The primary reactions of bacteriophytochrome studied with ultrafast mid-infrared spectroscopy

The early photochemistry of phytochromes involves the isomerization of their bilin chromophore at the C15=C16 methine bridge, a reversible process that can be triggered either by a red or far-red light. The photoproduct that is formed upon light absorption by the Pr state is characterized by a red-shifted absorption spectrum of the linear tetrapyrrrole chromophore. Bacteriophytochromes RpBphP2 and RpBphP3 from *R. palustris* show different responses to red light illumination. RpBphP2 protein gives rise to a state with classical far-red absorption upon light activation, whereas RpBphP3 shows the formation of a state with near-red absorption. By employing ultrafast time-resolved mid-infrared spectroscopy, we observed that the excited state of RpBphP2 decays in 58 ps, while the excited state of RpBphP3 decays in 293 ps. Excited state decay is followed by the formation of a primary intermediate that does not decay on the timescale of the experiment (3 ns), which shows a sharp induced absorption band at ~1540 cm⁻¹ that has not been observed before in previous time-resolved IR studies. Possible origins of this product band are discussed. By combining ultrafast mid-IR spectroscopy with FTIR spectroscopy on RpBphP2 and RpBphP3 wild type and mutant proteins, we demonstrate that the hydrogen bond strength at the ring D carbonyl of the bilin chromophore is significantly stronger in RpBphP3 as compared to RpBphP2. This result is consistent with the X-ray structures of bacteriophytochrome, which indicate one hydrogen bond from a conserved histidine to the ring D carbonyl for classical bacteriophytochrome, and one or two additional hydrogen bonds from a serine and a lysine side chain to the ring D carbonyl for RpBphP3.

This chapter is based on the manuscript:
5.1 Introduction

Phytochromes are red-light photoreceptors which play a critical role in regulating various cellular functions in plant, fungal and bacterial kingdoms (1-6). Genes from bacteriophytochromes (Bphs) RpBphP2 (P2) and RpBphP3 (P3) from Rhodoseudomonas palustris are found to be located in tandem downstream of the pucBAd encoding the apoproteins of the LH4 complexes and hence are crucial in the synthesis of light harvesting LH4 complexes (7). These proteins in general contain the PAS, GAF and PHY domains and have two meta-stable states. These states are inter-photoconvertible through an isomerization mechanism at the C15=C16 double bond of a linear tetrapyrrole cofactor. Both P2 and P3 bind biliverdin IXa (BV) auto catalytically at ring A through a covalent linkage with a conserved cysteine. P2 and P3 have a very distinctive light response manifested in their absorption spectra. P2 forms a light state that resembles the classical Pr – Pfr phytochrome light response, whereas P3 forms a Pnr state with a blue-shifted absorption spectrum, peaking at 645 nm. Like their counterparts in plant, the photochemistry of Bphs proceeds through several intermediate stages before attaining a conformation of 15Ea of BV in light state (e.g. Pfr state) upon light activation (8, 9).

![Figure 5.1: X-ray crystal structure of RpBphP3 (left) and the chemical structure of the biliverdin in Bphs (right).](image-url)
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The recent determination of crystal structures of various BPhs and the cyanobacterial phytochrome Cph1 has explored the light-activated function of phytochromes (8, 10-13). The linear tetrapyrrrole chromophore is bound to the PAS (BPh) or GAF (Cph1) domain at ring A through covalent linkage to a conserved cysteine. In the Pr state the chromophore assumes a ZZZssa configuration (Fig. 5.1) and is positioned in its binding pocket through steric interactions and hydrogen bonds from protein residues to the pyrrole rings and propionate side chains. Recent studies have indicated that 15Za to 15Ea isomerization of the chromophore at the C15-C16 double bond, which causes a flip of pyrrole ring D, accompanies formation of the Pfr state (8). The primary photoproduct, denoted Lumi-R, is formed on the 10–100 ps time scale and adopts the 15Ea configuration (14-19).

The X-ray crystal structure of a classical Bph from Deinococcus radiodurans DrBphP and cyanobacterial phytochrome Cph1 shows a single hydrogen bond between a conserved histidine residue and the BV chromophore ring D carbonyl in the chromophore binding pocket (10, 13, 20). In the P3 X-ray crystal structure (Fig. 5.1), besides the conserved histidine, Lys-183 and Ser-297 are within hydrogen bonding distance to the ring D carbonyl (12). These different aspects of classical Bph and P3 presents an opportunity to study the influence of the chromophore binding pocket on the phytochrome photochemistry. The understanding of their light activation mechanism and dynamics can be useful, such as leading to engineering of the phytochromes into an application as bio-luminescence tag in medical research (21).

In our previous femtosecond time-resolved absorption studies, we have shown that the excited state lifetimes and the spectra of P3 are very different from P2 and other BPhs, which we related to the hydrogen bond strength at ring D of the BV chromophore (19, 22). We determined that the two additional polar residues, lysine and serine located at the immediate vicinity of BV ring D are responsible for a lowering of the Lumi-R quantum yield and an increase of the BV excited-state lifetime.

The vibrational spectrum of a protein or a protein-bound chromophore contains a wealth of information about its structure, the interaction with the environment and electronic properties. Time-resolved IR spectroscopy is a powerful tool that can reveal many of the dynamic structural and physical-chemical properties of chromophores involved in (photo)biological reactions (23, 24). In addition, it can reveal the involvement of those parts of the protein that partake in the ongoing reactions. As the primary reactions in biological photoreceptors proceed on the ultrafast timescale, femtosecond IR spectroscopy is a method of choice to identify reaction mechanisms of biological photoreceptors (25-35). The femtosecond time-resolved infrared absorption (TRIR) study on Cph1 had shown that methine bridges, ring A/D of the BV were involved in structural changes in its primary photochemistry (16). The application of TRIR on another bacteriophytochrome, Agp1, had given a similar conclusion (35). Structural changes related to the methine bridges was also inferred by steady state resonance Raman studies on the intermediate states of various phytochromes that were cryo-trapped (36). However, information about the structural evolution of BV in the early photochemistry of P2 and P3 is lacking.
In this work, we continue our previous studies by comparing the early photochemistry of P2 and P3 using ultrafast IR spectroscopy. Our results support our earlier observation that excited state decay and Lumi-R formation is significantly slower in P3 as compared to P2. Comparison of time-resolved IR spectra and FTIR spectra of P2 and P3 confirms that hydrogen bonding to the ring D carbonyl is indeed significantly stronger in P3 than in P2. In addition, we observe a sharp product band at 1541 cm\(^{-1}\) in P2 and P3 that has not been previously observed before in ultrafast IR studies on phytochrome. Possible origins of this band are discussed.

5.2 Results and Discussion

**Ultrafast mid-IR spectroscopy of P2**

The reaction dynamics of P2 was investigated from a sub-picosecond timescale up to 3 nanoseconds by means of ultrafast mid-IR spectroscopy. The sample was excited at 680 nm and a spectral range of 1470 – 1780 cm\(^{-1}\) that covers the C=O and C=C vibration regions was monitored. The data were globally analyzed in terms of a kinetic scheme with sequentially interconverting species, where each species is characterized by an evolution-associated difference spectrum (EADS). Two decay lifetimes of 51 ps (solid line) and a non-decaying component (circle symbol line) were required for an adequate fit of the data. The EADS are shown in Fig. 5.2(a), Fig. 5.2(b) shows kinetic traces at selected frequencies. The 58 ps component is assigned to the BV excited state. In our visible pump-probe experiments, we observed a major excited state decay component of 50 ps, consistent with the present data. In addition, 0.4 ps, 4 ps and 250 ps components were observed with visible transient absorption spectroscopy (19). The absence of these components in our TRIR data is likely due to limited signal to noise.

The 58 ps EADS shows major bleach bands in the range of 1570 – 1640 cm\(^{-1}\), attributed to the BV chromophore C=C methine bridges vibrational bands (16, 35). These bands are located at 1591, 1613 and 1635 cm\(^{-1}\). Their frequencies closely resemble those in the Pr state of plant PhyA, DrBphP and Agp1, as observed with resonant Raman spectroscopy (37, 38). The largest bleach band at 1591 cm\(^{-1}\) in our TRIR data is assignable to the ring C – D methine bridge stretch band (39). The bleach band at ~1630 cm\(^{-1}\) is assigned to the ring A – B methine bridge (C4=C5) vibration band (39). Due to the reduction of bond orders in the excited states, these bleach bands are expected to be downshifted in the S\(_1\) state (16).
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Fig. 5.2: (a) Global analysis of P2. (b) Lifetime traces for the decay of the ring D C=O at 1702 cm\(^{-1}\), the decay of the C=C methine bridge stretching at 1591 cm\(^{-1}\) and the arising of the sharp early product band at 1540 cm\(^{-1}\).
The 58 ps EADS shows major bleach bands at 1728 and 1702 cm$^{-1}$ (Fig. 5.2(a)) which are assigned to the BV ring A C1=O and ring D C19=O vibrations, respectively. These C=O stretching bands have a lower frequency than those found in Cph1 (located at 1738 and ~1720 cm$^{-1}$ respectively), probably due to a different environment of the chromophore. Also, the different conjugation at ring A between BV and PCB may play a role. In ultrafast mid-IR data of Agp1, the ring D C19=O vibration was assigned to band at 1712 cm$^{-1}$ in the excited states (35). We note that there is some ambiguity in the assignment of the higher frequency carbonyl vibration as this band region is assigned to propionate chain B/C carbonyl stretching in a model pigment, whereas it was assigned to C19=O stretching in an $^{18}$O isotope labeled carbonyl group of ring A of PCB in PhyA (40, 41). The 58 ps EADS shows a bleach band at 1540 cm$^{-1}$ that was observed previously in TRIR spectroscopy on phytochromes but not interpreted (16, 35).

The final non-decaying component evolves in 58 ps and persists through our experimental timescale of 3 ns. It is assigned to the primary photoproduct Lumi-R. The nondecaying component has a low amplitude and most of its features do not rise above the noise level, in keeping with the low quantum yield of Lumi-R formation of 0.13 (19). Surprisingly, however, is the occurrence of a very sharp absorption band at 1541 cm$^{-1}$, at the same frequency as the bleach in the 58 ps EADS. Its possible origin will be discussed further on in the paper.
Fig. 5.3: (a) Global analysis of P3. (b) Time traces for the decay of the ring D C=O at 1678 cm\(^{-1}\), the decay of the C=C methine bridge stretching at 1588 cm\(^{-1}\) and the arising of the sharp early product band at 1541 cm\(^{-1}\).
Ultrafast mid-IR spectroscopy of P3

We investigated the reaction dynamics of P3 by means of ultrafast mid-IR spectroscopy. The sample was excited at 680 nm and a spectral range of 1470 – 1780 cm\(^{-1}\) that covers the C=O and C=C vibration regions was monitored. Global analysis indicated that three lifetime components of 9.5 ps, 293 ps and a non-decaying component were required for an adequate fit of the data. The EADS are shown in Fig. 5.3(a), whereas kinetic traces at selected frequencies are shown in Fig. 5.3(b).

The first EADS (solid line) has a lifetime of 9.5 ps. It corresponds to an unrelaxed form of the BV excited state (19) and shows strong bleach bands at 1588, \(\sim1603\), and 1630 cm\(^{-1}\) that represent the BV chromophore C=C methine bridges vibrational bands. The strongest bleach band at 1588 cm\(^{-1}\) is assigned to the BV ring C – D methine bridge (C15=C16) stretching. The 1630 cm\(^{-1}\) bleach band is assigned to the ring A – B methine bridge (C4=C5) stretching. These bands are similar to those observed in P2 (Fig. 5.2(a)), Cph1 and Agp1 (16, 35). Inspection of the carbonyl region (1680 – 1740 cm\(^{-1}\)) reveals a pattern that is quite different from that of P2: The ring A C=O stretching band is located at 1736 cm\(^{-1}\) [Fig. 5.3 (a), solid lines], a frequency comparable to those found in Cph1 and Agp1 (16, 35). Strikingly, the ring D C=O stretching mode of P3 is located at \(\sim1678\) cm\(^{-1}\) [Fig. 5.3(a), solid and dotted lines], a frequency much lower than other phytochromes. We will demonstrate below that the downshift of the ring D C=O frequency results from the increased hydrogen bond strength to ring D in P3. As in P2, a strong and broad bleach band at 1541 cm\(^{-1}\) is observed in the BV excited state of P3.

The bleach bands in the C=C and C=O regions of the first EADS evolve in 9.5 ps to the next EADS (dotted line) that has a lifetime of 293 ps. This EADS has minimal changes of bleach amplitudes when compared to the previous EADS. It represents a relaxed form of the BV excited state and has a lifetime similar to that of P3 in D\(_2\)O observed with ultrafast visible spectroscopy (500 ps) (19).

The BV excited state evolves in 293 ps to the non-decaying EADS, which is assigned to the primary photoproduct Lumi-R. This EADS has a very low amplitude, in keeping with the low Lumi-R quantum yield of P3 of 0.06 (19). As in P2, a sharp induced absorption band at 1541 cm\(^{-1}\) is observed. Figure 5.3(b) shows the rise of this product band. Other bands in this EADS are mostly buried in the noise, and will be neglected in the following.

FTIR spectroscopy

In P3, we observed that the ring D carbonyl has a particularly low frequency of 1678 cm\(^{-1}\) as compared to P2 and other classical (bacterio)phytochromes (16, 35). The lowering of the ring D carbonyl frequency is most likely due to the stronger hydrogen bonding at this site: the P3 X-ray structure shows 2 – 3 amino acids hydrogen bonding to the carbonyl of ring D in dark state, i.e. the conserved His299, Lys 183 and Ser297 (12). In classical phytochromes, only a conserved His hydrogen
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bonds to ring D \((10, 13, 42)\). To investigate this idea, we performed light-minus-dark FTIR spectroscopy on wild type P3 and the P3MA mutant \((19)\), where the two polar amino acids Lys-183 and Ser-297 are mutated to methionine and alanine respectively, eliminating the two hydrogen bonds. Also, FTIR spectra were taken on P2 and the P2KS mutant, where Met and Ala were replaced by polar residues Lys and Ser.

Fig. 5.4 shows the light-minus-dark FTIR spectra of P3 wild type (solid line) and the P3MA mutant (crossed symbol line). In P3 wild type, carbonyl bleaches are observed at 1734 cm\(^{-1}\) and 1685 cm\(^{-1}\) (Fig. 5.4, solid line), assigned to Ring A and Ring D carbonyls, respectively. This result is consistent with those of ultrafast IR spectroscopy, which indicated ring A and D carbonyl frequencies at 1736 and 1678 cm\(^{-1}\) [Fig. 5.3(a)] (note that the FTIR spectra were taken in \(H_2O\) and ultrafast IR spectra in \(D_2O\), giving rise to slightly different frequencies). In the P3MA mutant, the bleach at 1685 cm\(^{-1}\) has disappeared and a new bleach at 1711 cm\(^{-1}\) has appeared (Fig. 5.4, crossed symbol line), indicating that the BV ring D carbonyl shifts up by 26 cm\(^{-1}\) upon replacement of the hydrogen bonding amino acids Ser and Lys by nonpolar amino acids Met and Ala.

Fig. 5.5 shows the light-minus-dark FTIR spectra of P2 wild type (solid line) and the P2KS mutant (crossed symbol line). In P2 wild type, which forms only a single hydrogen bond from a conserved His to ring D, bleaches are observed at 1732 and 1703 cm\(^{-1}\) (Fig. 5.5, solid line), assigned to the ring A and D carbonyls, respectively. With ultrafast IR, similar frequencies are observed at 1728 and 1702 cm\(^{-1}\) (Fig. 5.2(a)). In the P2KS mutant, where M169 was replaced by Lys and A283 by Ser (the equivalent amino acids in P3), it is expected that two additional hydrogen bonds are formed to the ring D carbonyl. Indeed, the FTIR spectrum of the P2KS mutant (Fig. 5.5, crossed symbol line) shows a downshifting of ring D carbonyl stretching frequency from 1703 (in WT) to 1676 cm\(^{-1}\) (in P2KS) in the dark state.

We conclude that in P3 wild type in the dark, hydrogen bonding to the ring D carbonyl is significantly stronger than in P2, consistent with the X-ray structures of P3 and classical (B)ph \((12, 13, 42)\).
Fig. 5.4: FTIR result of P3MA mutant.

Fig. 5.5: FTIR result of P2KS mutant.
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The origin of 1541 band – possible implications for the isomerization reaction mechanism

A key observation of the present work is a band near 1540 cm⁻¹ that appears as a broad bleach in the BV excited state (thus corresponding to Pr) and a narrow induced absorption at essentially the same frequency in the primary photoproduct. The question arises what specific vibrational mode(s) of BV these bands belong to. We note that the negative and positive band do not necessarily relate to the exact same vibrational mode. In previous ultrafast mid-IR experiments on Cph1 and Agp1, a similar but smaller bleach band at ~1540 cm⁻¹ was observed (16, 35). With resonant Raman spectroscopy on plant PhyA in D₂O, Pr shows a band at 1547 cm⁻¹, and cryotrapped Lumi-R exhibited a sharp band at 1541 cm⁻¹ (36). In DrBphP, a band at 1546 cm⁻¹ was observed in D₂O with resonant Raman spectroscopy (38). In neither of these studies the bands near 1540 cm⁻¹ were interpreted. It does not belong to the N-H in-plane bending vibrational band [at ~1570 cm⁻¹ in PhyA, Agp1 and DrBphP in H₂O (37, 38, 43)] because this band downshifts to below 1100 cm⁻¹ upon deuteration (36, 38).

As a first possible origin of the 1540 cm⁻¹ band, calculations and IR absorption experiments on the model compound biliverdin dimethyl ester have indicated a mode at 1543 cm⁻¹ in D₂O that corresponded to the C=C ring stretch and C-vinyl stretch band of ring D (40). If this situation applies to our case, the ultrafast mid-IR experiments give the first evidence for a structural change in BV that affects vibrational modes of ring D. Previous ultrafast mid-IR, femtosecond stimulated Raman spectroscopy (FSRS) and cryotrapped resonance Raman studies on phytochromes have shown structural changes at the C – D and A – B methine bridges (16, 36, 44), but not at ring D itself. In our experiments, the broad bleach band at ~1541 cm⁻¹ in the excited state of P2 and P3 evolves to a sharp Lumi-R product band at 1540 cm⁻¹ in 58 and 293 ps, respectively. This sharp absorption band is not observed in an ultrafast IR study of Cph1 and points to different early chromophore ring D isomerization dynamics between Cph1 and Bphs (45). The narrow linewidth of the product band implies that ring D adopts a highly ordered structural state upon Z/E isomerization. Possibly, this band sharpening results from the absence of hydrogen-bond interactions with nearby amino acids, reducing dispersive interactions as compared with the Pr ground state. Possibly, ring D adopts a significantly twisted angle with respect to rings B and C, which will decrease π-electron conjugation and thereby decrease vibrational coupling with the rest of the chromophore. It should be noted that the latter idea is not consistent with the red-shifted absorption of Lumi-R with respect to Pr (19), which suggests an increase of π-electron conjugation upon isomerization.

As a second possibility for the origin of the ~1540 cm⁻¹ band, recent DFT calculations have indicated that in a pyrrole-N deuterated PCB chromophore in a ZZZssa configuration, a band near 1540 cm⁻¹ arises that includes mainly stretching coordinates from ring B and, to a minor content, from the B – C and A – B methine bridges. In the ZZEssa configuration, this band slightly shifts to 1542 cm⁻¹ (46). In case
this assignment underlies our observation of the 1540 cm$^{-1}$ band, it is difficult to understand how our observations relate with Lumi-R formation because no significant changes are thought to occur at ring B upon isomerization about the C15=C16 double bond. The DFT calculations indicate a slight difference in frequency by a few cm$^{-1}$ between ZZZssa and ZZEssa conformers (46) which can in principle give rise to a difference spectrum. However, it is not expected to result in a dominant feature in the Lumi-R-minus-Pr difference spectrum (recall that no other clear Lumi-R features rose above the noise level). Hence, it is unlikely that the positive 1540 cm$^{-1}$ band in the primary photoproduction spectrum relates to Lumi-R.

What, then, could be the origin of the 1540 cm$^{-1}$ band? First, it is important to note that the Lumi-R quantum yield is low, 0.13 in P2 and 0.06 in P3 (19). Thus, more than 85% of the BV excited states revert to Pr within at most a few hundred ps, implying that the energy of a 680 nm photon is dissipated in the protein on such a short timescale. Possibly, the narrow 1540 cm$^{-1}$ absorption arises from structural dynamics that are associated with re-formation of Pr. In this light it is interesting to consider the reaction model that we recently put forward for isomerization and excited state deactivation of the BV chromophore in Bph (19). There, we proposed that excited state deactivation to Pr proceeds through excited state proton transfer from pyrrole nitrogen at rings A, B or C to the backbone carbonyl of a conserved Asp or to a bound water, which brings the system to the molecular ground state through a conical intersection. Then, rapid re-protonation of BV takes place via proton backshuttling to the pyrrole nitrogen, resulting in re-formation of Pr. Before photon absorption, the BV chromophore, in Pr, was conformationally relaxed and in thermal equilibrium with the protein environment. After the reprotonation reaction that reforms Pr, the BV chromophore certainly is not in thermal equilibrium with the protein environment and may have a strained conformation at the deprotonation-reprotonation site. This scenario is consistent with the appearance of a sharp, positive band associated with a ring B stretch vibration, since ring B is one of the possible sites of the deprotonation-reprotonation reaction (19).

5.3 Conclusions

Here, we have reported an ultrafast mid-IR study of two bacteriophytochromes: P2, which shows classical Pr – Pfr photochemistry and P3, which shows an unusual Pr – Pnr photochemistry. In P2, BV excited-state decay occurs with a time constant of 51 ps, largely consistent with our results from visible transient absorption spectroscopy which indicated a biexponential decay with a main decay component of 60 ps. Excited-state decay in P3 is significantly slower with a time constant of 293 ps, which is also consistent with visible transient absorption results (19). In our previous work, we proposed that the slower excited state decay of P3 is related to an increased hydrogen bond strength at ring D, with three amino acid side chains (His, Lys and Ser) competing for hydrogen bonding to the ring D carbonyl in P3 (12, 19). In P2, only one such hydrogen bond is formed from His to ring D (10, 42). Here, we
obtained direct evidence for increased hydrogen bond strength at ring D in P3: ultrafast IR spectroscopy on P2 and P3, and FTIR spectroscopy on the P2 and P3 wild types and P3MA and P2KS mutants indicated that in P3, the ring D carbonyl has a low vibrational frequency at 1685 - 1678 cm⁻¹. In contrast, the ring D carbonyl vibration in P2 is located at 1703 cm⁻¹, which demonstrates that P3 has one or two additional hydrogen bonds to ring D.

For P2 and P3, we observed a pronounced positive and narrow band at 1540 cm⁻¹ in the difference spectrum of the primary photoproduc. At this stage the origin of this feature cannot be firmly established. It may correspond to a BV ring D – vinyl vibrational mode which narrows upon Lumi-R formation. Alternatively, it may be related to a BV ring B stretch vibration that becomes narrower upon re-formation of Pr upon excited-state decay through a deprotonation - reprotonation cycle at ring B.
Material and Methods

(i) Sample preparation

The detailed preparation of RpBphP2 and RpBphP3 bacteriophytochrome proteins are described previously (12) (See Chapter 3, Materials and Methods). For H/D exchange experiments, the proteins were dissolved in D$_2$O buffer (20mM TRIS•HCl, pH 8 at room temperature).

(ii) Time-resolved infra-red spectroscopy

The experimental setup is a home-built spectrometer based on a 1 kHz amplified Ti:Sapphire laser system operating at 1 kHz (Spectra Physics Hurricane) that allows visible pump/mid infrared probe in a time window from 180 femtoseconds to 3 ns, as previously described (25). The red excitation pulse was generated by means of a non-collinear optical parametric amplifier and centered around 680 nm, at an excitation energy of 150 - 250 nJ. The infrared probe had a spectral width of 200 cm$^{-1}$, was spectrally dispersed after the sample and detected with a 32-element array detector, leading to a spectral resolution of 6 cm$^{-1}$. Vibrational spectra between 1780 and 1450 cm$^{-1}$ were taken in intervals and simultaneously analyzed. Spectra were recorded at 100 time delay points between -20 ps and 2.8 ns. During the experiments, the sample cell was continuously translated with a Lissajous scanner, which ensured sample refreshment after each laser shot and a time interval of 1 minute between successive exposures to the laser beams. Background illumination to photorevert the Bph sample to Pr was provided with a LED with a center wavelength at 750 nm (P2) or 650 nm (P3).

(ii) Differential fourier-transform infra-red (FTIR) spectroscopy

The differential FTIR data were recorded at RT using a FTIR spectrometer (IFS 66s Bruker) equipped with a nitrogen cooled photovoltaic MCT detector (20 MHz, KV 100, Kolmar Technologies, Inc., USA). Two LEDs, emitting at 680 nm and 750 nm, were used to convert RpBphP2 to its light or dark states, respectively. For RpBphP3, LEDs emitting at 680 nm and 650 nm were used instead. The light minus dark FTIR data was obtained, by subtracting an initially recorded protein dark state spectrum as the background spectrum, from the light activated (using the 680 nm LED) protein spectrum. Background and sample interferogram data were averaged from 500 to 2000 interferogram scans, at 4 cm$^{-1}$ spectra resolution. Measurements were repeated by illuminating the sample with a 750 nm (on P2) or a 650 nm (on P3) light to deactivate the light state of the protein, and repeat the procedures by taking a background and a light activated spectrum. The FTIR sample was prepared using a drop of 2 mL of sample at OD$_{700nm}$ of ~100 (in 20 mM Tris/HCL pH8 buffer), and spread between two tightly fixed CaF2 windows.
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Acknowledgements

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BACTERIOPHYTOCHROMES

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Supporting Information

Fig. S5.1: (a) Global analysis of P2 PAS – GAF. (b) Lifetime traces for the decay of the ring D C=O at 1708 cm\(^{-1}\), the decay of the C=C methine bridge stretching at 1588 cm\(^{-1}\) and the arising of the sharp early product band at 1541 cm\(^{-1}\).
Fig. S5.2: (a) Global analysis of P3 PAS – GAF. (b) Lifetime traces for the decay of the ring D C=O at 1677 cm\(^{-1}\), the decay of the C=C methine bridge stretching at 1588 cm\(^{-1}\) and the arising of the sharp early product band at 1541 cm\(^{-1}\).