Structural snapshot of a bacterial phytochrome in its functional intermediate state

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Phytochromes are modular photoreceptors of plants, bacteria and fungi that use light as a source of information to regulate fundamental physiological processes. Interconversion between the active and inactive states is accomplished by a photoinduced reaction sequence which couples the sensor with the output module. However, the underlying molecular mechanism is yet not fully understood due to the lack of structural data of functionally relevant intermediate states. Here we report the crystal structure of a Meta-F intermediate state of an Agp2 variant from Agrobacterium fabrum. This intermediate, the identity of which was verified by resonance Raman spectroscopy, was formed by irradiation of the parent Pfr state and displays significant reorientations of almost all amino acids surrounding the chromophore. Structural comparisons allow identifying structural motifs that might serve as conformational switch for initiating the functional secondary structure change that is linked to the (de-)activation of these photoreceptors.

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Phytochromes are biliproteins of plants, bacteria and fungi that use red light as a source of information to regulate fundamental physiological processes, such as photosynthesis, seed germination, flowering and shade avoidance. The molecular architecture of phytochromes shares a common photosensory core module (PCM), which is composed of PAS (Per/Arnt/Sim), GAF (cGMP phosphodiesterase/adenyl cyclase/FhlA) and PHY (phytochrome-specific) domain. Despite the differences between the bilin-type chromophores (biliverdin (BV) vs. phytochromobilin) and their covalent binding sites (PAS vs. GAF domain) in bacterial and plant PCMs, the photoinduced processes that interconvert the two parent states Pr and Pfr follow similar pathways with spectrally distinguishable Lumi and Meta intermediates.

Bacterial phytochromes can be grouped into prototypical and bathy phytochromes, in which the red-absorbing Pr and far-red-absorbing Pfr are the thermodynamically stable states, respectively. The photoconversions between the parent states Pr and Pfr are coupled to downstream signalling processes via the modulation of an attached output module, typically a histidine kinase and comprises a secondary structure transition in a sensor-output interconnecting region, the PHY tongue (Fig. 1b), which probably represents a reaction pattern common to all phytochromes. Thus structural knowledge of the Meta intermediate is a prerequisite for a detailed understanding of the molecular mechanism of the signal transfer from the sensor to the output module (Fig. 1). In a previous study, irradiation of Pfr crystals of the bathy phytochrome PbBphP from Pseudomonas aeruginosa at different temperatures revealed insight into very early photoinduced structural changes of the chromophore in the Lumi-F state, i.e. the E→Z isomerization at the methine bridge C15–C16 between the pyrrole rings C and D of the BV chromophore.
chromophore\textsuperscript{21}. No structural models, however, are yet available for the later intermediates that, instead, have been characterized by vibrational spectroscopy indicating chromophore relaxation and proton transfer steps, in addition to the secondary structure transition of the PHY tongue\textsuperscript{17,22-24}.

Here we describe the crystal structure of a Meta-F intermediate of the PCM of a bathy phytochrome at 2.16 Å resolution formed during the Pfr→Pr phototransformation (Fig. 2). The intermediate has been generated by irradiating crystals of the parent Pfr state at ambient temperature and identified as Meta-F by resonance Raman (RR) spectroscopy. The conjugate Meta-F and Pfr crystal structures have been obtained from the engineered photoactivatable near-IR fluorescent protein (Agp2-PAiRFP2)\textsuperscript{35,36}, derived from the bathy phytochrome Agp2 from Agrobacterium\textit{fabrum}. The parent Pfr state of Agp2-PAiRFP2 (2.03 Å resolution) displays the same structure as the wild-type protein determined here as well (2.50 Å resolution) (Fig. 2).

**Results**

Crystal structure of the wild-type bathy phytochrome Agp2-PCM. Starting point of the present studies was the crystal structure of the PCM fragment of wild-type Agp2 (Agp2-PCM) in the dark adapted Pfr state, which crystallized in the orthorhombic space group P2\(_1\)2\(_1\)2\(_1\). The structure, solved at 2.5 Å resolution (Fig. 2a; Table 1), shows overall similar topologies to \textit{PaBphP-PCM}\textsuperscript{27}, including the characteristic, well-ordered helical conformation of a part of the PHY tongue (Fig. 2a). In contrast to \textit{PaBphP-PCM}, the tongue region of Agp2-PCM is not directly stabilized by crystallographic contacts to symmetry-related molecules within the crystal. The BV chromophore, \(\beta\)-facially attached to Cys13 via ring A, adopts a ZZEssa stereochemistry (Fig. 2a; Supplementary Figs. 1a, d, g, 2a, d, g). A hydrogen-bonding network around the propionate side chains of rings B and C involves the side chains of Tyr165, Arg211 and His278 and is extended via several ordered water molecules to include also Tyr205, Arg242, Ser245, His248, Ser260 and Ser262 (Fig. 2a; Supplementary Fig. 3). The N-H groups of the rings A, B and C interact with the backbone oxygen of Asp196 and a conserved water molecule (Wat722), whereas the N-H group of ring D forms a salt bridge with the carboxylate group of Asp196. These interactions are conserved in the Pfr state of other bathy phytochromes\textsuperscript{27}.

The Pfr Agp2-PCM crystals lose their diffraction capability immediately after irradiation with far-red light at ambient temperatures indicating major protein conformational changes in the crystal. We have, therefore, extended the studies to Agp2-PCM variants with substitutions (Supplementary Fig. 4), which do not affect essential structural and mechanistic properties.

Crystal structure of the Agp2-PAiRFP2 in the Pfr state. Preliminary crystallization and spectroscopic experiments identified the PCM fragment of the photoactivatable phytochrome Agp2-PAiRFP2 as a promising candidate. Ultraviolet–visible absorption spectra of Agp2-PAiRFP2 compared to the wild-type Agp2-PCM construct, the full-length Agp2 (Agp2-FL) and the prototypical full-length phytochrome Agp1 (Agp1-FL) display the characteristic spectrum of Pfr but an enhanced stability of the final photoproduct state, resulting in slower dark reversion to Pfr compared to Agp2-PCM (Supplementary Fig. 5). The half-lives of the photoinduced states of Agp2-FL, Agp2-PCM and the PCM fragment of Agp2-PAiRFP2 are 170 s\textsuperscript{11}, 20 s\textsuperscript{41} and 233 min\textsuperscript{25}, respectively. This Agp2-PCM variant includes 24 substitutions, which except for Val244Phe and Ala276Val do not participate in the interaction between the chromophore and chromophore-binding site (Supplementary Figs. 3, 4, 6).

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**Fig. 2** Overview of the crystal structures of bacterial phytochromes from \(A.\) \textit{fabrum}. Structures (ribbon representation) of the monomers are shown for the dark adapted Pfr state of a wild-type Agp2-PCM (PDB entry 6G1Y) and b Agp2-PAiRFP2 (PDB entry 6G1Z), c a Meta-F sub-state (Mol A) of the Agp2-PAiRFP2 (PDB entry 6G20) and d the Pr state of the Agp1-PCM (Agp1-PCMSER13 of \(A.\) \textit{fabrum}, PDB entry 5HSQ\textsuperscript{30}). Close-up view of the chromophore-binding pockets (a–d) including BV and amino acid residues within the hydrogen bond network of BV (stick representation) as well as selected water molecules (red spheres). PAS domains are displayed in grey, PHY domains in black and GAF domains are depicted in different colours.
Agp2-PAiRFP2 was crystallized in the dark adapted Pfr state (in the hexagonal space group P6₁,22) and the structure was solved at a resolution of 2.03 Å (Table 1). Remarkably, crystal packing and crystallization conditions differ significantly from those of Agp2-PCM. While Agp2-PCM shows a parallel orientation of the two crystallographic monomers, Agp2-PAiRFP2 monomers crystallize in a near anti-parallel arrangement (Fig. 3a–c). However, the overall topologies of the monomers in both structures (Fig. 2a, b) agree very well, as demonstrated also by superposition of the equivalent Cα atoms of both constructs yielding a root-mean-square deviation of only 0.6 Å (Fig. 3d). Also, structural details of the chromophore pocket are mirrored by the far-reaching similarities of the RR spectra of the Pfr states of Agp2-PCM and Agp2-PAiRFP2 in solution as well as in the crystalline form (Supplementary Fig. 7), demonstrating that neither amino acid substitutions nor crystallization conditions and water molecules, are almost identical for the Pfr states of both Agp2-PCM variants (Fig. 2a, b; Supplementary Figs. 1, 2 and 3). In both Pfr structures and in contrast to other bathyphytochrome structures such as PaBphP-PCM21, Gln190 of Agp2-PCM and Agp2-PAiRFP2 interacts with ring D of BV via a newly identified conserved water molecule (Fig. 2a, b; Supplementary Fig. 3). This good structural agreement is mirrored by the far-reaching similarities of the RR spectra of the Pfr states of Agp2-PCM and Agp2-PAiRFP2 in solution as well as in the crystalline form (Supplementary Fig. 7), demonstrating that neither amino acid substitutions nor crystallization conditions (e.g. crystal packing) do affect the structure of the chromophore pocket.

### Formation of the Meta-F state of Agp2-PAiRFP2

Next, the Pfr crystals of Agp2-PAiRFP2 were irradiated with 785 nm light at ambient temperature to generate the intermediate state (Supplementary Fig. 8; Methods). In this case, irradiation for several seconds did not lead to non-diffracting crystals. The identity of the resultant state was determined by RR spectroscopy (Fig. 4). The RR spectra of Pfr measured from the crystal (trace a) and frozen solution (trace b) are nearly identical. Upon irradiation of Pfr at 130 K in frozen solution, Lumi-F is obtained (trace c), the spectrum of which displays the loss of the 811 cm⁻¹ hydrogen-out-of-plane (HOOP) band and upshift of the C-D and A-B stretching modes by ca. 10 cm⁻¹ (Supplementary Fig. 10). The subsequent intermediate Meta-F (trace d, frozen solution), formed upon irradiation at ca. 240 K, is reflected by further upshifts of the C-D and A-B stretching modes by ca. 10 cm⁻¹ and the drastic intensity loss of the B-C stretching, indicating the

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**Table 1** Data collection and refinement statistics

|                      | Agp2-PCM          | Agp2-PAiRFP2       | Agp2-PAiRFP2       |
|----------------------|-------------------|--------------------|--------------------|
|                      | Pfr state         | Pfr state          | Meta-F state       |
|                      | (PDB entry 6G1Y)  | (PDB entry 6G1Z)  | (PDB entry 6G20)   |
| **Data collection**  |                   |                    |                    |
| Beamline             | ESRF, ID30A-3³⁶   | BESSY II (BL14.1)⁴⁰ | ESRF, ID23-1⁶²     |
| Wavelength           | λ = 0.9677 Å      | λ = 0.9184 Å       | λ = 0.9724 Å       |
| Space group          | P₂₁,2₁            | P₂₁,2₁             | P₂₁,2₁             |
| Cell dimensions      | a, b, c (Å)       | 74.52, 93.36, 174.42 | 182.38, 182.38, 179.88 | 183.11, 183.11, 179.65 |
| α, β, γ (°)          | 90.0, 90.0, 90.0   | 90.0, 90.0, 120.0  | 90.0, 90.0, 120.0  |
| Resolution (Å)       | 45.09–2.50        | 47.73–2.03         | 47.79–2.16         |
| Rmerge               | 0.187 (0.931)     | 0.143 (1.495)      | 0.138 (1.824)      |
| Rwork               | 0.132 (0.661)     | 0.040 (0.417)      | 0.031 (0.409)      |
| I/σ(I)              | 8.6 (1.9)         | 15.4 (1.9)         | 18.3 (1.9)         |
| CCA/2               | 0.989 (0.622)     | 0.999 (0.592)      | 0.999 (0.672)      |
| Completeness (%)     | 99.9 (99.9)       | 100.0 (100.0)      | 100.0 (100.0)      |
| Redundancy           | 5.4 (5.6)         | 13.3 (13.6)        | 20.1 (20.8)        |
| **Refinement**       |                   |                    |                    |
| Resolution (Å)       | 45.09–2.50        | 47.73–2.03         | 47.79–2.16         |
| No. of reflections   | 40755             | 107549             | 90118              |
| Rwork/Rfree (%)      | 20.1/24.1         | 18.0/21.3          | 20.9/23.2          |
| No. of atoms         |                   |                    |                    |
| Protein              | 7745              | 7750               | 7682               |
| Ligand/ion           | 86                | 244                | 262                |
| Water                | 202               | 853                | 311                |
| B-factors            |                   |                    |                    |
| Protein              | 37.00             | 32.70              | 51.30              |
| Ligand/ion           | 25.13             | 45.35              | 68.42              |
| Water                | 32.50             | 41.20              | 49.90              |
| R.m.s. deviations    |                   |                    |                    |
| Bond lengths (Å)     | 0.009             | 0.013              | 0.011              |
| Bond angles (°)      | 1.33              | 1.54               | 1.47               |
| Ramachandran plotc   |                   |                    |                    |
| Favour (%)           | 98.6              | 99.0               | 99.0               |
| Allowed (%)          | 1.4               | 1.0                | 1.0                |
| Outlier (%)          | 0.0               | 0.0                | 0.0                |

³⁶Root mean square
³⁷One crystal was used
³⁸Highest resolution shell is shown in parenthesis
³⁹Ramachandran plot calculated by MolProbity²⁸
relaxation of the ZZZssa chromophore structure. The spectrum of the irradiated Pfr crystals displays far-reaching similarities with that obtained from frozen solution irradiated under the same conditions and thus allows the unambiguous assignment to the Meta-F state. Minor differences exist between the RR spectra of the Meta-F states in frozen solution and in the crystalline form (trace e), such as the frequency upshift of the N-H in-plane (ip) mode of rings B and C. These deviations may point to a structural heterogeneity of the Meta-F state, presumably related to details of the hydrogen bonding network in the chromophore pocket. These findings point to the existence of Meta-F sub-states (vide infra). Note that, as for the solution spectra, the crystal spectra obtained after irradiation at 240 or 273 K were not different, implying that the chromophore adopts the fully relaxed structure of the final photoproduct already at 240 K (Supplementary Fig. 9).

The crystal structure of a Meta-F intermediate could be solved to a resolution of 2.16 Å (Table 1). The asymmetric unit includes two molecules (Mol A and B), which differ in terms of the intermolecular interactions of their N-termini (Fig. 5). Both show overall highly similar topologies. Compared to the Pfr structure, the overall fold of the protein and specifically the α-helical segment of the tongue remained unchanged but significant structural changes were observed for the chromophore and its binding pocket as reflected by the ΔFo (FoMeta-F – FoPfr) electron density maps (Fig. 6). The C-D methine bridge has undergone the expected E→Z isomerization as also shown by 2mFo–DFc and several types of omit electron density maps (Supplementary Figs. 1c, f, i and 2c, f, i). The resulting ZZZssa stereochemistry of the chromophore structure exhibits a deviation of the tilt angle between neighbouring C and D pyrrole rings (38.9° and 44.5° for Mol A and Mol B, respectively; Supplementary Table 1). In fact, the RR spectra measured from five different crystals, grown and irradiated under similar conditions showed some spectral variations, specifically for the C-D methine bridge stretching mode, which varied in frequency between 1621 and 1624 cm⁻¹. The frequency of this mode sensitively responds to changes of the torsion of the C-D methine bridge, which is different for the Meta-F sub-states in Mol A and Mol B. It is therefore tempting to relate the abovementioned frequency changes to variable contributions of Mol A and B to the spectrum, which in turn may result from the preferential Raman enhancement of the two chromophores depending on the orientation of the crystal with respect to the laser beam.

The isomerization and the subsequent relaxation process of the chromophore are associated with structural changes of the protein that, for the sake of simplifying the discussion, may be sorted into three groups.

Group I structural changes around the chromophore. The first group includes a rearrangement of the extended hydrogen bond network surrounding the chromophore, most likely initiated by the almost 180° rotation of ring D (Figs. 2a–c, 6a, b; Supplementary Figs. 1–3, 6, 10) and facilitated by conformational changes of the participating amino acid residues and movements of ordered water molecules. The ring D N-H group, which in Pfr forms a hydrogen bond with the Asp196 side chain, now interacts with the structural water Wat795. The C=O group of ring D, originally in close proximity to Wat738 and its hydrogen bonded partner Gln190, is now placed in hydrogen bonding distance to His278, which is rotated and slightly shifted compared to the Pfr state. Concomitantly, the ring C propionate side chain of BV (prop-C) is transferred into a different hydrogen bonding environment. It involves Arg211, which has undergone major rotational motions towards prop-C, as well as His248, Ser260, Ser262 and two water molecules (Wat707, Wat780) instead of Tyr165 and His278. The side chain of Phe244, which in wild-type Agp2 is a valine, has rotated to a position above the salt bridge between the ring B propionate side chain (prop-B) and Arg242, which together with Tyr205 has replaced Arg211 as the main hydrogen bonding partner (Fig. 2a, b, c; Supplementary Fig. 10). These changes are nearly identical for the Meta-F sub-states in Mol A and Mol B.

Group II structural changes with switching amino acids. The second group of structural changes in comparison to Pfr, as clearly seen in Mol A, involves Tyr165, Phe192 and Arg202, amino acid residues that are not connected to BV via a hydrogen bond network. These structural changes are accompanied by a transition from a β-facial to an α-facial orientation of the BV-binding Cys13 together with a conformational rearrangement of the N-terminus of the protein. In Mol A of the asymmetric unit, the N-terminus including Cys13 is completely free of inter-molecular interactions within the crystal packing (Fig. 5). In the BV chromophore, the hydrophobic side of ring D, facing the tongue of the PHY domain, forms a hydrophobic pocket with the re-positioned Phe192 and Tyr165 (Figs. 6a, f, 7a, b, d, e), thereby
obtained by irradiation at ca. 240 K, crystal. The RR spectra of Pfr measured
identical. Upon irradiation of Pfr at 130 K, Lumi-F is obtained (trace
whereas the subsequent intermediate Meta-F (trace
crystalline form (see also Supplementary Fig. 9)
between the RR spectra of the Meta-F states in frozen solution and in the
frozen solution; Meta-F obtained by irradiation at ca. 240 K, frozen solution; Meta-F
obtained by irradiation at ca. 240 K, frozen solution; Meta-F
obtained by irradiation at ca. 240 K, crystal. The RR spectra of Pfr measured
from the crystal (trace a) and from frozen solution (trace b) are nearly
identical. Upon irradiation of Pfr at 130 K, Lumi-F is obtained (trace c),
whereas the subsequent intermediate Meta-F (trace d, frozen solution) was
trapped upon irradiation at ca. 240 K. The spectrum of the irradiated
crystals displays far-reaching similarities with that of Agp2-PAiRFP2 in
frozen solution and irradiated under the same conditions and thus allows
the unambiguous assignment of the Meta-F state (trace e). Note that, as
for the solution spectra, there are essentially no spectral differences if the
crystals are irradiated at 240 or 273 K. Only minor differences exist
between the RR spectra of the Meta-F states in frozen solution and in the
crystalline form (see also Supplementary Fig. 9).

Fig. 4 Experimental resonance Raman spectra of Agp2-PAiRFP2, measured
from frozen solution and single crystals at 90 K. a Pfr, crystal; b Pfr, frozen
solution; c Lumi-F, frozen solution obtained by irradiation at ca. 130 K;
d Meta-F obtained by irradiation at ca. 240 K, frozen solution; e Meta-F
obtained by irradiation at ca. 240 K, crystal. The RR spectra of Pfr measured
from the crystal (trace a) and from frozen solution (trace b) are nearly
identical. Upon irradiation of Pfr at 130 K, Lumi-F is obtained (trace c),
whereas the subsequent intermediate Meta-F (trace d, frozen solution) was
trapped upon irradiation at ca. 240 K. The spectrum of the irradiated
crystals displays far-reaching similarities with that of Agp2-PAiRFP2 in
frozen solution and irradiated under the same conditions and thus allows
the unambiguous assignment of the Meta-F state (trace e). Note that, as
for the solution spectra, there are essentially no spectral differences if the
crystals are irradiated at 240 or 273 K. Only minor differences exist
between the RR spectra of the Meta-F states in frozen solution and in the
crystalline form (see also Supplementary Fig. 9).

replacing the conserved Wat758, accompanied by a positional
shift of its interaction partner Gln190 away from ring D in the
Meta-F state (Fig. 6a, g). Concomitantly, the side chain of Arg202
has rotated to accommodate two new water molecules (Wat722,
Wat732) as a hydrogen bond bridge to Tyr165 (Fig. 6a, b, d).
These conformational changes appear to be partially blocked in
Mol B although its chromophore adopts nearly the same ZZZssa
conformation and is involved in the same rearrangement of the
hydrogen bond network as described as the first group of struc-
tural changes in Mol A (Fig. 7d, e). However, in Mol B, the side
chains of both Tyr165 and Phe192 display two alternate con-
formations each, one conformation being similar to Pfr and the
other resembling Mol A. In Mol B, only one partially occupied
position of a water molecule (Wat809) is visible next to the
rotated Tyr165 conformer and, moreover, Arg202 does not
change its original position. In addition, Cys13 still adopts the
original β-facial orientation of the Pfr state accompanied by an
unchanged N-terminus, which is tightly packed to Mol A (Fig. 5).
In contrast, in the Pfr state of bathy phytochromes (e.g. wild-type
Agp2-PCM, Agp2-PAiRFP2, PaRphP-PCM (P. aeruginosa25))
Tyr165 is in hydrogen bond distance to prop-C of BV, Phe192
points away from ring D of BV and Arg202 is facing towards BV
(Fig. 7).

Group III structural changes within the PHY tongue region.
The third group of structural changes refers to the PHY tongue
(amino acids 433–471) that in Pfr adopts a-helical, loop and
cooled structural regions but is converted to a β-sheet and a β-
hairpin fold in Pr. In the structure of the Meta-F sub-states of
Mol A and Mol B, these Pr characteristic features including the
concomitant re-positioning of Trp440 away from the sterically
pressing Gln190 were not visible. Instead, the amino acid seg-
ments 439–448 in Mol A and 439–442 in Mol B were not resolved
in the electron density map, indicating a disordering of the
polypeptide backbone. In Mol B, an additional hydrophobic
contact in the dimer interface (Pro452B–Ser230A) is present and
the segment is slightly better resolved than in Mol A (Fig. 5).

Structural changes in the tongue region are also reflected by the
infrared (IR) difference spectrum of Agp2-PAiPFR2 in solution
(Fig. 8, red trace), generated by subtracting the spectrum of Pfr
(negative signals) from that of the final photoproduct (positive
signals). In the amide I band region, no negative band at ca. 1657
– cm⁻¹ is observed that in the Pr/Pfr difference spectrum of the
full-length wild-type Agp2-FL (grey trace) demonstrates the
rearrangement of the α-helical part of the tongue segment of Pfr17.
Instead, the negative signal at 1627 cm⁻¹ in the Agp2-PAiRFP2
difference spectrum (red trace) indicates the degradation of the
cooled loop structure of the tongue segment, whereas the positive
signal at 1642 cm⁻¹ points to the formation of a simpler β-
hairpin segment. However, a more complex β-sheet formation
can be ruled out due to the lack of a positive signal at ca. 1625 cm
–¹. In the region >1680 cm⁻¹, the Agp2-PAiRFP2 difference
spectrum agrees very well with that of the Meta-F/Pfr difference
spectrum of wild-type Agp2-PCM (black trace), including the
upsifts of the C=O stretching of the propionate side chain of
ring C (from 1749 to 1761 cm⁻¹ for Agp2-PAiRFP2) and the
C=O stretching of the ring D carbonyl from 1697 to 1709 cm⁻¹.
In contrast, no notable signals are detected in the amide I band
region of wild-type Agp2-PCM (black trace). Altogether, the IR
spectra indicate the high structural similarity of the chromophore
in the Meta-F states of Agp2-PAiRFP2 and wild-type Agp2-PCM,
with line in with the RR spectroscopic analysis.

However, unlike the wild-type Agp2-PCM in solution, the
Meta-F states of Agp2-PAiRFP2 display the onset of the
structural transformation of the tongue, documented by both the
Meta-F crystal structure and the IR difference spectrum (after irradiation of 273 K in solution). The IR difference spectrum of irradiated Agp2-PAiRFP2 constitutes the final product of the phototransformation of Pfr. The only deviation between IR spectroscopic and crystallographic data of Agp2-
PAiRFP2 refers to the formation of the small
β-hairpin segment detected in the solution IR spectra, which may be impaired or
not fully reached in the crystal. Nevertheless, the good overall
agreement of the crystallographic and IR spectroscopic data
also indicates that Agp2-PAiRFP2 in solution is not capable to
perform the complete structural transformation of the tongue.
To sum up, we assigned the crystallized intermediate to a
precursor of Pr, i.e. more precisely to a Meta-F intermediate
sub-state with a chromophore structure largely similar (albeit
not identical) to the wild-type Meta-F state but including the
onset of the restructuring of the tongue verified by our
spectroscopic data.
Discussion

In this work, we have presented the crystal structure of a Meta-F intermediate of a bathy phytochrome. This intermediate constitutes the starting point for the structural changes of the protein that eventually lead to the (de-)activation of the output module. The Meta-F state was generated from an Agp2 variant including 24 amino acid substitutions. Albeit mainly located outside the chromophore-binding pocket, the RR spectra of this intermediate marginally differs from that of Meta-F of the wild-type protein (Supplementary Fig. 9). Whereas spectroscopic data indicate nearly the same structure of the chromophore and its interactions with the immediate environment, Meta-F of Agp2-PAiRFP2 but not of the wild-type Agp2-PCM in solution displays already the onset of the restructuring of the tongue. This specific Meta-F state of Agp2-PAiRFP2, which cannot be easily trapped in the wild-type protein, provides valuable insight into the molecular mechanism by which chromophore isomerization is coupled to functional protein structural changes (Fig. 9). Furthermore, structural changes are more advanced in Mol A than in Mol B. Thus one may consider Mol B as a precursor of Mol A such that additional information about the sequence of the individual events can be derived. Accordingly, we propose the following mechanism for the Pfr-to-Pr photoconversion, including the Pr structure of Agp1 as a reference for the final state (Fig. 9).

1. After illumination, the chromophore isomerizes from ZZEssa to ZZZssa stereochmistry, which had previously been observed in PaBphP for Lumi-F sub-states. In contrast to Meta-F of Agp2-PAiRFP2, structural changes at the Lumi-F level are restricted to the chromophore and seem to propagate from the isomerization site at the C-D methine bridge to the B-C and eventually to the A-B methine bridge. The primary consequence of ring D rotation is the replacement of Asp196 (Asp194 in PaBphP) as the stabilizing group for ring D by His278 (His277 in PaBphP). This interaction persists in Meta-F of Agp2-PAiRFP2 but in this state the chromophore has adopted a more relaxed structure with a nearly 180° rotation of ring D compared to Pfr (Fig. 9). Chromophore relaxation is accompanied by a substantial rearrangement of the hydrogen bond network involving prop-B and prop-C and the rotated ring D, which is observed in both Mol A and Mol B. These group I structural changes cause (2) reorientations of amino acids and segments in the environment of both ends of the chromophore (i.e. rings A and D), which only take place in Mol A. On the one hand, the side chains of Phe192 and Tyr165 are reorientated in such a way that their aromatic rings form a hydrophobic pocket with ring D (Fig. 9). On the other hand, the BV-binding Cys13 undergoes a change from a β-facial to an α-facial orientation together with a conformational rearrangement of the N-terminus of the protein.

Fig. 5 Crystal packing details of Agp2-PAiRFP2 monomers in the asymmetric unit. Surface representation of Agp2-PAiRFP2 (PDB entry 6G20) Mol A and B per asymmetric unit in the Meta-F sub-states. a (PAS, GAF, PHY in grey, yellow, blue, respectively; N-terminus highlighted in red) shows the free N-terminus for molecule A (Mol A), whereas the N-terminus of molecule B (Mol B) is packed against the PAS domain of Mol A. Owing to the higher flexibility, the N-terminus of Mol A can undergo a β-facial to α-facial orientation transition of the BV-binding Cys13 in the Meta-F state. b Close-up view into the packed N-terminal region of Mol B dimer shown as surface and cartoon representation. c Cartoon representation of the dimer interface of Mol A and B, including the N-terminus of Mol B with the β-facial orientation of Cys13. Amino acids involved in the interface are highlighted as sticks. Compared to Mol A (Fig. 7d, e), some amino acids in Mol B display a positional heterogeneity as reflected by the alternate conformations of Tyr165 and Phe192.
Now we ask for the generality of the proposed mechanism. The group II structural changes seem to constitute the key process for coupling chromophore and protein structural changes. Here the formation of the hydrophobic pocket for ring D via reorientation of Tyr165 and Phe192 and the hydrogen bonding interactions of Tyr165 with the rotated Arg202 via two new water molecules is a structural motif that can be deduced from the various Pr state structures of prototypical bacterial (Agp1-PCM of *A. fabrum* [PDB entry 5HSQ30], DrBphP-PCM of *D. radiodurans* [e.g. PDB entry 4Q0118]) and cyanobacterial (Cph1-PCM of *Synechocystis* sp. [PDB entry 2VEA31]) phytochromes. Also in plant phytochrome AtPhyB-PCM of *A. thaliana* (PDB entry 4OUR32) in its dark adapted Pr state, very similar rotameric states of the corresponding amino acids were found although the two water molecules between the Tyr and Arg residues were not resolved in that structure due to the low resolution (3.4 Å) (Fig. 7f–i).

In the Pfr structures of the bathy phytochrome *PaBphP* and the prototypical phytochrome of *DrBphP*, the conformations of all three amino acids (Tyr165, Phe192 and Arg202 in Agp2) are very similar to those observed in the present Pfr structures of Agp2-PCM and Agp2-PAiRFP2. Consistent with these results, alternating conformations of Tyr165, Phe192 and Arg211 (numbering of Agp2) have been observed in the mixed Pr/Pfr state crystals of the Q188L *BphP* variant29. Moreover, the reorientation of the chromophore-binding Cys is not restricted to Agp2 since all known structures show an α-facial and β-facial orientation of the chromophore-binding Cys in Pr and Pfr, respectively (Fig. 7; Supplementary Fig. 11). In view of these findings, we hypothesize that the group II structural changes are a common mechanistic element for the Pfr→Pr phototransformations of all phytochromes.

The group III structural changes refer to the structural transition of the tongue and its initiation by Gln190/Trp440. Here we note similarities in the structural data for the prototypical
DrBphP\textsuperscript{18} since His201 undergoes a comparable conformational change as its counterpart Gln190 in Agp2. Trp451 of \textit{Dr}BphP (Trp440 in Agp2), which is resolved in three of the four molecules within the asymmetric unit of the Pfr-like structure, shows different conformations, which one may take as an indication for an increased flexibility at this position. Additionally, the tongue region is also not completely resolved. Highly flexible regions (unresolved regions in \textit{Dr}BphP Mol A 454–459, Mol B 452–459, Mol C 450–462, Mol D 452–463) comparable with those in the present Meta-F sub-state structures (Mol A 439–448, Mol B 439–442) support the view that restructuring of the tongue region starts (Pfr→Pfr photoconversion) in this region and subsequently propagates to the rest of the tongue.

After the secondary structure transition of the tongue is completed, the domain arrangement within the PCM is changed as well\textsuperscript{18–20}. These major conformational changes in the protein, which occur during the transfer from the Meta intermediate to the final product of the phototransformation, were proposed to affect the structure and thus the activity status of the output module. The present results, however, do not provide any information about these final steps of the photoinduced reaction cascade and the role of the output module in signalling, folding and back reaction as shown by other experiments (e.g. refs. \textsuperscript{8,32–35}). This is also true for the possible role of dimerization. In this context, we wish to point out that the organization of monomers within the crystal packing does not allow for any conclusions about functionally relevant and concentration-dependent protein–protein interactions in solution, specifically when, as in the present case, surface residues have been substituted (e.g. ref. \textsuperscript{30}).

Taken together, we have presented the crystal structure of an intermediate that is placed in a key position for coupling the photoconversion of the photoreceptor module with the (de-) activation of the output module. In view of similar structural motifs, the proposed general mechanistic model for the Pfr→Pfr photoconversion of the bathy phytochrome Agp2, as summarized in Fig. 9, may also hold for prototypical phytochromes, in line with previous suggestions for the Pfr→Pfr photoconversion\textsuperscript{18}. With the structural and spectroscopic data presented here, we are getting closer to identifying the mechanistic features that are common to the photocycles in both prototypical and bathy phytochromes towards a general scheme with small subtype-specific differences.

**Methods**

**Molecular cloning of wild-type Agp2-PCM.** The Agp2-PCM gene (NCBI GenBank ID AAK87910) was PCR-amplified from \textit{A. fabrum} genomic DNA and cloned into a pET21b expression vector with C-terminal His-tag by using the...
Correspond to the respective photoproduct spectrum, the negative signals refer to Pfr, whereas the positive signals following primers: forward primer sequence ATGTATATCTCCTTCTTA

![Figure 8](https://via.placeholder.com/150)

**Fig. 8** Photoinduced infra-red (IR) difference spectra of Agp2 variants in solution. Black trace (top): wild-type Agp2-PCM difference spectrum obtained after irradiation at ca. 240 K corresponding to the Meta-F-minus-Pfr difference spectrum (taken from ref. 17); red trace (bottom) Agp2-PAIRFP2, difference spectrum obtained after irradiation at 273 K. The grey trace (top) refers to the difference spectrum Pr-minus-Pfr of full-length wild-type Agp2, generated upon irradiation at 273 K (spectrum taken from ref. 17). Note that the Pr state could not be enriched for the wild-type Agp2-PCM due to the fast thermal back conversion to the Pfr state60. In each spectrum, the negative signals refer to Pfr, whereas the positive signals correspond to the respective photoproduct.

Methylation of wild-type Agp2-PCM

The DNA sequence of Agp2-PAIRFP2 (NCBI GenBank ID AGS83373.1)25 was codon optimized (Supplementary Fig. 4, 12). The gene encoding the PCR module derived from Agp2 (1-301 amino acids plus hexa-histidine tag) was transformed into *Escherichia coli* BL21-DE3 cells (Agilent Technologies).

**Molecular cloning of Agp2-PAIRFP2**

The DNA sequence of Agp2-PAIRFP2 was synthesized by GENEWIZ Inc. and cloned into a pET21b expression vector with C-terminal His-tag and transformed into *E. coli* BL21-DE3. The construct contains the following 24 substitutions: Lys65Arg, Arg83Lys, Gly119Lys, Glu120Asp, Ala123Thr, Met163Leu, Gln168Glu, Arg220Pro, Ser243Tyr, Ala246Glu, Ala249Arg, Met251Leu, Ala253Ser, Gly409Asp, Leu419Ile, Arg83Lys, Gly120Asp, Ala123Thr, Met163Leu, Gln168Glu, Arg220Pro, Ser243Tyr, Ala246Glu, Ala249Arg, Met251Leu, Ala253Ser, Gly409Asp, Leu419Ile, Thr469Ser, Ala487Thr, Glu494Gly (Supplementary Figs. 4, 12).

**Purification of wild-type Agp2-PCM and Agp2-PAIRFP2**

Agp2-PCM and Agp2-PAIRFP2 constructs were expressed using an autoduction medium (Overtree Express E. coli Medium, Novagen) for 48 h at 20°C. Cell pellets were washed and cell lysis was carried out using cell lyser (Microfluidics, Newton USA) in 50 mM Tris-HCl buffer containing 50 mM sodium chloride at pH 7.8. The lysozyme (Merck Millipore), 60 µg mL−1 was transformed into *Escherichia coli* (Overnight Express™ Instant TB Medium; Novagen) for 48 h and 20°C. Cell pellets were washed and cell lysis was carried out using cell lyser (Microfluidics, Newton USA) in 50 mM Tris-HCl buffer containing 50 mM sodium chloride at pH 7.8. The lysozyme (Merck Millipore), 60 µg mL−1 was transformed into *Escherichia coli* (Overnight Express™ Instant TB Medium; Novagen) for 48 h and 20°C.
and Pr (Agp1-PCMSER13, PDB entry 5HSQ30, blue) for comparison. Amino acid residues Tyr165, Gln190, Phe192 and Trp440
rearrangement of the hydrogen bond network and the rotated ring D induces chains of Phe192 and Tyr165 such that their aromatic rings form a hydrophobic pocket with ring D. Thus (c) the water molecule Wat758 (red sphere) at ring D is displaced, accompanied by (d) a positional shift of Gln190 in the Meta-F intermediate, which has a direct impact on the PHY tongue. In the Pfr state, the highly conserved Trp440 of the PHY tongue overlaps with the position of this shifted Gln190 (Meta-F state). Therefore, steric hindrance by Gln190 (5) would cause Trp440 to move and eventually affect the PHY tongue (Agp2-PAIRFP2) in Meta-F to induce refolding upon transition to the Pr state. In Agp2-PAIRFP2 (left panel), Trp440 is part of the tongue segment that is disordered in Agp2-PAIRFP2 Meta-F (middle panel). For comparison, Agp1-PCMSER13 shows an ordered segment and a β-sheet in this region (right panel).

**Data availability**

The atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 6G1Y, 6G1Z and 6G20. A reporting summary for this article is available as a Supplementary Information file. Other data are available from the corresponding authors upon reasonable request.

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Author contributions

P.S. and P.H. conceived, planned and designed the project; P.S. coordinated the project; L.S., B.M.Q. and N.M. performed wild-type Agp2-PCM and Agp2-PAiRFP2 preparation and functional analysis, B.M.Q, A.S., T.S. and P.S. performed wild-type Agp2-PCM crystallization and crystal optimizations; A.S., L.S. and M.S. performed Agp2-PAiRFP2 crystallization and crystal optimizations; I.S. and N.M. performed UV-vis spectroscopy on samples; A.S., L.S., M.S., B.M.Q., T.S., D.K., D.v.S. and P.S. performed X-ray diffraction data collections; M.F.L., F.V.E. and D.B. performed resonance Raman (RR) and infra-red spectroscopy on solution and crystal samples, M.A.M. and P.H. supervised Raman spectroscopic experiments; A.S., L.S. and P.S. performed structural analysis; A.S., L.S. and P.S. prepared all structure figures; T.L. provided phytochrome alignments; all authors contributed to data analysis and interpretation; A.S., L.S., N.K., T.L., P.H. and P.S. wrote the paper.

Additional information

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