

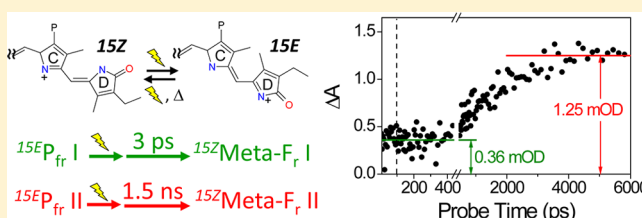
# Heterogeneous Photodynamics of the $P_{fr}$ State in the Cyanobacterial Phytochrome Cph1

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## Supporting Information

**ABSTRACT:** Femtosecond photodynamics of the  $P_{fr}$  form of the red/far-red phytochrome N-terminal PAS-GAF-PHY photosensory core module of the cyanobacterial phytochrome Cph1 (termed Cph1 $\Delta$ ) from *Synechocystis* were resolved with visible broadband transient absorption spectroscopy. Multiphasic generation dynamics via global target analysis revealed parallel evolution of two pathways with distinct excited- and ground-state kinetics. These measurements resolved two subpopulations: a majority subpopulation with fast excited-state decay and slower ground-state dynamics, corresponding to previous descriptions of  $P_{fr}$  dynamics, and a minority subpopulation with slower excited-state decay and faster ground-state primary dynamics. Both excited-state subpopulations generated the isomerized, red-shifted Lumi-F<sub>r</sub> photoproduct (715 nm); subsequent ground-state evolution to a blue-shifted Meta-F<sub>r</sub> population (635 nm) proceeded on 3 ps and 1.5 ns time scales for the two subpopulations. Meta-F<sub>r</sub> was spectrally similar to a recently described photoinactive fluorescent subpopulation of  $P_r$  ( $P_{fr}^{Fluor}$ ). Thus, the reverse  $P_{fr}$  to  $P_r$  photoconversion of Cph1 $\Delta$  involves minor structural deformation of Meta-F<sub>r</sub> to generate the fluorescent, photochemically refractory form of  $P_r$ , with slower subsequent equilibration with the photoactive  $P_r$  subpopulation ( $P_{PhotoP_r}$ ).



Phytochromes are photoswitching proteins found in plants, fungi, and bacteria.<sup>1–3</sup> In plants, phytochromes sense the ratio of red to far-red light to modulate light-induced responses such as seed germination, seedling establishment, flowering, and senescence.<sup>4–6</sup> Phytochromes utilize heme-derived linear tetrapyrrole (bilin) chromophores for light sensing. Photoexcitation initiates a rapid photoisomerization reaction around the C15,16 double bond of the bilin chromophore followed by a series of chromophore–protein relaxation events on the ground-state surface leading to changes in biological signaling activity. Phytochromes from cyanobacteria utilize phycocyanobilin [PCB (Figure 1A)] as a chromophore, while other phytochromes utilize phytychromobilin (PΦB) and biliverdin (BV) chromophores.<sup>3,7</sup>

The N-terminal PAS-GAF-PHY photosensory core module of the full length Cph1 protein (amino acids 1–514, here termed Cph1 $\Delta$ ) from *Synechocystis* sp. PCC6803 has served as an excellent model system for plant phytochromes because of its robust recombinant expression and known crystal structure.<sup>8–10</sup> The full length Cph1 protein consists of Cph1 $\Delta$  coupled to a C-terminal histidine kinase domain, and both proteins exhibit nearly identical photodynamics.<sup>11</sup> Red illumination of the dark-adapted  $^{15Z}P_r$  state of Cph1 $\Delta$  (Figure 1B, red curve) initiates forward photoconversion ( $P_r$  to  $P_{fr}$ ), generating the primary isomerized Lumi-R<sub>f</sub> intermediate.<sup>a 12–17</sup> Lumi-R<sub>f</sub> thermally evolves via several intermediates to generate the  $^{15E}P_{fr}$  photoproduct (Figure 1B, dark red curve) on a >100 ms timescale.<sup>18,19</sup> The dark-stable  $^{15Z}P_r$  state can be regenerated from  $^{15E}P_{fr}$  either

rapidly by far-red light (~700 nm) or via spontaneous dark reversion on a very slow (>24 h) timescale.<sup>7,20</sup>

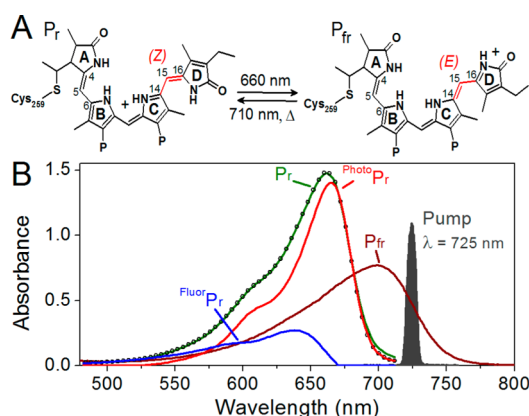
Despite numerous reports that address the primary forward photodynamics of Cph1 $\Delta$ ,<sup>13,15,16,21</sup> including multipulse transient absorption studies,<sup>14,17,22</sup> the reverse primary photodynamics ( $^{15E}P_{fr}$  to  $^{15Z}P_r$ ) are less well characterized. The reverse excited-state photodynamics of Cph1 $\Delta$  are appreciably faster than those of the forward photoreaction,<sup>12,23</sup> as has been reported for other phytochromes.<sup>24–27</sup> Diller and co-workers resolved the biphasic decay of the  $P_{fr}$  excited state of Cph1 $\Delta$  with lifetimes of 540 fs and 3.2 ps.<sup>12</sup> More recently, we resolved 260 fs and 2.8 ps decay lifetimes using broadband transient absorption spectroscopy.<sup>23</sup> On the basis of the evidence that the  $P_{fr}$  ground state of Cph1 $\Delta$  is homogeneous,<sup>28,29</sup> we attributed these components of  $P_{fr}$  excited state decay to the formation and decay of a photochemically nonproductive vibrationally hot ground state relaxing back to the  $P_{fr}$  ground state.<sup>23</sup> Subsequent intermediates in reverse photoconversion have been characterized via cryo-trapping methods,<sup>30,31</sup> but an equivalent study using time-resolved spectroscopy at physiological temperature has not been reported for Cph1 $\Delta$ .

More recently, the circular dichroism (CD) spectra of phytochrome  $P_{fr}$  states have been interpreted as arising from a heterogeneous ground state.<sup>32</sup> There is growing evidence that

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**Figure 1.** (A) Phycocyanobilin (PCB) chromophore in 15Z and 15E states. (B)  $P_{fr}$  pump–probe experiment with the 725 nm pump pulse spectrum (gray area) and  $P_{fr}$  (brown curve) spectrum compared. The spectrum of  $P_{fr}$  was computed to account for the residual absorbance of  $P_r$  at photoequilibrium, and both  $P_r$  and  $P_{fr}$  spectra represent equimolar concentrations of each state. Also, the decomposition of the  $P_r$  spectrum (green) into fluorescent and photoactive  $P_r$  populations (blue and red, respectively) based on SVD analysis of temperature-dependent  $P_r$  absorbance bands is shown.<sup>21</sup> The empty circles show the simulated  $P_r$  spectrum with fluorescent and photoactive  $P_r$  populations as bases.

observed multiphasic excited-state dynamics in phytochromes and related PCB-incorporating GAF domains cyanobacteriochrome (CBCR) photosensors arises due to ground-state heterogeneity.<sup>21,33,34</sup> In recent temperature- and excitation wavelength-dependent studies of forward photoconversion of Cph1 $\Delta$ ,<sup>21</sup> we resolved five  $P_r^*$  excited-state decay pathways arising from photoactive ( $^{Photo}P_r$ ) and blue-shifted fluorescent ( $^{Fluor}P_r$ ) subpopulations (Figure 1B, red and blue curves, respectively).  $^{Photo}P_r$  productively formed the primary Lumi- $R_F$  photoproduct on a 20 ps timescale, whereas  $^{Fluor}P_r$  did not yield Lumi- $R_F$  and exhibited longer excited-state lifetimes. The spectral properties, low quantum yield, and long-lived excited states of  $^{Fluor}P_r$  were all remarkably similar to the properties of Y176H variant Cph1 $\Delta$ .<sup>35,36</sup> The ground-state bleach bands observed in transient absorption studies of the CBCRs NpR6012g4 from *Nostoc punctiforme* and RcaE from *Fremyella diplosiphon* exhibited pronounced excitation wavelength dependence.<sup>34,37</sup> Ground-state heterogeneity may thus be more widespread in the phytochrome and CBCR family of photosensors than previously appreciated.<sup>38,39</sup>

We here extend our recent study of the ultrafast dynamics of the reverse reaction of Cph1 $\Delta$ <sup>23</sup> by using a narrowband excitation pump system (Figure 1B and Figure S1 of the Supporting Information) with an improved signal-to-noise ratio and greater temporal range (7 ns vs 100 ps). The new narrowband excitation data resolve clear multiphasic decay of the  $P_{fr}$  excited-state population, demonstrating heterogeneity of the  $P_{fr}$  ground state. Measurement up to 6 ns resolves multiphasic secondary photoproduct formation, not previously observed. We interpret the Cph1 $\Delta$  reverse reaction as arising from the parallel evolution of two productive, kinetically distinct  $P_{fr}$  subpopulations arising via ground-state heterogeneity. Our studies also suggest that the  $P_{fr}$  photoreaction initially produces the fluorescent  $^{Fluor}P_r$  subpopulation, which then slowly equilibrates with the photoactive  $^{Photo}P_r$  population. This study thus illustrates the utility of transient absorption techniques to elucidate the underlying ground-state heterogeneity in photoreceptors.

## EXPERIMENTAL PROCEDURES

**Protein Purification.** Cph1 $\Delta$  protein was purified after recombinant expression in *Escherichia coli* cells engineered to produce phycocyanobilin (PCB) as described previously.<sup>10,40</sup>

**Ultrafast Experimental Setup.** The ultrafast laser source consisted of an amplified Ti:sapphire laser system (Spectra Physics Spitfire Pro) that delivered 800 nm pulses with a 2.3 mJ pulse energy at a 1 kHz repetition rate and a 40 fs full width at half-maximum (fwhm) pulse duration.<sup>41</sup> The laser output was split into two separate pathways for generating pump and probe pulses. Broadband white light probe pulses were generated by focusing the 800 nm pulses into a slowly translating 2 mm CaF<sub>2</sub> crystal. The resulting probe light was then focused onto the sample and dispersed by a commercial spectrograph (Oriel MS125) to be detected with a linear 256-pixel photodiode array (Hamamatsu S3901 and C7884).

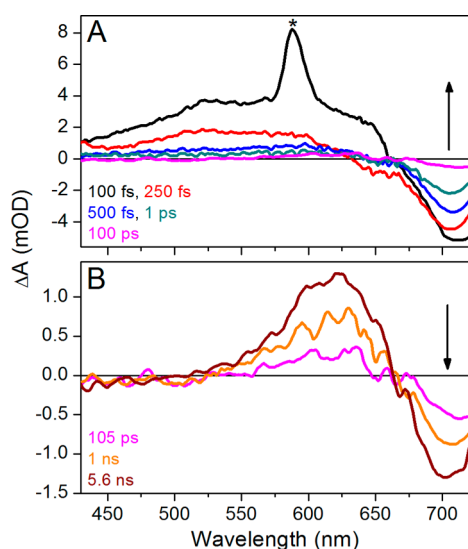
Excitation pulses (Figure 1B, gray shape) were generated by a two-stage home-built noncollinear optical parametric amplifier (NOPA) that produced tunable visible excitation pulses.<sup>42</sup> The first NOPA stage generated a seed beam that was spectrally filtered ( $\lambda_{center} = 725$  nm; 10 nm bandwidth) and amplified by a second NOPA. This amplified light was filtered again by a second interference filter of identical specifications. An instrument response function (IRF) of 120 fs was estimated by the rise time of the excited-state absorption (ESA) band of the pump–probe signals of the IR-140 laser dye. The energy of the pump pulses was 900 nJ/pulse at the sample.

The pump beam was chopped at 500 Hz to generate difference spectra with respect to the nonpumped probe spectrum. The probe beam was optically delayed with respect to the pump pulse with a computer-controlled linear motor stage (Newport IMS600LM), which allowed up to 6 ns temporal separation. Pump pulses were linearly polarized and set to 54.7° (magic angle) with respect to probe pulse polarization. Pump pulse spot diameters of 250–360  $\mu$ m were estimated using a micrometer stage and a razor blade; the broadband probe pulses were focused to  $\sim$ 50  $\mu$ m. The appreciably greater pump pulse volume minimizes artifactual contributions to the signals due to varying spatial overlap between pump and probe beams. This minimization was confirmed by monitoring the signal amplitude and spectral shape while dithering the pump beam with respect to the probe beam.

The sample was passed continuously in a closed circuit to ensure fresh sample for each excitation pulse. The sample was continuously illuminated with a red light-emitting diode (Epitex Inc., L650-66-60;  $\lambda_{center} = 648$  nm) through a quartz window to shift the  $P_r \rightleftharpoons P_{fr}$  equilibrium to favor the  $P_{fr}$  state in the sample cell. Because the 725 nm excitation light is not resonant with the  $P_r$  spectrum (Figure 1), no interfering  $P_r$  signals were observed in the data. The path length of the quartz cuvette was 2 mm, and the optical density at the red absorbance band was 0.4–0.5 at that path length. All experiments were performed at room temperature.

## RESULTS

**Biphasic Photodynamics of  $P_{fr}$ .**  $P_{fr}$  to  $P_r$  primary transient absorption (TA) spectra at selected times are contrasted in Figure 2. These spectra can be decomposed into four overlapping contributions: (1) negative ground-state bleach originating from the loss of ground-state population due to the excitation pulse, (2) negative stimulated emission (SE) signals arising from the probe pulse, (3) positive excited-state absorption (ESA) signals

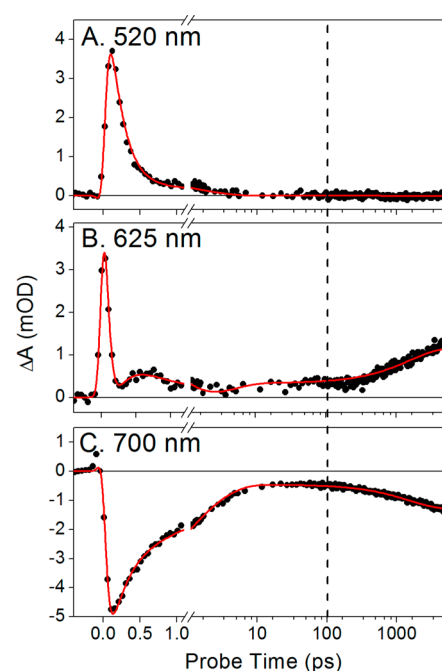


**Figure 2.** Transient absorption spectra of the  $P_{fr}$  to  $P_r$  photoreaction in the  $<100$  ps time range (A) and  $>100$  ps to 6 ns time range (B). The asterisk in panel A refers to the water Raman peak. The 100 ps transient spectrum (magenta) is shown in both panels.

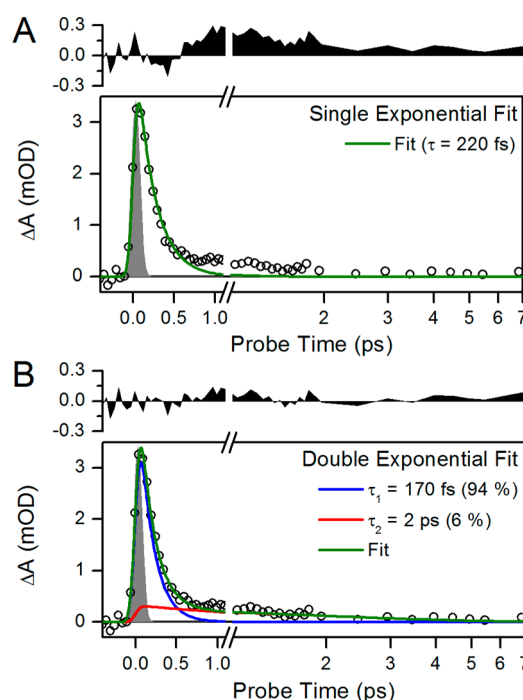
arising from  $S_1 \rightarrow S_n$  transitions, and (4) positive photoproduct absorption that arises from the  $S_0 \rightarrow S_1$  transition of photoproduct or ground-state intermediates (GSI). The ground-state bleach contribution is spectrally identical to the inverted ground-state absorption in a homogeneous population. However, in a situation with multiple ground-state subpopulations, the bleach is the sum of the inverted ground-state absorptions from the fraction of each subpopulation actually excited by the pump pulse. While both SE and ESA bands are markers of excited-state  $P_{fr}^*$  populations, interpretation of the ESA is a simpler way of characterizing excited-state kinetics due to less complicated overlap with photoproduct, bleach, and SE bands.

At times of  $<100$  ps, transient spectra (Figure 2A) are nearly identical to the previously reported broadband pump data<sup>23</sup> (Figures S2 and S3 of the Supporting Information), with a slight difference in bleach amplitude attributed to differences in excitation overlap.<sup>34,37</sup> The 100 fs transient spectrum (black curve) exhibits a broad ESA band from 430 to 675 nm, a negative band arising from bleach and/or SE beyond 675 nm, and a sharp water Raman peak at 580 nm. This spectrum rapidly decays within 100 fs (Figure 2A), reflecting the evolution of populations outside the Franck–Condon region.<sup>23</sup> The 250 fs spectrum (Figure 2A, red curve) exhibits a broad positive band from 430 to 640 nm and a negative bleach and/or SE at  $>640$  nm. The 520 nm signal (Figure 3A) exclusively tracks ESA and hence reports the decay of the excited-state population with biphasic relaxation kinetics with a subpicosecond component and a slower  $\sim 2$  ps component. The 520 nm kinetics are fit to both single- and double-exponential decay functions convolved over a 120 fs IRF in Figure 4. The single-exponential fit extracts a 220 fs lifetime for the best fit of the data, but the residual exhibits a systematic deviation from the data. Such a deviation is not seen with a biexponential fit, consisting of two lifetimes at 170 fs (94%) and 2 ps (6%). This may indicate either a complex bifurcating excited-state potential energy surface or the two  $P_{fr}^*$  populations coexisting upon photoexcitation with a 94%/6% occupation.

Other regions of the spectrum do not exhibit time scales comparable to that seen for the excited state at 520 nm (Figure



**Figure 3.** Kinetics traces of the  $P_{fr}$  to  $P_r$  reaction at selected probe wavelengths as indicated. The vertical dashed line marks the 100 ps probe time, which is the maximal probe time from the previous experiment.<sup>23</sup> The traces are fit (red) with the target model in Figure 8A.



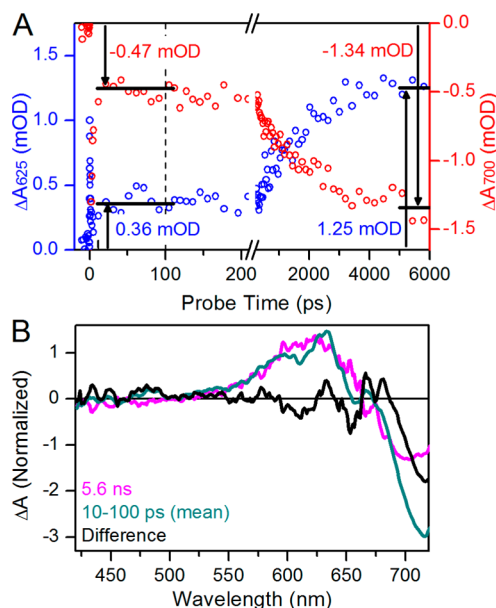
**Figure 4.** (A and B) Single- and double-exponential fitting of the 520 nm ESA kinetic traces, respectively. The fit is convolved with a 120 fs IRF (gray area). The residual of each fit is plotted above with the same y-axes for comparison of the single- and double-exponential fits.

3B,C). At 625 nm, the amplitudes of the positive absorption signals begin to increase at approximately 200 ps (Figure 3B), clearly indicating the appearance of a species with absorption at shorter wavelengths on this time scale. The amplitude of the ground-state bleach band peaking at 700 nm steadily decreases until approximately 50 ps (Figure 3C). At later times ( $>200$  ps),



the amplitude of the bleach apparently increases even in the absence of repeated photoexcitation. An isosbestic point is observed at  $\sim 660$  nm (Figure 2B). The 625 nm (Figure 3B) and 700 nm (Figure 3C) kinetic traces thus reveal secondary dynamics at  $>100$  ps that were not resolved in the small time windows of previous studies.<sup>12,23</sup>

As a first step in interpreting the apparent increasing ground-state depletion at later times, we compared the evolution at 700 and 625 nm in more detail (Figure 5). After  $\sim 10$  ps, both traces



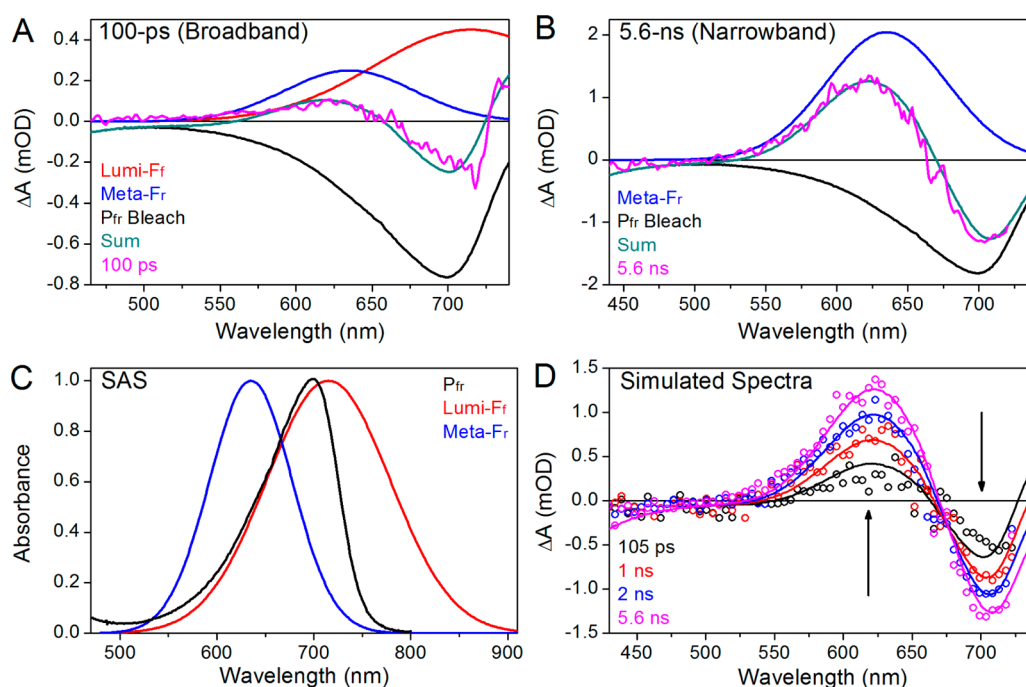
**Figure 5.** (A) Comparison of amplitudes between earlier and later probe time ranges at 625 and 700 nm probe wavelengths (blue and red and left and right y-axes, respectively). Horizontal bars indicate plateaus; the earlier time was averaged from 10 to 100 ps, and the later time was averaged from 4 to 6 ns. (B) Comparison between 5.6 ns and 10–100 ps spectra (magenta and dark cyan, respectively). The 10–100 ps spectra are averaged and scaled 5-fold for review. The difference between them is obtained (black).

plateau to preterminal values that remain unchanged to 200 ps (Figure 5A). The preterminal signal amplitudes are 0.36 and  $-0.47$  mOD at 625 and 700 nm, respectively, after which the signals increase their respective amplitudes and mirror each other, again reaching stable terminal plateau values with amplitudes of 1.25 and  $-1.34$  mOD, respectively. The ratios of the late to early signal amplitudes are 3.47 and 2.85 at 625 and 700 nm, respectively. Were these wavelengths reporting evolution of a homogeneous population between two species, these ratios would be the same. The observed discrepancy in these ratios is likely due to the presence of multiple spectral species contributing to the negative band around 700 nm. We therefore compared earlier and later probe time spectra (Figure 5B). Comparison of the experimental 5.6 ns spectrum to the mean spectrum of the plateau region (10–100 ps) shows good agreement in the positive absorption band peaking at 625 nm (Figure 5B, magenta and teal curves, respectively). However, the bleach region peaking at 710 nm differs in amplitude, also shown by the difference spectrum between the two curves (Figure 5B, black curve), which has a negative band peaking around 720 nm. This difference spectrum is attributed to that of the primary photoproduct Lumi-F<sub>f</sub> (see below).<sup>a</sup>

The increasing magnitude of the ground-state bleach band at later times (Figure 5A, red circles) is not physically possible in a photoinitiated system under continuous flow, as the increased amplitude of the bleach signal indicates an increasing photo-reaction quantum yield at later times, as if the initially photoexcited but nonproductive population becomes photo-excited again. Thus, this phenomenon suggests the presence of an intermediate population with positive absorption spectrally overlapping the  $P_{fr}$  ground-state bleach. The bleach band is a primary contributor to the negative signal in sub-10 ps dynamics (Figure 3C), and the preterminal amplitude at 700 nm is a permanent bleach that cannot increase its amplitude. Thus, as the spectrally overlapping population decays, the permanent bleach is restored to give the mistaken impression of a growing bleach. The rise of the 625 nm kinetics and an observed isosbestic point at 660 nm (Figure 2B) thus are consistent with evolution of Lumi-F<sub>f</sub> into a blue-shifted secondary photoproduct on a subnanosecond time scale.

**Spectral Simulation of Primary and Secondary Photoproducts.** The spectral similarity between the plateau region spectrum (10–100 ps) and the terminal spectrum (5.6 ns) on the 625 nm absorption band (Figure 5B) and the presence of two photoproduct generation phases ( $<10$  and  $>100$  ps) suggest parallel reactions (i.e., two Lumi-F<sub>f</sub>  $\rightarrow$  the blue-shifted intermediate), and reflects a heterogeneous  $P_{fr}^*$  excited state with different time scales. It appears that the plateau region spectrum contains both Lumi-F<sub>f</sub> and the blue-shifted intermediate, whereas the terminal spectrum contains just the blue-shifted intermediate (Figure 5B). To test this interpretation further and facilitate global analysis of the data (see below), we conducted a spectral decomposition of the data (Figure 6). Spectra of the two photoproducts were estimated from the decomposition of both narrowband and broadband excitation spectra. The preterminal plateau spectrum is simulated from the 100 ps transient spectrum of the broadband excitation data, which exhibits positive absorption in both far-red and red regions (Figure 6A, magenta curve).<sup>23</sup> This spectrum was previously assigned to the “Lumi-F” (distinct from Lumi-F<sub>f</sub>) photoproduct under the assumption that Lumi-F has a broadened spectrum with absorption to both the blue and the red of  $P_{fr}$ .<sup>23</sup> A similar absorption band in the far-red spectral region was also resolved in plant phytochrome reverse primary dynamics.<sup>24,25</sup> However, absorption at 625 nm after 5.6 ns is similar to this band (Figure 5B and Figure S5F of the Supporting Information), and the red-shifted component has decayed by this point as shown by recovery of the bleach (Figure S4 of the Supporting Information). Furthermore, the rise of the blue-shifted component occurs with distinct kinetics, after decay of the excited state (Figure 3B). These data all suggest that the 100 ps spectrum (or Lumi-F) consists of two photoproducts with a shared  $P_{fr}$  bleach.

We assumed that the  $P_{fr}$  bleach is an inverted  $P_{fr}$  spectrum (Figure 1B, dark red curve), implying  $P_{fr}$  subpopulations exhibiting nearly identical absorption spectra but different kinetics. The spectrum of the blue-shifted intermediate was estimated from the inverted  $P_{fr}$  spectrum and the 5.6 ns spectrum recorded after narrowband excitation (Figure 6B) because the red-shifted intermediate Lumi-F<sub>f</sub> has largely decayed by 5.6 ns (Figure 5B) as shown by depletion of the negative band peaking at 720 nm. Combination of a simple Gaussian with a center wavelength of 635 nm and a 100 nm bandwidth (fwhm) and the fixed  $P_{fr}$  bleach (Figure 6C, blue and black curves, respectively) simulates the 5.6 ns transient spectrum well (Figure 6B, cyan and

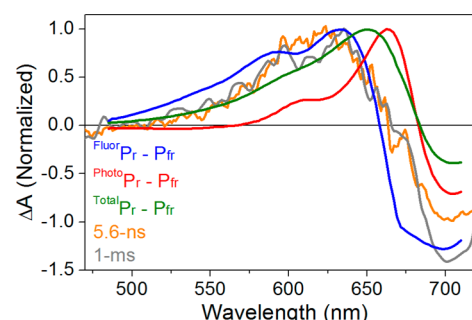


**Figure 6.** Model simulations of  $>100$  ps transient spectra. (A and B) Spectral decomposition of 100 ps and 5.6 ns transient absorption difference spectra by Lumi-F<sub>r</sub>, Meta-F<sub>r</sub>, and P<sub>fr</sub> bleach spectra. Both Lumi-F<sub>r</sub> and Meta-F<sub>r</sub> are estimated as Gaussians with a  $\lambda_{\text{center}}$  values of 715 and 635 nm and  $\Delta\lambda$  values of 150 and 100 nm, respectively. The P<sub>fr</sub> bleach is the inverted P<sub>fr</sub> spectra from Figure 1B. (C) Species-associated spectra (SAS) of P<sub>fr</sub> (black), Lumi-F<sub>r</sub> (red), and Meta-F<sub>r</sub> (blue). (D) Simulation of  $>100$  ps difference spectra with three basis sets in panel C. The P<sub>fr</sub> bleach is fixed.

magenta). Following the nomenclature protocols recently proposed for CBCRs (and also presented here),<sup>37</sup> we designate the red-absorbing species at 635 nm as Meta-F<sub>r</sub>, a secondary intermediate arising from far-red illumination and absorbing red light.<sup>a</sup> The combination of Meta-F<sub>r</sub> and P<sub>fr</sub> bleach spectra allowed extraction of the red-shifted primary photoproduct Lumi-F<sub>r</sub> from the broadband excitation data; for simulation, Lumi-F<sub>r</sub> was deduced by a simple Gaussian spectral shape (Figure 6A) with a center wavelength of 715 nm and a 150 nm bandwidth (fwhm).

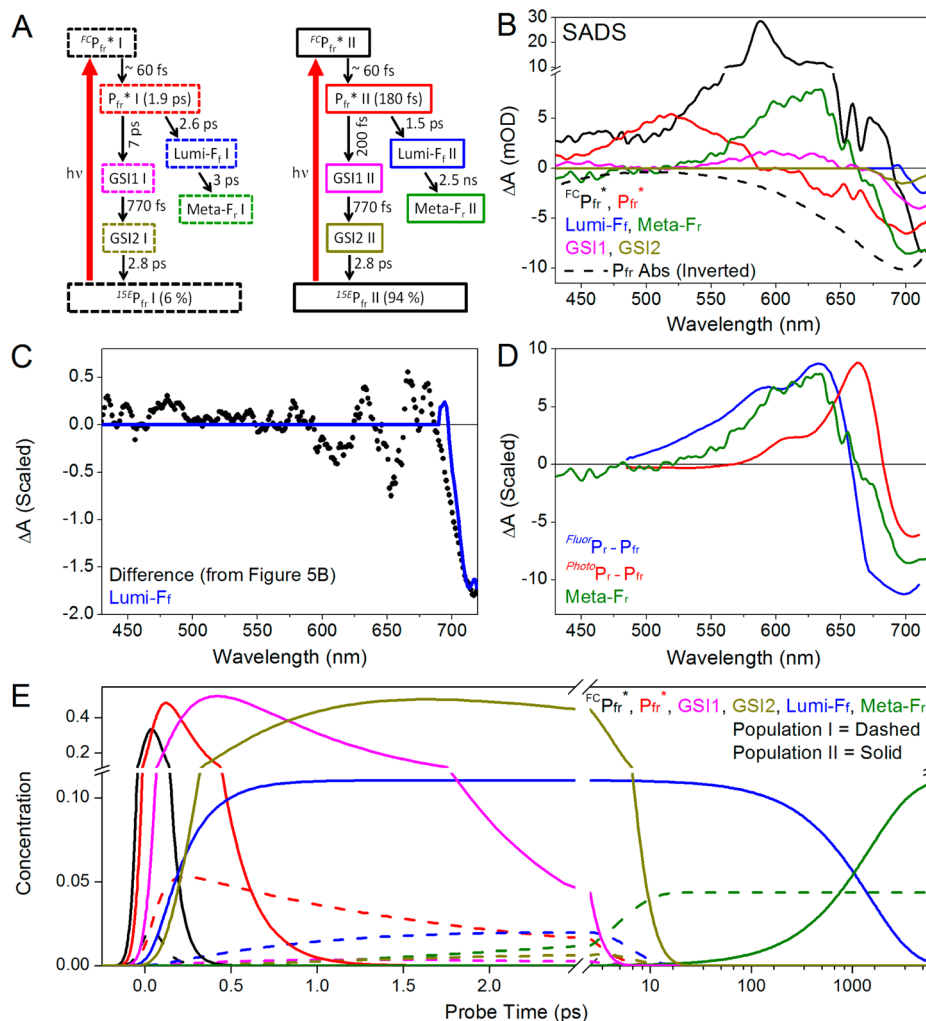
The Gaussians used to simulate the Lumi-F<sub>r</sub> and Meta-F<sub>r</sub> intermediates are contrasted with the P<sub>fr</sub> ground-state absorption spectra in Figure 6C. Combining these two Gaussians allowed us to simulate difference spectra at varying probe times for comparison to experiment (Figure 6D). A slight deviation from experiment was observed at approximately 100 ps, but excellent agreement was obtained at later probe times. The simulated spectra also predicted the experimentally observed 660 nm isosbestic wavelength observed for the underlying two-population kinetics (Figures 2 and 6D). The difference at 100 ps is likely to indicate that Lumi-F<sub>r</sub> does not have a Gaussian line shape and hence could be more similar to P<sub>fr</sub> or P<sub>r</sub> with a higher-energy vibronic tail (Figure 1B).

**Evolution between 100 ps and 1 ms.** Figure 7 contrasts 5.6 ns and 1 ms transient spectra (orange and gray curves, respectively). The previous broadband excitation (Figure S1 of the Supporting Information) data,<sup>23</sup> collected with a different probe window (450–740 nm), exhibit a positive photoproduct absorption band peaking around 730 nm at 100 ps that has decayed by the 1 ms spectrum (Figure S4B of the Supporting Information, arrow). This region of the spectrum is not detected in this study but is satisfactorily modeled in the spectral decomposition (see above) and global analysis (see below).



**Figure 7.** Comparison between the 6 ns and 1 ms difference spectra (orange and gray curves, respectively) in P<sub>r</sub> to P<sub>fr</sub> dynamics and the P<sub>r</sub> – P<sub>fr</sub> difference spectra. Three kinds of P<sub>r</sub> – P<sub>fr</sub> difference spectra are constructed on the basis of fluorescent and photoactive P<sub>r</sub> (blue and red, respectively) and complete P<sub>r</sub> spectra (green) represented in Figure 1B. The  $\text{FluorP}_r - \text{P}_{fr}$  spectrum is scaled to match the 5.6 ns spectrum. The  $\text{PhotoP}_r - \text{P}_{fr}$  and  $\text{TotalP}_r - \text{P}_{fr}$  difference spectra used the same P<sub>fr</sub> amplitude, and  $\text{PhotoP}_r$  and  $\text{TotalP}_r$  have the same respective ratio to the  $\text{FluorP}_r$  amplitude in Figure 1B. The 1 ms spectrum is scaled to the 5.6 ns spectrum.

Discrepancies among the P<sub>r</sub> – P<sub>fr</sub> difference spectrum, the transient difference spectrum after 100 ps, and the transient difference spectrum after 1 ms were previously interpreted as evidence that evolution occurs after 100 ps for the Meta-F intermediate to generate P<sub>r</sub>.<sup>23</sup> This interpretation rests on the assumption that the P<sub>r</sub> subpopulations are spectrally similar, such that photoconversion of an ensemble of P<sub>r</sub> subpopulations in the static difference spectrum will provide an accurate comparison for transient difference spectra. However, we have recently resolved spectrally distinct  $\text{FluorP}_r$  and  $\text{PhotoP}_r$  (Figure 1B) subpopulations of P<sub>r</sub> in Cph1Δ.<sup>21</sup> We therefore compared 5.6 ns and 1 ms spectra with narrowband excitation to three P<sub>r</sub> – P<sub>fr</sub> difference spectra (Figure 7):  $\text{FluorP}_r - \text{P}_{fr}$  (blue curve;  $\lambda_{\text{peak}} = 633$



**Figure 8.** Global analysis of  $P_{fr}$  to  $P_r$  dynamics. (A) Proposed target model with two  $P_{fr}$  subpopulations ( $P_{fr}$  I and  $P_{fr}$  II). Each spectral species is represented in a box, and its apparent time constant is given in parentheses. (B) Estimated SADS of the target model.  $^{FC}P_{fr}^*$ ,  $P_{fr}^*$ , GSI1, GSI2, Lumi-Fr, and Meta-Fr from populations I and II have identical respective SADS. (C) Comparison between Lumi-Fr SADS and the difference spectra in Figure 5B. (D) Comparison between Meta-Fr SADS (green) and  $^{Fluor}P_{fr} - P_{fr}$  and  $^{Photo}P_{fr} - P_{fr}$  difference spectra (blue and red, respectively). (E) Concentration profile of each constituent population. The color scheme is the same as that in panel A, with population I represented by the dashed curves.

nm),  $^{Photo}P_{fr} - P_{fr}$  (red curve;  $\lambda_{peak} = 663$  nm), and  $^{Total}P_{fr} - P_{fr}$  (green curves;  $\lambda_{peak} = 650$  nm). Neither the  $^{Total}P_{fr} - P_{fr}$  spectrum nor the  $^{Photo}P_{fr} - P_{fr}$  difference spectrum is in good agreement with the observed transient spectra. The  $^{Fluor}P_{fr} - P_{fr}$  difference spectrum is in better agreement with both transient spectra, suggesting that the secondary photoproduct Meta-Fr formed upon photoexcitation of  $P_{fr}$  either is very similar to the  $^{Fluor}P_{fr}$  subpopulation or is the same species within the resolution of our data.

#### Global Analysis of Cph1Δ Reverse Photoconversion.

We analyzed the narrowband transient signals using a global analysis formalism to decompose the transient signals into the constituent evolution of a finite number of species.<sup>43,44</sup> Within this framework, transient difference spectra are described by eq 1:

$$\Delta A(\lambda, t) = \sum_{l=1}^n c_l(t) \times \Delta \varepsilon_l(\lambda) \quad (1)$$

where the change in absorption  $\Delta A$  at probe time  $t$  is the summation of  $n$  species with distinct time-dependent concentrations,  $c_l(t)$ , and fixed difference spectra,  $\Delta \varepsilon_l(\lambda)$ . The time-

dependent concentration of each species is given by solution of the first-order linear differential equation shown in eq 2:

$$dc(t)/dt = Kc(t) + I_{pump}(t)[x_1 x_2 \dots x_n]^T \quad (2)$$

where  $c(t)$  is the  $1 \times n$  vector describing the time-dependent concentration of the respective  $l^{th}$  species,  $K$  is the matrix describing the connectivity scheme among species dictated by the chosen target model,  $I_{pump}(t)$  is the IRF of the pump pulse, and  $x_l$  is the initial fractional occupation of  $l^{th}$  species by the excitation pulse. This analysis extracts the number of species, their connectivity scheme, and the extracted spectra. If the model successfully describes the underlying sample dynamics, then the extracted spectra,  $\Delta \varepsilon_l(\lambda)$ , represent the true difference spectra of the constituent transient populations and are called species-associated difference spectra (SADS). If the analysis unsuccessfully models the data, then the estimated spectra are linear combinations of the SADS.

In constructing a target model, we first used a simpler scheme that describes the transient signals as a sequential flow of spectral species with single-exponential kinetics (i.e., species  $1 \rightarrow$  species  $2 \rightarrow \dots \rightarrow$  species  $n$ ). The extracted difference spectra from this



analysis are termed sequential evolution-associated difference spectra (EADS).<sup>45</sup> This procedure estimates experimentally observed time scales, the number of species ( $n$ ), and the general spectral evolution of the signals. Sequential analysis also provides valuable model-independent constraints for target model construction.<sup>34,45</sup>

Both narrowband and broadband excitation data sets were initially subjected to a sequential analysis (Figure S5 of the Supporting Information), which give an excellent fit to the respective signals (Figures S6 and S7 of the Supporting Information, respectively). The broadband excitation signals have one less EADS ( $n = 5$  vs  $n = 6$ ) because these data were only collected to 100 ps and do not resolve the second phase of Meta- $F_r$  growth observed in the narrowband excitation data. The estimated EADS lifetimes are consistent between the two data sets at 60 fs, 180 fs, 770 fs, 2.8 ps, and 1.5 ns for the first five EADS. EADS5 for the broadband excitation data is constrained to a 1.5 ns lifetime to be consistent with the lifetime extracted from narrowband excitation signals, while the narrowband EADS6 are stable to the end of the experiment ( $\tau = \infty$ ). The EADS5 to EADS6 transition in the narrowband excitation signals corresponds to the experimentally observed dynamics observed after 100 ps (Figure 5A).

Side-by-side comparison of the sequential EADS for broadband and narrowband excitation data shows good agreement (Figure S8 of the Supporting Information), with a greater bleach magnitude observed for broadband excitation data. This difference is tentatively attributed to excitation wavelength-dependent dynamics, which have been reported for the Cph1 $\Delta$   $P_r$  state<sup>21</sup> and in CBCR systems.<sup>34,37</sup> EADS6 estimated from the narrowband excitation signals is nearly identical to the 1 ms spectrum, demonstrating negligible spectral evolution from 6 ns to 1 ms (Figure S5E of the Supporting Information). EADS6 of the narrowband excitation signals is also comparable to EADS5 of the broadband signals, with good spectral overlap at the 625 nm positive band (Figure S5F of the Supporting Information).

The biphasic decay of ESA, the sequential analysis described above, and the simulation of  $P_{fr}$  CD spectra<sup>32</sup> argue for the adoption of a parallel evolving target model for interpreting the narrowband transient data (Figure 8A). By analogy to other phytochrome and CBCR systems,<sup>21,22,33,34,45</sup> we modeled this as arising because of an inhomogeneous ground state rather than bifurcation on a homogeneous population on an excited-state surface. In the absence of evidence to the contrary, we assumed that the two subpopulations possess identical ground-state and excited-state spectra. Kinetic analysis of the ESA band at 520 nm (Figure 4) extracted fractional occupancies for the 1.90 ps and 180 fs components of 6 and 94%, respectively. These subpopulations are termed  $P_{fr}$  I and  $P_{fr}$  II, respectively, using the nomenclature adopted for CBCR systems.<sup>37</sup> The overall quantum yield was set at ~15% [15.4% for the target model (Table 1)], based on the previously reported quantum yield for reverse photoconversion of Cph1 $\Delta$ .<sup>8</sup> The low quantum yield indicates that nonproductive regeneration of the  $P_{fr}$  ground state is the dominant route for de-excitation of  $P_{fr}^*$ . Branching between productive and nonproductive  $P_{fr}^*$  is posited to occur at progression through the conical intersection and not during subsequent evolution of the ground-state photoproducts (discussed below).

The target model includes branching between productive and nonproductive components in each  $P_{fr}^*$  population (Table 1), with only the overall quantum yield constrained. The raw data at 625 nm provide an independent estimate of the branching ratios.

**Table 1. Kinetic Parameters and Branching Ratios of the  $P_{fr}^*$  State Based on the Target Model from Figure 8A<sup>a</sup>**

	initial occupancy (%)	apparent $\tau$	% ( $P_{fr}^*$ to GSI)	% ( $P_{fr}^*$ to Lumi- $F_r$ )	$\Phi$ (Lumi- $F_r$ ) (%)
$P_{fr}^*$ I	6	180 fs	27	73	4.4
$P_{fr}^*$ II	94	1.9 ps	88	12	11

<sup>a</sup>The apparent  $\tau$  is the observed lifetime of the spectral species. % ( $P_{fr}^*$  to GSI) is the branching ratio of the respective  $P_{fr}^*$  population to GSI, and % ( $P_{fr}^*$  to Lumi- $F_r$ ) is the branching ratio from  $P_{fr}^*$  to Lumi- $F_r$ . The addition of both parameters makes up 100% for respective subpopulations.  $\Phi$  (Lumi- $F_r$ ) is the final quantum yield of Lumi- $F_r$  in reference to the total ( $P_{fr}$  I +  $P_{fr}$  II) excited  $P_{fr}$  molecules.

Two phases of Meta- $F_r$  photoproduct formation are clear in the 625 nm kinetics: a fast formation phase completed by approximately 10 ps and a slower phase proceeding on a 1.5 ns timescale (Figure 5A). The difference in time scales results in steady plateaus at 625 nm, allowing the ratio of absorption intensities for the early and later plateau regions to be 0.36:1.25. Assuming that Meta- $F_r$  does not decay on the experimental timescale, the ratio of Meta- $F_r$  yield between the earlier and later formation would thus be 0.36:0.89, which translates to absolute  $\Phi$  values of 4.3 and 10.7%, respectively, if total yield is 15%.<sup>8</sup> Because the initial excited-state occupancy of  $P_{fr}$  I is only 6%, it cannot account for the slowly arising component of the Meta- $F_r$  yield, which requires at least 10.7% occupancy at 100% quantum yield. Given the initial occupancies and absolute  $\Phi$ , we can set the branching ratio of the  $P_{fr}$  ground state to photoproduct at ~28:72 and ~89:11 for  $P_{fr}$  I and  $P_{fr}$  II, respectively. The calculated values derived from the target model (Table 1) are 27:73 and 88:11 to be consistent with the experimental estimate.

In the target model, each of the two  $P_{fr}^*$  populations generates one component of Lumi- $F_r$  that then decays into Meta- $F_r$  (Figure 8A). The extracted Lumi- $F_r$  SADS compares well with the difference spectrum calculated from the 5.6 ns transient spectrum and the mean transient spectrum from 10 to 100 ps (Figures 5B and 8C). The decay of Lumi- $F_r$  after 100 ps is thus sufficient to explain the spectral evolution observed in the bleach region (Figure 6). The Meta- $F_r$  SADS is in reasonable agreement with the  $P_{fr}^{fluor} - P_{fr}$  difference spectrum (Figure 8D) and is consistent with the final 5.6 ns and 1 ms spectrum (Figure 7). The concentration of each species is plotted in Figure 8E ( $P_{fr}$  I in dashed lines and  $P_{fr}$  II in solid lines). The final Lumi- $F_r$  concentration associated with  $P_{fr}$  I is 4.4% and with  $P_{fr}$  II is 11% (Table 1), in good agreement with the independent estimate derived from analysis of spectral evolution at 625 nm (see above).

The sequential EADS analysis estimated lifetimes of 60 fs, 180 fs, 770 fs, and 2.8 ps (Figure S7 of the Supporting Information). The 60 fs component is assigned to Franck–Condon relaxation as reported previously.<sup>23</sup> The 180 fs EADS exhibits a clear ESA band peaking around 520 nm, indicating the presence of  $P_{fr}^*$ .  $P_{fr}^*$  decay is biphasic as discussed above, so the 2.8 ps component is likely to be a superposition of  $P_{fr}^*$  decay and subsequent dynamics. The 770 fs component was not resolved in the previous broadband excitation data because of the lower signal-to-noise ratio in this data set (compare Figures S6 and S7 of the Supporting Information); however, a spectrally similar component can be extracted from these data using the current analysis (Figure S5D of the Supporting Information). In the narrowband data, this component is necessary to describe the <1 ps dynamics (Figure 3B). The 770 fs component (EADS3) is spectrally

distinct from the  $P_{fr}^*$  and  $^{Hot}P_{fr}$  spectra resolved in the previous study, which are also resolved here as EADS2 and EADS4 (Figure S5 of the Supporting Information). In the current target model, the 770 fs component is assigned as the first GSI population [GSI1 (Figure 8A)], with spectral features qualitatively similar to those of the Meta- $F_r$  photoproduct (Figure 8B, magenta and green curves, respectively). Because this component appears before the appearance of the primary Lumi- $F_r$  photoproduct, we assign the 770 fs component to a nonisomerized GSI (GSI1) that adopts a structurally twisted, blue-shifted conformation.<sup>46–48</sup> The 2.8 ps component (EADS4) is spectrally and kinetically analogous to the  $^{Hot}P_{fr}$  GSI extracted from the previous broadband data, with red-shifted product absorption (Figure S5C,D of the Supporting Information). We therefore interpret this species as a second GSI species (GSI2) arising from the earlier GSI1 via vibrational relaxation before decaying back to the  $P_{fr}$  ground state.<sup>23,46</sup>

We tested this interpretation by fitting the narrowband data to a target model with just one GSI having a lifetime of 2.8 ps (Figure S9 of the Supporting Information). This model does not accurately describe the <1 ps dynamics (Figure S10B of the Supporting Information). By contrast, including two GSI populations in the final target model (Figure 8A) allows a good fit to the 625 nm kinetic trace (Figure 3B). The decay of nonproductive  $P_{fr}^*$  back to the  $P_{fr}$  ground state is therefore postulated to occur via two resolved ground-state intermediate (GSI) populations.<sup>46–49</sup> The final target model (Figure 8A) gives good agreement with the narrowband data set on all time scales examined (Figure 3).

We next applied the heterogeneous target model to the broadband excitation signals (Figure S11 of the Supporting Information), with excellent results (Figures S11 and S12 of the Supporting Information). Because the broadband excitation signals were collected only up to 100 ps, the 1.5 ns Lumi- $F_r$  II to Meta- $F_r$  II evolution was not modeled. The side-by-side comparison with respective SADS between the narrowband and broadband excitation signals (Figure S11 of the Supporting Information) shows consistencies in  $P_{fr}^*$  and two GSI populations, albeit with variation in the bleach-band amplitude (~700 nm). The initial Franck–Condon relaxation  $^{FC}P_{fr}^*$  SADS are expected to vary between these data sets, because the location of the initially excited wavepacket is strongly affected by the excitation energy. The Lumi- $F_r$  and Meta- $F_r$  SADS show the most notable discrepancy (panels E and F, respectively, of Figure S11 of the Supporting Information). Unfortunately, analysis of the broadband excitation signals could not extract Lumi- $F_r$  as a separate component because of the limited time window, and the Meta- $F_r$  SADS was the mixture of Lumi- $F_r$  and Meta- $F_r$  populations. The concentration of Meta- $F_r$  at 100 ps (Figure 8E) is too low for separation of Lumi- $F_r$  and Meta- $F_r$  by global analysis.

## DISCUSSION

Our previous interpretation of Cph1Δ primary reverse reaction dynamics was based on a homogeneous model that attributed a “broadened” spectrum to the Lumi- $F_r$  primary photoproduct in comparison with the  $P_{fr}$  ground state.<sup>23</sup> That study neither considered nor excluded the possibility that the Lumi- $F_r$ ’s broadened spectrum reflects simultaneous photogeneration of blue- and red-absorbing photoproducts.<sup>23</sup> The main limitation of that study was the short temporal window examined. In this study, we have extended the analysis to longer times and have used a narrower excitation pulse, with a higher signal-to-noise

ratio in the transient spectra. This allowed us to observe spectral evolution from the red-shifted Lumi- $F_r$  primary photoproduct to a blue-shifted Meta- $F_r$  species, a process described well by a target model with a heterogeneous  $P_{fr}$  ground state. Resonance Raman intensity analysis of the Cph1Δ  $P_{fr}$  state<sup>28</sup> supported a homogeneous  $P_{fr}$  ground state, consistent with characterization of  $P_{fr}$  by solid-state NMR<sup>29</sup> but inconsistent with a recent re-evaluation of  $P_{fr}$  CD spectra<sup>32</sup> and with the transient absorption signals we report here. In comparable analyses of the Cph1Δ  $P_r$  state, solid-state NMR<sup>29</sup> and transient absorption<sup>21</sup> both revealed considerable heterogeneity, consistent with an earlier analysis of temperature and excitation wavelength effects<sup>50</sup> but at odds with resonance Raman intensity analysis.<sup>51</sup> For  $P_{fr}$ , the two kinetically distinct phases of Meta- $F_r$  generation reported here strongly support the presence of at least two subpopulations. Other transient absorption studies of phytochromes and CBCRs have also reported multiphasic excited-state relaxation, typically interpreted as arising from coevolving subpopulations.<sup>33,34,45,47,52</sup> This is consistent with characterization of other photoreceptor systems such as PYP and phototropin, which also exhibit multiexponential kinetics attributed to inhomogeneity.<sup>46,53</sup>

An inverse correlation between excited-state lifetime and quantum yield for primary photoproduct formation of red/green CBCR has been proposed,<sup>54</sup> under the assumption that rapidly decaying excited states will be less prone to competing reactions. Two bacteriophytochromes from *Rhodospseudomonas palustris* also demonstrated an inverse correlation between excited-state lifetime and isomerization efficiency.<sup>55</sup> However, Cph1Δ provides a counterexample to this trend, because  $P_{fr}^*$  decays much more rapidly than  $P_r^*$  despite comparable quantum yields.<sup>8,22</sup> Interestingly, the Cph1Δ  $P_{fr}$  excited-state dynamics for the two subpopulations described here demonstrate such a counterexample within a single reaction:  $P_{fr}^*$  I exhibits a slower lifetime but a higher branching ratio to the isomerized photoproduct when compared to  $P_{fr}^*$  II (Table 1). It is not yet clear whether there is a general physical interpretation for such correlations.

In *R. palustris* bacteriophytochromes, excited-state proton transfer was proposed as a nonproductive excited-state quenching mechanism.<sup>55</sup> For the forward reaction of the red/green CBCR NpR6012g4, pump–dump–probe experiments supported a twisted GSI population as the dominant pathway of excited-state decay.<sup>47</sup> Such failed attempts at photoisomerization usually decay back to the original ground state,<sup>37</sup> but the twisted GSI chromophore conformation in NpR6012g4 was shown to partition between the original ground state and the primary photoproduct.<sup>47</sup> Such second-chance mechanisms would enhance quantum yield with no change in excited-state lifetime. In the Cph1Δ  $P_{fr}$  state, which has a low quantum yield, the need for two GSI populations in the target model (Figure 8A) suggests a rugged ground-state potential energy surface and raises the possibility of unproductive depopulation of the excited state to generate GSI species.

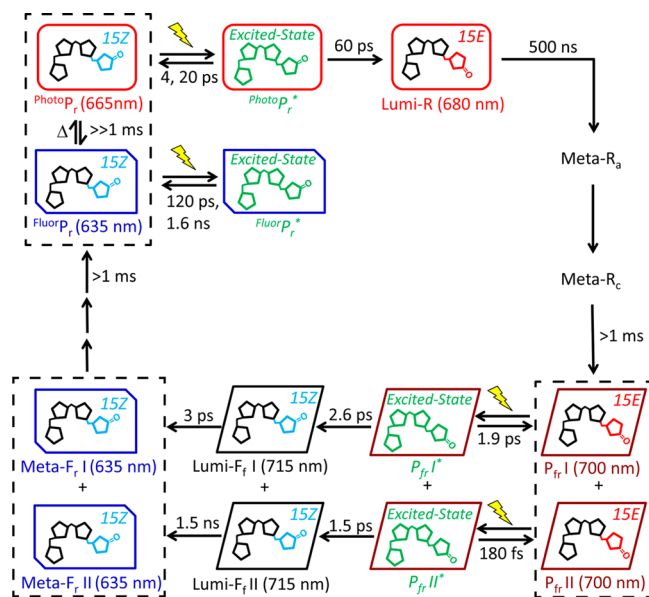
This study demonstrates that the primary photoproduct is a mixture of red-shifted Lumi- $F_r$  species that decay to the secondary intermediate Meta- $F_r$  on vastly different time scales. Lumi- $F_r$  exhibits a red-shifted absorption maximum at ~715 nm (Figure 6C), consistent with the red-shifted primary photoproducts seen in other phytochromes and CBCRs.<sup>12,24,34,45,56</sup> The Lumi- $F_r$  to Meta- $F_r$  transition exhibits a significant blue shift from 715 to 635 nm, suggesting a major structural rearrangement. A combination of cryo-trapping and solid-state NMR



allowed characterization of two intermediates in the Cph1Δ reverse reaction, “Lumi-F” and “Meta-F”.<sup>30</sup> The Lumi-F to Meta-F conversion triggered more significant chemical shift changes than those observed for the Meta-F to  $P_r$  conversion, especially for atoms associated with the D-ring of the chromophore. These observations led to the conclusion that Lumi-F possesses an isomerized 15Z configuration but is structurally more constrained than Meta-F. Two intermediates were also resolved in a temperature-scan crystallographic study of  $P_{fr}$  to  $P_r$  photo-conversion in a bacteriophytochrome from *Pseudomonas aeruginosa*.<sup>57</sup> The first intermediate exhibited D-ring isomerization, whereas the second intermediate exhibited a more twisted chromophore conformation but a more relaxed protein environment. These structural changes are consistent with the blue shift observed upon the analogous transition from Lumi-F<sub>r</sub> to Meta-F<sub>r</sub> in Cph1Δ.

In our previous study of the Cph1Δ reverse reaction photodynamics,<sup>23</sup> the  $P_{fr}$  spectrum was used to calculate the static  $P_r - P_{fr}$  difference spectrum used as a comparison (Figure 7, green curve). Since then we resolved two spectrally distinct subpopulations with the Cph1Δ  $P_r$  state,  $^{Fluor}P_r$  and  $^{Photo}P_r$ .<sup>21</sup> We therefore compared the individual  $^{Fluor}P_r - P_{fr}$  and  $^{Photo}P_r - P_{fr}$  difference spectra both to the transient spectra taken at 5.6 ns and 1 ms (Figure 7) and to the Meta-F<sub>r</sub> SADS (Figure 8D). Remarkably, the  $^{Fluor}P_r - P_{fr}$  difference spectrum (blue curve) is the best match to the experimental transient spectra. This is consistent with the lack of major structural changes of the chromophore during the formation of  $P_r$  from Meta-F in Cph1Δ,<sup>30,57</sup> so the Meta-F<sub>r</sub> species resolved in this study seems to be consistent with the Meta-F intermediate resolved by cryo-trapping. The strong spectral similarity between Meta-F<sub>r</sub> and  $^{Fluor}P_r$  also suggests that reverse photoconversion initially forms  $^{Fluor}P_r$  and the regeneration of the red-shifted, photoactive  $^{Photo}P_r$  subpopulation occurs on a slower timescale via thermal equilibration of the  $^{Fluor}P_r$  and  $^{Photo}P_r$  subpopulations. This observation may also imply the existence of a short refractory period within the Cph1Δ photocycle, during which newly regenerated  $P_r$  cannot be photoconverted to  $P_{fr}$  because it is in the less active  $^{Fluor}P_r$  substate.

The schematic of the photocycle of Cph1Δ is demonstrated in Figure 9, including both ultrafast and secondary dynamics of the  $P_r$  and  $P_{fr}$  states. The dark-adapted  $P_r$  state consists of two coexisting populations in thermal equilibrium:  $^{Fluor}P_r$  and  $^{Photo}P_r$ . Upon red light excitation, only  $^{Photo}P_r$  generates Lumi-R photoproduct, which then proceeds through Meta-R<sub>a</sub> and Meta-R<sub>c</sub> intermediates before forming the  $P_{fr}$  state.<sup>58</sup> We have now demonstrated that  $P_{fr}$  is also heterogeneous, with at least two subpopulations that exhibit parallel primary and secondary dynamics upon far-red light excitation. The red-shifted primary photoproduct Lumi-F<sub>r</sub> undergoes further structural relaxation to generate the blue-shifted Meta-F<sub>r</sub> intermediate with relaxation time scales of 3 ps and 1.5 ns for the two subpopulations. These time constants are much faster than those observed for structural relaxations in forward photoconversion of Cph1Δ and the related cyanobacterial phytochrome CphA.<sup>19,59</sup> In contrast to the productive and nonproductive  $P_r$  subpopulations, both  $P_{fr}$  subpopulations can generate Meta-F<sub>r</sub> populations that strongly resemble the  $^{Fluor}P_r$  subpopulation. In Cph1Δ, further evolution may involve formation of an equilibrium with the photoactive  $^{Photo}P_r$  subpopulation, but whether other phytochromes have similar  $P_r$  subpopulations is not yet clear. It will also be interesting to probe the molecular details underlying the



**Figure 9.** Scheme of the total Cph1Δ photocycle including both forward ( $P_r$  to  $P_{fr}$ ) and reverse ( $P_{fr}$  to  $P_r$ ). The spectrally and kinetically distinct fluorescent ( $^{Fluor}P_r$ ) and photoactive ( $^{Photo}P_r$ )  $P_r$  subpopulations are in slow equilibrium ( $\gg 1$  ms), which make up the overall  $P_r$  state. The light-activated pathways are indicated by photoexcitation (yellow light sign) and subsequent evolution via thermally activated steps (solid arrows). The evolution of  $P_{fr}$  to Lumi-F<sub>r</sub> to Meta-F<sub>r</sub> occurs via heterogeneous pathways with different reaction kinetics and yields. Populations enclosed within black boxes indicate inhomogeneous coexisting populations.

dynamics observed in this study via transient CD and vibrational experiments to elucidate the molecular nature of phytochrome inhomogeneity.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Sequential EADS analysis of both narrowband and broadband excitation signals and extra target model analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

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## ■ ABBREVIATIONS

Cph1Δ, PAS-GAF-PHY photosensory core module of Cph1; BV, biliverdin; CBCR, cyanobacteriochrome; CD, circular dichroism; EADS, evolution-associated difference spectrum; GSI, ground-state intermediate; ESA, excited-state absorption;

SE, stimulated emission; IRF, instrument response function; NOPA, noncollinear optical parametric amplifier; PCB, phycocyanobilin; PΦB, phytychromobilin;  $P_r$ , red-absorbing dark state of red/far-red phytychromes;  $P_{fr}$ , far-red-absorbing photoproduct state of red/far-red phytychromes;  $P_{fr}^{Fluor}$ , non-photoactive, fluorescent  $P_r$  subpopulation;  $P_{fr}^{Photo}$ , photoactive  $P_r$  subpopulation;  $P_{fr}^*$ , excited-state population(s) derived from photoexcitation of  $P_{fr}$ ; PYP, photoactive yellow protein; SADS, species-associated difference spectra; SNR, signal-to-noise ratio;  $\Phi$ , photocycle quantum yield.

## ADDITIONAL NOTE

<sup>a</sup>For the nomenclature of phytyochrome photostates, in Lumi-X<sub>y</sub>, Lumi refers to the primary intermediate formed within 10 ns of photoexcitation, the uppercase X refers to the spectral range of the parent state (R for  $P_r$  and F for  $P_{fr}$ ), and lowercase subscript y refers to the spectral range of the intermediate-state difference absorption peak. For the intermediates after 10 ns time windows, the intermediate is termed Meta-X<sub>y</sub>. The color code for the spectral range is v for violet (380–450 nm), b for blue (450–495 nm), g for green (495–570 nm), y for yellow (570–590 nm), o for orange (590–620 nm), r for red (620–710 nm), and fr for far-red (710–750 nm). More details can be found in the Supporting Information of reference <sup>60</sup>.

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