Photochemical & Photobiological Sciences

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/pps

Influence of a chromophore analogue in the protein cage of photoactive yellow protein

Norio Hamada[a], Zhe Tan[a], Yasuo Kanematsu[a], Naoya Inazumi[b], and Ryosuke Nakamura[a]

[a] *Science & Technology Entrepreneurship Laboratory (e-square), Osaka University, 2-1 Yamadaoka, Suita, Osaka, Japan. E-mail: hamada@uic.osaka-u.ac.jp; Tel: +81 6 6879 7755*

[b] *Department of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Japan*

Time-resolved spectra of photoactive yellow protein (PYP) containing cyano-*p*-coumaric acid (CHCA) were measured. To understand the mechanism of photo-isomerization, an electron-withdrawing CN group was introduced into PYP to alter the C=C double bond character. Free CHCA chromophore in aqueous solution underwent photo-isomerization whereas PYP with a bound CHCA (PYP-CN) exhibited no photocycle at acidic or alkaline pH or urea and other solutions. Furthermore, no photocycle was observed with PYP mutants after illumination. This phenomenon cannot be fully explained by the electron-withdrawing property of the CN group. We conclude that the CHCA chromophore in PYP was locked in the protein cage and that the CN group interacted with the protein residues.

Introduction

Photoactive yellow protein (PYP) is a water-soluble photoreceptor protein containing a *p*-coumaric acid (*p*CA) chromophore as a light absorption antenna[1-3]. After PYP absorbs a blue-wavelength photon, *trans-cis* photo-isomerization of the *p*CA chromophore leads to sequential chemical steps involving protein conformational changes during the photocycle (Fig. 1(a))[4-6]. Photoreceptor proteins are highly controlled by their

chromophore, and the chromophore in turn is controlled by its protein environment. This environment is called the "protein cage" or "protein nano-space"[7-9]. Thus, in order to understand the mechanisms controlling the chromophore, it is important to investigate the protein cage in detail. Photo-isomerization of the chromophore is a key to the functional photocycle of PYP. Previous studies have demonstrated that free chromophore photo-isomerizes through a simple one-bond-flip pathway, whereas the chromophore in a protein cage photo-isomerizes through several steps called the "hula-twist" model or "bicycle-pedal" model[10-12]. The more complicated mechanism observed in a protein milieu is due to the hydrogen-bonding network between the chromophore and several protein residues, as well as the unique environment surrounding the chromophore, such as the electrostatic field inside the protein cage. To elucidate the influence of the protein cage around a chromophore, we examined chromophore analogues with various properties such as the presence of an electron-donating or -withdrawing group, and sterically hindered chromophores. Many chromophore analogues have been studied to understand the mechanisms underlying photo-isomerization and protein conformational change[13-17]. From among these, here we investigated analogues of the cyano-*p*-coumaric acid (CHCA) chromophore (Fig. 1(b)) because there are no protein vibrational bands in the 2200 cm^{-1} region corresponding to the CN stretching mode. Consequently, changes in the CN stretching mode during the PYP photocycle should allow detailed probing of the state of the protein cage. In a previous study, Sigala et al. reported that the dark state in PYP-CN and the photoactivation state in wild-type (WT) PYP have similar electronic structures of chromophore, based on NMR spectroscopic data[18]. We predicted that PYP-CN comprised a population of photo-intermediates due to the low potential energy of its *trans*/*cis*

isomerization.

Experimental

Samples

Cyano-*p*-coumaric acid, *p*-coumaric acid and other analogues were purchased from Sigma Aldrich, USA. WT PYP and mutants were expressed and purified from *Escherichia coli* and reconstituted with chromophores as described previously[19].

UV/vis spectroscopy

UV/vis spectra were obtained using a multichannel fiber optics system (S2000, Ocean Optics, USA) with a deuterium lamp (Hamamatsu Photonics, Japan) as the monitoring light source. Millisecond time-resolved spectra were acquired by exciting with a 460 nm laser pulse or a 430 nm light-emitting diode with an electro-gated-shutter, controlled by a delay pulse generator (DG535, Stanford Research Systems, USA).

Ultrafast time-resolved spectroscopy

Ultrafast time-resolved spectroscopy was performed using a femtosecond dispersed transient absorption spectroscopy setup based on an amplified mode-locked Ti:Sapphire laser system operating at 1 kHz. Pump pulses at 400 nm were generated by frequency doubling the fundamental pulses in a beta barium borate crystal and the femtosecond supercontinuum generated in a CaF_2 plate was used as broadband probe pulses. The polarization of the pump pulse was set at the magic angle (54.7°) with respect to the probe pulse. After passing through the sample, the dispersed probe pulse was analyzed using a diode array detector. The output signals were digitized and collected at the repetition rate of the laser system (1 kHz). The pump beam was modulated at 500 Hz

with a mechanical chopper frequency locked to the laser pulse train. The group velocity dispersion (GVD) of the broadband probe pulses was estimated from measurements of the cross-phase modulation in 1.0-mm-thick quartz glass and the data were corrected for GVD. The full width at half-maximum of the cross-correlation traces between the pump and probe pulses was 0.15 ps.

Results and discussion

Properties of free CHCA

pH titrations were conducted (Fig. 2a) to elucidate the state of the phenolic OH on the chromophores. The pKa value of the phenolic OH in CHCA in aqueous solution was determined from the pH titration curve and by solving Hill formula (Fig. 3b). The pKa value of CHCA in aqueous solution is 8.1, about 1 pH unit lower than that of free *p*CA chromophore (pKa 9.0) due to the electron-withdrawing CN group. This result was in good agreement with a previous study[18]. At alkaline pH, the OH of the phenolic ring and the carbonic group are thus both deprotonated. Spectra of the CHCA chromophore in solution at pH 10.8 were measured after illumination with 380 nm light (Fig. 4). An increase in exposure time resulted in a decrease in the intensity of the peak around λ_{380nm} and an increase in the intensity of the peak around λ_{349nm}. A study on *p*CA found that a red-shifted peak corresponded to *trans-p*CA and a blue-shifted peak corresponded to *cis-p*CA[20]. The effects of steric and electronic character of CN cause the larger red-shifted in analogue chromophore. The configuration of CHCA was assigned by NMR (Fig.3)[21] and UV/vis (Fig. 5) spectroscopic analysis of the chromophore. We concluded that the deprotonated *cis*-form of CHCA exhibits a peak at 343 nm and that this peak is slightly blue-shifted in the protonated *trans*-form (Fig. 5), thereby confirming that free

deprotonated CHCA chromophore in aqueous solution undergoes photo-isomerization.

UV/vis spectra of PYP-CN and E46Q-CN

Results obtained previously using a halogenated chromophore (unpublished data) suggested that incorporation of the electron-withdrawing CN group would affect the recovery process in the PYP photocycle. There has been a controversial discussion regarding the isomerization steps occurring in the PYP E46Q mutant[22]. The CHCA chromophore was covalently anchored in the binding pocket of WT PYP and E46Q to provide PYP-CN and E46Q-CN, respectively (Fig. 6). The absorbance maximum of PYP-CN was red-shifted 20 nm compared to WT, to 466 nm. As shown in Table 1, the absorbance maxima of PYP-CN and E46Q-CN were red-shifted and the half bandwidth was narrower than that of WT PYP and E46Q, respectively. These and previous spectral data[18] suggest that PYP-CN and E46Q-CN may have a structure and hydrogen bond network around the chromophore similar to WT PYP. The pH stabilities of PYP-CN and E46Q-CN were measured by pH titration experiments (Fig. 7a) and the pKa values were estimated to be 3.9 in PYP-CN and 6.0 in E46Q-CN by fitting with Hill equation. Although the CN group might alter the strength of hydrogen bonding, we concluded that the hydrogen bond network around the chromophore, and particularly the configuration between E46, Thy42, and the chromophore, were not drastically affected by the CN group.

Millisecond time-resolved spectroscopy

The photocycle was observed by measuring time-resolved spectra of WT PYP under various conditions (Fig. 8). The time course of the absorbance at 350 nm provided a lifetime estimate of 500 ms for the M intermediate, consistent with previous studies. In

contrast, no photocycle on the order of hundreds of milliseconds was observed for PYP-CNs; typical spectra (Fig. 9) for the acidic form of PYP-CN (pH 5.0) at 80 ms after illumination with 470 nm light. Many studies have reported a long-lived M intermediate for the PYP photocycle under low pH conditions[23, 24], in contrast to our current findings for PYP-CN. PYP-CN were observed under conditions long-lived M indermediate was stabilized (supplementary Fig.1). We examined the photocycle of PYP-CN under various conditions such as 2M urea, alkaline conditions, guanidinium HCl, and low and high temperature because WT and E46Q were observed long-lived M intermediate at such conditions, but none of the spectra showed a difference between the dark state and the state following illumination, although Fig. 9 shows a slight change around 500 nm. Since the magnitude of this change depends on the timing of data acquisition, we assumed that the illumination came from irradiation source. We therefore examined a new mutant construct, M100K-CN, because M100K exhibited a long-lived M intermediate[25, 26] and found that M100K-CN had no photocycle (supplementary Fig.1).

Ultrafast time-resolved spectroscopy

Ultrafast time-resolved spectra were measured at room temperature to confirm the presence or absence of a photocycle. The left panel in Fig. 10 shows the transient absorption spectra for WT at selected delay times ranging from 0.3 ps to 1.0 ns after photoexcitation. A positive band below 400 nm and two negative peaks at 445 and 500 nm appeared immediately after photoexcitation and are ascribed to excited-state absorption, ground-state bleach, and stimulated emission, respectively. Both the excited-state absorption and the stimulated emission rapidly disappear, with a time constant of the excited-state lifetime of 2 ps, followed by the appearance of a new

positive peak at 500 nm at 1.0 ns, ascribed to the intermediate state. In contrast, the ground-state absorption remained at 1.0 ns. These characteristic features of the transient absorption spectra indicate that PYP enters the photocycle reaction[27, 28].

The right-hand panel in Fig. 10 shows the transient absorption spectra for PYP-CN. As with WT, excited-state absorption (<400 nm), ground-state bleach (465 nm), and stimulated emission (500 nm) are observed at 0.3 ps. However, in contrast to WT, the three bands disappear simultaneously and no positive peak originating from the intermediate species could be recognized at 1.0 ns.

Note that the ground-state bleach (465nm) at 0.3 ps for PYP-CN is much narrower than the ground-state absorption shown in Fig. 6. The half band width of the ground-state bleach and absorption spectra are 1304 and 1869 cm^{-1}, respectively. The narrowing behavior is presumably due to the spectral hole burning, suggesting a considerable contribution of inhomogeneous broadening to the ground-state absorption spectrum for PYP-CN.

Protein cage

We concluded that PYP-CN exhibited either an abnormal photo-reaction or no photocycle. Sigala et al. reported that the CN group led to charge delocalization in the dark state based on UV/vis spectroscopic and NMR data, with no mention of photo-reaction and photocycle. We predicted that charge delocalization led to alter the double bond property and this caused easy photo-isomerization. Free deprotonated CHCA chromophore had easy photo-isomerization (Fig.4). Importantly, we confirmed that PYP containing a halogenated chromophore[29] had a photocycle similar to that of native PYP (data not shown). We previously[29] pointed out that the F atom in the

chromophore may form a hydrogen bond with the β carbon hydrogen of Phe96. Phe96 is highly conserved in the PYP family and Morishita et al. noted that Phe96 and a phenol ring carbon of the chromophore may form a CH/π hydrogen bond[30]. In the present study, a CN stretching mode was observed at 2230.8 cm^{-1} for free CHCA and a very weak peak due to strong fluorescence was observed at 2203.1 cm^{-1} for PYP-CN in Raman spectroscopy (data not shown). This shift is supposed to originate from thioester bonding. It might reflect interaction between the CN group and the Phe96 residue or interference of the CH/π interaction between Phe96 and the chromophore. Consequently, the protein cage providing the environment around the chromophore in PYP is specifically suited to the photoreaction and photocycle. This highly controlled protein cage might be achieved a low-barrier hydrogen bond[31].

Photo-isomerization

Several theoretical studies predict that the hydrogen out-of-plane (HOOP) bending mode plays an important role in photo-isomerization during the PYP photo-reaction[32], and the function of HOOP in PYP was experimentally confirmed by Raman spectroscopy[33]. In the present study, HOOP may have been restricted due to the CN group. The effect of the CN group will be investigated in the future.

Conclusions

We conclude that PYP-CN exhibits no photocycle while the CHCA chromophore bound to PYP has a normal configuration involving the phenolic OH of the chromophore and the E46 and Tyr42 residues. According to WT PYP crystal structure, the closest residues to C=C double bond was Ala67 and its distance were about 3.5 A (Supplementary Fig.2). The size of CN is about 2.8 A. It seems

not to be steric direct contact with side chain. It is unclear whether the CN group interacts with other residues or not; however, the CN group has a drastic effect on the photo-reaction. This behavior, similar to that of locked chromophores, might be due to both electron-withdrawing and steric factors. This behaviour was not disrupted at low and high pH and mild denaturing conditions. The protein cage tunes the chemical properties of the chromophore, such as the double-bond character, to stabilize the electrostatic and steric condition of the chromophore.

Acknowledgements

We thank Dr. Kumauchi for helpful comments.

This work was supported by JSPS KAKENHI Grant Number 24540435.

References

1 T. E. Meyer, Isolation and characterization of soluble cytochromes, ferredoxins and other chromophoric proteins from the halophilic phototrophic bacterium Ectothiorhodospira halophile, *Biochem. Biophys. Acta.* 1985, **806**, 175-183.

2 W. W. Sprenger, W. D. Hoff, J. P. Armitage, and K. J. Hellingwerf, The eubacterium Ectothiorhodospira halophila is negatively phototactic, with a wavelength dependence that fits the absorption spectrum of the photoactive yellow protein, *J. Bacteriol.* 1993, **175**, 3096-3104.

3 K. J. Hellingwerf, J. Hendriks, and T. Gensch, Photoactive Yellow Protein, a new type of photoreceptor protein: Will this "yellow lab" bring us where we want to go? *J. Phys. Chem. A*, 2003, 107, 1082-1094.

4 M. Kim, R. A. Mathies, W. D. Hoff and K. J. Hellingwerf, Resonance Raman evidence that the thioester-linked 4-hydroxylcinnamyl chromophore of photoactive yellow protein is deprotonated, *Biochemistry*, 1995, **34**, 12669-12672.

5 R. Kort, H. Vonk, X. Xu, W. D. Hoff, W. Crielaard, K. J. Hellingwerf, Evidence for trans-cis isomerization of the p-coumaric acid chromophore as the photochemical basis of the photocycle of photoactive yellow protein, *FEBS Lett.* 1996, **382**, 73-78.

6 G. Rubinstenn, G. E. Vuister, F. A. Mulder, P. E. Dux. R. Boelenns, K. J. Hellingwerf and R. Kaptein, Structural and dynamic changes of photoactive yellow protein during its photocycle in solution, *Nat. Struct. Biol.* 1998, **5**, 568-570.

7 H. Chosrowjan, N. Mataga, Y. Shibata, Y. Imamoto and F. Tokunaga, Environmental effects on the femtosecond-picosecond fluorescence dynamics of

photoactive yellow protein: Chromophores in aqueous solutions and in protein nanospaces modified by site-directed mutagenesis, *J. Phys. Chem. B*, 1998, **102**, 7695-7698.

8 N. Mataga, H. Chosrowjan, S, Taniguchi, N. Hamada, F. Tokunaga, Y. Imamoto and M. Kataoka, Ultrafast photoreactions in protein nanospaces as revealed by fs fluorescence dynamics measurements on photoactive yellow protein and related systems, *Phys. Chem. Chem. Phys*. 2003, **5**, 2454-2460.

9 A. Espagne, D. H. Paik, P. Changenet-Barret, P. Plaza, M. M. Martin and A. H. Zewail, Ultrafast light-induced response of photoactive yellow protein chromophore analogues, *Photochem. Photobiol. Sci.*, 2007, **6**, 780-787.

10 Y. Imamoto, M. Kataoka and R. S. Liu, Mechanistic pathways for the photoisomerization reaction of the anchored, tethered chromophore of the photoactive yellow protein, *Photochem. Photobiol.*, 2002, **76**, 584-589.

11 R. S. Liu, L. Y. Yang and J. Liu, Mechanisms of photoisomerization of polyenes in confined media: from organic glasses to protein binding cavities, *Photochem. Photobiol.*, 2007, **83**, 2-10.

12 L. Wei, H. Wang, X, Chen, W. Fang and H. Wang, A comprehensive study of isomerization and protonation reactions in the photocycle of the photoactive yellow protein, *Phys. Chem. Chem. Phys*. 2014, **16**, 25263-25272.

13 R. A. Kroon, W. D. Hoff, H. P. M. Fennea, J. Gijzen, G. Koomen, J. W. Verhoeven, W. Crielaard and K. J. Hellingwerf, Spectral tuning, Fluorescence, and Photoactivity in hybrids of Photoactive Yellow Protein, reconstituted with native or modified chromophores, *J. B. C*. 1996, **271**, 31949-31956.

14 S. Devanathan, U. K. Genick, E. D. Getzoff, T. E. Meyer, M. A. Cusanovich and G. Tollin, Preparation and properties of a 3,4-dihydroxyxinnamic acid chromophore variant of the photoactive yellow protein, *Arch. Biochem. Biophys*. 1997, **340**, 83-89.

15 R. Cordfunke, R. Kort, A Pierik, B. Gobets, G. J. Koomen, J. W. Verhoeven, and K. J.Hellingwerf, Tran/cis (Z/E) photoisomerization of the chromophore of photoactive yellow protein is not a prerequisite for the initiation of the photocycle of this photoreceptor protein, *Pro. N. A. S*. 1998, **95**, 7396-7401.

16 D. M. van Aalten, W. Crielaard, K. J. Hellingwerf and L. Joshua-Tor, Structure of the photoactive yellow protein reconstituted with caffeic acid at 1.16 Å resolution, *Acta. Crystal. D Biol. Crystal*. 2002, **5**8, 585-590.

17 N. Hamada, K. Matsumoto, K. Soda, R. Nakamura, H. Ichida, F. Tokunaga and Y. Kanematsu, Inhomogeneity observed in the photocycle of photoactive yellow protein, *J. Lumin*. 2006, **119**, 122-126.

18 P. A. Sigala, M. A. Tsuchida and D. Herschlag, Hydrogen bond dynamics in the active site of photoactive yellow protein, *Pro. N. A. S*. 2009, **106**, 9232-9237.

19 Y. Imamoto, T. Ito, M. Kataoka and F. Tokunaga, Reconstitution photoactive yellow protein from apoprotein and p-coumaric acid derivatives, *FEBS Lett*. 1995, **374**, 157-160.

20 A. Espagne, D. H. Paik, P. Changenet-Barret, P. Plaza, M. M. Martin and A. H. Zewail, Ultrafast light-induced response of photoactive yellow protein chromophore analogues, *Photochem. & Photobiol. Sci*. 2007, **6**, 780-787.

21 B. Danylec and M.N. Iskander, 1H NMR measurement of the trans-cis photoisomerization of cinnamic acid derivatives, *J of Chem. Educ.* 2002, **79**, 1000-1001.

22 S. Tripathi, V. Srajer, N. Purwar, R. Henning and M. Schmidt, pH dependence of the Photoactive Yellow Protein photocycyle investigated by time-resolved crystallography, *Biophys. J.* 2012, **102**, 325-332.

23 E. D. Demchuk, U. K. Genick, T. T. Woo, W. D. Getzoff and D. Bashford, Protonation states and pH titration in the photocycle of photoactive yellow protein, *Biochemistry*, 2000, **39**, 1100-1113.

24 B. C. Lee, M. Kumauchi, and W. D. Hoff, Modulating native-like residual structure in the fully denatured state of photoactive yellow protein affects its refolding, *J. Biol. Chem*, 2010, **285**, 12579-12586.

25 S. Devanathan, U. K. Genick, I. L. Canestrelli, T. E. Meyer, M. A. Cusanovich, E. D. Getzoff and G. Tollin, New insights into the photocycyle of Ectothiorhodospira halophila Photoactive Yellow Protein: Photorecovery of the long-lived photobleached intermediate in the Met100Ala mutant, *Biochemistry*, 1998, **37**, 11563-11568.

26 M. Kumauchi, N. Hamada, J. Sasaki and F. Tokunaga, A role of Methionine100 in facilitating PYP_M-decay process in the photocycle of Phtoactive Yellow Protein, *J. Biochem*. 2002, **132**, 205-210.

27 L. Uji, S. Devanathan, T. E. Meyer, M. A. Cusanovich, G. Tollin and G. H. Atkinson, New photocycyle intermediates in the Photoactive Yellow Protein from Ectothiorhodospira halophile: picosecond transient absorption spectroscopy, *Biophy. J.* 1998, **75**, 406-412.

28 Y. Imamoto, M. Kataoka, F. Tokunaga, T. Asahi and H. Masuhara, Primary photoreaction of Photoactive Yellow Protein studied by subpicosecond-nanosecond spectroscopy, *Biochemistry*, 2001, **40**, 6047-6053.

29 H. Yamada, M. Kumauchi, N. Hamada, XG. Zheng, II. H. Park, K. Masuda, K. Yoshihara and F. Tokunaga, Analogue chromophore study of the influence of electronic perturbation on color regulation of Photoactive Yellow Protein, *Photochem. Photobiol*. 2006, **82**, 1422-1425.

30 T. Morishita, M. Harigai, Y. Yamazaki, H. Kamikubo, M. Kataoka and Y. Imamoto, Array of aromatic amino acid side chains located near the chromophore of photoactive yellow protein, *Photochem. Photobiol*. 2007, **83**, 280-285.

31 S. Yamaguchi, H. Kamikubo, K. Kurihara, R. Kuroki, N. Niimura, N. Shimizu, Y. Yamazaki and M. Kataoka, Low-barrier hydrogen bond in photoactive yellow protein, *Pro. N. A. S*. 2009, **106**, 440-444.

32 E. V. Gromov, Unveiling the mechanism of photoinduced isomerization of the photoactive yellow protein (PYP) chromophore, *J. Chem. Phys*. 2014, **141**, 224308.

33 M. Creelman, M. Kumauchi, W. D. Hoff and R. A. Mathies, Chromophore dynamics in the PYP photocycle from femtosecond stimulated Raman spectroscopy, *J. Phys. Chem. B*, 2014, **118**, 659-667.

Figure legends

Fig. 1 The photoactive yellow protein (PYP) photocycle. The olefin group of the chromophore in the dark state is in the *trans* form and the hydroxyl group is deprotonated. (a) Absorption of light isomerizes the *trans* form to the *cis* form, causing structural changes and protonation of the phenolic hydroxyl group and a large blue-shift in the absorption maximum (λ_{max}) from 446 nm to 355 nm. Subsequently, the M intermediate changes into the dark state within 1 s. (b) The chemical structure of *p*-coumaric acid (*p*CA) above, and cyano-*p*-coumaric acid (CHCA) below.

Fig. 2 Titration of free CHCA chromophore, using HCl or NaOH to adjust the pH. (a) Plot of titration data. (b) Titration analyzed at 386 nm. The solid line is the theoretical curve fit using Hill formula.. pKa value, 8.1. OD, optical density.

Fig.3 1H-NMR data for CHCA. Upper panel shows spectra for dark state of CHCA, lower one after 10min illumination. Arrows indicate signals originated from *cis*-form CHCA, as monitored by 600MHz NMR spectroscopy with fiber-coupled system.

Fig.4 Absorption spectra for free CHCA after 410 nm light-emitting diode illumination at pH 10.8. Illumination times were 0, 1, 2, 4, 8, 16, 32, 64,128, 256, 300, 400, and 512 min.

Fig. 5 Absorption spectra of free CHCA chromophore under acidic conditions (left), the absorption spectrum of the photo-product (middle), and the spectrum of free CHCA

chromophore at pH 7.4 conditions (right).

Fig. 6 Absorption spectra for wild-type (WT), PYP with a bound CHCA (PYP-CN), E46Q and E46Q-CN at pH 7.4, 10 mM MOPS buffer.

Fig. 7 (a) Acid titration of PYP-CN over the pH range 2.0-7.5. (b) Titration curves of WT, PYP-CN, E46Q and E46Q-CN. The solid lines were obtained by fitting with Hill formula.

Fig. 8 Absorption spectra of WT PYP, 1. dark state, 2. 100 ms after illumination. Inset; time course at 446 nm absorbance fit with a single exponential curve.

Fig.9 Absorption spectra of PYP-CN at pH 5.0, 1. dark state, 2. 80msec after illumination.

Fig. 10 Ultrafast time-resolved spectra. (a) Transient absorption spectra for WT at selected delay times from 0.3 ps to 1.0 ns after photoexcitation. (b) Transient absorption spectra for PYP-CN.

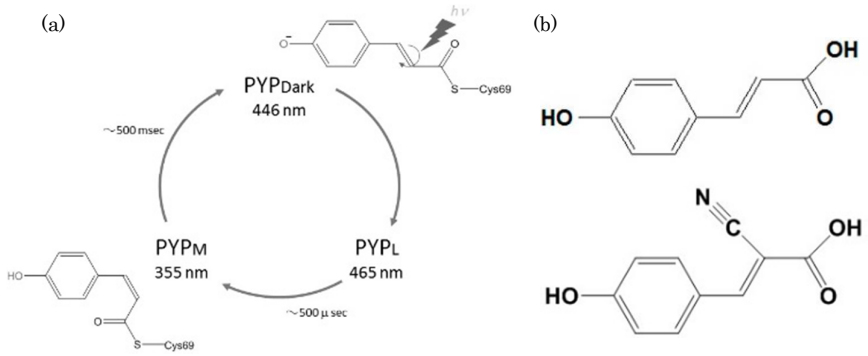

Fig.1

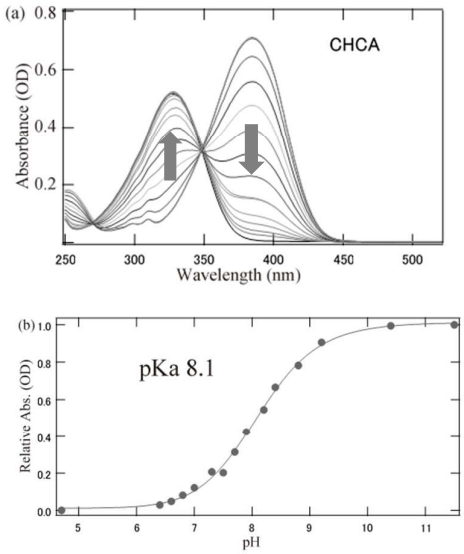

Fig.2

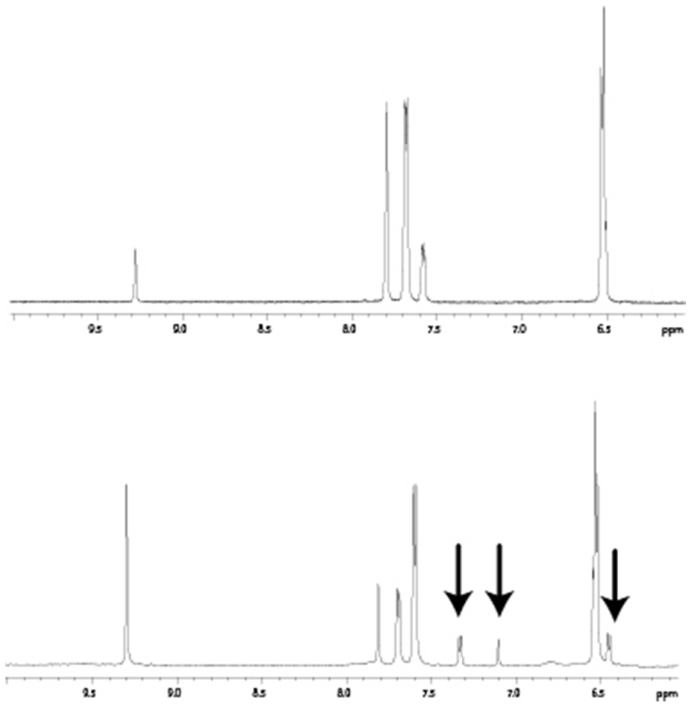

Fig.3

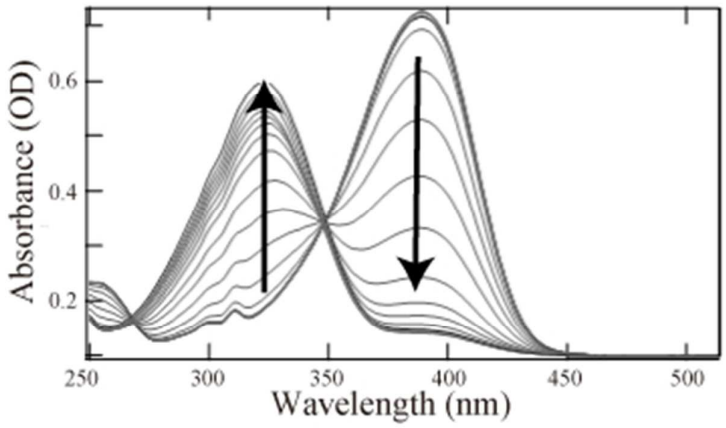

Fig.4

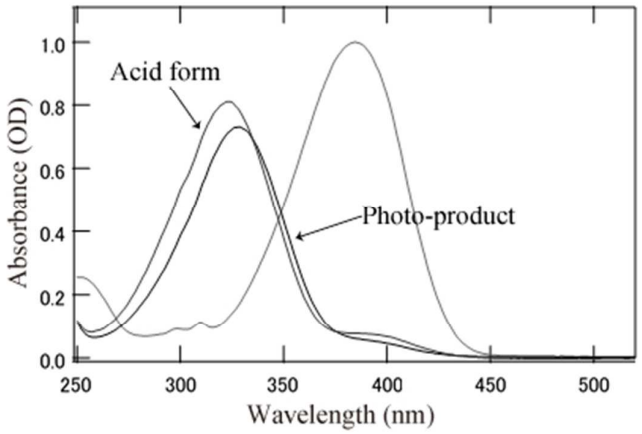

Fig.5

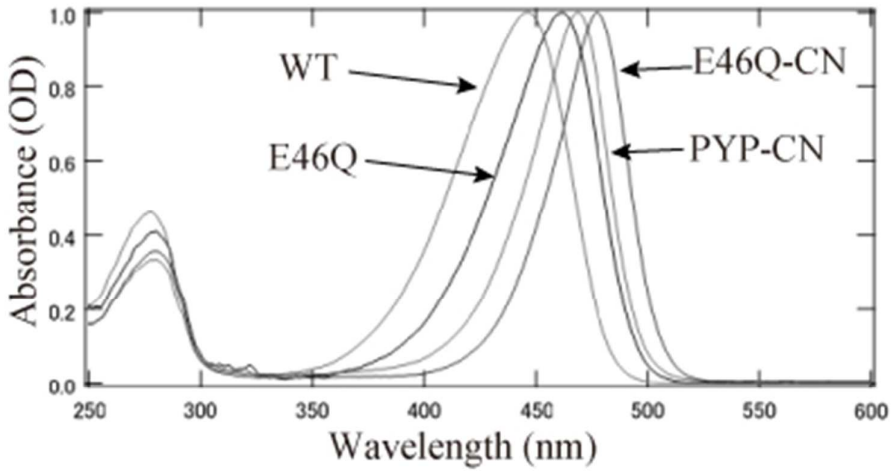

Fig.6

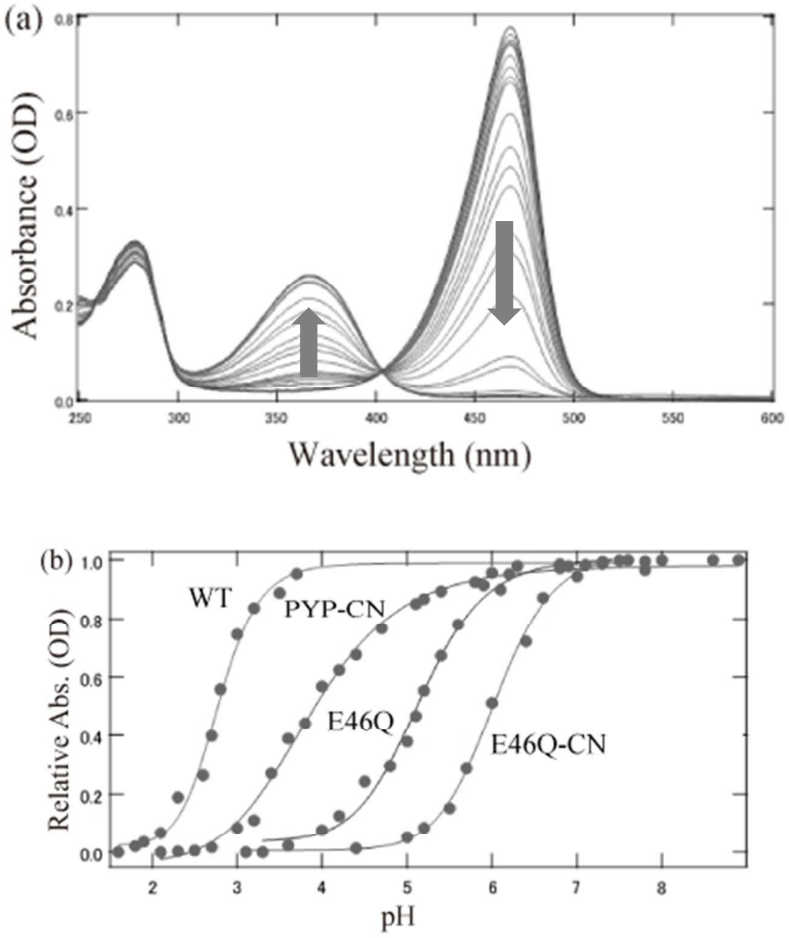

Fig.7

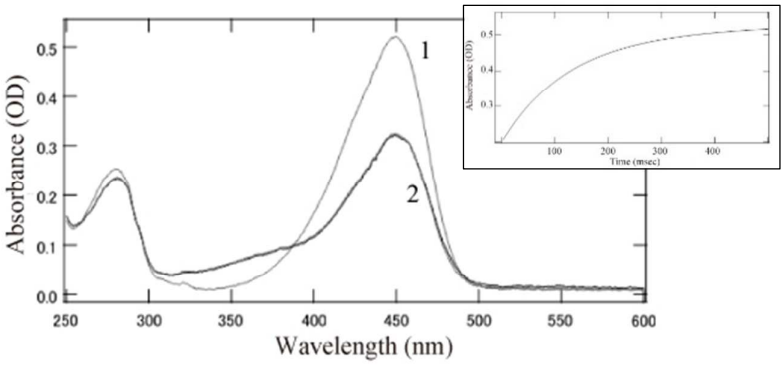

Fig.8

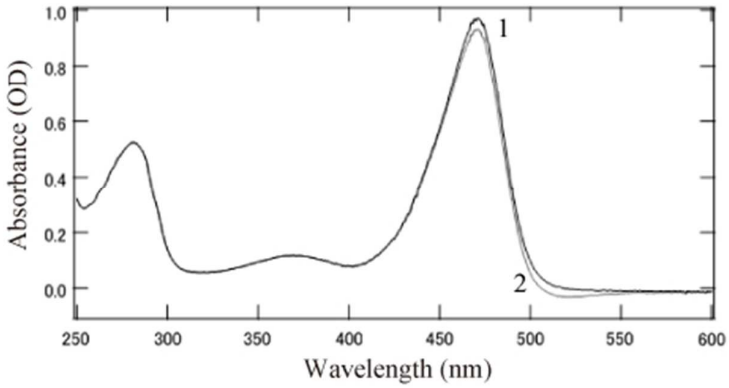

Fig.9

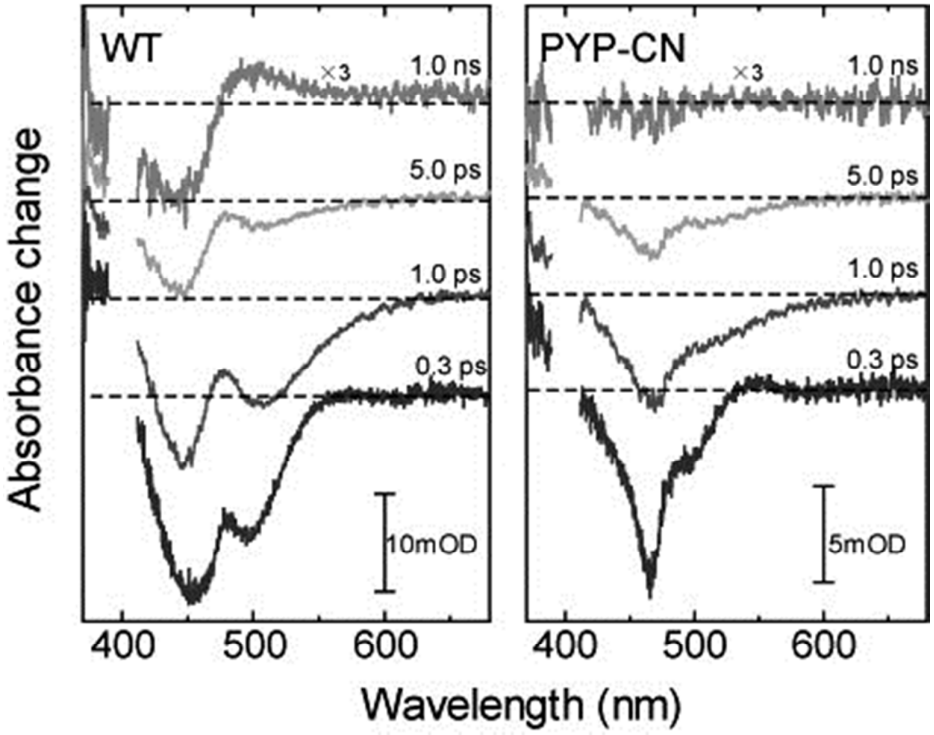

Fig.10

Table 1. Summary of observed data

	λ_{Max} (nm)	HBW (cm^{-1})*	pKa
PYP-CN	466	1869	3.9
E46Q-CN	477	1750	6.0
WT	446	3023	2.9
E46Q	460	2531	5.0

*Half bandwidth