Enzymatic Activity and Excited State Processes in Protochlorophyllide Oxidoreductase

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~ CHAPTER 2 ~

Conformational changes in an ultrafast light-driven enzyme determine catalytic activity

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The role of conformational changes in explaining the huge catalytic power of enzymes is currently one of the most challenging questions in biology[13, 28-32, 34]. Although it is now widely regarded that enzymes modulate reaction rates by means of short- and long-range protein motions[29-32, 34], it is almost impossible to distinguish between conformational changes and catalysis. We have solved this problem using the chlorophyll biosynthetic enzyme NADPH: protochlorophyllide (Pchlide) oxidoreductase, which catalyses a unique light-driven reaction involving hydride and proton transfers[5]. Here we report that prior excitation of the enzyme-substrate complex with a laser pulse induces a more favourable conformation of the active site, enabling the coupled hydride and proton transfer reactions to occur. This effect, which is triggered during the Pchlide excited-state lifetime and persists on a long timescale, switches the enzyme into an active state characterized by a high rate and quantum yield of formation of a catalytic intermediate. The corresponding spectral changes in the mid-infrared following the absorption of one photon reveal significant conformational changes in the enzyme, illustrating the importance of flexibility and dynamics in the structure of enzymes for their function.

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2.1. Introduction

Dehydrogenase enzymes catalyse proton and hydride transfer reactions with rate enhancements of up to 10^{17} in comparison with the equivalent reaction in solution[13]. However, understanding the role of conformational changes in this catalytic power is challenging, as the hydrogen transfer processes are much faster than the structural changes associated with switching of an enzyme from an 'inactive' to an 'active' conformation[25, 26, 35].

Here we study an enzyme from the family of alcohol dehydrogenases, NADPH:protochlorophyllide oxidoreductase (POR), which has provided a unique opportunity to observe the light-driven formation of an activated enzyme conformation before catalysis. The requirement of light for initiation of catalysis by POR makes it an excellent model system for studying the mechanism and timescales of enzymatic proton and hydride transfers, as the enzyme-substrate complex can be pre-formed in the dark and catalysis initiated by a short pulse of light[5]. POR catalyses the *trans* addition of hydrogen from NADPH across the C17=C18 double bond of the D ring of Pchlide to produce chlorophyllide (Chlide)[8], which is an important regulatory step for chlorophyll biosynthesis and the subsequent assembly of the photosynthetic apparatus[5, 8, 36]. It is proposed that a conserved Tyr residue donates a proton to the C18 position[14] and a hydride is transferred from the *proS* face of the NADPH nicotinamide ring to the C17 position of the Pchlide molecule[21, 22] (figure 1.2 in chapter 1). The involvement of the Pchlide excited state in the reduction suggests that significant parts of the reaction may occur on the picosecond timescale. Indeed, we have previously shown that catalysis can be triggered with a 50-fs laser pulse, and, using ultrafast pump–probe absorption spectroscopy, observed that the formation of a reaction intermediate in the excited state proceeds with time constants[20] of 3 ps and 400 ps. In addition, following the formation of the POR–Chlide–$NADP^+$ state there is a series of ordered product release and cofactor binding events in which domain movements and/or reorganization of the protein have an important role[18, 19, 37, 38].

2.2. Methods Summary

Composition of the samples

POR from the cyanobacterium *Thermosynechococcus elongatus* BP-1 was produced as previously described[19]. The samples contained 0.5 mM Pchlide, 0.5 mM POR and 2.5 mM NADPH in activity buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 1% Genapol, 0.1% β-

mercaptoethanol) and were kept in the dark at all times. For FTIR spectroscopy experiments the H_2O in the buffer was replaced by D_2O.

Visible pump–probe experiments

Absorption difference spectra at 48 different time points between -10 ps and 5 ns were collected with a set-up based on a 1-kHz amplified laser system, which has been described previously[39]. Briefly, the output of a regenerative Ti:sapphire amplifier operating at 1 kHz (Hurricane, Spectra Physics), producing 85-fs pulses of ~0.8 mJ, was used to pump a non-collinear optical amplifier. This amplifier was tuned to 475 nm to excite the complexes at the red edge of the Soret band (S0→S2 transition) of Pchlide, where the absorption of the product Chlide is minimal. The excitation energy per laser flash was 100 nJ, focused to a spot of about 150-µm diameter. A small part of the 800-nm output was used to generate a white light continuum in a sapphire plate and, after interrogating the sample, was dispersed in a spectrograph and detected on a 256-element diode array read out at 1 kHz. Detection was in the wavelength region 600–700 nm, probing the S0↔S1 transition of Pchlide and products. The polarization of the probe light was at the 'magic angle' (54.7°) with respect to the excitation light. The instrument response function was about 130 fs. The sample was put in a CaF_2 cell of 200-µm path length, contained in a Lissajous sample scanner. The scanner was moved at such speed that a new part of the sample was illuminated at every shot (the shots taken at the corners of the scanned area, where the scanner velocity is lower, were discarded). The same volume was illuminated again after 1-2 min. One scan of the pump-delay line, recording data from -15 ps to 6 ns, took ~40 s, so each scan corresponds to one laser pulse with an energy density of ~0.03 photons per enzyme. Hence, during one scan each part of the sample was illuminated by a single shot of the laser, in subsequent scans the sample was illuminated by more laser pulses. Previous[20] ultrafast transient absorption measurements on POR have used a flow cell for the sample (~4 mL) and the acquisition time of one scan was approximately 10 min. All experiments were performed at room temperature (22 °C). The data was analyzed using a global, and a target fit algorithms[40]. The spectra shown in figure 2.1 are the averages of spectra recorded on three freshly prepared samples. A full description of the global and target analysis is given in section 2.4. Supplementary information.

Temperature effects

A simple calculation shows that negligible water heating occurs in our experiment. If we suppose that half of the laser energy (E = 100 nJ) is absorbed by the sample, it can be calculated that with a volume of 0.1 ml (the water thermal capacity being 4 J ml^{-1} K^{-1}), we

would need 8×10^6 laser shots to heat the water by 1 K, which at 500 Hz takes 4 h. This is neglecting thermalization with the environment. In a similar way, we can calculate with convection equations that if the sample is at 5 °C when it is put into the cell, it takes only about 77 s to reach room temperature. Thus, we may consider the enzymes to be at room temperature during the experiments.

FTIR spectroscopy experiments

Light-minus-dark difference spectra were measured by means of an FTIR Bruker IFS 66/S spectrometer in rapid-scan mode. First we collected infrared steady-state absorption spectra of the POR:Pchlide:NADPH complex in the dark, as the background, and then we recorded the induced absorption changes every second while the whole area of the sample cell was excited at 444 nm by the 20-Hz output of a Continuum Panther optical parametric oscillator pumped by a Continuum Surelite I-20 Nd:YAG laser.

2.3. Results

Here we use ultrafast pump–probe absorption difference spectroscopy to study the initial catalytic steps in more detail, which reveals that a critical light-driven activation of the enzyme is required before catalysis. The use of a Lissajous sample scanner in combination with very high detection sensitivity facilitates quantitative measurements under single-pulse conditions, allowing us to measure reaction rates and quantum yields as functions of the total number of laser shots previously seen by the sample. Our spectrometer ensures that in each scan the sample is illuminated by only one shot of the laser, with an excitation density of (0.03 absorbed photons per enzyme-substrate complex. The time-dependent absorption difference spectra obtained after a laser pulse are shown in figure 2.1.A, B, C for a number of subsequent scans in the form of evolution-associated difference spectra (EADS). The EADS result from a global analysis of the data in which the time-dependent behaviour of the absorption difference spectra is fitted to three exponentials that evolve in a sequential manner, A→B→C→, with increasing lifetimes τ_1, τ_2 and τ_3. The EADS represent the spectra of the states A, B and C. Inspection of the absorption difference spectra shows that, although the enzyme shows spectral evolution on an ultrafast timescale, it is clear that prior exposure to light has a marked influence on the dynamics and, more profoundly, the yield of the reaction.

All scans show a negative band that peaks at 640 nm and originates in the laser-excited, enzyme-bound Pchlide substrate. However, in the later scans a significant negative band also appears at ~675 nm, owing to a Chlide-precursor species formed in its excited state (figure 2.1.B, C) [(20)]. Recent theoretical studies [(41)] together with preliminary

experimental data (Supplementary figure S1) suggest that this precursor species (I675*) is likely to represent a state in which Pchlide forms a strongly hydrogen-bonded complex with residues in its direct environment and/or NADPH, which is essential for the subsequent hydride and proton transfer steps to proceed on a microsecond timescale. In the initial scans there is a progressive blue shift of the signal at 640 nm, in combination with a loss of stimulated emission at the red end of this band (figure 2.1.A), and only a very small amount of negative signal at ~675 nm is formed in 700 ps. The formation time and yield of the negative 675-nm signal, indicative of the I675* precursor, is completely dependent upon the scan number; in later scans I675* formation occurs with 4-ps and 540-ps time constants and with a much higher yield (figure 2.1.C). In addition, direct excitation of the Chlide product, formed during previous scans, is observed in the later scans, as the signal at ~670 nm is negative in the time-zero spectra (black spectra in figure 2.1.B, C).

To analyse the data in more detail, we fit the full set of spectra to a minimal model (top and bottom panels in figure 2.1.D). In this model we divide the POR enzyme population into an 'inactive' fraction, which describes the Pchlide* dynamics (between the states denoted Pchlide*I, Pchlide*II and Pchlide*III, in that order), and an 'active' fraction, in which there is an additional reaction path from each of the Pchlide*I, Pchlide*II and Pchlide*III states to I675*. Also, the directly excited Chlide formed in previous scans is included. The results of this target analysis are shown in figure 2.1.E, and representative traces and fits can be found in section 2.4. Supplementary Information.

The size of the fraction of active enzyme is dependent on the number of laser pulses and increases in a similar manner to the proportion of enzymes that have been excited at least once. In fully light-activated enzymes, the quantum yield of transformation of Pchlide to the Chlide precursor I675* is 0.85, of which 0.35 occurs by means of the single-step mechanism, with an effective rate of ~300 ns^{-1} (top panel in figure 2.1.D), and 0.5 by means of the two-step mechanism, with an effective rate of 3.7 ns^{-1}. Also, the accumulating concentration of Chlide (middle panel figure 2.1.D) can be described with a yield of 0.3 for the I675*-to-Chlide reaction, consistent with previous[19] quantum yield measurements of 0.21.

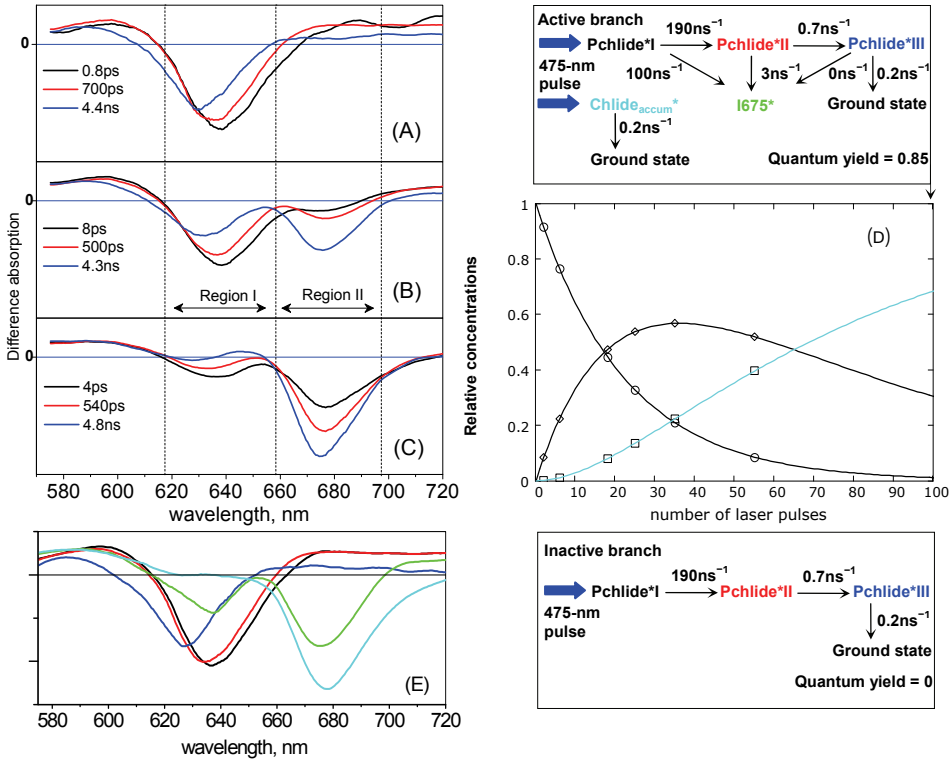

Figure 2.1. (A, B, C) Evolution-associated difference spectra resulting from a global analysis, as a function of illumination time. A sequential model with increasing lifetimes was used. Excitation of the POR:Pchlide:NADPH complex was at 475 nm, the induced absorption changes were recorded at 48 different time points between −10 ps and 5 ns, and one spectrum consists of 256 spectral points. Region I is composed of bleached absorption and stimulated emission from the Pchlide substrate, and in region II the stimulated emission signal is observed from the I675* product formed in the excited state. (A) Scans 1 and 2; (B) scans 6–12; (C) scans 26–55. The vertical scale corresponds to an absorption change of -30×10^{-3}.

(D) Model for the ultrafast catalytic reactions in POR applied in a target analysis[40] of the illumination-dependent POR pump–probe data sets. **Top panel:** Kinetic scheme for active enzymes that can form the photoproduct I675*; **Middle panel:** concentration of inactive (circles), active (diamonds) enzymes and accumulated Chlide (squares) as a function of applied laser pulse. The concentrations at $t = 0$ of inactive and active enzymes and Chlide accumulated in previous scans ($Chlide_{accum}$*) are assumed to follow the populations of unexcited, singly excited and twice-excited enzymes, respectively (solid lines drawn for an excitation density of 0.045/pulse); **Bottom panel:** kinetic scheme for inactive enzymes, showing only Pchlide* photochemistry (from Pchlide*I to Pchlide*II to Pchlide*III).

(E) Species-associated difference spectra of the Pchlide*I, Pchlide*II, Pchlide*III, I675* and $Chlide_{accum}$* states obtained from the target analysis; the spectra are colour coded to match the states in the kinetic scheme.

Because we exclude any possible effects of temperature or changing sample conditions during each scan (Supplementary figure S2), there must be a direct relationship between the rate of the reaction and the level of illumination, in terms of the number of laser pulses used for prior activation of the enzyme-substrate complexes. Therefore, it appears that catalysis cannot proceed until the enzyme-substrate complex has been excited at least once, suggesting that the first photon turns the enzyme 'on', whereas the second photon induces catalysis. Additional experiments, in which we place an activated enzyme sample in the dark for several periods of time, reveal that the active conformation persists for at least 19 hours (Supplementary figure S4), suggesting that the enzyme-substrate complex has a 'memory' which lasts for a long time relative to the timescale of the catalytic events. In addition, we also show that, upon completion of a single turnover and mixing with fresh Pchlide, POR retains between 80 and 100% of its catalytic activity (Supplementary figure S5).

These results demonstrate that the rate and quantum yield of formation of the intermediate state I675* is significantly enhanced after the Pchlide substrate has cycled through the excited state at least once. This effect may arise from a more favourable catalytic configuration of the enzyme-substrate complex, caused by the changed electron distribution in the Pchlide excited state. To investigate whether conformational changes in the enzyme are induced upon the absorption of a photon, we recorded absorption difference spectra in the mid-infrared region under illumination conditions similar to those used in figure 2.1. The difference spectra (light-minus-dark) are recorded every second using a Fourier transform infrared (FTIR) spectrometer in rapid-scan mode, while flashing with 5-ns laser pulses at a 20-Hz repetition rate to excite the enzyme-substrate complex. The traces recorded between 1800 and 1250 cm^{-1} (figure 2.2.A) clearly show different saturation behaviours at different frequencies, suggesting the occurrence of two or more different light-induced processes. The time- or photon-flux-dependent behaviour of the data can be described with a sequential model, A→B→C→, with increasing lifetimes/photon fluxes τ_1, τ_2 and τ_3, resulting in the spectra depicted in figure 2.2.B.

The presence of relatively large signals in the amide I and II regions shows that brief illumination of the enzyme-substrate complex (corresponding to 20 flashes of 5-ns laser pulses) yields conformational changes in the secondary structure of the enzyme. The amplitude of these signals is increased by further illumination of the sample (figure 2.2.B, red spectrum), and the conformational change in POR has an extremely long lifetime of many minutes (Supplementary figure S4). However, in neither case are these signals accompanied by catalytic activity; only continued illumination produces the mid-infrared signatures for the disappearance of Pchlide and NADPH and the formation of Chlide and $NADP^+$ (figure 2.2.B, blue spectrum).

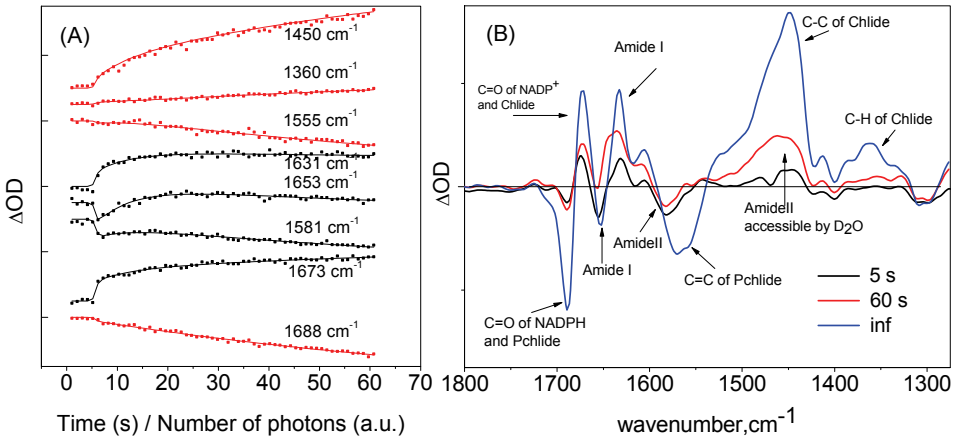

Figure 2.2. Mid-infrared absorption difference data. (A) Selected time/photon flux traces; a.u., arbitrary units. (B) corresponding evolution-associated difference spectra. The EADS result from a global analysis of the light-minus-dark difference spectra using a sequential model with increasing lifetimes, A→B→C→. Negative bands arise from the disappearance of infrared absorption associated with Pchlide and NADPH substrates and the POR protein in the dark state, and positive bands are due to the appearance of new species in the reaction upon illumination, such as Chlide, NADP+ and POR in its active conformation.

The two distinct phases in the mid-infrared spectral evolution clearly indicate that the enzyme undergoes two separate processes upon illumination: there is an initial structural optimization, and then − only after continued illumination − is Pchlide converted into Chlide. Combining the results from the femtosecond visible pump-probe and those in the mid-infrared, we conclude that absorption of the first photon activates the enzyme, which results in a high quantum yield formation of I675* on the picosecond timescale when a second photon is absorbed. The structural changes are currently difficult to quantify using the spectroscopic changes in the infrared, and may involve minor structural rearrangements, optimizing the alignment of the NADPH-nicotinamide ring and the Tyr residue with the D ring of Pchlide to increase the rate of the hydride and proton transfers. On the other hand, the long lifetime and the fact that activation is not reversed upon turnover of the enzyme may suggest an irreversible process in the POR protein. It would appear that the conformational change is both highly efficient (that is, it needs only one photon) and very specific, resulting in a high catalytic quantum yield. It is likely that such a directional

conformational change underlies efficient hydrogen and proton transfers in many other enzymes.

Several other enzymes have been found to have a 'rest' conformation and a second conformation in which the enzyme is 'active'[25-27]. In POR, however, we find that the active conformation is retained during and after turnover. This light-adaptation of POR may have an important role in green plants and other photosynthetic organisms, as it allows the enzyme to capitalize on very low or erratic photon fluxes by remaining in an activated, catalytically efficient state for long periods.

2.4. Supplementary Information

2.4.1. Global analysis

For each illumination condition, all 48 time-gated spectra (measured at 256 wavelengths) were collated in a matrix, which was globally fitted using a sequential kinetic scheme with increasing lifetimes[40]. From this the lifetimes and the EADS were estimated. The quality of the fit was judged by inspection of the singular vectors of the matrix of residuals, which had to be structureless. The instrument response function was described by a Gaussian shape, and the white-light dispersion over the spectral range was modelled as a second-order polynomial. With increasing lifetimes, and thus decreasing rates, the first of the EADS decays with the first lifetime and corresponds to the difference spectrum at time zero with an ideal, infinitely small instrument response function. The second of the EADS is formed with the first lifetime and decays with the second lifetime. The third of the EADS (and in our case the final one) represents the difference spectrum of the longest-living species. It is formed with the second lifetime and decays with the third lifetime. The error in the lifetimes obtained from the fitting procedure does not exceed 10%. EADS may not represent pure species, and they are interpreted as a weighted sum (with only positive contributions) of species-associated difference spectra (SADS). Referring to figure 2.1.A, B, C, we note that in the blue spectral region (612–656 nm) an evolution of the Pchlide* bleaching and stimulated emission is observed, whereas in the red spectral region (656–700 nm) Pchlide* excited-state absorption, as well as Chlide* bleaching and stimulated emission, contributes.

2.4.2. Target analysis

To resolve the SADS from the EADS, a target analysis was performed simultaneously on data from all seven illumination conditions (~98 000 data points in total). In this target analysis, the kinetic scheme from figure 2.1.D was used to estimate the microscopic rate

constants and SADS of the three successive Pchlide* species (black, red and blue, figure 2.1.E), the accumulated Chlide* ($Chlide_{accum}$*, cyan) bleaching and stimulated emission, and the stimulated emission of the I675* product (green). We assume that the Pchlide* photochemistry is intrinsic for Pchlide* and therefore independent of the activation state of the protein.

The concentrations of inactive and active enzymes and of $Chlide_{accum}$* at $t = 0$ for each successive scan are assumed to depend on the number of applied laser pulses according to the analytical expression for the concentration of unexcited, singly excited and twice-excited enzymes obtained by solving the coupled differential equations belonging to the following rate equation:

$$inactive \underset{k1}{\rightarrow} active \underset{k2}{\rightarrow} (I675^*)Chlide$$

Here k_1 is the excitation rate per pulse and k_2 is the excitation rate per pulse multiplied by the quantum yield of Chlide formation. I675* is set within parentheses because it is a short-lived intermediate and, therefore, its concentration at the moment of excitation will be zero. Fitting this model to the data, we obtain the estimated rate constant for each reaction, as indicated in the schemes, 0.045 per pulse for the excitation rate and 0.3±0.1 for the quantum yield (QY) of Chlide formation. The quantum yield of I675* product formation in activated enzymes is 0.85, which is obtained from the following expression: $QY = k_{product}/(k_{product}+k_{loss})$. Here $k_{product}$ and k_{loss} refer to the rates towards the I675* state and to the Pchlide*II (or Pchlide*III) state, respectively. The quantum yield for product formation in inactive enzymes is zero. Therefore, in this experiment, by measuring the dynamics on a picosecond timescale and the concentrations of the different populations at time zero, we independently determine the quantum yield of formation of both I675* and Chlide formation and find them to be 0.85 and 0.3, respectively.

The estimated difference spectra of the Pchlide*I, Pchlide*II and Pchlide*III states (respectively black, red and blue in figure 2.1.E) are flat in the 670-720-nm region and represent pure Pchlide spectra. The Pchlide*III spectrum most likely represents a fraction of Pchlide not bound to the POR enzyme, whereas the Pchlide*I-to-Pchlide*II dynamic process is probably due to vibrational relaxation. In previous work, we showed that formation of the state emitting at 675 nm depends on the presence of NADPH and the Tyr189 proton donor[20]. H^- transfer most likely occurs with a rate of 2×10^6 s^{-1} (Supplementary figure S1); therefore, we believe that the state emitting at 675 nm is an intermediate in the catalytic reaction and represents the formation of a hydrogen-bonded complex. The spectrum resolved for the I675* state (green) shows negative bands at 675 nm and 640 nm, consistent with stimulated emission from I675* and bleached absorption of Pchlide. Finally, the spectrum of the state $Chlide_{accum}$* (cyan) is consistent

with that of directly excited Chlide, which is formed in previous scans on a timescale longer than nanoseconds, outside the measuring window of the current experiment.

The root-mean-square error of the fit was 0.9×10^{-3}, which means that, with a maximum signal of 26×10^{-3}, the signal-to-noise ratio is almost 30. The matrices of residuals resulting from the target analysis were further analysed using a singular value decomposition[39]. The first left-singular vectors from all seven experiments (Supplementary figure S3) show no structure, indicating that all kinetics are described satisfactorily. The structure in the first right-singular vector (Supplementary figure S3) resembles the Chlide spectrum, which probably indicates that the noise in the measurement arises from small fluctuations in the transmission of the sample (due to varying relative amounts of Pchlide and Chlide caused by the fact that the movement of the Lissajous scanner is not locked to the acquisition), rather than from noise in the laser pulses.

2.4.3. Assignment of bands in FTIR spectra

The infrared absorption difference signals in the frequency range 1660–1630 cm^{-1} can be assigned to the amide I protein band[42] and the bleaches of absorption at both 1580 cm^{-1} and at 1470 cm^{-1} to amide II[43], the former representing amide groups not accessible by D_2O and the latter representing amide groups that are accessible by the solvent. Differential bands between 1690 and 1670 cm^{-1} were assigned to carbonyl vibrations[43] of NADPH/$NADP^+$ and Pchlide/Chlide[44-46]. The 1570–1450 cm^{-1} differential signal is assigned to the Pchlide-to-Chlide conversion process representing disappearance of a C=C mode and appearance of a C–C mode[44-46]. The signal around 1360 cm^{-1} was assigned to the appearance of a chlorin C–H mode[44-46], and therefore signals of Chlide formation.

2.4.4. Supplementary figures

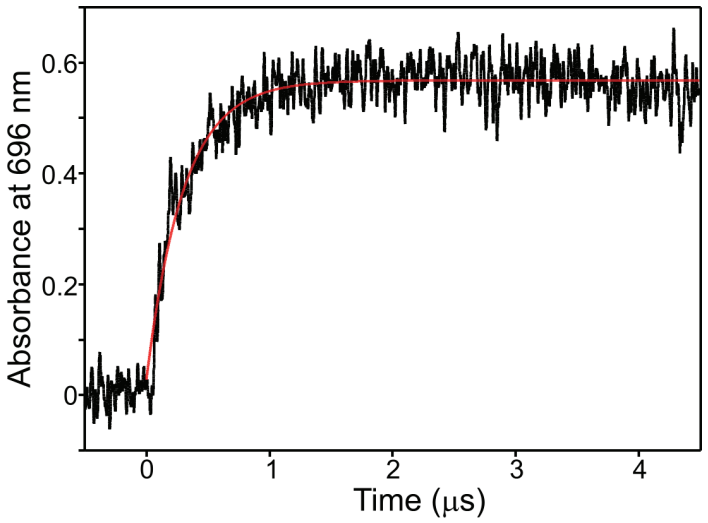

Figure S1. In order to detect further intermediates in the reaction, 1 mL samples containing 30 μM POR, 100 μM NADPH and 10 μM Pchlide in 50 mM Tris-HCl, pH 7.5, 0.1% Genapol, 0.1% β-mercaptoethanol were excited at 450 nm by using an OPO of a Q-switched Nd-YAG laser (Brilliant B, Quantel) in a cuvette of 1 cm pathlength. The energy output of each laser pulse was ~60 mJ and pulses were 6-8 ns in duration. Spectral transients were recorded at 696 nm using an Applied Photophysics LKS-60 flash photolysis instrument with the detection system at right angles to the incident laser beam. The probe light (150 W xenon lamp) was passed through a monochromator before and after passage through the sample. Absorbance changes were measured using a photomultiplier tube and kinetic transients were typically collected over 5 μs. Consequently, an intermediate at 696 nm was shown to form within 2-3 μs. As this intermediate has previously been shown to represent a charge-transfer species formed by hydride transfer from the NADPH it confirms that hydride transfer will only proceed after the formation of I675*.

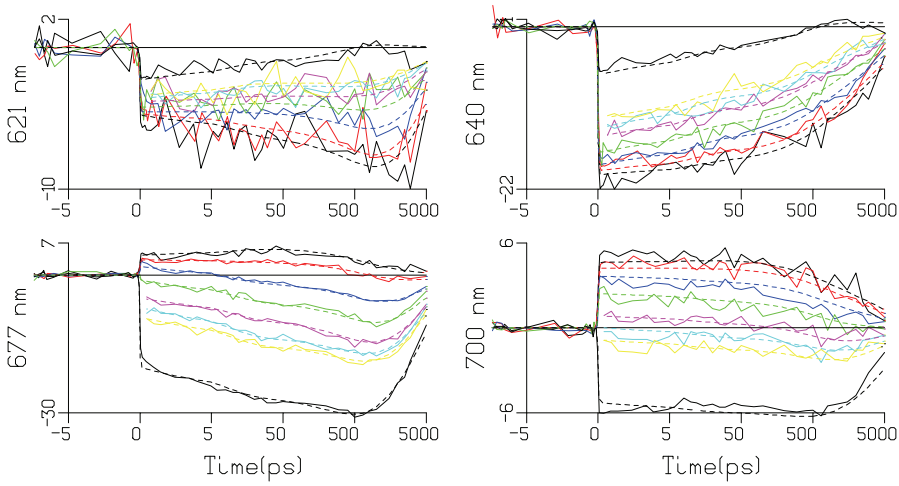

Figure S2. Time traces recorded at 621, 640, 677 and 700 nm. Scans 1-2 (black), 3-5 (red), 6-18 (blue), 19-25 (green), 26-35 (magenta), 36-45 (cyan), 46-55 (yellow), 55-100 (black). Scans 36-45 were measured in reversed-time direction to exclude that the illumination effect is caused by changing sample conditions during a scan. The time traces are the average of traces recorded on three freshly prepared samples. The dotted lines are the fit resulting from the model described in figure 2.1.D. Note that the time axis is linear from -5 to 5 ps relative to the maximum of the instrument response, and logarithmic thereafter.

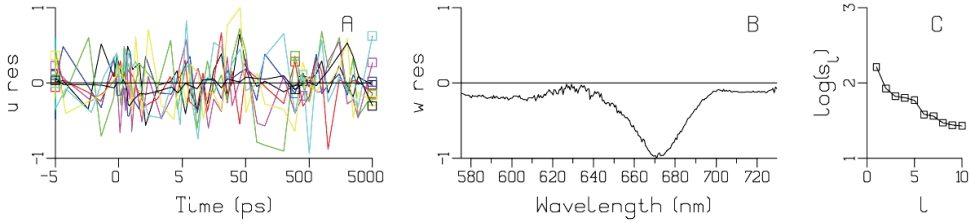

Figure S3. Singular Value Decomposition of the matrix of residuals resulting from the target analysis. (A) first left singular vectors res,1 u , key to colors and time axis as in figure S2. (B) first right singular vector res,1 w; (C) first ten singular values res,l s on a logarithmic scale.

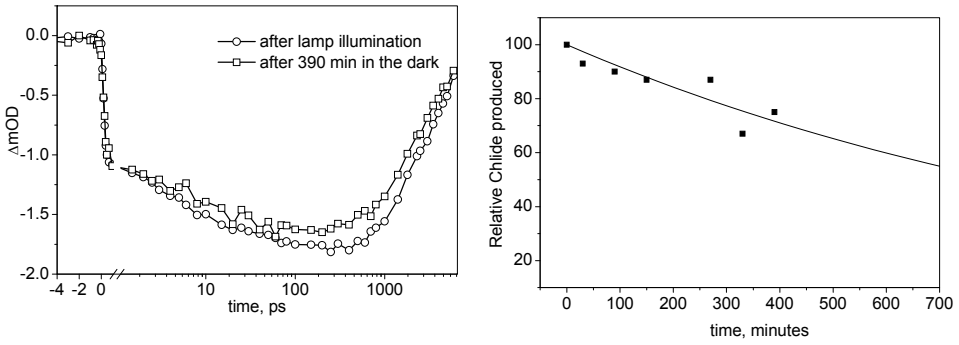

Figure S4. In order to determine the lifetime of the activated POR:Pchlide complex transient absorption experiments were performed on samples homogeneously preilluminated with a 40 W tungsten lamp for 30 seconds, which were then placed in the dark for several periods of time. Samples (a mix of protein, substrate and cofactor was prepared in the dark with concentrations 0.5 mM, 0.5 mM and 3 mM respectively) were in a 1-mm path quartz cuvette, placed 50 cm from the lamp, no heat or spectral filter was used. The time of pre-illumination was chosen such as to obtain a highly activated sample where the majority of enzyme complexes have absorbed at least one photon.

(A). Transient absorption traces recorded at 670 nm, upon excitation at 475 nm, (black curve), and after a subsequent period of 390 mins in the dark (red curve). The traces were normalized on the amount of directly excited Chlide, i.e. on the amplitude of the signal at zero time delay.

(B) Relative reduction in active enzymes as a function of dark period obtained by integration of the area under the recorded time traces, after subtraction of contribution of directly excited Chlide. The solid line is a single exponential fit with a time constant of 1170 minutes (19.5 hrs).

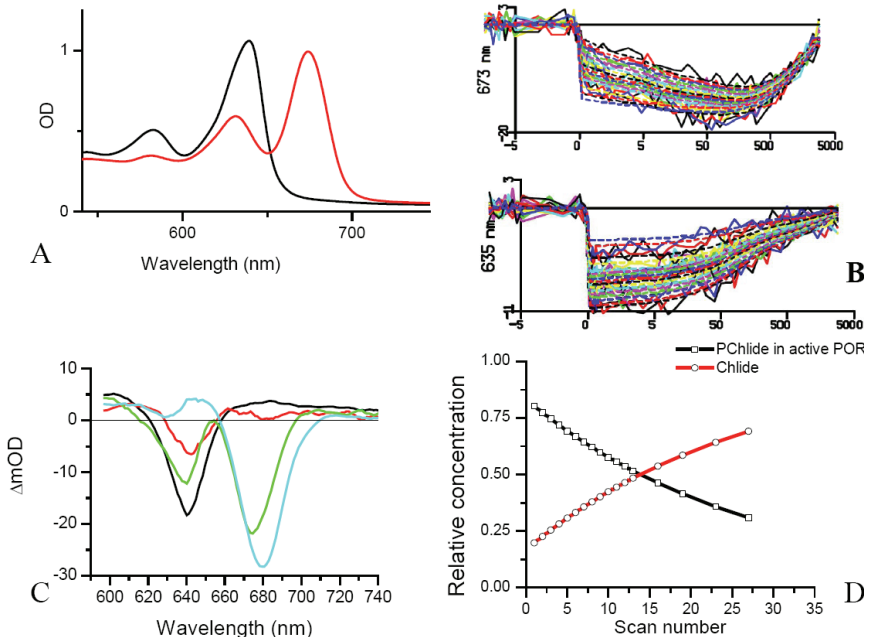

Figure S5. To determine whether the enzyme retains its activity after turnover and release of the Chlide product, experiments were performed on converted samples that were mixed with fresh Pchlide.

(A) The visible absorption spectrum of a sample that has never seen light is shown in black. The absorption maximum is at 639 nm corresponding to Pchlide bound to the enzyme. The absorption spectrum of the sample after it has been illuminated with a CW lamp for 5-10 minutes until a large fraction of the Pchlide was converted into Chlide (peak at 674 nm) is shown in red. This sample was remixed with fresh Pchlide and NADPH and was immediately injected in the experimental cell.

(B) The time traces of 27 consecutive scans, recorded at 635 nm and 673 nm. The negative offset at t = 0 at 673 nm is indicative of the amount of Chlide present in the sample, and increases with each scan. The dynamics at 673 nm are indicative of the amount of I675* formed on the timescale of the experiment. Remarkably, already in the first scan, a lot of I675* is produced, and the dynamics seems fairly similar in each consecutive scan.

(C) Using the analysis as detailed in the manuscript, we can obtain a satisfactory fit of the full dataset (see panel B), without including an inactive fraction of POR. This fit results in spectra of the Pchlide*I (black), Pchlide*II and Pchlide*III states (restricted to have the same spectrum, red), the I675* state (green) and accumulated Chlide (cyan), that are very similar to those reported in figure 2.1.E of the manuscript.

(D) The conversion of Pchlide into Chlide can be described with the parameter k = 0.036, which is the excitation rate per pulse multiplied by the quantum yield of Chlide formation. A fit of comparable quality could also be obtained with a fraction of inactive POR-Pchlide of 20%. These analyses therefore consistently show that the activity of the sample is very high, much higher than that of a sample that has not turned over, where the fraction of Pchlide in inactive POR is between 90 and 100%. We conclude therefore that the activity of POR is retained upon release of Chlide and rebinding of Pchlide.

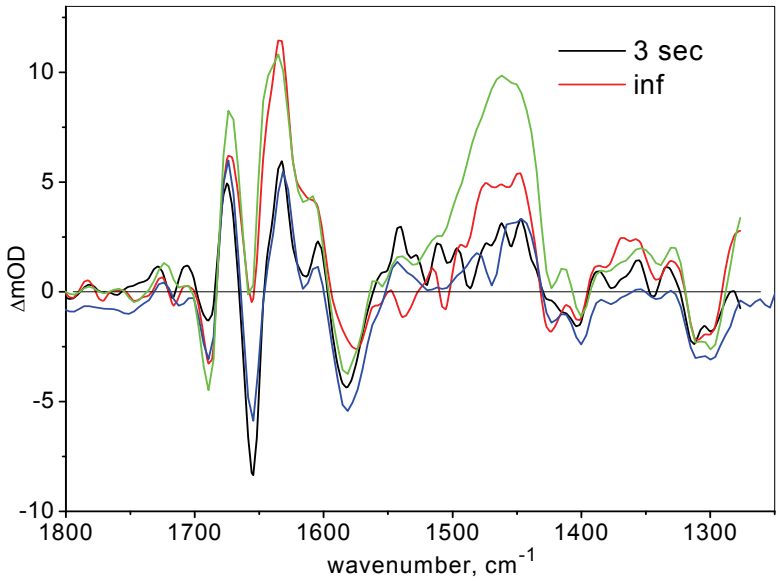

Figure S6. Evolution-associated difference spectra resulting from a global sequential kinetic analysis of FTIR light-minus-dark difference spectra. In this experiment the pump laser was turned off after 5 seconds whereas spectra were collected during 30 seconds. The black spectrum transforms into the red spectrum with a time constant of ~3 s. The red spectrum has an (on the time scale of the experiment) infinite lifetime. For comparison the first two spectra of figure 2.2.B are also shown, in blue and green respectively.