

Initial Photoinduced Dynamics of the Photoactive Yellow Protein

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The photoactive yellow protein (PYP) is the photoreceptor protein responsible for initiating the blue-light repellent response of the *Halorhodospira halophila* bacterium. Optical excitation of the intrinsic chromophore in PYP, *p*-coumaric acid, leads to the initiation of a photocycle that comprises several distinct intermediates. The dynamical processes responsible for the initiation of the PYP photocycle have been explored with several time-resolved techniques, which include ultrafast electronic and vibrational spectroscopies. Ultrafast electronic spectroscopies, such as pump-visible

probe, pump-dump-visible probe, and fluorescence upconversion, are useful in identifying the timescales and connectivity of the transient intermediates, while ultrafast vibrational spectroscopies link these intermediates to dynamic structures. Herein, we present the use of these techniques for exploring the initial dynamics of PYP, and show how these techniques provide the basis for understanding the complex relationship between protein and chromophore, which ultimately results in biological function.

1. Introduction

Biological photoreceptors are light-sensing protein complexes comprising a protein with a bound light-absorbing chromophore. Their study provides an opportunity to resolve and characterize how nature has tuned proteins to convert photon energy (that is, information) into biological function. Such photoreceptor proteins can be triggered with (laser) flash illumination and because modern ultrafast lasers offer exceptional control of laser light pulses, excellent time resolution is achievable in spectroscopic studies. Photoreceptors are also signal transduction proteins, so one may anticipate that the conformational transitions would be significant to allow participation in a macroscopic signal transduction pathway.^[1] Herein, we discuss the results of the application of ultrafast fluorescence, UV/Vis, and vibrational pump-probe spectroscopies to the exploration of the initial photodynamics of the photoactive yellow protein (PYP) photoreceptor.

PYP was first discovered as a low-abundant protein in *Halorhodospira halophila* and a number of related halophilic purple bacteria.^[2] The similarity of PYP to the sensory rhodopsins from archaeobacteria suggested that PYP might function in a photosensory pathway.^[3,4] *H. halophila* is repelled when exposed to blue light, and the wavelength dependence of this negative phototaxis response follows the absorption spectrum of PYP. Thus, the current consensus on the function of PYP in extremophilic eubacteria is that of a photoreceptor in a light-induced behavioral response that allows the bacterium to avoid regions with high intensities of (blue) light.^[5]

The three-dimensional structure of PYP from *H. halophila* was resolved with X-ray crystallography to 0.82 Å.^[6] In 1994, the chromophore bound to PYP was shown, with NMR spectroscopy and other biophysical techniques, to be the *trans*-4-hydroxycinnamic acid molecule (also known as *p*-coumaric acid).^[7,8] This chromophore is present in the anionic form (that is, with a phenolate moiety) in the dark-adapted ground state of the protein^[9] and is buried within the major hydrophobic core, where it is stabilized via a hydrogen-bonding network

(Figure 1A).^[10] On the other side of the chromophore, the carbonyl oxygen atom forms a hydrogen bond with the backbone nitrogen atom of C69. At neutral pH, the chromophore, with a pK_a in solution of 8.9,^[11] is deprotonated. In addition to studying the wild-type PYP (wt-PYP), mutants can be generated to study the effects of changing specific residues. The wt-PYP apoprotein can also be combined with chromophores with altered structure^[11] (referred to as hybrids) and, moreover, mixed-mutant hybrids can be created that further tune the spectral and functional properties of the protein.^[12]

2. PYP Photocycle

In the past decade, PYP has become a popular model system for studying the photoinitiation and ensuing dynamics of photoreceptor proteins. Functional activation of PYP can be measured in vitro through the analysis of its photocycle with a wide range of biophysical techniques,^[13,14] which led to the observation that initially a number of red-shifted intermediates are formed, with the chromophore in its *cis* configuration (Figure 1B). On the millisecond timescale, the chromophore is transiently and reversibly protonated by the neighboring carboxyl group of E46. As a result, the protein partially and transiently unfolds (and subsequently refolds), while shifting its color to the blue (350 nm).^[3,15,16]

The rich photoinduced dynamics observed in PYP include multiple chemical reactions (e.g., isomerization and proton

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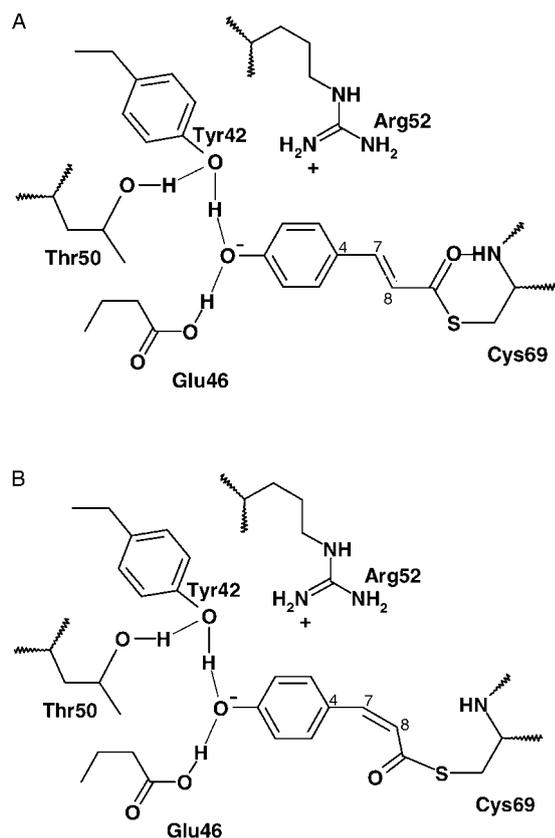


Figure 1. Active site of PYP depicting the chromophore covalently linked to Cys69 and amino acids E46, Y42, T50, and R52 near the phenolate ring of the chromophore, in the ground state (A) and in the pR state (B). Figure reprinted with permission from M. L. Groot, L. van Wilderen, D. S. Larsen, M. A. van der Horst, I. H. M. van Stokkum, K. J. Hellingwerf, R. van Grondelle, *Biochemistry* **2003**, *42*, 10054. Copyright 2003 American Chemical Society.

transfer) and many transient intermediates. As a consequence, different photocycle schemes have been proposed for the evolution of PYP (Figure 2), which depend on the environmental conditions (e.g., temperature) and excitation wavelengths. The PYP photocycle encompasses over 14 orders of magnitude in time at room temperature; however, when cooled below -50°C , the intermediates in the photocycle can be trapped and then explored. Imamoto et al. proposed a branched cycle that entails two pathways that separate early in the photocycle before combining to produce a sequential model (Figure 2B).^[17] A similar model was proposed for the room-temperature time evolution extracted from ultrafast pump-probe signals (Figure 2A).^[18] However, in contrast, a completely sequential model was proposed for the room-temperature evolution by Devanathan et al. (Figure 2C).^[19] In this Minireview, we limit the discussion to the early red-shifted intermediates accessible with ultrafast spectroscopy, in particular to the formation of I_0 and pR₁. To date, a consistent nomenclature is yet to emerge for the photocycle intermediates;^[13,14] here, we use the Hoff et al. naming system with the rationale discussed elsewhere.^[13]

A principal goal in investigating the ultrafast photophysics of PYP, and of photoreceptors in general, is to build a microscopic picture of how the photocycle is initiated that includes

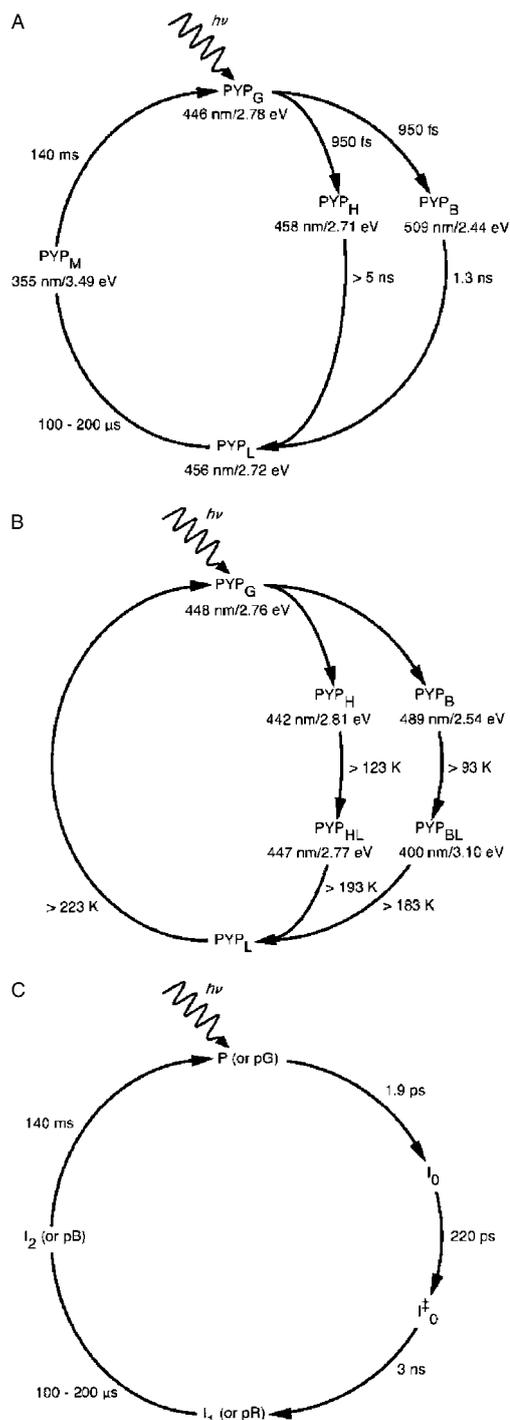


Figure 2. Photocycle intermediates observed by UV/Vis spectroscopy: A,C) at room temperature with time-resolved techniques and B) at low temperature with cryogenic trapping techniques. A) and C) depict alternative room-temperature photocycle kinetics reported by Imamoto et al.^[18] and Devanathan et al.,^[19] respectively, with time constants for conversion between species. B) depicts photocycle intermediates observed at low temperature^[17] with temperatures (K) of spontaneous (thermal) conversion between species. A) and B) provide absorption maximum [in nm] and equivalent excitation energy [in eV]. C) contains the nomenclature of Hoff et al.^[16] in parentheses. Please note that several of the slow intermediates of the PYP photocycle have been omitted for clarity.^[18,61] Figure reprinted with permission from M. J. Thompson, D. Bashford, L. Noodleman, E. D. Getzoff, *J. Am. Chem. Soc.* **2003**, *125*, 8186. Copyright 2003 American Chemical Society.

effects from the inherent dynamics of the PYP chromophore and surrounding protein environment, and then characterize the ensuing photocycle responses. The experimentalist has several different ultrafast techniques available to explore the initial photochemistry of PYP, which encompass both electronic and vibrational spectroscopies. All the ultrafast measurements discussed herein require the optical excitation of PYP with ultrafast laser light with a wavelength that has a significant overlap with the absorption spectrum of PYP. In Figure 3, the ab-

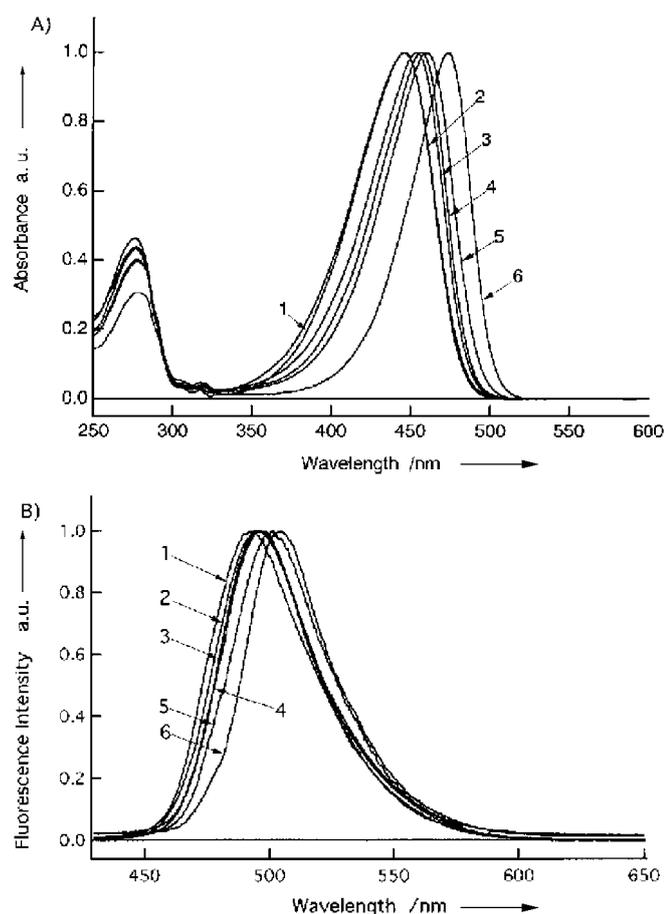


Figure 3. A) Absorption and B) fluorescence spectra of wild-type and mutant PYP samples: 1) Wild-type, 2) R52Q, 3) T50A, 4) T50V, 5) E46Q, and 6) E46Q/T50V. Figure reprinted with permission from N. Mataga, H. Chosrowjan, Y. Shibata, Y. Imamoto, F. Tokunaga, *J. Phys. Chem. B* **2000**, 104, 5191. Copyright 2000 American Chemical Society.

sorption spectra of wt-PYP and several mutants are compared with the corresponding fluorescence spectra. The absorption spectrum for wt-PYP has a particularly large bandwidth of 2800 cm^{-1} (full width half maximum), which has led to speculations about the involvement of overlapping multiple electronic transitions.^[20] Ultrafast laser pulses around 400 and 470 nm are relatively easy to generate with widely available ultrafast Ti:sapphire laser systems, therefore many experimental studies involve excitation on the high-energy and low-energy sides of the visible absorption band of PYP.

3. Time-Resolved Fluorescence Signals

PYP is not a strongly fluorescing protein, with a fluorescence yield of $\approx 2 \times 10^{-3}$; however, emission can be observed at times immediately following excitation.^[21,22] Fluorescence is sensitive to excited-state properties, so many interfering features observed in other ultrafast spectroscopic techniques are excluded, but collection of the time-resolved fluorescence signals is useful in characterizing the excited-state dynamics of PYP.^[23] In 1997, Mataga and co-workers reported the first fluorescence upconversion measurement on wt-PYP.^[24] The following year, Glasbeek and co-workers compared the upconverted signals obtained for wt-PYP with those of several hybrid PYP samples that were reconstituted with modified chromophores.^[25] Both upconversion studies identified distinct multi-exponential excited-state decay dynamics of wt-PYP with three characteristic quenching timescales: 600 fs, 4 ps, and 45 ps. Moreover, these measurements showed that the excited-state decay is distinctly faster on the high- and low-energy sides of the fluorescence band. This narrowing is directly ascribed to evolution along the reaction coordinate and is not observed in hybrid samples, reconstituted with nonisomerizing chromophores.^[26,27]

Mataga and co-workers further performed upconversion measurements on other PYP mutants and hybrids.^[28-31] Figure 4 shows the observed upconversion signals for wt-PYP

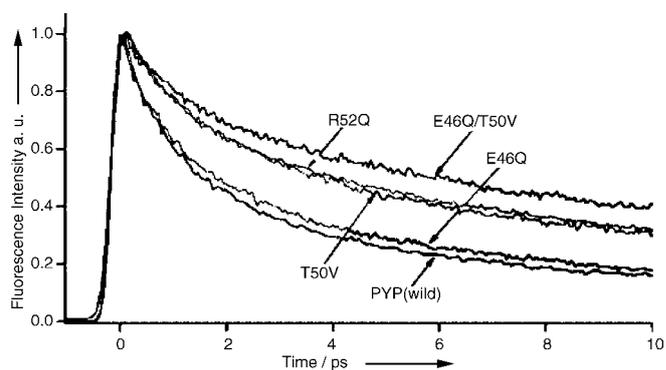


Figure 4. Fluorescence dynamics of wild-type PYP and its site-directed mutants excited at 410 nm and observed at the wavelength of the maximum of the steady-state fluorescence spectrum. The fluorescence decay curves are normalized at the peak intensity. Figure reprinted with permission from H. Chosrowjan, N. Mataga, Y. Shibata, Y. Imamoto, F. Tokunaga, *J. Phys. Chem. B* **1998**, 102, 7695. Copyright 1998 American Chemical Society.

and several mutants. The observed differences between the quenching times were ascribed to the “looseness” of the different protein environments around the chromophore in the wild-type protein and the various site-directed mutants. It is interesting to note that in all studied PYP mutants and hybrids, the observed excited-state lifetimes are distinctly longer than those in wt-PYP (the connection between quenching time and photocycle initiation yield is further discussed below). The Mataga group further improved the upconversion measurements on PYP to identify oscillations in the fluorescence signals that are ascribed to excited-state vibrational wavepack-

ets.^[29,32,33] These vibrations may act as promoter modes in initiating the PYP chromophore twisting that occurs during the ensuing photocycle, which suggests that the PYP system is tuned to enhance isomerization of the chromophore. Comparisons between dynamics of the PYP chromophore in the protein and isolated in solution corroborate this viewpoint (see below).

Other time-resolved fluorescence techniques that have also been used to study PYP systems include optical Kerr gating^[34] and streak camera detection.^[35] Although these measurements have poorer time resolution (> 500 fs) than upconversion, they are advantageous because they almost simultaneously collect multiple wavelength signals. Hence, evolution such as shifting and narrowing can be more clearly observed without the need for spectral reconstruction techniques from the single-wavelength traces.^[36,37] Recently, the upconversion technique has been expanded to include the near-simultaneous collection of multiple wavelengths,^[38,39] which combines high time resolution (< 150 fs) with dispersed wavelength detection. Vengris et al. used this technique to contrast the fluorescence dynamics in the PYP protein and in isolated model chromophores in solution.^[27] They ascribed the multiexponential decay in wt-PYP in terms of a heterogeneous model, with the ground state consisting of subpopulations, each with a different quenching time. This heterogeneous model is discussed further in the following sections.

4. Electronically Resonant Transient Absorption Signals

Fluorescence measurements are sensitive only to the excited-state dynamics, but transient absorption spectroscopies are sensitive to dynamics on both the excited and the ground electronic states. The term transient absorption encompasses several subpicosecond techniques, which include both pump-probe (PP)^[23] and pump-dump-probe (PDP) spectroscopies, among others.^[40,41] The central theme in these techniques is that the observed signal is a consequence of the change in the transmission of a weak probe pulse induced by some combination of the preceding laser pulses. The PDP technique builds upon the PP technique by introducing a third pulse between the pump and probe pulses with a wavelength that is resonant *only* with the stimulated emission of the sample. This additional “dump” pulse will near-instantaneously de-excite a portion of the sample by shifting the excited-state population back to the ground electronic state (though perhaps in a non-equilibrium part of the potential).

4.1 Pump-Probe Measurements

Early microsecond^[3] and nanosecond^[16,42] time-resolved transient absorption measurements on wt-PYP have characterized many aspects of the slower dynamics of the PYP photocycle. The extension of these studies into the ultrafast regime for wt-PYP was first published by van Grondelle and co-workers in 1997 and the following year Devanathan et al. published similar ultrafast dispersed PP results.^[43,44] The data collected in

both measurements were interpreted within a sequential model (Figure 2C). The Masuhara and Tokunaga groups, in contrast, modeled similar wt-PYP dispersed PP signals with a kinetic scheme that includes a branching in the photocycle.^[18]

Figure 5 shows selected PP traces of wt-PYP from 426 to 525 nm after excitation at 460 nm. These dispersed PP signals, measured by Ujj et al., exhibit complex dynamics across the probe wavelengths.^[44] The initial dynamics attributed to the excited-state lifetime (observed in the time-resolved fluorescence experiments) are readily observed in the first 10 ps, whilst the photocycle intermediates are observed at later times. The ensuing dynamics include the generation of a red-shifted I_0 intermediate (Figure 2) that is formed on a similar timescale to the relaxation of the excited state of the chromophore. This intermediate then evolves into pR (also referred to as I_1) on a ≈ 3 -ns timescale and the successive photocycle dynamics are outside the typical temporal range for ultrafast measurements. An additional intermediate I_0^\ddagger , temporally located between I_0 and pR₁, was proposed by Ujj et al.^[44] However, interference by the long-living stimulated emission (SE) contributions may question this proposition.^[25]

Representative ultrafast PP transient spectra of wt-PYP, excited with ultrafast 395-nm excitation pulses, are shown in Figure 6 and span probing times from 180 fs to 4 ns. The collected spectra and dynamics of the PYP system are similar to the data obtained in other dispersed PP measurements.^[18,19,43,44] All transient spectra show ground-state bleach, which peaks at the maximum of the absorption spectrum (446 nm). The 180-fs and 2-ps spectra also exhibit a broad negative band at 500 nm and a pronounced positive band at 370 nm, which are ascribed to the SE and the excited-state absorption (ESA) bands, respectively. In contrast, the 35-ps, 500-ps, and 4-ns spectra do not exhibit clear SE bands, but instead show positive product-state absorption bands peaking at 500 and 480 nm, respectively, which are ascribed to the product-state absorptions of the initial red-shifted intermediates in the PYP photocycle (Figure 2), namely, I_0 and pR.^[14,45] The 35-ps, 500-ps, and 4-ns PP spectra also exhibit a noticeable, positive, sharply peaked band at 360 nm that has not been previously ascribed to either the I_0 or the pR product-state band. Since the ultrafast fluorescence signals in Figure 4 show that no appreciable excited-state population remains at this time,^[24,25,46] this sharply featured band is not ascribed to the similar UV ESA band observed in the 180-fs and 2-ps spectra. Furthermore, a weak broad absorption is observed in the 4-ns spectrum, which extends beyond 550 nm.

The excitation wavelength dependence of the excited-state dynamics can be investigated by measuring the PP signals after exciting PYP at the high- and low-energy sides of its main absorption band (Figure 3). Gensch et al.^[47] and Devanathan et al.^[19] previously addressed this excitation wavelength dependence of the wt-PYP sample and a representative comparison of these signals is shown in Figure 7. The bleach in the high-energy excitation is noticeably enhanced for the 395-nm excitation data. The observation of a weak red tail and the UV band in the 395-nm excited PP signals in Figure 6 suggests, however, that electron photodetachment from the phenolate

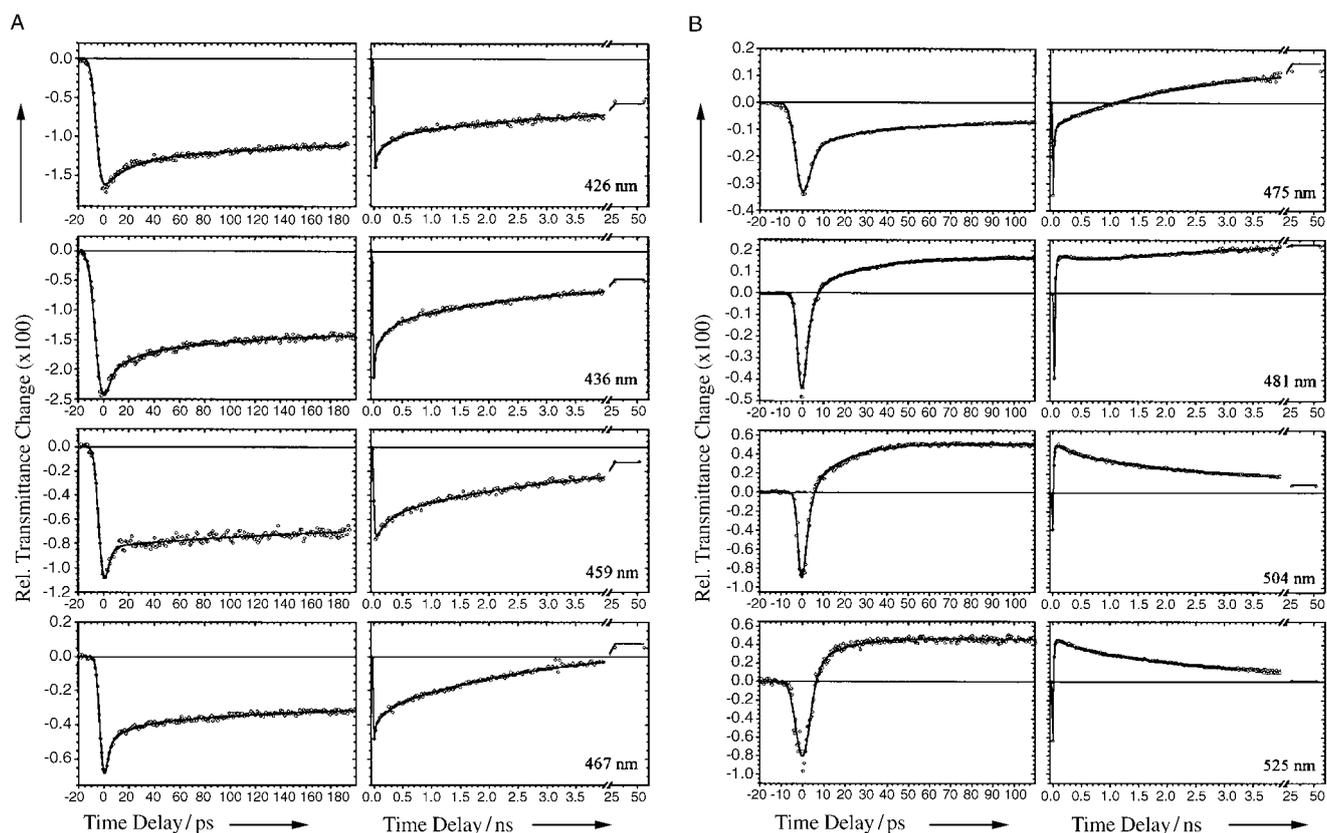


Figure 5. PP traces obtained for eight wavelengths: A) 426, 436, 459, and 467 nm; B) 475, 481, 504, and 525 nm. Time delays of -20 to $+200$ ps are shown in the left-hand panels, and delays of up to 50 ns are shown in the right-hand panels. Solid lines correspond to global fit curves. Figure reprinted with permission from L. Uji, S. Devanathan, T. E. Meyer, M. A. Cusanovich, G. Tollin, G. H. Atkinson, *Biophys. J.* **1998**, *75*, 406. Copyright © 1998 by the Biophysical Society.

chromophore may also be one of the possible results of excitation of PYP. Larsen et al. therefore measured the power dependence of the 200-ps transient PP spectrum after 395-nm excitation and observed a complex behavior that cannot be classified as either linear or quadratic.^[48] The decomposition and characterization of these data into contributions from the two pathways is achieved with the aid of global analysis, where the measured power-dependent spectrum is decomposed into a sum of two spectra with intensity-dependent amplitudes, with one spectrum associated with photocycle intermediates and the other connected to a photoionization pathway.

The obtained concentration curves can be modeled with a five-state model. In this model, one 395-nm photon from the pump pulse excites the PYP system into the first excited electronic state; however, since a strong ESA band overlaps the 395-nm excitation wavelength (Figure 6), an additional photon can also be absorbed from the pump pulse, which promotes the already-excited PYP system into a highly excited electronic state. From this high-energy electronic state, the $50\,000\text{ cm}^{-1}$ of imparted excitation energy is sufficient to ionize the chromophore and eject an electron into the protein pocket, thus leaving a radical behind. Consequently, the observed photodynamics of PYP, after excitation with 395-nm ultrashort laser pulses, can be separated into a one-photon photocycle and a stepwise sequential two-photon ionization process. Both pho-

toinitiated reactions result in spectrally and temporally overlapping photoproducts. The activation of this competing pathway with higher-energy excitation light explains the observed increased ground-state bleach observed by Devanathan et al. in the 395-nm versus 460-nm excited PP signals (Figure 7).

4.2 Pump-Dump-Probe Measurements

Although a powerful technique, dispersed PP signals are not definitive in determining the details behind the initiation of the PYP photocycle, mainly because of the overlapping spectral and temporal properties of the transient species involved (Figure 6). For example, the near-complete overlap of the stimulated emission band with the absorption of the first photoproduct, I_0 , precludes directly correlating the quenching timescales of the excited state with the timescale of photocycle initiation.^[18,43,49] Hence, different kinetic schemes have been proposed for modeling the initial dynamics of the PYP photocycle.^[18,44] Recently, the PDP technique has been used to further address the initial dynamics of PYP.^[48]

Representative kinetic traces from two PDP data sets dumped at 500 fs (\circ) and 2 ps (\bullet) are shown in Figure 8, which overlap the PP signals (Δ). The dump pulse shifts part of the population from the excited state to the ground state, which is observed as a loss of SE (550 nm) and ESA (375 nm) bands with a concomitant recovery in the bleach region

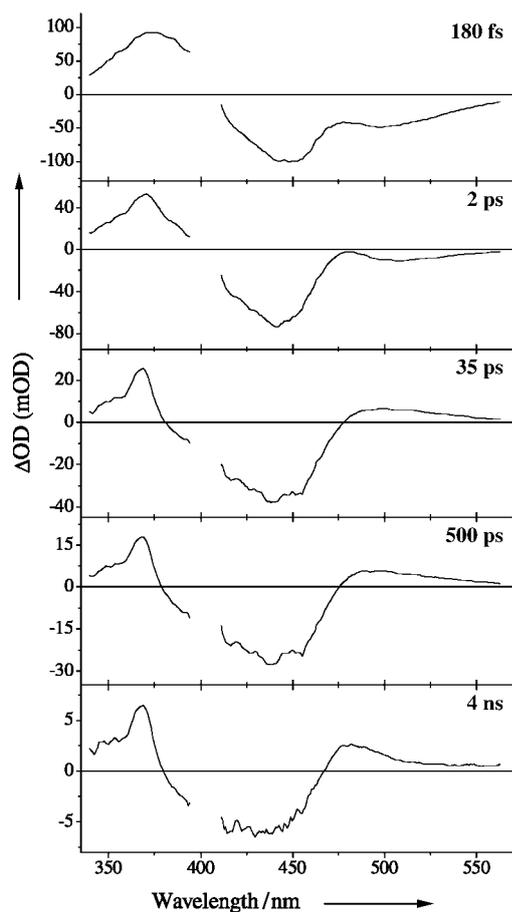


Figure 6. Representative dispersion-corrected transient PP spectra. The region around the pump excitation wavelength (395 nm) is corrupted because of scatter from the pump pulse. Figure reprinted with permission from Delmar S. Larsen, Mikas Vengris, Ivo H. M. van Stokkum, Michael A. van der Horst, Frank L. de Weerd, Klaas J. Hellingwerf, Rienk van Grondelle, *Biophys. J.* **2004**, *87*, 1848. Copyright © 2004 by the Biophysical Society.

(445 nm). However, the relative magnitude of the bleach recovery at 445 nm ($\approx 25\%$) is not comparable with the depletion of the SE and ESA ($\approx 50\%$), which suggests the involvement of a third intermediate state that temporarily stores the ground-state population before refilling the bleach on a longer time-scale. This ground-state intermediate (GSI) is more clearly observed as an increase in the dump-induced absorption in the 480- and 490-nm traces, where a local maximum in both the PP and the PDP signals is observed. The structural basis of this intermediate state is not known; it may originate from a competing structural rearrangement motion (e.g., rotation about one of the sigma bonds, instead of the double bond of the chromophore) or from a local minimum in the ground-state potential energy surface of the isomerization coordinate. The smaller dump in the 350-nm trace is indicative of the failure to remove the overlapping radical contribution by the dump pulse and is useful for characterizing its properties.

On the basis of these PDP data and the PP data discussed in the previous section, two self-consistent kinetic models can be constructed to describe the observed dynamics (Figure 9). Both the proposed homogeneous and inhomogeneous kinetic

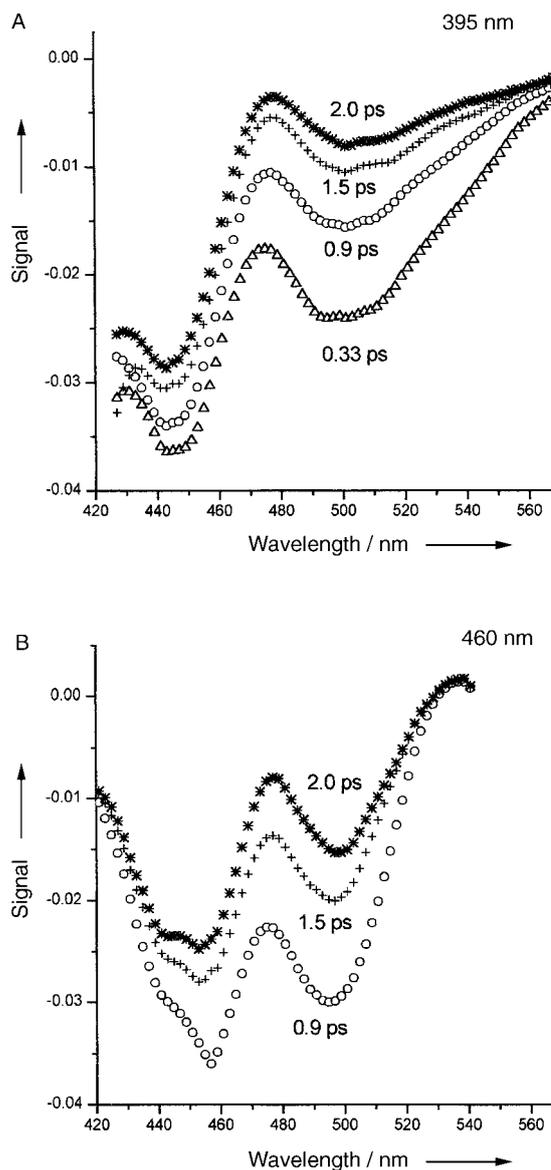


Figure 7. Femtosecond time-resolved difference spectra for excitation wavelengths 395 nm (A) and 460 nm (B) in the 427–570-nm spectral region; ground-state repopulation occurs in the 420–470-nm region, and stimulated emission from 470 to 530 nm. Experimental data points after dispersion correction are shown at 0.33, 0.9, 1.5, and 2.0 ps after excitation. A minor laser-scattering artifact appears at ≈ 458 nm in the panel B. Figure reprinted with permission from S. Devanathan, A. Pacheco, L. Ujj, M. Cusanovich, G. Tollin, S. Lin, N. Woodbury, *Biophys. J.* **1999**, *77*, 1017. Copyright © 2004 by the Biophysical Society.

schemes model the observed PDP and PP dynamics as an evolution between discrete interconnected transient states that are separated into four categories: 1) excited-state dynamics, 2) ground-state dynamics, 3) photocycle dynamics, and 4) an ionization channel. Both models produce similar-quality fits to the measured PDP data, but their respective interpretations differ. The kinetic models differ primarily in the connectivity scheme for the excited-state evolution, and with respect to which state(s) is (are) initially excited by the applied laser pulse (thick black lines). The inhomogeneous model ascribes the multiexponential excited-state behavior to a superposition of

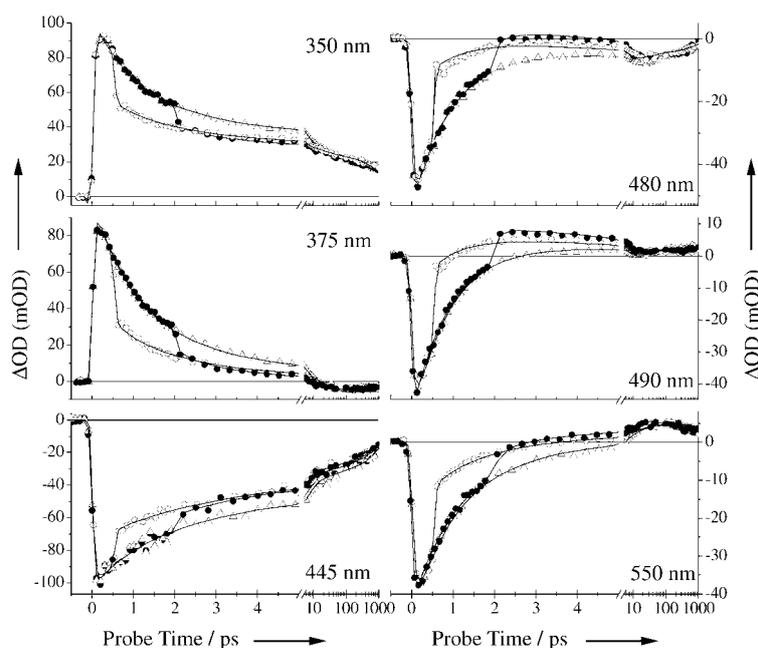


Figure 8. Selected PP (Δ) and PDP traces dumped at 500 fs (\square) and 2 ps (\bullet). Symbols are the experimental data and the solid lines are the results of the global fits to these data. Note that the time axis is linear up to 5 ps, and then log-arithmic up to 1 ns. Figure reprinted with permission from Delmar S. Larsen, Mikas Vengris, Ivo H. M. van Stokkum, Michael A. van der Horst, Frank L. de Weerd, Klaas J. Hellingwerf, Rienk van Grondelle, *Biophys. J.* **2004**, *87*, 1848. Copyright © 2004 by the Biophysical Society.

multiple subpopulations with differing decay times, whereas the homogeneous model ascribes the multiexponential decay to evolution along the excited-state potential energy surface.

The PDP technique probes the reaction yield of each excited-state intermediate (ESI) by dumping the ESI at different times and then probing the magnitude of the change of the I_0 photoproduct absorption.^[48,50] The PDP data favor the inhomogeneous over the homogeneous model, since each ESI does not have the same yield in initiating the PYP photocycle. This observation, in combination with the global analysis of the kinetic trace PDP signals, supports the observation that ESI1 has the highest yield ($\approx 40\%$) followed by ESI2 (20%), whereas ESI3 has a near-negligible yield ($\approx 1\%$) in initiating the photocycle. A homogeneous model would require that the excited-state population would have a time-dependent yield and evolve across a complex potential energy surface with quenching pathway(s) that compete with photocycle generation. The inhomogeneous model, in contrast, is simpler to construct and interpret.

5. Vibrationally Resonant Ultrafast Signals

The ultrafast electronic spectroscopic data discussed in the previous sections provide a useful platform for characterizing the timescales and applicability of underlying kinetic models in the ensuing PYP dynamics. However, these time-resolved signals are sensitive only to electronic transitions and can probe structural changes in the chromophore only when these changes are mirrored in the electronic spectrum (e.g., the *cis*

conformation exhibits a different absorption spectrum than the *trans* conformation). The time-dependent structure of the chromophore and surrounding residues can only be directly addressed with structure-sensitive techniques such as vibrational spectroscopies (Raman and IR) or diffraction studies (with X-rays or neutrons).

Once the vibrational spectroscopic data have been collected, the proper interpretations require knowledge about which vibrational mode is observed in which part of the spectrum. This assignment information can be obtained either by (site-specific) isotope enrichment, site-directed mutagenesis, or the use of specific hybrids, or by theoretical calculations. Based on first principles, Yamauchi and co-workers investigated the vibrational spectra of select PYP conformations, which compared favorably to Raman data obtained at room

temperature and after cryotrapping.^[51,52] Although many of the assignments of different researchers agree, there are some vibrational modes for which a consensus has yet to be established. This must be considered when contrasting the data collected with different techniques.

Atkinson and co-workers suggest, from a comparison of coherent anti-Raman Stokes (CARS) signals with the dispersed PP signals on wt-PYP and its E46Q mutant, that the chromophore does *not* adopt an isomerized structure within the first ≈ 200 ps of the photocycle.^[53] This hypothesis centered on the observed temporal differences between the PP signals for the two PYP samples (specifically with respect to the proposed I_0^+ evolution) and the localization of the vibrational differences (from CARS signals) on the phenolic moiety. Consequently, the observed temporal differences (< 200 ps) are ascribed to changes in the phenolic group and not to the coumaryl tail of the chromophore. Although the CARS technique is explicitly a time-resolved technique and thus, in principle, could reveal the initial structural dynamics, this study was based on exploring the equilibrium pG-state structure, and does not involve time resolution of the structural photoinduced changes in PYP.

Time-resolved Fourier-transform infrared (FTIR) spectroscopic measurements, with 50-ns and 10- μ s time resolution, have been successfully used to directly explore the structural dynamics of both wt-PYP and its E46Q mutant.^[54,55] On account of this limited time-resolution, both studies explored only the later stages of the photocycle, which include the deprotonation of the PYP chromophore and the subsequent unfolding of the protein. The extension of these UV/Vis-PP studies to the

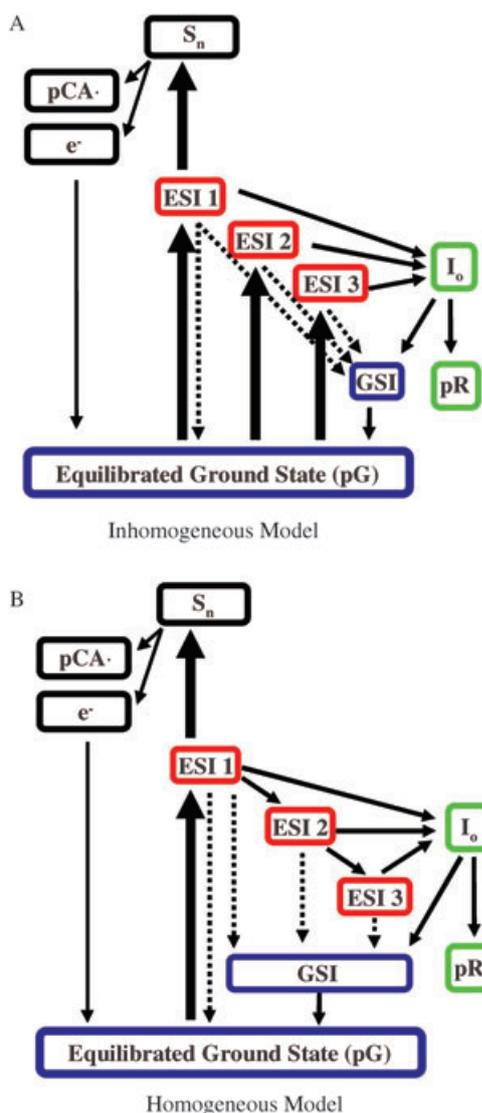


Figure 9. Connectivity schemes compatible with the data and used in the global analysis for the PDP: A) inhomogeneous model and B) homogeneous model. Dynamical states are separated into four classes: excited state (red), ground state (blue), photocycle products (green), and two-photon ionization dynamics (black). ESI1, ESI2, and ESI3 refer to the excited-state intermediates 1, 2, and 3, respectively. pG is the equilibrated ground-state species, and GSI is the ground-state intermediate. Thick solid arrows represent the initial excitation process from the laser pulse and thin solid arrows represent the "natural" PP population dynamics. The dashed arrows represent the population transfer dynamics that may be enhanced with the dump pulse. S_n is a higher-lying electronic state and pCA \cdot is the p-coumaric acid PYP chromophore radical after ionization. Figure reprinted with permission from Delmar S. Larsen, Mikas Vengris, Ivo H. M. van Stokkum, Michael A. van der Horst, Frank L. de Weerd, Klaas J. Hellingwerf, Rienk van Grondelle, *Biophys. J.* **2004**, *87*, 1848. Copyright © 2004 by the Biophysical Society.

ultrafast regime, and specifically to the PYP system, was recently published by Groot et al.^[56] Global analysis of the UV/Vis-IR PP data revealed three lifetimes: 2 ps, 9 ps, and 1 ns with a nondecaying component (> 10 ns). The estimated species-associated difference spectra (SADS) from these data (Figure 10) highlight the utility of this type of measurement, where the complete (< 1000 cm⁻¹) vibrational structure can be

resolved as a function of time. The difference spectra associated with the 2- and 9-ps timescales are nearly identical and most likely originate from the change in vibrational structure upon the decay of the excited state and the formation of the first product, because of the similarity in timescales with the upconversion data (compare Figure 4). The weak 40-ps component observed in upconversion and PP measurements is not observed in this time-resolved IR study.

These measurements show that the ultrafast (< 10 ps) quenching of the PYP excited state is directly connected to the photoisomerization of the bound chromophore. This finding is revealed by the disruption of the hydrogen bond between the carbonyl oxygen atom of the chromophore and the backbone N-H moiety of C69 (Figure 1), which upshifts the frequency of the C=O bond from 1633 to 1666 cm⁻¹; additional isomerization features in the transient spectra corroborate this conclusion. Earlier Vis-IR PP measurements on bacteriorhodopsin yielded a similar conclusion about the initial isomerization dynamics of its retinal chromophore.^[57] Additionally, no deprotonation is observed for the chromophore within the first 5 ns, as shown in time-resolved FTIR data.^[54,55] These conclusions are in agreement with recent time-resolved resonance Raman measurements by Mathies and co-workers.^[58]

The Groot et al. UV/Vis-IR PP data further suggest that upon photoexcitation of PYP, a significant charge translocation occurs from the phenolic oxygen atom toward the ethylene chain, which may be important for the weakening of the isomerizable double bond and/or the breaking of the hydrogen bond of the chromophore's carbonyl group with the N-H group of C69. Similarly, large charge-transfer contributions to the excitation in PYP were observed in recent electro-optical Stark measurements,^[59,60] which suggests that charge transfer contributes significantly to the initiation of the photocycle.

6. Concluding Remarks

Light absorption by the intrinsically bound chromophore within PYP leads to the initiation of a photocycle, which evolves on multiple timescales and comprises several distinct intermediates. The ultrafast dynamical processes responsible for the initiation of the PYP photocycle have been explored with different time-resolved techniques. We presented here a review of the results of these techniques in exploring the initial dynamics of PYP, and show how these techniques provide the basis for understanding the complex relationship between protein and chromophore that ultimately results in biological function.

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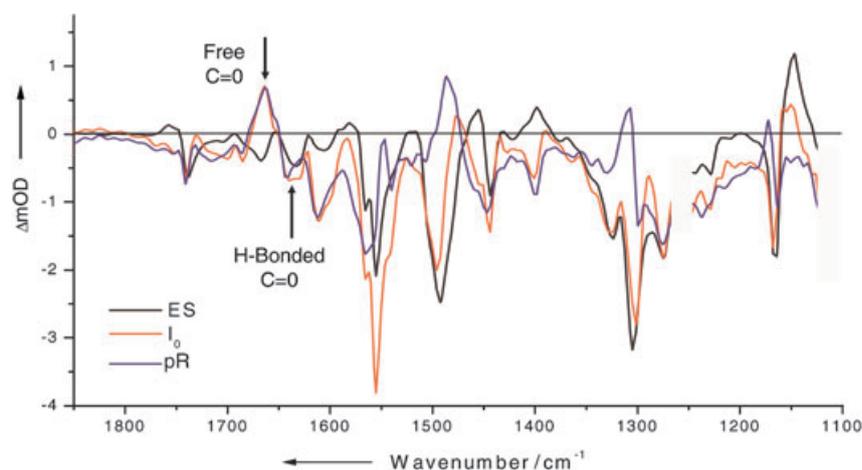


Figure 10. Species-associated difference spectra of wt-PYP induced by excitation of the chromophore with a short 475-nm laser pulse. The excited-state decay was fitted with a biexponential rate of 1/2 and 1/9 ps⁻¹; the *I*₀ to pR transition rate was ≈ 1 ns⁻¹. The quantum yield of the *I*₀ and pR states was only 24%, so their presence in the raw data was ≈ four times smaller than that of the excited-state spectrum. Arrows show the breaking of the hydrogen bond of the chromophore to the C69 residue. Figure reprinted with permission from M. L. Groot, L. van Wilderen, D. S. Larsen, M. A. van der Horst, I. H. M. van Stokkum, K. J. Hellingwerf, R. van Grondelle, *Biochemistry* **2003**, *42*, 10054. Copyright 2003 American Chemical Society.

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