Published in final edited form as:

J Photochem Photobiol A Chem. 2020 October 1; 401: . doi:10.1016/j.jphotochem.2020.112738.

Structural and spectroscopic characterization of photoactive yellow protein and photoswitchable fluorescent protein constructs containing heavy atoms

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Abstract

Photo-induced structural rearrangements of chromophore-containing proteins are essential for various light-dependent signaling pathways and optogenetic applications. Ultrafast structural and spectroscopic methods have offered insights into these structural rearrangements across many timescales. However, questions still remain about exact mechanistic details, especially regarding photoisomerization of the chromophore within these proteins femtoseconds to picoseconds after photoexcitation. Instrumentation advancements for time-resolved crystallography and ultrafast electron diffraction provide a promising opportunity to study these reactions, but achieving enough signal-to-noise is a constant challenge. Here we present four new photoactive yellow protein constructs and one new fluorescent protein construct that contain heavy atoms either within or around the chromophore and can be expressed with high yields. Structural characterization of these constructs, most at atomic resolution, show minimal perturbation caused by the heavy atoms compared to wild-type structures. Spectroscopic studies report the effects of the heavy atom identity and location on the chromophore's photophysical properties. None of the substitutions prevent photoisomerization, although certain rates within the photocycle may be affected. Overall, these new proteins containing heavy atoms are ideal samples for state-of-the-art time-resolved crystallography and electron diffraction experiments to elucidate crucial mechanistic information of photoisomerization.

Graphical abstract

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Author Contributions: Methodology, M.G.R. and C.-Y.L.; Investigation, M.G.R. and C.-Y.L.; Formal Analysis, M.G.R. and C.-Y.L.; Writing – Original Draft Preparation, M.G.R.; Writing – Review & Editing, M.G.R., C.-Y.L., and S.G.B.; Supervision, S.G.B.; Project Administration, M.G.R., C.-Y.L., and S.G.B.; Funding Acquisition, S.G.B.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of Interest. The authors declare no conflict of interest.

Keywords

photoisomerization; photophysics; optogenetics; x-ray crystallography; noncanonical amino acid incorporation

1. Introduction

Photoisomerization around specific carbon-carbon double bonds is central to many biological processes and has been exploited to develop valuable experimental tools and sensors. For example, different families of rhodopsins have been repurposed as optogenetic tools to control neural excitation with light [1], while engineered photoswitchable fluorescent proteins have allowed for imaging beyond the diffraction limit with super-resolution microscopy [2]. Photoactive yellow protein (PYP), a member of the PAS domain family responsible for initiating light-activated signal transduction [3], has also been adapted as an optogenetic tool with a variety of functions [4–7]. A thorough mechanistic understanding of the photoisomerization process in these systems is essential to further develop tools with new and improved functionality. While many theoretical and experimental studies have focused on elucidating mechanistic information (kinetics, structures, reaction pathways and intermediates) for many of these systems, the detailed mechanisms, and importantly, specific factors that influence those mechanisms, are still heavily debated [8–10].

Technological and instrument advancements have contributed to the development of experimental techniques capable of visualizing photoisomerization in new ways. One method ideally suited to reveal mechanistic information of photoisomerization within protein samples is time-resolved x-ray crystallography. While the Laue crystallography method has been the workhorse technique for obtaining time-resolved structural data of biological samples, the development of a new x-ray source (x-ray free electron laser) has led to an improved technique called time-resolved serial femtosecond crystallography (TR-

SFX), which has improved temporal resolution, limited radiation damage effects, and less stringent sample requirements [11]. The photocycles of PYP and a reversibly switchable fluorescent protein, rsEGFP2, have been investigated recently with this technique [12–14], which has revealed structural intermediates femtoseconds to picoseconds after photon absorption. Incorporation of heavy atoms onto the chromophore in these two systems would both improve the TR-SFX signal and break the symmetry of the phenolate ring to discriminate between different possible photoisomerization mechanisms (e.g. hula twist *vs* one-bond-flip, Fig. 1A) [15]. Another new method, ultrafast electron diffraction (UED) [16], offers high structural and temporal resolution for the observation of light-induced atomic motion. Although studies thus far have been limited to small molecules in the gas phase [17,18] and thin films [19], the development of free-flowing sub-micron liquid sheets stable in vacuum have extended the applicability of this method to solution-phase samples without requiring any protein crystallization [20]. Since the UED signal scales with atomic number, the presence of heavy atoms substantially improves the signal-to-noise ratio and facilitates data analysis. Finally, UED may be useful for proteins that undergo substantial structural changes that may be inhibited in crystals.

In this work, we have used a variety of synthetic chemistry and molecular biology techniques to incorporate heavy atoms site-specifically at multiple locations within PYP and rsEGFP2. We present high resolution structures of all PYP (atomic resolution) and rsEGFP2 (1.5 Å) constructs that highlight the minimal impact of the heavy atom incorporation on the global tertiary protein structure and the local chromophore environment. Spectroscopic characterization of these proteins reveals the extent of perturbation to the chromophore's photophysical properties. Based on these results, heavy atom incorporation within PYP and rsEGFP2 should provide minimally perturbed protein samples that can produce high signal-to-noise ratios for techniques like UED and TR-SFX to obtain meaningful mechanistic insights into photoisomerization.

2. Results and Discussion

2.1 Incorporation of Heavy Atoms

We chose the locations for heavy atom incorporation in both PYP and rsEGFP2 based on two main criteria. First, the UED signal reflects changes in absolute atom pair distances with the signal proportional to the product of the two atoms' atomic numbers. The signal also scales with the inverse of atom distance [16], so in order to monitor light-induced movements of the chromophore, the heavy atom(s) must be located either on or near the chromophore to selectively amplify the chromophore's signal over that from the rest of the protein background. A subset of protein constructs that obey this first criterion includes those with a heavy atom substituent on the chromophore's phenolate ring, which breaks its symmetry (Fig. 1A, center) [15]. TR-SFX studies on these constructs would also provide useful mechanistic information. Second, there must be a feasible route to express sufficient quantities of each modified protein containing the heavy atom at a specific site. With these criteria in mind, we successfully expressed four different PYP variants and two different rsEGFP2 variants (Fig. 1B), in addition to the wild-type proteins for each as comparisons. All except the selenium-containing PYP could be produced on the gram scale.

For PYP, we first focused on modifying the *para*-coumaric acid (pCA) chromophore by introducing a bromine atom at the 3 position of the phenolate ring (Fig. 1B). Synthesis of the 3-bromo-pCA (3-Br-pCA) chromophore derivative followed by functionalization to the corresponding acid anhydride and linkage to the thiol of C69 yielded a PYP construct with the modified chromophore (PYP 3-Br-pCA, see Materials and Methods). Similar approaches in our lab and others have produced PYP variants with a less electron-rich substituent on the phenolate ring of pCA such as a hydroxy group [21–23], methoxy group [21,24], and chlorine atom [25]. Note that substituents have also been placed at the α position of the carboxylic acid of pCA (C3 in Fig. 1A) [26, 27]; however, synthesis of these analogs requires harsh conditions and the substituents have been shown to prevent the photocycle from occurring.

We next aimed to introduce a heavy atom near to, but not as part of, the chromophore. This heavy atom can act as a (relatively) static reference as the chromophore rotates while providing greatly enhanced signal due to its size. Based on the crystal structure of PYP (PDB: 1NWZ [28]), taking into account proximity to the chromophore and likelihood for minimal structural and photophysical perturbations, we chose to target F96 (Fig. 1A). Using an amber suppression system originally engineered to incorporate *para*-cyanophenylalanine [29], we successfully introduced an iodine atom as *para*-iodophenylalanine at residue 96 (PYP F96(4-IF), Fig. 1B). Since the signal for UED relies on atom pair distances and is proportional to atomic number, we also expressed a hybrid PYP construct that contains both the brominated chromophore and the iodinated phenylalanine (PYP 3-Br-pCA F96(4-IF)). Producing a construct with an iodinated chromophore is possible in principle, would lead to an even greater UED and TR-SFX signal enhancement, and has been done in fluorescent proteins [30,31]; however, limited availability of the iodinated precursor prevented synthesis of the pCA chromophore derivative. The final PYP construct expressed in this work replaces the sulfur of C69 with selenium (PYP C69U, Fig. 1B), resulting in a selenoester linkage between the pCA chromophore and the rest of the protein. C69 is an ideal residue to target to selectively modify chromophore properties, but to our knowledge, this selenoester-containing variant has not been previously produced. The selenocysteine was incorporated using a cysteine auxotrophic *E. coli* strain with defined growth and expression media as previously described (see Materials and Methods) [32,33].

For rsEGFP2, we sought a construct similar to PYP 3-Br-pCA, which contains a heavy atom to break the chromophore symmetry. The chromophore within rsEGFP2, 4-(*p*-hydroxybenzylidene)-5-imidazolinone (HBI), is formed via an intramolecular autocatalytic reaction between three residues (A66, Y67, and G68) within the protein β -barrel. To introduce a substituent on the chromophore's phenolate ring, we used amber suppression to replace residue Y67 with the noncanonical amino acid, 3-chlorotyrosine (3-ClY), which results in an asymmetrically chlorinated chromophore (rsEGFP2 Y67(3-ClY), Fig. 1B), as previously described [15,34]. To provide two heavy atoms within the rsEGFP2 protein, similar to the reasoning behind the hybrid PYP 3-Br-pCA F96(4-IF), we expressed a double amber suppression construct that, in addition to a chlorinated chromophore, has 3-ClY at residue 107 (Fig. 1B, rsEGFP2 Y67(3-ClY) Y107(3-ClY)). Residue Y107 was chosen as the second site for amber suppression since this tyrosine has been previously shown to occupy only one rotational conformer based on NMR studies of 3-fluorotyrosine incorporation [35].

2.2 X-ray Crystallography

Structural analysis of all PYP and rsEGFP2 constructs discussed above is important to determine the extent of perturbation caused by the heavy atoms and to provide starting coordinates for model building and data analysis in UED and TR-SFX experiments. The heavy-atom-containing PYP constructs were readily crystallized following similar protocols as previously described [25], and the crystals diffracted to atomic resolution (between 0.85 and 0.97 Å resolution, Table 1). The tertiary structures of all constructs align well with that of the wild-type protein (1NWZ, overall RMSD of less than 0.7 Å for the α -carbons, Table 1), with only slight deviations appearing in loop regions far from the chromophore, suggesting that the heavy atom incorporation does not alter the overall fold of the protein (Fig. 2A). The chromophore and the residues in its immediate environment also are largely unchanged (Fig. 2B), which is consistent with a previously reported PYP structure containing a substituted chromophore (a hydroxy group at the 3 position of the phenolate ring) [23]. The most substantial observed deviations come from R52, V57, and V66 upon bromination of the chromophore. These three residues form a pocket around the bromine atom, although none of the deviations are more than 0.8 Å (Fig. 2C). The chromophore itself remains approximately planar in each PYP construct. The dihedral angle ($C_3-C_4-C_5-C_6$) deviates at most by 6.3° from wild type (Table 1). Introducing iodine at the *para* position of F96 has a minimal impact on the dihedral angle of the side chain ($C_\alpha-C_\beta-C_\gamma-C_{\delta 1}$, Table 1) and causes an approximately 0.4 Å displacement of the β -carbon of V122, towards which the iodine points (Fig. 2D). For the PYP C69U variant, the C_1-Se and the $Se-C_2(=O)$ bonds increase by ~0.12 Å compared to those of the wild-type PYP containing the sulfur atom (Table 1), which agrees with reported values [36] and the general trend of weakening bonds between carbon and heavier, more polarizable atoms, further validating the successful incorporation of selenium (see also Fig. S2 and Table S2). The electron density from each solved structure clearly displays the presence of the heavy atoms (Fig. 2E). To more clearly represent the extra density of the selenium in PYP C69U, Figure 2E shows a difference map (green positive density) between the electron density of PYP C69U and a model that instead contains a sulfur atom. The presence of positive density means that a sulfur atom is not electron-rich enough to fit the observed density. This positive density is completely eliminated when a selenium-containing model is used. For further validation of selenium incorporation, Figure S2 shows the x-ray fluorescence spectrum of PYP C69U protein crystals with clear selenium K α and K β peaks.

The rsEGFP2 Y67(3-ClY) Y107(3-ClY) construct crystallized in similar conditions as previously described [15], and crystals diffracted to 1.5 Å resolution (Table 2), which matches the resolution of wild-type rsEGFP2 (5DTX) [37] and the previously reported structure of rsEGFP2 Y67(3-ClY) (6PFR) [15]. As with the PYP constructs, the tertiary structure, the chromophore, and its local environment for both mutant constructs align well with those of wild type (Figs. 3A and 3B). The only residue noticeably displaced is V151, which occupies part of the pocket surrounding the chlorine atom on the chromophore. The β -carbon of V151 is pushed back by ~1 Å due to the presence of the chlorine, and the side chain adopts an alternative rotamer (Fig. 3C). The displacement of a single residue to accommodate a chromophore substituent has been observed in other fluorescent proteins as well [30], while the rest of the structure remains largely unchanged. The dihedral angles of

the chromophore's P and I bonds, as defined in Figure 1A as C₁-C₂-C₃-C₄ and N₁-C₁-C₂-C₃, respectively [38], span a range of 24° and 6°, respectively (Table 2). Similar to the degree of perturbation around the chromophore, the environment around Y107(3-ClY) remains largely unchanged except for one residue: V23, towards which the chlorine directly points. The α- and β-carbons of V23 are pushed back by 0.7 Å compared to wild type, and the side chain adopts a different rotamer (Fig. 3D). The aromatic ring of Y107(3-ClY) tilts with respect to that of Y107 in the wild-type protein (Fig. 3D, ~12° change in the X² dihedral angle, C_α-C_β-C_γ-C_{δ1}). The electron density showing the presence of heavy atoms in each rsEGFP2 construct is shown in Figure 3E.

Overall, the introduction of heavy atoms into PYP and rsEGFP2 have very minor structural consequences, which is an important criterion if these modified proteins are to be used in place of their wild-type versions to gain useful insights into photophysical and time-resolved structural properties of interest.

2.3 UV-vis Absorption and Fluorescence Spectroscopy

We next characterized the effect of heavy atom incorporation on the photophysical properties of each construct. The absorption spectra of the PYP constructs are shown in Figure 4A with peak values listed in Table 3. Bromination of the pCA chromophore within PYP causes a small blue shift of 1 nm, similar to the observed blue shift for other halogenated chromophores in PYP [25] and other fluorescent proteins [30,31]. Franck-Condon excitation is purely an electronic process that reflects the electronic properties of the chromophore and the effect of the environment at the instantaneous moment of photon absorption before any atomic rearrangements. The direction of the shift is simply due to the electron-withdrawing nature of the substituent, while the small magnitude reflects the weak electronic effect of halogen substituents. This result further supports the conclusion that the substituent causes minimal structural perturbation, since the shift is caused by the electronic effect of the substituent rather than any substantial protein structural changes. Incorporation of iodine near the chromophore has no effect on the absorption properties of the PYP chromophore, which is consistent with the minimal structural perturbations observed from the iodine addition depicted in Figure 2. The bandshape and peak maxima of the fluorescence emission spectra similarly remain largely unchanged (Fig. 4B). The PYP C69U construct shows a 7 nm red shift in the absorption peak maximum, which is due to the greater electron-donating capability of the heavier selenium atom compared to sulfur. Similar red shifts in absorption have been observed in organic dyes after replacement of oxygen and/or sulfur with heavier chalcogen atoms, such as selenium and tellurium [39–42]. This trend can also be validated by comparing the absorption properties of pCA model chromophore derivatives. For example, the absorption peak of the thiomethylester derivative is red-shifted compared to the methylester derivative [43]. The fluorescence emission maximum of PYP C69U is similarly red shifted with respect to that of wild type.

The absorption spectra of all rsEGFP2 constructs, displayed in Figure 4C, show that the electron-withdrawing chlorine substituent on the chromophore causes a small blue shift of 2 nm, analogous to the PYP constructs with 3-Br-pCA discussed above. Despite the small blue shift of the absorption maximum, the fluorescence emission maximum for both the Y67(3-

CIY) and the Y67(3-CIY) Y107(3-CIY) constructs is red shifted by 5 nm (Fig. 4D), resulting in an increased Stokes shift. Although this increased Stokes shift is not present in the PYP variants, it has been previously observed in other fluorescent proteins with substituted chromophores [31] and may be due to increased flexibility of the β -barrel upon incorporation of the bulky substituent.

The fluorescence quantum yields (FQYs) for each construct are reported in Table 3. The FQY measured in this work for wild-type PYP of 0.2% agrees with the previously reported value [21]. Bromination of the chromophore in PYP 3-Br-pCA leads to an almost 3-fold increase in FQY, while iodination of F96 leads to a 1.2-fold increase. Combining the two modifications in the hybrid PYP construct leads to a 5-fold increase in FQY, which is greater than what would be expected if the results were simply additive. Steric confinement, whether within the protein environment or artificially constructed [43], is thought to rigidify the chromophore, hinder bond rotation, and consequently raise the FQY. Previous work on photoswitchable fluorescent proteins has suggested that the size of the chromophore pocket can influence the isomerization, and in turn, the fluorescence, quantum yield. Mutating M159 within the chromophore pocket of Dronpa, another reversibly photoswitchable fluorescent protein also containing the HBI chromophore, to a smaller threonine residue decreases the FQY by more than a factor of 2 while increasing the isomerization quantum yield by orders of magnitude [45]. PYP constructs with other chromophore substituents, such as a hydroxy and methoxy group [21], also have increased FQYs. However, there is both theoretical and experimental evidence that suggests the electrostatic environment of the protein and the electronic properties of the chromophore can also influence the competition between fluorescence and isomerization [30,46]. In fact, the hydroxy and methoxy substituents, which have much more of an electronic effect on the chromophore than a halogen, increase PYP's FQY by as much as 20-fold [21], while bromination only leads to an almost 3-fold change despite the large size of the substituent. Replacing the sulfur with selenium in the PYP C69U construct actually decreases the FQY by about 25%. Similar effects on FQY have been observed in various dyes with increasing sizes of chalcogen atoms and can be attributed to decreased fluorescence lifetimes and increased intersystem crossing rates to the triplet state [39–42].

Interestingly, contrary to the results from the PYP variants, the FQYs for the rsEGFP2 variants are constant and agree with the reported literature value [47]. Introducing a chlorine onto the chromophore and at residue 107 has no effect on the FQY, even though chlorinated chromophores within other fluorescent proteins, such as superfolder GFP and Dronpa2, have been previously shown to lead to increases in FQYs by as much as 25% [30]. These differences further highlight that a chromophore's photophysical properties are highly dependent on its specific local environment.

Despite some differences in absorption, fluorescence, and FQY caused by the introduction of heavy atoms, the chromophores within these constructs still undergo photoisomerization. The spectroscopic changes associated with chromophore isomerization and subsequent protonation are observed for the PYP 3-Br-pCA, PYP F96(4-IF), and PYP 3-Br-pCA F96(4-IF) constructs (Fig. 5A depicts a representative example with PYP 3-Br-pCA F96(4-IF)). Minimal spectral changes were observed for the wild-type and C69U PYP constructs, but

the thermal relaxation rates were too fast to accurately measure, so the chromophores had already completed the photocycle by the time the spectra were collected. Based on these results, the photocycle still occurs upon bromination of the chromophore and iodination of F96, but the kinetics of the photocycle are altered (Fig. 5B). See the Supplementary Material for a more thorough discussion of the effect of heavy atom substitution on the PYP photocycle. No time-resolved studies have been previously performed on PYP constructs with a halogen at the 3 position of the phenolate ring, but pCA variants containing hydroxy and methoxy substituents were also shown to photoisomerize but with slowed kinetics compared to wild-type PYP [21]. Structural and spectroscopic evidence reported recently confirms that a chlorinated chromophore within rsEGFP2 still photoisomerizes from the *cis* to *trans* conformation upon irradiation with blue light [15]. Additionally, photoisomerization still occurs in various point mutants of PYP and rsEGFP2 [47–49], suggesting the chromophore's switching behavior is quite robust. Since the constructs presented in this work retain the ability to photoisomerize upon photon absorption, TR-SFX and UED experiments will be able to provide valuable insights into the photoisomerization mechanism. Even if certain constructs deviate from wild-type behavior, these experiments would reveal information about the factors that influence a chromophore's response to light.

3. Conclusions

In this work, we have successfully produced five new protein variants of PYP and rsEGFP2 in high yields that contain heavy atoms in and around the chromophore. High resolution x-ray structures of each protein show minimal structural perturbation due to these heavy atoms. Photophysical characterization highlights the effect of heavy-atom size, location, and identity on different light-induced processes, although none of the perturbations studied are large enough to eliminate the photocycle. The heavy-atom-containing protein variants presented here are ideal samples for elucidating important mechanistic information of chromophore photoisomerization within a protein environment using time-resolved x-ray crystallography and ultrafast electron diffraction.

4. Materials and Methods

4.1 Plasmid Construction

The gene for PYP from *Halorhodospira halophila* was generously provided by Professor Marius Schmidt at University of Wisconsin Milwaukee and cloned into pET-15b at the NdeI and XhoI restriction sites. The pUltra plasmid encoding the amber suppression machinery for incorporation of *para*-iodophenylalanine (originally developed for *para*-cyanophenylalanine, pUltra-CNF) was acquired from Addgene [29]. The pET-15b plasmid containing the gene for wild-type rsEGFP2 was prepared as previously described [15]. The aminoacyl-tRNA synthetase for incorporation of 3-chlorotyrosine [50] was transferred into the pUltra plasmid [51] using NEBuilder HiFi DNA assembly (New England Biolabs) to improve incorporation efficiencies for the double rsEGFP2 mutant. Point mutations were made using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's protocol.

4.2 Synthesis of 3-Br-pCA

201 mg (1 mmol) 3-bromo-4-hydroxybenzaldehyde (Sigma-Aldrich) and 229 mg (2.2 mmol) malonic acid (Sigma-Aldrich) was added to a 10 mL round-bottom flask containing a stir bar. 450 μ L pyridine (J.T. Baker) and 18 μ L piperidine (Sigma-Aldrich) was then added with stirring. The flask was submerged in a water bath held at 80°C and refluxed for 5 hours. The flask was then cooled to room temperature before dropwise addition of 9 mL 2 M hydrochloric acid to produce a white precipitate. The solid was isolated with vacuum filtration, washed with additional 2 M hydrochloric acid, and dried completely under vacuum, producing 181 mg of the desired 3-Br-pCA product (75% yield). Any residual water in the product will prevent proper subsequent chromophore functionalization. LC-MS and ^1H NMR data confirmed the identity and purity (Fig. S1). The residual pyridinium salt detected in the ^1H NMR spectrum does not interfere with any downstream reactions.

4.3 Functionalization of pCA and 3-Br-pCA

To facilitate incorporation into the PYP protein, pCA and 3-Br-pCA must be functionalized into an acid anhydride. The functionalization should be performed the same day as chromophore incorporation to minimize hydrolysis. 82 mg pCA (Sigma-Aldrich) or 122 mg 3-Br-pCA (0.5 mmol) was added to a dry round-bottom flask with a stir bar along with 91 mg (0.56 mmol) carbonyldiimidazole (Sigma-Aldrich). The flask was flushed with argon before addition of 12 mL dry tetrahydrofuran (Sigma-Aldrich). The mixture was stirred for 1 hour at room temperature under argon, then the solvent was removed with rotary evaporation to yield a yellow solid. The flask with the functionalized chromophore was then used during the protein purification process as described below.

4.4 Protein Expression

All proteins (wild-type PYP, PYP 3-Br-pCA, PYP F96(4-IF), PYP 3-Br-pCA F96(4-IF), wild-type rsEGFP2, rsEGFP2 Y67(3-CIY), and rsEGFP2 Y67(3-CIY) Y107(3-CIY)) were expressed using a similar protocol with minor variations, except for PYP C69U which is described separately below. The pET-15b plasmid containing the gene of interest was transformed into chemically competent BL21(DE3) *Escherichia coli* cells (Invitrogen). For proteins requiring noncanonical amino acid incorporation, the relevant pUltra plasmid was co-transformed into the BL21(DE3) cells. The appropriate antibiotics were purchased from Sigma-Aldrich and used at each expression step at the following concentrations: ampicillin (100 mg/L, for pET-15b, all proteins) and spectinomycin (50 mg/L, for pUltra, only for noncanonical amino acid incorporation). Single colonies were used to inoculate 10 mL of LB Miller (25 g/L). After overnight incubation at 37°C with shaking at 200 rpm, the starter cultures were used to inoculate 1 L of modified Terrific Broth (47.6 g/L supplemented with 8 mL/L glycerol, Fisher) at a 1:100 dilution in a 3 L baffled flask. For proteins requiring noncanonical amino acid incorporation, the relevant noncanonical amino acid (3-CIY or 4-IF, Chem-Impex) was added to the media to a concentration of 0.5 mM prior to autoclaving. The flasks were shaken at 200 rpm and 37°C until an OD_{600} of 0.6–0.8 at which point they were cooled to room temperature on ice. Protein expression was then induced with 0.24 g/L of isopropyl β -D-1-thiogalactopyranoside (IPTG, ChemCruz), and the flasks were shaken at 200 rpm and 20°C overnight.

For incorporation of selenocysteine at residue 69 of PYP, we followed the protocol as previously described [32,33]. This protocol results in global incorporation of selenocysteine at native cysteine residues. Since PYP has only a single cysteine (C69, responsible for conjugation to the pCA chromophore), this protocol yields the single point mutant: PYP C69U. Note that an amber suppression system with an aminoacyl tRNA synthetase/tRNA pair has been evolved for site-specific incorporation of selenocysteine [52]. Since PYP only contains a single cysteine, we chose the global incorporation approach described below, although amber suppression could have also led to a similar result.

The cysteine auxotroph *E. coli* strain (BL21(DE3) selB::kan cys51e) was kindly provided by Dr. Marie-Paule Strub at the NIH. Recipes for the defined growth media and the defined expression media are described in Table S1. The auxotrophic behavior of BL21(DE3) selB::kan cys51e was tested by growing the cells in defined growth media containing 25 mg/L kanamycin with and without cysteine. As expected, no growth was observed without cysteine. The auxotrophic BL21(DE3) selB::kan cys51e cells were made chemically competent in CaCl₂ according to standard protocols (New England Biolabs).

After transformation with the pET-15b plasmid containing the PYP gene and selection on plates made from the defined growth media, a single colony was picked and grown overnight to saturation in 10 mL of defined growth media. 1 L of defined growth media containing ampicillin (100 mg/L) and kanamycin (25 mg/L) was inoculated with the overnight starter culture, and the flask was shaken at 200 rpm and 37°C until an OD₆₀₀ of ~1.2 was reached. IPTG was added to 1 mM. After 5 minutes of shaking, 10 mg/L chloramphenicol (Sigma-Aldrich) was added. After another 5 minutes of shaking, the cells were pelleted at 5000 rcf for 15 minutes at 25°C. The cell pellet was resuspended in a wash buffer containing 10 g/L potassium monohydrogen phosphate (Sigma-Aldrich) and 1 g/L sodium acetate (Sigma-Aldrich). The cells were then pelleted again, the supernatant was removed, and this cycle was repeated for a total of 3 washes. Finally, the cells were resuspended in the defined expression media, which contains 100 mg/L of selenocysteine (Sigma-Aldrich) added fresh. The flask was then shaken for 30 minutes at 30°C before the addition of IPTG to 1 mM. Protein expression then continued overnight with shaking at 200 rpm and 30°C. Over time, the cells turn a deep orange-red color, likely from the production of elemental selenium, Se⁰ [53].

4.5 Protein Purification

After overnight expression, cells were pelleted at 5000 rcf for 30 minutes and then resuspended in a buffer containing 50 mM Tris-HCl (Fisher) at pH 8.0 and 250 mM NaCl (Fisher). Cells were lysed at high pressure with a homogenizer (Avestin EmulsiFlex-C3), and the lysate was centrifuged for two rounds of 90 minutes each at 25000 rcf and 4°C. The supernatant was filtered through a 0.45 µm Millex-HV syringe filter (Millipore).

At this stage, for all PYP proteins, either the pCA or the 3-Br-pCA chromophore was incorporated by adding the filtered supernatant to the flask containing the solid functionalized chromophore and stirring at room temperature for at least 1 hour. This step could also be performed at 4°C overnight. The mixture was then centrifuged at 25000 rcf to remove residual solid.

The supernatant (after chromophore incorporation for PYP proteins or after lysing for rsEGFP2 proteins) was then loaded onto a column of Ni-NTA agarose resin (QIAGEN) equilibrated with the Tris buffer described above. The column was washed with 2 column volumes of Tris buffer containing 20 mM imidazole (Sigma-Aldrich), and then the protein was eluted with Tris buffer containing 200 mM imidazole. The eluted proteins were exchanged into Buffer A (20 mM Tris-HCl at pH 8.0, 10 mM NaCl) with spin filtration (10K Amicon Ultra-15 Centrifugal Filters, Millipore) for purification via anion-exchange chromatography (HiTrap 5 mL Q HP, GE Healthcare) with a gradient of Buffer A and Buffer B (20 mM Tris-HCl at pH 8.0, 1 M NaCl). The final purified proteins were stored in a buffer containing 50 mM Tris-HCl at pH 8.0 and 250 mM NaCl at 4°C. The identity and purity of all proteins were confirmed with electrospray ionization mass spectrometry (ESI-MS) measured with LC-MS (Waters 2795 HPLC with ZQ single quadrupole MS in Stanford University Mass Spectrometry (SUMS) facility). The expected and observed masses are summarized in Table S2.

The incorporation of 3-Br-pCA and selenocysteine was further characterized using x-ray fluorescence spectroscopy on crystalline samples with excitation at the K-edge. Figure S2 confirms the presence of selenium and bromine in PYP C69U and PYP 3-Br-pCA F96(4-IF), respectively, compared to a GFP control protein without heavy atoms based on matching peaks to reported values [54].

4.6 UV-vis Absorption Measurements

UV-vis absorption measurements for each protein were performed with a PerkinElmer 365 UV-vis spectrophotometer. Data were obtained every 1.0 nm with a scan rate of 480 nm/min. The slit width was set to 1.0 nm. For the photocycle testing and thermal relaxation experiments, samples were diluted to 2 μ M with a buffer containing 50 mM Tris-HCl at pH 8.0 and 250 mM NaCl. A 27 mW 488 nm diode laser (85-BCD-030-115, Melles Griot) was used to irradiate the continuously stirring sample until a photostationary state had been reached as determined by a plateau of the peak absorption as a function of irradiation time. The cuvette was then transferred to a PerkinElmer Lambda 25 UV-Vis spectrometer to monitor the thermal relaxation kinetics in the dark. Absorption at the room temperature peak maximum of each protein variant was monitored every 1.0 s. The time evolution of peak absorbance for each construct was fit to an exponential to extract the thermal relaxation rate k .

$$A(t) = A_0 e^{-kt} + C \quad (1)$$

Applying transition state theory to k yields:

$$k = \frac{k_B T}{h} \kappa \exp\left(-\frac{\Delta G^\ddagger}{RT}\right) \quad (2)$$

where k_B is the Boltzmann constant, T is the temperature, h is Planck's constant, κ is the transmission coefficient assumed to equal 1 (one-time barrier crossing), R is the gas constant, and $\Delta G^\ddagger$ is the desired energy barrier height for thermal relaxation.

4.7 Fluorescence Quantum Yield Determination

The absolute fluorescence quantum yield for each protein was determined as previously described [30,55].

4.8 Protein Crystallization

Prior to protein crystallization of all PYP proteins, the His tag was cleaved using 2 U thrombin per mg PYP for 4 hours at room temperature. Crystal formation of PYP proteins with the His tag still attached was observed to be substantially hindered. The proteins were then purified again using anion exchange chromatography as described above, exchanged into buffer containing 20 mM potassium phosphate pH 6.0 (Sigma-Aldrich), and concentrated to 20 mg/mL. Crystals were grown using the hanging drop method in a 24-well VDX plate with sealant (Hampton Research) and 22 mm plastic cover slips (Ted Pella) using a mother liquor of 20 mM potassium phosphate pH 6.0, 1 M NaCl, and 1.9 M ammonium sulfate (Sigma-Aldrich). 1 μ L of the protein solution was mixed with 1 μ L of mother liquor on the plastic slide, which was then sealed above a well containing 500 μ L of mother liquor. The sample within the sealed well was incubated overnight at room temperature in the dark. The next day, each drop was streak seeded with a cat whisker using various dilutions of crushed wild-type PYP crystals. For any subsequent rounds of crystal growth, we used crushed crystal seeds of the desired PYP protein rather than wild-type PYP. Crystals were visible a few hours after the forced nucleation and continued to grow for one day. Crystals were looped, dipped into perfluoropolyether oil (Hampton Research), and flash cooled in liquid nitrogen.

rsEGFP2 Y67(3-CIY) Y107(3-CIY) was exchanged into buffer containing 50 mM HEPES (Sigma-Aldrich) pH 7.5 and concentrated to 12 mg/mL. Hanging drop trays were prepared in the same way as described above but with a mother liquor of 0.08 M HEPES pH 8.1 and 1.84 M ammonium sulfate. Seeding was not required. Crystals formed within a week at room temperature in the dark. They were looped, dipped into a cryoprotectant containing 0.1 M HEPES pH 8.1, 1.7 M ammonium sulfate, and 1 M sucrose (Sigma-Aldrich), and then flash cooled in liquid nitrogen.

4.9 Diffraction Data Collection and Structure Determination

The x-ray diffraction data for all proteins were collected at 100 K at the Stanford Synchrotron Radiation Lightsource (Menlo Park, CA) at BL 9–2, 12–2, and 14–1 (Table S3). To collect diffraction spots with the highest possible resolution for PYPs, the x-ray source was tuned to the shortest available wavelength at each beamline, and the detector was offset from the center to the furthest possible distance from the mounted crystals (Table S3). Owing to the high space-group symmetry of PYP crystals, data completeness remains excellent with the off-center detector configuration. Given the high dynamic range of the PILATUS 6M PAD detector, no overexposure was observed from the low-resolution diffraction spots.

The data were indexed, reduced, and scaled using X-ray Detector Software (XDS) [56,57] using the autoxds script [58]. The structures were solved by molecular replacement with PHENIX [59] using the coordinates from wild-type PYP (for the PYP proteins, PDB ID:

1NWZ [28]) and wild-type rsEGFP2 (for rsEGFP2 Y67(3-CIY) Y107(3-CIY), PDB ID: 5DTX [37]). The chromophore restraint file for the 3-Br pCA was built using REEL and eLBOW in PHENIX. Numerous rounds of model building and refinement were performed with Coot [60] and PHENIX until the R-free score converged. The resulting data collection and refinement statistics along with PDB ID codes are summarized in Table S3.

4.10 Accession Numbers

Protein structures and associated diffraction data have been deposited to the Protein Data Bank under the following accession numbers: PYP 3-Br-pCA, PDB ID: **6UMZ**; PYP F96(4-IF), PDB ID: **6UMY**; PYP 3-Br-pCA F96(4-IF), PDB ID: **6UN0**; PYP C69U, PDB ID: **6UN2**; rsEGFP2 Y67(3-CIY) Y107(3-CIY), PDB ID: **6UN4**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Use of SSRL, SLAC National Accelerator Laboratory, is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by the National Institutes of Health, National Institute of General Medical Sciences (including P41GM103393). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of NIGMS or NIH. M.G.R. was supported by a Center for Molecular Analysis and Design graduate fellowship. C.-Y.L. was supported by a Kenneth and Nina Tai Stanford Graduate Fellowship and the Taiwanese Ministry of Education. This work was supported, in part, by NIH Grant GM118044 (to S.G.B.) and NSF CCI Phase I: Center for First Principles Design of Quantum Processes (CHE-1740645).

Abbreviations:

3-Br-pCA	3-bromo- <i>para</i> -coumaric acid
3-CIY	3-chlorotyrosine
4-IF	<i>para</i> -iodophenylalanine
FQY	fluorescence quantum yield
HBI	4-(<i>para</i> -hydroxybenzylidene)-5-imidazolinone
pCA	<i>para</i> -coumaric acid
PYP	photoactive yellow protein
rsEGFP2	reversibly switchable enhanced green fluorescent protein 2
TR-SFX	time-resolved serial femtosecond crystallography
UED	ultrafast electron diffraction

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Highlights

- Photoisomerization converts light energy into motion, but mechanism still debated
- Proteins containing heavy atoms on or near chromophore can be expressed in high yields
- Heavy atoms minimally perturb chromophore's structure and photophysical properties
- Time-resolved crystallography and electron diffraction can elucidate mechanistic information

Fig. 1.

Summary of photoactive yellow protein (PYP) and rsEGFP2 constructs containing heavy atoms. A) Cartoon representation of the tertiary structure of each wild-type protein, highlighting the location and atomic structure of each chromophore and aromatic residue of interest (F96 and Y107 for PYP and rsEGFP2, respectively). The center schematic depicts how a substituent on the phenolate ring of the chromophore breaks the symmetry and allows for the isomerization mechanism to be discerned based on the structure [15]. B) Structures of the chromophores and amino acids containing heavy atoms used in this study.

Fig. 2.

Structure alignment and electron density for the photoactive yellow protein (PYP) constructs. The color code matches that in Figure 1B. Note that all hydrogen atoms have been removed from the models for clarity. PDB IDs are as follows: wild-type PYP: 1NWZ [28], PYP 3-Br-pCA: 6UMZ, PYP F96(4-IF): 6UMY, PYP 3-Br-pCA F96(4-IF): 6UN0, PYP C69U: 6UN2. A) Ribbon structures for each PYP construct showing that the tertiary structure is well conserved. B) The chromophore pocket within PYP, including several nearby residues, remains largely unchanged upon addition of heavy atoms. C) Bromination of the pCA chromophore leads to small deviations from the wild-type structure at residues V57 and V66. D) Iodination of F96 causes a minor structural rearrangement of V122. E) Electron density (gray, $2mF_o - DF_c$ contoured at 1σ) for the pCA chromophore and F96 for each PYP construct clearly shows successful incorporation of the specified heavy atoms. The bottom right figure in gray shows positive electron density (green, $mF_o - DF_c$ contoured at $\pm 3\sigma$) for the PYP C69U construct when using a sulfur-containing model, confirming the presence of the larger selenium atom.

Fig. 3.

Structure alignment and electron density for the rsEGFP2 constructs. The color code matches that in Figure 1B. Note that all hydrogen atoms have been removed from the models for clarity. PDB IDs are as follows: wild-type rsEGFP2: 5DTX [37], rsEGFP2 Y67(3-ClY): 6PFR [15], rsEGFP2 Y67(3-ClY) Y107(3-ClY): 6UN4. A) Ribbon structures for each rsEGFP2 construct showing that the tertiary structure is well conserved. B) The chromophore pocket within rsEGFP2, including several nearby residues, remains largely unchanged upon addition of heavy atoms. The two chlorines on the HBI chromophore represent the two conformations, shown in detail in E. C) Chlorination of the HBI chromophore pushes V151 back by ~1 Å, while D) chlorination of Y107 similarly affects V23. E) The chlorine of the chromophore occupies a single conformation for the Y67(3-ClY) construct, but 10% of the alternative conformation exists in the Y67(3-ClY) Y107(3-ClY) construct. The chlorine of Y107 occupies only a single conformation.

Fig. 4.

Normalized absorption (A) and fluorescence (B) spectra of the photoactive yellow protein (PYP) constructs. Despite substantial signal averaging, the signal-to-noise ratios for the PYP fluorescence spectra are low due to their inherently low fluorescence quantum yields (Table 3). Normalized absorption (C) and fluorescence (D) spectra of the rsEGFP2 constructs. The color coding follows that of Figure 1B.

Figure 5.

Spectroscopic and kinetic evidence for the photocycle in the heavy-atom-containing PYP constructs. A) Absorption spectra depicting the photocycle in PYP 3-BrpCA F96(4-IF). After irradiation with 488 nm light, denoted $h\nu$, the peak at 444 nm decreases in intensity and is replaced by a peak around 350 nm, which corresponds to the photoisomerized protonated state. The magnitude of the absorbance change is proportional to the extinction coefficients of the two peaks. After 15 minutes in the dark, denoted Δ , the spectrum resembles that of the initial state, indicating completion of the photocycle. Note that the irradiated spectrum does not represent the true photostationary state, since thermal relaxation occurs during the measurement. B) Thermal relaxation kinetics for the PYP 3-Br-pCA (dark red), PYP F96(4-IF) (light green), and PYP 3-Br-pCA F96(4-IF) (dark green) constructs. The color coding matches that in Figure 1B. Table S4 contains the thermal relaxation rates which were obtained by fitting the data to Equation 1. The absorbance was measured at the peak maximum for each construct, as listed in Table 3.

Table 1.

Structural properties of PYP constructs. See Table S3 for full x-ray data collection and refinement statistics.

Construct (PDB ID)	Resolution (Å)	RMSD of α -carbons to WT (Å)	Chromophore Dihedral Angle (C ₃ -C ₄ -C ₅ -C ₆)	F96 Dihedral Angle (C _{α} -C _{β} -C _{γ} -C _{δ1})	C ₁ -S/Se Bond Distance (Å)	S/Se-C ₂ (=O) Bond Distance (Å)
WT (1NWZ)	0.82	0	-2.5°	-19.0°	1.80	1.77
3-Br-pCA (6UMZ)	0.90	0.622	3.8°	-22.7°	1.81	1.77
F96(4-IF) (6UMY)	0.92	0.275	-3.2°	-18.2°	1.80	1.78
3-Br-pCA F96(4-IF) (6UN0)	0.85	0.690	1.5°	-21.3°	1.80	1.79
C69U (6UN2)	0.97	0.410	-6.7°	-22.1°	1.93	1.89

Table 2.

Structural properties of rsEGFP2 constructs. See Table S3 for full x-ray data collection and refinement statistics.

Construct (PDB ID)	Resolution (Å)	RMSD of α -carbons to WT (Å)	P-bond Dihedral Angle (C ₁ -C ₂ -C ₃ -C ₄)	I-bond Dihedral Angle (N ₁ -C ₁ -C ₂ -C ₃)	Y107 Dihedral Angle (C _{α} -C _{β} -C γ -C _{δ1})
WT (5DTX)	1.45	0	-3.1°	5.6°	-17.5°
Y67(3-CIY) (6PFR)	1.51	0.767	-12.9°	11.5°	-17.5°
Y67(3-CIY) Y107(3-CIY) (6UN4)	1.50	0.865	10.7°	10.8°	-29.2°

Table 3.

Photophysical properties of PYP and rsEGFP2 constructs. SD: standard deviation.

PYP Constructs	Absorption Maximum (nm)	Fluorescence Emission Maximum (nm)	Fluorescence Quantum Yield (%)	Fluorescence Quantum Yield SD (%)
WT	446	492	0.20	0.02
3-Br-pCA	445	492	0.54	0.04
F96(4-IF)	446	493	0.24	0.03
3-Br-pCA F96(4-IF)	444	493	0.98	0.07
C69U	453	502	0.14	0.03
rsEGFP2 Constructs	Absorption Maximum (nm)	Fluorescence Emission Maximum (nm)	Fluorescence Quantum Yield (%)	Fluorescence Quantum Yield SD (%)
WT	483	501	25.7	0.3
Y67(3-CIY)	481	506	26.5	0.4
Y67(3-CIY) Y107(3-CIY)	481	506	24.4	0.5