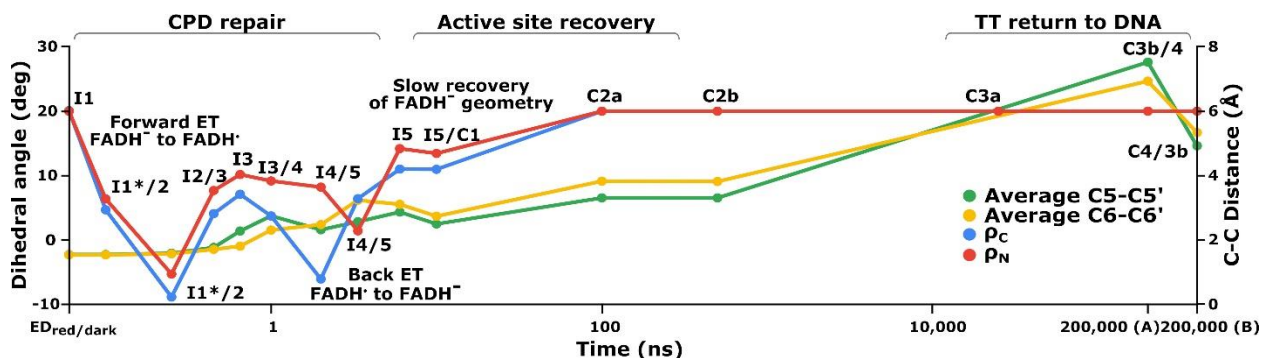
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Fig. 4. Post-repair events leading to thymine base return and DNA release. (A) Structures of the newly repaired thymines (T||T) as well as the isoalloxazine moiety from the second TR-SFX series (orange), from

1109 N10ns, N100ns, and N500ns, each superposed over the reduced dark structure (Ndark CPD shown in
1110 green, while its isoalloxazine moiety is depicted in gold). Each panel also shows 3σ -contoured $\Delta F_o(Y\text{-dark})$
1111 maps, with positive peaks in cyan and negative ones in magenta. **(B)** Peak distance plots along the C5-C5'
1112 (green) and C6-C6' (yellow) axes, and **(C)** ρ_C (blue) and ρ_N (red) angles plots over time for the second TR-
1113 SFX time-series. **(D)** Structures of the newly repaired thymines from the second TR-SFX series (orange)
1114 during their return to the dsDNA, including N25 μ s and N200 μ s, each superposed over the reduced dark
1115 structure (Ndark in green). Because the two *Mm*CPDII-DNA complexes in N200 μ s are at two different
1116 DNA release stages, they are both shown here. Each panel also shows 3σ -contoured $\Delta F_o(Y\text{-dark})$ maps as
1117 in (A). **(E)** Difference maps at the dsDNA, from left to right: N10ns, N500ns, N25 μ s and complex B from
1118 N200 μ s. For all complexes, 3σ contoured $\Delta F_o(Y\text{-dark})$ maps are superposed over the entire DNA molecule.
1119



1120
 1121 **Fig. 5. Roles of the 5WC/R256 locus during DNA repair and the BIR locus during DNA release. (A-**
 1122 **C)** Detailed structures of the 5WC/R256 locus (orange, F100ps for A, F650ps for B and N500ns for C)
 1123 superposed with the corresponding dark structure (green). The FAD coenzyme is depicted in gold for both
 1124 structures in each panel. The R256 side chain is dynamic with two alternative conformations at 100 ps. 3σ
 1125 contoured ΔFo(Y-dark) maps are shown as well, with positive peaks in cyan, and negative ones in magenta.
 1126 The characteristic dark five-water cluster (5WC) is shown as green spheres, while the remaining water
 1127 molecules for each specific time-resolved snapshot in red. Here, blue dotted lines highlight the 5WC
 1128 arrangement in each time-resolved snapshot. Notably, already at 100 ps (A), the square bipyramidal
 1129 interaction between 5WC and R256 is broken, resulting in a square pyramidal arrangement. R256 and the
 1130 CPD are shown as stick figures, with interatomic distances between the center of mass of the R256
 1131 guanidinium moiety and the O2 carbonyl of each of the CPD thymine highlighted with black dotted lines.
 1132 Distances in Ångstrom are provided nearby and are color-coded as the structures themselves. (D-F)
 1133 Detailed view of the unpaired bubble and the BIR residues D428, R429, W431 and R441 with Ndark (green)
 1134 superposed onto three individual time-resolved snapshots (D: N500ns, E: N200μs complex A, F: N200μs
 1135 complex B). dT7 and dT8, corresponding to the CPD bases, as well as all BIR residues are shown as stick
 1136 models, while all other bases and amino acids as cartoon representations. 3σ contoured ΔFo(Y-dark) maps
 1137 as above are shown as well. To highlight BIR lock opening, marked by the movement of R441, the R441
 1138 to CPD phosphate center of mass distance is shown as a red dashed line, with color-coded distances in
 1139 Ångstrom provided nearby.
 1140



1141
 1142 **Fig. 6. The complete timeline for major events and video presentation.** Evolution of the average C5-
 1143 C5' and C6-C6' atomic distances of the CPD, and dihedral angles of the isoalloxazine ring of FAD, as
 1144 derived from our structural models. The intermediate number(s) for each time point, and the catalytic
 1145 hallmarks of different time periods, are indicated. The intermediates up to 10 ns are reaction intermediates
 1146 for CPD repair (**Int1 to Int5**), while the intermediates from 10 ns upward are conformational intermediates
 1147 (**Con1 to Con4**) in the processes of active site recovery and product release. Importantly, since the refined
 1148 structural models may represent an average structure derived from the composite of different intermediates
 1149 present at any given time-point, the evolving C5-C5' and C6-C6' distances should not be simply considered
 1150 as bond elongation, but rather, as shifts in the populations of bond split/unsplit intermediates. In any case,
 1151 these values visualize the progress along the reaction coordinate for CPD cleavage over time.

1152
 1153
 1154
 1155 Movie 1: A molecular movie showing the key structural components of all intermediates highlighted in
 1156 Fig. 6.

1157
 1158 <https://vimeo.com/802945501/90b53c6781>

1159

1160 **Table 1.** Properties of the static and time-resolved structures of *Mm*CPDII complexes with damaged DNA.

1	2	3	4	5	6	7	8	9	10	11
Structure ^a	Interm. ^a	TT state	FAD state	ρ_C / ρ_N (deg)	5WC /R256 ^f	BIR ^g D428/W431/ R441	CC peak distance C5/C6 (Å) ^c	Negative densities C5/C6 (e ⁻ /Å ³) ^e	PDB code	Resol (Å)
Oxidized photolyase:DNA complex										
ox/ss		T<>T	FAD _{ox}	2.0/2.1	Dynamic /CPD ^f	Locked	-/-	0 / 0	7YE0	2.75
ps-ns time series (SwissFEL)										
Fdark	Int1	T<>T	FADH [•]	20.1/20.0	Ordered /5WC	Locked	-/-	0 / 0	7YC7	1.95
F100ps	Int1*2	FET ^b T<>T / (T<>T) [•]	FET ^b FADH ^{•*} /FADH [•]	4.6/6.3	Ordered /CPD		-/-	0.00/0.00	7YCM	2.00
F250ps	Int1*2			-8.9/-5.3	Dynamic /CPD		-/-	0.19/0.00	7YCP	2.08
F450ps	Int2/3	(T<>T) [•]	FADH [•]	4.0/7.6			1.5/-	0.18/0.02	7YCR	2.15
F650ps	Int3	(T_T) [•]		7.1/10.1			1.9/-	0.21/0.03	7YD6	2.15
F1ns	Int3/4	(T+T) [•]		3.7/9.1			1.9/1.9	0.19/0.03	7YD7	2.25
F2ns	Int4/5	BET ^b (T+T) [•] /T+T	FADH [•] /FADH [•]	-6.1/8.2			2.3/2.7	0.21/0.08	7YD8	2.15
F3.35ns				6.4/1.4			2.7/2.3	0.25/0.08	7YEB	2.20
F6ns	Int5	T+T	FADH [•] recovering	11.0/14.2			2.3/1.9	0.25/0.24	7YEC	2.20
F10ns				7.1/8.3			2.3/1.9	0.19/0.18	7YEE	2.15
ns-μs series (SACLA)										
Ndark		T<>T	FADH [•]	20.0/20.0	Ordered /5WC	Locked	-/-	n.d./n.d.	7YDZ	2.23
N10ns	Con1	T+T	FADH [•] recovering	10.9/13.4	Dynamic /CPD		1.9/1.9		7YEI	2.70
N100ns	Con2a	T+T	FADH [•] recovered	20.0/20.0	Dynami /5WC		1.9/2.3		7YEJ	2.55
N500ns	Con2b				Ordered /5WC		2.3/2.7		7YEK	2.40
N25μs	Con3a	T/T					Unlocking	-/- ^d	7YEL	2.55
N200μsA ^a	Con 3b/4	T/T			N/A ^f			-/- ^d	7YEM	2.60
N200μsB ^a	Con 4/3b	-T-T-					Unlocked	-/- ^d	7YEM	2.60

1161
1162 ^aThe definitions of structure names and intermediate numbers are explained in the text. Each structure has
1163 a PDB code, except that N200μsA and N200μsB share the same PDB code (7YEM) since they correspond
1164 in N200μs to the two independent complexes, A and B, of the asymmetric unit. When multiple

1165 intermediates coexist in a structure, the bolded number designates the major form. F250ps may also contain
1166 a contribution by Int3 based on negative density at C5-C5' only.

1167 ^bFET and BET designate forward electron transfer and back electron transfer, respectively. Here, no
1168 dominant CPD, TT and FAD intermediates can be defined. These structures should be hence considered as
1169 mixtures.

1170 ^cC-C peak distances corresponding to the distance between the maxima of the characteristic CPD repair
1171 signals, i.e. the cyclobutane negative density and the 5'-T positive density, along the C5-C5' and the C6-
1172 C6' axes. The position of the maximum was calculated as described in **Materials and methods summary**
1173 and **fig. S5**.

1174 ^dThe thymine bases have started to flip back to pair with adenine bases.

1175 ^eThe average integrated negative electron densities from difference maps around C5 and C6 atoms
1176 calculated as described in **Supplementary Text S4, fig. S13B**.

1177 ^fThe conformations of active site moieties 5WC and R256 sidechain. 5WC is designated as ordered (intact)
1178 or dynamic. R256 is designated as pointing toward 5WC or CPD. N/A for 200 μ s because the thymine
1179 bases have moved out of the active site.

1180 ^gBIR: The bubble intruding region composed of amino-acids D428, W431, and R441 acts as a lock. In its
1181 locked state, BIR stabilizes the unpaired bubble and prevents CPD flip-back before repair. Upon completion
1182 of CPD repair, flip-back of the repaired bases unlocks BIR, displaces it and initiates product release.

1183