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Phytochrome as Molecular Machine: Revealing Chromophore Action during the Pfr → Pr Photoconversion by Magic-Angle Spinning NMR Spectroscopy

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Abstract: The cyanobacterial phytochrome Cph1 can be photoconverted between two thermally stable states, Pr and Pfr. The photochemically induced Pfr → Pr back-reaction has been followed at low temperature by magic-angle spinning (MAS) NMR spectroscopy, allowing two intermediates, Lumi-F and Meta-F, to be trapped. Employing uniformly ^{13}C- and ^{15}N-labeled open-chain tetrapyrrole chromophores, all four states—Pfr, Lumi-F, Meta-F, and Pr—have been structurally characterized. In the first step, the double bond photoisomerization forming Lumi-F occurs. The second step, the transformation to Meta-F, is driven by the release of the mechanical tension. This process leads to the break of the hydrogen bond of the ring ***D*** nitrogen to Asp-207 and triggers signaling. The third step is protonically driven allowing the hydrogen-bonding interaction of the ring ***D*** nitrogen to be restored. Compared to the forward reaction, the order of events is changed, probably caused by the different properties of the hydrogen bonding partners of N24, leading to the directionality of the photocycle.

Introduction

Phytochromes comprise a well-characterized family of red-/far-red light sensitive photoreceptors found in plants, cyanobacteria, bacteria, and fungi.[1,2] Their photochemically active chromophore is an open-chain tetrapyrrole covalently bound to the protein via a thioether linkage (Figure 1). The chromophore triggers the conversion between two thermally stable states, Pr and Pfr. In the Pr ground state, the chromophore absorbs red light ($\lambda_{max} \sim 658$ nm in the case of Cph1) triggering a *Z*-to-*E* photoisomerization of the C15═C16 double bond (for numbering see the Supporting Information, Figure S1), and thus converting the protein into the far-red light absorbing Pfr state ($\lambda_{max} \sim 702$ nm for Cph1). The Pfr state reverts to Pr either in the dark (on a time scale of weeks in the case of Cph1) at ambient temperature or instantaneously by a light-induced sequence of reactions.[3] Recently, the three-dimensional structure of the cyanobacterial phytochrome Cph1 in the Pr state has been solved.[4] In addition, Yang et al. presented the crystal structure of the bacteriophytochrome of *Pseudomonas aeruginosa* PaBphP in its unusual Pfr ground state.[5] Based on these X-ray studies, the geometry of the tetrapyrrole methine bridges has been shown to be *ZZZssa* and *ZZEssa* in the Pr and Pfr states, respectively (Figure S1). Magic-angle spinning (MAS) NMR

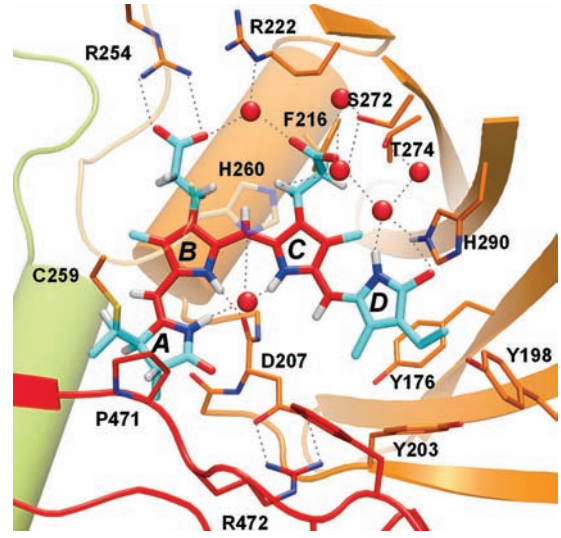

Figure 1. Chromophore binding pocket in Cph1Δ2 phytochrome (pdb 2 VEA).

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has revealed that the forward phototransformation (Pr → Pfr) is linked with a change of the hydrogen-bonding interaction on the ring ***D*** carbonyl and an increase of the length of the conjugated π-system.[6] Very recently, however, it has been claimed that in the ~20-kDa GAF-domain fragment of "SyB-Cph1" phytochrome from the thermotolerant cyanobacterium *Synechococcus* OSB' the photoisomerisation occurs on the C5 methine group.[7]

In both Pr → Pfr and Pfr → Pr phototransformations, intermediate states can be distinguished (for review, see ref 3). Their structural characterization may allow detailed insight into the mechanism of the phototriggered processes. The photochromic photocycle of various phytochromes has been studied by absorption and vibrational spectroscopy. For the Pr → Pfr conversion, absorption spectroscopy has identified at least two intermediates called Lumi-R and Meta-R.[8,9] For the Pfr → Pr back-reaction, two intermediates (Lumi-F and Meta-F) have been observed[8] (for review see ref 10). These intermediates in various phytochromes have also been studied by vibrational spectroscopy.[11−14] For the Pr → Pfr transformation it has been shown that the formation of Lumi-R is linked to the double-bond isomerization, whereas the single-bond rotation occurs upon formation of Pfr from Meta-R.[11,13,15−17]

Because both the parent states absorb significantly in the red region, the maximal Pfr occupancy attainable at photoequilibrium is 70−80%.[18] The remaining >20% Pr complicates studies of Pfr photolysis. Consequently much less is known about the back-reaction. It has been suggested that the back-reaction occurs essentially in a single step, directly after the photoexcitation, followed by minor relaxation steps rearranging the cofactor−protein interactions.[13,19] The occurrence of such concerted double-bond and single-bond rotation has been proposed.[20] In any case, forward- and back-reactions are different processes and not simply mirror-symmetric transformations. The intermediates of the back-reaction, Lumi-F and Meta-F, have been shown in phytochrome of the cyanobacterium *Calothrix* to absorb at 635 and 640 nm at low temperatures, respectively,[14] although the specific characteristics of both of these intermediates has not yet been described.

In order to explore these transformation processes, we investigated the intermediates of the back-reaction of phytochrome Cph1 by cross-polarization (CP) MAS NMR, exploiting our ability to prepare Pfr at 100% occupancy via size-exclusion chromatography. The intermediates were trapped directly in the magnet under illumination at low temperature. Light-triggered low-temperature MAS NMR investigations of intermediates have recently also been presented on rhodopsin,[21] bacteriorhodopsin,[22] and adenylyl transfer reaction of T4 DNA ligase.[23]

Experimental Section

Sample Preparation for MAS NMR Spectroscopy. Preparation of u-[^{13}C,^{15}N]-PCB and Cph1Δ2 is described in ref 24. Mixtures at the Pfr/Pr photoequilibrium mixtures (~70% Pfr) were produced by saturating irradiation at 660 nm using appropriate LEDs (Roithner Lasertechnik GmbH, Vienna, Austria). Pfr Cph1Δ2 phytochrome at 100% occupancy was obtained by size-exclusion chromatography of the photoequilibrium mixture using Superdex 200 (Pharmacia/GE).[25] The intermediates were produced from Cph1Δ2 in the Pfr state directly in the magnet by irradiating with light filtered through a far-red cutoff filter (λ_{max} = 730 nm). Chemical synthesis of the ^{15}N21-PCB-Cph1Δ2 sample is described in the Supporting Information.

MAS NMR Spectroscopy. About 15 mg of u-[^{13}C,^{15}N]-PCB-Cph1Δ2 protein in the Pfr state were placed in a 4 mm zirconium rotor. The Lumi-F and Meta-F intermediate states were thermally trapped in the magnet by irradiation from a xenon lamp via a far-red interference filter (λ_{max} = 730 nm) at 173 and 203 K, respectively. The illumination setup used has been specially designed for a Bruker MAS probe.[26]

All 2D ^{13}C−^{13}C dipolar-assisted rotational resonance (DARR) experiments[27] were performed in a field of 17.6 T with an Avance-WB750 spectrometer, equipped with a 4-mm triple resonance CP/MAS probe (Bruker, Karlsruhe, Germany). The ^{13}C−^{1}H dipolar interaction has been recovered by continuous wave irradiation on ^{1}H radio frequency field intensity to satisfy the n = 1 condition. Typical ^{1}H $\pi/2$ and ^{13}C π pulse lengths were set at 3.1 and 5 μs, respectively. The ^{1}H power was ramped 80−100% during CP. Mixing times of 5 and 50 ms were used to maximize homonuclear recoupling between ^{13}C nuclei. ^{1}H decoupling was about 80 kHz continuous wave (CW) during the proton mixing and about 43 kHz TPPM during acquisition. The DARR spectra were recorded with 1536 scans and with 8 μs evolution in the indirect dimension, leading to experimental times of about 80 h. The data were

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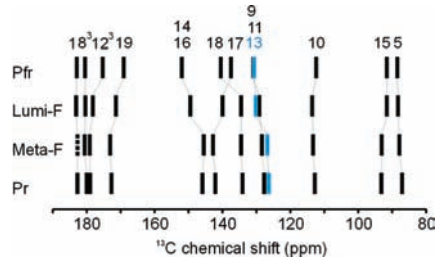

Figure 2. ^{13}C chemical shifts for selected carbon atoms during the Pfr → Pr back-reaction. For sake of clarity, the C13 resonance is noted in blue.

processed with the Topspin software version 2.0 (Bruker, Karlsruhe) and subsequently analyzed using the program Sparky version 3.100 (T. D. Goddard and D. G. Kneller, University of California).

1D ^{15}N CP/MAS spectra were recorded using an AV-750 spectrometer, equipped with 4-mm CP/MAS probe. ^{1}H−^{15}N heteronuclear experiments were performed at the Avance-WB750, using a frequency switched Lee−Goldburg pulse sequence.[28] The proton $\pi/2$ pulse was set to 3.1 μs, and the spinning frequency was 8 kHz.

For the liquid-state NMR, 500 μmol of u-[^{13}C,^{15}N]-PCB was dissolved in 0.7 mL methanol-d_4 and placed in a 5-mm NMR tube. The ^{15}N−^{1}H HMBC spectrum was obtained with a 600 MHz DMX spectrometer.

Results

Conformational Changes of the Cofactor Carbon Atoms. In a previous study, we investigated the two parent states of the phytochrome system Pr and Pfr by ^{1}H, ^{13}C, and ^{15}N CP/MAS NMR.[6] Here we report the complementary information on the intermediates of the back-reaction from Pfr to Pr, i.e., Lumi-F and Meta-F. Figures S2 and S3 show the two-dimensional (2D) ^{13}C−^{13}C DARR spectra of the Lumi-F and Meta-F intermediates. From these data, almost complete sets of ^{13}C assignments have been obtained for both intermediate states (Table S1). Calculations of ***D***-ring models confirm the assignments (see the SI). The carbon atoms C9 and C11 as well as C14 and C16 overlap in both intermediate states (for numbering, see Figure S1).

The changes of chemical shifts during the three transitions of the back-reaction (Pfr → Lumi-F, Lumi-F → Meta-F, Meta-F → Pr) for selected carbons are shown in Figure 2. The general features are as follows: (i) The major changes occur at the carbons between C13 and C19 and (ii) the transformations during the two first transitions are significantly larger than during the third transition. There are unexpectedly large chemical shift changes during the second transition. Hence, the transition from Lumi-F to Meta-F appears to be more than small rearrangements in the chromophore-binding pocket. Spatial details can be better recognized in Figure 3. At the C19 carbonyl group, the changes occur during the first two transitions to roughly the same extent. Since the Pfr state is characterized by stronger hydrogen-bonding interactions than in Pr,[6] it appears that the weakening of these interactions occurs in two steps. During the first transition, a significant change occurs selectively at position C18, and also a change of the chemical shift of the C12^{3} carboxylic group of ring ***C*** occurs already during this transformation. C18 shows no further changes, whereas during the second transition, in addition to a large C13 change, both ring carbon neighbors of the C10 and C15 bridges are strongly affected. It appears that

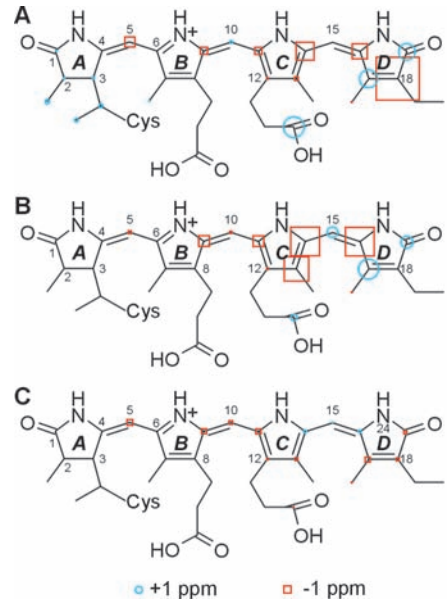

Figure 3. Changes in ^{13}C and ^{15}N chemical shift of the u-[^{13}C,^{15}N]-PCB chromophore in Cph1Δ2 during the Pfr → Lumi-F (A), Lumi-F → Meta-F (B), and Meta-F → Pr (C) transitions. For each transition, the starting state is taken as reference and the size of the circles and squares refers to the difference in ^{13}C and ^{15}N chemical shift in the next state.

the thermally driven single bond transformation following the light-induced double bond isomerization affects the electronic structure even stronger. The ***A***/***B*** ring unit shows less extensive effects, those that are seen affecting the C3^{1} thioether linkage to Cys-259 (Cph1 numbering of residues is used throughout the article). In fact, perhaps the biggest surprise is that only very weak shifts were found to be associated with C15.

Nitrogen Atom Assignments. The one-dimensional (1D) ^{15}N spectra of ^{15}N21- and u-[^{13}C,^{15}N]-PCB-Cph1Δ2 labeled phytochrome in the Pr, Pfr, and intermediates states are presented in Figure 4, columns A and B, respectively. ^{15}N21 resonates at around 158 ppm and appears to be weakly affected during the Pfr to Pr back-reaction. This evidence, forcing us to correct previous assignments,[6,29] is in line with the weak changes along ring ***A*** during the Pfr → Pr conversion deduced from the ^{13}C chemical shifts. Further insights into the ^{15}N chemical shift assignment are obtained by liquid-state NMR, which reveals that the ring ***D*** nitrogen (N24) resonance occurs at 130.9 ppm, supporting our assignment of the ^{15}N signals at 131.9 and 138.0 ppm to N24 in the Pr and Pfr states, respectively.[29] In addition, we assume that the ^{15}N resonances at 136.5 and 127.1 ppm arise from N24 in the Lumi-F and Meta-F intermediates states, respectively. The ^{15}N assignments on ring ***D*** have been confirmed by theoretical calculations (see the SI). The two remaining ^{15}N signals originate from N22 and N23. Due to the strong hydrogen-bonding interaction between N22 and the carbonyl backbone of Asp-207, the resonance of N22 is likely low-field shifted relative to the one of N23 (see the SI). Hence, we tentatively assign the peaks at 155.8, 157.9, 161.1, and 160.5 to N22 and the signals at 142.6, 146.0, 144.8, and 147.0 to N23, in the Pfr, Lumi-F, Meta-F, and Pr states, respectively (Table S2). This implies that the chemical shifts of the two inner nitrogens are different in the Pr state despite the fact that the ^{13}C chemical shifts of these rings show minimal differences. It

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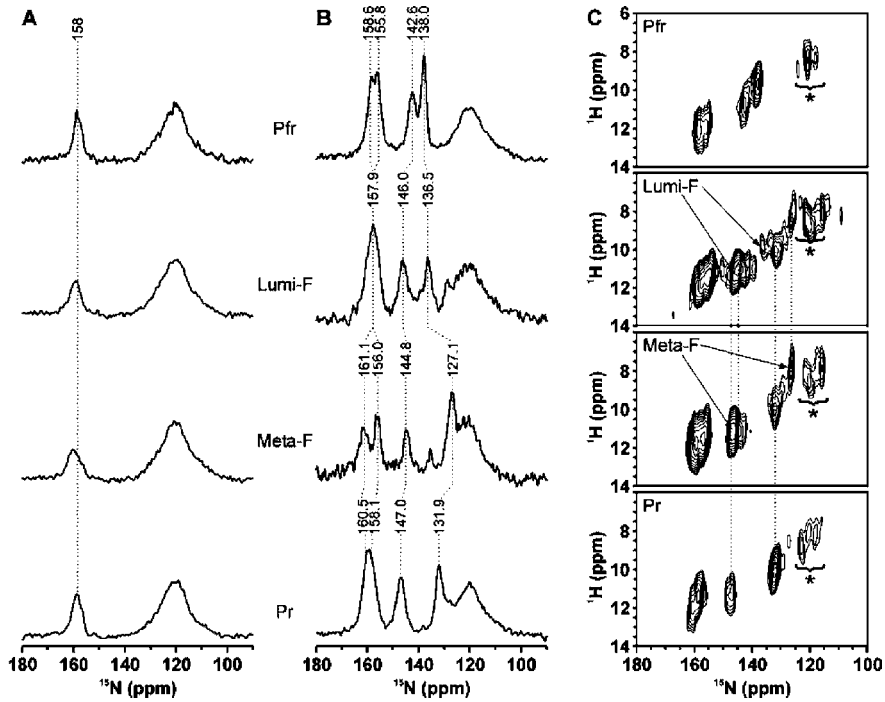

Figure 4. 1D ^{15}N spectrum of ^{15}N21-PCB-Cph1Δ2 (column A) and u-^{15}N-PCB-Cph1Δ2 (column B) in the Pfr, Lumi-F, Meta-F, and Pr recorded at 17.6T with a spinning speed of 8 kHz and at 243, 173, 203, and 243 K, respectively. 2D ^{1}H−^{15}N FSLG heteronuclear correlation spectra (column C) of Lumi-F and Meta-F were obtained from a Pfr:Pr (1:1) mixture.

appears that the electronic interaction between the conjugated π-system and the near-by nitrogens is limited.

Changes of the Nitrogen Atoms. The Pfr → Lumi-F transition causes the major effect on N22 and N23 shifting by +2.1 and −3.4 ppm, respectively (Figures 3 and 4, column B), whereas changes of the other nitrogen atoms are rather limited. Also, the proton chemical shifts (Figure 4, column C) do not undergo any significant change during this step. The Lumi-F → Meta-F transition shows stronger changes, especially on N24, which shifts 9.4 ppm downfield. Interestingly, this change is associated with a significant upfield shift of its pyrrole proton from ∼10 to ∼8 ppm (Figure 4, column C), indicating a modification of hydrogen-bonding interaction in the Meta-F intermediate state. In addition, while the signal assigned to N22 shifts from 157.9 to 161.1 ppm, no significant proton shift is observed. The Meta-F → Pr transition is again associated with strong chemical shift changes of both N24 and its pyrrole proton, implying that a hydrogen-bonding interaction at this position is restored. N21 and N23 are also affected during this transition (+2.1 and +2.4 ppm, respectively).

Discussion

Pfr → Lumi-F Transition. It has been proposed that the chromophore in the Pfr state is conformationally strained,[6,13] requiring a strong fixation of the ring ***D*** in the pocket. To this end, a strong hydrogen bond of the ring ***D*** carbonyl has been proposed, leading to enhanced conjugation from ring ***A*** to ring ***D*** which increases the stiffness of the cofactor.[1,6] Our ^{13}C data show a very strong chemical shift change at C18 specifically associated with the Pfr → Lumi-F primary photoreaction. This can be related to changes in the electronic structure directly induced by the photoisomarization and the change of hydrogen-bonding interaction at the C19 carbonyl.[6,15] Hence, we expect that the extended conjugation is already broken in the Lumi-F state, as also demonstrated by its strongly blue-shifted absorption.[14] In addition, the strong effect on C18 might also result from a mechanical interaction of the C18 ethyl group associated with the tensed cofactor state. Indeed, the thermal stability of oat phytochrome A in the Pfr state is improved when the size of the C18 side chain group is increased.[30] In recent X-ray structure on the Pfr-like state of *Pseudomonas aeruginosa* PaBphP,[5] however, no residues close to the vinyl group are observed.

The ring ***D*** nitrogen N24 has been shown to be protonated and hydrogen bound in the Pfr state (Figure 4, column C). Surprisingly, as demonstrated by the small changes in the ^{15}N shift at N24 as well as at the ^{1}H resonance of its bound proton, this hydrogen-bonding interaction remains even after the photoisomerization, implying that the cofactor is not yet fully relaxed in Lumi-F. The fact that the hydrogen bond of N24 remains almost unchanged in Lumi-F suggests that the hydrogen-bonding partner is very strong. The crystal structure of Cph1Δ2 in the Pr state indicates that the hydrogen-bonding partner is a water molecule.[4] Comparing the ^{15}N chemical shifts of Pfr, Pr, and the intermediate Meta-F, where this hydrogen bond is broken, we further conclude that the hydrogen-bonding partner in Pfr is stronger than water. It has been shown by mutational studies that Asp-207 is crucial for the formation of the Pfr state.[31] As in the X-ray structure of the PaBphP Pfr state,[5] Asp-207 could indeed be the strong hydrogen-bonding partner of N24 in the Pfr state of Cph1.

The maintenance of the hydrogen-bond of the ring ***D*** nitrogen is difficult to reconcile with a full 180° rotation. The interruption of the conjugation in the Pr state and the presence of an extended conjugation of the π system in the Pfr state also suggest a rotation less than 180°. Studying a phytochrome model com-

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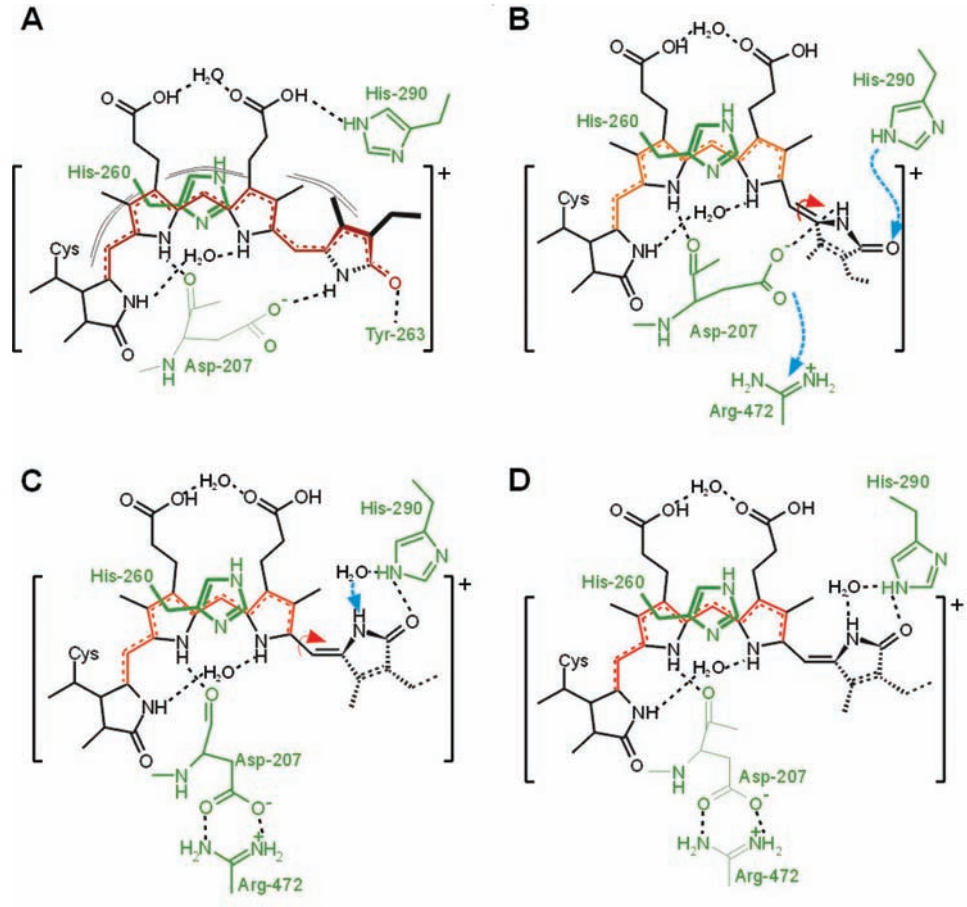

Figure 5. Structures of the tetrapyrrole chromophore and its binding pocket in the Pfr (A), Lumi-F (B), Meta-F (C), and Pr (D) states. The conjugated system is shown in dark red (Pfr), orange-red (Lumi-F, Meta-F) and red (Pr). Protein residues are indicated in green.

pound in solution, Stanek and Grubmayr[32] showed that the full *E/Z* isomerization of the C15═C16 double bond dramatically affects the ^{13}C chemical shift of the C15 carbon (7 ppm upshift upon *E/Z* isomerization). For the Pfr → Lumi-F transition, however, only a very weak effect is observed at C15, although we cannot rule out that other effects might mask the expected large change at this position. In any case, the first photochemical event which has been trapped is definitely not a classical full 180° rotation of ring ***D*** associated with a complete *E/Z* isomerization as observed in solution.

Interestingly, the protein pocket around the C12^3 carboxylic group of the ring ***C*** propionic side chain appears to be already rearranged in the Lumi-F intermediate. In particular His-290 may be in its Pr position and already interacts with the ring ***D*** carbonyl group.

Lumi-F → Meta-F Transition. Whereas the Pfr → Lumi-F transition is characterized by the photochemical *E*-to-*Z* C15═C16 double bond isomerization, the Lumi-F → Meta-F transition is a thermal process. This transition is characterized by the full release of ring ***D*** from its tensed state as indicated by two significant changes: (i) the loss of hydrogen-bonding interaction at N24 and (ii) a second stronger change associated with the methine bridges of C10 and C15. It appears that the chromophore is fully relaxed in Meta-F, whereas in Lumi-F the hydrogen bridge at N24 still prevents any significant rotation of ring ***D***. It is surprising that during the Lumi-F to Meta-F transition the changes around methine bridges C10 and C15 are stronger than in the Pfr → Lumi-F transition. Since the strong change in chemical shift observed at C13 specifically at this step is difficult to rationalize in terms of steric interactions, the combined upshift of the C13 and C14 resonances, interrupting the alternating pattern of up- and downshifts, may indicate a conjugation defect occurring after the initial *E*-to-*Z* photoisomerization.

Meta-F → Pr Transition. As the chromophore is already relaxed in the Meta-F state, the changes along the conjugated system are very minor with respect to the ^{13}C chemical shifts. Interestingly, the main effects occur at C3^1, the cofactor-matrix link, and at N24 which is rebound by a hydrogen-bonding interaction to a water molecule as seen in the X-ray Pr structure.[4] Additional minor changes indicate an overall rearrangement of the chromophore−protein interactions, potentially involving, not observable, movements of the protein environment.

Model for the Back-Reaction. We infer from our data that the tensed chromophore in Pfr is associated with three important effects (Figure 5A): (i) increased conjugation associated with C−C single bonds changing to partially higher bond orders and double bonds to partially lower bond orders, (ii) the strong hydrogen-bond of the C19 carbonyl to Tyr-263, as shown in the crystal structure of PaBphP in its Pfr-like ground state, and (iii) the strong hydrogen-bonding of ring ***D*** nitrogen, presumably to Asp-207, as also implied by the PaBph3 structure,[5] although the latter is not likely to be an entirely reliable model for the Pfr state in Cph1 and other plant-type phytochromes.

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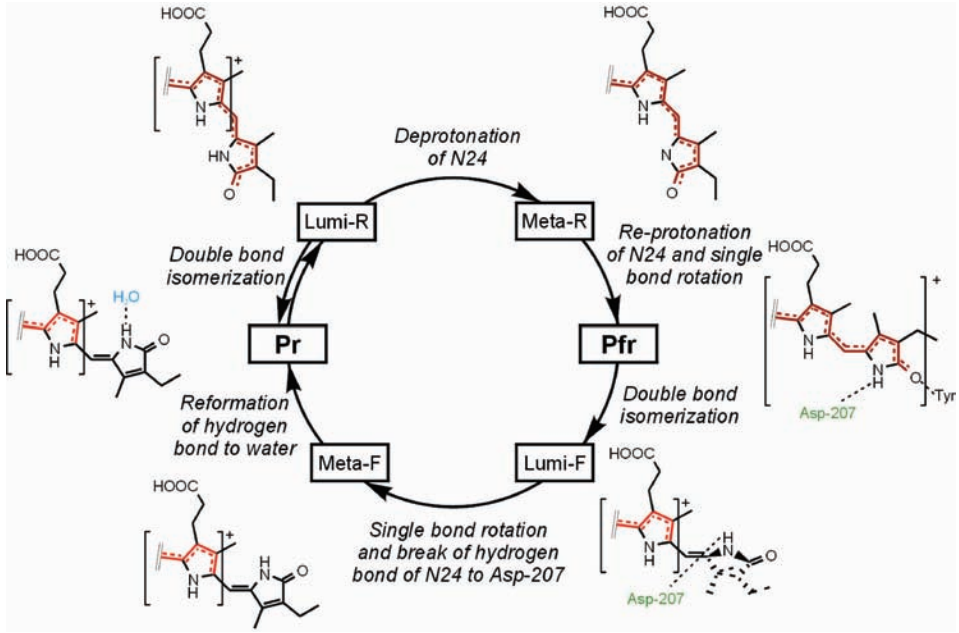
Scheme 1. Changes of the Chromophore during the Photocycle of Phytochrome



The Lumi-F state (Figure 5B) is characterized by (i) a shorter and weaker conjugation of the π-system, (ii) a partial relaxation of the cofactor due to incomplete rotation of the ring ***D*** caused by the weakening of the C19 carbonyl hydrogen-bonding interaction and possibly by the release of the C18 blocking, as well as (iii) a matrix rearrangement around the carboxylic group of the ring ***C*** propionic side chain.

The Meta-F state (Figure 5C) is described by (i) the full relaxation of the chromophore after breaking the hydrogen-bond of the ring ***D*** nitrogen to the carboxylic group of the side chain of Asp-207 allowing rotation around the C15 single bond to be completed, (ii) the appearance of a conjugation defect localized around C13 as an effect of shortening of the conjugated system, and (iii) the formation of a new hydrogen-bond between C19 carbonyl and His-290.

In the Pr state (Figure 5D), in which the cofactor is relaxed and hydrogen bound, (i) a new hydrogen-bond at N24 is formed by a water molecule and (ii) protein relaxations have occurred.

Our data show clear evidence that the chromophore isomerization during the back-reaction occurs in two steps and not in a single concerted step. The first step is linked to the C15═C16 double-bond isomerization, the second occurs at the C14−C15 single bond, and the last step is a pure relaxation process. Hence, both transformations, Pr → Pfr and Pfr → Pr, are two-step processes.

Model for Photoconversion and Signal Transduction. The data presented here describe the changes of the Cph1 chromophore from Pfr to the Pr ground state. Thus, the observed changes of the chromophore and the protein during the transition from Pfr to Lumi-F, followed by the Meta-F to Pr states, reflect changes that are connected to both photoconversion and signal transduction. The Pfr → Pr photoconversion is linked to separate chromophore−protein interactions at opposite sides of the chromophore, i.e., at the C12[3] carboxylic group of ring ***C*** and the entire ring ***D*** via its two hydrogen bonds. The highly conserved His-290, bound to the C19 carbonyl in Pr [4] and to the C12[3] carboxylic group in the Pfr-like ground state of PaBph3[5], faces inward from a β-sheet at the surface of the molecule. Although the imidazole of its equally well conserved neighbor, His-291, is exposed to the solvent in both X-ray structures, a signaling mechanism is not obvious. Similarly, from the C12[3], no molecular signal pathway is apparent although the interactions implied by the X-ray structures are very different in the Pr and Pfr states.

On the other hand, our data interpretation suggests a break of the hydrogen bond between N24 and Asp-207 during the formation of Meta-F (Figure 5B,C). The release of Asp-207 would allow for the formation of the Asp-207/Arg-472 salt bridge connecting the PHY domain tongue to the chromophore region.[4] All of these residues are highly conserved, Asp-207 being particularly important for stabilizing the Pfr state.[31] The tongue region comprises a most unusual hairpin structure inserted into the otherwise GAF/PAS-like PHY domain, making intimate contact with the extreme N-terminus of the molecule, itself unusual in forming a knot by passing through a loop in the GAF domain. Hence, the present data indicate that the Asp-207/Arg-472 association is likely to couple light-induced changes in chromophore geometry to the tongue and probably the N-terminus.

Phytochrome As Molecular Machine. The Pfr → Pr transformation occurs in two separated isomerization steps, and is linked to a switch of the hydrogen-bonding partner of N24 from Asp-207 to water (Scheme 1). Although the initial double-bond isomerization can be interpreted as process to remove the blockade of the back-reaction, the subsequent single-bond rotation is related to the release of the tension and the mechanism of signaling. The formation of the N24-water hydrogen bond requires both rotations of C14−C15 single and C15═C16 double bond. We have shown that the single-bond rotation is linked to the break of the hydrogen bond to Asp-207, leading to an intermediate Meta-F having no hydrogen-bonding interaction at N24.

As the different absorption properties suggest, the order of events may be different on the pathway from Pr to Pfr, although also this well characterized reaction course is initiated by an isomerization around a double bond and must include a subsequent single bond rotation as well as the change of the hydrogen bonding partner of N24 (Scheme 1). It is possible

that the formation of Meta-R is not only linked to a break of a hydrogen bond to water but to a deprotonation process on N24 leading to a neutral chromophore and therefore a bleached first electronic transition.[33] The single bond rotation occurs during the final transformation, the formation of Pfr from Meta-R.[17] Thus, after double-bond isomerization, the reaction course from Pr to Pfr is driven protonically, and the final single bond rotation seems to be a reaction on this charge reorganization. On the other hand, in the reaction course from Pfr to Pr, it is a mechanical force of the single-bond rotation which initiates the change of the hydrogen bonding partner. Hence, phytochrome is not a simple switch but appears to be a molecular machine able to channel light excitation to drive nonlinear processes. This nonlinearity leads to a unidirectionality of the photocycle.

The directional symmetry break of the photocycle may be required for signaling and therefore be linked to the difference in matrix interaction in Pr and Pfr. In particular, the chemical properties of the hydrogen-bonding partners of N24 are distinguished and may prearrange the direction of the reaction course. The geometrically fixed Asp-207 provides sufficient tension on the ring ***D*** to store mechanical tension on the chromophore in the Pfr state. The rather mobile water molecule may not be able to fix ring ***D*** in the Pr state to a similar extent. On the other hand, Asp-207, as negatively charged aspartate, would have sufficient force to deprotonate N24 on the reaction course to Pfr. A water molecule would not be able to lead to the same process.

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Supporting Information Available: Figures S1−S4 and Tables S1 and S2. A description of the ^{15}N tentative assignment, DFT calculation on model compounds, chemical synthesis of ^{15}N21-PCB, and biochemical synthesis of the Cph1 phytochrome. This material is available free of charge via the Internet at http://pubs.acs.org.

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