Phototransformation of the Red Light Sensor Cyanobacterial Phytochrome 2 from Synechocystis Species Depends on Its Tongue Motifs*

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Phytochromes are photoreceptors using a bilin tetrpyrrole as chromophore, which switch in canonical phytochromes between red (Pr) and far red (Pfr) light-absorbing states. Cph2 from Synechocystis sp., a noncanonical phytochrome, harbors besides a cyanobacteriochrome domain a second photosensory module, a Pfr/Pr-interconverting GAF-GAF bidomain (SynCph2(1-2)). As in the canonical phytochromes, a unique motif of the second GAF domain, the tongue region, seals the bilin-binding site in the GAF1 domain from solvent access. Time-resolved spectroscopy of the SynCph2(1-2) module shows four intermediates during Pr → Pfr phototransformation and three intermediates during Pfr → Pr back-conversion. A mutation in the tongue's conserved PRXSF motif, S385A, affects the formation of late intermediate R3 and of a Pr-like state but not the back-conversion to Pr via a lumi-F-like state. In contrast, a mutation in the likewise conserved WXE motif, W389A, changes the photocycle at intermediate R2 and causes an alternative red light-adapted state. Here, back-conversion to Pr proceeds via intermediates differing from SynCph2(1-2). Replacement of this tryptophan that is ~15 Å distant from the chromophore by another aromatic amino acid, W389F, restores native Pr → Pfr phototransformation. These results indicate large scale conformational changes within the tongue region of GAF2 during the final processes of phototransformation. We propose that in early intermediates only the chromophore and its nearest surroundings are altered, whereas late changes during R2 formation depend on the distant WXE motifs of the tongue region. Ser-385 within the PRXSF motif affects only late intermediate R3, when refolding of the tongue and docking to the GAF1 domain are almost completed.

Within the broad range of photoreceptors existing in photosynthetic and nonphotosynthetic bacteria, bilin-binding GAF domain-containing proteins like phytochromes and cyanobacteriochromes (CBCRs) cover the whole light spectrum. Despite different spectral characteristics between the red/far red-light-absorbing phytochromes and CBCRs, which switch between all kinds of colors, they both harbor the same kind of chromophore, a covalently attached linear tetrpyrrole. Classical phytochromes photoconvert between two conformations, the red and far red light-absorbing Pr and Pfr state. Upon red light illumination of Pr, the excited state Pr* is formed, which decays and converts into the primary red-shifted photoproduct lumi-R (also termed I700) within picoseconds (1). This step involves the Z → E isomerization of the C15, C16 double bond of the bilin chromophore. The further steps are light-independent and proceed thermally driven in longer time scales. Accordingly, phytochrome photoconversion starts within picoseconds and can last up to seconds (2, 3). The light-triggered reaction from Pfr → Pr undergoes different intermediates (4, 5).

Full-length oat PhyA shows pairs of intermediates in the Pr → Pfr phototransformation with similar spectroscopic signatures but different lifetimes (2). This is caused by at least two distinct Pr conformations each leading to one set of subsequent intermediates (6). Cph1 from Synechocystis sp., a bacterial phytochrome commonly used as a model for plant phytochromes, exhibits a multistep photoconversion reminiscent of the PhyA Pr → Pfr reaction. However, SynCph1 displays a different kinetics (5, 7) with an intermediate that has no counterpart in any other phytochrome.

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In contrast to canonical phytochromes from plants or bacteria, the domain architecture of the second phytochrome from *Synechocystis* sp., *Cph2*, exhibits some remarkable differences. First of all, *Cph2* is a bimodule photosensor module (PAS (Period/ARNT/Single-minded), GAF (cGMP phosphodiesterase/adenylyl cyclase/FhIA), and PHY (phytochrome)), which defines canonical group I phytochromes (10), is here altered to a GAF-GAF bimodule (group II phytochromes) as the N-terminal photosensory module. This *Cph2*(1-2) module exhibits $P_{fr}/P_{pr}$ photochromicity known from group I phytochromes (9), where the PHY domain has been structurally recognized as a GAF domain as well (11). *Cph2* covalently attaches phycocyanobilin (PCB) via a thioether linkage that is partly solvent-exposed due to the missing PAS domain (8). Similar to plant phytochrome B, unlike *Cph1* and PhyA, the $P_{fr}$ state of this photosensory module undergoes dark reversion to its $P_{pr}$ state (9). Besides the UV-visible spectra, resonance Raman spectra of *Cph2*(1-2), which are highly sensitive to structural changes at the chromophore-binding site, resemble closely the spectral features of *Cph1* in both states, $P_{pr}$ and $P_{fr}$. Given the lack of a PAS domain, one may consider the *Cph2*(1-2) module as a minimal model of $P_{fr}/P_{pr}$ photoconversion in canonical phytochromes. Besides the red/far red light-sensitive GAF-GAF bimodule, *Cph2* harbors a CBCR-like GAF3 domain as its second photosensory module with a blue/green photochemistry. This photochromic switch controls light-regulated cyclic di-GMP levels (12) by tuning the catalytic activity of the GGDEF2 domain. GGDEF and EAL domains (named after their conserved motifs) produce and degrade, respectively, cyclic di-GMP, a eubacterial second messenger (13). A GGDEF1*-EAL module with an inactive GGDEF domain is found in *Cph2* following the N-terminal GAF bimodule sensor module (Fig. 1A).

The crystal structure (8) of the red/far red phytochrome region confirmed that the GAF2 domain is directly related to the PHY domains of canonical phytochromes. Like the latter, it includes a tongue-like extension that covers the bilin-binding site of the GAF1 domain and may hence be involved in intramolecular signal transduction. Despite significant sequence divergence between the core GAF2 and PHY domains, three motifs within the tongue region, W(G/A)G, PRXSF, and WXE, are almost invariant in group I and II phytochromes and were hence suggested to be crucial for phototransformation and stability of the resulting $P_{fr}$ state. The significance of the bulky aromatic character of the tryptophan residues in the W(G/A)G and WXE motifs for $P_{fr}$ formation was derived from site-directed mutagenesis and structural comparison with the tongue conformation of bathyphytochromes in their $P_{fr}$ ground state (8). The resulting model of a “tryptophan switch” during photoconversion implies that the tongue undergoes a red light-triggered conformational change thus altering the conformation and/or orientation of the conserved PRXSF motif and thereby its interactions with the chromophore-binding site. In this context, the tryptophans of the W(G/A)G and WXE motifs are supposed to swap their positions and play an important role in the stabilization of a $P_{fr}$-specific conformation of the tongue region (8). This model was recently corroborated by the findings in *Deinococcus radiodurans* phytochrome that shows the predicted features of the tryptophan switch model by the structures of its $P_{fr}$ and $P_{pr}$ states (14).

Here, we characterize *Cph2*(1-2) and three of its tongue mutants by time-resolved absorption spectroscopy to track slow conformational changes, which are mostly indicative of structural changes within the protein moiety. Whereas the native *Cph2*(1-2) module and its W389F mutant exhibit both a cognate spectral behavior under steady state conditions, the W389A and S385A mutants were found to fail in the formation of a $P_{fr}$-like state upon red light illumination (Fig. 1B). We analyzed the light-induced $P_{fr} \rightarrow P_{pr}$ and $P_{pr} \rightarrow P_{fr}$ photoconversions in the micro- to millisecond time range by laser flash photolysis and time-resolved absorption spectroscopy. The data were used in global fit analysis to obtain lifetime-associated difference spectra (LADS) of the intermediates.

**MATERIALS AND METHODS**

*Cph2*(1-2) and mutants were produced and purified as described (9); the measurements with the PCB-assembled proteins were performed in Tris buffer (50 mM Tris, 300 mM NaCl, 5 mM EDTA, pH 8.0). For flash photolysis, the protein sample was diluted to an absorbance of 0.5 at the P$_{max}$ maximum using a cuvette of a 1-cm path length. Absorption spectra were recorded before and after the measurements (spectrophotometer UV-2401 P, Shimadzu) to exclude denaturation of the protein. The sample was kept in a quartz cuvette with a 1-cm path length was kept at 288 K (15 °C). Photoconversion of the proteins to 100% P$_{fr}$ or the highest P$_{pr}$ occupancy was accomplished by irradiation with a 720 and a 625 nm LED light source (high power LED, Roitner; 720 nm, 350 mA; 1.8 V; 625 nm, 350 mA, 2.2 V), respectively. An optical parametric oscillator, coupled to a Spitlight 300 Ne-YAG laser (Versason HB, GWU-Lasertechnik), was used for excitation (pulse length, 9 ns; energy output 640 nm, 70 μJ; 700 nm, 45 μJ) with $\lambda_{ex} = 640$ nm for the $P_{fr} \rightarrow P_{pr}$ conversion and $\lambda_{ex} = 700$ nm for the reverse reaction. The resulting absorption changes were detected at various wavelengths between 520 and 720 nm. For *Cph2*(1-2), the S385A and W389A mutant, the entire spectral range was recorded in 10-nm steps. The variant W389F was measured with seven wavelengths (590, 640, 660, 665, 690, 700, and 720 nm) that display the highest absorbance changes throughout the intermediate spectra, allowing the prediction of the photocycle according to the previously recorded spectra and lifetimes. Absorbance changes were detected via a continuous wave xenon lamp (Amiko) and two matched monochromators (A 1020) placed before and behind the sample. Absorbance changes were recorded by a photomultiplier mounted to the second monochromator, from which the signals were read into a computer for further data handling and fitting. The detection range covered times between a few microseconds to 100 ms. The resolution is limited to ~0.1 μs at the lower time limit and ~1 μs at the higher time limit. Beyond 80 μs, the photoconversion was complete for *Cph2*(1-2) as well as for its mutants. For each wavelength and single time traces, 10 measurements were averaged to improve the signal to noise ratio. Between individual measurements, the sam-
ple was irradiated with the LED light sources for 8 and 5 s, respectively, to revert photochemically generated products and to achieve maximal Pr or Pfr state occupancy.

For data analysis, the single time traces were baseline-corrected if necessary and assembled (usually three time windows with different scaling were measured separately and later combined into a single wavelength recording trace). Global fit analysis was performed by fitting the curves with a sum of exponential functions (MATLAB) (15–17), yielding lifetimes for the individual transitions between intermediates and the lifetime-associated difference spectra (LADS). Care was taken to minimize laser or scattering light artifacts during the short time windows that might impair detection of early intermediates. Derived lifetimes have a variance of about \( \pm 10% \) and do not influence selectively one or the other’s lifetime. A more detailed study on canonical phytochromes (CphA) considers variations between consecutive measurements, temperature effects, and accumulation problems such as continuous back-irradiation or back-irradiation after one set of 3–5 single laser shots (18).

The acid denaturation assay was performed by addition of 8 M urea/HCl, pH 2, to a preliminarily irradiated protein sample in the dark at room temperature (19); subsequently, absorption spectra were recorded.

RESULTS

We hereby report the first kinetic study of the late intermediates of the \( P_r \rightarrow P_{fr} \) phototransformation and the \( P_{fr} \rightarrow P_r \) back reaction of a Cph2-type phytochrome module. We also address the influence of the highly conserved PRXSF and WXE motifs within the tongue on photoconversion. For that purpose, the S385A, W389A, and W389F as shown in Ref. 8 after far-red (Pfr state, black line) and red light illumination (red line). Difference spectra are calculated with \( A_p - A_{photoequilibrium} \) and shown in blue. Deconvoluted, pure \( P_r \) (or red light-adapted) spectra are presented in green (9). At the photoequilibrium, a portion of 59% \( P_{fr} \)-like state for S385A, 75% \( P_{al} \) for W389A, and 59% \( P_r \) state for W389F are obtained in comparison with SynCph2(1-2) (60% (9)).

C. UV-visible absorbance spectra of SynCph2(1-2) as well as S385A, W389A, and W389F after denaturation with acidic urea (denatured \( P_r \), state, black line; denatured red light-adapted state, red line; difference spectrum, blue line).
Syn
in
Denatured Pr states show absorbance maxima at 663 nm like other PCB-cysteine adducts with 15
maximum at 15
The denatured red light-adapted states show a mixture of the flash triggered the photoconversion.
absorption as the Pfr conformation (approximate appearance of intermediates is indicated by arrows). The intermediate lifetimes are also presented. The final intermediate R3 exhibits similar absorption as the Pr, conformation (D). The initial 10% of the time traces prior to the laser pulse are recorded to determine the baseline level; at \( t = 0 \) the laser flash triggered the photoconversion. E, individual time traces (A–D) were assembled. Here, the fit of the resulting data is shown in green as well as the residuals (below).

**FIGURE 2.** Single time traces of the SynCph2(1-2) \( \text{Pr} \rightarrow \text{Pfr} \) transition measured at 665 nm. Here, the intermediates show alternately lower or higher absorbance than the Pr, reference state and can be observed without further data analysis. A–D show the time traces with increasing time scales; the approximate appearance of intermediates is indicated by arrows. The intermediate lifetimes are also presented. The final intermediate R3 exhibits similar absorption as the Pr, conformation (D). The initial 10% of the time traces prior to the laser pulse are recorded to determine the baseline level; at \( t = 0 \) the laser flash triggered the photoconversion. E, individual time traces (A–D) were assembled. Here, the fit of the resulting data is shown in green as well as the residuals (below).

denaturation assay (19) of the Pr, and red light-adapted state. Denatured Pr, states show absorbance maxima at 663 nm like other PCB-cysteine adducts with 15Z conformation (19, 20). The denatured red light-adapted states show a mixture of the 15Z and 15E states (Fig. 1C). Here, the 15E state absorbance maximum at \( \approx 640 \text{ nm} \) corresponds to other PCB-15E states as in SynCph1 (21).

**Photokinetics of the SynCph2(1-2) Module**—The \( \text{Pr} \rightarrow \text{Pfr} \) transition was triggered by a 9-ns laser pulse of red light (\( \lambda = 640 \text{ nm} \)) that was tuned close to the \( \lambda_{\text{max}} \) of the Pr, state of SynCph2(1-2) (\( \lambda_{\text{max}} = 644 \text{ nm} \)). During photoconversion, SynCph2(1-2) forms distinct intermediates that can all be followed in single time traces at 665 nm (Fig. 2). The formation of the first intermediate, lumi-R, occurs in phytochromes in the picosecond time range (1) and is hence too fast to be resolved in this study. Accordingly, the decay of a lumi-R-like state (lumi-R, \( \lambda \)) represents the first observable process. For SynCph2(1-2) four intermediates, lumi-R, -R1, -R2, and -R3, were found for the \( \text{Pr} \rightarrow \text{Pfr} \) conversion with lifetimes of 1.3 \( \mu \text{s} \), 299 \( \mu \text{s} \), 2.56 ms, and 17.1 ms (Table 1). Their spectral features are reflected by their LADS shown in Fig. 3. Notably, these LADS are defined as differencespectra, where positive amplitudes indicate a decaying species, i.e., the absorbance at the specific wavelength decreases between the preceding state and the observed one, whereas negative LADS amplitudes specify an absorbance rise.

During \( \text{Pr} \rightarrow \text{Pfr} \) phototransformation of SynCph2(1-2), the first intermediate corresponds to the lumi-R-like state with an increase in absorbance at about 670 nm and a calculated lifetime of 1.3 \( \mu \text{s} \). The bathochromic shift of the lumi-R, absorbance as compared with the initial Pr, state is continued by the next intermediate, R1 (lifetime 299 \( \mu \text{s} \)), due to a further rise of absorbance at 690 nm. The LADS of the R1 intermediate is bimodal as it shows also a shallow decrease of absorbance at 620 nm. Furthermore, its absorbance changes related to this lifetime show relatively small positive and negative amplitudes. The third intermediate, R2 (lifetime of 2.56 ms), reveals an absorbance at 690 nm. The LADS of the R1 intermediate, lumi-R, occurs in phytochromes in the picosecond time range (1) and is hence too fast to be resolved in this study. Accordingly, the decay of a lumi-R-like state (lumi-R, \( \lambda \)) represents the first observable process. For SynCph2(1-2) four intermediates, lumi-R, -R1, -R2, and -R3, were found for the \( \text{Pr} \rightarrow \text{Pfr} \) conversion with lifetimes of 1.3 \( \mu \text{s} \), 299 \( \mu \text{s} \), 2.56 ms, and 17.1 ms (Table 1). Their spectral features are reflected by their LADS shown in Fig. 3. Notably, these LADS are defined as difference specta, where positive amplitudes indicate a decaying species, i.e., the absorbance at the specific wavelength decreases between the preceding state and the observed one, whereas negative LADS amplitudes specify an absorbance rise.

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**TABLE 1**
Intermediate lifetimes of SynCph2(1-2), S385A, W389A, and W389F photocycles

|                  | Wild type | S385A | W389A | W389F |
|------------------|-----------|-------|-------|-------|
| \( \text{Pr} \rightarrow \text{Pfr} \) |           |       |       |       |
| Lumi-F           | 0.9 \( \mu \text{s} \) | 1.3 \( \mu \text{s} \) | 1.2 \( \mu \text{s} \) | NA    |
| -R1              | 255 ms    | 284 ms| 214 ms| 255 ms|
| -R2              | 2.56 ms   | 1.57 ms| 2.28 ms| 2.0 ms|
| -R3              | 17.1 ms   | 8.10 ms| 33.3 ms|       |
| \( \text{Pfr} \rightarrow \text{Pr} \) |           |       |       |       |
| Lumi-R           | 1.3 \( \mu \text{s} \) | 1.3 \( \mu \text{s} \) | 1.4 \( \mu \text{s} \) | 1.5 \( \mu \text{s} \) |
| -R1              | 299 \( \mu \text{s} \) | 112 \( \mu \text{s} \) | 414 \( \mu \text{s} \) | 380 \( \mu \text{s} \) |
| -R2              | 2.56 ms   | 1.57 ms| 2.28 ms| 2.0 ms|
| -R3              | 17.1 ms   | 8.10 ms| 33.3 ms|       |

The lifetimes of SynCph2(1-2), S385A, and W389A are calculated via global fit analysis. The lifetimes of W389F are averages from fits of single time traces. The lifetimes of the first two intermediates of the \( \text{Pfr} \rightarrow \text{Pr} \) photoconversion of W389F could not be derived (NA means not available) due to the low absorbance changes between its early intermediates.
lifetimes of 0.9 μs, 798 μs, and 6.20 ms. This makes the reverse photoconversion about three times faster than the Pr → Pfr phototransformation. Like other phytochromes, SynCph2(1-2) undergoes different intermediates than during the “forward” Pr → Pfr conversion. The absorbance changes between the intermediates are not as pronounced as in the Pr → Pfr photo-transformation (Fig. 4), which is reflected in the LADS by displaying only small changes for the associated amplitudes (Fig. 3). Already the early lumi-F intermediate formed in the Pfr → Pfr phototransformation resembles the Pr state, and the further F1 and F2 intermediates show only modest absorption changes. The last intermediate, F2, with a lifetime of 6.20 ms, displays an absorbance rise at about 640 nm and leads finally to the Pr state.

The global analysis of the time-resolved data from the S385A mutant reveals, as in the native SynCph2(1-2), four intermediates with lifetimes of 0.9 μs, 798 μs, and 6.20 ms. This makes the reverse photoconversion about three times faster than the Pr → Pfr phototransformation. Like other phytochromes, SynCph2(1-2) undergoes different intermediates than during the “forward” Pr → Pfr conversion. The absorbance changes between the intermediates are not as pronounced as in the Pr → Pfr photo-transformation (Fig. 4), which is reflected in the LADS by displaying only small changes for the associated amplitudes (Fig. 3). Already the early lumi-F intermediate formed in the Pfr → Pfr phototransformation resembles the Pr state, and the further F1 and F2 intermediates show only modest absorption changes. The last intermediate, F2, with a lifetime of 6.20 ms, displays an absorbance rise at about 640 nm and leads finally to the Pr state.

Light-induced Kinetics of SynCph2(1-2) Tongue Mutants—Ser-385 is located in the conserved PRXSF motif that is situated in the common tongue region of group I and II phytochromes. In the SynCph2(1-2) Pr crystal structure (8), this polar residue shows no interaction with the GAF1 domain and its embedded PCB chromophore and points away from the chromophore binding pocket, whereas in bathyphytochromes and the Pfr state of D. radiodurans phytochrome (14), this residue was found to form a hydrogen bond with the aspartate of the conserved DIP motif within the chromophore-binding site. Accordingly, the S385A mutant of SynCph2(1-2) exhibits a Pfr steady state absorbance spectrum that deviates significantly from that of the native SynCph2(1-2) module (Fig. 1B). After red light illumination of the Pr state, the Pfr spectrum shows a broadened peak in the red region between 500 and 620 nm. At the photodynamic equilibrium, the resulting spectrum exhibits a bleaching at 646 nm and an isosbestic point at 556 nm relative to the Pr state with its Abs max at 646 nm. At wavelengths shorter than the isosbestic point, the mutant shows increased absorbance upon photoconversion unlike native SynCph2(1-2).

The global analysis of the time-resolved data from the S385A mutant reveals, as in the native SynCph2(1-2), four intermedi-
ates (lumi-R₁, R₁, R₂, and R₃') with lifetimes of 1.3 μs, 112 μs, 1.57 ms, and 8.10 ms for the forward reaction, i.e., Pᵣ → Pₚᵣ. The first three intermediates are similar to SynCph₂(1-2) but with up to 3-fold shorter lifetimes. Accordingly, the overall photoconversion is about two times faster than in native SynCph₂(1-2) and leads to a final state with a difference spectrum akin that of native SynCph₂(1-2), but with a 2.6-fold reduced overall amplitude. Likewise, the late R₂ and R₃ intermediates exhibit similarly lowered overall amplitudes, whereas the lumi-R₁ and R₁ intermediates are almost unaffected. As in SynCph₂(1-2), the lumi-R₁-like intermediate decays with a lifetime of 1.3 μs and exhibits an increase in absorbance in the long wavelength range at about 690 nm. This effect is continued in the second intermediate (R₁, lifetime of 112 μs) that also displays a reduced absorbance at 620 nm. The highest impact on the absorbance change takes place during the formation of the R₂ intermediate with a lifetime of 1.57 ms, also nearly two times faster than in native SynCph₂(1-2). Here, a pronounced absorbance decrease around 655 nm takes place. The last intermediate, R₃’, with a lifetime of 8.10 ms is distinct from the R₃ intermediate of native SynCph₂(1-2). Instead of an absorbance increase at 700 nm as detected for the native photosensor, the R₃’ intermediate of the S385A mutant displays a broader increase above 590 nm with maximal changes at 660 nm. This spectral signature reflects the broad absorbance band of the Pₚᵣ state, as was seen before in steady state difference spectra.

The signals in the S385A measurements are 3-fold diminished compared with the native SynCph₂(1-2) module not only during the Pᵣ → Pₚᵣ but also the Pₚᵣ → Pᵣ photoconversion. Like SynCph₂(1-2), S385A shows three intermediates for the Pₚᵣ → Pᵣ conversion, lumi-F’, F₁, and F₂ with lifetimes of 1.3 μs, 619 μs and 3.53 ms, which all exhibit the spectral signatures of a state close to Pᵣ. The lumi-F’ intermediate is the only one that differs from lumi-F of intact SynCph₂(1-2), because it displays an absorbance rise also at wavelengths above ~640 nm. The greatest absorbance changes occur during the formation of intermediate F₂ with an absorbance rise at 640 nm. The lumi-F’ and F₁ intermediates decay similarly as their SynCph₂(1-2) counterparts; only the F₂ intermediate decays nearly two times faster.

The SynCph₂(1-2) mutant W389A addresses the role of the conserved WXE motif in the tongue region. This motif is about 15 Å distant from the bilin chromophore and is suggested as being part of a tryptophan switch during photoconversion (8). Like the S385A mutant, the W389A mutant shows a spectrum after red light illumination that lacks the signatures of the Pₚᵣ state (Fig. 1B). This alternative steady state spectrum is highly broadened with an unusual absorbance rise in the 450–600 nm region that surpasses the one of S385A. The Pₚ peak at 644 nm is bleached; the isosbestic point at 573 nm is red-shifted compared with S385A because of the broader absorbance.

Global analysis of the time-resolved data reveals three instead of four intermediates (lumi-R₁, R₁, and R₂’) with lifetimes of 1.4 μs, 414 μs, and 2.28 ms (Fig. 3). Interestingly, these intermediates resemble the native intermediates not only in their lifetimes but also in the global features of their difference spectra. The first lumi-R-like intermediate with a lifetime of 1.4 μs shows an absorbance rise at 700 nm that is continued by the intermediate R₁. The 2.28-ms R₂’ intermediate exhibits an absorbance decrease not only in the range of SynCph₂(1-2) (Aₘₚₓ ~660 nm) but also at longer wavelengths, where intermediate R₁ already shows signatures of the Pₚᵣ state. Unlike the wild type and the S385A mutant, this tongue mutant shows a pronounced rise of absorbance below 580 nm for the R₂’ intermediate. A fourth
intermediate R3, as in SynCph2(1-2) and S385A, which is indicative of late conformational changes of the photoreceptor module, is missing. The signal intensities of the late R2’ intermediate and the final state of the W389A variant are again three to four times smaller than in SynCph2(1-2) and hence are in accordance with the S385A variant.

The Pfr → Pr back conversion of W389A reveals three intermediates (lumi-F, F1, and F2) with lifetimes of 1.2 μs, 2.21 ms, and 19.5 ms. Although the intermediates already show the known P-like features, their LADS differ from native SynCph2(1-2) and its S385A mutant. Furthermore, the back reaction takes three times longer, and the constant difference spectrum (Fig. 3) corresponding to the final Pr conformation shows a comparatively broadened peak.

For the variant W389F with native SynCph2(1-2)-like steady state spectra, only a few wavelengths were measured to allow the prediction of the intermediates and their lifetimes. W389F behaves like SynCph2(1-2) in all observed wavelengths (Fig. 5) and reveals four observable intermediates in the forward reaction. The lifetimes of the intermediates calculated from the single time traces and not by global analysis are 1.5 μs, 380 μs, 2.0 ms, and 33.3 ms. The lifetimes of lumi-R, R1, and R2 are in good agreement with those of the native SynCph2(1-2) photosensor. Only the intermediate R3 differs in its lifetime that is two times longer than in SynCph2(1-2). In the reverse reaction only the lifetime of intermediate F2 could be assigned via fit of the single time traces. Without global analysis, the absorbance changes between the intermediates are too small to allow reliable fitting of the curves. The lifetime of intermediate F2 (3.1 ms) is two times faster than that of SynCph2(1-2).

**DISCUSSION**

Photoconversion of SynCph2(1-2), a PAS-less Phytochrome—Cyanobacterial phytochromes of group I (e.g. Cph1 and CphA) are well characterized with respect to their photochemical activity (5, 7, 18, 22, 23). In contrast, photoreceptors such as SynCph2(1-2), composed of only two GAF domains in a tandem array and lacking the N-terminal PAS domain, have not been investigated before by time-resolved spectroscopy. Despite the deviant domain arrangement, the tandem-GAF Cph2 photoreceptor highly resembles canonical phytochromes by undergoing a photochromic switch between a red-absorbing and a far red-absorbing state. Here, the photocycles of recombinant SynCph2(1-2) and three mutants within the PRXSF and WXE motif of the tongue region were characterized in the micro- to millisecond time scale, which all share the primary 15Z → 15E photochemistry. Following other studies on various phytochromes (18), we applied a sequential, unidirectional model for the generation and decay of intermediates. Even if equilibria between intermediates were involved, the final reaction products in these measurements will be the photostates, i.e. Pfr, for the forward reaction or Pr, if the back conversion was followed. In this case, the kinetics and reaction rates that evolve from global fit analysis correspond to the ratios k'/k". For native SynCph2(1-2), three intermediates, R1 to R3, could be observed after lumi-R formation. While the decay time of 2.56 ms, assigned to intermediate R2, identifies the decay of short wavelength intermediates, the dominant contribution to Pfr formation is found for R3 with an observed lifetime of 17.1 ms.

In the canonical phytochromes from plants, the Pfr → Pfr phototransformation was found to be extremely complex with
up to six intermediates (22), which can include a further series of \( P_{\text{r}} \)-like states prior to the final formation of \( P_{\text{r}} \). For \( \text{SynCph2}(1-2) \) similar slow reactions cannot \textit{per se} be excluded, because the reported late spectral changes were too red-shifted (>700 nm) to be in the range of this study. However, the absorbance properties of \( \text{SynCph2}(1-2) \) are blue-shifted compared with these canonical phytochromes (9).

It is intriguing to compare the detected absorbance changes for \( \text{SynCph2}(1-2) \) with those from group I phytochromes, especially in the picosecond to microsecond time range. The excitation/absorption detection setup as employed here yields a step function in the wavelength range of the first intermediate (around 700 nm), as “lumi-R” or \( I_{\text{700}} \) is formed within picoseconds (1) and remains constant far into the microsecond time range. The decay of this intermediate is described for group I phytochromes with a lifetime of \( -80-100 \mu s \) (7, 23). In group I phytochromes, the following intermediates (meta-R or \( I_{\text{bleach}} \)) show reduced oscillator strength \( (I_{\text{bleach}}) \), and the decay of the first intermediate follows monoexponential kinetics. In \( \text{Cph2} \), however, the absorbance around 700 nm, after being formed as a step function, remains positive over the entire time range of detection. Accordingly, one may assume that \( \text{SynCph2}(1-2) \) intermediates following the lumi-R-like state show a larger oscillator strength and thus do not cause a transient bleaching. For the reverse \( P_{\text{r}} \to P_{\text{s}} \) reaction, three intermediates, lumi-F, \(-F_1\), and \(-F_2\), are detected that all show the signatures of the \( P_{\text{s}} \) state and undergo only minor changes. These findings, especially the spectral similarity of all intermediates with the final photoproduction, \( P_{\text{s}} \), are in accordance to the behavior of group I phytochromes. For comparison, laser flash photolysis studies of \( \text{SynCph1} \) revealed five intermediates with time constants of at 4.5 \( \mu s \), 270 \( \mu s \), 3.8 ms, 30 ms, and 280 ms at pH 8.0 during the \( P_{\text{s}} \to P_{\text{r}} \) phototransformation (24). The fifth intermediate with a comparable long lifetime therefore cannot be found in \( \text{SynCph2}(1-2) \). Initial \( Z \to E \) isomerization in \( \text{SynCph1} \) occurs in the electronically excited \( P_{\text{s}}^* \) state with a time constant of 30 ps according to an infrared spectroscopy analysis (25). A complementary Raman spectroscopy study concluded that 85% of the molecules relax back from the excited \( P_{\text{s}}^* \) state to the \( P_{\text{s}} \) ground state explaining the low quantum yield of phytochrome phototransformation. After isomerization, the residual 15% were supposed to reach a product-like electronically excited Lumi-R* state that decays to the Lumi-R ground state, which is formed within 30 ps (26). Recent multipulse pump-probe data indicate that isomerization proceeds, most likely via a conventional conical intersection, between the excited and ground state energy surfaces (27). In any case, parts of the kinetic data of \( \text{SynCph1} \) (5, 7, 24, 25, 28) combined with structural NMR data (29, 30) allow the assignment of structural changes to the various intermediates. Unfortunately, only the nearest chromophore environment in the chromophore-binding site was addressed, which excludes most of the tongue region, which is very similar in \( \text{SynCph1} \) and \( \text{SynCph2} \) (8).

Another cyanobacterial phytochrome of group I, the \( \text{SynCph1} \) orthologue CphA from \( \text{Calothrix} \) sp. PCC7601 (also known as \( \text{Fremyella diplosiphon} \) or \( \text{Tolypothrix sp.} \)) revealed four intermediates with lifetimes of 8 \( \mu s \), 330 \( \mu s \), 3.2 ms, and 23 ms for the \( P_{\text{s}} \to P_{\text{r}} \) conversion and four intermediates with lifetimes of 1.5 \( \mu s \), 1.5 ms, 6 ms, and 50 ms for the backward reaction (18). These results are again in good agreement with the \( \text{SynCph2}(1-2) \) lifetimes apart from the fourth intermediate of CphA in the \( P_{\text{r}} \to P_{\text{s}} \) phototransformation. Cryotraping experiments of CphA in combination with UV-visible and Fourier transform infrared difference spectroscopy revealed the presence of three intermediates in the forward and two intermediates in the backward reaction that show remarkable similarities with the intermediates of \( \text{SynCph1} \) (31).

The assignment of intermediates to structural changes can be achieved for at least two intermediates in the \( P_{\text{s}} \to P_{\text{r}} \) conversion (29). The lumi-R state is initially formed by double bond \( Z \to E \) isomerization between C15 and C16 (26, 32). In the meta-R state, the chromophore is transiently deprotonated at the D-ring nitrogen and is then reprotonated upon \( P_{\text{s}} \) formation (24).

In \( \text{SynCph2}(1-2) \), the lumi-R state can be assigned to the first intermediate of this study. Because of its fast formation below the resolution limit of the measurements, only its decay can be followed. The remaining intermediates reflect structural changes in the protein as well as adaptations of the chromophore. The assignment of the transient protonation to the intermediates is not possible at the current state. Model compounds exhibit a red-shift upon protonation (33–35). For \( \text{SynCph1} \), the transient proton release and a kinetic isotope effect were assigned to an intermediate with the 320-\( \mu s \) lifetime (7). Interestingly, its difference spectrum and lifetime resembles intermediate R1 of \( \text{SynCph2}(1-2) \); both exhibit a red-shifted absorbance.

The \( P_{\text{s}} \to P_{\text{r}} \) conversion starts like the forward reaction with the double bond isomerization between C15 and C16 leading to the lumi-F intermediate. Subsequent conformational changes in the range of rings C and D result in the formation of the intermediate meta-F. The meta-F \( \to P_{\text{s}} \) transition is accompanied by the generation of a new hydrogen bond between the D-ring nitrogen and a water molecule (30).

\textit{Tongue Variants and Their Impact on the Photocycle—}The tongue region of the GAF2 domain seals the chromophore binding pocket in GAF1 and is supposed to be involved in signal transduction (8, 36). This arrangement is comparable with the group I phytochromes where the tongue protrudes from the PHY domain, which is actually a degenerated GAF domain. Ser-385 is located in the PRXSF motif within a loop region of the tongue (Fig. 1A). The preceding residue Arg-383 of this motif is involved in the arginine to aspartate salt bridge, thereby generating a tight interaction between the GAF1 and GAF2 domain. Ser-385 points out of the binding pocket in the crystal structure of the \( P_{\text{s}} \) conformation, whereas Trp-389 is part of the conserved WXE motif in the stem region of the tongue consisting of two \( \beta \)-strands (Fig. 1A). Although Ser-385 and Trp-389 are \( \sim 13 \) and \( \sim 16 \) \( \AA \), respectively, apart from the D-ring of the chromophore in the \( P_{\text{s}} \) state, they affect formation of the \( P_{\text{s}} \) state. Steady state absorbance spectra of their alanine variants reveal that they fail to form substantial amounts of a \( P_{\text{s}} \) state like native \( \text{SynCph2}(1-2) \) (Fig. 1B).

In this study we could show that S385A exhibits four intermediates in the \( P_{\text{s}} \to P_{\text{r}} \) reaction like native \( \text{SynCph2}(1-2) \). Both share the first three intermediates lumi-\( \text{R}_{\text{L}, \text{1}} \), -\( \text{R}_{\text{1}} \), and -\( \text{R}_{\text{2}} \) of...
the $P_r \to P_{fr}$ reaction. The fourth intermediate $R_3'$ differs in S385A (Fig. 3), but nevertheless, it decays to a $P_{fr}$-like state (Fig. 6). The $P_{fr} \to P_r$ conversion is very similar to $Syn\text{Cph2}(1-2)$, suggesting that the red light adapted state of S385A decays into a comparable intermediate like the $F_3$ state of $Syn\text{Cph2}(1-2)$ and that the $P_{fr}$ and $P_{fr}$-like states correspond to each other. From this first intermediate lumi-$F'$, the protein decays via the common intermediates $F_1$ and $F_2$ of the $Syn\text{Cph2}(1-2)$ $P_{fr}$ to $P_{fr}$ photoconversion.

In contrast, the photocycle of the W389A variant at first glance differs from native $Syn\text{Cph2}(1-2)$. The forward $P_r \to P_{fr}$ reaction proceeds through the first two $Syn\text{Cph2}(1-2)$-like intermediates lumi-$R_{-R}$ and $-R_1$. The third intermediate $R_2'$ deviates and decays without a fourth intermediate like in $Syn\text{Cph2}(1-2)$ to the red light-adapted state that shows a very broad absorbance band, including an absorbance rise around 550 nm (Fig. 1B, bottom, left panel). This state represents an alternative photoprotein and is therefore named here $P_{alt}$. W389A apparently misses the last intermediate $R_3$ (Fig. 3, panel bottom, left panel), although a meta-$R$-like decay with a lifetime of $\approx 2.3$ ms (akin those of the wild type and the S385A mutant) can clearly be identified. The inspection of the steady state $P_r/P_{fr}$ difference spectrum of this mutant (Fig. 1B, bottom, left panel) clarifies this confusion, as it shows a red light-adapted state with an apparently small oscillator strength that cannot be detected in the lifetime-associated difference spectrum. Interestingly, any conversion processes of intermediates of the entire detection range are covered from the contributions of the very broad absorbance of the $P_{alt}$ form.

The difference between the red light-adapted states of S385A and W389A is also reflected by their circular dichroism (CD) spectra. Here, W389A shows increased ellipticity at 550 nm, whereas S385A exhibits only a smaller effect and corresponds to $Syn\text{Cph2}(1-2)$ (8). Interestingly, the W389A steady state absorbance difference spectrum corresponds to that of the $Syn\text{Cph2}(1)$ fragment that consists only of the GAF1 domain and therefore lacks the tongue region altogether (37). The difference spectrum of W389A also resembles that of a 45-kDa fragment (amino acids 1–425) of the plant phytochrome A from *Avena sativa*. This recombinant PhyA fragment was designed to study the properties of the proteolytic 39-kDa fragment of PhyA of *A. sativa*; however, trypsin digestion of the native protein also causes loss of the N-terminal 65 amino acids. Accordingly, this PhyA fragment that lacks most of the PHY domain forms upon irradiation a red light-adapted state (38) with a very broad absorbance band at 550 nm and only moderate thermal stability, thus phenomenologically indicating the importance of the PHY domain and its tongue region. The spectral similarity between phytochrome fragments, which either lack their second GAF/PHY domain or bear disruptive mutations within their PRXF motif like R383D (9) and the W389A mutant of $Syn\text{Cph2}(1-2)$, now clearly highlights the role of the tryptophan in the WXE motif for tongue rearrangement and stability of the $P_{fr}$ form. Accordingly, W389A undergoes three intermediates in the back reaction to $P_r$ that differ significantly from the intermediates of $Syn\text{Cph2}(1-2)$ (Fig. 6), and the overall time elapsed to arrive at the $P_r$ state is three times longer than for $Syn\text{Cph2}(1-2)$ (19.5 versus 6.20 ms, cf. Table 1).

From these findings, one can conclude that although the Ser-385 residue contributes to a native-like $P_{fr}$ formation, it is not essential for the formation of the first three intermediates of the $P_r \to P_{fr}$ reaction and the intermediate two and three of the $P_{fr} \to P_r$ photoconversion. Only the formation of the final $P_{fr} \to P_{fr}$ intermediate $R_3$ and of lumi-$F$ is affected by this serine. Accordingly, the S385A variant shows the same conformational changes at the chromophore level during photoconversion and only slight differences at the late stages of the protein environment’s adjustment, i.e. the steps leading to and from $P_{fr}$, as compared with $Syn\text{Cph2}(1-2)$. Interestingly, Ser-385 apparently stabilizes the intermediates because its alanine mutation exhibits about 5-fold faster photoconversions.

The alanine mutation of Trp-389, however, shows major implications on the photocycle; it only shares the first two intermediates with $Syn\text{Cph2}(1-2)$. This suggests that this amino acid
plays a key role in the structural rearrangements during the late photoconversion. Interestingly, the phenylalanine mutation suffices to restore the native SynCph2(1-2) behavior.

Mutagenesis studies in the photosensory module of oat phytochrome A (39) with amino acids in close proximity to the chromophore in the GAF domain revealed different effects on the decay of I_{\text{doo}} (lumi-R). Some mutants displayed faster decays and accelerated P_{ir} formation, whereas a proline to alanine mutation resulted in a slower P_{ir} formation due to a more rigid conformation in the mutant. The acceleration was assigned to an increase in polarity or a weakened interaction between two protein domains enabling faster conformational changes (39). In SynCph2(1-2), the S385A mutation accelerated the formation of the red light-adapted state, where the decrease of polarity inhibits interactions that stabilize the intermediates. W389F shows a slower P_{ir} formation due to the less bulky amino acid substituent that is crucial to allow formation of the new tongue conformation.

Lessons from the Photocycle, a Tryptophan Switch for Signaling—We previously suggested a conformational change during photoconversion that involves a tryptophan switch of residues in the tongue region (8). We suggested the tryptophan residues of the W(G/A)G and WXE motif act as anchors within the structure for stabilizing either the P_{r} or the P_{ir} state. It can be concluded that bulky aromatic residues like phenylalanine can substitute the function of tryptophan and preserve the SynCph2(1-2) behavior, whereas small residues such as alanine fail to do so.

In this study, we demonstrate that the W389A variant has indeed major implications on the photocycle in comparison with S385A. On their way to the red light-adapted state, the alanine mutation of the former prevents structural changes important for P_{ir} formation. Assuming that these structural changes implicate rearrangements of the tongue, one can postulate that of the tryptophan residue in intermediate R3. In bathyphytochrome, the involvement of the serine residue in the interactions occurs later than that of the tryptophan residue in intermediate R3. The S385A variant shows that the movement of the serine residue of the PRXSF motif and hence the formation of the hydrogen bond network occurs during the formation of intermediate R3.

During P_{r} → P_{i} photoconversion, the S385A mutation only affects lumi-F, whereas photoconversion of W389A proceeds via a different set of intermediates. The implications of the mutations on the back reaction are not as big as in the forward reaction, because in lumi-F the protein already shows P_{r}-like characteristics and the following intermediates reflect only minor changes. This finding again demonstrates the paramount importance of the tryptophan residues in the Trp switch for the P_{ir} formation of phytochromes.

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