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Original Citation

Song, C., Psakis, Georgios, Kopycki, J., Lang, C., Matysik, J. and Hughes, J. (2014) The D-ring, Not the A-ring, Rotates in *Synechococcus* OS-B' Phytochrome. *Journal of Biological Chemistry*, 289 (5). pp. 2552-2562. ISSN 0021-9258

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The *D*-ring, not the *A*-ring, rotates in *Synechococcus* OS-B' phytochrome*

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*Running title: *D*-ring photoflip in *Synechococcus* phytochrome

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Keywords: Photoreceptors; Nuclear Magnetic Resonance; Protein structure; Biophysics

Background: Phytochrome photoreceptors are activated by light-induced isomerization of the chromophore cofactor.

Results: An unusual chromophore *D*-ring hydrogen bond is broken in association with *Z/E* isomerization upon photoactivation while other changes occur at the *A*-ring linkage to the protein.

Conclusion: Activation arises from a photoflip of a strongly-tilted *D*-ring.

Significance: The hypothesis that the *A*-ring rotates upon photon absorption is wrong.

ABSTRACT

Phytochrome photoreceptors in plants and microorganisms switch photochromically between two states, controlling numerous physiological processes of great biological and agricultural significance. Generally, this phototransformation is associated with a rotation of the terminal ring *D* of the tetrapyrrole chromophore. Here, we apply solid-state cross-polarization magic-angle spinning (CP/MAS) NMR on the two parent states of the 23-kDa GAF-domain fragment of phytochrome from *Synechococcus* OS-B'. Major changes are associated with the *A*-ring covalent linkage to the protein as well as the protein residue contact of ring *D*. Conserved contacts associated with the *A*-ring nitrogen rule out an *A*-ring photoflip, whereas loss of contact of the *D*-ring nitrogen to the protein implies movement of ring *D*. Although none of the methine bridges showed a chemical shift

change comparable to those characteristic of the *D*-ring photoflip in canonical phytochromes, denaturation experiments showed conclusively that the same occurs in *Synechococcus* OS-B' phytochrome upon photoconversion. The results are consistent with the *D*-ring being strongly tilted in both states and the C15=C16 double bond undergoing a *Z/E* isomerization upon light absorption. More subtle changes are associated with the *A*-ring linkage to the protein. We disprove the proposal that the *A*-ring rotates (Uliasz *et al.* (2010) *Nature* 463, 250–254). The findings are discussed in relation to the position of the *D*-ring, photoisomerization and photochromicity in the phytochrome family.

Phytochromes are photochromic biliprotein photoreceptors controlling development in plants but also represented in diverse microorganisms. In general, red-light absorption by the Pr dark state leads to formation of the Pfr state which is photoconverted by far-red light back to Pr, physiological signaling being switched by state transition. The structural characteristics of Pr and Pfr, the photoconversion mechanism and the physiological signals generated are all subjects of intense research (1-3).

The photosensory module of Class I phytochromes comprises a PAS (Period / Arnt / Single-minded) – GAF (cGMP phosphodiesterase / adenylyl cyclase / FhlA) – PHY (phytochrome-specific) tridomain. In plants and cyanobacterial phytochrome Cph1, the bilin chromophore

[structure with pyrrole ring and atom labels shown in Fig. 3 inset] is covalently bound to a Cys residue in the GAF domain, whereas in bacteriophytochromes (BphP's) the attachment site is near the N-terminus. Crystal structures of BphP's and *Synechocystis* Cph1 (Cph1) in the Pr state show the chromophore in a periplanar ZZZssa geometry associated with an intricate network of conserved hydrogen bonds, the ring **D** being poised at 20–40° relative to the **B/C**-ring plane (4–7). Early work (8) had suggested that photoconversion involved a **D**-ring photoflip and indeed the Pfr conformation was shown to be ZZEssa in the unusual BphP from *Pseudomonas aeruginosa* in which Pfr is the lowest energy dark state (9–11). Studies with sterically-locked chromophores also supported these conclusions (12). From MAS NMR of Pr and Pfr of Cph1 we showed unambiguously that they are associated with the ZZZssa and ZZEssa geometries, respectively (13). How and even whether the Z→E photoisomerization and associated **D**-ring photoflip itself leads to red/far-red photochromicity and triggers signaling remains uncertain, however. In any case it is clear that photoconversion involves changes in the molecule in addition to the **D**-ring flip, including major salt bridge rearrangements associated with the chromophore and more widespread changes in rigidity (1,2,13,14). Proton transfer is also involved, but although the chromophore probably deprotonates transiently prior to Pfr formation, all four pyrrole nitrogens are protonated in both Pr and Pfr (13,15–20).

Liquid NMR studies of phytochrome have been hampered by both the large size of the photochemically-functional PAS–GAF–PHY module and the high mobility of the chromophore within it (19–21). Vierstra and co-workers described a phytochrome from the thermotolerant cyanobacterium *Synechococcus* OS-B' [here termed SyB.Cph2 as suggested (3)] which showed orange/far-red photochromicity resembling that of canonical phytochromes despite missing the N-terminal PAS domain (22). Photochromicity was retained by the isolated 23-kDa GAF-domain fragment [SyB.Cph2(GAF)], a remarkable finding, as phytochromes generally lose their ability to form *bona fide* Pfr if PHY domain function is compromised. However, the "Pfr" state of SyB.Cph2(GAF) not only showed a ~30 nm blue shift [λ_{\max} at 630 nm and 690 nm (see Fig. 1G), thus

here the parent states are termed P630 and P690, respectively] but also lost the Pfr-typical resonance Raman (RR) band at 815 cm⁻¹ (22). Despite these unusual properties, the 23-kDa SyB.Cph2(GAF) fragment was a potentially valuable object for liquid NMR structural studies, and indeed from such data model ensembles for both P630 and P690 were published (23,24). These led to the proposal that a photoflip of ring **A** rather than ring **D** occurred in SyB.Cph2(GAF), and indeed it was argued that this might be the case in all phytochromes (24). Whereas changes in addition to **D**-ring rotation are well known, the notion of an **A**-ring photoflip as the primary photochemical event conflicts with a large body of published data. This as well as various experimental and theoretical problems associated with the work itself (discussed below) prompted us to apply the MAS NMR methods which we have established for canonical phytochromes (2) to SyB.Cph2(GAF).

Although, as in canonical phytochromes, major ¹³C chemical shift (δ^C) changes are associated with the **A**-ring linkage to the protein at C3 and C3¹ (for atom numbering, see Fig. 3 inset), an **A**-ring photoflip can be ruled out as the ¹H–¹⁵N heteronuclear correlations between the **B**-ring nitrogen (N22) and the proton attached to **A**-ring nitrogen (H^{N21}) are seen in both states. Although significant δ^C changes associated with ring **D** are seen (for example at C19), these are quite different from and much smaller than those in canonical phytochromes. Denaturation experiments showed conclusively, however, that ring **D** does undergo a Z→E photoflip in the native holoprotein. The discrepancy can be resolved on the basis of a strongly-twisted C15=C16 double bond, however. These findings together with others imply that phytochrome photochromicity involves a complex interplay between ring **D** and the surrounding protein, changes associated with ring **A** including the covalent linkage possibly playing an important role in intramolecular signaling.

Experimental procedures

Sample preparation—The SyB.Cph2(GAF) sequence was synthesized following codon optimization for expression in *E. coli* (residues 1–200 plus Ser-Leu linker and His₆ tag, see supplemental Fig. S1). The apoprotein was overproduced from pPROLar in BL21pro for 4 hours at 37 °C following induction with 1 mM

IPTG and 0.2% arabinose. The cells were lysed into TES β (50 mM Tris/HCl pH 7.8, 5 mM EDTA, 300 mM NaCl, with 1mM β -mercaptoethanol added freshly) and the crude extract clarified at 45000 g prior to *in vitro* autoassembly with ~20% excess uniformly ^{13}C - and ^{15}N -labeled phycocyanobilin (*u*-[^{13}C , ^{15}N]-PCB) prepared as described (13). The holoprotein was precipitated with ammonium sulfate, redissolved in 50 mM Tris/HCl pH 7.8, 1 mM IDA, 300 mM NaCl, 10 mM imidazole with 1 mM β -mercaptoethanol added freshly) and affinity-purified over Ni-NTA eluting with a similar buffer containing 100 mM imidazole. Peak fractions were pooled, precipitated with ammonium sulfate again and resuspended at ~2 mg/ml in TES β . Working in darkness using infra-red visualization, the sample was then photoconverted to P630 by saturating irradiation with far-red light (>700 nm), purified by size-exclusion chromatography over Superdex 200, rebuffered into 50mM Tris/HCl pH7.8 with 5% glycerol, concentrated to ~4 mM by ultrafiltration, transferred to a MAS rotor and frozen at -80 °C. A photoequilibrium mixture containing ~50% P690 was obtained by saturating irradiation of the concentrated sample at 525 nm in a ~100 μm diameter capillary prior to freezing in the rotor. UV-vis absorbance spectra (Agilent 8453) of the samples (Fig. 1G) were measured by scratching ~3 μL of frozen material from the rotor followed by dilution into TES β .

PCB and phytochromobilin (P Φ B) adducts of SyB.Cph2(GAF) and Cph1(PAS-GAF-PHY) were produced *in vivo* by coexpressing the appropriate phytochrome gene with cyanobacterial hemoxygenase and *pcyA* or *Arabidopsis HY2* in *E. coli*. Working in darkness, the purified holophytochrome at ~10 μM was photoconverted with an appropriate LED source and diluted ten-fold into 8 M urea/HCl, pH 1.5, the absorbance spectrum thereby indicating the proportion of Z- and E- isomers present (25,26).

MAS NMR studies—All solid-state MAS NMR spectra were performed on a Bruker AV-750 WB spectrometer (Karlsruhe, Germany) equipped with a 4-mm triple-resonance CP/MAS probe (Bruker). The ^1H channel was tuned to 750.13 MHz for broadband decoupling, and the X channel was tuned to 188.62 MHz for ^{13}C and 76.01 MHz for ^{15}N . In each experiment, a 4-mm diameter zirconia MAS rotor was loaded with ~8 mg protein in ~90 μL . The MAS spinning frequency in all

experiments was set to 13 kHz and controlled to ± 3 Hz with a Bruker MAS control unit. The temperature over the sample volume of *in situ* MAS probe at the $\nu_{\text{R}} = 13$ kHz was calibrated via the T_1 of ^{79}Br in KBr (27). In this study, 233 ± 0.5 K was maintained throughout the measurements. Optimized ^1H $\pi/2$ and ^{13}C π pulse lengths were 3.1 and 8.0 μs , respectively. The ^1H power was ramped 100–80% during CP and ^1H r.f. field strength of 72 kHz was employed for two-pulse phase modulation (TPPM) decoupling during acquisition. NMR spectra for P690 were recorded with a photoequilibrium mixture of P630 and P690. Spectra for P690 were obtained by subtraction of the P630 spectra from the P630+P690 mixture with an appropriate weighting constant of 0.5. The raw spectra for P630 and the mixture were processed and phased identically prior to subtraction.

2D ^{13}C - ^{13}C DARR experiments were used to assign ^{13}C chemical shifts of the PCB chromophore. The DARR spectra shown in Fig. 1 were recorded with two ^{13}C homonuclear recoupling mixing times of 5 and 50 ms, respectively. During the mixing period, the ^1H - ^{13}C dipolar interaction was recovered by ^1H continuous wave irradiation with the intensity satisfying the $n = 1$ rotary-resonance condition (28). The data were obtained with the following acquisition parameters: an 8-ms evolution in the indirect dimension, a relaxation delay of 1.5 s for each t_1 slice, and 128 slices of 2048 scans each, leading to a total experimental time of ~110 h. The 2D ^1H - ^{15}N heteronuclear dipolar correlation spectra shown in Fig. 2 were measured with 2152 transients, a relaxation delay of 1.8 s for each t_1 slice, and a total of 128 t_1 slices. An optimized CP contact time of 2.048 ms was used. The spectrum was recorded over a period of ~139 h. Prior to Fourier transformation the data were zero-filled to 4096 points and an exponential apodization of 15 Hz was applied. Other acquisition parameters are given in the figure legends.

For the 2D ^1H - ^{15}N HETCOR experiment acquired with an LG-CP contact time of 2.0 ms, the maximum detection limit for heteronuclear transfers can be set to ~3.5 Å. This limit is not overestimated, as experimentally determined by the poorly-resolved P630 correlation signals between the pyrrole nitrogens of rings B-C and the pyrrole water at δ^{H} of ~7.9 ppm (red, Fig. 2). In the Cph1 2VEA structure (5), this pyrrole water is associated with the rings A-C at distances of ~3.3

Å. Also, the 2D ^1H - ^{15}N MELODI-HETCOR spectrum of cationic histidine with an 800- μs LG-CP period revealed two intermolecular hydrogen bonds between the imidazole nitrogens and water protons at distances up to 3.5 Å (extracted from the crystal structure of L-histidine hydrochloride monohydrate) (29).

^{13}C and ^{15}N chemical shifts were externally referenced with respect to backbone CO resonance of solid glycine-HCl at 176.04 ppm and the backbone N^{α} resonance of solid histidine-HCl at 49.04 ppm, respectively. ^1H chemical shifts were calibrated by running a ^1H - ^{13}C HETCOR experiment on the histidine-HCl through its predominantly down- and up-field peaks centered at 9.21 and 18.42 ppm, respectively. The NMR data were processed with Bruker Topspin 3.0 and further analyzed by using the Sparky 3.114 (T. D. Goddard and D. G. Kneller, SPARKY 3. University of California, San Francisco, CA). The ^{13}C and ^{15}N CP/MAS spectra were transferred to WaveMetrics Igor Pro 6.10 (Lake Oswego, OR) for the FWHM (full-width at half-maximum) measurements using the integrated multiple-peak fitting package.

Results

2D homo- and heteronuclear MAS NMR dipolar correlation spectroscopy on SyB.Cph2(GAF) holophytochrome assembled *in vitro* with u - $[^{13}\text{C}, ^{15}\text{N}]$ -PCB enabled complete and unambiguous ^{13}C and ^{15}N assignments of the chromophore for both P630 and P690 (Figs. 1 and 2).

2D ^{13}C - ^{13}C dipolar-assisted rotational resonance (DARR) spectra—Contour plots of ^{13}C - ^{13}C DARR spectra are shown in Figs. 1A and B for P630 and P690, respectively. The spectra were recorded with two ^{13}C homonuclear recoupling mixing times of 5 and 50 ms (red and purple for P630, orange and cyan for P690, respectively) for ^{13}C assignment of the chromophore: initial assignments were obtained from the well-defined correlation networks connecting directly-bonded carbons and confirmed by the paired peaks originated from weak polarization transfers among indirectly-bonded carbons. For example in P630, paired peaks of the D-ring ethyl side-chain (C18–C18¹–C18²) are clearly present in the spectrum recoded with a mixing time of 5 ms (red, Fig. 1A), and the assignment of C18¹ (15.0 ppm) and C18² (11.3

ppm) is verified by the correlations involving neighboring D-ring carbons, like C17¹ (8.5 ppm)–C18¹/C18², and C19 (171.7 ppm)–C18¹/C18², resolved in the spectrum at the 50-ms dipolar mixing time (purple, Fig. 1A). Also, the correlation network in the A-ring region shows a single set of δ^{C} for C1 (182.0 ppm), C2 (35.2 ppm), and the ethylidene side-chain (C3¹, 47.4 ppm and C3², 20.6 ppm), while revealing a correlation network split for its methyl side-chain (C1–C2–[C2^{1a} (17.2 ppm)/C2^{1b} (18.1 ppm)]–[C3^b (53.6 ppm)], Fig. 1C) as well as a doubling for the C3 response with a δ^{C} change of 0.3 ppm (C3^a (53.3 ppm)/C3^b–C3¹, Fig. 1C) and a signal tripling for C4 (C3^b–C4^c (157.9 ppm)/C4^b (157.4 ppm)/C4^a (156.9 ppm), Fig. 1D). Similarly, a 1.1-ppm resonance separation is observed for C5 (85.4/86.5 ppm, Fig. 1D) and a 0.7-ppm difference for its B-ring neighbor, C6 (151.4/152.1 ppm, Fig. 1E). Such signal doubling is also observed in P690 for C2¹ and C3 (17.6/18.0 and 51.0/52.7 ppm, respectively, Fig. 1F), whereas C4–C6 splitting was not apparent. These observations indicate at least two different conformations of the chromophore in both states and loss of order in P690 (for example in the C4–C6 region).

The complete ^{13}C chromophore assignments in both P630 and P690 states are listed in Table 1. The overall similarity between the ^{13}C chemical shifts of the chromophore of SyB.Cph2(GAF) as P630 and of Cph1(PAS–GAF–PHY) as Pr implies that the chromophore adopts a ZZZ_{ssa} geometry in both proteins. The 1D ^{13}C CP/MAS NMR spectra of SyB.Cph2(GAF) as P630 and P690 are shown in Fig. 3 alongside the associated difference spectrum generated by P630→P690 photoconversion (bottom). Most ^{13}C resonances of the pyrrolic quaternary carbons show only subtle changes. In particular those lying between 120 and 160 ppm, including C6–C9 and C11–C14 of rings B and C as well as C4 and C16–C18 of rings A and D show minimal changes in δ^{C} (Fig. 3, inset) and line-widths (expressed as FWHM, $\nu_{1/2}$, Table 2). Consequently, the data provide little support for C15=C16 photoisomerization, calling the expected Z/E photoflip of ring D into question. By way of comparison, the D-ring photoflip in the canonical Cph1 and plant phytochromes is associated with striking δ^{C} changes of ~6 ppm for C14 and most D-ring pyrrolic carbons (see Table 1). Similarly, however, there is also little support for C4=C5 isomerization and the proposed A-ring photoflip

(24): the C4 and C6 atoms associated with the *A–B* methine bridge show a δ^C change less than 1.3 ppm, far smaller than that from C14 and C16 of the *C–D* bridge associated with the *D*-ring photoflip in canonical phytochromes (13,17,18). Furthermore, δ^C changes seen at ring *A* [C3 (−2.3/−0.9 ppm), its side-chain C3¹ and C3² (+1.0 and −1.3 ppm, respectively) linkage to Cys138, and pyrrolic carbons C1 (+1.3 ppm) and C2 (+1.2 ppm)] are also too small for an *A*-ring photoflip. On the other hand, the *D*-ring side-chain carbons C17¹ and C18¹ (+0.3 and +0.4 ppm, respectively) and C18 (−0.1 ppm) show essentially no change during photoconversion. Thus we confirm the larger effect on the *A*-ring C3² relative to that on the *D*-ring C17¹ seen in liquid NMR studies of SyB.Cph2(GAF) with the chromophore selectively labeled using 1,2-[¹³C]ALA (α -aminolevulinic acid), a difference that *by itself* might be construed as evidence for the proposed *A*-ring photoflip (24).

2D ¹H–¹⁵N heteronuclear correlation (HETCOR) spectra—Both P630 and P690 spectra presented in Fig. 2 are dominated by correlations between directly-bonded NH groups of the chromophore. The four pyrrole nitrogens are fully protonated in both P630 and P690, evidenced by the narrow δ^N dispersions (~30 ppm) and all δ^N values lying below 161 ppm. These findings are in line with those from studies of Cph1 and plant phytochromes (13,17,20). In P630 (red, 1D trace), the areas of the four isolated peaks at 161.7, 156.6, 145.4, and 131.9 ppm correspond to a single nitrogen in each case. All four P630 peaks occur at δ^N positions almost identical to those observed for Cph1(PAS–GAF–PHY) (Table 1), the resulting tentative assignments being validated by their correlations with the NH protons. In P630, three different ¹H sites at $\delta^H = 9.9, 9.3,$ and 8.8 ppm in the ¹H spectral region of 8–11 ppm (red, Fig. 2) are seen. As indicated by the ¹⁵N assignment of Cph1 (18), ¹H partners of two tetrapyrrole nitrogens of the inner rings *B* (N22) and *C* (N23) should resonate at the same frequency of 9.3 ppm, while N22 (161.7 ppm) should show a correlation with another H^N at ~9.9 ppm in close proximity (H^{N21} of ring *A*). Indeed, an intense correlation signal at 9.9 ppm is seen, thus the ¹⁵N peak at 156.6 ppm can be assigned unambiguously to N21. The ¹⁵N assignment is validated by resolved P690 correlations (blue, Fig. 2): H^{N23} is at 8.9 ppm, and at the same time N23 (145.6 ppm) shows a weak interaction with its neighboring proton at $\delta^H = 9.8$

ppm, which can be directly assigned to H^{N22}. In the N22 slice centered at 161.5 ppm, a much more intense signal at this ¹H frequency is observed. Also seen in this slice, the weak signal at $\delta^H = 10.6$ ppm is readily attributed to the intramolecular magnetization transfer between N22 and H^{N21}. In the N21 slice (156.0 ppm), two correlations centered at the same ¹H frequencies as N22 (9.8 and 10.6 ppm) are seen, but their intensities are reversed. Moreover, in both states, we attribute the remaining ¹⁵N resonance locating at the most high-field position to the *D*-ring nitrogen (N24), and its directly-bonded ¹H partner (H^{N24}) resonates at $\delta^H = 9.4$ and 8.8 ppm in P630 and P690, respectively.

Intriguingly, the P630-state N24 also correlates with an acidic proton with δ^H of 14.4 ppm associated with a hydrogen bond to the protein. This unique signal can be only be associated with the ring nitrogens of Trp, His or perhaps Pro (13,29). From available phytochrome structures, the only such residue within 6 Å of N24 is the perfectly-conserved homolog of His169^{SyB.Cph2}. In Cph1 and bacterio-phytochromes in the Pr state (5), however, this residue is hydrogen-bonded to the *D*-ring carbonyl oxygen at ~2.8 Å rather than N24. Such a difference could easily arise by reorientation of the imidazole side-chain (30). Indeed two His290 isoforms were observed for Cph1 Pr (13). Moreover, the liquid NMR data for SyB.Cph2(GAF) are also compatible with a His169N δ 1/N ϵ 2-H \cdots N24 contact, seven of the twenty lowest-energy models in the corrected P630 ensemble (2LB9) showing this contact within 3.5 Å. As our detection limit of ~3.5 Å was calibrated (see Experimental procedures), this bond is clearly broken in P690.

The ¹⁵N chemical shifts of the pyrrole nitrogens in both states are summarized in Table 1. The strongest δ^N change upon P630→P690 photoconversion was seen for the *A*-ring nitrogen N21 but amounted to only −0.6 ppm, whereas the pyrrole nitrogens of rings *B–D* were essentially unaffected (<0.2 ppm). The small change in δ^N does not support the idea that P630→P690 photoconversion in SyB.Cph2(GAF) is associated with a *Z/E* isomerization of C4=C5 and an *A*-ring photoflip as proposed (24). Crucially, the interaction between the *B*-ring N22 and *A*-ring H^{N21} (10.6 ppm) is retained in P690 (blue, Fig. 2), ruling out an *A*-ring photoflip. In contrast to the δ^N change observed for all four nitrogens, their directly-bonded protons show prominent δ^H

changes (0.4–0.7 ppm, Fig. 2) associated with photoconversion, since they are all involved in hydrogen bonding with the protein. Thus a 0.6-ppm δ^{H} change seen for H^{N24} provides additional qualitative support for the putative H169N δ 1/N ϵ 2-H \cdots N24 hydrogen bond and its rupture in P690, implying significant changes at ring **D**. Also, δ^{H} changes correlate well with those of δ^{C} for the PCB methyls, like C3², C7¹, and C18² (Table 1), reflecting the light-driven changes at the periphery of the chromophore.

Denaturation—The findings described above not only rule out an **A**-ring photoflip: other than the breakage of the His169 \cdots N24 hydrogen bond, they provide no evidence for **D**-ring photoflip either. Indeed, the pattern of chemical shift changes contrasts with findings for Cph1(PAS–GAF–PHY) in which Pr/Pfr photoconversion Pfr is clearly associated with a *Z/E* isomerization of C15=C16 and a **D**-ring photoflip (13,17). We obtained independent evidence that *Z/E* isomerization nevertheless also occurs in the case of SyB.Cph2(GAF) by denaturation under acidic conditions (25,26). In acidic urea the bilin *Z*-isomer shows a photostable absorbance peak at about 665 nm whereas the *E*-form absorbs at about 580–610 nm and can be converted to *Z* in light. The data (Table 3) show conclusively that SyB.Cph2(GAF)-PCB in the P630 state comprises the *Z*-isomer whereas the P690+P630 mixture following 630 nm irradiation consists predominantly (~80%) of the *E*-isomer. Experiments with Cph1(PAS–GAF–PHY)-PCB gave similar results (as reported earlier (26)). Equivalent measurements were also carried out on P Φ B adducts which differ from PCB in carrying a vinyl instead of an ethyl side-chain at C18 of ring **D**. A bathochromic shift was seen in all cases, although the shifts in the case of native SyB.Cph2(GAF) were much smaller (Table 3).

Discussion

The state-related changes associated with the **D**-ring atoms we report for SyB.Cph2(GAF) are very different from those for canonical phytochromes (2). Given the dramatic hypsochromic absorbance shift and the loss of the Pfr-typical 815-cm⁻¹ RR band associated with deletion of the PHY domain ((22) and Fig. 1G), the unusual findings reported here might relate to specific properties of the GAF fragment, a more complete SyB.Cph2 molecule perhaps behaving

more similarly to canonical phytochromes. In the latter case, a functional PHY domain seems necessary for Pfr formation or stabilization, the photoconversion process apparently arresting at a bleached, probably deprotonated meta-R intermediate if the PHY domain is damaged or deleted.

In the apparent absence of the expected **D**-ring rotation in SyB.Cph2(GAF), the Vierstra group proposed an **A**-ring photoflip as the basis for photoconversion not only in SyB.Cph2(GAF) but in all phytochromes (24). Although in an earlier publication (23) they described a structural ensemble (2K2N) for SyB.Cph2(GAF) P630 from liquid NMR in which rings **A–C** were predominantly in roughly coplanar *ZZ**ss* geometries with almost perpendicularly-orientated **D**-rings, a refined P630 ensemble (2KOI) was presented subsequently (24) showing highly contorted chromophores with both rings **A** and **D** often almost perpendicular to **B** and **C**. This was accompanied by a P690 ensemble (2KLI) in which again the rings **A–C** were approximately coplanar with the **D**-rings approximately perpendicular. It was thus proposed that photoconversion is associated with an **A**-ring photoflip, not just in SyB.Cph2(GAF) but in all phytochromes (24). There are several reasons for doubting this:

(i) The NMR data obtained (24) are consistent with a wide variety of model structures, especially in the case of P690 which was necessarily derived by subtraction of the P630 spectra from the P690+P630 mixture. In particular, the limited data for the pyrrolic carbons apparently allowed the algorithm considerable freedom in calculating possible structural models. Indeed, various predicted chromophore geometries show, for example, a **C**-ring which is flipped such that the propionate points in the opposite direction to that of the **B**-ring.

(ii) A likely source of error is associated with the P690+P630 mixture itself. As SyB.Cph2(GAF) P690 shows significant dark reversion (22), the sample was continuously irradiated at 620 nm within the magnet, ostensibly to maintain photoequilibrium. With an extinction coefficient of *ca.* 100 mM⁻¹ cm⁻¹ (Fig. 1G), the actinic light would penetrate less than 0.1 mm into the ~2 mM sample, leading to a significant P690 gradient in the microcell during measurement. [In the present study, the sample was passed slowly through a 0.1 mm capillary irradiated omnidirectionally with 525

nm actinic light establishing P630/P690 photoequilibrium prior to freezing.]

(iii) The 2K2N, 2K0I and 2KLI ensembles (23,24) all showed significant “puckering” of the chromophore pyrrole rings although aromatic pyrroles are known to be planar (1,31). Bilin chemistry also has generally concluded that the **B**- and **C**-rings are coplanar, in contrast to the picture presented by the 2K2N, 2K0I and 2KLI ensembles.

(iv) Corrected/refined model ensembles for P630 and P690 (2LB9 and 2LB5, respectively) deposited after the planarity problems were pointed out (1), although it would appear that appropriate energy minimization of the methine bridges has not been carried out. These latest ensembles provide little support for an **A**-ring photoflip as originally proposed on the basis of the same data (24). Although 2LB9 and 2LB5 are marked “to be published”, no correction has yet appeared.

In the present study, although we found major changes in δ^C for C3, C3¹ and C3² associated with the **A**-ring linkage to the protein, none of the effects that would necessarily accompany an **A**-ring photoflip is seen. In particular, the fact that the correlation between N22 and H^{N21} is retained upon photoconversion rules out an **A**-ring rotation. We suggest that changes in the linkage apparent from C3¹ together with the problems detailed above might have favored models in which the **A**-ring was positioned differently in P630 and P690, thereby implying an **A**-ring photoflip.

The Ulijasz *et al.* paper (24) attracted particular attention because it contradicted the general consensus that Pr and Pfr geometries are *ZZZssa* and *ZZEssa*, respectively, and indeed that the primary photochemical event in phytochrome photoconversion is a **D**-ring photoflip. The latter conclusion is based on a large body of published data for plant/Cph1-type phytochromes and bacteriophytochromes, culminating in both X-ray crystallographic and MAS NMR-based structural studies (4,5,7,9-11,13,17,18,32). Although *Za*→*Ea* isomerization at C15 is not in doubt, some reports have nevertheless implied isomerization at the C5 methine. Studies with BphP's assembled with synthetic biliverdin derivatives in which the **D**-ring was locked by covalent bridges to the **C**-ring yielded Pr- and Pfr-like spectra associated with *Za* and *Ea* geometries, respectively, at the C15 methine (12). However, further work with locked

15*Ea* in which the **A**-ring too was locked implied *Zs*→*Za* or even *Zs*→*Ea* hula-twist isomerization at C5 (33). Although our data exclude the possibility of the **A**-ring rotation associated with *Zs*→*Za*, we cannot rule out a hula-twist in which the **A**-ring only shifts slightly. Planar *Za* geometry at C5 would be associated with clashes between **A**- and **B**-rings, however, requiring that the **A**-ring be strongly tilted. The same applies to the **D**-ring in both Pr (*ZZZssa*) and Pfr (*ZZEssa*) – except that ample space around the **D**-ring for the tilt and photoflip exists, whereas close packing of the protein side-chains around the **A**-ring in Pr makes such a tilt unlikely in Pfr. Of course, the PaBphP Pfr crystal structure shows *Zs* at C5 anyhow (9). We suggest that side-effects of the locking modifications to the chromophore might explain the discrepancy, however. There is little room in the pocket for the atoms of the synthetic bridges between the **A**- and **B**-rings. Moreover, we would expect that major changes would follow removal of the protons associated with the pyrrole nitrogens. The appropriate controls for this are in any case missing.

On the other hand, there is no doubt that changes in the **A**-ring are associated with photoconversion. In liquid NMR studies of Cph1 from *Synechocystis* 6803, although a photoflip was only evident for ring **D**, significant state-induced changes were also seen at the C2¹ and C3² methyl groups associated with the **A**-ring and its linkage to the protein (20). We suggest that these effects arise indirectly from post-photochemical relaxation processes involving cofactor and protein. The δ^C changes seen here for C3, C3¹ and C3² are quantitatively similar to changes at these atoms in the case of both Cph1 and plant phytochromes during photoconversion, although there the changes associated with ring **D** predominate (17). X-ray crystallographic data for the PaBphP Q188L mutant also imply major changes at the bilin–protein linkage associated with photoconversion (10). As these effects seem to be widespread in the phytochrome family, it might be that the covalent linkage is an important component of the molecular mechanism of phytochrome action. Nevertheless, at least photochromicity is retained in phytochromes in which the Cys attachment site has been blocked or mutated (34-37). In any case, several other carbon atoms (e.g., C1, C2, C4, C7¹, C12¹, and C18²) also show ~1 ppm δ^C changes,

indicating that the structural changes in the protein are not confined to the linkage.

The **D**-ring changes associated with photoconversion seen in the present study are clearly distinct from those in canonical phytochromes, thus we were surprised to find that the P630 and P690 states indeed represent *Z* and *E* isomers, respectively (see Table 3). Whereas it might be that the photoconversion mechanism in the SyB.Cph2(GAF) fragment is unusual, it is sobering to note that a satisfactory explanation for the ~50 nm bathochromic shift in λ_{\max} characteristic of Pr→Pfr photoconversion in canonical phytochromes has yet to be offered. It is certainly not a direct consequence of the *Z*→*E* isomerization of C15=C16 and the associated rotation of ring **D**, as in the absence of protein interactions the isomerization is associated with a ~50 nm shift *in the opposite direction* (as apparent from Table 3). Thus, whereas photochromicity arises from the bilin, the direction of the spectral shift is reversed by the protein, perhaps in association with changes in the hydrogen-bonding network modifying the charge distribution. Counter ion changes might control orbital energies and conjugation defects. Interactions with the protein leading to strains and torsions of the chromophore methine bridges would also have major effects on the absorbance properties, as extensive studies of free bilins have shown (38). Recent work on PaBphP bacteriophytochrome indicates that the C15 methine bridge between rings **C** and **D** is significantly stretched in the Pfr state (11).

The His169...N24 contact we observe specifically for P630 is intriguing as the equivalent His interaction in the crystal structures of canonical phytochromes is to the **D**-ring carbonyl (5). A contact to the nitrogen might arise if the ring were strongly tilted, however. Such a situation is indeed seen in several models derived from the

liquid NMR studies (2LB9 cf. (24), see Fig. 4). Such a geometry might derive from hydrophobic interactions with Phe82 and/or Val167. It might also reconcile the unexpectedly small δ^C changes at C14 and C16 with a *Z*→*E* photoisomerization as well as the missing 815 cm⁻¹ Raman band in P690 of the SyB.Cph2(GAF) fragment (22). However, substituting the **D**-ring ethyl side-chain with a vinyl group leads to bathochromic shifts in SyB.Cph2(GAF), albeit much smaller than those seen in canonical phytochromes (Table 3), implying that ring **D** is weakly conjugated with the rest of the chromophore in both states. According to Hückel theory, conjugation is proportional to \cos^2 of the angle between the π -electron axes, thus **D**-ring tilts up to 70° or beyond 110° would still allow significant conjugation. When the **D**-ring is almost orthogonal, however, conjugation is lost, as in α -phycoerythrocyanin and certain cyanobacteriochromes (39,40). Hence, we suggest that in SyB.Cph2(GAF) the C15=C16 double bond twisted before and after photoconversion, the dihedral angle changing from ~70° over 90° to ~110° in association with the **D**-ring photoflip: this would explain all the current data and imply that the missing PHY domain significantly influences the geometry of the **D**-ring in the case of P690. The PHY domain is in any case essential for physiological signaling.

ACKNOWLEDGMENTS

We thank Peter Schmieder (FMP, Berlin-Buch, Germany), Hugo Scheer (LMU Munich, Germany) and J. Clark Lagarias (UC Davis) for valuable discussions, Fons Lefeber, Kees Erkelens and K. B. Sai Sankar Gupta (University of Leiden, The Netherlands) for instrumental assistance, and the DFG (Hu702/8) and the NWO (DN 89-190 and ALW 822.02.007) for funding.

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Figure Legends

Figure 1. 2D MAS NMR ^{13}C - ^{13}C homonuclear dipolar correlation spectra of u - $[^{13}\text{C},^{15}\text{N}]$ -PCB-SyB.Cph2(GAF). Polarization-transfer times of 5 and 50 ms were employed for the P630 (A, red and purple, respectively) and P690 (B, orange and cyan, respectively) states. The blue lines in two full contour plots indicate sequences of nearest-neighbor correlations (for numbering see Fig. 3 inset; for full assignments including indirect-bonded correlations for P630 and P690 and 1D spectra, see supplemental Figs. S2 and S3, respectively). The observed ^{13}C signal splittings of a subset of carbon resonances are illustrated in (C–E) and (F) for P630 and P690, respectively (expansions are inset). The data were collected with an 8 ms evolution in the indirect dimension; 1434 complex t_2 and 128 real t_1 points with 2048 scans. A relaxation delay of 1.5 s was applied. (G) UV-vis absorbance spectra of the SyB.Cph2(GAF) preparations used in this study. The spectra of P630 and of the P690+P630 photoequilibrium mixture were measured following irradiation with >700 nm and 525 nm light, respectively, as described.

Figure 2. 2D MAS NMR ^1H - ^{15}N FSLG-decoupled dipolar correlation spectra of u - $[^{13}\text{C},^{15}\text{N}]$ -PCB-SyB.Cph2(GAF) as P630 (red) and P690 (blue). The regions without resonances are omitted. Two 1D traces onto the ^{15}N dimension are shown. ^{15}N resonances (N21–N24) are indicated by vertical lines. All four NH protons ($\text{H}^{\text{N}21\text{--}\text{N}24}$) are fully resolved and marked by horizontal lines. The asterisk indicates protein backbone signals in natural abundance. The spectra were acquired with 1536 complex t_2 and 128 real t_1 points, and 2152 scans. A LG–CP contact time of 2.048 ms was applied. The relaxation delay was 1.8 s.

Figure 3. 1D ^{13}C CP/MAS NMR spectra of u - $[^{13}\text{C},^{15}\text{N}]$ -PCB-SyB.Cph2(GAF) as P630 (red) and P690 (blue) and the P690–P630 difference spectrum (bottom). ^{13}C resonances as P630 and P690 are labeled. Resonances of the natural abundance glycerol carbons are indicated by asterisks. The inset shows the changes in ^{13}C shifts of the chromophore during photoconversion. The P630 state is taken as reference, and the size of the circles is proportional to the difference as P690–P630. Carbons showing splittings are labeled with multiple circles.

Figure 4. Stereopair illustrating the proposed His169···N24 hydrogen bond associated with a strongly-tilted D-ring. In this model (#5 from the corrected 2LB9 P630 ensemble, cf. (24)) the hydrogen bond length is 3.2 Å and the C15=C16 dihedral angle 69°. Hydrophobic interactions with Phe82 and Val167 might also be important. (PyMol image).