Flavin photoreduction mechanism in a DNA photolyase elucidated by time-resolved X-ray crystallography

DNA photolyases, members of the photolyase-cryptochrome family [1], are light-driven enzymes containing a flavin adenine dinucleotide (FAD) coenzyme (Fig. 1). In the process of photoactivation, two light-triggered single-electron photoreduction steps convert the oxidized chromophore (FAD_{ox}), via the radical semiquinone state ($FAD^{\bullet-}$ and its subsequently protonated form $FADH^{\bullet}$), to the reduced hydroquinone state ($FADH^{-}$) [2]. The electrons fueling the two photoreduction steps are provided by an electron transfer chain consisting of a tryptophan triad, which ultimately extracts an electron from the environment [3], although the precise identity of the *in vivo* electron donor is still unknown [4]. Upon photoactivation, DNA photolyases catalyze blue light-driven DNA repair of UV photolesions, such as cyclobutane pyrimidine dimers (CPD) [1] (Fig. 1).

We determined the 3D structures of reaction intermediates in the light-dependent reduction of a photolyase enzyme from *Methanosarcina mazei*, at atomic resolution [5] (Fig. 2). By using time-resolved crystallography at the SACLA **BL2** beamline, we collected snapshots at different times ranging from 10 ns to 5 ms, resulting in a 3D movie describing these FAD transformations in detail [5] (Fig. 3).

The 3D movie shows that, after the reaction is initiated by blue light, the flat geometry of FAD becomes distorted within nanoseconds, in a motion reminiscent of the fluttering wings of a butterfly (Fig. 2). Meanwhile, nearby amino-acids from the enzyme stabilize and support these movements, allowing the FAD_{ox} to settle into the first semi-stable intermediate, $FAD^{\bullet-}$, which is characterized by a strong twist (Fig. 3). Unless the protein environment quickly supports the strained geometry of $FAD^{\bullet-}$, by donating a proton and yielding $FADH^{\bullet}$, photolyase reverts back to its original FAD_{ox} state within milliseconds. However, if $FADH^{\bullet}$ is produced, then a second reaction cycle elicits renewed fluttering of the FAD, resulting in the final product, $FADH^{-}$ (Fig. 3).

The protonation of $FAD^{\bullet-}$ at the *N5* position is essential for photoactivation, as only the neutral semiquinone $FADH^{\bullet}$ can be further reduced to the active $FADH^{-}$ state (Fig. 3). However, the proton donor and molecular mechanism remain elusive [3]. Our structural data points toward the close involvement of the Arg378-Asp409 salt bridge in this step, as its geometry is strongly affected by the $FAD^{\bullet-}$ protonation. The Arg378 guanidinium moiety moves away from the isoalloxazine ring after the $FAD^{\bullet-}$ to $FADH^{\bullet}$ conversion, reverting to a position similar to the oxidized state (Fig. 2). However, the geometry of the bifurcated salt bridge is altered by this $FAD^{\bullet-}$ to $FADH^{\bullet}$conversion, causing the Arg378 sidechain to break its salt bridge with Asp409 by a swivelling motion (Fig. 2). Our results also provide structural evidence for the stabilization of the protonated state by hydrogen bonding between isoalloxazine *N5* and Asn403 *Oδ1*, supporting the unusually high pKa for the $FADH^{\bullet}$ *N5* position in photolyase (Fig. 3). Furthermore, the intensity of the above-mentioned negative difference map peak remained approximately constant, and additional positive and negative peaks surrounding the guanidinium-carboxylate salt bridge appeared. These results, along with the absence of other efficient proton donors or water molecules within 6.5 Å of the isoalloxazine *N5* nitrogen, suggest that Arg378 is the donor for the protonation of $FAD^{\bullet-}$ to $FADH^{\bullet}$ (Fig. 3).

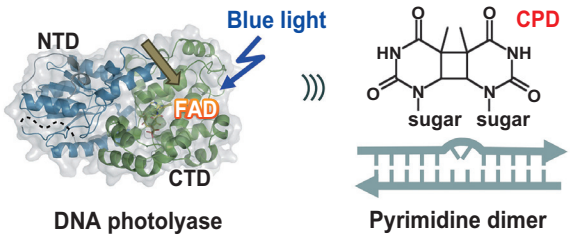

Fig. 1. Light-driven enzymatic catalysis of DNA repair by DNA photolyase. The FAD cofactor, located in the conserved folded structure of photolyase, should be fully reduced by photoreduction by the same blue light, prior to the DNA repair reaction.

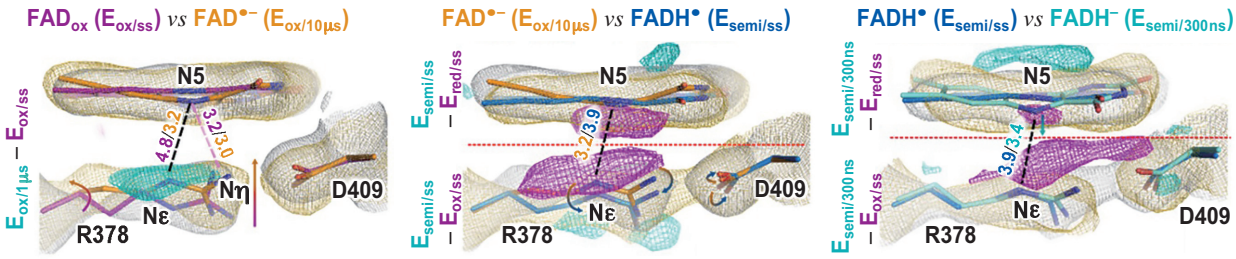

Fig. 2. Details of the FAD reaction in photolyase. Three frames of the 3D molecular movie are shown. Here, gray and yellow meshes represent the electron clouds surrounding the structures of the flavin and neighboring amino-acids, respectively. Meanwhile, the structures are shown as sticks with different colors (purple for the oxidized state, orange for the $FAD^{\bullet -}$ state, dark blue for the $FADH^{\bullet}$ state, and light blue for $FADH^{-}$). Differences between these structures are emphasized by superimpositions. Subtle differences are also highlighted by difference density clouds (magenta for negative clouds, cyan for positive clouds). Difference density clouds result from subtracting the simple electron clouds shown in yellow and gray. Thus, a negative difference density corresponds to regions where electrons disappear during the reaction, while in positive density regions, electrons appear.

Overall, our results show the atomic details of time-dependent structural changes in the photoreduction of FAD, mediated by an FAD redox sensor triad (Arg378, Asn403 and Asp409). The results also demonstrate that an enzyme-catalyzed redox reaction can take nano- to micro-seconds to complete the orbital re-hybridization processes, even though the electron transfer itself is usually faster by at least one order of magnitude. Both represent novel findings for photolyases and redox enzymes in general.

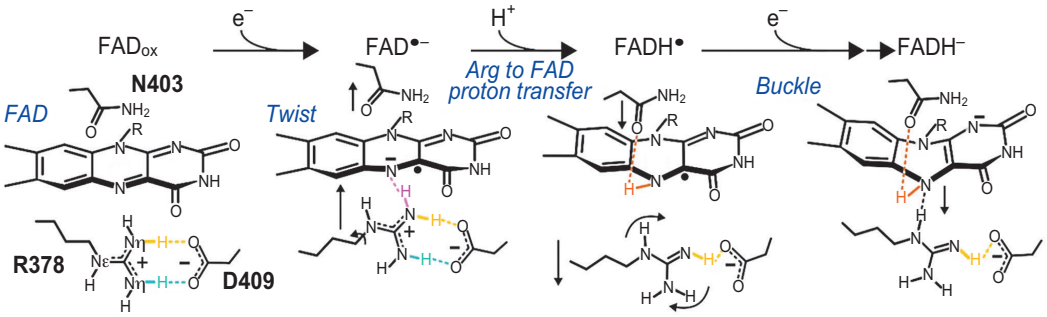

Fig. 3. Schematic representation of the 3D molecular movie. Structural formulas of the FAD and surrounding three amino-acids (R378, N403 and D409) are presented. Covalent interactions are shown as black lines, and non-covalent ones are dotted, colored lines. As the reaction progresses, note how the shape of FAD changes and its interaction network switches between different elements.

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