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# Vibrational Couplings between Protein and Co-factor in Bacterial Phytochrome Agp1 revealed by 2D-IR Spectroscopy

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Phytochromes are ubiquitous photoreceptor proteins that undergo a significant refolding of secondary structure in response to initial photoisomerisation of the chromophoric group. This process is important for the signal transduction through the protein and thus its regulatory function in different organisms. Here, we employ 2D-IR spectroscopy, an ultrafast spectroscopic technique that is sensitive to vibrational couplings, to study the photoreaction of bacterial phytochrome Agp1. By calculating difference spectra with respect to the photoactivation, we are able to isolate sharp difference crosspeaks that report on local changes in vibrational couplings between different sites of the chromophore, the protein and between them. These results indicate i.a. that a dipole coupling between the chromophore and the so-called tongue region plays a role in stabilizing the protein in the light-activated state.

## A. Significance Statement

2D-IR spectroscopy is severely limited in its application to larger proteins due to broad and overlapping signals in the amide I region. Here, we overcome this limitation and isolate couplings between pairwise two single molecular groups in the biotechnologically relevant phytochrome Agp1 (510 aa) by calculating light-induced difference spectra. In phytochromes, the photoactivation of a co-factor with a relatively small structural change triggers a large-scale refolding of big parts of the protein, but the mechanism of that interaction is not understood. We observe crosspeaks in the 2D-IR spectra that are directly related to the changing dipole coupling between the co-factor and the part of the protein that refolds, suggesting that both sites stabilize each other mutually.

## I. INTRODUCTION

Phytochromes are photoreceptive proteins with variable domain architecture<sup>1</sup> that many different organisms, such as plants,<sup>2</sup> fungi,<sup>3</sup> cyanobacteria,<sup>4</sup> and bacteria<sup>5</sup> use to sense light. All phytochromes bind open-chain tetrapyrrole chromophores, like e.g. biliverdin X $\alpha$  (BV, mainly in bacterial variants)<sup>6</sup> and convert between two “parent” states with distinct absorption spectra. Bacterial phytochromes (Bphs) are of specific interest within this diverse class of proteins, because they can be easily expressed, purified, modified and used in biotechnological applications such as optogenetics or fluorescence microscopy.<sup>7,8</sup>

Bphs act as light-driven enzymes in bacterial two-component signaling, e.g. light-activated histidine kinases,<sup>9</sup> phosphatases,<sup>10</sup> or cyclases.<sup>11</sup> They photoswitch between a red-absorbing (Pr) and a far-red absorbing (Pfr) state, thereby regulating the according C-terminal catalytic domain, which is typically located several nanometers away from the chromophore. Due to this large distance, the light-regulation of enzymatic activity involves an intricate mechanism: an initial light-triggered

*Z/E* isomerisation of BV causes refolding of a nearby secondary structure element (the “tongue” region, fig. 1), resulting in functional tertiary structure rearrangements of the catalytic domain.<sup>8</sup> The light-induced transition of the tongue is a rather dramatic structural change as it comprises the repositioning of all involved amino acids and the breaking and formation of tens of hydrogen bonds. Due to its magnitude, it is often viewed as the critical step in intramolecular signal amplification.<sup>12</sup>

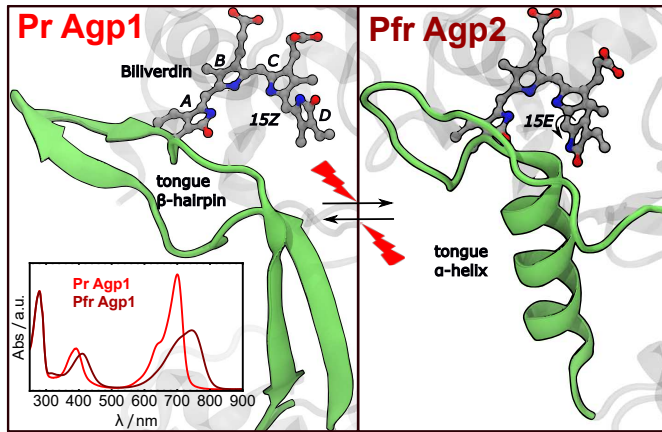


FIG. 1. The red/far-red photochemistry of canonical Bphs. Left: detailed view of the crystal structure of Agp1 in the Pr state (PDB code 5HSQ), with the BV chromophore in the *15Z* configuration and the tongue region as a hairpin fold. Right: The crystal structure of the Pfr-stabilized phytochrome Agp2 can be used as a model for Agp1 in the Pfr state (PDB code 6G1Y). In Pfr, BV adopts the *15E* configuration and the tongue forms a helix. Inset: vis absorption spectra of Agp1 in Pr and Pfr.

Over the last decades, many analytic techniques such as x-ray crystallography,<sup>13,14</sup> NMR spectroscopy,<sup>15</sup> cryo electron microscopy,<sup>16</sup> time-resolved x-ray solution scattering,<sup>12,17</sup> and molecular dynamics simulations<sup>18</sup> were used to probe the molecular details of this complex reaction cascade. On top of that, vibrational spectroscopies (resonance Raman, RR, and infrared absorp-

tion, IR) had a strong contribution to the current understanding of the phytochrome photoreactions due to their high intrinsic time resolution, sensitivity to subtle changes in molecular geometries, and applicability to solution samples at physiological temperatures.<sup>19–23</sup> IR difference spectroscopy was mainly employed in the amide I region, where the frequency changes of C=C and C=O stretching modes of the chromophore<sup>24</sup> can be followed in the same spectral window as the backbone amide I modes, which report e.g. on the tongue transition between  $\beta$ -hairpin and  $\alpha$ -helical states.<sup>19,25,26</sup>

Since the different contributions in this information rich region overlap strongly and partly cancel each other, clear assignments and quantitative measurements are unfortunately seriously aggravated in IR difference spectroscopy. Here, we attempt to partially overcome this problem by spreading the spectrum out into two dimensions using 2D-IR difference spectroscopy. 2D-IR is an advanced femtosecond IR-pump/IR-probe technique with a frequency resolved pump axis that provides additional observables such as 2D-lineshape and crosspeaks that are inaccessible by linear spectroscopy.<sup>27,28</sup> Furthermore, 2D-IR spectroscopy is, in contrast to linear techniques, sensitive to vibrational couplings. Due to these advantages, 2D-IR offers a direct way to study the connectivity between different functional groups in phytochromes via crosspeaks as a function of the photocycle, and thus reveal new insights in the molecular mechanism of the signal transduction in solution with high time resolution.

We selected the canonical bacterial phytochrome Agp1 from *Agrobacterium fabrum*<sup>9,29</sup> as a model system, because this specific Bph has been studied extensively with x-ray crystallography and linear vibrational spectroscopy, providing a solid reference for the 2D measurements.<sup>20,23,30,31</sup> Since no crystal structure of Agp1 in the Pfr state is available, the crystal structure of the Pfr-stabilized phytochrome Agp2 is used as a model for the Pfr state of Agp1 throughout the manuscript.<sup>32</sup> Within that frame, we find that the 2D-IR measurements provide a large amount of interesting information such as changes in lineshapes and crosspeaks that allow us to dissect the IR spectrum and propose a model for the kinetic stabilisation of the Pfr state.

## II. RESULTS

**2D-IR spectroscopy in the Pfr and Pr states** To set the stage, absolute 2D-IR spectra of Agp1 were measured in the Pr and Pfr states by illuminating the sample inside the spectrometer. While the Pr state can be populated with this procedure essentially pure, the Pfr spectrum can contain a residual Pr contribution (compare UV-vis spectra in fig. 1). We estimated it to be  $< 20\%$  by performing an iterative subtraction procedure on the vis absorption spectra  $Pfr_{pure} = Pfr_{exp} - s \cdot Pr_{exp}$ , varying  $s$  until negative bands appear as a clear sign of oversubtraction. In contrast to most other 2D-IR studies in the amide I region, the data presented here were taken in H<sub>2</sub>O buffer

solution (not D<sub>2</sub>O). This was possible due to the very high solubility of the protein and resulting high concentration of the sample (ca. 10 mM), and further demonstrates that the sensitivity of 2D-IR has advanced over the last years to the point where it is feasible now to perform experiments on protein samples in H<sub>2</sub>O. The choice to stay with H<sub>2</sub>O ensures that in a large and complex protein like Agp1 all buried protic residues are protonated instead of producing a mixed protonation/deuteration pattern due to a partial inaccessibility for D<sub>2</sub>O exchange, alleviating the assignment of vibrational bands.

In the absolute 2D-IR spectra of Agp1, four local maxima are detected on the main diagonal between 1600 and 1730 cm<sup>-1</sup> (Fig. 2, roman numerals). Peaks I-III can be observed in 2D-IR spectra of most proteins and assigned to the dominant amide I modes of different secondary structure elements of the entire protein. Specifically, peak I (ca. 1630 cm<sup>-1</sup>) corresponds to the so-called  $\beta$ -sheet a- vibration, II (1660 cm<sup>-1</sup>) to  $\alpha$ -helices, and III (1685 cm<sup>-1</sup>) to the  $\beta$ -sheet a+ mode. This assignment is based on reference spectra from pure  $\beta$ -sheet proteins like concanavilin A or  $\alpha$ -helical proteins like myoglobin, which display these bands.<sup>27,33</sup> Furthermore, the  $\beta$ -sheet a- and a+ vibrations are coupled, giving rise to crosspeaks I/III that can be observed in  $\beta$ -sheet proteins in the form of a “Z”-shape (Fig. 2, dotted line). In the case of Agp1, the dominant diagonal  $\alpha$ -helix signal overlaps strongly with these crosspeaks, leading to a complex shape similar to other proteins with mixed secondary structure like e.g. ubiquitin.<sup>27</sup> Furthermore, a water background from the HOH bending mode<sup>34</sup> is overlapping at with the amide I signals at 1660 cm<sup>-1</sup>, as evidenced by a more homogeneous lineshape of this band. Since the 2D IR response scales quadratically with the extinction coefficient  $\epsilon$ , the signal of the amide I band, whose extinction coefficient is larger, is enhanced relative to that of the water background. Consequently, the signal sizes of both are in the same order of magnitude, despite the much higher concentration of water.

Diagonal peak IV is found outside the amide I region and is assigned to the C=O stretching mode of the BV ring D carbonyl (see also fig. 1 for nomenclature, CO<sub>D</sub> at 1711 cm<sup>-1</sup> in Pr), in agreement with the literature, while CO<sub>A</sub> is expected at higher frequencies.<sup>23,24,31</sup> Since peak IV does not overlap with other bands, the absolute 2D-lineshape can be analysed directly in both photostates (Fig. 2 C and D). CO<sub>D</sub> thus acts as a local vibrational probe inside the protein environment, and reports on the changes of its immediate surroundings, such as hydrogen bond dynamics.<sup>35</sup> First, CO<sub>D</sub> is downshifted in Pfr by about 2 cm<sup>-1</sup> in good agreement with previously published linear IR data.<sup>36,37</sup> Additionally, this mode is significantly weaker in Pr compared to Pfr. The round lineshape and nearly horizontal nodal line (thick black line) in Pfr indicate that this functional group senses an ensemble of slightly different micro-environments, that interchange at a rate equal or faster than the t<sub>2</sub>-delay of 200 fs (homogeneous broadening). In contrast, the steeper nodal line and weaker peak signal in Pr indicate that CO<sub>D</sub> is found in a more heterogeneous environment with longer inter-

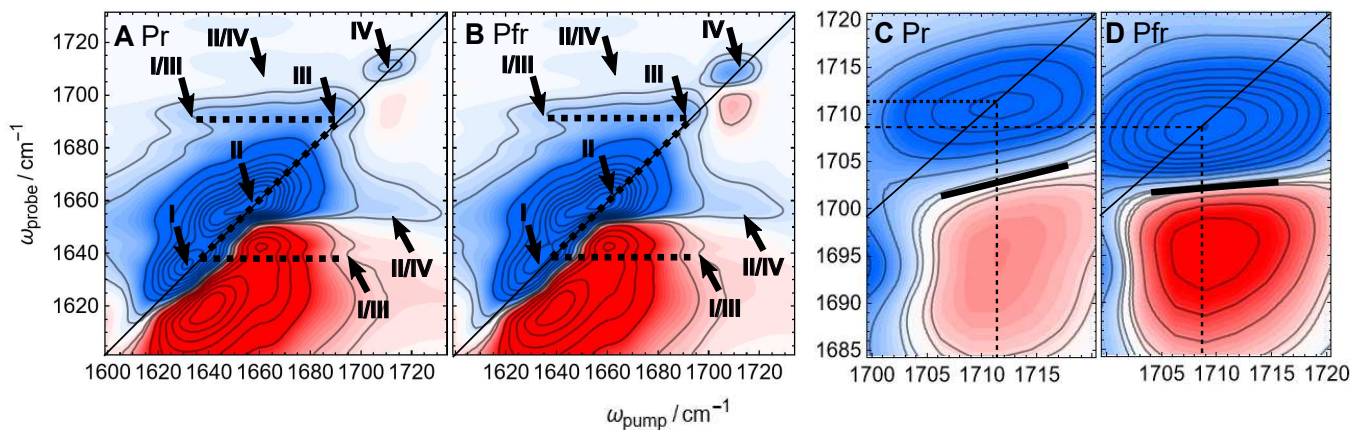


FIG. 2. 2D-IR spectroscopy of Agp1 in the amide I region. A: 2D-IR spectrum in the Pr state, B: 2D-IR spectrum in the Pfr state. Diagonal and off-diagonal signals marked with roman numerals are discussed in detail in the text. C and D: Detailed view of the  $\text{CO}_D$  region in the absolute Pfr and Pr spectra, with nodal line slope indicated as thick black line.

change dynamics  $> 200$  fs. However, the actual downshift and the changes in intensity and nodal line slope might be even stronger than we report here due to a residual Pr contribution in our spectra.

Furthermore, the 2D-IR spectra reveal broad crosspeaks between  $\text{CO}_D$  and the amide I region (labeled II/IV in figure 2 B), indicating vibrational coupling between  $\text{CO}_D$  and amide I in both states. Although these broad crosspeaks in the absolute spectra cannot be assigned to specific interactions with secondary structure elements, they are direct markers for protein-chromophore interactions. The subtle differences between Pr and Pfr in the amide I region are hard to analyse in the absolute spectra due to the overwhelming static amide I signal and water background. To enhance the spectral changes and simultaneously subtract out the background and the residual Pr contributions to the Pfr spectrum, the Pfr-minus-Pr difference 2D-IR spectrum was calculated.

**Difference 2D-IR spectroscopy** Figure 3 shows the difference 2D-IR spectrum compared with the linear IR difference spectrum (top) and the resonance Raman (RR) spectra of Pfr and Pr (middle panel). The difference spectrum was obtained by subtracting the Pr spectrum from Pfr in a 1:1 ratio without any scaling or the like. The linear data were taken from a previous publication.<sup>37</sup> The RR spectra allow for an assignment of vibrational modes that originate from BV, while the linear IR difference spectra selectively probe changes in vibrational frequencies between the Pfr and Pr states for both BV and protein. The dominant features on the diagonal coincide with both linear data:

- I A large signal with negative sign at  $1635 \text{ cm}^{-1}$  is mainly attributed to the loss of  $\beta$ -sheet a- mode due to the refolding of the tongue region, which corresponds to the largest signal in the FTIR.
- II A large positive signal is detected at  $1660 \text{ cm}^{-1}$  due to folding of the tongue region into an  $\alpha$ -helix in Pfr.

III The  $\beta$ -sheet a+ mode is weak and consequently the difference upon refolding of the tongue is small. No clear difference signal is visible for this mode in either of the spectra which is possibly also obscured by neighboring signals.

IV The change of the  $\text{CO}_D$  lineshape upon photoconversion produces a difference-pattern that closely resembles the absolute Pfr lineshape, with a small negative Pr contribution at higher frequencies. Since the  $\text{CO}_D$  is part of the conjugated system, this mode is also weakly detected in the RR spectra.

The assignment of the dominant FTIR signals that correspond to the diagonal difference bands is based on a large body of literature.<sup>23,25,26,37,38</sup> In these studies it was shown that signals from other, less defined, structural changes overlap with these signals, that are typically smaller by a factor of  $2 \times 10^x$  than the helix refolding contribution. A closer look at the off-diagonal region reveals weak difference crosspeaks that connect the major features on the diagonal, e.g. I/III or II/IV.

**Difference crosspeaks** The plot in fig. 3 reveals a complex pattern of small crosspeaks, which reports on changes of vibrational couplings upon photoconversion. Another possible origin is a change in transition dipole strength, which would alter the magnitude of a crosspeak and would therefore also produce a difference signal. Contributions from different parts of the protein are marked in purple (BV), orange ( $\beta$ -sheet) and green ( $\alpha$ -helix) and discussed in the following:

IV/V A pair of positive crosspeaks that connects  $\text{CO}_D$  with a diagonal feature (V) outside the amide I region around  $1600 \text{ cm}^{-1}$  is detected. This signal coincides precisely with the position of the dominant C=C stretching peak in the Pfr RR spectrum, indicating intramolecular vibrational coupling between

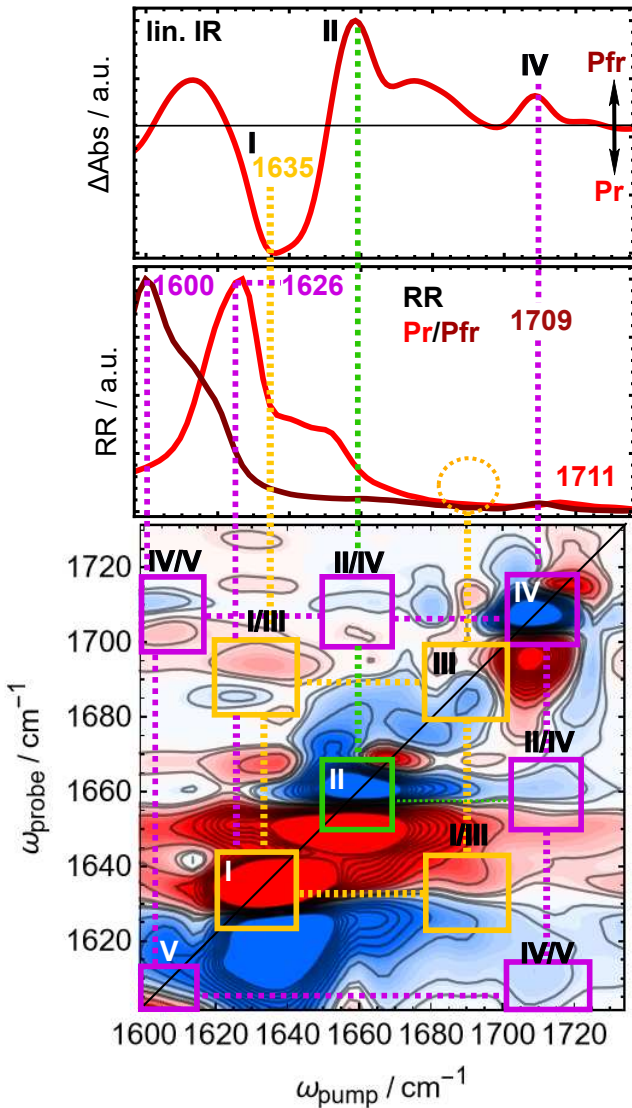


FIG. 3. Top panel: Linear IR difference spectrum of Agp1. Middle panel: RR spectra of Agp1 in the Pr and Pfr states, data for this part of the figure is taken from a previous publication.<sup>37</sup> Lowest panel: Pfr-minus-Pr difference 2D-IR spectrum. Roman numerals are discussed in detail in the text, signals originating from the  $\beta$ -hairpin are highlighted in orange,  $\alpha$ -helix in green and BV modes in purple.

the  $\text{CO}_D$  and C=C modes in Pfr. While this positive crosspeak is quite strong, a negative counterpart would be expected at  $1711/1626 \text{ cm}^{-1}$  due to Pr C=C and  $\text{CO}_D$  bleach at this frequency. Such a feature is barely visible, probably due to the broadened and therefore weaker  $\text{CO}_D$  band in Pr.

I/III Crosspeaks with a negative sign are found at the position where the coupling between the  $\beta$ -sheet a- and a+ modes is expected, and therefore assigned to the loss of  $\beta$ -sheet content due to tongue re-folding. However, diagonal peak I also contains a contribution from the BV C=C stretching signal at  $1626 \text{ cm}^{-1}$ , the strongest signal in the Pr

RR spectrum. In contrast to peak I, crosspeak I/III likely does not contain contributions from the C=C stretching mode and solely originates from the  $\beta$ -sheet, because there are no BV modes around  $1690 \text{ cm}^{-1}$  which could couple to the C=C and cause an off-diagonal feature at this position (dotted orange circle). Another option would be a highly localized protein mode at this position that couples with the C=C stretching only in Pr, which also appears unlikely.

II/IV A positive crosspeak is observed between  $\text{CO}_D$  and the  $\alpha$ -helix signal, indicating that  $\text{CO}_D$  couples stronger specifically to helix amide I modes in Pfr. In contrast to the broad feature in the absolute spectrum which extends over the entire amide I region, the difference crosspeak is quite sharp and localized at the  $\alpha$ -helix position. While the increased dipole strength of  $\text{CO}_D$  increases the magnitude of the diagonal  $\text{CO}_D$  band and all related crosspeaks, the relative sharpness and localisation of this peak at  $1660 \text{ cm}^{-1}$  indicates that it truly originates from a coupling with an  $\alpha$ -helical element in Pfr, in contrast to the broad crosspeaks in the absolute spectra in both states.

### III. DISCUSSION

**2D-Lineshapes and crosspeaks** The oscillator strength of the amide I modes of regular secondary structure motifs such as  $\alpha$ -helices and  $\beta$ -sheets concentrates into a smaller number of normal modes, in which the C=O groups oscillate in-phase, resulting in an increase in transition dipole strength by a factor of more than  $\times 2$ .<sup>39</sup> Together with the quadratic dependence of the 2D-IR signal, this results in more narrow 2D-IR spectra compared to linear IR spectra for these secondary structure motifs. In Agp1, this narrowing of the amide I band significantly reduces the overlap with bands in adjacent regions and thus allows to investigate the absolute  $\text{CO}_D$  lineshape in Pr and Pfr directly, which is not possible in linear IR experiments. Generally speaking, the amide I narrowing makes it possible to study all other vibrational modes with frequencies  $>1700 \text{ cm}^{-1}$  in this way. In photoreceptors, especially the C=O stretchings of protonated carboxylic sidechains that appear e.g. in bathy phytochromes,<sup>40</sup> retinal proteins,<sup>41</sup> cryptochromes,<sup>42</sup> or photoactive yellow protein<sup>43</sup> are functionally relevant and therefore of high interest. 2D-IR spectroscopy at low temperatures<sup>44</sup> or transient 2D-IR spectroscopy<sup>45,46</sup> could potentially reveal dynamic changes in the lineshape as these photoreceptors progress through their functional cycles. Here, these modes would act as local probes for the changing dynamics of their environment during the photocycle.

Furthermore, the 2D-IR difference technique spreads the congested linear spectra out into two dimensions, which is helpful for distinguishing between contributions of overlapping bands. For example, crosspeak I/III exclu-

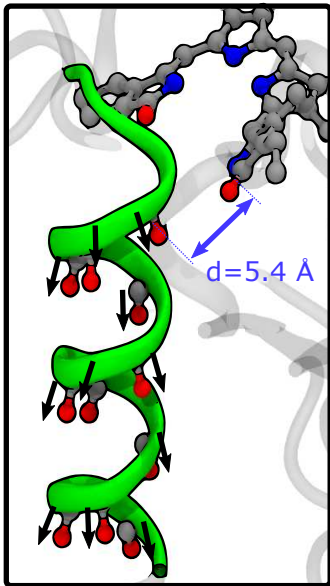


FIG. 4. Model for dipole stabilisation of the  $15E$  configuration of BV and the helical structure of the tongue region in the Pfr state: The alignment of the C=O dipoles results in increased vibrational coupling.

sively serves as a marker for the  $\beta$ -sheet loss, in contrast to diagonal feature I that overlaps with the C=C stretching vibration. Altogether, three systems of crosspeaks were observed in the difference 2D-IR spectrum that appear at consistent positions above and below the main diagonal. These three systems report on different types of coupling. Crosspeak IV/V originates from two localized and adjacent chromophore modes, which are very likely mechanically coupled to each other. I/III originates from two modes that are likely delocalized over the entire length of the tongue hairpin structure.<sup>47</sup> II/IV is particularly interesting because here, a localized mode from the chromophore couples to a delocalized  $\alpha$ -helix mode of the protein. Mechanical coupling through bonds appears unlikely because the CO<sub>D</sub> group is ca. 20 bonds away from the closest amino acid, a cysteine residue that is covalently linked to BV pyrrole ring A.<sup>30,48</sup> Therefore the most probable explanation for this crosspeak is an increased dipole coupling through space with the  $\alpha$ -helical tongue region in the Pfr state.

**Helix stabilisation in Pfr** The increased dipole coupling that leads to crosspeak II/IV can be rationalized by looking at the crystal structure of the Pfr-stabilized phytochrome Agp2, which we use as a model for the Pfr state in Agp1 (fig. 4).<sup>32</sup> The strength of dipole coupling depends on the distance and angles between the individual dipoles, and CO<sub>D</sub> is in close proximity to the tongue in Pfr. All peptide CO groups in  $\alpha$ -helices point into one direction, and thus their individual dipoles add up and couple strongly between each other. In case of Agp2, 11 peptide groups (amino acids PRO459-THR469) contribute to the overall helix backbone dipole. The distance of CO<sub>D</sub> to the closest CO group of that helix (PRO459) is only 5.4 Å in Pfr (fig. 4), while ring D and the tongue

region do not interact directly in Pr (compare fig.1).

Studies of a truncated Agp1 variant lacking the tongue region showed that Pfr is not stabilized properly,<sup>23,49</sup> as indicated by a strongly broadened vis absorption and accelerated dark reversion to Pr. Similar results were found for other bacterial phytochromes, indicating that this is a general effect in canonical Bphs.<sup>37,50</sup> Therefore, we propose that the interaction between the tongue helix and CO<sub>D</sub> we detect here in form of a crosspeak might play a role in stabilizing the position of pyrrole ring D in Pfr, and thus contributes to the kinetic stabilization of Pfr on timescales between minutes and days. The timescale of dark reversion is a highly variable parameter in different phytochromes, and important for fine-tuning of the amount of active state in photostationary equilibrium and therefore the biological function. Here, it is important to recall that it is the transition dipole coupling that gives rise to crosspeaks in a 2D-IR spectrum, while it is dipole interactions that may electrostatically stabilize a structure. The amide I mode is in essence the vibration of a diatomic molecule, i.e., the polar C=O group of the protein backbone, in which case the transition dipole is directly related to the dipole. The idea of dipole stabilisation is further supported by the homogeneous 2D-lineshape of CO<sub>D</sub> in Pfr, which indicates a well-defined single structure in this state.

The other way around, one might also speculate that isomerisation and repositioning of CO<sub>D</sub> could act as a driving force for the refolding of the tongue into the helical configuration. It has for example been shown that coupling of an external electronic dipole to a peptide can function in this way and trigger the folding of a random coil into a helix.<sup>51</sup> However in this example, the electronic dipole was much larger than in CO<sub>D</sub>. On the other hand, that study considered and isolated peptide that did not interact with the rest of a larger protein. We estimated the dipolar interaction energy between CO<sub>D</sub> and the helix backbone carbonyls by extracting the coordinates of all relevant C=O groups from the Agp2 Pfr crystal structure (Fig. 4) and assuming charges of 0.5  $e$  and -0.5  $e$  for the C and O atoms, respectively. This model calculation yielded a high interaction energy with the first C=O group of ca. 2 kJ/mol, while the 10th C=O group contributes only with an energy of 50 J/mol due to the strong distance dependence. The entire helix adds up to ca. 3.8 kJ/mol or 1.5  $k_B T$ . It is clear that many strongly competing forces are responsible for the structure of a protein, and energies on this order of magnitude might have large net effects. Overall, we suggest that the observation of the dipolar interaction is related to the evident mutual stabilization of these two sites and the corresponding crosspeak might be used as a marker for the interaction between them in future studies.

#### IV. METHODS

Agp1 (PAS-GAF-PHY) samples were prepared according to published protocols in a Tris buffer containing 50 mM Tris-Cl, 5 mM EDTA, and 300 mM NaCl and adjusted

with HCl and NaOH to pH 7.8.<sup>48</sup> 2D-IR spectra were collected with a setup based on a 100kHz Yb-fiber laser, mid-IR OPA and a pulse shaper as described before in detail<sup>35,46</sup>. Here, the 2D-IR spectra were collected in the rotating frame with a reference frequency of 1450 cm<sup>-1</sup>, while the step size for scanning the  $t_1$  delay was 40 fs, and the  $t_2$ -delay was 200 fs for all measurements. During data collection, the sample was illuminated inside the setup with two different light sources to photoswitch between the Pr and Pfr states. Here, a red LED (LIU630A Thorlabs, Newton, MA, USA) was used to populate the Pfr state, while a far-red diode laser (L785P25) was used for Pr. The individual absolute spectra of each state were collected for 30 min, corresponding to an averaging of 10<sup>6</sup> individual 2D-IR spectra. This process for Pr and Pfr was repeated ten times and the average of all data was used for analysis. UV-vis absorption spectra were recorded before and after the 2D-IR measurements, and no signs of photodegradation were found in these spectra or in 2D-IR spectra at different time points during the accumulation. All measurements were performed in aqueous (H<sub>2</sub>O) buffer solution, the pathlength of the sample cell was 5  $\mu$ m. The concentration of the sample was 10 mM, as determined by measuring the OD at 280 nm in the same cell used for the 2D-IR measurements and using an extinction coefficient of  $\epsilon_{280} = 78505 \text{ mol}^{-1} \text{ cm}^{-1}$  (from protein data bank) to calculate the concentration.

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### B. Author contributions

D.B. conceptualization, spectroscopic measurements and formal analysis. N.M protein expression and purification. P.H methodology, supervision, funding acquisition. D.B. writing with contributions from all authors.

### C. Conflict of interest

The authors declare no conflict of interest.

### D. Data availability statement

The data that support the findings of this study were deposited on zenodo under the DOI 10.5281/zenodo.6757659.

## REFERENCES

- <sup>1</sup>K. Anders and L. O. Essen, "The family of phytochrome-like photoreceptors: Diverse, complex and multi-colored, but very useful," *Curr. Opin. Struct. Biol.* **35**, 7–16 (2015).
- <sup>2</sup>W. L. Butler, K. H. Norris, H. W. Siegelman, and S. B. Hendricks, "Detection, assay, and preliminary purification of the pigment controlling photoresponsive development of plants," *Proc. Natl. Acad. Sci. U. S. A.* **45**, 1703–8 (1959).
- <sup>3</sup>A. Froehlich, B. Noh, and R. Vierstra, "Genetic and molecular analysis of phytochromes from the filamentous fungus *Neurospora crassa*," *Eukaryot. Cell* **4**, 2140 (2005).
- <sup>4</sup>T. Lamparter, F. Mittmann, W. Gärtner, T. Börner, E. Hartmann, and J. Hughes, "Characterization of recombinant phytochrome from the cyanobacterium *Synechocystis*," *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11792–7 (1997).
- <sup>5</sup>Z. Y. Jiang, L. R. Swem, B. C. Rushing, S. Devanathan, G. Tollin, and C. E. Bauer, "Bacterial photoreceptor with similarity to photoactive yellow protein and plant phytochromes," *Science* **285**, 406–409 (1999).
- <sup>6</sup>S. H. Bhoo, S. J. Davis, J. Walker, B. Karniol, and R. D. Vierstra, "Bacteriophytochromes are photochromic histidine kinases using a biliverdin chromophore." *Nature* **414**, 776–779 (2001).
- <sup>7</sup>D. M. Shcherbakova, M. Balaban, and V. V. Verkhusha, "Near-infrared fluorescent proteins engineered from bacterial phytochromes," *Curr. Opin. Chem. Biol.* **27**, 52–63 (2015).
- <sup>8</sup>G. Gourinchas, S. Ettl, and A. Winkler, "Bacteriophytochromes – from informative model systems of phytochrome function to powerful tools in cell biology," *Current Opinion in Structural Biology* **57**, 72–83 (2019).
- <sup>9</sup>B. Karniol and R. D. Vierstra, "The pair of bacteriophytochromes from *Agrobacterium tumefaciens* are histidine kinases with opposing photobiological properties," *Proc. Natl. Acad. Sci.* **100**, 2807–2812 (2003).
- <sup>10</sup>E. Multamäki, R. Nanekar, D. Morozov, T. Lievenen, D. Golonka, W. Y. Wahlgren, B. Stucki-Buchli, J. Rossi, V. P. Hytönen, S. Westenhoff, J. A. Ihalainen, A. Möglich, and H. Takala, "Comparative analysis of two paradigm bacteriophytochromes reveals opposite functionalities in two-component signaling," *Nature Communications* **12** (2021), 10.1038/s41467-021-24676-7.
- <sup>11</sup>G. Gourinchas, S. Ettl, C. Göbl, U. Vide, T. Madl, and A. Winkler, "Long-range allosteric signaling in red light-regulated diguanylyl cyclases," *Sci. Adv.* **3**, 1–12 (2017).
- <sup>12</sup>H. Takala, A. Björling, O. Berntsson, H. Lehtivuori, S. Niebling, M. Hoernke, I. Kosheleva, R. Henning, A. Menzel, J. a. Ihalainen, and S. Westenhoff, "Signal amplification and transduction in phytochrome photosensors." *Nature* **509**, 245–8 (2014).
- <sup>13</sup>J. R. Wagner, J. S. Brunzelle, K. T. Forest, and R. D. Vierstra, "A light-sensing knot revealed by the

- structure of the chromophore-binding domain of phytochrome,” *Nature* **438**, 325–331 (2005).
- <sup>14</sup>X. Yang, E. A. Stojković, J. Kuk, and K. Moffat, “Crystal structure of the chromophore binding domain of an unusual bacteriophytochrome, RpBphP3, reveals residues that modulate photoconversion.” *Proc. Natl. Acad. Sci. U. S. A.* **104**, 12571–12576 (2007).
- <sup>15</sup>C. Song, T. Rohmer, M. Tiersch, J. Zaanen, J. Hughes, and J. Matysik, “Solid-state NMR spectroscopy to probe photoactivation in canonical phytochromes,” *Photochem. Photobiol.* **89**, 259–273 (2013).
- <sup>16</sup>E. S. Burgie, T. Wang, A. N. Bussell, J. M. Walker, H. Li, and R. D. Vierstra, “Crystallographic and electron microscopic analyses of a bacterial phytochrome reveal local and global rearrangements during photoconversion,” *J. Biol. Chem.* **289**, 24573–24587 (2014).
- <sup>17</sup>H. Takala, S. Niebling, O. Berntsson, A. Björling, H. Lehtivuori, H. Häkkänen, M. Panman, E. Gustavsson, M. Hoernke, G. Newby, F. Zontone, M. Wulff, A. Menzel, J. A. Ihalainen, and S. Westenhoff, “Light-induced structural changes in a monomeric bacteriophytochrome,” *Struct. Dyn.* **3** (2016).
- <sup>18</sup>G. Battocchio, R. González, A. G. Rao, I. Schapiro, and M. A. Mroginiski, “Dynamic Properties of the Photosensory Domain of *Deinococcus radiodurans* Bacteriophytochrome,” *Journal of Physical Chemistry B* **124**, 1740–1750 (2020).
- <sup>19</sup>J. A. Ihalainen, E. Gustavsson, L. Schroeder, S. Donnini, H. Lehtivuori, L. Isaksson, C. Thöing, V. Modi, O. Berntsson, B. Stucki-Buchli, A. Liukkonen, H. Häkkänen, E. Kalenius, S. Westenhoff, and T. Kottke, “Chromophore-Protein Interplay during the Phytochrome Photocycle Revealed by Step-Scan FTIR Spectroscopy,” *Journal of the American Chemical Society* **140**, 12396–12404 (2018).
- <sup>20</sup>D. Buhrke, U. Kuhlmann, N. Michael, and P. Hildebrandt, “The Photoconversion of Phytochrome Includes an Unproductive Shunt Reaction Pathway,” *ChemPhysChem* **19**, 566–570 (2018).
- <sup>21</sup>J. Dasgupta, R. R. Frontiera, K. C. Taylor, J. C. Lagarias, and R. A. Mathies, “Ultrafast excited-state isomerization in phytochrome revealed by femtosecond stimulated Raman spectroscopy.” *Proc. Natl. Acad. Sci. U. S. A.* **106**, 1784–1789 (2009).
- <sup>22</sup>J. J. van Thor, K. L. Ronayne, and M. Towrie, “Formation of the early photoproduct Lumi-R of cyanobacterial phytochrome Cph1 observed by ultrafast mid-infrared spectroscopy,” *J. Am. Chem. Soc.* **129**, 126–132 (2007).
- <sup>23</sup>P. Piwowarski, E. Ritter, K. P. Hofmann, P. Hildebrandt, D. von Stetten, P. Scheerer, N. Michael, T. Lamparter, and F. Bartl, “Light-induced activation of bacterial phytochrome Agp1 monitored by static and time-resolved FTIR spectroscopy,” *ChemPhysChem* **11**, 1207–1214 (2010).
- <sup>24</sup>H. Foerstendorf, C. Benda, W. Gärtner, M. Storf, H. Scheer, and F. Siebert, “FTIR studies of phytochrome photoreactions reveal the C=O bands of the chromophore: Consequences for its protonation states, conformation, and protein interaction,” *Biochemistry* **40**, 14952–14959 (2001).
- <sup>25</sup>E. A. Stojković, K. C. Toh, M. T. A. Alexandre, M. Barclayon, K. Moffat, and J. T. M. Kennis, “FTIR spectroscopy revealing light-dependent refolding of the conserved tongue region of bacteriophytochrome,” *J. Phys. Chem. Lett.* **5**, 2512–2515 (2014).
- <sup>26</sup>A. Kraskov, A. D. Nguyen, J. Goerling, D. Buhrke, F. Velazquez Escobar, M. Fernandez Lopez, N. Michael, L. Sauthof, A. Schmidt, P. Piwowarski, Y. Yang, T. Stensitzki, S. Adam, F. Bartl, I. Schapiro, K. Heyne, F. Siebert, P. Scheerer, M. A. Mroginiski, and P. Hildebrandt, “Intramolecular Proton Transfer Controls Protein Structural Changes in Phytochrome,” *Biochemistry* **59**, 1023–1037 (2020).
- <sup>27</sup>Z. Ganim, H. S. Chung, A. W. Smith, L. P. Deflores, K. C. Jones, and A. Tokmakoff, “Amide I Two-Dimensional Infrared Spectroscopy of Proteins,” *Accounts of Chemical Research* **41**, 432–44 (2007).
- <sup>28</sup>P. Hamm and M. T. Zanni, *Concepts and Methods of 2D Infrared Spectroscopy* (Cambridge University Press, 2011).
- <sup>29</sup>T. Lamparter, N. Krauß, and P. Scheerer, “Phytochromes from *agrobacterium fabrum*,” *Photochem. Photobiol.* **93**, 642–655 (2017).
- <sup>30</sup>S. Nagano, P. Scheerer, K. Zubow, N. Michael, K. Inomata, T. Lamparter, and N. Krauß, “The crystal structures of the N-terminal photosensory core module of *Agrobacterium* phytochrome Agp1 as parallel and anti-parallel dimers,” *J. Biol. Chem.* **291**, 20674–20691 (2016).
- <sup>31</sup>A. Takiden, F. Velázquez Escobar, J. Dragelj, A. L. Woelke, E. W. Knapp, P. Piwowarski, F. Bartl, P. Hildebrandt, and M.-A. Mroginiski, “Structural and Vibrational Characterization of the Chromophore Binding Site of Bacterial Phytochrome Agp1,” *Photochem. Photobiol.* **93**, 713–723 (2017).
- <sup>32</sup>A. Schmidt, L. Sauthof, M. Szczepek, M. Fernández López, F. Velázquez Escobar, B. M. Qureshi, N. Michael, D. Buhrke, T. Stevens, D. Kwiatkowski, D. von Stetten, M. Mroginiski, N. Krauss, T. Lamparter, P. Hildebrandt, and P. Scheerer, “Structural snapshot of a bacterial phytochrome in its functional intermediate state,” *Nat. Commun.* **9**, 1–13 (2018).
- <sup>33</sup>N. Demirdöven, C. M. Cheatum, H. S. Chung, M. Khalil, J. Knoester, and A. Tokmakoff, “Two-dimensional infrared spectroscopy of antiparallel  $\beta$ -sheet secondary structure,” *Journal of the American Chemical Society* **126**, 7981–7990 (2004).
- <sup>34</sup>L. Chuntonov, R. Kumar, and D. G. Kuroda, “Non-linear infrared spectroscopy of the water bending mode: Direct experimental evidence of hydration shell reorganization?” *Physical Chemistry Chemical Physics* **16**, 13172–13181 (2014).
- <sup>35</sup>J. Ruf, P. Hamm, and D. Buhrke, “Needles in a Haystack: H-bonding in an Optogenetic Protein observed with Isotope Labeling and 2D-IR Spectroscopy,” *Physical Chemistry Chemical Physics* (2021), 10.1039/d1cp00996f.
- <sup>36</sup>C. Schumann, R. Groß, N. Michael, T. Lamparter, and R. Diller, “Sub-picosecond mid-infrared spectroscopy of



- phytochrome Agp1 from *Agrobacterium tumefaciens*,” *ChemPhysChem* **8**, 1657–1663 (2007).
- <sup>37</sup>D. Buhrke, G. Gourinchas, M. Müller, N. Michael, P. Hildebrandt, and A. Winkler, “Distinct chromophore–protein environments enable asymmetric activation of a bacteriophytochrome-activated diguanylate cyclase,” *Journal of Biological Chemistry* **295**, 539–551 (2020).
- <sup>38</sup>G. Merga, M. Fernandez Lopez, P. Fischer, P. Piwowarski, Z. Nogacz, A. Kraskov, D. Buhrke, F. Velazquez Escobar, N. Michael, F. Siebert, P. Scheerer, F. Bartl, and P. Hildebrandt, “Light- and Temperature-dependent Dynamics of Chromophore and Protein Structural Changes in Bathy Phytochrome Agp2,” *Physical Chemistry Chemical Physics* (2021), 10.1039/d1cp02494a.
- <sup>39</sup>M. Grechko and M. T. Zanni, “Quantification of transition dipole strengths using 1D and 2D spectroscopy for the identification of molecular structures via exciton delocalization: Application to  $\alpha$ -helices,” *Journal of Chemical Physics* **137** (2012), 10.1063/1.4764861.
- <sup>40</sup>F. Velázquez Escobar, P. Piwowarski, J. Salewski, N. Michael, M. Fernández López, A. Rupp, B. Muhammad Qureshi, P. Scheerer, F. Bartl, N. Frankenberg-Dinkel, F. Siebert, M. Andrea Mroginski, and P. Hildebrandt, “A protonation-coupled feedback mechanism controls the signalling process in bathy phytochromes.” *Nat. Chem.* **7**, 423–30 (2015).
- <sup>41</sup>K. Gerwert, G. Souvignier, and B. Hess, “Simultaneous monitoring of light-induced changes in protein side-group protonation, chromophore isomerization, and backbone motion of bacteriorhodopsin by time-resolved Fourier-transform infrared spectroscopy,” *Proceedings of the National Academy of Sciences of the United States of America* **87**, 9774–9778 (1990).
- <sup>42</sup>T. Kottke, V. A. Lórenz-Fonfría, and J. Heberle, “The grateful infrared: Sequential protein structural changes resolved by infrared difference spectroscopy,” *Journal of Physical Chemistry A* **121**, 335–350 (2017).
- <sup>43</sup>Y. Imamoto, Y. Shirahige, F. Tokunaga, T. Kinoshita, K. Yoshihara, and M. Kataoka, “Low-temperature fourier transform infrared spectroscopy of photoactive yellow protein,” *Biochemistry* **40**, 8997–9004 (2001).
- <sup>44</sup>A. Shalit, F. Perakis, and P. Hamm, “Communication: Disorder-suppressed vibrational relaxation in vapor-deposited high-density amorphous ice,” *The Journal of Chemical Physics* **140**, 151102 (2014).
- <sup>45</sup>S. C. Hoi, Z. Ganim, K. C. Jones, and A. Tokmakoff, “Transient 2D IR spectroscopy of ubiquitin unfolding dynamics,” *Proceedings of the National Academy of Sciences of the United States of America* **104**, 14237–14242 (2007).
- <sup>46</sup>P. Hamm, “Transient 2D IR Spectroscopy from Micro- to Milliseconds,” *J. Chem. Phys* **154**, 1–6 (2021).
- <sup>47</sup>A. W. Smith, H. S. Chung, Z. Ganim, and A. Tokmakoff, “Residual native structure in a thermally denatured  $\beta$ -hairpin,” *Journal of Physical Chemistry B* **109**, 17025–17027 (2005).
- <sup>48</sup>T. Lamparter, N. Michael, O. Caspani, T. Miyata, K. Shirai, and K. Inomata, “Biliverdin Binds Covalently to *Agrobacterium* Phytochrome Agp1 via Its Ring A Vinyl Side Chain,” *Journal of Biological Chemistry* **278**, 33786–33792 (2003).
- <sup>49</sup>S. Noack, N. Michael, R. Rosen, and T. Lamparter, “Protein conformational changes of *Agrobacterium* phytochrome Agp1 during chromophore assembly and photoconversion,” *Biochemistry* **46**, 4164–4176 (2007).
- <sup>50</sup>J. R. Wagner, J. Zhang, D. von Stetten, M. Günther, D. H. Murgida, M.-A. Mroginski, J. M. Walker, K. T. Forest, P. Hildebrandt, and R. D. Vierstra, “Mutational analysis of *Deinococcus radiodurans* bacteriophytochrome reveals key amino acids necessary for the photochromicity and proton exchange cycle of phytochromes,” *J. Biol. Chem.* **283**, 12212–12226 (2008).
- <sup>51</sup>C. Y. Huang, S. He, W. F. DeGrado, D. G. McCafferty, and F. Gai, “Light-induced helix formation,” *Journal of the American Chemical Society* **124**, 12674–12675 (2002).