Signaling mechanism of phytochromes in solution

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Abstract

Phytochrome proteins guide the red/far-red photoresponse of plants, fungi, and bacteria. The proteins change their structure in response to light, thereby altering their biochemical activity. Crystal structures suggest that the mechanism of signal transduction involves refolding of the so-called PHY tongue, but the two other notable structural features of the phytochrome superfamily, the helical spine and a figure-of-eight knot, have not been implied in the signaling mechanism. Here, we present solution NMR data of the complete photosensory core module from *D. radiodurans* (*Dr*BphP). Photoswitching between the resting and active states induces changes in amide chemical shifts, residual dipolar couplings, and relaxation dynamics. All observables indicate a photoinduced structural change in the knot region and lower part of the helical spine. Supported by functional studies of plant phytochromes, the new mechanism explains how the conformational signal is directed through the protein to the signaling spine. The new pathway explains photo-sensing by phytochromes with atomic precision under biological conditions.

Introduction

Phytochromes are universal photoreceptors found in plants, fungi, and various microorganisms.[1] They detect red and far-red light, thereby controlling many aspects of growth, development, and movement. The functions range from phototaxis and pigmentation in bacteria to seed germination, shade avoidance and flowering in higher plants.[2–8] Phytochromes work by switching between two photochemical states. In prototypical phytochromes, the resting state absorbs red light (Pr state), and the activated state absorbs far-red light (Pfr state). The proteins can be reversibly switched between the states with red light (Pr→Pfr) and far-red light (Pfr→Pr). Phytochromes are important for life, since they ensure that microbes and vegetation can adapt to light and thrive on earth.

Similar to other signaling proteins,[9,10] phytochromes are modular proteins. They are divided into three groups according to the domain architecture. Group I phytochromes share a highly conserved photosensory module consisting of the domains PAS-GAF-PHY (Per/Arndt/Sim-cGMP phosphodiesterase/adenyl cyclase/Fh1A-phytochrome specific).[11,12] Plant, fungal and bacterial phytochromes belong to this group. The photosensory module of the cyanobacterial group II and III phytochromes consist of GAF-PHY and GAF, respectively.[13] A covalently linked bilin chromophore (biliverdin in bacterial phytochromes) is attached via a thioether linkage to a conserved cysteine in the GAF domain (cyanobacteria and plants) or PAS domain (bacteria).[11] Output domains vary and can consists of a C-terminal histidine kinase in bacteria, so-called N-terminal extensions, and C-terminal PAS domains connected to a kinase-like domain in plants and fungi.[12] Phytochromes are usually homodimers.

The photosensory module of phytochromes contains three characteristic structural elements. These are a conserved loop region in the PHY domain, called "tongue" (residue 444-476, group I and II), which extends from the PHY domain back towards the chromophore binding pocket, a figure-of-eight "knot" in the PAS/GAF domain (residue 27-38 and 228-256, group I), and a long helix called "spine" (residue 300-345, groups I-III), which

extends through the entire photosensory module (Fig. 1). The spine helix is coupled to another helix in the PHY domain, which forms a coiled-coil with the equivalent helix in the sister-monomer.[14,15] The tongue refolds from a β-hairpin conformation in Pr to an α-helical structure in Pfr.[16–18] Interestingly, crystal structures have not indicated significant structural changes of the knot or in the helical spine region in the PAS and GAF domains upon photoactivation. As a result, the tongue is currently considered to be the main signaling route in phytochromes.[16,19] This is not fully satisfying given that many fully functional phytochromes exist, which lack the tongue.[13] Therefore, another signaling pathway may exist.

The knot in group I phytochromes is formed at the interface between the PAS and GAF domains spanning the space between the chromophore and the helical spine (residue 27-38 and 228-256) (Fig. 1a).[7] The sequence between Cys24 and the start of the PAS domain (residue 27-38) is passed through a loop formed by a large insertion within the GAF domain (residue 228-256).[7] The knot is stabilized by a small but critical hydrophobic core, centered on the conserved Ile35. The region contains many conserved residues (Ile35, Gln36, Leu41, Ala225, Val232, Leu234, Leu248, Leu253 and Arg254) corroborating its importance for the protein.[7] It has been speculated that the knot stabilizes contacts between the PAS and GAF domains, or that it severely restricts movement of the N-terminal domain and thereby orients Cys24 for efficient conjugation to biliverdin. Another theory is that the knot limits the flexibility of the protein in order to prevent undesirable loss of entropy due to large domain movements upon photoisomerization of the chromophore.[7] However, as current crystal structures doe not reveal any changes in the knot region between Pr and Pfr,[15,17,18,20–23] these ideas remain speculative.

The helical spine consists of two long helices, one connecting the PAS/GAF domain with the PHY domain ('lower' spine helix) and one connecting the PHY to the output domains ('upper' spine helix, Fig. 1a). Many sensory proteins contain helical spines, where they are actively involved in signaling.[10,24,25] This also seems likely for phytochromes.[18,26] A bending of the spine in between the PAS-GAF and PHY domains has been inferred from

crystal structures, X-ray solution scattering, and DEER spectroscopy (*SI Appendix,* Fig. S2a),[16,21,27–29] but more detailed changes in the spine have not been reported.

In order to uncover the signal transduction mechanism, the protein should be studied in solution. Solution Nuclear Magnetic Resonance (NMR) is an ideal tool to characterize dynamic molecules at atomic resolution. Chemical shifts are very sensitive probes for locating and detecting conformational changes of amino acids or their environment.[30,31] Residual dipolar coupling (RDCs) provide information on the orientation of chemical bonds in relation to a molecular frame, defined by the protein. They provide long-range orientational information on the single-residue level.[32,33] T1 and T2 relaxation measurements give information on changes in structural dynamics between states. Of special interest is the T1/T2 ratio, which in a significantly non-spherical molecule indicates the relative orientation of each amide bond with respect to the molecular frame.

The investigation of phytochromes by NMR spectroscopy is difficult, because of their large size. Therefore, solution-state NMR has only been applied to a short phytochrome constructs, which consists of a single GAF domain,[34–37] and residues around the labelled chromophore in PAS/GAF constructs.[38] However, the knotted PAS/GAF domain or the complete photosensory module have not been investigated, because they were considered to be too large.[34] Solid state NMR has been used to study the chromophore binding pocket or larger phytochrome fragments.[39–41]

We have recently presented the first solution NMR assignment of the complete photosensory module of a uniformly triple-labelled (^{2}H,^{13}C,^{15}N) phytochrome (BMRB Entries 27783 and 27784 for Pr and Pfr states, respectively),[42] using the monomeric version of the *D. radiodurans* bacteriophytochrome (PAS-GAF-PHY) [43,44] (*SI Appendix,* Fig. 1). The assignments open up for a detailed investigation of the photoinduced changes in DrBphP$_{mon}$ in the present study. We present chemical shift perturbation analysis, RDCs, and ^{15}N T1 and T2 relaxation data for Pr and Pfr state for DrBphP$_{mon}$. The observables describe a clear difference between the Pr and the Pfr state in the knot region and the lower part of the

helical spine, identifying a new signaling route in phytochromes.

Results

Chemical shifts in Pfr and Pr indicate remodelling of the PAS and GAF domains

In Fig. 1, we present the changes of the amide chemical shift between Pr and Pfr for DrBphP$_{mon}$. Most residues show only small changes, but a few residues show significant differences of > 2.5 standard deviations (σ). These residues are located in the PAS and GAF domains and cluster in the knot region and the lower part of the helical spine (Fig. 1b). Residue Phe61, which also has a significant change in chemical shift, is located close to the bottom part of the helical spine. The chemical shift perturbation analysis does not cover the tongue region and the middle part of the helical spine, because assignment is lacking (*SI Appendix,* Fig. 1).[42] Chemical shift changes indicate chemical or structural changes in the near surrounding of the relevant amide groups. We therefore suggest that, in solution, previously unrecognized structural or chemical changes occur in the PAS/GAF domain.

The secondary structure remains unchanged in the knot and the spine region.

Next, we calculated the secondary chemical shifts for CA (*SI Appendix,* Fig. S4a), CO (*SI Appendix,* Fig. S4b) and CB ((*SI Appendix,* Fig. S4c). These shifts collectively report on the secondary structure of the residues. However, the sign and the intensity for the secondary chemical shifts did not change between Pr and Pfr and we conclude that the secondary structure of the PAS, GAF, and PHY domains is retained in both states. We note that we cannot probe the secondary structure changes in the tongue region, because the tongue is not assigned in the Pr state.

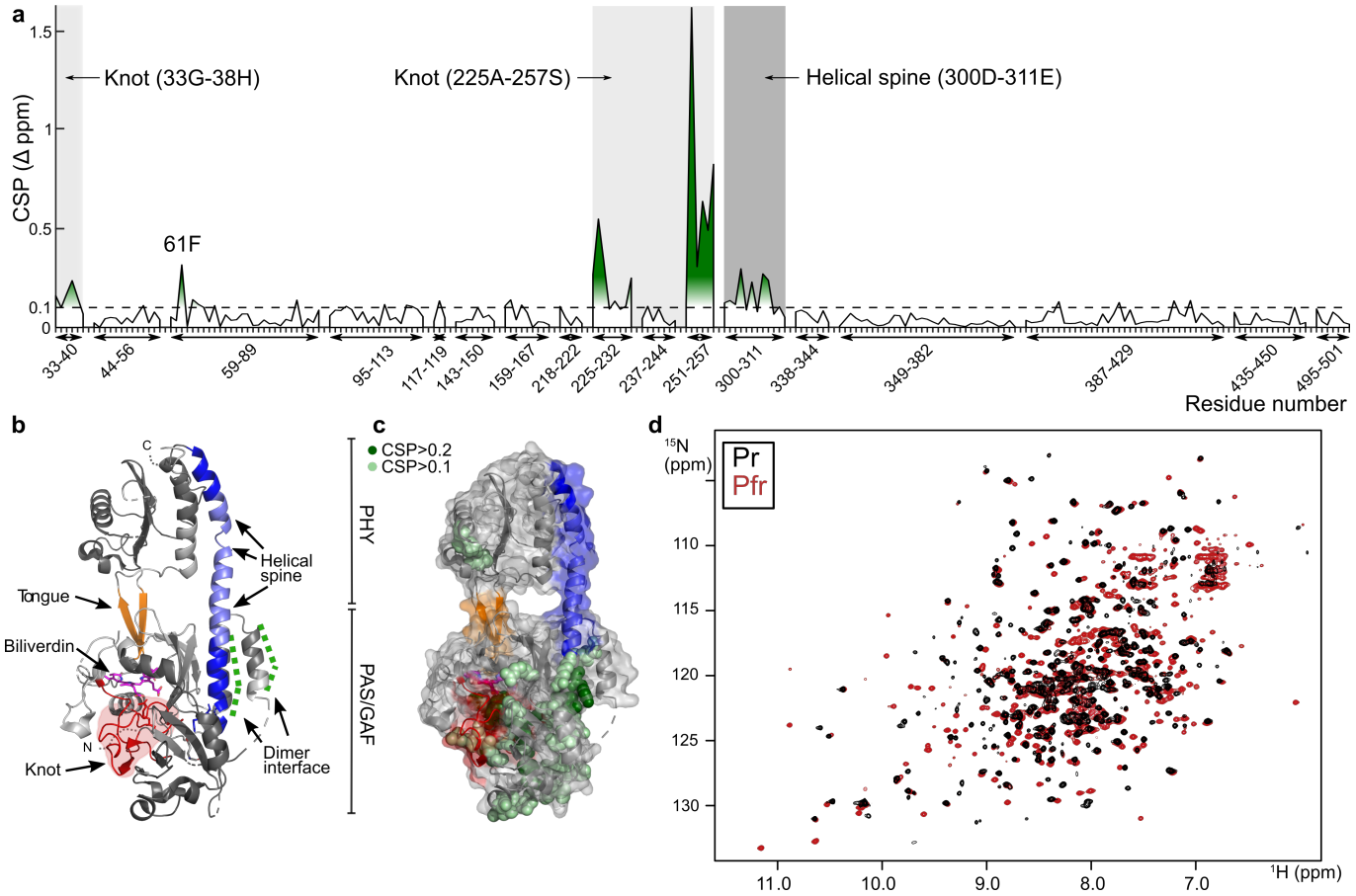

Figure 1: **Chemical shift perturbation data for N and NH show large changes in chemical shifts in the knot and the helical spine. a** The chemical shift perturbation (CSP) of the amide chemical shift is shown. The knot and lower helical spine are indicated. Only residues which are assigned in Pr and Pfr are displayed. For full set of CSP data, see *SI Appendix,* Fig. 7. The chemical shift perturbation was calculated as $d = \sqrt{(\frac{\delta_H^2 + (\alpha * \delta_N^2))}{2}}$, with $\alpha = 0.15$. **b** The structure of the photosensory module of *Dr*BphP (PDB ID: 4O0P) is displayed with the tongue (orange), knot (red), biliverdin (magenta), and helical spine (blue) indicated. The lighter colors mark residues which have not been assigned in both states. **c** The same structure is shown with chemical shift changes indicated in green colors. **d** 2D [^{1}H^{15}N]-TROSY spectra for the dark (Pr) and light (Pr/Pfr mixture) state are displayed. The assignment for the pure Pfr state was obtained in Ref.[42]

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Residual dipolar couplings (RDC) indicate changes in the knot and helical spine

RDCs are NMR observables, which report on long-range structure. We measured RDCs on the amide N-H bonds using Pf1 phages for partial molecular alignment of the proteins. To test if the Pr and Pfr crystal structures represent the solution structures, we inspected correlation plots between experimental RDCs and RDCs computed from the crystal structures of DrBphP$_{PSM}$ using the software PALES (Fig. 2 and Table 1).[45,46] We performed the assessment for RDCs in the complete photosensory core (PAS/GAF/PHY), in the PAS/GAF domain, and for the knot and the spine region. We classified the agreement into three categories according to the Q-factor:[47] $Q < 0.4$ for excellent agreement,[48] $0.4 \leq Q < 0.8$ for intermediate agreement, and $Q \geq 0.8$ for poor agreement. All results are visually confirmed in Fig. 2, where the gradient reports on correlation.

Table 1: Summary of RDC analysis

Structure	Number	RDC	PCC	Q	Agreement
Pr (4Q0J)	176	PAS/GAF/PHY (Pr)	0.85	0.653	intermediate
Pr (4Q0J)	94	PAS/GAF (Pr)	0.93	0.361	excellent
Pr (4Q0J)	21	Knot/Spine (Pr)	0.945	0.248	excellent
Pfr (5C5K)	190	PAS/GAF/PHY (Pfr)	0.762	3.093	poor
Pfr (5C5K)	105	PAS/GAF (Pfr)	0.764	4.484	poor
Pfr (5C5K)	36	Knot/Spine (Pfr)	0.757	4.747	poor

Structure: The crystal structures used in the RDC analysis. **Number:** Number of experimental RDCs. **RDC:** The RDC set used in the analysis. **PCC:** The Pearson correlation coefficient indicates the linear correlation between observed and calculated RDC:s without re-scaling. **Q:** The Q factor ($Q = \frac{rms(D_{obs}-D_{calc}}{rms(D_{obs})}$) is an estimate of the average disagreement between observed and calculated RDC:s.[47,48] See main text for classification of the "agreement" column.

For Pr, we find intermediate agreement for PAS/GAF/PHY and excellent agreement for PAS/GAF and the knot/spine region. This indicates that the crystal structure for the PAS/GAF domain resembles the solution structure well, but that adding the PHY domain causes some discrepancy. We attribute this to floppiness of the PHY domain with respect

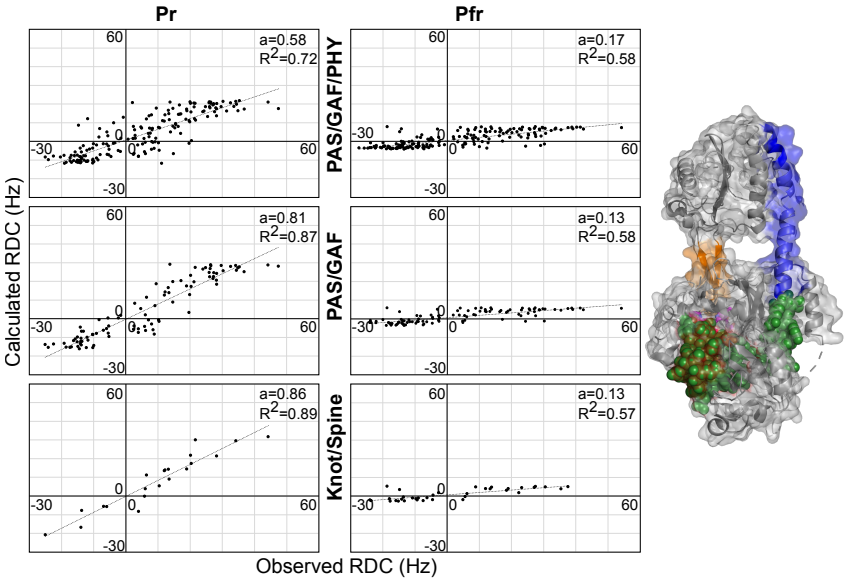

Figure 2: **RDC data indicate changes in the PAS/GAF domains of *Dr*BphP .** Calculated RDCs are plotted against observed RDCs for Pr and Pfr. We used the PDB code 4Q0J for the Pr state calculations and 5C5K for Pfr state calculations. A linear fit to the data is shown with a as the gradient and R^2 for the goodness of the fit. The computations were run for different fragments of the protein as indicated. The color code in the structure is as in Fig.1 and the knot and spine region (residues 27 to 38, 228 to 256 and 299 to 344) is marked in green.

to the PAS/GAF domain. This is expected from solution X-ray scattering,[28] molecular dynamics simulations,[44] and the variations in the position of the PHY domain in different crystal structures of the photosensory core module.[16,17,21,49]

For Pfr, all fragments yield poor agreement. This means that the solution structure of the PAS/GAF domain in Pfr diverts from the crystal structure,[7,16,21] implying that the crystal structures have not captured the structural change that occurs in the PAS/GAF domain. Using RDCs, we track the origin of this disagreement down to the knot/spine region, where poor correlation is observed in Pfr (Fig. 2 and Table 1). We conclude that, upon photoconversion in solution, structural changes occur in the helical spine and the knot region leading to a rearrangement of the PAS/GAF domains.

Relaxation dynamics indicate structural changes in the knot/spine region

The rotational correlation time of a entire protein (τ_c) is related to its molecular size and shape. τ_c was calculated from the average ratio of ^{15}N T1 (longitudinal) and ^{15}N T2 (transverse) relaxation times. They were similar for the Pr and the Pfr states: 21.9±3 ns and 22.2±3 ns, respectively. This indicates that the quaternary structure of DrBphP$_{mon}$ is not changed dramatically upon photoactivation, which is consistent with the time-resolved solution scattering structures of the same protein fragment.[27]

We also determined ^{15}N T1 and T2 relaxation times for each residue (Fig. 3a, b and *SI Appendix,* Fig. S3). Similar to RDC measurements, the photoinduced change of the T1/T2 ratio for each residue indicates a structural reorientation of the amide bond in the molecular frame (Fig. 3c). Many of the residues, which have large differences in T1/T2 ratios (Fig. 3d) cluster in the knot and spine region of the protein. One of the regions with large changes (Ala251, Arg254-Ala255-Thr256-Ser257) corresponds well with the Val252-Leu253-Arg254 amino acid stretch, where the largest changes in chemical shifts were observed (see Fig. 1). The relaxation data is in agreement with the chemical shift perturbations and the RDC

measurements, indicating a change of the proteins structure in the knot and helical spine regions upon photoactivation.

The NMR observables collectively reveal a signaling route through the knot and spine region

The analysis of chemical shifts, RDCs, and T1/T2 ratios indicate that significant structural changes occur when *Dr*BphP is photoconverted from Pr to Pfr in solution. The changes in chemical shift are residue-specific and they clearly pinpoint the changes to the knot region and the helical spine (Fig. 1c). RDCs report directly on the orientation of the amide bonds. They show that the changes are a *structural* perturbation of the protein in the same region as indicated by the chemical shifts (Fig. 2). Moreover, the RDCs show that the crystal structures in Pfr do not represent the solution structure well, in particular in the PAS/GAF domain. T1/T2 is also an indicative of structural changes, indicating changes in the same region (Fig. 3). The secondary structure is not changed, precluding complete unfolding of the domains. Thus, we conclude that significant structural changes arise in the PAS/GAF domain and that these changes are focused on the knot region and the helical spine.

Fig. 4 illustrates this new proposed signaling pathway. The NMR data indicate alterations spanning from the chromophore to the helical spine. Arg254 is the entry point to the signal transduction chain. In the Pr state, Arg254 has a salt bridge to the propionate group of the B ring of the biliverdin,[16,17,50] which is broken in Pfr.[21,39] The signal continues through Val252, which is located next to the conserved Leu253, and via the conserved Glu127 and Arg302, which form a salt bridge in the crystal structures. Arg302 is located in the bottom part of the helical spine. Phe61 is located within 10Åof the salt bridge and shows a significant change in chemical shift. We propose that the new pathway transports the signal through the spine to the C-terminal PHY and output domains and that it is an integral part of the photoresponse of phytochromes.

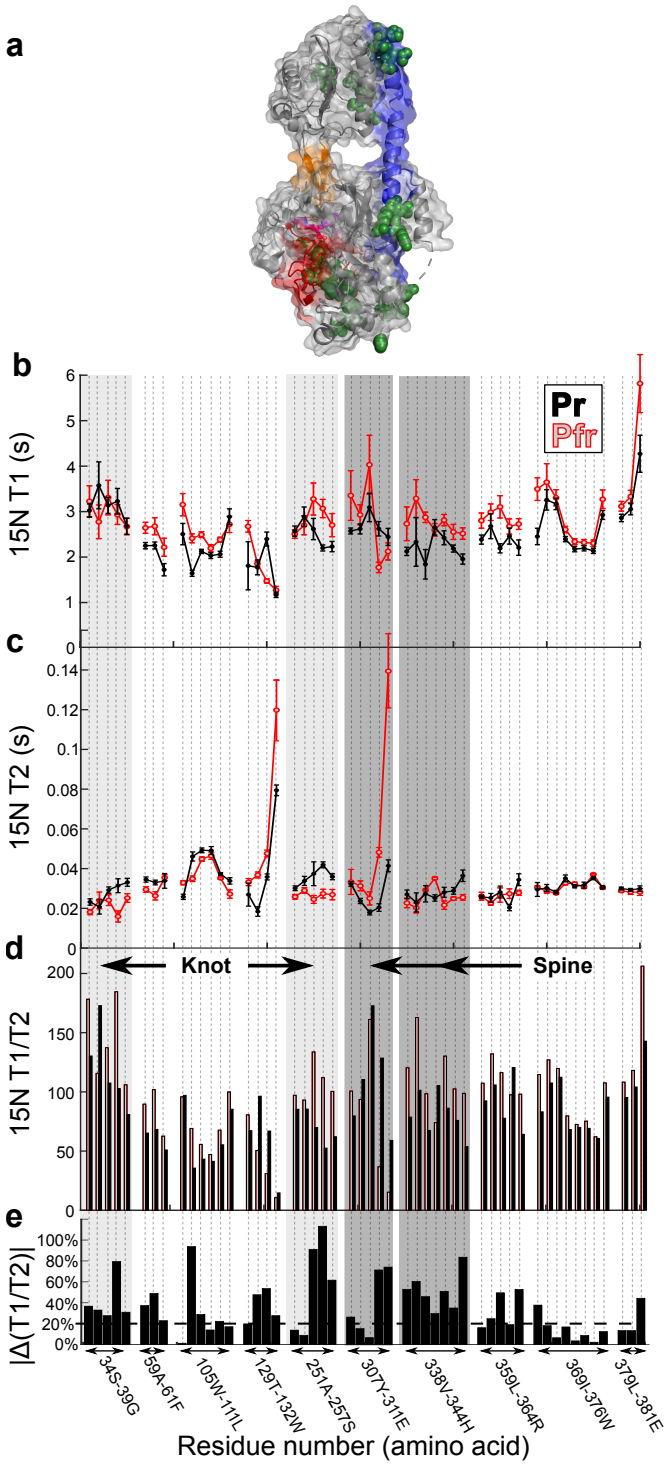

Figure 3: **Large differences in the ^{15}N T1/T2 ratios of the relaxation times indicate structural changes in the knot and spine region.** **a** shows the *Dr*BphP structure (pdb ID: 4O0P) with the residues with large changes in the ^{15}N T1/T2 ration marked in green. The other colors mark structural elements as in Figure 1. **b** displays the ^{15}N T1 relaxation, **c** the ^{15}N T2 relaxation and **d** the ^{15}N T1/T2 ratio for Pr (black) and Pfr (red). In **e**, the change of the T1/T2 ratio is shown in percent of T1/T2 of the Pr state. Changes larger than 20% (dashed line) where mapped on the structure in **a**.

Discussion

It seems reasonable that all three outstanding and conserved structural elements of the phytochrome photosensory module are involved in the function of the protein. Here, we show that a signal transduction pathway exists, which spans form the chromophore via the knot to the lower part of the helical spine. Conceivably, these changes may translate to the C- and N-terminal output domains. Previous crystallographic work has only shown that the PHY-tongue is involved in the transduction of the light signal (Fig. 4), but changes in the knot and spine region inside the PAS/GAF domains were not observed (illustrated in *SI Appendix,* Fig. S2) .[7,16–18,21,29]

Generally, knotted polypeptide chains are expected to have an evolutionary rationale.[7,51] The phytochrome knot contains many highly conserved residues.[7] It has been demonstrated that the knot region is important for signaling in a plant phytochrome as mutations of R110Q, G111D, G112D, and R352K in the *Arabidopsis* PhyB (corresponding to Ile31, Pro32, Gly33 and Arg254 in *Dr*BphP) have normal photochemistry, but are defective in intracellular signal transfer.[52] One end of the knot is the start of the N-terminal extension (Fig. 1a), which is known to be of functional relevance in plant phytochromes.[53–56]

Helical spines are common structural elements in many signaling proteins and critical for signal transduction.[10,24,25,57] While the helical spine exists in all phytochromes, the PHY-tongue does not, because PHY-less phytochromes exist.[43] Coiled-coil interactions of the C-terminal extension of the helical spine to the output domain have been shown to be critical for controlling the signaling output in phytochromes,[58] resembling the converged view for other bacterial histidine kinases.[10,25] These considerations provide support for our proposed new pathway for signal transduction from the chromophore via the knot region to the lower part of the helical spine.

With the presented NMR experiments, we were not able to characterize structural changes in the tongue region, because the peaks of the tongue are absent in the 3D trosy-type H-N-C spectra in the Pr state. However, based on the crystallographic evidence,[16,21] we assume

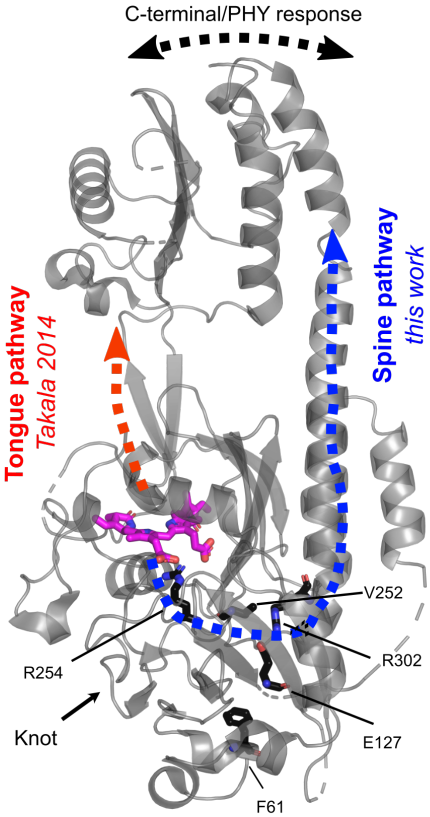

Figure 4: **The proposed "spine" signaling pathway for phytochromes.** The structure of *Dr*BphP (PDB ID: 4O0P) is shown together with the two pathways, which guide the signal to the PHY-domain and further to the C-terminal output domains. The "tongue" pathway (red) was proposed in 2014 based on crystal structures by Takala *et al.* and involved refolding of the tongue region.[16] The new signaling pathway, which is proposed here with solution NMR spectroscopy, proceeds through the knot and spine region. Conserved amino acids, which participate in the pathway, are indicated.

that this region changes. As such, there would be two pathways for signal transduction in the (PAS/GAF/PHY) photosensory module of phytochromes; through the PHY-tongue[16] and through the knot and helical spine. It is difficult to say which part of the protein is affected first. We consider that the two signaling routes may work in concert to stabilize each other, or that they are redundant signaling pathways.

An overlay of the Pr and Pfr structures for *Dr*BphP$_{PSM}$ shows virtually no change in the PAS/GAF domain, but the NMR data clearly indicate substantial changes in these domains.[16–18,21] The crystal structure resemble the solution structure well in Pr, but not in Pfr. How can that this be rationalized? It is known that the photophysical state of the chromophore can be uncoupled from the conformation of the phytochrome in crystal structures.[15,22] Crystal packing can alter protein conformations, with packing energies on the order of tertiary or quarternary interactions. It could be that the Pfr conformation of PAS/GAF in solution has a disordered connection between PAS and GAF domains. Such a 'loose' conformation would not be favoured in tightly packed crystals. It could also be that one of the conformations causes a disruption of the dimer packing, which would also not be preferred in crystals. The unnatural situation of tight packing in crystals then pushes the protein back into the Pr conformation. We note that a difference signal (Pfr minus Pr) was recorded with solution x-ray scattering for the PAS/GAF fragment of *Dr*BphP .[28] This signal indicates structural changes in solution in agreement with the present NMR data.

Multiple conformations have been observed in the chromophore binding regions of phytochromes. Solid-state and solution NMR on the sensory module of Cph1 phytochrome and a GAF fragment from a red/green cyanobacteriochrome has revealed two isoforms of the red-light absorbing Pr state.[34,39] Double conformations have never been observed in crystal structures of phytochromes, emphasizing the detrimental effect that crystal packing can have. Even though we do not detect multiple conformations in the backbone chemical shifts near the chromophore, the presence of such heterogeneity is not in conflict with our model of signal transduction.

Although the collective NMR observables pinpoint the structural changes to the knot and spine region of the PAS/GAF domain, they do not reveal an atomistic model of structural changes. One plausible mechanism is that the spine helix rotates and with that transduces the signal to the PHY and output domain. This would be consistent with structural changes, refined against time-resolved X-ray scattering data for *Dr*BphP$_{mon}$ and for the full length *Dr*BphP.[27,28] The studies identified a twist and bending of the photosensory core[27] and an overall twist of the output domains with respect to PAS/GAF.[28] This is also consistent with how other sensory proteins are thought to work.[24,57] An alternative mechanism could be that the changes in the lower part of the spine helix lead to a modification of the dimer interface. The PAS/GAF part of the helical spine forms a significant fraction of the dimer interface. A rotational or translational motion could change the interactions across the dimer, thereby changing the dimeric arrangement. This would lead to modification of the coiled-coil connector from the PHY domain to the output domain, as proposed.[58] Indeed, the PAS/GAF fragment of *Dr*BphP crystallize with different dimer arrangements in different crystal forms[7,59,60] and phytochromes typically have different dimerization constants in Pr and Pfr.[20,61]

To conclude, we propose a previously unrecognized signaling pathway along the knot and the spine helix in *Dr*BphP (Fig. 4), connecting the chromophore and the output domains. The work opens up for more detailed investigations of phytochromes with NMR spectroscopy, which we hope will refine the signal transduction cascade in the protein superfamily with even greater precision.

Online Methods

Detailed information for the cloning, protein expression, protein purification, NMR sample preparation, NMR experiments, and data analysis can be found in *SI Appendix*.

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Author contributions

LI, EG and SW designed the project. LI and EG planned the experiments together with CP, UB, GK, SW, and VO. LI and EG produced and purified all samples and performed the measurements and analysis together with CP, UB, VO, and SW. LI, EG, VO and SW wrote the manuscript with input from all authors.

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