Supporting Information

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SI Materials and Methods

Cloning, Expression, and Protein Purification. The PCR-amplified BglII/EcoRI fragment encoding PAmCherry1 was cloned into pBAD/HisB vector (Invitrogen). The recombinant PAmCherry1 protein with the N-terminal His_6-tag was expressed in the LMG194 bacterial strain (Invitrogen) by overnight culture in RM minimal medium at 37 °C in the presence of 0.004% arabinose. The culture was then centrifuged at 5,000 rpm at 4 °C for 15 min, the cell pellet resuspended in 50 mM NaH_2PO4, 300 mM NaCl, pH 8.0, buffer and lysed by sonication on ice. The recombinant protein was purified with Ni-NTA resin (Qiagen).

Protein Crystallization. Before crystallization, PAmCherry1 protein was dialyzed against 10 mM Tris-HCl, 100 mM NaCl, pH 8.0 (at 25 °C) and diluted to concentration of 17 mg/mL. Diffraction quality crystals were grown using the hanging drop vapor diffusion method by mixing 1 μL of protein and 1 μL of reservoir solution and equilibrating the samples against reservoir solution. The reservoir solution contained 30% PEG 4000, 0.1 M Tris-HCl, pH 8.5, 0.2 M $MgCl_2$. Crystals reached maximal size in 1 week. Diffraction from the PAmCherry1 crystals in dark state was consistent with the space group *I222* with unit cell dimensions as follows: a, 69.17 Å; b, 83.75 Å; c, 99.03 Å; and $\alpha = \beta = \gamma = 90°$.

Protein Photoactivation. The photoactivation of PAmCherry1 crystals was performed with 50mW/cm^2 laser beam (Chameleon Ultra Ti-Sapphire laser; Coherent) of 405 nm in their crystallization drops in the same reservoir where the crystals were grown, at room temperature for 28 min until the red fluorescence reached plateau. The increase in red fluorescence of the crystals was detected by using a Leica MZ16F fluorescence stereomicroscope equipped with custom red (570/30 nm exciter, 615/40 nm emitter) filter set (Chroma) and ½-inch CCD camera (Imaging Source). PAmCherry1 crystals in the fluorescent (i.e., ON) state were isomorphous to the crystals in the non-fluorescent dark (i.e., OFF) state; their diffraction properties were not impacted by photoactivation procedure even though crystals changed color to light pink.

X-Ray Diffraction Data Collection and Crystallographic Refinement. Crystals of PAmCherry1 in the OFF and ON states with dimensions $0.2 \times 0.2 \times 0.1$ mm^3 were mounted in cryo-loops directly from the crystallization droplet and flash-cooled in liquid nitrogen. Diffraction data were collected on a Quantum 315 CCD detector (Area Detector Systems) with 1.08 Å wavelength radiation on the X29A beam line (National Synchrotron Light Source, Brookhaven National Laboratory) or at the LRL-CAT (Advanced Photon Source, Argonne National Laboratory) using Mar-CCD 165 mm detector and 0.979 Å wavelength radiation. Intensities were integrated using the program HKL2000 and reduced to amplitudes using the program TRUNCATE (see Table S1 for statistics) (1, 2). The structures were solved using the molecular replacement method with Phaser (3). Model building and refinement were performed with the programs REFMAC and COOT (2, 4); see Table S1 for statistics. The quality of the final structure was verified with composite omit maps, and the stereochemistry was checked with the programs WhatCheck (5) and ProChek (6). The LSQKAB and SSM algorithms were used for structural superimpositions (2, 7). Quaternary structures were analyzed using the PISA server (8).

Chymotrypsin Digestion and Chromopeptide Purification. Two aliquots of 20 μg non-illuminated PAmCherry1 (OFF state) and 2 aliquots of 20 μg PAmCherry1 illuminated with 405 nm light (ON state) were subjected to an in-solution digestion as described (9) with chymotrypsin. The samples were evaporated to near dryness in a Speed Vac and resuspended in 3 μL guanidinium hydrochloride and heated to 80 °C for 30 s. The samples were cooled to room temperature and 1.0 μL 0.33 M hydrochloric acid was added to neutralize the digestion buffer. Chymotrypsin buffer (30 μL) containing 0.1 M Tris, pH 7.8, and 10 mM $CaCl_2$ was added to the remaining 2 tubes and chymotrypsin was added at an enzyme:protein ratio of 1:60. The digests were incubated at room temperature for 22 h and quenched with 0.1% TFA. The peptides were desalted and isolated using a C18 ZipTip (Millipore).

MS Analysis of Chromopeptides. Mass spectra were acquired on a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems). The instrument was equipped with a Nd:YAG laser (PowerChip JDS; Uniphase) operating at 200 Hz and controlled by Applied Biosystems 4000 Series Explorer version 3.6 software. Each spectrum was accumulated with 500 shots in positive ion mode. MS/MS were acquired in PSD mode with mass isolation window ±3 Da. Note that we do not report data on all possible peptides that could be derived from PAmCherry1 in the OFF or ON states in this article; we report the chromophore-containing peptides that ionized well and gave data with a high signal-to-noise ratio.

1. Otwinowski W, Minor F (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276:307–326.
2. Storoni LC, McCoy AJ, Read RJ (2004) Likelihood-enhanced fast rotation functions. *Acta Crystallogr D Biol Crystallogr* 60:432–438.
3. Anonymous (1994) The CCP4 suite: Programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* 50:760–763.
4. Emsley P, Cowtan K (2004) Coot: Model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60:2126–2132.
5. Hooft RW, Vriend G, Sander C, Abola EE (1996) Errors in protein structures. *Nature* 381:272.
6. Laskowski R, MacArthur M, Moss D, Thornton J (1993) PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Cryst* 26:283–291.
7. Krissinel E, Henrick K (2004) Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr D Biol Crystallogr* 60:2256–2268.
8. Krissinel E, Henrick K (2007) Inference of macromolecular assemblies from crystalline state. *J Mol Biol* 372:774–797.
9. Gross LA, Baird GS, Hoffman RC, Baldridge KK, Tsien RY (2000) The structure of the chromophore within DsRed, a red fluorescent protein from coral. *Proc Natl Acad Sci USA* 97:11990–11995.

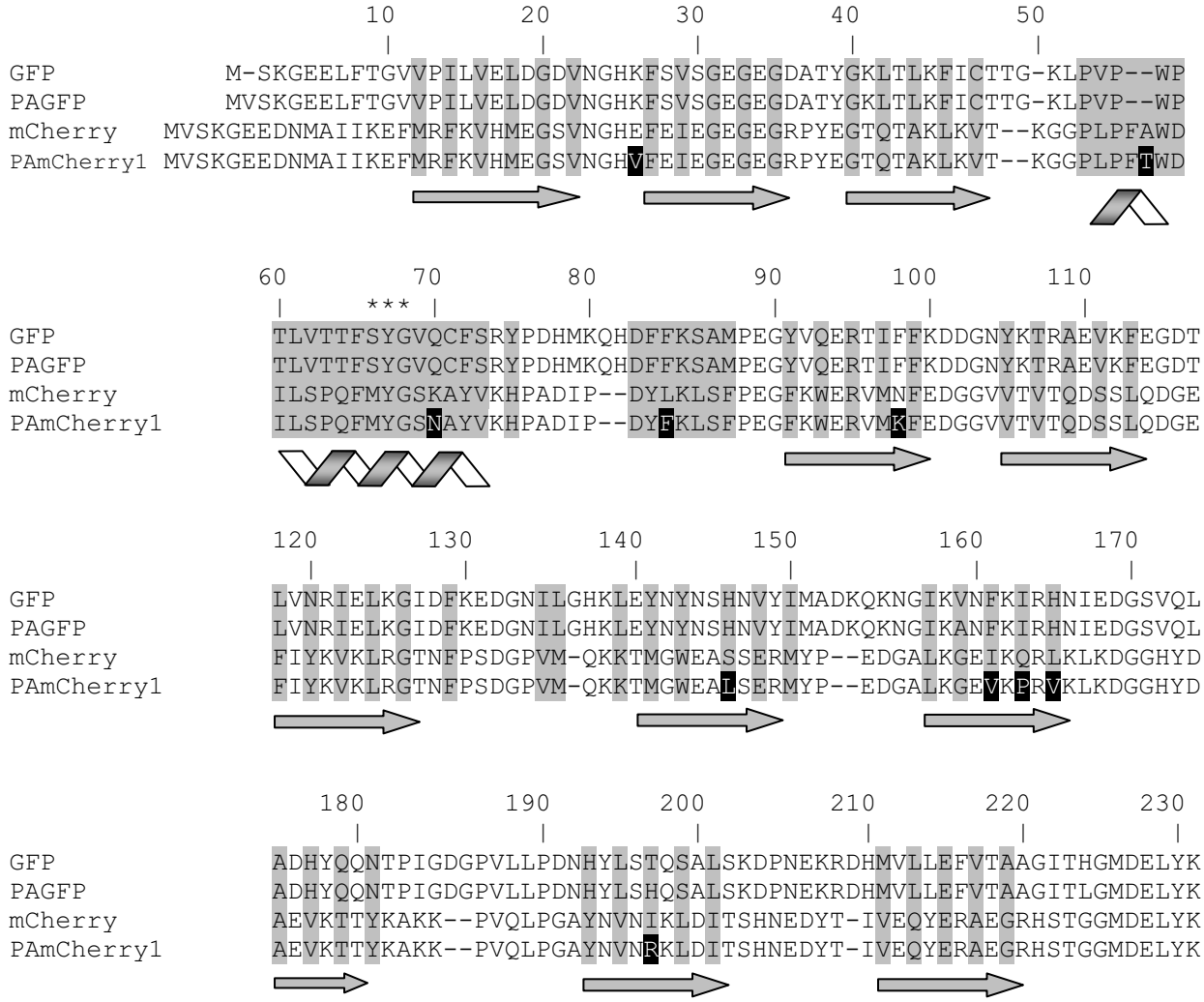


Fig. S1. Amino acid sequence of PAmCherry1 aligned with GFP, PAGFP, and mCherry. Amino acid residues that differ in PAmCherry1 from mCherry are indicated in white and highlighted in black. Residues buried in β-can are shaded. Asterisks indicate residues that form the chromophore. β-sheet-forming regions and α-helixes are denoted as arrows and ribbons, respectively. Alignment numbering follows that of the PAmCherry1 polypeptide.

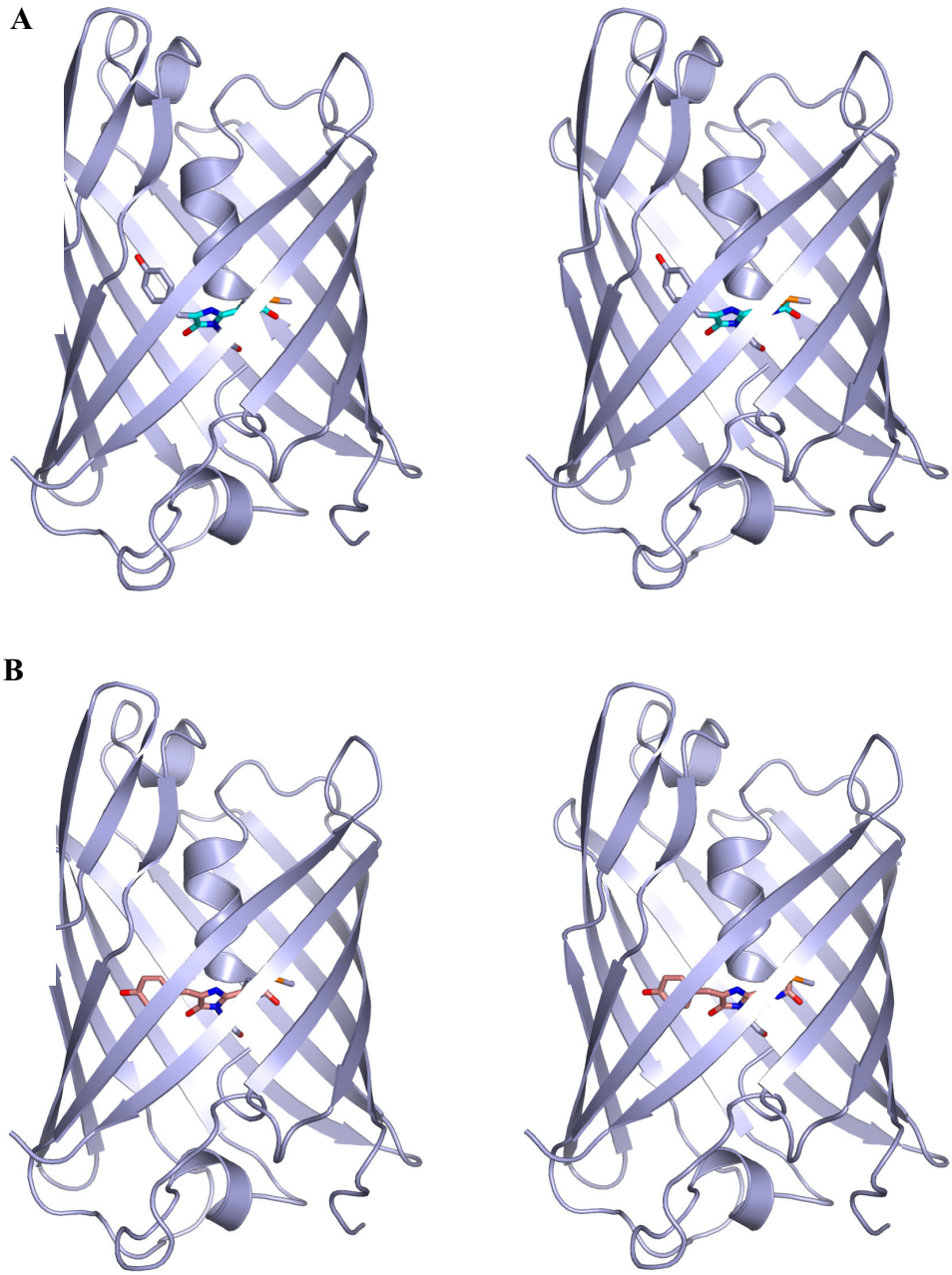


Fig. S2. Overall structures of PAmCherry1 in the OFF and ON states. (*A*) PAmCherry1 in the OFF state with the chromophore colored in cyan. (*B*) PAmCherry1 in the ON state with the chromophore colored in pink.

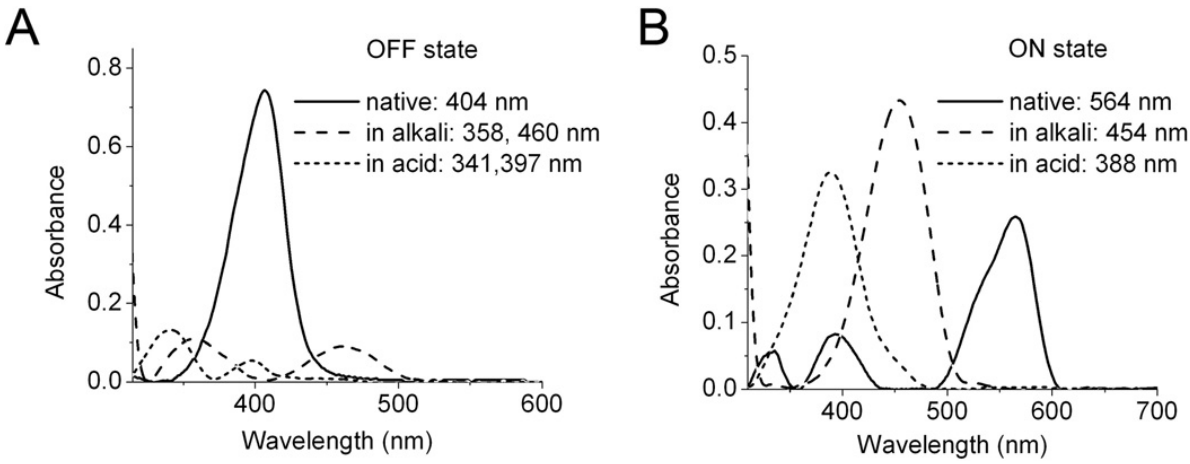


Fig. S3. Absorbance spectra of PAmCherry1 in the OFF (*A*) and ON (*B*) states are shown for the protein in PBS solution (solid lines), denatured in 1 M NaOH (dashed lines) or denatured in 0.3 M HCl (dotted lines).

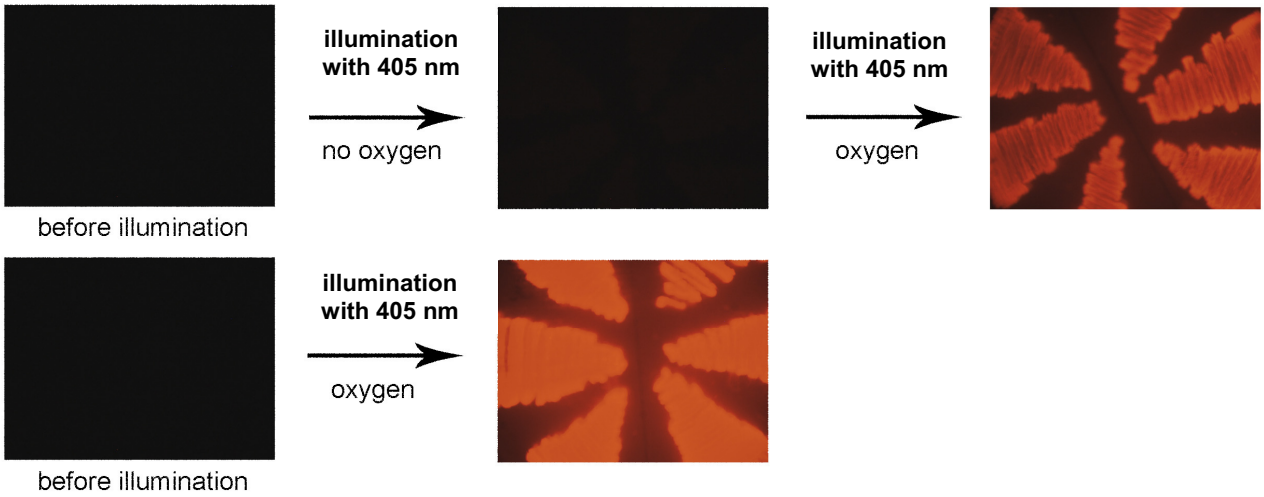
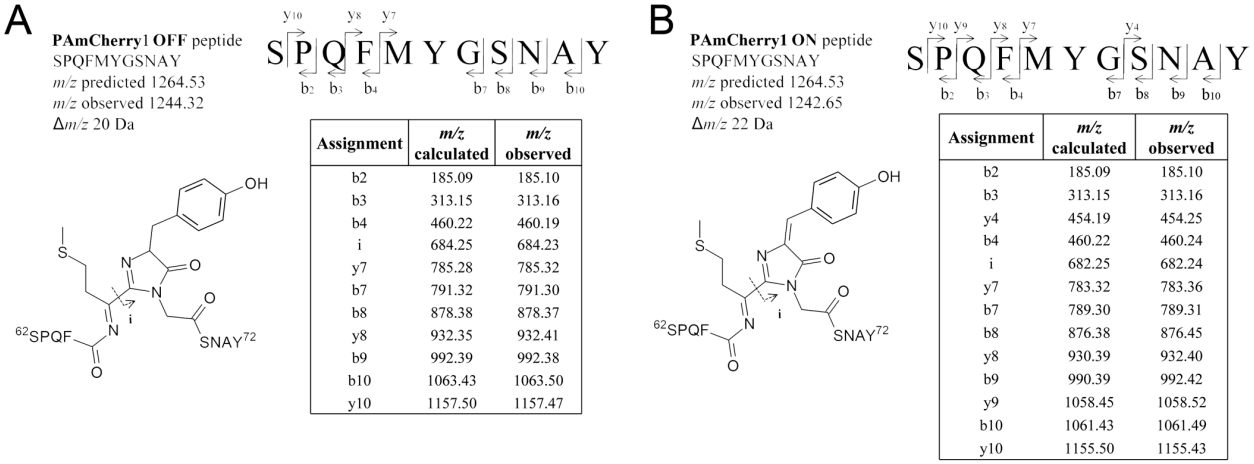


Fig. S4. Photoactivation of PAmCherry1-expressing bacteria in ambient and anaerobic conditions. Streaks of bacteria expressing PAmCherry1 were imaged using a Leica MZ16F fluorescence stereomicroscope equipped with 570/30 nm excitation and 615/40 nm emission filters (Chroma). The images were taken before and after the 10-min illumination with 405-nm LED array (40 mW/cm^2) either in ambient atmospheric conditions or in anaerobic conditions with oxygen replaced with CO_2.



Assignment	*m/z* calculated	*m/z* observed
b2	185.09	185.10
b3	313.15	313.16
b4	460.22	460.19
i	684.25	684.23
y7	785.28	785.32
b7	791.32	791.30
b8	878.38	878.37
y8	932.35	932.41
b9	992.39	992.38
b10	1063.43	1063.50
y10	1157.50	1157.47

Assignment	*m/z* calculated	*m/z* observed
b2	185.09	185.10
b3	313.15	313.16
y4	454.19	454.25
b4	460.22	460.24
i	682.25	682.24
y7	783.32	783.36
b7	789.30	789.31
b8	876.38	876.45
y8	930.39	932.40
b9	990.39	992.42
y9	1058.45	1058.52
b10	1061.43	1061.49
y10	1155.50	1155.43

Fig. S5. MS analysis of the chromophore-bearing peptide fragments from the PAmCherry1 in the OFF (*A*) and ON (*B*) states. The amino acid sequences and suggested structures of the PAmCherry1 chymotrypsin-derived peptides are shown. The sites of the MS/MS fragmentation are shown with solid arrows (*Top Right*). The N- and C-terminal ions are denoted as ''yn'' and ''bn,'' respectively, where ''n'' indicates the number of amino acids in the respective fragment. Distinctive peaks in the MS/MS spectra of the PAmCherry1 chromophore-bearing peptide ions are summarized in the tables. The N-terminal fragments ''i,'' which result from cleavage at the C^{α}-C bond in Met-66 of the peptides, are indicated with dashed arrows on the chemical structures. Note that the hydroxyl group of Tyr-67 in the ON state (*B*) is protonated because of the MS/MS experimental conditions.

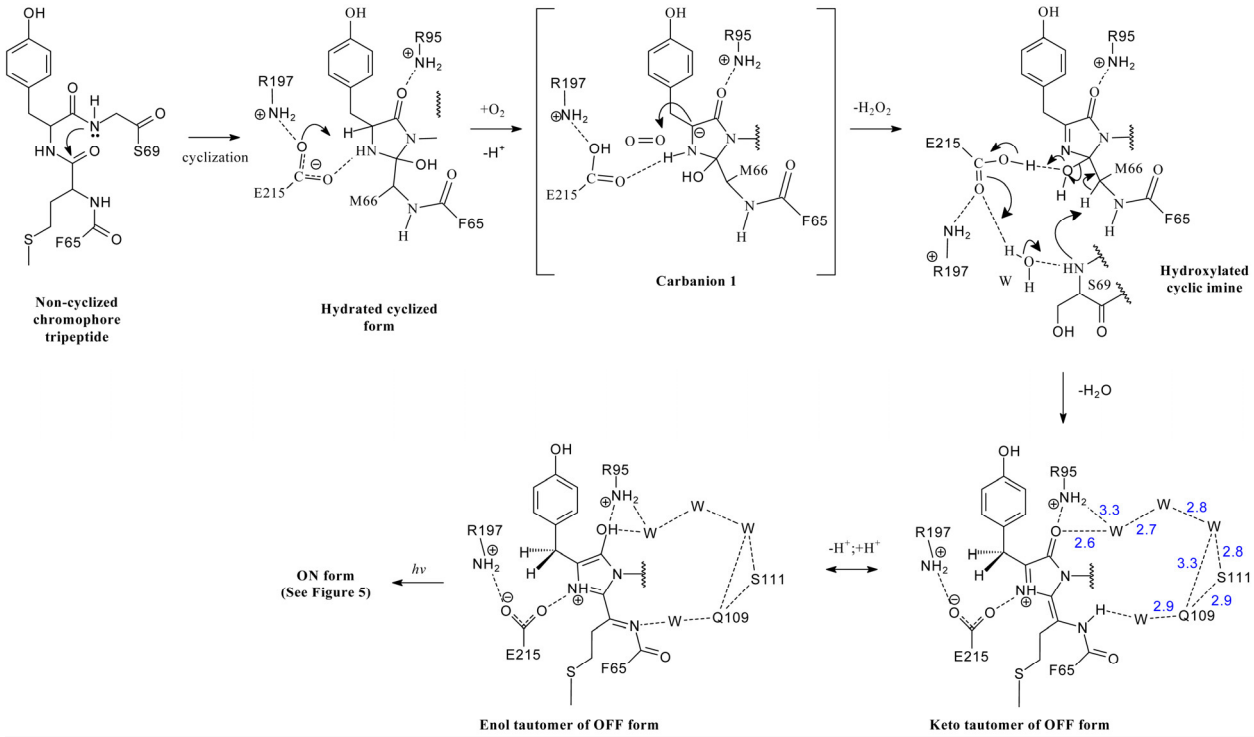


Fig. S6. Alternative mechanism for the formation of the PAmCherry1 dark chromophore (OFF state). A hydrated cyclized form is the chromophore form with the non-oxidized bond between *p*-hydroxyphenyl and non-oxidized, hydrated 5-member ring and without N-acylimine group. A hydroxylated cyclic imine form is the chromophore form with the non-oxidized bond between *p*-hydroxyphenyl and oxidized, hydrated 5-member ring and without N-acylimine group. The hydrogen bonds are shown with dashed lines; their lengths are indicated in angstroms. Intermediate unstable compounds are shown in parenthesis. *hv*, illumination with violet light. Migration of the electron density is shown with curved arrows.

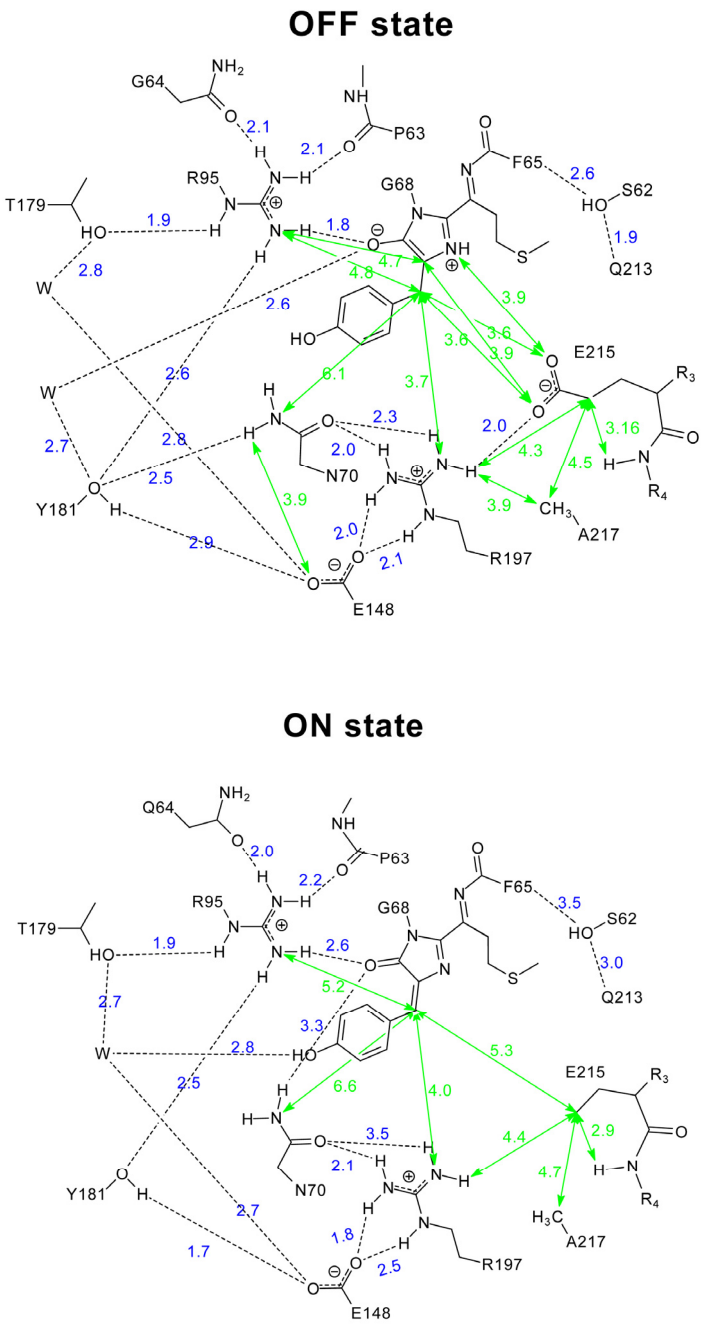


Fig. S7. Immediate environment of the PAmCherry1 chromophore in the OFF and ON states. Distances between the atoms of the amino acid residues and the chromophore are shown with green arrows. Suggested hydrogen bonds are shown with dashed lines. Lengths of the distances and hydrogen bonds are indicated in angstroms.

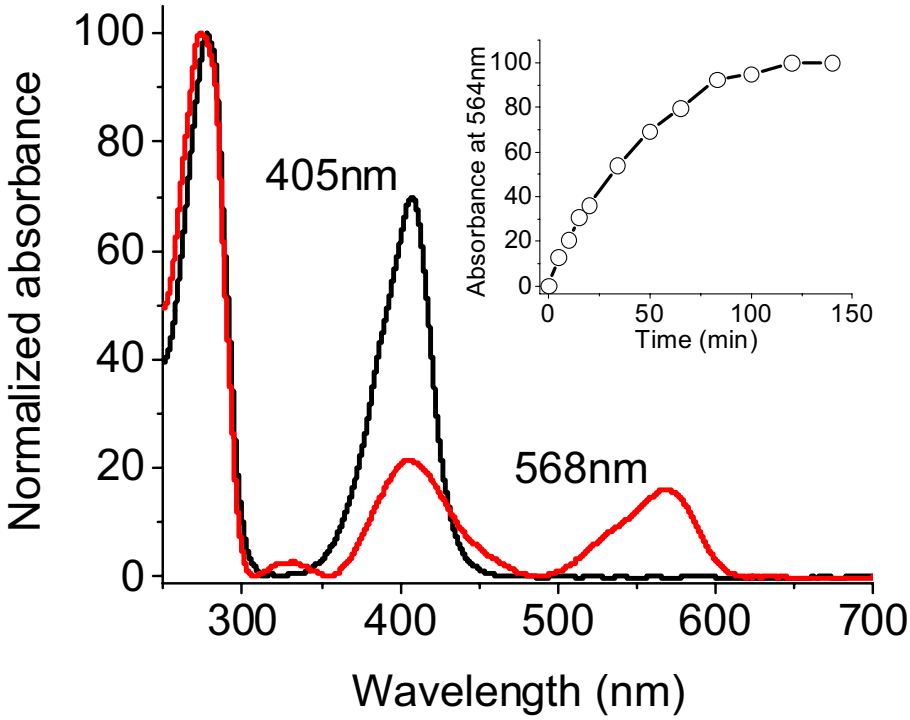


Fig. S8. Absorbance spectra of the PAmCherry1/A217S mutant before and after the photoactivation. Absorbance spectra of the PAmCherry1/A217S mutant before (black line) and after (red line) the photoactivation with 405 nm LED array (40 mW/cm^2) for 120 min. The photoactivation kinetics for the PAmCherry1/A217S mutant detected by the absorbance at 568 nm is also shown (*Inset*).

Table S1. Data collection and refinement statistics for PAmCherry1 in the OFF and ON states

Measurement	OFF state	ON state
Data collection		
Beamline	APS-LRL-CAT	NSLS-X29A
Wavelength, Å	0.979	1.08
Resolution limits, Å	20–1.50	20–1.65
Observed reflections	269.100	183,942
Unique reflections	45,776	31,288
Completeness, %	98.6 (89.1)*	90.3 (45.3)*
R_{merge}[†]	0.087 (0.431)*	0.089 (0.484)*
Refinement statistics		
Protein non-hydrogen atoms	1,753	1,747
Water molecules	324	250
R_{cryst}[‡]	0.174 (0.251)*	0.177(0.357)*
R_{free}[‡]	0.195 (0.281)*	0.209(0.435)*
Average B-factor, Å^2	21.6	27.8
RMSD from ideality		
Bond length, Å	0.017	0.022
Bond angles, °	1.87	2.04
Torsion angles, °	15.3	16.1
Ramachandran plot		
Core, %	98.1	98.1
Allowed, %	1.8	1.8
Generous, %	0.1	0.1

*Values in parentheses indicate statistics for the high-resolution bin.

[†]$R_{merge} = \Sigma\Sigma_j |I_j(hkl) - \langle I(hkl)\rangle| / \Sigma\Sigma_j < 341 \langle I(hkl)\rangle|$, where I_j is the intensity measurement for reflection j and $\langle I\rangle$ is the mean intensity over j reflections.

[‡]$R_{cryst}/(R_{free}) = \Sigma||F_o(hkl)| - |F_c(hkl)||/\Sigma|F_o(hkl)|$, where F_o and F_c are observed and calculated structure factors, respectively. No σ-cutoff was applied; 5% of the reflections were excluded from refinement and used to calculate R_{free}.

Table S2. Absorbance maxima for PAmCherry1 and other fluorescent proteins denatured in alkali or in acid

	Absorbance, nm	
Protein/Condition	Major peak	Minor peaks
PAmCherry1 in OFF state		
1 M NaOH	358	460
0.2 M HCl	341	397
PAmCherry1 in ON state		
1 M NaOH	454	None
0.2 M HCl	388	None
EGFP		
1 M NaOH	447	None
0.2 M HCl	385	None
mCherry		
1 M NaOH	457	None
0.2 M HCl	446 (420*, 395†)	None
TagRFP		
1 M NaOH	448	None
0.2 M HCl	387	None
mKate		
1 M NaOH	448	None
0.2 M HCl	387	None

*Heating at 95°C for 5 min.
†Heating at 95°C for 60 min.

Table S3. Spectral properties of the purified PAmCherry1, PAmCherry1 mutants, and mCherry/K70N//I197R before and after illumination with 405 nm light

	Absorbance (excitation/emission), nm	
Protein/illumination with 405 nm	Major peak	Minor peaks
PAmCherry1		
Before	407	None
After	563	330, 390
PAmCherry1/Y67L		
Before	407 (404/463)	386
After	407	386, 556
PAmCherry1/Y67Q		
Before	407 (405/490)	386
After	407	386
PAmCherry1/Q213M		
Before	386, 395	None
After	386	474 (466/505), 557 (549/591)
PAmCherry1/Q213E		
Before	405	322, 387
After	560	387
PAmCherry1/Q213I		
Before	402	323, 386
After	552	384, 487
PAmCherry1/Q213L		
Before	400	386
After	556	386
PAmCherry1/Q42L		
Before	398	None
After	564	None
PAmCherry1/Q42V		
Before	413	None
After	565	None
PAmCherry1/Q42R		
Before	413	None
After	564	None
PAmCherry1/Q109A,V		
Before	none	None
After	none	None
PAmCherry1/Q109L		
Before	none	354
After	none	483
PAmCherry1/Q111L		
Before	383	None
After	383	466
PAmCherry1/Q111V		
Before	384	None
After	384	469
PAmCherry1/Q111I		
Before	382	354
After	382	462
PAmCherry1/Q109R/S111E		
Before	385 (367/470)	None
After	385	486 (467/506) 508
PAmCherry1/Q42L/Q109F/S111A		
Before	358 (366/435)	None
After	358	478 (466/503)
mCherry/K70N/I197R		
Before	486	None
After	486	575 (575/601)