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MAS NMR study of the photoreceptor phytochrome

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Chapter 3

MAS NMR on the Pr and Pfr parent states

3.1 Introduction

A characteristic feature of all phytochromes is the photoreversibility between two states: the absorption of red light initiates photochemical activity of the thermally stable Pr state ($\lambda_{\text{max}} \sim 660$ nm) which travels through a series of intermediates [96] and eventually generates the far-red absorbing Pfr state. The Pfr state ($\lambda_{\text{max}} \sim 710$ nm), with a moderate thermal stability of several hours to days, is converted back to Pr upon absorption of a far-red photon. The origin of this red shift is not known. All phytochromes bind an open-chain tetrapyrrole (bilin) as a chromophore, whose photochemistry triggers the conversion between the Pr and Pfr states. The X-ray structures of the chromophore binding PAS-GAF bi-domain of *Deinococcus radiodurans* [18] and *Rhodopseudomonas palustris* [52], both assembled with biliverdin, have been reported. The structure of a complete PAS-GAF-PHY sensory module of Cph1 from *Synechocystis* sp. PCC 6803 phytochrome in its Pr state demonstrated that the PCB cofactor is completely sealed from access to bulk solvent [53]. Although a comparison between these 3D structures reveals some differences of the chromophore binding pocket, most of the chromophore-protein interactions are conserved. In all crystal structures, the open-chain tetrapyrrole chromophore has been reported to adopt a *ZZZssa* geometry of the three methine bridges.

The chromophore in different phytochromes has been intensively studied by various spectroscopic methods. It is commonly accepted that the conversion from Pr to Pfr is initiated by a $Z \rightarrow E$ photoisomerization of the methine bridge between rings **C** and **D** [29, 40, 43, 97]. However, the exact geometry of the chromophore in the Pfr state and the role of the chromophore binding pocket in the phototransformation have yet to be established.

Little is known about the details of the photochemical machinery that allows for intramolecular signal transduction from the chromophore to the protein surface. Mutational studies on Cph1 [19, 98] have demonstrated the crucial role of Asp-207 (Cph1 numbering of residues is used throughout the chapter) for intramolecular signal transduction (Fig. 1.6). It has been proposed that in Cph1 Tyr-176, whose side-chain is close to ring **D**, acts as a molecular gate to photoisomerization of the C15=C16 double bond [44]. In the bacteriophytochrome RpBphP2, two tyrosine residues, Tyr-207 and Tyr-272 that equivalent to Tyr-198 and Tyr-263 in Cph1, respectively, are thought to play an important role during the Pr $\rightarrow$ Pfr conversion [52].

CP/MAS NMR has evolved into a uniquely versatile tool for the structure elucidation in systems of high molecular mass and solid materials. In conjunction with selective isotope labeling, CP/MAS NMR allows for the study of large protein complexes down to the atomic level [99]. In this chapter, the N-terminal sensory modules of the cyanobacterial phytochrome Cph1 and the 65-kDa fragment of oat phytochrome A have been studied by ^{13}C and ^{15}N CP/MAS NMR. These phytochromes were assembled *in vitro* with uniformly ^{13}C - and ^{15}N -labeled PCB cofactor. In this way, selective observation of the chromophore in the protein matrix can be achieved. A full NMR analysis of the cofactor in both Pr and Pfr states is presented. Significant changes around ring **D** and the propionic side-chain of ring **C** are observed and explained by a model for signal transduction.

3.2 Results

3.2.1 Assignments of ^{13}C CP/MAS NMR spectra of Pr and Pfr states in Cph1 Δ 2

The 2D ^{13}C - ^{13}C DARR spectrum of the u- ^{13}C , ^{15}N -PCB-Cph1 Δ 2 in the Pr state was recorded with two ^1H - ^1H mixing times of 5 ms and 50 ms (Fig. 3.1, black and grey, respectively). Using a 50 ms mixing time, the carbon

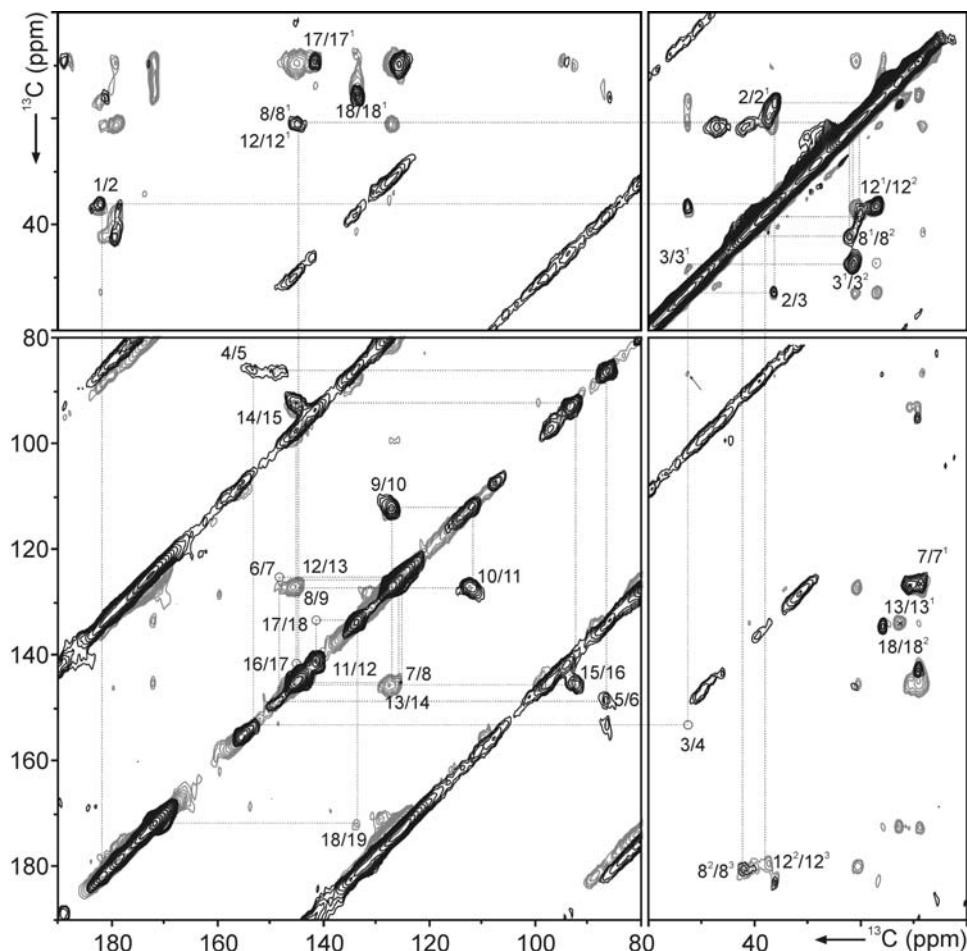


Figure 3.1: Contour plot of the 2D ^{13}C - ^{13}C DARR NMR spectra of u - $[^{13}\text{C}, ^{15}\text{N}]$ -PCB-Cph1 Δ 2 in the Pr state. Proton mixing times of 5 and 50 ms were used for the Pr datasets (black and grey, respectively) recorded at 233 K with spinning frequencies of 9 and 10 kHz. The lines indicate sequences of nearest neighbor correlations.

resonance at 182.5 ppm correlates with two aliphatic carbons allowing for an unambiguous assignment of carbons C1, C2 and C2¹ (see Table 3.1). For carbons C1 and C2, a slight doubling is observed. The well resolved correlation peaks in the aliphatic region (60 to 0 ppm) reveal the correlation network between the C2, C2¹, C3, C3¹ and C3² carbon atoms. None of the DARR spectra displays the C3/C4 cross-peak, however, the C3/C5 correlation is visible using a mixing time of 50 ms. The assignment of position C5 has been

Position	Cph1Δ2			<i>phyA65</i>		
	Pr	Pfr	Pfr-Pr	Pr	Pfr	Pfr-Pr
C1	182.5	182.8	0.3	182.6	182.5	-0.1
C1 ^a	184.2	—	1.4	—	—	—
C2	37.1	37.2	0.1	36.8	37.0	0.2
C1 ^a	38.1	—	-0.9	—	—	—
C2 ¹	17.5	18.5	1.0	17.7	17.7	0.0
C3	53.4	54.3	0.9	53.9	53.9	0.0
C3 ¹	47.6	50.0	2.4	47.6	49.1	1.5
C3 ^{1a}	—	—	—	45.8	—	3.3
C3 ²	21.8	21.4	-0.4	21.7	21.3	-0.4
C3 ^{2a}	—	—	—	23.0	—	-1.7
C4	153.9	154.0	0.1	n.d.	n.d.	—
C5	87.1	88.5	1.4	85.8	87.1	1.3
C6	149.1	148.8	-0.3	n.d.	n.d.	—
C7	126.1	126.1	0.0	124.7	124.6	-0.1
C7 ¹	9.2	9.3	0.1	7.2	7.5	0.3
C8	144.5	143.6	-0.9	146.4	143.4	-3.0
C8 ¹	22.7	23.1	0.4	21.8	23.0	1.2
C8 ^{1a}	21.5	—	1.6	—	—	—
C8 ²	43.1	41.7	-1.4	41.5	41.1	-0.4
C8 ^{2a}	41.1	—	0.6	—	—	—
C8 ³	180.5	180.5	0.0	179.9	179.7	-0.2
C8 ^{3a}	179.7	—	0.8	—	—	—
C9	127.7	131.0	3.3	127.7	130.6	2.9
C10	112.7	112.3	-0.4	113.0	111.9	-1.1
C11	127.7	131.0	3.3	127.7	130.6	2.9
C12	145.5	145.8	0.3	146.4	144.5	-1.9
C12 ¹	21.1	20.6	-0.5	21.8	21.0	-0.8
C12 ²	37.5	38.3	0.8	41.5	39.0	-2.5
C12 ³	179.5	175.2	-4.3	177.5	174.7	-2.8
C13	126.3	130.6	4.3	123.7	130.1	6.4
C13 ¹	11.3	11.3	0.0	10.0	12.1	2.1
C14	145.7	151.5	5.8	146.6	150.5	3.9
C15	93.5	91.7	-1.8	93.3	92.3	-1.0
C16	145.7	151.1	5.4	146.6	150.5	3.9
C17	142.2	137.7	-4.5	142.6	138.6	-4.0
C17 ¹	9.8	10.1	0.3	10.3	10.3	0.0
C18	134.3	140.4	-6.1	133.3	140.1	6.8
C18 ¹	16.1	15.6	-0.5	16.6	n.d.	—
C18 ²	13.3	13.3	0.0	n.d.	n.d.	—
C19	172.8	168.9	-3.9	172.4	169.6	-2.8

Table 3.1: ^{13}C chemical shifts obtained for Cph1Δ2 and *phyA65* containing an u-[^{13}C , ^{15}N]-PCB chromophore.

confirmed by a 1D ^{13}C CP/MAS NMR spectrum of the $^{13}\text{C5}$ -PCB-*phyA65* (Appendix B, Fig. B.1). The C4/C5 and C5/C6 correlations are visible in the spectrum recorded with a short proton mixing time of 5 ms. The two

neighbors of carbon C5 are difficult to distinguish although a weak correlation with C7 suggests that $^{13}\text{C}6$ resonates with a chemical shift of 149.1 ppm. The ^{13}C - ^{13}C correlation network along the pyrrole rings **B** and **C** is shown in Figure 3.1 and allows for the assignment of the carbon atoms up to position C16. The C16/C17 and C17/C18 correlation peaks are weakly visible, the assignments of C17 and C18 have been obtained from the C18/C19 correlation as well as from the methyl and ethyl side-chain (C17¹, C18¹ and C18²). The ^{13}C assignment reveals that the central rings **B** and **C** of the chromophore are highly symmetrical in the Pr state. The ^{13}C - ^{13}C correlations in the aliphatic domain show a single correlation network for one of the propionic side-chains (23.1, 41.7 and 180.5 ppm) and a split correlation network for the second propionic side-chain (22.5, 42.8, 180.6 ppm, and 21.5, 41.1, 179.7 ppm). Since the C8/C8¹ and C12/C12¹ correlation signals overlap, the two propionic side-chains cannot be assigned unambiguously. However, the chemical shift differences occurring in comparison with the Pfr state (see below) make an assignment of the propionic side-chain with the single correlation network to carbon C12 more reasonable.

The 2D ^{13}C - ^{13}C DARR spectra of Cph1Δ2 in the Pfr state have been recorded with two ^1H - ^1H mixing times (5 and 50 ms) and are depicted in Figure 3.2 (black and grey, respectively). The ^{13}C assignments have been obtained in the same manner as for the Pr state. In the Pfr state, the C8/C8¹ and C12/C12¹ correlation peaks do not overlap. In addition, the C12/C13, C13/C13¹ and C13¹/C14 cross-peaks are visible in the 2D spectrum and allow for the unambiguous assignment of the two propionic side-chains. While in the side-chain of ring **B** two sets of chemical shifts appear for the Pr state, only a single set is detected for the Pfr state. This is similar to the observation of doubling at the ring **A** carbons C1 and C2. Since also optical spectroscopy, operating on a fast timescale, observes two distinguished forms of the PCB cofactor in the Pr state [100,101], it can be assumed that the two conformers coexist in solution. As the origin of this heterogeneity, mobility of the nearby N-terminal-helix (Thr-4 to Leu-18) that seals parts of the chromophore binding site along ring **A** and **B** can be postulated. The disappearance of the doublings in the Pfr state may be caused by a decreased mobility in this region, thus leading to conformational homogeneity.

A ^1H - ^{13}C heteronuclear 2D spectrum of the region of the methine carbons of u-[^{13}C , ^{15}N]-PCB in Cph1Δ2 is shown in Figure B.2. The signal labeled

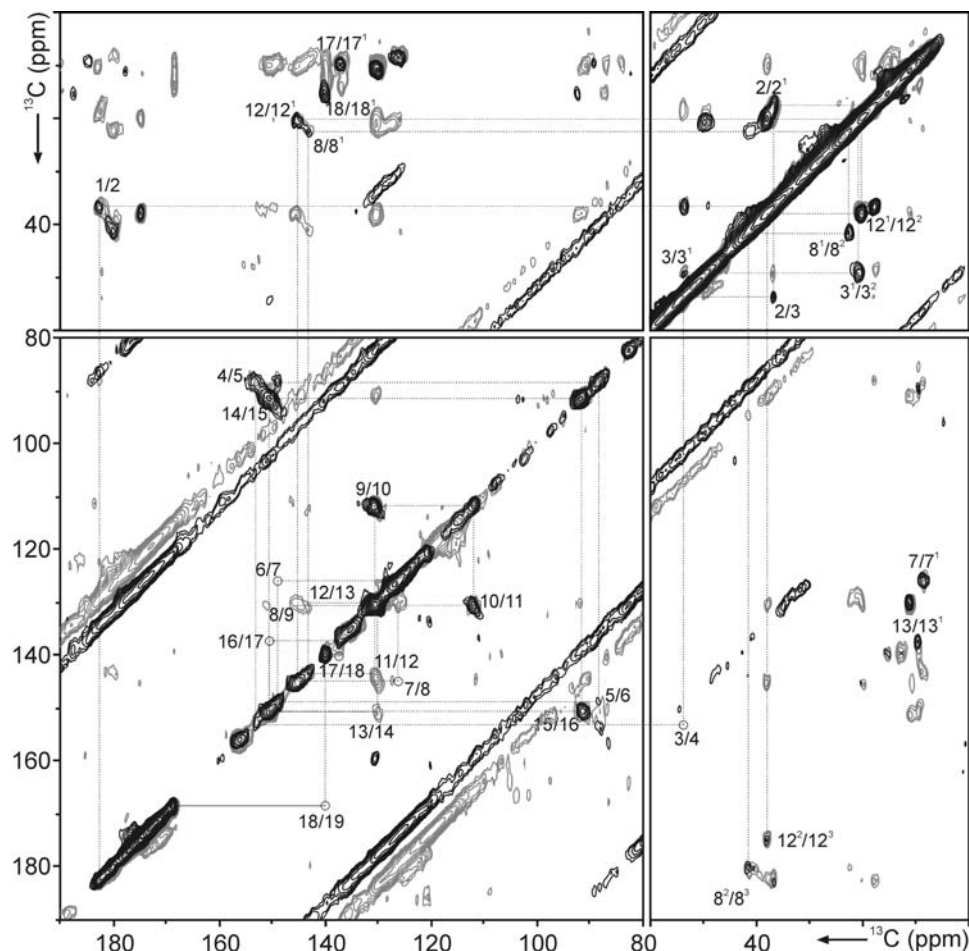


Figure 3.2: Contour plot of the 2D ^{13}C - ^{13}C DARR NMR spectra of u - ^{13}C , ^{15}N -PCB-Cph1 Δ 2 in the Pfr state recorded at 233 K in a field of 17.6 T using proton mixing times of 5 and 50 ms (black and grey, respectively) and spinning frequencies of 9 and 10 kHz, respectively.

with an asterisk originates from the protein. The low-field response of H10 has also been observed previously for the Pr state by liquid-state NMR [102] and appears to be an intrinsic property of open-chain tetrapyrrole compounds [103]. Upon photoconversion to the Pfr state, the chemical shift of H5 remains unchanged, while at H10 and H15 high-field shifts of 0.4 ppm are observed. A shift for H15 can in principle be expected due to the isomerization of the C15=C16 double bond [103].

Position	Cph1Δ2			<i>phyA65</i>		
	Pr	Pfr	Pr-Pfr	Pr	Pfr	Pr-Pfr
N21	158.1	158.6	-0.5	159.5, 156.8	159.7, 157.3	
N22	160.5	155.8	4.7			
N23	147.0	142.6	4.4	146.7	143.5	3.2
N24	131.9	138.0	-6.1	133.6	137.7	-4.1

Table 3.2: ^{15}N chemical shifts obtained for Cph1Δ2 and *phyA65* containing an u- ^{13}C , ^{15}N -PCB chromophore.

3.2.2 ^{15}N CP/MAS NMR of u- ^{13}C , ^{15}N -PCB-Cph1Δ2

The ^{15}N CP/MAS NMR spectra of the u- ^{13}C , ^{15}N -PCB-Cph1Δ2 in the Pr and Pfr states are presented in Figure 3.3, column A. The $^{15}\text{N21}$ -PCB was chemically synthesized and assembled to Cph1Δ2 to assign the ^{15}N response of the ring **A** nitrogen (Fig. 3.3B). The broad resonance at ~ 120 ppm is due to the amide nitrogens of the protein backbone originating from ^{15}N nitrogens in natural abundance. The spectrum of the Pr state shows three distinct ^{15}N maxima at 159, 147 and 132 ppm, while the spectrum of pure Pfr exhibits three cofactor maxima at 158, 143 and 138 ppm. The asterisks indicate the position of protein backbone signals in natural abundance. A deconvolution using a Voigt function with a Lorentzian:Gaussian ratio of 1:1 was applied to resolve the overlapping signals and to obtain the accurate chemical shifts (Fig. B.3). The chemical shifts of the simulated chromophore peaks have been determined at (i) 160.5, 158.1, 147.0 and 131.9 ppm for Pr and (ii) 158.6, 155.8, 142.6 and 138.0 ppm for Pfr (Table 3.2).

The 1D ^{15}N spectra of $^{15}\text{N21}$ -PCB-Cph1Δ2 (Fig. 3.3B) reveal that the ring **A** nitrogen atom exhibits a chemical shift at around 158 ppm in both Pr and Pfr states. Thus the peaks at 158.1 and 158.6 ppm are assigned to the ring **A** nitrogen in the Pr and Pfr states, respectively. As shown in **Chapter 2**, the ring **D** ^{15}N of the u- ^{13}C , ^{15}N -PCB in its free state resonates with a chemical shift of 133.1 ppm. The ^{15}N signals at 132 and 138 ppm in the Pr and Pfr state are tentatively assigned to N24, while the peaks at 160.5 and 147.0 ppm in the Pr state and at 155.8 and 142.6 ppm in the Pfr state are attributed to the ^{15}N responses of rings **B** and **C**. In both states, one of the inner ring ^{15}N signals appears downshifted by about 10 ppm in comparison with the other.

Since all nitrogens of the cofactor are protonated [39, 94, 104], the tetrapyrrole is positively charged in both Pr and Pfr states. The X-ray structure

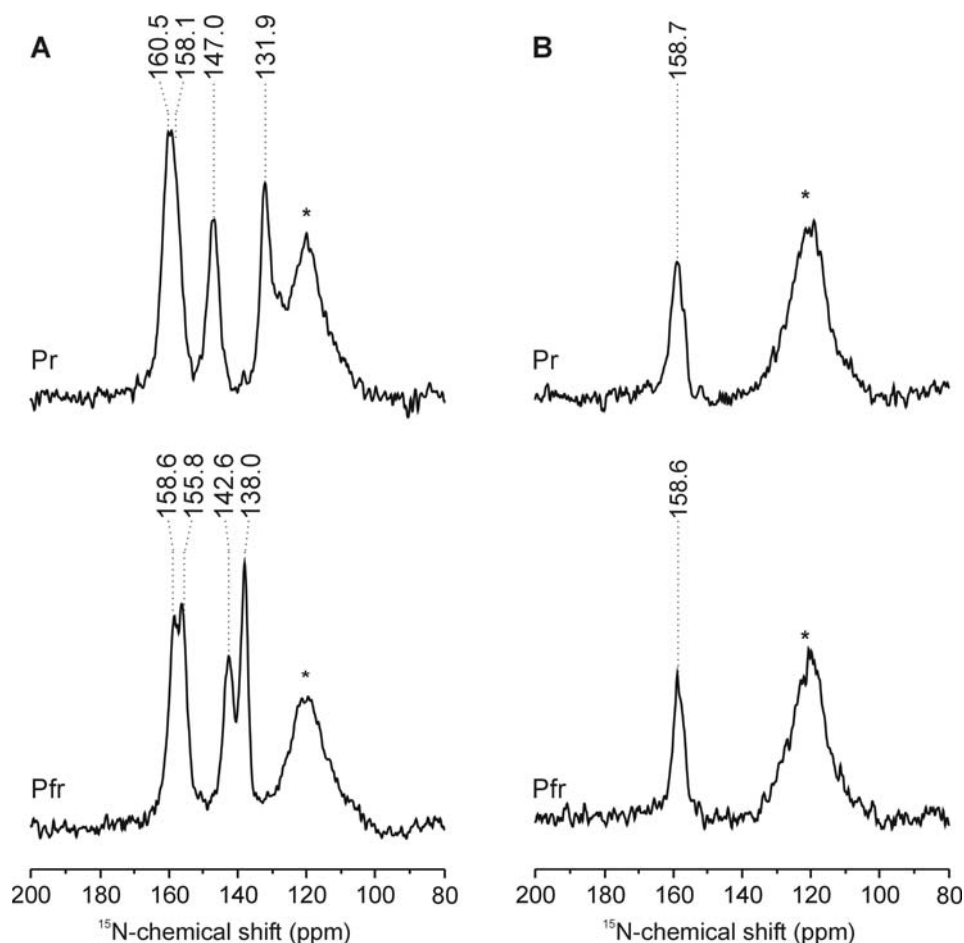


Figure 3.3: 1D ^{15}N CP/MAS NMR spectra of $u\text{-}[^{13}\text{C}, ^{15}\text{N}]\text{-PCB-Cph1}\Delta 2$ (A) and $^{15}\text{N}21\text{-PCB-Cph1}\Delta 2$ (B) recorded at 243 K in a field of 17.6 T and a spinning frequency of 8 kHz. The asterisks indicate the signal due to the protein backbone originating from ^{15}N nitrogens in natural abundance.

of Cph1 (PDB ID 2VEA) shows that the amide oxygen of Asp-207 is at hydrogen-bonding distance to N21, N22 and N23. The distance between this oxygen and N22 is as short as 2.72 Å, *i.e.*, three time shorter than the standard deviation of the average $\text{N}\cdots\text{O}$ distance in a $\text{N}\text{—H}\cdots\text{O}$ hydrogen-bonding interaction [105]. The asymmetry of the hydrogen-bonding environment may explain that the ^{15}N signal of N22 is shifted relative to the response of N23. The peaks at 160.5 and 147.0 ppm are tentatively assigned to N22 and N23,

respectively. In analogy, the signal at 155.8 and 142.6 are respectively assigned to N22 and N23 in the Pfr state.

3.2.3 Pr $\rightarrow$ Pfr conversion in Cph1 Δ 2

The change in ^{13}C chemical shifts generated by the Pr $\rightarrow$ Pfr conversion are depicted in Figure 3.4A. After photoconversion mostly the rings **C** and **D** are affected, while smaller changes are observed also in rings **A** and **B**. It is generally accepted that the first step of the Pr $\rightarrow$ Pfr conversion is the photoisomerization of the C15=C16 double bond. The change in chemical shift for the carbons at the C15 methine bridge and at N24 bring further evidence for a photoisomerization occurring at the C15=C16 double bond. In addition, the entire ring **D** shows considerable changes in its ^{13}C shifts, denoting a modification of its interaction with the protein surroundings. The Pr $\rightarrow$ Pfr transformation affects also slightly the two methine bridges between rings **A**–**B** and **B**–**C**. At the **B**–**C** methine bridge, the resonances of C9 and C11 are equally downshifted by 3.3 ppm. The significant downshift at the C3¹ position by 2.4 ppm suggests a modification of the chromophore–protein linkage. The C12³ carbon atom exhibits a significant 4.3 ppm upfield shift after photoconversion pointing to a change of the environment of the carboxylic moiety. According to the crystal structures this carboxyl group of the propionic side-chain of ring **C** interacts *via* two water molecules with the conserved His-290, which in turn interacts with ring **D** [51–53]. On the other hand, there are no indications for changes at the propionic side-chain of ring **B**, which forms a salt-bridge *via* two hydrogen-bonds to the conserved Arg-254 in the Pr state [53]. Apparently, this interaction remains unaffected in the Pfr state.

3.2.4 Pr $\rightarrow$ Pfr conversion in the plant phytochrome *phyA*

The 1D ^{13}C CP/MAS NMR spectra of u- ^{13}C , ^{15}N -PCB-Cph1 Δ 2 in the Pr (black) and Pfr (grey) states are shown in Figure 3.5A. The Pr-Pfr difference spectrum (Fig. 3.5B) reveals the changes of chemical shifts generated by the Pr $\rightarrow$ Pfr photoconversion. Positive signals represent the Pr state and the negative signals arise from the Pfr state. In comparison, Figures 3.5C and 3.5D show the corresponding spectra for the plant-derived phytochrome u- ^{13}C , ^{15}N -PCB-*phyA*65. The Pr (black) and Pfr (grey) states are displayed in

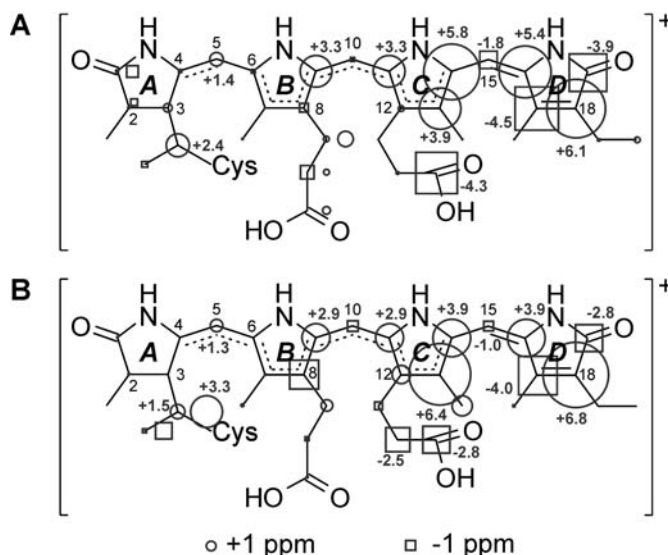


Figure 3.4: Changes in ^{13}C chemical shifts of the u- ^{13}C , ^{15}N -PCB chromophore in Cph1Δ2 (A) and *phyA65* (B). The Pr state is taken as reference and the size of the circles and squares refers to the difference in ^{13}C chemical shift in the Pfr state. The carbons showing two resonances are labeled with two symbols. The positive charge due to the protonation of the ring B nitrogen is thought to be delocalized mainly over rings B and C, and the non planarity of the C14–C15 single bond breaks the conjugation with ring D.

Figure 3.5C and the Pr–Pfr difference spectrum is presented in Figure 3.5D. The two difference spectra (Fig. 3.5B and D) show similarities, in particular in the methine and aromatic carbons regions. A small but clearly detectable difference however is noted in the spectral region around 125 ppm. The 1D ^{15}N CP/MAS NMR spectra of the u- ^{13}C , ^{15}N -PCB-Cph1Δ2 in the Pr and Pfr states are presented in Figure B.4. The similarity of the ^{13}C and ^{15}N spectra of Cph1Δ2 and *phyA65* suggests that the chromophore geometry and the immediate protein environment are comparable in both species.

The 2D ^{13}C - ^{13}C DARR NMR spectra of the Pr state and of the Pfr/Pr mixture (ratio $\sim 1:1$) of *phyA65* (Fig. B.5) lead to the ^{13}C assignments in both Pr and Pfr states (Table 3.1). The change in chemical shift accompanying the Pr $\rightarrow$ Pfr conversion is shown in Figure 3.4B. Also, the comparison of Figures 3.4A and B demonstrates that the phototransformation affects the chromophore in Cph1Δ2 and *phyA65* in a similar manner. In *phyA65*, however, the C13 and C13¹ signals shift more than for Cph1Δ2. This explains the

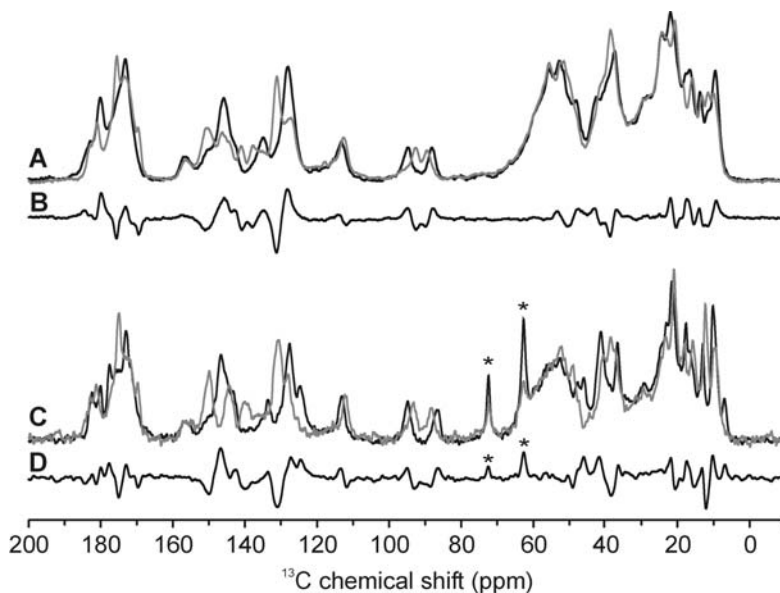


Figure 3.5: 1D ^{13}C CP/MAS NMR spectra of $u\text{-}[^{13}\text{C}, ^{15}\text{N}]\text{-PCB}$ in Cph1 Δ 2 and *phyA65* in the Pr (**A** and **C**, black, respectively) and Pfr (**A** and **C**, grey, respectively) states. The Pr-Pfr difference spectra in Cph1 Δ 2 and *phyA65* are shown in **B** and **D**, respectively. Positive signals refer to Pr and negative signals to Pfr. The asterisks indicate the position of glycol signals in natural abundance.

difference observed at 123 ppm between the difference spectra 3.4B and D.

As shown by vibrational techniques, the conformations of the bilin chromophore in the Pr and Pfr states in native plant phytochrome (*phyA124*) resemble those in the photosensory modules *phyA65* of oat phytochrome [39,41] and Cph1 of *Synechocystis* [106]. The NMR data presented in this chapter provide additional clear evidence that the chromophore and its interactions with the protein are conserved in both states, possibly across the entire Cph1/plant phytochrome family. In view of these similarities, the observations for the whole Cph1 and plant phytochrome will be discussed in a common framework.

3.3 Discussion

3.3.1 Chromophore photoconversion

As shown in Figures 3.4A and B, the photoconversion of PCB mostly affects rings **C** and **D**. This is in line with a photoisomerization occurring along the C15=C16 double bond. During this process the chromophore is involved throughout its entire structure, hence, the photoisomerization is not a local event but modifies the entire chromophore/protein interaction. A significant effect is also seen at the C10 methine carbon that is situated between the almost coplanar rings **B** and **C**. In contrast, the shifts around methine carbon C5 that links rings **A** and **B** are small. The observed pattern can be rationalized by the assumption of five effects: (i) The chromophore is tensely fixed in the Pfr state, (ii) the conjugation increases in the Pfr state, (iii) the hydrogen-bonding interaction of the ring **D** carbonyl increases in the Pfr state, (iv) a local change of the electronic structure around ring **C** is identified, and (v) a significant change of the protein interacting with the ring **C** carboxylic group takes place.

(i) A loss of conformational heterogeneity at the ring **B** propionic side-chain implies an improved fit of the chromophore in the protein. Several cross-peaks at rings **A** and **B** become sharper in the Pfr spectrum, *e.g.*, carbons C4 and C6. The signal doubling observed for carbons C1 and C2 in the Pr state disappears in the Pfr state. These observations suggest that an induced fit mechanism balances an increase of mechanical tension occurring in the Pfr state, as is also observed by ^{15}N MAS NMR [104] and vibrational spectroscopy [43]. Such tension would also explain the shift observed at C3¹ that covalently links the chromophore *via* a thioether linkage to Cys-259.

(ii) The entire conjugation pattern undergoes a significant modification. In Figure 3.4 on the left side of ring **C**, even numbered carbons are marked for a downfield shift (circle), while odd numbered carbons are up-shifted (square). On the right side of ring **C**, however, the opposite pattern appears. Such pattern change, involving the entire conjugated chain implies a change in bond order. Assuming enhanced tension in the Pfr state, it can be proposed that an increase of bond-order for single bonds, increasing their rotational energy and providing the required stiffness, takes place concomitantly with a decrease in bond order for the double bonds.

(iii) There is a clear upshift at the carbonyl of ring **D** associated with

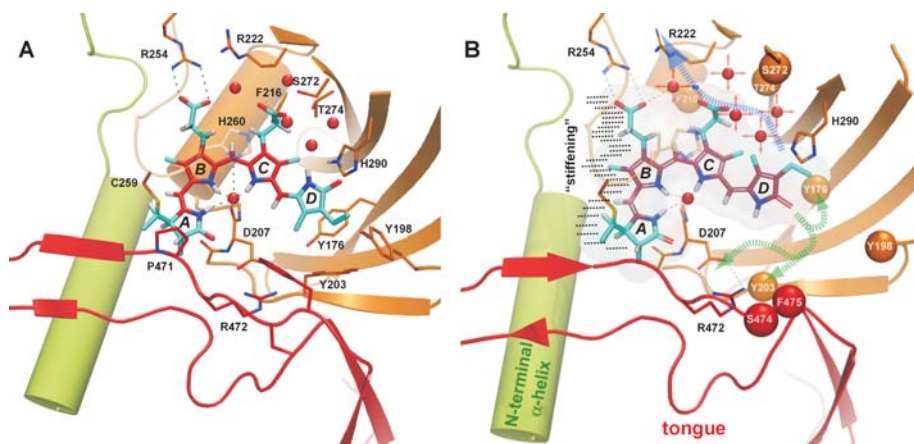


Figure 3.6: The PCB binding site of Cph1 Δ 2. Structural view on the protein environment of the PCB chromophore in the Pr state [53] highlighting the hydrogen-bonding network (A). Overview of putative structural changes caused by Pfr state formation (B). The dashed arrows indicate potential paths of signal transmission within the chromophore binding site. The observed loss of conformational freedom along rings A and B is highlighted by dotted lines. This figure was made by PyMol [107].

decreased electron density at carbons C17 and C19. A clear change of this group has also been shown by FTIR spectroscopy [39, 40]. In the Pr state of Cph1 (Fig. 3.6A) a weak hydrogen-bond is formed between the ring D carbonyl and His-290 due to its energetically unfavorable angle of 102° ($O-H\epsilon-N\epsilon$) caused by the low tilt of ring D *vs.* ring C (26.3°). An increase of hydrogen-bonding interactions can take place for the ring D carbonyl during Pfr state formation. Such an increased polarization at the terminal group may cause an increasing conjugation throughout the entire chain of the chromophore and hence the red-shift of Pfr absorption. It is reasonable to assume that a strong hydrogen-bonding involving the carbonyl of ring D stabilizes the chromophore in the Pfr state.

(iv) While the chemical shifts of carbons in rings B and C are almost mirror symmetrical around C10 in the Pr state, this symmetry is partially broken in the Pfr state. While in the other rings an alternating pattern along the conjugated chain occurs, solely in ring C all carbons are downshifted in the Pfr state. This is indicative of an increase of electron density in the Pfr state at this ring. The perturbation of the alternating pattern at ring C suggests that the origin of the change of the conjugation pattern is localized

here. It is possible that in the Pr state the conjugation is interrupted at or around ring **C** due to the interplanar tilt between rings **C** and **D**, and that the observed alternating pattern is caused by the extension of the conjugated system beyond ring **C**. Such an effect could also explain the red-shift of the absorption spectrum upon generating the Pfr state. In any case, ring **C** appears to be the hotspot for the change of the electronic structure.

(*v*) At the propionic side-chain of ring **C**, a significant up-shift of the ^{13}C resonance at the carboxylic group occurs. It can be assumed that this group faces strongly altered interactions with the protein environment. This change may correspond to a modification previously observed by FTIR spectroscopy [39,40] which has been interpreted as an alteration of either a protein amide or carboxyl group. In contrast to the ring **B** propionic side-chain, the ring **C** propionic group is well hydrated within a cluster of five water molecules and makes only an indirect interaction with the Arg-222 *via* a water molecule (Fig. 3.6A). Interestingly, a structural comparison between Cph1 and the BphPs shows that Arg-222 may either adopt an outward-oriented conformation towards the GAF-PAS interface or point into the core region of the GAF domain itself.

Hence, from this analysis, the following picture of the Pr $\rightarrow$ Pfr photo-transformation emerges: The chromophore forms a strong hydrogen-bond *via* its ring **D** carbonyl, increasing both the strength and length of the conjugation network and stabilizing the chromophore in a tensed shape (Fig. 3.6B). The rings **A** and **B** are tightly packed in the protein pocket. The observed changes in ^{15}N chemical shifts in the rings **C** and **D** may be explained by such tension linked to traction at this side of the chromophore, leading to a banana-shaped cofactor in the Pfr state. In addition, it is also possible that the changes in ^{13}C and ^{15}N chemical shifts are linked to a redistribution of the positive charge along the chromophore during the conversion from Pr to Pfr to mediate the bond order alteration and promote an induced fit in the Pfr state.

3.3.2 Charge localization in phytochrome

As shown by the MAS NMR study of the free PCB in **Chapter 2**, solely the nitrogen of ring **B** is unprotonated. Therefore, the ring **B** is a pyrroline ring with a "pyridine" kind of nitrogen and ring **C** is a pyrrole ring. The nitrogen atom of the pyrrole ring is protonated, the hydrogen being in plane

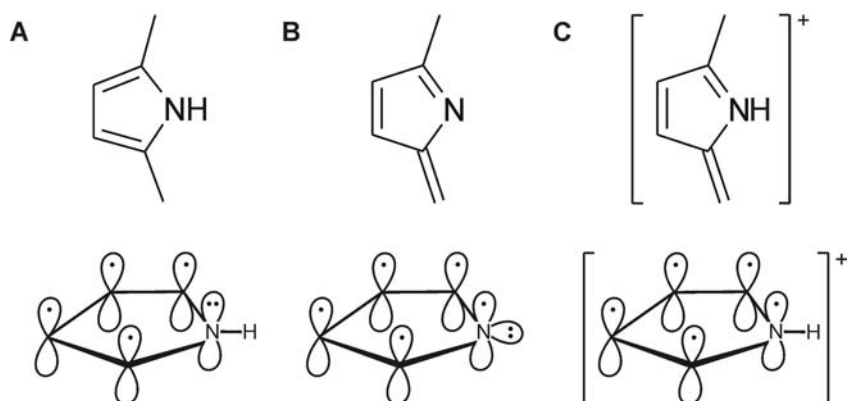


Figure 3.7: Structure of pyrrole (**A**), pyrrolenine (**B**) and protonated pyrrolenine rings (**C**).

with the ring and, according to the Hückel rule, the two π -electrons of the lone pair of the nitrogen atom are delocalized in the aromatic ring (Fig. 3.7A). The two π -electrons of the lone pair can be considered as a conjugation defect in the sense of polymer physics [108]. In the case of the pyrrolenine ring, the nitrogen is conjugated with the π -system (Fig. 3.7B). The assembly of PCB in the protein pocket leads to the protonation of the nitrogen of the pyrrolenine ring. The protonated pyrrolenine ring is positively charged and the nitrogen is conjugated with the π -system (Fig. 3.7C).

Due to the conjugation of the π -system in open-chain tetrapyrrole, the positive charge can be potentially delocalized over the four the nitrogen atoms/rings (Fig. 3.8). The crystal structures of phytochromes do not show the presence of a strong counterion in the vicinity of the chromophore, therefore the positively charged chromophore appears to be in an unstable state. In the Pr state, the two inner rings have very similar ^{13}C chemical shifts, suggesting a symmetric electronic structure of the conjugated π -system in the rings **B** and **C**. This suggests that the positive charge is mainly spread over the two inner rings **B** and **C** and that the mesomeric forms **II** and **III** are prevailing in the Pr state (Fig. 3.8). In addition, studies of model compounds of phytochrome have shown that the an appreciable positive charge resides on the central atom C10 [109].

The conversion to the Pfr state is accompanied by a general downshift of the ^{13}C resonances at the ring **C**. This indicates a modification of the electro-

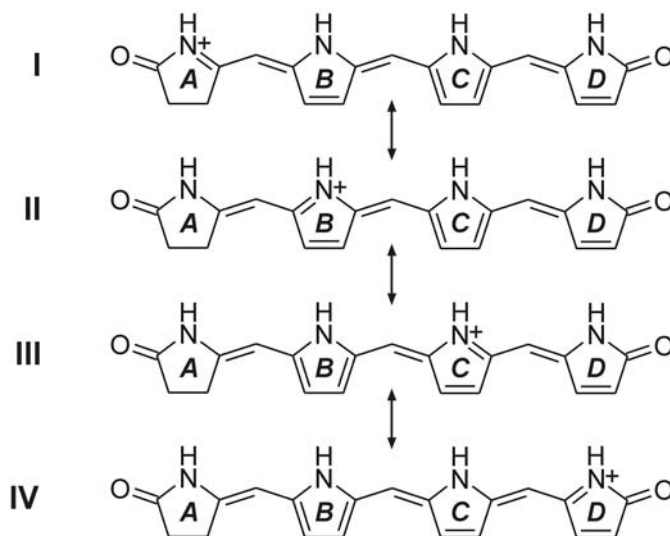


Figure 3.8: Resonance structures of protonated PCB chromophore in phytochrome. The positively charged nitrogen is conjugated and the others are representing conjugation defects in the conjugated system.

nic structure along this ring, which may be associated with a redistribution of the positive charge along the chromophore. Indeed an extended delocalization of the positive charge over the **B** and **C** rings as well as the **D** ring, *i.e.* Figure 3.8 forms **II**, **III** and **IV**, may contribute to increase the stability of the chromophore in the Pfr state. By switching with light, the protein can conveniently balance the charge in such a system and mediate an induced fit by the charge polarization of the chromophore and the polar protein surroundings in the Pfr state.

3.3.3 Signal transduction pathway

The question arises at which positions the chromophore dynamics is coupled to the protein environment to allow signal transduction to the protein surface. Important information can be extracted from Figures 3.4A and B. The changes occurring on the left side of ring **C** can be explained by a change of conjugation. On the other hand, on the right side of ring **C** multiple effects are overlaying and two of them are clearly due to changed chromophore-protein interaction. There are significant changes localized at the carboxylic

group of the propionic side-chain of ring **C** as well as at the carbonyl group of ring **D**. The X-ray structures of Cph1 and the DrBphP bacteriophytochromes show that His-290 (His-372 in *phyA65* and His-299 in DrBphP) is bridged *via* two conserved water molecules to the carboxyl group of ring **C** and forms a hydrogen-bond to the carbonyl of ring **D** [18, 52, 53] (Fig. 3.6). Hence, it can be suggested that this highly conserved His-290 couples the photochemistry of the chromophore to the protein.

Since the ^{13}C signal of the ring **A** carbonyl and the ^{15}N signal of the ring **A** nitrogen exhibit only minor changes during the Pr $\rightarrow$ Pfr photoconversion, only local conformational changes of the protein matrix surroundings are likely to occur. On the other hand, changes of the hydrogen-bonding network by ring **D** photoisomerisation could alter the conserved salt bridge between Asp-207 and Arg-472 and thus transmit the signal to the protein surface, *e.g.*, by rearrangement of the tongue region (Fig. 3.6B). As possible conserved partners for strong hydrogen-bonding to the ring **D** carbonyl in the Pfr state nearby located hydrogen-bonding donors such as Asp-207, Tyr-198, Tyr-203, Tyr-263 and Ser-474 may be considered. Based on the crystal structure of PaBphP in its Pfr-like ground state [55], Tyr-263 may indeed be hydrogen-bonded to the C19 carbonyl (see also **Chapter 4**).

3.4 Materials and Methods

3.4.1 Sample preparation for MAS NMR spectroscopy

The u- ^{13}C , ^{15}N -PCB was prepared following published methods [94]. Preparation of Cph1 Δ 2 and *phyA65* apo- and holoproteins were performed as described [21, 110]. For the measurements of the Pr state, samples were irradiated with light filtered through a far-red cut-off filter ($\lambda_{\text{max}} \sim 730$ nm). Pfr/Pr mixtures were produced by saturating irradiation at 660 nm. Cph1 Δ 2 phytochrome in its pure Pfr state was obtained by size-exclusion chromatography using Superdex 200 (Pharmacia/GE-Healthcare, USA) [111].

3.4.2 MAS NMR spectroscopy

1D ^{13}C CP/MAS spectra were recorded using a DMX-400 spectrometer, equipped with 4-mm CP/MAS probe. All data were recorded at 243K with a spinning frequency of 10 kHz. The proton $\pi/2$ pulse was set to 3.6 μs . The

^1H power was ramped 80-100% during CP. During the data acquisition, the protons were decoupled from the carbons by use of the TPPM decoupling scheme [65]. For u- ^{13}C , ^{15}N -PCB-Cph1 Δ 2 measurements in the Pr and Pfr states, approximately 15 mg of protein were placed in a 4-mm zirconia rotor. About 12 mg of u- ^{13}C , ^{15}N -PCB-*phyA*65 was used for the measurements of the Pr state and Pfr/Pr (1:1) mixture.

All 2D ^{13}C - ^{13}C DARR experiments 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). ^1H $\pi/2$ - and ^{13}C π -pulse lengths were set at 3.1 μs and 5 μs , respectively. The ^1H power was ramped 80-100% during CP. Spin diffusion periods of 5 and 50 ms were applied. ^1H CW decoupling was about 80 kHz during the proton mixing and about 43 kHz for TPPM decoupling during acquisition. The 2D ^{13}C - ^{13}C spectra of Cph1 Δ 2 were recorded with 1536 scans, and with 8 ms evolution in the indirect dimension, leading to experimental times of 80 hours. The spectra of the *phyA*65 protein were recorded with 2048 scans in 2.5 days. The data were processed with the Topspin software version 2.0 (Bruker, Karlsruhe, Germany) and subsequently analyzed using the program Sparky version 3.100 (T. D. Goddard & D. G. Kneller, University of California, San Francisco, USA).

1D ^{15}N CP/MAS spectra were recorded using an AV-750 spectrometer, equipped with 4-mm CP/MAS probe. The proton $\pi/2$ - pulse was set to 3.1 μs , temperature was 243 K and the spinning frequency 8 kHz. ^1H - ^{13}C heteronuclear experiments were performed at a DMX-400 spectrometer (Bruker, Karlsruhe, Germany) using a frequency switched Lee-Goldburg pulse sequence [68].