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Filming DNA repair at the atomic level

Dissection of multistep catalysis by a photoenzyme could inspire green chemistry applications

By Marten H. Vos

Enzymes catalyze biochemical reactions at high rates and specificity. Structural characterization of transition states and intermediates of enzymatic catalysis at the atomic level provides fundamental understanding and could reveal bioengineering possibilities. For most enzymes, real time studies are limited by substrate binding kinetics. By contrast, in natural photoenzymes, enzyme-substrate complexes can be pre-formed and catalysis initiated by ultrashort light pulses. The photoenzyme CPD DNA photolyase repairs the potentially mutagenic DNA cyclobutane pyrimidine dimers (CPDs) that are induced by ultraviolet light. On pages XXX and YYY of this issue, Maestre-Reyna *et al.* (1) and Christou *et al.* (2), respectively, report time-resolved crystallographic studies of photolyase, characterizing a wealth of functional steps spanning the picosecond to microsecond timescale, including electron transfer, substrate bond breaking and product migration. Beyond establishing the order of these events, these findings may inspire engineering of sunlight driven biobased catalysis.

Natural photoenzymes form stable complexes with substrate molecules in the dark (3). Catalytic transformation of substrates to products then occurs upon photon absorption by a molecule within this complex. Very few natural photoenzymes are known, but these are important in several aspects. Using sunlight directly to drive catalysis, they are intrinsically “sustainable” bioreactors. Moreover, the breadth of photoproducts can be expanded by engineering photoenzymes for green chemistry photocatalysis, or by constructing enzyme variants inspired by photoenzymes. On a more fundamental level, using ultrashort light pulses allows visualization of photoenzyme catalytic intermediates in real-time with femtosecond resolution, i.e., down to the timescale of intrinsic nuclear motions. Dissecting such natural catalytic pathways is also informative about non-photochemical enzymes.

Three different photoenzymes are known to date. Catalysis by light-dependent protochlorophyllide oxidoreductase (involved in chlorophyll biosynthesis) is initiated by excitation of

the substrate itself. The two other photoenzymes, photolyase and the algal enzyme fatty acid photodecarboxylase (FAP), both harbor a blue-light absorbing flavin-adenine dinucleotide (FAD) co-enzyme, but their photochemical mechanisms are markedly different. FAD can adopt three different redox states (4), and both enzymes produce semi-reduced FAD radical intermediates. FAP-mediated photoreduction of the oxidized flavin leads to oxidation of the nearby substrate followed by bond breaking, whereas photolyase-mediated photooxidation of the fully reduced FADH⁻ leads to CPD reduction followed by (double) bond breaking (5). Of the three enzymes, only photolyase is also found in non-photosynthetic organisms, although not in placental mammals.

The mechanisms of the three photoenzymes have been studied by time-resolved optical spectroscopy. However, structural snapshots of intermediates with atomic resolution have hitherto only been obtained in FAP (6). Upon oxidation of the fatty acid substrate by the excited flavin, the bond between the hydrocarbon chain and the CO₂ moieties breaks. These structural studies helped to reveal that this bond breaking occurs in hundreds of picoseconds, concomitant with the preceding electron transfer, in full agreement with spectroscopy.

The method of choice for time-resolved structural studies is serial femtosecond crystallography (TR-SFX) using X-ray Free Electron Lasers (XFEL) (7), which became available about a decade ago. Five facilities offering TR-SFX are now operational worldwide, three of which have contributed to the work of Maestre-Reyna *et al.* and Christou *et al.* XFEL sources provide extremely bright and short (sub-10 fs) X-ray pulses allow diffraction from protein microcrystals before any radiation damage (8) (diffract-before-destroy). Using many individual microcrystal diffraction patterns enables the determination of reliable steady-state structures of proteins that are prone to alteration by X-ray radiation (1, 6, 9). When combined with ultrafast visible pulses, intermediate structures in light-induced processes can be determined.

Finding appropriate conditions for the experiments described by Maestre-Reyna *et al.* and Christou *et al.* was challenging. Photolyase is often unstable, and the enzyme from the

anaerobic archaeon *Methanosarcina mazei* appears most suitable to form photolyase-DNA complex microcrystals, which must be produced at the XFEL facilities, under anaerobic and light-shielded conditions. Time-resolved spectroscopy on photocatalysis in solution has been previously performed on other photolyases (10, 11), but not on the *M. mazei* enzyme, presumably due to its low solubility. It is remarkable that these TR-SFX studies are not preceded by any time-resolved spectroscopic characterization of the same process, as with other colored proteins.

The experiments were performed in an intensity regime of visible light just above the level where differences with the structures obtained without illumination become assignable. Yet this regime is far above the linear, physiological, regime where less than one photon is absorbed per protein. The necessity of working in this regime, common to nearly all TR-SFX studies in proteins, is not fully understood and presently of intense interest (12-14). Maestre-Reyna *et al.* and Christou *et al.* argue that in their studies this issue has limited effect, because the catalytic processes are slower than the expected non-linear effects and in general kinetic agreement with linear-regime spectroscopic studies in other photolyases.

The CPD substrate, which consists of a pair of doubly cross-linked adjacent thymine or cytosine bases, initially bulges from the damaged DNA strand into the enzyme. Although the emphasis of the findings differs, both studies come up with a coherent picture of the order and timing of events leading to the restoration of the intact DNA double helix (see the figure). Upon population of the FADH^{*} excited state, four chemical reactions are distinguished within the ~10⁻¹⁰ – 10⁻⁹ s timespan. Flavin to CPD electron transfer occurs. Reducing the CPD breaks its two crosslinks (also without photolysis present). In the *M. mazei* photolyase, the CPD crosslinks were found to break one after the other with sizeable delay both upon reduction and between the bond ruptures. Subsequently the electron returns to the flavin. These events occurred non-concomitantly, which allowed the structural reorganizations associated with the subsequent processes to be distinguished. Whether the two bonds break simultaneously or sequentially has been intensely discussed based on spectroscopy and quantum

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yields (QY, the number of products formed per absorbed photon; sub-unity QY arises from back reactions) for other photolyases (10, 11). The results of Maestre-Reyna *et al.* and Christou *et al.* settle the issue for the *M. mazei* enzyme; yet, given the close timescales, and the enzyme-dependent QY, the situation could vary between different photolyases.

Photolyase catalysis in the strict chemical sense is completed within a few nanoseconds. Two distinct steps occur on a much slower timescale ($>10^7$ s), corresponding to sequential migration out of the active site and partial disordering of the two DNA bases. As pointed out in both studies, this trajectory may be influenced by the crystalline rather than solution environment, hindering full restoration of the repaired DNA double helix structure.

Intriguingly, Christou *et al.* reported a striking 'flip' of the three-ring flavin chromophore along the butterfly bend angle at 3 ps, suggested to functionally stabilize the FADH* excited state. At such short times, the heat from multiple-photon absorption is not dissipated and future studies should investigate this feature under linear excitation conditions. A related open question is the origin of the sub-unity QY, reported by Maestre-Reyna *et al.* at ~ 0.25 , lower than for other photolyases. In neither of the two studies a corresponding strong decay of the product states is reported. This suggests that wasteful back reactions compete with the primary catalytic electron transfer on the picosecond timescale, an issue that can be addressed by time-resolved spectroscopy.

Together, the studies of Maestre-Reyna *et al.* and Christou *et al.* give detailed structural insight in a complex catalytic mechanism and at the same time open up important new avenues for structural, spectroscopic as well as extended quantum computational investigations to map out the entire reaction pathway and energetics based on the observations. Moreover, they are expected to promote further development of engineered flavin-dependent light-driven biocatalysts inspired by photolyase photochemistry initiated by excitation of reduced flavin. These green chemistry systems catalyze a rapidly expanding range of reactions, including highly specific radical cyclizations and polymerizations (15), but are yet far less efficient than native systems (4).

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Light-induced DNA repair monitored by TR-SFX

Figure Legend: Structures of catalytic intermediates of were determined by photolyase microcrystals diffracting intense X-ray pulses arriving at known delays after visible light excitation pulses. At least six distinct intermediates are distinguished between the photolyase-CPD enzyme-substrate complex (left) and the recovered intact double helix (right) spanning more than six orders of magnitude in time. Cartoons representing the four temporally distinct chemical steps occurring on the hundreds of picoseconds to a few nanoseconds time range are shown. These are based on PDB 5ZCW and figures in (1), but do not correspond directly to the structural models. Also reported Intermediates on the microsecond timescale along the trajectory of the repaired bases out of the active site towards the intact double helix are not represented. Details of the intermediates in (1, 2).