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Dynamic Nuclear Polarization Provides New Insights into Chromophore Structure in Phytochrome Photoreceptors

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Abstract: *Phytochromes are red/far-red photochromic photoreceptors acting as master regulators of development in higher plants, thereby controlling transcription of about 20% of their genes. Light-induced isomerization of the bilin chromophore leads to large rearrangements in protein structure, whereby the role of protonation dynamics and charge distribution is of particular interest. To help unravel the inherent mechanisms, we present two-dimensional dynamic nuclear polarization (DNP) enhanced solid-state magic-angle spinning (MAS) NMR spectra of the functional sensory module of the cyanobacterial phytochrome Cph1. To this end, the pyrrole ring nitrogen signals were assigned unequivocally, enabling us to locate the positive charge of the phycocyanobilin (PCB) chromophore. To help analyze proton exchange pathways, the proximity of PCB ring nitrogen atoms and functionally relevant H_2O molecules was also determined. Our study demonstrates the value of DNP in biological solid-state MAS NMR spectroscopy.*

Phytochromes are red/far-red photochromic biliprotein photoreceptors, in plants regulating about 20% of all genes and thereby mediating fundamental effects of light on development such as germination, de-etiolation and flowering.[1] The cyanobacterial phytochrome Cph1 is a valuable model for structure/functional studies of the family, its PAS-GAF-PHY sensory module (Cph1Δ2) having been investigated by X-ray crystallography (Figure 1),[2] conventional solution and solid-state magic-angle-spinning (MAS) NMR,[3,4] infrared, and Raman spectroscopy.[5] X-ray crystallography has provided structures of both the Pr and Pfr states in various phytochromes.[2,6] In conjunction with spectroscopic methods, it was found that ring ***D*** of the bilin chromophore rotates at the beginning of the photocycle,[4d,6e,7] leading to changes in the protonation states of the pyrrole nitrogen atoms prior to Pfr formation (Figure 1b).[8] Open questions include the localization of the positive charge on the chromophore and the protonation dynamics associated with the four pyrrole nitrogen atoms,[6d,8] information that can be provided by solid-state NMR spectroscopy. In particular, the charge distribution is potentially accessible from analysis of

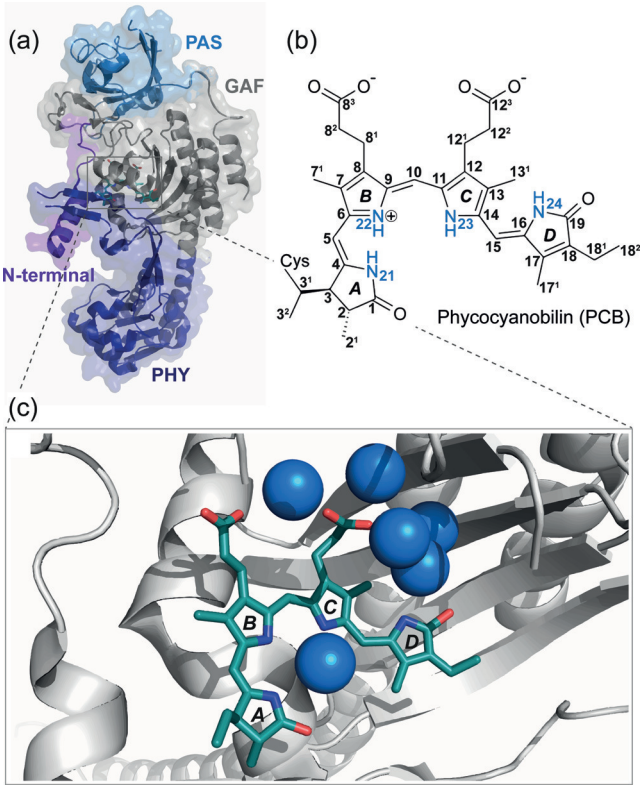

Figure 1. a) Cph1Δ2 (pdb: 2VEA) consists of a N-terminal region (purple), the PAS (light blue), GAF (gray) and PHY (dark blue) domains. b) The bilin is covalently bound via a thioether link to Cys 259. The geometry is *ZZZssa* as Pr. c) The chromophore is coordinated with six H_2O molecules (blue spheres) in the binding pocket. Nitrogen atoms are depicted in blue, carbon in cyan, and oxygen in red.

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chemical shifts and the chemical shift anisotropy of the pyrrole nitrogen resonances in conjunction with QM/MM calculations.[9] Site-specific proton exchange rates provide information about protonation dynamics. In previous NMR studies of Cph1Δ2, the nitrogen resonances were not unambiguously assigned because of the low signal-to-noise ratio (S/N).[4a–c]

To resolve the S/N issue, we apply here dynamic nuclear polarization (DNP) enhanced MAS NMR spectroscopy to Cph1Δ2 reconstituted with u-[^{13}C,^{15}N]-PCB. It has been shown in previous studies of photo-active proteins that applying DNP can be crucial to obtain further insights into their function.[10] The recently introduced biradical bcTol (Figure 2a) is used for our DNP-enhanced MAS NMR studies, profiting from its high solubility in aqueous solutions.[11] We determined the enhancement (ε; Figure 2b) as an approximation of the improvement in S/N, keeping in mind that paramagnetic relaxation enhancement (PRE) and depolarization effects can additionally influence sensitivity measurements.[12] At 100 K, the carbonyl, aliphatic and aromatic ^{13}C signals of u-[^{13}C,^{15}N]-PCB in Cph1Δ2 showed an $\varepsilon = 38 \pm 8$. The resolved signals of the three methine carbons (C5, C10, and C15) and the nitrogen signals indicate moderate homogeneous and heterogeneous line broadening, with 120–150 Hz observed linewidths after subtracting scalar couplings involved (see the Supporting Information). In DNP-enhanced MAS NMR spectroscopy, deceleration of motional processes at around 100 K may lead to heterogeneously broadened NMR lines,[13] whereas homogenous line broadening is caused by short distances between the analyte and the radical through PRE effects.[12] In case of PCB in Cph1Δ2, the small number of possible rotations around single bonds may help to minimize heterogeneous broadening whereas the protein around the chromophore protects from homogeneous broadening through PRE effects. A 2D ^{13}C-^{13}C dipolar assisted rotational resonance (DARR) spectrum was recorded as a reference (Figure 2c) and the assignment of the ^{13}C spectrum obtained earlier by Matysik and co-workers was mapped onto this spectrum.[4b,14]

In order to assign the signals of the four nitrogen atoms in PCB, we recorded a two-dimensional ^{15}N-^{13}C-^{13}C correlation spectrum (Figure 2d). Now, with the improved S/N provided by DNP, a spectrum with all expected relayed cross peaks could be obtained. Some natural abundance signals of Cph1Δ2 shine through at the typical protein backbone ^{15}N (115–130 ppm) and ^{13}CO (170–180 ppm) or ^{13}Cα (40–60 ppm) chemical shifts (solid circles), presumably due to a constructive superposition of some of the 514 residues. Three ^{15}N-^{13}C cross peaks with low intensity potentially due to another PCB form were identified

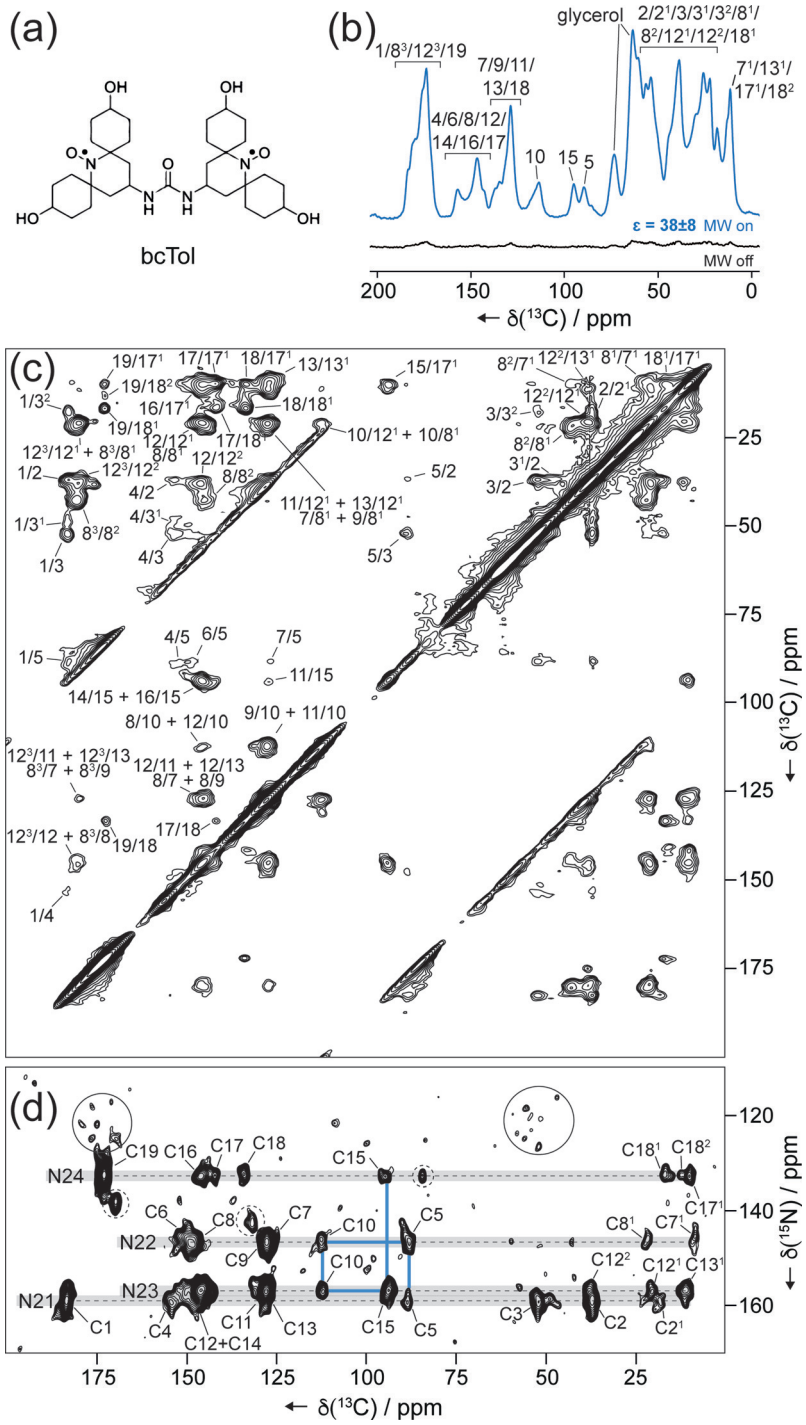

Figure 2. a) bcTol was used as a polarizing agent. b) An enhancement of $\varepsilon = 38 \pm 8$ at 100 K was determined. c) DNP-enhanced ^{13}C-^{13}C solid-state MAS NMR spectrum with 25 ms DARR mixing of u-[^{13}C,^{15}N]-PCB in Cph1Δ2 Pr. d) 2D DNP-enhanced ^{15}N-^{13}C-^{13}C solid-state MAS NMR spectrum of u-[^{13}C,^{15}N]-PCB in Cph1Δ2. The blue lines indicate the sequential connection of the ^{15}N resonances via methine carbon atoms C5, C10, and C15. The cross peaks in solid circles correspond to natural abundance signals of the protein background. The signals in dashed circles are discussed in the text.

(dashed circles) perhaps resulting from a small proportion (< 5 %) of Pfr in the sample. The Pfr form may occur because of a short, accidental exposure to light during sample handling (see the Supporting Information). An unequivocal assignment of the ^{15}N signals was achieved by making use of correlations involving the carbonyl signals of C19 and C1 (Figure 1 b), and of cross peaks to the methine carbons C5, C10 and C15 (see also the Supporting Information). Rings ***A*** and ***D*** are distinguished by correlations involving the aliphatic sites in ring ***A***, and the sequential walk between them, correlating N21, N22, N23 and N24 via the methine carbons is indicated in Figure 2 d.

The chromophore carries a positive charge whose localization could not yet be demonstrated experimentally and which is supposedly delocalized within the aromatic/polyene system. In PCB, the structural symmetry between the similar rings ***B*** and ***C*** is generally reflected in almost identical ^{13}C chemical shifts.[4b] In contrast, distinctly different ^{15}N chemical shifts were observed (Figure 2 d and 3). The signal of N22 in ring ***B*** may serve as an indication of charge distribution, since it is shifted upfield by 10.6 ppm compared to the signal of N23 originating from ring ***C***. The more drastic upfield shift of N24 in ring ***D*** by 26.7 ppm compared to its structural equivalent N21 in ring ***A*** can be explained by the difference in chemical structure of the two rings and the large hydrogen bond network to which N24 is connected. Furthermore, N21 is close to the negatively charged counter-ion Asp 207, consistent with an additional downfield shift. Taken together, our experimental data suggest that in the Pr state the positive charge is somewhat stronger localized at ring ***B***, since the resonance of a positively charged and protonated nitrogen nucleus generally shows an upfield shift of about 10 ppm compared to the neutral case.[9a] QM/MM calculations of the PCB nitrogen chemical shifts, however, are in agreement with a delocalization of the positive charge between rings ***B*** and ***C*** in the Pr state (see the Supporting Information). Test calculations also suggest that the chemical shift difference between N22 and N23 is not due to interactions with the pyrrole water, but is strongly depending on the geometry (see the Supporting Information). In summary, a considerable fraction of the positive charge we assume to be delocalized between rings ***B*** and ***C*** in the Pr state (Figure 1). Earlier studies suggested that the positive charge is located at N24 in the Pfr state.[8] Consequently, the positive charge must move from rings ***B*** and ***C*** to N24 in the Pr→Pfr transition. In the back-reaction, it returns to them, enabled through changes in the hydrogen-bonding networks involving N24, Asp 207, Arg 472 and H_2O molecules.[4c,6d,8]

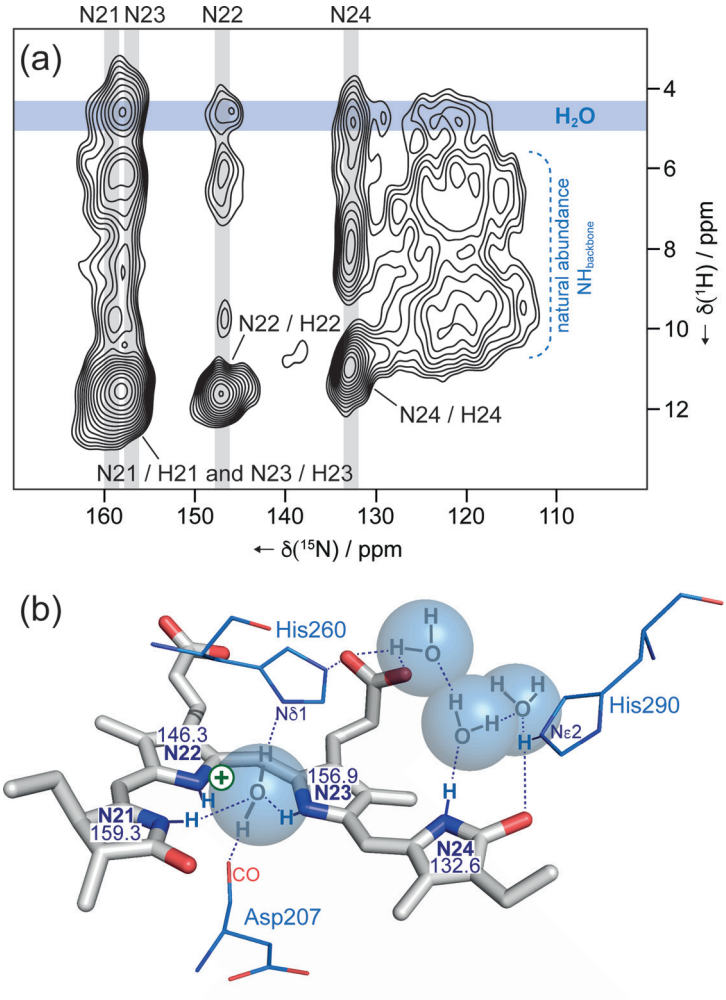

Figure 3. a) Interactions between ^{15}N and ^{1}H in u-[^{13}C,^{15}N]-PCB in Cph1Δ2 monitored by DNP-enhanced ^{1}H-^{15}N solid-state MAS NMR spectroscopy. Cross peaks from natural abundance amide groups are annotated in blue. b) Structural view on the potential hydrogen-bonding networks in the chromophore binding pocket and ^{15}N chemical shifts of the pyrrole nitrogen atoms (pdb 2VEA). The positive charge is mainly localized at ring ***B*** in Pr.

Interactions between water protons and the chromophore nitrogens of Cph1Δ2 were monitored by DNP enhanced ^{1}H-^{15}N correlation spectroscopy (Figure 3 a). The spectrum contains strong cross peaks of the cofactor nitrogen atoms and their directly bound protons (see labeled cross peaks) as well as a substantial number of signals resulting from natural abundance ^{15}N nuclei in amide groups of the protein backbone (^{15}N 110–130 ppm and ^{1}H 6–11 ppm, respectively). Some of the cross peaks between the four pyrrole nitrogen nuclei and protons at around 5.5–7 ppm ^{1}H chemical shift may originate from protons at the methine carbons C5, C10 and C15. Of particular interest are the correlations between nitrogen nuclei and protons at the water frequency. A qualitative analysis of spectra with different cross polarization contact times allowed the interaction of the N22 and N24 nitrogen spins with protons of neighboring H_2O molecules to be estimated (see the Supporting Information). Compared to the corresponding N22/H_2O signal, the cross peak between H_2O and N24 strengthens faster and reaches a higher maximum intensity as seen in the DNP enhanced ^{1}H-^{15}N spectrum. This correlates with a higher density of H_2O molecules in vicinity to N24 than in the case of N22 in agreement with the X-ray structure (Figure 1 c). Here, three H_2O molecules are located close to N24 whereas only one can contribute to a N22/H_2O cross peak. Both distances between N22 or N24 and the closest water

molecules are the same (3.2 Å) as suggested by the crystal structure.[2b]

In conclusion, with the help of DNP we were able to assign the ^{13}C and ^{15}N NMR signals of PCB in Cph1Δ2 unequivocally (Figure 3b). By means of chemical shift comparison, our measurements imply that the positive charge carried by the chromophore is mainly localized at ring ***B***. Chemical shift calculations reveal a more comprehensive view and suggest the delocalization of charge to include ring ***C***. In addition, we could detect interactions to functionally relevant H_2O molecules. The assignment of the nitrogen resonances is thus a fundamental step for NMR studies elucidating the mechanism of phytochrome molecular action since they mediate protonation and charge dynamics and subsequent structural changes during the photocycle.

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