The Photoactivated PYP Domain of *Rhodospirillum centenum* Ppr Accelerates the Recovery of the Bacteriophytochrome Domain after White Light Illumination

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ABSTRACT: Ppr from the purple phototrophic bacterium, *Rhodospirillum centenum* (also known as *Rhodocista centenaria*), is a hybrid of photoactive yellow protein (PYP), bacteriophytochrome (Bph), and histidine kinase (HK) domains. The holo-Ppr (containing both chromophores) exhibits characteristic absorption maxima at 435 nm due to the PYP domain and at 400, 642, and 701 nm due to the Bph domain. Illumination of the Ppr with white light causes a bleach of both PYP and Bph absorbance; weak blue light primarily bleaches the PYP, and red light activates only the Bph. When excited by blue light, the PYP domain in Ppr recovers with biphasic kinetics at 445 nm (32% with a lifetime of 3.8 min and the remainder with a lifetime of 46 min); white light primarily results in fast recovery, whereas the 130-residue PYP construct shows only the faster kinetics in both blue and white light. Furthermore, there is a slight red shift of the ground state Bph when the PYP is activated; thus, both spectroscopy and kinetics suggest interdomain communication. When Ppr is illuminated with red light, the recovery of the Bph domain to the dark state is significantly slower than that of PYP and is biphasic (57% of the 701 nm decay has a lifetime of 17 min and the remainder a lifetime of 50 min). However, when illuminated with white light or red followed by blue light, the Bph domain in Ppr recovers to the dark-adapted state in a triphasic fashion, where the fastest phase is similar to that of the fast phase of the PYP domain (in white light, 25% of the 701 nm recovery has a lifetime of ∼1 min) and the slower phases are like the recovery after red light alone. Apo-holo-Ppr (with the biliverdin chromophore only) recovers with biphasic kinetics similar to those of the slower phases of holo-Ppr when activated by either red or white light. We conclude that the photoactivated PYP domain in Ppr accelerates recovery of the activated Bph domain. Phytochromes can be reversibly switched between Pr and Pfr forms by red and far-red light, but the consequence of a bleaching phytochrome is that it cannot be photoreversed by far-red light. We thus postulate that the function of the PYP domain in Ppr is to act as a blue light switch to reverse the effects of red light on the Bph.

The detection of light quantity and quality is crucially important for all photosynthetic organisms and for those nonphototrophic species whose habitat is routinely exposed to light. In particular, detection of harmful UV and potentially beneficial red light is physiologically relevant, but only a limited number of photoreceptors have been characterized in detail. Photoactive yellow protein (PYP)[1] (*1*, *2*) is a small bacterial blue light sensor that uses *p*-hydroxycinnamic acid as a chromophore. The maximum absorbance is species-dependent but is at 446 nm for the prototypic PYP from *Halorhodospira halophila* (Hh). Light excitation results in a *trans*−*cis* isomerization, protonation of the chromophore, and subsequent protein conformational changes. PYP has a high quantum efficiency (between 30 and 60%), and it is readily bleached and usually recovers in the dark in less than a second, although it can also be photoreversed by near-UV light in milliseconds (*2*, *3*). In a broader context, PYP is important as the structural prototype for the widespread family of PAS domain family sensory proteins known to bind a variety of ligands (*4*).

Ppr, from the purple phototrophic bacterium, *Rhodospirillum centenum*, is a hybrid photoactive yellow protein, bacteriophytochrome, histidine kinase (*5*). Ppr with only the PYP domain reconstituted has a slightly more blue-shifted absorption maximum than Hh PYP (λ_{max} = 434 nm), and the kinetics for recovery after a light flash [lifetime of ∼1 min (*5*)] is nearly 400 times slower than that for Hh PYP. The three-dimensional structure of the 130-residue PYP construct is very similar to that of *Hr. halophila* PYP, but the different conformation of the M100 loop relative to Hh PYP explains the slow recovery kinetics (*6*). Although in most cases, the function of PYP remains speculative, it has

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[1] Abbreviations: apo-holo-Ppr, full-length protein with the biliverdin chromophore only; Bph, bacteriophytochrome; BV, biliverdin; CHS, chalcone synthase; DGC, diguanylate cyclase; HemO, heme oxygenase; HK, histidine kinase; holo-Ppr, full-length protein containing both chromophores; IPTG, isopropyl β-D-thiogalactoside; MCP, methyl-accepting chemotaxis protein; PAS, acronym formed of the names of the first three proteins recognized as sharing this sensor motif (periodic clock protein of *Drosophila*, aryl hydrocarbon receptor nuclear translocator of vertebrates, and single-minded protein of *Drosophila*); PCR, polymerase chain reaction; PKS, polyketide synthase; Ppr, PYP-phytochrome related or PYP-Bph-HK; PYP, photoactive yellow protein; WT, wild type.

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been shown that Ppr regulates the expression of a polyketide synthase gene (PKS) homologous to that for chalcone synthase (*5*). The exact enzymatic activity of the PKS has not yet been determined, but its expression is correlated with desiccation-resistant cyst formation (*7*). In *Azotobacter vinelandii*, cysts contain a special membrane lipid which is synthesized by a PKS (*8*); thus, a cyst-specific lipid may be the product of the *Rs. centenum* PKS.

In plants, phytochromes have been well studied as red light sensors since their discovery in 1959 (for a recent review, see ref *8*). Until recently, phytochromes were thought to be restricted to plants and algae; however, it is now clear that a widespread family of phytochromes is present in both photosynthetic and nonphotosynthetic bacteria and fungi (*10*−*14*). The biological function of the bacteriophytochromes (Bph) is still not fully understood, but they may play rather diverse roles depending on the species, including phototaxis, chromatic adaptation, resetting circadian clocks, regulation of pigment biosynthesis, etc.

The bacteriophytochromes contain biliverdin, a linear tetrapyrrole chromophore, which normally switches between a red (Pr, $\lambda_{max} \sim 700$ nm) and a far-red (Pfr, $\lambda_{max} \sim 750$ nm) form as a result of photoisomerization around the C15−C16 double bond. Plant (Phy) and cyanobacterial (Cph) phytochromes contain more reduced tetrapyrrole chromophores that absorb at shorter wavelengths, and the absorption spectra are species-dependent, as are the photoproducts. While photoconversion between Pr and Pfr can be rapid, dark recovery can take a few minutes to several hours or days. Nevertheless, the quantum efficiency is remarkably low, typically less than 10% (*15*), which suggests that strong illumination is necessary to switch from one state to the other. Recently, the three-dimensional structure of the chromophore-binding portion of bacteriophytochrome from *Deinococcus radiodurans* was determined for the Pr state (*16*, *17*). The structure shows that Bph is composed of two PAS domains, the first of which includes the cysteine, to which the chromophore is covalently attached, and the second of which provides most of the chromophore environment. A portion of the two PAS domains is intertwined, and the Bph N-terminus is in the proximity of the biliverdin chromophore. Although these results give a first glimpse into phytochrome structure, the sensory mechanism is still undetermined.

We previously reported the reconstitution of holo-Ppr with both the PYP and the Bph chromophores (*18*) and showed that instead of a light-induced red shift, as in most phytochromes, the Bph of Ppr is bleached at 701 nm with no apparent red-shifted form. We now describe the characterization of Ppr and the interplay between the two chromophore domains.

MATERIALS AND METHODS

Plasmid Construction. The genes for *Rs. centenum* Ppr, the 130-residue PYP domain construct, and *Rhodopseudomonas palustris* CG009 HemO (the biosynthetic enzyme for biliverdin) were provided by C. Bauer (Indiana University, Bloomington, IN) as constructs of pET28a (pZJ137) (*5*), pET28a (pZJ191), and pET11a, respectively. Construction of the pACYC(TALpCL) plasmid that contains the two biosynthetic genes for holo-PYP formation was described previously (*19*). To coexpress all four genes in *Escherichia coli*, the Ppr and HemO genes were recloned into a single plasmid (pETDuet-1, Novagen) which was compatible with pACYC(TALpCL) when coexpressed in *E. coli*.

The full-length Ppr gene was cut from the pZJ137 Ppr construct using XbaI and BamHI, thus retaining a plasmid-based N-terminal six-histidine tag, and ligated into the MCS1 region of pETDuet-1. The HemO gene was isolated from pET11a(HemO) and modified using PCR with primers atatatatcatatggtggtggaagcagc and atatgacgtctaggcgtcgagatgctg, to attach NdeI and AatII sites to the 5′ and 3′ ends, respectively, for ligation into the MCS2 site of pETDuet-1. Underlined portions are restriction enzyme recognition sites. This assembly yielded the construct pH_6-Ppr/HO-Duet in which both genes are under the control of their individual T7lac promoters. To produce Ppr with only the Bph chromophore, we omitted the pACYC(TALpCL) plasmid.

Protein Production and Purification. Expression of Ppr was performed using *E. coli* BL21(DE3) (Novagen), LB medium, and selective antibiotics chloramphenicol (25 μg/mL) and carbenicillin (200 μg/mL). *E. coli* was cotransformed with pACYC(TALpCL) and pH_6Ppr/HO-Duet. A single colony was used to inoculate 100 mL medium, then grown overnight at 30 °C, and harvested, and the cell pellet was resuspended in 100 mL of fresh medium, 15 mL of which was then used to inoculate 1.5 L in 2.8 L Fernbach flasks. In a typical preparation, the contents of two flasks were grown in the dark for 3 days at 20 °C, at 95 rpm, without any IPTG induction. Cell pellets were resuspended in 20% glycerol, 40 mM NaCl, and 50 mM phosphate (pH 7.2) to a volume at least 3 times that of the pellet weight and disrupted on ice using a Branson model 550 sonicator for a total of 5 min, with 30 s pulses at 70% power. The suspension was then centrifuged for 1 h at 200000*g* or 40K rpm in a Spinco Ti45 rotor. The best yield prior to purification was 30 mg of soluble Ppr/L of culture with much more remaining in the pellets. In some preparations, virtually all the Ppr was in the insoluble pellet, especially when cells were grown at higher temperatures and/or levels of aeration. Attempts to solubilize this fraction (including mild detergents, urea, and mild sonication) were unsuccessful.

Supernatants were applied directly to a 30 mL TALON (Clontech) IMAC(Co^{2+}) resin column, which was then rinsed with at least 10 column volumes of 20% glycerol, 400 mM NaCl, and 50 mM phosphate buffer (pH 7.2) until there was no significant 280 nm absorbance eluted. Ppr was then eluted from the resin, collecting 1 mL fractions, using 150 mM imidazole, 20% glycerol, 400 mM NaCl, and 50 mM phosphate (pH 7.2). The Ppr was contaminated with large amounts of a proteolytic fragment of the N-terminal domain which was easily detected by its PYP spectrum. Fortunately, it was bound to the TALON resin more tightly and eluted only after one-third to one-half of the holoPpr had been collected. Observing the absorption spectrum or just the 400 nm (Bph) to 434 nm (PYP) ratio allowed segregation of Ppr that was free of the contaminating excess PYP (the proteolytic fragments of Bph and HK were not adsorbed to the column since they did not have His tags). If Ppr was not separated from the proteolytic fragments upon elution from the TALON resin, we found it to be difficult to separate them by other means such as gel filtration or ion exchange, indicating that they are involved in complex formation. When carefully performed, the protein was essentially pure after

elution from the TALON column as indicated by a single band on SDS−PAGE in the vicinity of 96 kDa. The purest fractions had a 280 nm (protein) to 701 nm (Bph) ratio of 1.35 and a 400 nm to 434 nm ratio of ~1.3. The Ppr was used for further experiments in the elution buffer without removing the His tag. Preparation of apo-holo-Ppr was similar except that *E. coli* was transformed with the pH_6-Ppr/HO-Duet only.

Preparation of the 130-residue PYP construct was less dependent on growth conditions than that of Ppr, although there was some insoluble PYP in the pellet. For production of the PYP from pZJ191 and pACYC(TALpCL), we used kanamycin (25 μg/mL) and chloramphenicol (25 μg/mL). Fractions of the PYP construct with the lowest 280 nm to 434 nm ratios from the TALON column (eluted with 150 mM imidazole) were pooled and diluted 12-fold with 5% glycerol, 0.5 mM EDTA, and 20 mM MES (pH 6.2) and then chromatographed on CM-Sepharose (GE Biosciences) with a 0 to 500 mM NaCl gradient in the same buffer. Although pure, the PYP construct readily separated into two distinct bands which, when analyzed by electrospray MS, have monoisotopic masses of 16 850 and 16 807 Da, consistent with partial acetylation, both forms with the attached six-His tag. The buffer conditions used for kinetics measurements were the same as those used for Ppr.

UV−Vis Spectroscopy. Absorption spectra and kinetics were obtained using a CARY 300 spectrophotometer. All studies were carried out in 20% glycerol, 50 mM phosphate, 400 mM NaCl, and 150 mM imidazole (pH 7.2) unless otherwise indicated. The protein was first bleached via a 30 s exposure to a 40 W tungsten lamp; subsequent recovery was measured in the dark in the spectrophotometer. Experiments were also carried out with filters to excite only the 701 nm region (Corning K-7), the 400 and 434 nm region (filter 4-72), or more selectively the 434 nm region (filter K2 + 4-72). Absorbance changes were measured for up to 240 min, and the kinetic data were fit using Sigmaplot. To obtain difference spectra in the fastest possible time without significantly sacrificing resolution, samples were scanned with a CARY 50 spectrophotometer at 4800 nm/min with 1 nm intervals, using the same illumination method that was used for the kinetics. Some of the experiments were repeated by illuminating the sample with monochromatic light (ORIEL monochromator 77250) at the appropriate wavelength. Although this light was of lower intensity and resulted in a smaller bleach amplitude, it allowed us to exclude photoreversal due to excitation at multiple wavelengths.

RESULTS

Production and Purification of Ppr. The gene for *Rs. centenum* Ppr was previously cloned, and the apoprotein was produced in *E. coli* and chemically reconstituted with the PYP chromophore (*5*). We were also able to produce the Ppr holoprotein with both PYP and Bph chromophores when the three biosynthetic enzymes were supplied on plasmids (*18*). Figure 1 shows that *E. coli* cells containing both plasmids are colored blue-green. Note that, after lysis of the cells, a significant amount of color remained in the insoluble fraction. In some of the preparations, where the cells were grown at room temperature with vigorous aeration and induced, there was virtually no soluble Ppr produced. The best yields of soluble protein were obtained when cells were grown for 3 days at 20 °C in Fernbach flasks at 95 rpm without induction. SDS−PAGE and mass spectrometric analysis showed that a significant amount of the 96 kDa protein was degraded with time, even when stored at 4 °C, into relatively stable 13 480.1, 49 287.6, and 54 134.7 Da components. It was clear from the UV−vis spectrum and peptide mapping after a tryptic digest of the stable components that they had lost the PYP domain and at least parts of the histidine kinase domain. Therefore, freshly prepared protein was used for the spectral and kinetic analysis. This observation is consistent with the degradation pattern in *Synechocystis* Cph1 where major products of 52 and 49 kDa resulted from cleavage within the N-terminal PAS domain and between the Cph and kinase domains (*20*). The Ppr is also unstable to shear due to magnetic stirring and to freeze−thaw cycles. It was stabilized to an extent by addition of 20% glycerol. The major spectral characteristics are stable even when the protein is partially degraded by proteolysis. Nevertheless, it was difficult to establish kinetic reproducibility. After 1 week at 4 °C in glycerol, the recovery of the Bph following illumination was noticeably slower, which we believe is an indication of partial denaturation. There are relatively minor changes in the kinetics of the PYP construct without glycerol, but the Ppr denatured more rapidly in the absense of glycerol as indicated by increased turbidity even after a single kinetics experiment, less reproducibility, and additional slower kinetic phases. However, the major findings that we report are present without glycerol and are likely to be physiologically relevant.

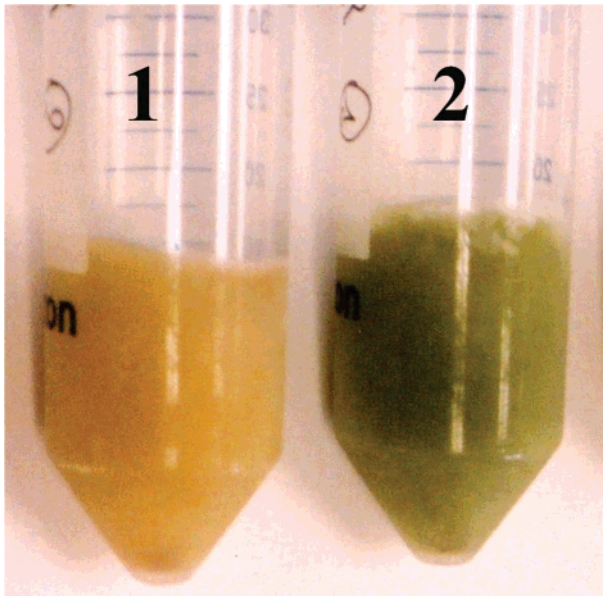

FIGURE 1: *E. coli* cell pellets, resuspended in Tris-HCl (50 mM, pH 7.2). Tube 1 contained cells without plasmids. Tube 2 contained cells grown at 28 °C, with plasmids and 0.5 mM IPTG induction.

Spectral Characterization of Ppr. The dark-adapted UV−vis spectrum of apo-holo-Ppr, containing the biliverdin chromophore only, is shown in Figure 2A. The absorption maxima are at 396 and 701 nm with a shoulder at 642 nm. When illuminated with weak blue light (using the Corning 4-72 filter; blue line in Figure 2A), the Bph is hardly affected by excitation of the Soret (~2% bleach at 701 nm). However, when illuminated with red (Corning K7 filter) or white light, each of which appears to be equivalent to the other, the Bph

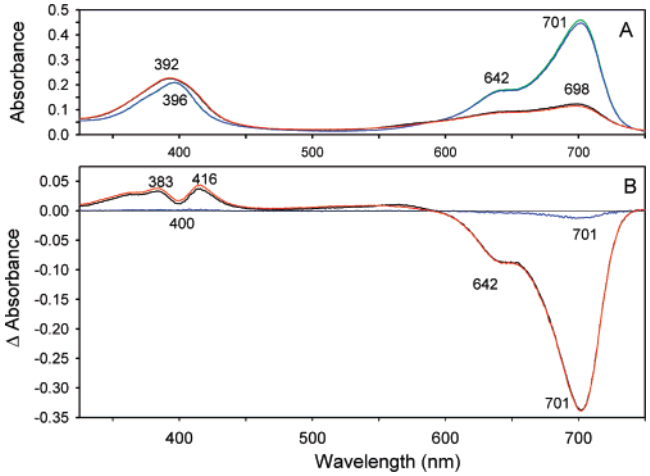

FIGURE 2: (A) Absorption spectra of dark-adapted apo-holo-Ppr (green) and apo-holo-Ppr after a 30 s blue light illumination (blue), after a 30 s red light illumination (red), and after a 30 s white light illumination (black). (B) Difference spectra for blue minus dark (blue), red minus dark (red), and white minus dark (black).

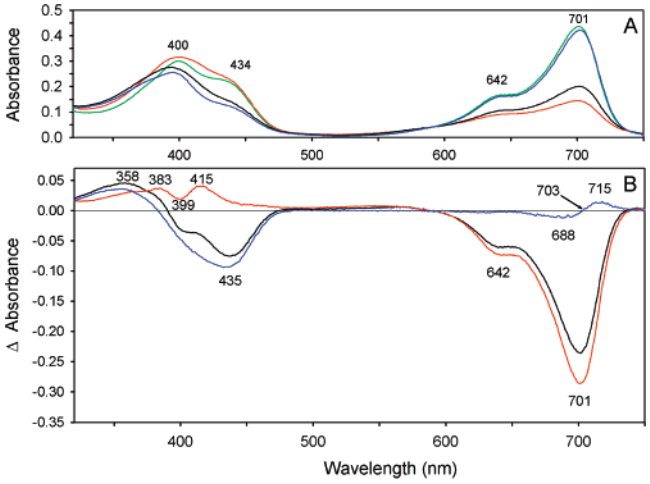

FIGURE 3: (A) Absorption spectra of dark-adapted holo-Ppr (green) and holo-Ppr after a 30 s blue light illumination (blue), after a 30 s red light illumination (red), and after a 30 s white light illumination (black). (B) Difference spectra for blue minus dark (blue), red minus dark (red), and white minus dark (black).

is bleached by at least 80% at 701 nm (red and black lines in Figure 2). The Soret band increases in intensity and is broadened with a characteristic difference spectrum (Figure 2B) having peaks at 383 and 416 nm and a valley at 400 nm. By comparison of the dark-adapted holo-Ppr containing both PYP and Bph chromophores in Figure 3A with apo-holo-Ppr in Figure 2A, it is apparent that the 434 nm shoulder is due to the PYP and that the 358 nm peak and 435 nm valley in the difference spectrum of Figure 3B also result from PYP. By comparison of the spectrum of holo-Ppr to that of holo-apo-Ppr with only the PYP chromophore (*5*) and to that of the PYP construct, it is clear that the PYP domain within the Ppr is spectrally similar to the 130-residue PYP construct (data not shown). Red light activation of holo-Ppr is very similar to that of apo-holo-Ppr in that it appears to affect only the Bph (red line in Figure 3). Blue light illumination of holo-Ppr primarily activates the PYP but also affects the Bph (Figure 3A), although in this case, the Bph is slightly red-shifted rather than bleached which is evident from the valley at 688 nm and peak at 715 nm with an isosbestic point at 703 nm (Figure 3B). Because Bph in apo-

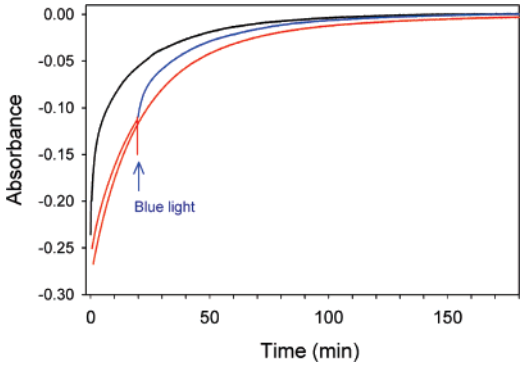

FIGURE 4: Kinetics of recovery at 701 nm following a 30 s red light illumination (red), following a 30 s red light illumination, a 20 min recovery, and then a 30 s blue light illumination (red and blue), and following a 30 s white light illumination (black).

Table 1: Lifetimes (minutes) and Magnitudes (percentage of total spectral change in italics) for Dark Recovery after Different Light Excitation of Holo-Ppr, Apo-Holo-Ppr, and the 130-Residue PYP Construct from Ppr[a]

	λ_{det}[b] (nm)	white	red	blue	red and blue
apo-holo-Ppr	701	12 (*20*), 47	12 (*41*), 29	nd[c]	14, 50[d]
holo-Ppr	701	1.1 (*25*), 11 (*25*), 33	17 (*57*), 50	nd[c]	1.3, 13, 40[d]
	434	2.1 (*77*), 12	N/A[e]	3.8 (*32*), 46	nd[c]
PYP construct	434	2.8 (*79*), 11	N/A[e]	4.4 (*100*)	N/A[e]

[a] All data were taken at pH 7.0. [b] Detection wavelength. [c] Not determined. [d] Magnitudes depend on the time delay of the blue flash after the red light illumination. [e] Not applicable.

holo-Ppr is not significantly activated by blue light under these conditions (less than 2%; cf. Figure 2A), it is apparent that the ground state Bph domain recognizes the changes in the PYP, either through direct contact of PYP with the Bph chromophore or indirectly via conformational changes in the peptide chain. Note that the magnitude of the red shift is much smaller than that observed with other phytochromes where the biliverdin is photoactivated. When holo-Ppr was illuminated with white light, neither the Bph nor the PYP bleached as much as in red or blue light (see below). Although most of the absorbance can be bleached at 701 and 642 nm by red light, there is some residual absorbance centered at ~650 nm as indicated by the 701 nm to 642 nm ratio of 2.6 in the dark and ~4.0 in the difference spectrum. Thus, there could be formation of a small amount of a blue-shifted form of the Bph domain similar to the Pnr form of *Rp. palustris* Bph3 which absorbs at 645 nm (*12*).

Kinetics of Recovery. As indicated above, red light illumination of holo-Ppr bleached the Bph domain at 701 nm, which then recovered in the dark in a biphasic manner with rate constants of ~0.06 ± 0.01 m^{-1} (~57 ± 10% amplitude) and 0.02 m^{-1} (lifetimes of 17 and 50 min, respectively) as shown in Figure 4 and Table 1. We consistently observed that older preparations have slower kinetics (rate constants shifting closer to 0.01 m^{-1}) with larger amplitudes. We therefore consider the slowest kinetics to be characteristic of a partially degraded protein. When Ppr was illuminated with blue light, only the PYP domain was bleached and recovery at 434 nm was biphasic with rate constants of 0.26 and 0.022 m^{-1} [~32% with a 3.8 min lifetime and the remainder with an ~46 min lifetime (Figure

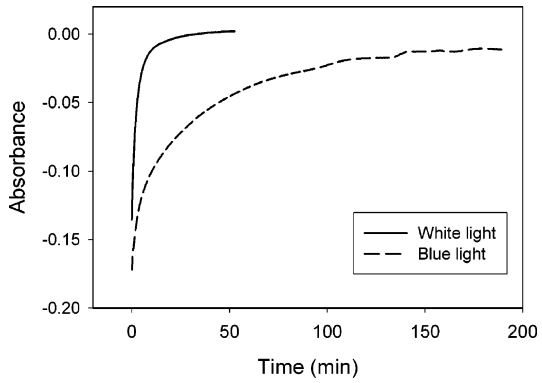

FIGURE 5: Kinetics of recovery of holo-Ppr monitored at 440 nm after a 30 s white light (—) and a 30 s blue light (– – –) illumination.

5)]. With white light, recovery was mainly fast phase (~80%), which is consistent with the monophasic recovery we previously reported (*18*). The analysis presented here has a better signal-to-noise ratio and consequently allowed a more detailed study, which shows that there is a small-amplitude slower phase with a lifetime of ~12 min in the PYP recovery. The 130-residue PYP construct, when illuminated with either blue or white light under the same conditions (data not shown), had a lifetime of 3−4 min for recovery. Recovery of the PYP construct following white light illumination also had an additional slower phase (lifetime of ~11 min) with a small amplitude (~15−20%). However, it should be kept in mind that the experiments are performed under steady state conditions, generating intermediates that are themselves photoactive. Depending on conditions, there may be more or less biphasic character. In essence, the construct has the same lifetimes as the fast phase of the PYP in Ppr. Thus, the very slow phase of the photoactivated PYP domain (46 min), which is obvious in the results of the blue light experiment depicted in Figure 5, is unique to Ppr and suggests that interaction with ground state Bph alters the kinetics.

When Ppr was illuminated by white light, recovery at 701 nm was triphasic with rate constants of 0.9, 0.09, and 0.03 m^{-1} (25% with a lifetime of 1.1 min, 25% at 11 min, and 50% at 33 min). The latter two rate constants are essentially the same as in red light (17 and 50 min), whereas the fastest rate constant (1 min lifetime) is clearly new and suggests acceleration of recovery via an interaction with PYP, which has a similar fast phase. Apo-holo-Ppr, when illuminated with either red or white light (kinetic data not shown), behaved like holo-Ppr with red light (Table 1). However, when the holo-Ppr sample was illuminated with red light and allowed to recover for a variable length of time and then illuminated with blue light, there was a clear acceleration of recovery at 701 nm as shown in Figure 4 for the case of a 20 min post-red light recovery. The resulting rate constants are similar to those from the white light experiment (0.75, 0.08, and 0.025 m^{-1}). It is apparent that the fast recovery of Bph after white light illumination is due to acceleration from interaction with blue light-activated PYP. The kinetics of apo-holo-Ppr after bleach by red light was unaffected by additional illumination with blue light. Thus, the blue or white light acceleration is unique to holo-Ppr.

DISCUSSION

We previously showed that a functional Ppr with both photoactive chromophores could be heterologously produced in *E. coli*, and illumination of fully reconstituted Ppr with white light results in a reversible bleach of both the PYP domain at 434 nm and the Bph domain at 701 nm (*18*). This is very different from the usual bacteriophytochrome which undergoes a reversible red shift to ~750 nm following illumination but similar to the Asp207Ala and His260Ala mutants of *Agrobacterium tumefaciens* Bph1, for which the pK_a of the pyrrole nitrogen is lowered, resulting in deprotonation and bleach of the chromophore (*21*). However, amino acid sequence alignment shows that both residues are conserved in Ppr; hence, naturally occurring mutations at these positions are not responsible for the bleach as opposed to red shifts, although it is conceivable that substitutions at other positions could produce similar results. In the same regard, residues Tyr263 and Tyr176 have been described as being important for the maintenance of efficient photoisomerization; however, these residues are also conserved in Ppr (*16*, *22*). The 700 nm peak of the *Ab. tumefaciens* Bph mutants is not completely bleached, but there is residual absorbance at ~650 nm, which is consistent with our observations with Ppr (Figure 3A). *Rh. palustris* Bph3 also shows a bleached Bph form; however, the 645 nm peak is more prominent in comparison to Ppr and *Ab. tumefaciens* Bph1 and has been labeled the Pnr or near-red form (*12*). One explanation for the observed bleach with formation of weak 650 nm absorbance could be that the 180° rotation of the D ring of BV to form Pfr is not completed but is arrested at an intermediate stage that is deprotonated and has a decoupled conjugation. A way that might occur is through migration of the C15−C16 double bond to the C14−C15 pair with corresponding free rotation of ring D. At the same time, the charge on the C ring nitrogen may migrate to the D ring nitrogen with a change in the pK_a. Any steric or other hindrance to free 180° rotation would trap the chromophore in an intermediate state, the structure of which would be species-specific. The reason for such a hypothetical bottleneck in D ring rotation in Ppr and Bph3 is currently unknown.

To date, there have not been any studies published on the interaction of PYP with its native reaction partner. Thus, a naturally occurring hybrid protein combining PYP, Bph, and HK domains like Ppr provides such potential reaction partners. Moreover, on the basis of naturally occurring combinations of domains, it appears that Bph is the normal reaction partner for HK, DGC, and MCP in hybrid proteins containing these modules (*10*, *13*, *18*, *23*, *24*). However, the PYP in Ppr is not necessarily the direct reaction partner for Bph but could interact with either the Bph or the HK domains. Jiang et al. (*5*) obtained evidence for interaction between PYP and HK with Ppr reconstituted with *p*-hydroxycinnamic acid; however, they did not study holo-Ppr, and it is not possible to either confirm or eliminate a direct interaction between PYP and HK in the holoprotein. On the other hand, our data indicate an interaction between activated PYP and both ground state and activated Bph as described above. When considering the interaction between the blue light-activated PYP domain and the ground state Bph domain, the slower recovery of PYP and the resulting

spectral shift of Bph suggest either direct interaction of PYP near the Bph chromophore or a through-bond conformational change that alters the environment of the Bph chromophore. We cannot distinguish between the two possibilities, but it is known that PYP undergoes a light-activated conformational change that exposes a hydrophobic region to solvent (*25*, *26*). An intriguing possibility is that this hydrophobic patch in PYP binds at or near the biliverdin binding pocket of Bph, causing the observed red shift. We also have evidence of an interaction between blue light-activated PYP and red light-activated Bph from the kinetics of recovery of the Bph in white light. In this case, the Bph recovery is accelerated in the presence of activated PYP. At this time, it is unclear how it may differ from the ground state interaction.

With typical phytochromes, which are photoactivated switches, the stable Pr form can be converted to the Pfr form by red light. The Pfr form decays back to the Pr form in the dark on a time scale of minutes to hours or can be immediately reversed by far-red light. Because *Rs. centenum* Ppr is bleached upon red light illumination, it cannot be reversed by far-red light. Thus, there should be another mechanism for rapid recovery of the Bph to achieve fast adaptation to changes in the environment. We have presented evidence that PYP may fill that role by accelerating the recovery of Bph. Thus, when holo-Ppr is illuminated by white light or by red followed by blue light, there is a rapid recovery of Bph with a lifetime of ~1 min, which is in addition to what is observed following red light alone. The kinetics for recovery of Bph at 701 nm, following red light activation, are the same for holo-Ppr and for apo-holo-Ppr, which suggests that there is no interaction between activated Bph and ground state PYP. We therefore conclude that PYP functions as a blue light switch to accelerate recovery of the Bph when both are activated by white light.

The environmental significance of a red−blue light switch is apparent from the requirement of red light for photosynthesis. *Rs. centenum* is a motile aquatic photosynthetic bacterium that would move toward red light and avoid blue light or shading. Red light would be quickly attenuated in moving from the surface down the water column, whereas blue light would travel much further. Although Ppr has not been implicated in phototaxis, it is indirectly connected to cyst formation, which is a response to starvation brought about by a lack of nutrients or a decreasing level of red light (*27*, *28*).

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