Enzyme Catalysis

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Enzyme Catalysis "Reilluminated"

*Wolfgang Gärtner**

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Enzymes are unique products of evolution owing to their capability to accomplish chemical reactions with outstanding efficiency and under extremely mild conditions. Special attention has always been paid to those proteins that succeed in performing either outstandingly complex reactions (considered chemically to be practically impossible) or extremely fast processes that are limited only by diffusion. Hunter et al. have addressed the properties of one such enzyme in a recent publication in *Nature*.[1] The authors were particularly attracted by the question of what regulates enzymes: Is it the change in conformation upon insertion of the substrate into the active site that initiates the reaction, or is it the beginning of the chemical reaction (changes of the electrostatic environment or changing polarization of chemical bonds) that causes conformational changes in the active site?

The target of the investigations by Hunter et al. is the NADPH:protochlorophyllide (Pchlide) oxidoreductase (POR), which was investigated in great detail in the 1960s and 1970s[2,3] (for a recent overview, see Ref. [4]). In this case, the POR from *Thermosynechococcus* elongates was investigated, making use of a homology model for the POR from *Synechocystis* (Figure 1).[5] POR is a key enzyme in the biosynthesis of the photosynthetic units, and it mediates the light-induced generation of the chlorophyll precursor chlorophyllide (Chlide, from protochlorophyllide), thereby constituting the basis for the biosynthesis of the other components of the photosynthetic apparatus. This function encompasses all features required for its classification as a biological photoreceptor. The light induction of the enzymatic activity was described in early work.[2,3] In continuously irradiated preparations, a remarkably high quantum yield of 0.85 for the conversion of Pchlide is found. POR carries one molecule of NADPH (nicotinamide adenine dinucleotide phosphate in reduced form) as cofactor that takes part in the hydrogenation of a substrate's double bond (C17–C18). The initial reaction consists of a hydride transfer to position 17 and subsequent *trans* addition of a proton to position 18. This proton is provided by the phenolic hydroxy group of a tyrosine residue in close proximity (as demonstrated by site-directed mutagenesis).[6] Dehydrogenases such as POR are amongst the fastest enzymes known, in some cases accomplishing an acceleration of the reaction by seventeen orders of magnitude compared to the uncatalyzed reaction.[1,7]

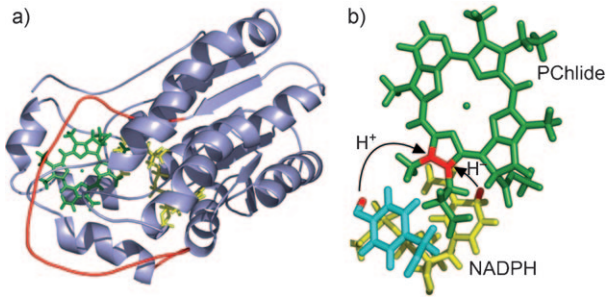

Figure 1. Homology model for POR from *Synechocystis* (left).[5] In contrast to other oxidoreductases, POR carries a 33 amino acid extension (red) assumed to be involved in binding of Pchlide. Right: detailed three-dimensional model showing the arrangement of Pchlide (green), NADPH (yellow, positioned below), and the proton-transferring tyrosine residue (blue). The reactive positions (hydride, proton, and C17–C18 double bond) are shown in red. The arrows indicate the *trans* addition of the two hydrogen atoms.

Interestingly, a preincubated complex of NADPH, Pchlide, and apo-POR is entirely stable in the dark and requires light for activation and maintenance of the reaction, making POR an excellent system for the examination of the questions described above. The authors even indicate that a substrate-loaded enzyme preactivated by a single flash remains in the activated state for at least 19 h and catalysis can still be triggered by additional irradiation. Accordingly, it should be possible to detect conformational changes during the reaction and to break down the catalytic process into its fundamental steps, if only sufficiently short flashes of extremely low intensity can be applied.

Indeed, irradiation of a POR sample with ultrashort, weak laser flashes ($\lambda_{exc} = 475$ nm, ca. 0.03 photons per POR molecule with a pulse duration of ca. 50 fs), combined with appropriate detection methods, yielded surprising results (irradiation was performed using the Soret band; detection was in the Q_y band). The low light intensity required accumulated series of scans to obtain a readable signal. Examination of scans 1 and 2 in comparison to scans 6–12 and 26–55 revealed different structures for the absorption changes in each scan series (Figure 2). Three time domains (ca. 1 ps, several hundred picoseconds, and ca. 5 ns) are presented for each series of scans. Within approximately 1 ps of the laser flash, a practically instantaneous bleaching of the Pchlide absorption band around 640 nm is detected; this band later (beyond the observation time

[*] W. Gärtner
Max-Planck-Institut für Bioanorganische Chemie
Stiftstrasse 34-36, 45470 Mülheim (Germany)
E-mail: gaertner@mpi-muelheim.mpg.de

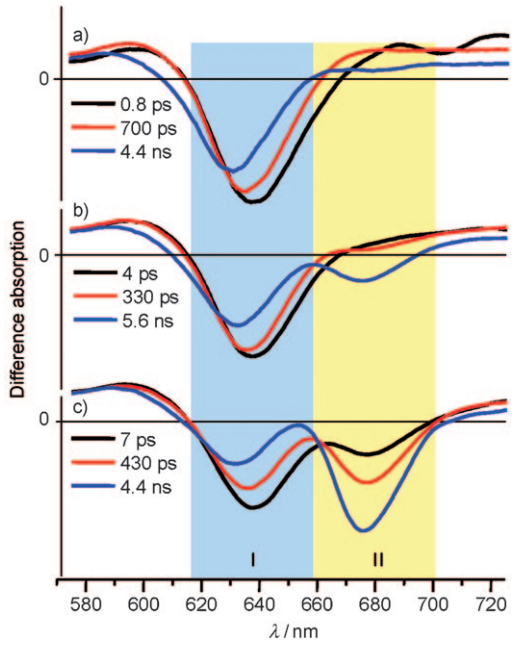

Figure 2. Course of light-induced absorption changes near the Q_y band of Pchlide. Two regions of maximal absorption changes are marked in blue and yellow. Shown are three series of scans for data acquisition: a) scans 1 and 2, b) scans 6–12, and c) scans 26–55. Each series was acquired for an interval from ca. 1 ps to 5 ns. Absorption changes in the time domains of a few picoseconds (black curves), several hundred picoseconds (red), and several nanoseconds (blue) are shown for all three scan series (relative to the spectrum at time $t = 0$ for each series).

window) converts into the absorption of the Chlide product. At 0.8 ps, a clearly detectable stimulated emission is found together with the bleaching in scans 1 and 2. In all three series of scans, the initially observed bleaching at 640 nm shifts to shorter wavelengths when the detection time window is extended to 4–5 ns. This finding clearly demonstrates that reactions or structural rearrangements take place on this short time scale. For the longest observation period (5.6 ns), the evaluation of scans 6–12 shows the emission of a second species around 675 nm, which becomes dominant in scans 26–55. The quantum yield and the rate constants for the generation of this active species I_{675}* were calculated separately for all three scan series. Both parameters increase as the duration of light exposure of the sample increases. From these EADS (evolution-associated difference spectra), the authors developed a three-state reaction scheme, employing a (model-related) target-analysis, in full accordance with the observed spectral changes: the continuous application of the short, weak laser flashes causes accumulation of activated Pchlide, which travels through a state of maximal concentration and finally converts into the product (Chlide). The model includes several intermediate states that the Pchlide molecule adopts in the excited state.

This observed accumulation of a reactive species caused by consecutive laser flashes then made possible the application of (rapid-scan) vibrational spectroscopy (FTIR), allowing simultaneous detection of the changes in conformation of substrate *and* enzyme. It was then possible to assign IR bands that gain or lose intensity with successive laser flashes to each of the three states of the model derived from the ps/ns detection. For example, the intensity decrease of the NADPH and Pchlide C=O (carbonyl) bands and the corresponding intensity increase of the carbonyl bands for $NADP^+$ and the product Chlide can be clearly observed. The intensity increase of the C–C stretching vibrations of the product are also readily assigned. Most importantly, these changes in the intensity of substrate and cofactor bands occur simultaneously with changes in the amide I and amide II bands assigned to protein backbone vibrations. This congruence unambiguously connects changes of the substrate with changes of the protein.

The observed requirement of continuous irradiation to maintain a high turnover rate led the authors to propose a reaction mechanism for the enzyme activity of POR. The primary absorption of a photon generates the active state of the protein, from which the intermediate I_{675}* is formed. This already induces changes of the enzyme's electronic properties and potentially also changes the acidity of the reactive positions. Apparently, these changes move the C17–C18 region of the substrate, the NADPH molecule, and the tyrosine side chain into more favorable positions. Absorption of additional photons is required to drive the reaction until the product is formed, but the molecular nature of these changes is not yet fully understood. The authors mention that the additional light activation is probably essential for the hydride transfer step. Only after this reaction, probably after accumulation of negative charge at position 18, does the proton transfer become possible. Both transfer processes are extremely fast and are considered to be tunneling processes (supported by quantum chemical calculations),[8] probably requiring the light energy to adjust the relative conformation of the reaction partners in the excited state

Surely, POR is a unique model for the investigation of catalytic processes in enzymes, but the obtained results clearly reveal generally valid reaction mechanisms. Light activation allows an instantaneous start of reactivity—in contrast to a diffusion-limited start when a substrate has to be added. The further sequence of reactions, however, can only be resolved by application of state-of-the-art spectroscopy with ultrashort laser flashes. Although originally meant in a different context, Goethe's last request is clearly also valid for POR: "More light!"[9]

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