Initial Steps of Signal Generation in Photoactive Yellow Protein Revealed with Femtosecond Mid-Infrared Spectroscopy‡

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ABSTRACT: Photoactive yellow protein (PYP) is a bacterial blue light sensor that induces Halo rhodospira halophila to swim away from harmful exposure to intense blue light (see, for a review, ref 1). Due to its rich and complex photocycle and its excellent (photo)chemical stability, PYP has become a model system for the study of biological signal generation in photoreceptor proteins. Light-induced signal generation in biology involves the amplification of an initially small configurational change generated in an active site into a conformational change of a larger scale. The initial configurational change in the photocycle of PYP (2), i.e., trans-cis isomerization of its intrinsic chromophore, p-coumaric acid (3, 4 depicted in Figure 1], is similar to that in other photosensors such as (bacterio)rhodopsin and (bacterio)phytochromes. A key characteristic of the PYP photocycle is a partial unfolding of the protein (4) on a submillisecond time scale, triggered by the absorption of a photon.

The PYP photocycle has been characterized by visible transient spectroscopy: the excited state of the chromophore decays multieexponentially in a few picoseconds to a red-shifted intermediate, denoted I0 and absorbing around 500 nm (6–11), followed by the formation of an intermediate absorbing at 480 nm in 1–3 ns (6–11), denoted I1 (also called pR or PYP1). A blue shift of the chromophore absorption due to protonation (13, 14) occurs after ~0.3 ns, accompanied by a partial unfolding of the protein [pB or PYPM state (4, 14)]; this is probably the signaling state of PYP. Upon deprotonation and reisomerization of the chromophore, the ground state is recovered on a ~200 ms time scale. The structural changes of the chromophore and in the protein occurring during the photocycle have been studied with a wide range of techniques, such as time-resolved X-ray crystallography (15, 16) and time-resolved FTIR spectroscopy (17), from ~10 ns after excitation onward, and by the application of these techniques on photocycle intermediates trapped by lowering the temperature (18, 19). It was concluded that a few nanoseconds after light absorption, i.e., when PYP is in the I1 state, important structural changes have already occurred. The chromophore has adopted the cis configuration (15–18), and changes in the hydrogen-bonding network and structural rearrangements of nearby amino acids are observed. The chromophore phenolate ring has moved only slightly in the new configuration, and its

1 Abbreviations: PYP, photoactive yellow protein; mid-IR, mid-infrared; ES, excited state; SADS, species-associated difference spectra; FTIR, Fourier transform infrared; OD, optical density; WT, wild type.
hydrogen bonds with the nearby E46 and Y42 residues remain intact (16). Together with the covalent bond with Cys69, this limits the freedom of motion of the chromophore appreciably; therefore, isomerization can take place only by simultaneous rotation around the dihedral angle of multiple bonds [e.g., \( \theta_{7=8} \) and \( \theta_{4=7} \), as has been suggested previously (20, 21)]. However, the time scale of these initial events has not yet been identified.

In the present study, we provide for the first time the “missing link” between ultrafast optical information on one hand and “slower” structural information on the other by applying mid-infrared transient absorption spectroscopy with \( \sim 200 \) fs time resolution. In contrast to UV–vis spectroscopy, vibrational spectroscopy is sensitive to precise structural configurations and can even have a suboptical cycle time resolution of \( \sim 10 \) fs if a coherent emission technique is used (22). Our data for the first time yields information on the structural changes that occur in the excited state and in the first two ground-state intermediates \( I_0 \) and \( I_1 \).

MATERIALS AND METHODS

PYP was prepared as described previously (23). The sample was placed between two 2 mm thick CaF\(_2\) plates separated by a 6 \( \mu \)m Teflon spacer. The use of concentrated PYP solutions (OD\(_{466} \approx 1.0\) and thin spacers diminished the presence of the intense water absorption bands in the IR absorption spectrum.

The experimental setup consists of an integrated Ti:sapphire oscillator–regenerative amplifier laser system (Hurricane, SpectraPhysics) operating at 1 kHz and 800 nm, producing 85 fs pulses of 0.8 mJ. A portion of this 800 nm light was used to pump a noncollinear optical parametric amplifier to produce the excitation pulses with center wavelength of 475 nm, i.e., at the red edge of the absorption spectrum. To ensure a fresh spot for each laser shot, the sample was moved by a home-built Lissajous scanner. In a single experiment a spectral probe window of about 200 cm\(^{-1}\) was covered, so five partly overlapping regions were measured between 1850 and 1100 cm\(^{-1}\); experiments were repeated at least two times.

RESULTS AND DISCUSSION

Figure 2 shows time traces collected over the 1100–1850 cm\(^{-1}\) region upon excitation of the PYP chromophore at 475 nm. A global analysis of the data revealed that three lifetimes are present in our data, 2 ps, 9 ps, 0.9–1 ns, and a nondecaying component (>10 ns). Before \( t = 0 \), a small perturbed free induction decay signal is present; therefore, no attempt was made to extract any kinetic component in the order of the instrument response, i.e., \( \leq 200 \) fs. The state associated with the 0.9–1 ns lifetime and the nondecaying state can be identified as the \( I_0 \) and \( I_1 \) states, respectively. The spectra of the 2 and 9 ps components are due to the excited state (ES) of PYP, which is known to decay multie expositionally (6–11). We performed a target analysis (12, 13) of the data, using a specific kinetic model based on results from visible femtosecond pump–probe experiments (10, 11). This allows us to extract the species-associated difference spectra (SADS). The spectra of the two ES were virtually identical, which is why in a second round the data was fitted to a model in which the initial decay is biexponential (see Figure 3). The relative amplitudes of the 2 and 9 ps time constants were determined to be 0.7 and 0.3, respectively. The overall quantum yield of \( I_0 \) (and \( I_1 \)) formation was estimated in the analysis to be 0.24 ± 0.05, of which the major part is formed with the 2 ps time constant, in good agreement with the yield of \( I_1 \) formation determined from visible data (10, 11). The SADS of the states are shown in Figure 4. Note that in these spectra negative bands are

![Figure 1: Active site of PYP, depicting the chromophore covalently linked to Cys69 and amino acids Glu46, Tyr42, Thr50, and Arg 52 near the phenolate ring of the chromophore, in the ground state (a) and in the \( I_1 \) state (b), after refs 16 and 19.](image-url)
The I₁ spectrum shows a very good resemblance to the by a glutamine (data not shown) and is given in Table 1. The assignment of the dynamic band shifts for each of the ES, I₀, and I₁ states is based on literature reports (17, 18, 20, 21, 25–31) and on experiments performed on a mutant in which residue Glu46 (glutamate) has been replaced with the exception of bands 9 and possibly 10. Apparently the protein starts to respond on the 10 ns scale. Recently, on the basis of time-dependent DFT calculations, it was suggested (38) that in the I₀ state [i.e., the cryotrapped structure reported by Genick et al. (19)] was used in these calculations] a subpopulation of the chromophore is protonated, which led to a much smaller electronic blue shift than that of the PYPₘ state in line with visible pump–

Figure 3: Schematic depiction of the first part of the PYP photocycle. Indicated are the rate constants, the relative yield for I₀ formation from each of the excited states, and the quantum yield for I₁ formation obtained from fitting this model to the data.

Ground state

Figure 2: Eight of the 120 time traces collected over the 1100–1850 cm⁻¹ range upon excitation of the chromophore at 475 nm with a 60 fs pulse, plotted on a linear scale up to 3 ps and on a logarithmic scale for later delay times. The y-axis is in mOD units. The dotted line represents the fit to the data; some figures show data from two different experiments, indicated by the different colors. Before time zero a small perturbed free induction decay is observed.
The frequency upshift of the chromophore’s C=O stretch from \(\sim 1640\) to \(1663\) \text{cm}^{-1}\) (feature 8a,b) in the ES to \(I_0\) transition clearly demonstrates the breaking of the hydrogen bond with Cys69 (25), probably due to the flipping of the C=O around the ethylene chain of the chromophore. This flipping of the C=O group has been observed during the first few nanoseconds of the \(I_1\) intermediate in time-resolved X-ray data (16) and in an early cryotrapped (PYP_{BL}) intermediate (19). Furthermore, an increase of bleaching of trans C=C modes at \(1607\) \text{cm}^{-1}\) is observed and the appearance of an upgoing C=C product band at \(1289\) \text{cm}^{-1}\) typical for the formation of the cis isomer (18). The complete disappearance of the C=C (and C=C) trans modes and the appearance of C=C cis mode, together with the decrease in distance between Glu46 and the chromophore and the breaking of the H-bond with Cys69, show that the cis ground state is formed on the picosecond time scale. Probably the chromophore assumes in \(I_0\) a stretched cis configuration, similar to that observed in the cryotrapped PYP_{BL} structure.

The changes in the region of the C\text{=}C(\text{=}S\text{=})O vibrational modes (“feature 2”), in particular, the rise of the positive band at \(\sim 1310\) \text{cm}^{-1}\), show that a further cis relaxation around these bonds takes place on the time scale of the \(I_0\) to \(I_1\) transition. The quantum yield for the \(I_0\) to \(I_1\) transition is estimated to be 90–100\%, indicating that \(I_0\) is a stable cis intermediate from which no return to the trans ground state occurs. Time-resolved X-ray studies show that in the \(I_1\) intermediate the chromophore is in a slightly stretched cis conformation. In the first few nanoseconds the carbonyl oxygen is in a flipped position on the opposite side of the chromophore (16; see structure depicted in Figure 1b).

On no time scale is breaking of the hydrogen bond with Glu46 observed in our spectra, excluding the possibility of a large movement of the phenol ring of the chromophore during isomerization. Therefore, isomerization does seem to occur via a double isomerization mechanism about the vinyl bond and the thioester linkage in which the connections of the chromophore with the protein remain intact (20, 21).
Table 1: Assignment of Features Observed in the Mid-IR Difference Spectra for States ES, I₀, and I₁

<table>
<thead>
<tr>
<th>Frequency/Assignment</th>
<th>ES</th>
<th>I₀</th>
<th>I₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1633/+1621/-1607 C=C pCA trans→cis markers 17,18,26,27,29</td>
<td>1a-c</td>
<td>~</td>
<td>+</td>
</tr>
<tr>
<td>-1326/-1302/-1274/+1308/+1289 2a-e C-C(S)=O pCA trans→cis markers 17,18,27,29</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-1188 Phenol ring Y9a pCA trans→cis and ionic markers 16,27,29</td>
<td>3a,b</td>
<td>+</td>
<td>1149 b</td>
</tr>
<tr>
<td>-1495 Phenol ring Y19a, ring-O’ symmetric stretch ionic marker 28,29</td>
<td>4a,b</td>
<td>+</td>
<td>1457 b</td>
</tr>
<tr>
<td>+(1580-1585)/-1565/-1555 5a-c Phenol ring Y8b ionic marker 26,27,30</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>-1540 Phenol ring Y8a 28,30</td>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-1444 Phenol ring Y19b 28,29</td>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+1665/-1635/-1640 C=O of pCA 25</td>
<td>8a,b</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-1740 COOH stretch of Glu46 16,20</td>
<td>9a,b</td>
<td>+</td>
<td>1755</td>
</tr>
<tr>
<td>+1693/-1685 Arg52 C=N 17</td>
<td>10a,b</td>
<td>~</td>
<td>+</td>
</tr>
</tbody>
</table>

* Frequencies are in cm⁻¹. In the left column +/- denotes a positive and negative band, respectively, and in the right three columns +/- denotes the presence/absence of these bands in the different states. If present and different for each of the states, the frequency of the product band is indicated in the right three columns.

The small +1693/-1685 cm⁻¹ band shift (“10”) observed in all spectra we tentatively assign to Arg52, of which the C=NN stretch lies in this frequency region (31). Groenhof et al. (32) found in molecular dynamics simulations that upon excitation partial charge transfer from the chromophore to Arg52 takes place. These changes of Arg52 are in line with the time-resolved X-ray data of Ren et al., who observe, within a few nanoseconds, significant changes in residues not in direct contact with the chromophore (16). Here we show that these changes, probably due to the changed electron distribution/polarization of the chromophore, are sensed within ~200 fs and remain present during the nanosecond time scale of the experiment.

In conclusion, using time-resolved mid-IR spectroscopy, we have resolved the structural changes in the very early part of the PYP photocycle. Our measurements are the first to characterize the vibrational features of the excited state from which isomerization occurs with a 20−30% overall quantum yield. We have found additional evidence for a charge translocation upon excitation from the phenolic oxygen toward the ethylene chain, which may be important for the weakening of the isomerizable C7−C8 double bond. Isomerization occurs on the 2 ps time scale and is accompanied by breaking of the hydrogen bond of the carbonyl oxygen with the protein. The transition from I₀ to I₁ occurs with a yield of 90−100%, and we therefore conclude that I₀ is a stable long-living cis ground-state configuration, which structurally relaxes on the nanosecond time scale of the I₀ to I₁ transition. We have further shown that the H-bond of the chromophore with Glu46 remains intact on all time scales relevant for this investigation. The combination of charge translocation and isomerization upon light absorption seems to be a common theme in photosensors. The protein appears to play an active role in combining the two and, thereby, in directing the photocycle, since it stabilizes the negative charge on the phenolic oxygen in the ground state by an extensive hydrogen bond network.

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REFERENCES


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