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Transient Protonation and Conformational Dynamics in Prototypical and Fluorescent Phytochromes

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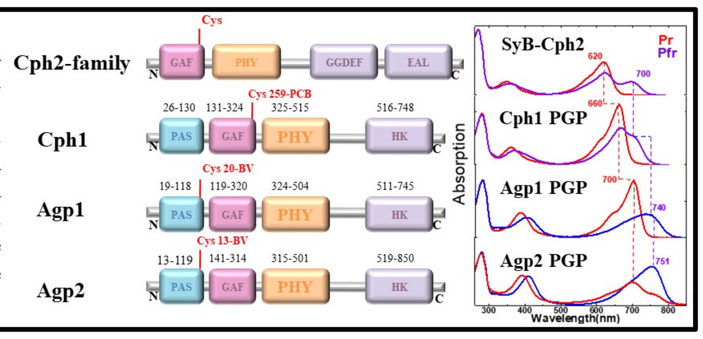
Introduction

Phytochromes are red-light photoreceptor proteins that regulate a variety of responses and cellular processes in plants, bacteria, and fungi. They share similarities in their molecular mechanism, amongst which are light absorption by the chromophore, transient protonation/deprotonation and protein structural changes, eventually leading to activation and signal transduction. Phytochromes act as photochemical switches, which interconvert between a red (Pr) and a far-red (Pfr) absorbing state [1]. We previously showed a different chromophore protonation behavior dependent on the Cph1 assembly in vitro or in vivo [2], a study which is now extended to Agp1-PGP. We also show that modifications in the distant PHY domain of Cph1-PGP and GAF domain of Agp1-PGP affect the chromophore pK_a. Further, we observed conformational changes of PHY and GAF domains as a result of the chromophore deprotonation for Cph1-PGP and Agp1-PGP, respectively. Modern biology investigation on phytochrome as a near-infrared (NIR) photoreceptor is important to design an optogenetic tools (OTs). Recently, near-infrared (NIR) fluorescent proteins (FPs) engineered from bacterial phytochromes that bind to biliverdin IXα (BV) and PCB binding iRFP series and cyanobacterial phytochromes, have become invaluable probes for multicolor fluorescence microscopy and in vivo imaging. However, all current NIF FPs have short lifetime and low quantum yield. Here we applied a rational approach to combine mutations known to enhance fluorescence in Cph1, a cyanobacterial phytochrome, to derive a series of highly fluorescent mutants. The mutants were analysed by biochemical, fluorescence steady-state and time-resolved spectroscopy and were shown to feature high extinction coefficient and fluorescence quantum yields, and long fluorescence lifetime, contributing to overall high brightness of the fluorophores.

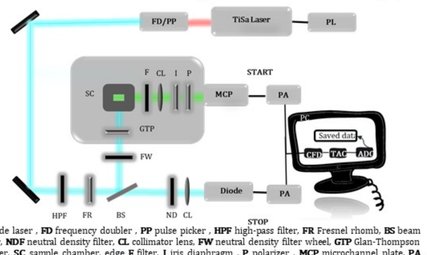


Methods

- **Quick Exchange Mutagenesis** Site-directed mutagenesis was carried out according to the Quik Change™ mutagenesis protocol (Agilent Technologies) albeit individual enzymes were purchased separately from different manufactures (DNA polymerase: Finnzymes, DpnI endonuclease: New England Biolabs).
- **Protein Labeling with IAF** The dye bounds covalently to cysteine in protein. For this purpose, dyes are selected in which the molecular group allows the dye to bind to a group of the protein or an amino acid. Examples of linkers are the iodoacetamide or maleimide groups. These linkers bind specially to the SH group of cysteines. The labeling stereometry is calculated using:

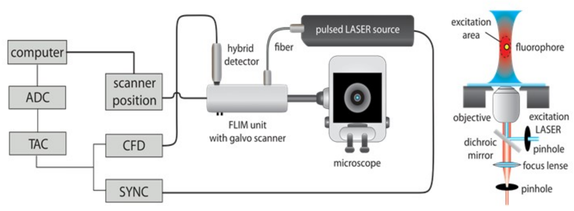
$$\frac{c_{Label}}{c_{Protein}} = \left(\frac{\Delta A_{Label}}{\varepsilon_{Label}}\right)\left(\frac{\varepsilon_{Protein,Pr}}{A_{Protein,Pr}}\right)$$

- **Time-Correlation Single Photon Counting (TCSPC)**



DL diode laser , **FD** frequency doubler , **PP** pulse picker , **HPF** high-pass filter, **FR** Fresnel rhomb, **BS** beam splitter, **NDF** neutral density filter, **CL** collimator lens, **FW** neutral density filter wheel, **GTP** Glan-Thompson polarizer, **SC** sample chamber, edge **F** filter, **I** iris diaphragm , **P** polarizer , **MCP** microchannel plate, **PA** preamplifier, **CFD** constant fraction discriminator , **TAC** time-to-amplitude converter , **ADC** analog -to-digital converter.

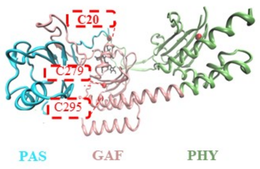
- **Fluorescence lifetime imaging microscopy (FLIM)**



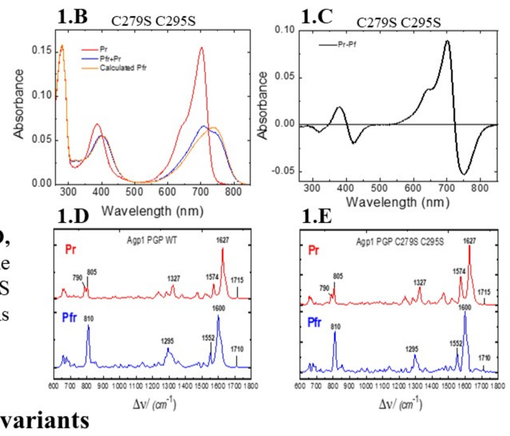
Results 1: Conformation Dynamic in Agp1 PGP

- **Results 1-1 : Cys less mutant behaves similar to the WT**

1.A

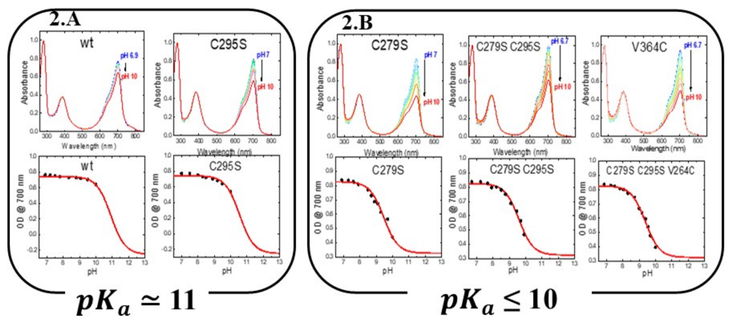


- Photosensor module PAS-GAF-PHY (PGP) of Agp1 has three native cysteines, 20, 279 and 295 (**Fig 1.A**). C20 in PAS domain is covalently bond to the chromophore, therefore is not available for dye labeling.



- As the UV-vis (**Fig 1.B and 1.C**) and Raman Resonance (RR) (**Fig 1.C and 1.D, Anastasia Kraskov B2**) spectroscopy show, the photoconversion and the chromophore binding pocket (CBP) interaction with BV in cysteine less (C279S C295S) mutant of Agp1 PGP and WT are identical, at the standard conditions (50 mM Tris, 150 mM Nacl pH 7.8).

- **Results 1-2: Chromophore deprotonation in Agp1 PGP mutant variants**

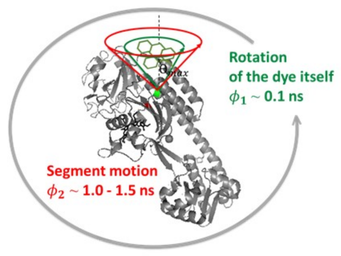


- UV-vis titration of Agp1 PGP WT and the mutant variants indicated two groups in which one group has pK_a around 11 (WT and C295S, **Fig 2.A**) and the other group with *pKa* lower than 10 (C279S mutant variants **Fig 2.B**).

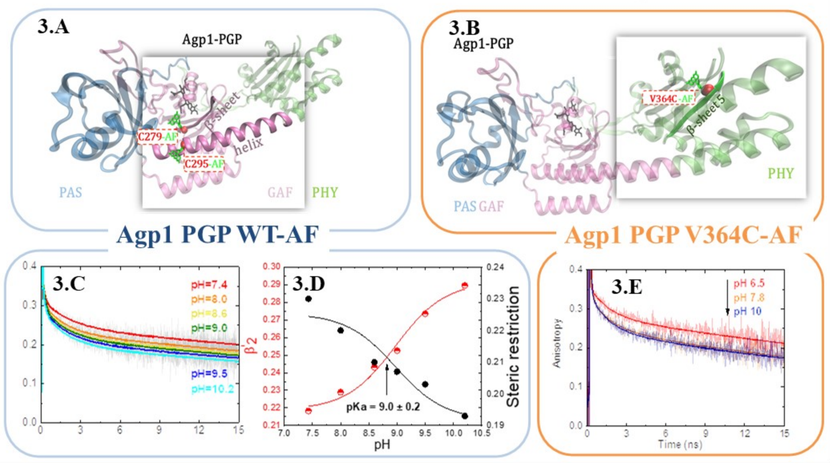
- **Results 1-3: Conformation dynamic at the GAF domain**

Dye **Loop/Segment** **Steric restriction**

- $r(t)/r_0 = \beta_1 e^{-t/\phi_1} + \beta_2 e^{-t/\phi_2} + \beta_3 e^{-t/\phi_3}$ **Fit model function**
- $\beta'_2 = \beta_2/(\beta_2 + r_\infty)$ **Loop relative mobility**
- $r_\infty/r_0 = (1/2\cos[\Theta_{max}(1+\cos[\Theta_{max}])]^2$ **Steric restriction**

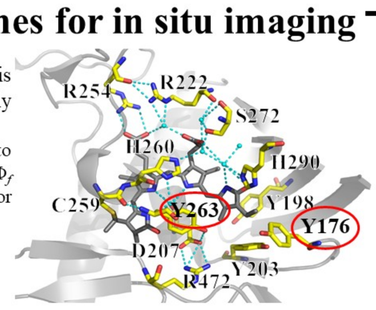


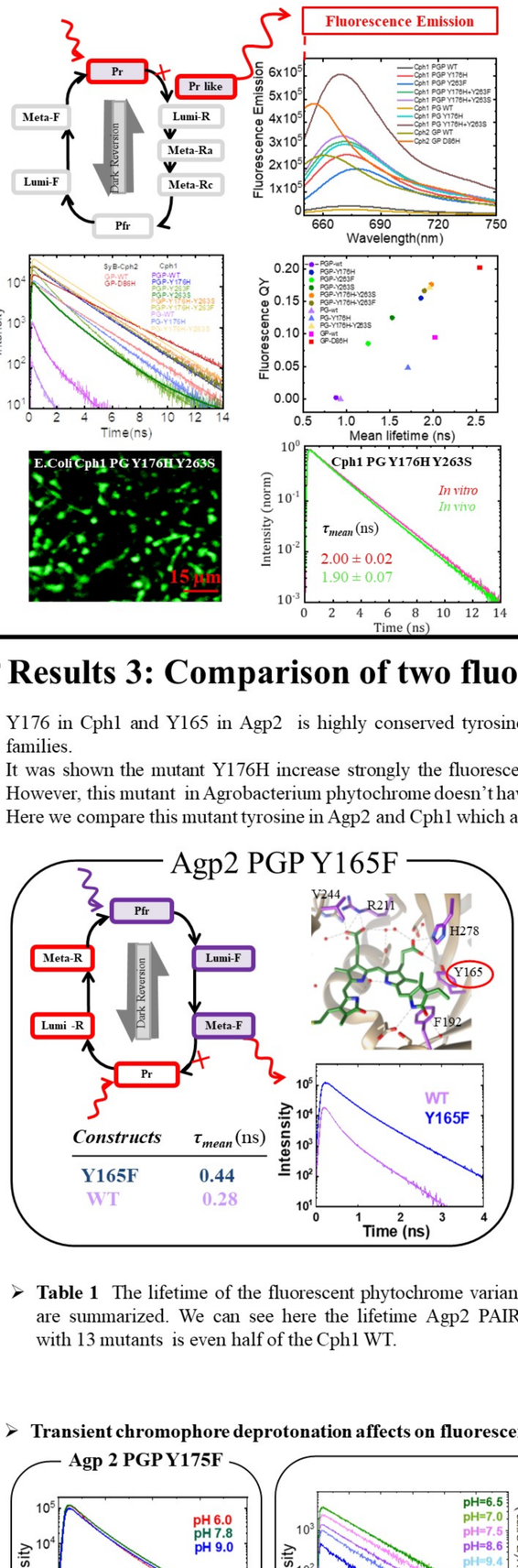
- **Labeling at the GAF domain:** in the WT C279 and C295 are accessible for IAF labeling **(Fig 3.A)**.
- **Labeling at the PHY doamin**: the single cysteine mutant V364C (the same position as C371 in Cph1) was prepared **(Fig 3.B)** to bond to IAF.
- **Conformation dynamic in the GAF domain:** the pH titration fluorescence anisotropy in Agp1 wt **(Fig 3.C)** and the global fit of β'_2 and β_3 show the pK_a 9.0 ± 0.2 **(Fig 3.D)** which is around the chromophore pK_a of UV-vis titration of wt-AF.
- **Conformation dynamic in the PHY domain:** the pH titration fluorescence anisotropy in V364C doesn't show any significant changes regard to chromophore deprotonation (see **Fig 3.E**).



Results 2: Improved fluorescent phytochromes for in situ imaging

- The popular method to reach the high fluorescence quantum yield is the mutagenesis of critical amino acids in chromophore binding pocket to screen for a highly fluorescent mutant.
- Highly fluorescent mutants of Cph1 achieved by combining the single mutations known to enhance fluorescence in various phytochromes. The effect of Y176H and Y263F/S on Φ_f appear synergistic in the photosensory module, values of 17.6% and 17.1% were achieved for Cph1 PGP and PG constructs, respectively





- Photoconversion is disturbed in all mutant variants.
- There is a linear correlation between the quantum yield and lifetime except for two variants PG Y176H and GP wt.

Construct	Φ_F	τ_{mean} (ns)
Cph1 PGP WT	0.024	0.86 ± 0.05
Cph1 PGP Y176H	0.144	1.90 ± 0.05
Cph1 PGP Y263F	0.082	1.26 ± 0.02
Cph1 PGP Y263S	0.125	1.46 ± 0.01
Cph1 PGP Y176H Y263F	0.160	1.92 ± 0.01
Cph1 PGP Y176H Y263S	0.176	2.00 ± 0.01
Cph1 PG WT	0.018	0.70 ± 0.12
Cph1 PG Y176H	0.108	1.74 ± 0.08
Cph1 PG Y176H Y263S	0.171	2.00 ± 0.01
Cph2 GP WT	0.124	2.07 ± 0.01
Cph2 GP D86H	0.202	2.60 ± 0.01

- Stable fluorescence lifetime *in vitro* and *in vivo*

Results 3: Comparison of two fluorescent phytochrome

- Y176 in Cph1 and Y165 in Agp2 is highly conserved tyrosine in the chromophore binding pocket of the most phytochrome families.
- It was shown the mutant Y176H increase strongly the fluorescence quantum yield and quantum emission (Fischer 2004-2005). However, this mutant in Agrobacterium phytochrome doesn't have the same effect as it has in cyanobacterial Phytochrome.
- Here we compare this mutant tyrosine in Agp2 and Cph1 which are Y165F and Y176H, respectively.

- **Table 1** The lifetime of the fluorescent phytochrome variants are summarized. We can see here the lifetime Agp2 PAIR2 with 13 mutants is even half of the Cph1 WT.

Constructs	τ_{mean} (ns)	χ_{red}^2
Agp2 PGP WT	0.28	1.0
Agp2 PGP Y165F	0.44	1.03
Agp2 PGP PAIR2	0.44	0.99
Cph1 PGP WT	0.86	1.03
Cph1 PGP Y176H	1.90	1.01

- **Transient chromophore deprotonation affects on fluorescence intensity in Cph1 Y176H**

- Cph1 Y176H lifetime **1.80 ± 0.14 ns** remain stable for all pH points, while the intensity is strongly pH dependent . The pK_a **8.9 ± 0.1** is around the chromophore pK_a 9.
- The same residue mutant in Agp2 has a different effect, neither the lifetime and nor the intensity are pH dependent.

Conclusions and Discussion

- **The mutant C279S in Agp1 PGP reduces the chromophore pK_a from 11 to lower than 10.** Although the UV-vis and RR show the identical spectra of the C279S and wt, the UV/vis titration of C279S shows different chromophore deprotonation. The C279 is the closest cysteine to the CBP, one amino acids before H280. Recent work showed the H250A reduced the chromophore pK_a to around 8.8 (von Stetten 2007). It seems the mutant of cysteine to serin has such this effect on chromophore de-protonation equilibrium.
- **Conformation changes at the PHY domain due to chromophore deprotonation behave different in Cyanobacterial Cph1 and Agrobacterial Agp1 phytochrome:** The results from the pH titration anisotropy in Agp1 PGP shows the flexibility of GAF domain (**Fig 3.C**) due to the chromophore deprotonation as we proved earlier for Cph1 PGP [3], while the PHY domain (**Fig 3.E**) doesn't seem to be flexible as Cph1. These results from two members of phytochrome family which is one (Agp1) is more primitive than the other (Cph1), indicates the more advance mechanism of Cph1 compare to Agp1, in which the PHY domain is getting more flexible as a reaction to the chromophore deprotonation, and this might facilitate the protein signaling and protein respond after chromophore photoconversion.
- **High fluorescence quantum yield and lifetime in Cyanobacterial phytochrome, which make it to be used as an optogenetic tools and *in vivo* imaging.**
- **Mutant of the conserved tyrosine in Agp2 and Cph1 shows different results in their fluorescence quantum yield and lifetime:** The flourescence lifetime of Agp2 PGP Y165F (0.42 ± 0.02 ns) which is the highest flourecnce lifetime among the Agp2 mutant variants is even half of the Cph1 PGP wt (0.86 ± 0.05 ns). The mutant Y176H in Cph1 PGP significantly increase the lifetime to 1.90 ± 0.05 ns which is almost five times longer than the mutant Y165F in Agp2 PGP. This significant difference in fluorescence lifetime is might related to the mechanisms of photoconversion between BV-utilising bacteriophytochromes and phytobilin-binding phytochromes.
- **The chromophore deprotonation affects on fluorescence quantum yield in Cph1 Y176H and not in Agp2 Y165F.**

Construct	Chromophore pK_{DPC}	pK_a of conformational change
Cph1 PGP wt	9.04± 0.02	-
Cph1 PGP-C371S	8.37± 0.03	-
Cph1 PG	8.12± 0.06	-
Cph1 PGP-AF (labeling in PHY/PAS)	8.28 ± 0.02	8.5± 0.2
Cph1 PGP- C371S-AF (labeling in GAF)	8.10± 0.08	8.0± 0.2
Cph1 PG-AF (labeling in PAS-GAF)	< 7.5	7.5± 0.2
Agp1 PGP wt	≃11	-
Agp1 PGP V364C	9.38± 0.03	-
Agp1 PGP wt –AF (labeling in GAF)	9.06 ± 0.07	9.0 ± 0.2
Agp1 PGP V364C-AF (labeling in PHY)	9.40 ± 0.20	-

- **Table 2** The chromophore deprotonation pK_a (pK_{DPC}) and the pK_a from the conformation changes of Cph1 PGP WT and the mutant variants, Agp1 WT and the mutant V364C are summarized as well as their labeled with IAF

References

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Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (DFG) through collaborative research center 1078 (Project A2) is gratefully