Title:

Photocycle characterization of a blue-orange cyanobacteriochrome from *Synechococcus* sp. PCC 7002

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ABSTRACT

Cyanobacteria employ photoreceptors called cyanobacteriochromes (CBCRs) to sense the colour and intensity of light. The information extracted from the solar spectrum is used for adaptive responses such as optimizing photosynthesis, phototaxis and cell aggregation. GAF domains are the principal light sensors in cyanobacteriochromes. They contain a conjugated bilin chromophore and boast an impressive spectral diversity. Characterizing the spectral characteristics of GAF domains in model strains, such as *Synechococcus* sp. PCC 7002, can open new avenues for optogenetics and biotechnology. Based on sequence analysis we predicted several different GAF domains in this strain. The 7GP-03 gene encodes a single GAF domain with two Cys residues: one in the conserved α3 helix; one in the conserved DXCF motif. Spectral analysis of recombinant 7GP-03 with phycocyanobilin (PCB) showed that the protein cycles between two states, Po and Pb, which absorb orange and blue light, respectively. Acid-denaturation analysis showed that the 15E isomer of PCB was bound in the (dark) Po state, whereas 15Z is in the (photoproduct) Pb state. Site-directed mutagenesis of the DXCF motif and iodoacetamide treatments showed that Cys73 in the DXCF motif is essential for the conversion from Po to Pb. The predicted thiol link formation between Cys73 and C10 of PCB is the first sequence of events in the photocycle, followed by PCB isomerization from 15E to 15Z. 7GP-03 is interesting from the perspective of colour perception in *Synechococcus* sp. PCC 7002 but also due to its 15E state being the holoprotein's dark state.

INTRODUCTION

Cyanobacteriochromes (CBCRs) are a family of highly tunable photoreceptors that serve as sensors of light colour and intensity in cyanobacteria (Rockwell et al., 2012). In their simplest form, CBCRs consist of a single *cGMP-phosphodiesterase/adenylate cyclase/FhlA* (GAF) domain (Rockwell et al., 2015). However, most putative CBCRs are multidomain proteins consisting of several GAFs and other sensory/effector domains, such as the Per-Arnt-Sim (PAS) and histidine kinase (HisK). While several GAF domains and signaling pathways have been characterized (Bezy and Kehoe, 2010; Bhaya, 2016; Hirose et al., 2010, 2013a; Kehoe and Gutu, 2006) the full range of photo-perception mechanisms and signaling targets in cyanobacteria remain to be explored.

The GAF domain is a small globular structure of about 19 kDa. GAF domains can be evolutionarily adapted to sense a variety of environmental parameters (Aravind and Ponting, 1997; Ho et al., 2000; Schultz, 2009), which explains their great ubiquity in nature. CBCR GAF domains usually contain a covalently attached phycocyanobilin (PCB) molecule (Burgie and Vierstra, 2014; Ikeuchi and Ishizuka, 2008; Rockwell et al., 2011, 2013a). The natural spectral properties of the bilin are further tuned by the protein environment and/or by isomerisation (Ishizuka *et al.*, 2011; Rockwell *et al.*, 2011; Chen *et al.*, 2012; Rockwell et al., 2013; Yuu Hirose *et al.*, 2013; Fushimi *et al.*, 2020). The result is a myriad of unique spectral sensitivities: CBCR GAF domains can sense spectral colours in the UV, visible and far-red regions (Fushimi and Narikawa, 2019). Both CBCRs and phytochromes make use of the GAF domain (Rockwell and Lagarias, 2010). These domains cycle between a P_1 and a P_2 spectral state. In doing so, CBCR GAF domains tend to measure the ratios of two different colors of light. Unlike phytochromes, CBCRs are not restricted to red/far red-absorbing states (Pr and Pfr). The transition between the two holoprotein states is founded upon rotation of the fourth chromophore ring (Chen et al., 2012; Rockwell et al., 2013b; van Thor et al., 2006). Depending on the extent of this rotation around C15, the chromophore itself cycles between a 15Z and a 15E isomer, each being associated with one of the holoprotein P states. In solution, the 15Z state is the more stable of the two (Fushimi et al., 2016). In all currently known CBCRs, the 15Z state of the chromophore seems to be associated with the dark/ground state of the holoprotein. We define the term "chromotype" to denote the specific combination of spectral colours sensed by a GAF domain, relative to the 15Z/15E chromophore state. For example, a GAF domain with a Pr (15Z) and Pg (15E) configuration will have a Red/Green (R/G) chromotype. Similarly, a Pb (15Z) and Po (15E) GAF domain will have a Blue/Orange (B/O) chromotype.

There is great evolutionary value in measuring the ratios of spectral colours in the environment. These ratios result in a mixed population of P_1 and P_2 states, depending on their sensitivity and dark reversion kinetics. Cyanobacteria make use of this readout to tell the time, depth in water column, and presence of other (photosynthetic) organisms. Some of the major responses elicited by CBCRs are: 1) chromatic acclimation, 2) phototaxis and 3) cell aggregation. Chromatic acclimation is the process of adapting the type and abundance of light-harvesting pigments to make better use of the current light environment (Kehoe and Gutu, 2006). The classic example is the control of ratio of phycocyanin (PC) and phycoerythrin (PE) in *Fremyella diplosiphon* depending on the ratio of green to red light. This process is partially controlled by the RcaE CBCR in a transcriptional manner (Bezy and Kehoe, 2010). Phototaxis is the process of moving towards or away from a specific light stimulus. The example is the phototactic motion of *Synechocystis* sp. PCC 6803 in response to blue light (Yoshihara and Ikeuchi, 2004). In this example the

process is at least partially controlled by the PixJ CBCR, possibly by means of dimerization and interaction with the pili on the membrane. Cell aggregation, biofilm formation and buoyancy changes are all under the control of c-di-GMP levels in *Synechocystis* sp. PCC 6803 (Agostoni et al., 2016). The c-di-GMP levels are linked with the activity of a cellulose synthase, which affects biofilm accumulation (Kawano et al., 2011). This process has been tied to blue light (Agostoni et al., 2013), possibly by an unknown CBCR. The c-di-GMP levels are known to be boosted by GGDEF domains (Galperin et al., 2001; Romling et al., 2013) and diminished by EAL domains (Dow et al., 2006), both of which are common in CBCR proteins. Possible crosstalk between CBCR and other physiological or developmental responses pathways remains to be elucidated.

In addition to the fundamental knowledge gained from studying the physiological roles of CBCR signaling mechanisms, the spectral characterization of CBCRs has potential for applications in biotechnology of cyanobacteria. *Synechococcus* sp. PCC 7002 is already a well characterized, transformable metabolic model organism and a potential chassis for biotechnology. Currently, nothing is known about the spectral properties of its CBCRs. Charting the organism's responses to different combinations of colours could allow the organism's metabolism to be controlled without chemical additives by tapping into existing pathways or engineering new ones. The first step is identifying putative CBCRs and characterizing them. Here we describe the identification and characterization of a novel single-GAF domain encoding gene from *Synechococcus* sp. PCC 7002. We show that 7GP-03 is a Blue-Orange GAF domain with an unusual photocycle where the 15E state is the dark state and 15Z is the photoproduct.

RESULTS

Recombinant 7GP-03 is a blue and orange sensor

A BLAST-P search was performed using the GAF domain amino acid sequence from the CBCR PixJ from *Themosynechococcus elongatus*. The results listed 16 putative proteins from the genome of *Synechococus* sp. PCC 7002 containing at least one GAF domain (Figure 1). Only three GAF domains from these putative proteins exhibited high sequence similarity to known CBCRs: 7GP-01g, 7GP-03g, and 7GP-04g. 7GP-01g is likely a member of the well-studied Red-Green (RG) family. 7GP-03g is likely a member of the underrepresented Blue-Orange (BO) family. 7GP-04g is a predicted member of the CikA (CA) family. Both CA and RG GAF domains seem to be linked with HisK or HAMP-MCP effector domains. The CA family members are currently known to only absorb blue light and have been linked with the cell clock (Schmitz et al., 2000). The RG family is spectrally diverse. Not much is known about the photocycle mechanism of BO GAF domains, all of which currently have the B/O chromotype and occur as single domain proteins. Here, 7GP-03 was selected for experimental characterization in the interest of learning more about BO CBCRs.

To assess the spectral properties of 7GP-03 recombinant protein was generated. Initial light treatments were used to monitor photocycling in 7GP-03 and the first observations indicated that the protein was reversibly photochromic (Figure 2a). The recombinant protein eluted from the Ni^{2+} resin as a deep blue solution (ground/dark state) at room temperature. The colour changed from blue to green (photoproduct)

when exposed to a high intensity halogen bulb through a yellow filter (for filter specifications see Suppl. Figure 2). The photoproduct seemed stable under ambient light conditions and temperature. Reversion to the blue/dark state was achieved by replacing the yellow filter with a blue filter (Suppl. Figure 2) or by subjecting the protein to darkness at 4 °C overnight. Absorbance spectroscopy was employed to further investigate the different protein states (Figure 2b,c). The results indicate that the dark state has a maximal absorbance (λ_{max}) around 600 nm, which corresponds to monochromatic yellow/orange (orange hereafter) (Figure 2 d,e). The photoproduct state has a λ_{max} at 440 nm which falls within the blue spectral region (Figure 2 d,e). In the following, the two states are referred to as Po (orange absorbing dark state) and Pb (blue absorbing photoproduct state), respectively.

The dark (Po) state contains 15E PCB

Acid-denaturation spectroscopy was used to interrogate the chromophore species and configurations in the Po and Pb states (Figure 3). The assay involves denaturing the protein allowing direct spectral observation of the chromophore and assigning C15 double-bond configurations (15Z/15E). Under blue light the absorbance spectrum of the denatured protein was reminiscent of the native protein in Po with two absorbance peaks at 358 and 580 nm. Subsequent exposure of the denatured Po sample to white light shifted the orange light absorption from 580 nm to red at 666 nm. This observation is indicative of PCB relaxation from 15E to 15Z as inferred by acid-denaturation assays from other CBCRs (Fushimi et al., 2016). Under orange light the protein is in the Pb state. The denatured Pb protein showed the same absorbance spectrum as the denatured Po sample following white light illumination. The spectrum of the denatured Pb sample did not change upon subsequent exposure to white light. These results indicate that the Po state contains phycocyanobilin as 15E isomer (15E PCB), whereas the Pb state contains 15Z PCB. The data from the deconvoluted peaks is shown in Suppl. Figure 3. The chromotype of 7GP-03, expressed as the 15Z/15E colour combination, is therefore blue/orange (B/O). This is in accord with other CBCRs from the same gene family. However, 7GP-03 is unusual insofar as the Po (15E) state is the dark state of the holoprotein. In solution, the 15E isomer of PCB eventually decays into the 15Z isomer (van Thor et al., 2006) and accordingly the dark states of all CBCRs characterized to date, including other members of the BO family, contain 15Z PCB. The novel result for 7GP-03 suggests the presence of one or more stable bonds between of 7GP-03 and the chromophore in the 15E state.

Cysteine 73 is required for Po to Pb transition

Blue light absorption in photoreceptors is known to be achieved by means of a Cys residue interacting with the conjugated system of the chromophore, forming a thiol bridge to C10 of the bilin (Rockwell, 2015). 7GP-03 contains only two cysteine residues. One, Cys101, is in a conserved position within helix α3. This residue is generally associated with constitutively binding to PCB at ring A (Ikeuchi and Ishizuka, 2008). The second residue, Cys73, is located within the DXCF motif and is suspected to yield the blue light sensitivity of the Pb state. Recombinant C73S and C73A protein was generated as before and both proteins eluted as purple solutions. Absorbance spectroscopy of the mutant proteins (Figure 4) indicated a Po dark state that

cannot convert to Pb. The results from the deconvoluted peaks are shown in Suppl. Figure 4. The results indicate that Cys73 is essential for the transition between photostates. However, Cys73 offers no explanation to why the dark state of 7GP-03 is 15E as opposed to 15Z in all other proteins of the Blue-Orange family: all other B/O CBCRs have a cysteine in this position.

Cys73-C10 link is the first event in the photocycle

B/O CBCRs contain the DXCF motif. The 15Z/15E interconversion is thought of as the main and first photocycle event in DXCF CBCRs, followed by the formation of a second Cys linkage at C10 of PCB (Hardman et al., 2020).The exact sequence of transitions in the photocycle of a B/O CBCR remains to be fully characterized. To better understand the photocycle order of events in 7GP-03 we applied acid-denaturation spectroscopy to the mutant proteins 7GP-03_C73S and 7GP-03_C73A (Figure 5). The results from the peak deconvolution are shown in Suppl. Figure 5. The inability of the mutants to form the Pb state (shown in Figure 4,5) is now accompanied by the denatured protein shifting its absorption from orange to red upon white light treatment. The results of this experiment indicate that PCB remains as the 15E isomer in both mutants of the protein, meaning that removal of Cys73 abolishes the ability of the chromophore to isomerize. Thus, formation of the Cys73-C10 link is likely to be the first event of the photocycle and needed to stabilize or facilitate the first event the ring rotation.

To interrogate this hypothesis in the wild-type protein, we used a pharmacological approach. Iodoacetamide (IAM) alkylates any free Cys residues and therefore prevents formation of thiol bridges with other residues (Gurd, 1972). This has been used with CBCRs before (Enomoto et al., 2012; Narikawa et al., 2014). Absorbance spectroscopy showed that treatment with IAM decreased the ability of the proteins to transition from Po to Pb (Figure 6), again suggesting that coupling to the Cys residues are required for the first event in the photocycle. The deconvoluted peak data from this experiment is shown in Suppl. Figure 6. Acid-denaturation spectroscopy was performed on IAM-treated 7GP-03 (Figure 7a). The control experiment (Figure 7b) contained the Po form of 7GP-03, treated with IAM and subsequently denatured with urea. The peak deconvolution is shown in Suppl. Figure 7. The lack of significant relaxation of 15E PCB to 15Z in the control indicated the presence of the 15E isomer in most of the proteins and ruled out any unexpected effect from IAM. Interestingly, the Po to Pb transition was mostly associated with 15Z PCB in the presence of IAM. In contrast, during the equivalent experiment with Cys73 mutants the chromohphore remained in the 15E state. It seems that the presence of the Cysteine alone is what correlates with 15E -> 15Z transition. This indicates that ring D rotation may be facilitated by bending of the chromophore due to Cys73's presence (and size) more than its reactivity. A similar effect was seen in denatured 7GP-03_C73S (Figure 5).

DISCUSSION

In this study we characterized a novel photoreceptor of the cyanobacterium *Synechococcus* sp. PCC 7002 based on the identification of a gene encoding a single GAF domain, named here 7GP-03. We characterized the photocycling properties of recombinant 7GP-03-PCB holoprotein using absorbance spectroscopy, site-directed mutagenesis, and pharmacological treatments. The results show that 7GP-03 is a reversibly photochromic CBCR with a blue/orange (B/O) chromotype (expressed as the colour sensitivity in 15Z/15E forms). Akin to all other CBCR GAF domains of the Blue-Orange family, 7GP-03 occurs in two states, Pb and a Po. A unique feature of 7GP-03, different to all previously characterized CBCRs, including Blue-Orange ones, is that the dark (ground) state of the holoprotein contains PCB as the 15E isomer. In the Blue-Orange family of CBCRs the Po state also contains 15E PCB, just like in 7GP-03. However, the Po state has not yet been observed as the dark state of the holoprotein. Site-directed mutagenesis showed that blue light sensitivity of the Pb state is linked with the activity of Cys73 in the DXCF motif as is the case for other CBCRs with similar wavelength sensitivity and the same motif. Additional pharmacological treatments and mutagenesis studies indicated that thiol bridge formation between Cys73-C10 of PCB in the Pb state does not explain why the dark state (Po) is the more energetically favorable despite containing 15E PCB and no thiol bridge. The combined results suggest that additional residues and/or bonds stabilize this isomer in Po of 7GP-03.

The relative heights and positions of the absorbance spectra of 7GP-03 (439 nm, 604 nm) are almost identical to those of NpF4973g (434 nm, 602 nm). NpF4973g from *Nostoc punctiforme* is a member of the BO family thus also having a Po and Pb state (Rockwell et al, 2015). Similarly, the Po state also contains 15E PCB and the Pb state contains 15Z PCB with a thiol link. In the case of NpF4973g the Pb state is the dark state of the holoprotein. The two proteins have over 80% amino acid sequence similarity. Most of the difference is due to the N-terminal extension in NpF4973g, which is unlikely to participate in the photocycle. If this region is excluded from the analysis, the sequence similarity is 92%. The remaining 8% difference probably accounts for the opposing energy landscape of the Po states. Fushimi *et al.* (2020) identified six key residues in the binding pocket of GAF domains with an effect on the chromotype. The positions of these key residues can be mapped to all currently characterized CBCR GAF domains. 7GP-03 is unique among all currently characterized GAF domains in having an aspartate residue (Asp 114) in one of these six key positions (Asn 109 equivalent in NpF4973g and Asn 109 TePixJg). The net negative charge of the aspartic acid under physiological conditions may create a charge attraction with the fourth ring of PCB, thus stabilizing the 15E isomer in Po. In turn, this may explain why the interaction between the insert Cys residue and C10 of PCB is the required for first event in the photocycle to occur. Additional site-directed mutagenesis in both proteins should be carried out in the future to test this hypothesis.

At this stage, the data presented here form the basis of a photocycle model for 7GP-03 which is similar in other BO CBCRs (Figure 8). Orange light of about 600 nm is absorbed by the Po state. An interaction between Cys73 and C10 of PCB is followed, resulting in the formation of a thiol bridge. This event probably causes changes in the shape of the bilin and the binding pocket of the protein, resulting in rotation of the fourth ring and isomerization 15E->15Z. The resulting Pb state has been observed to be quite stable with a half-life of about 4 days. This can be explained by it already having a 15Z PCB.

Further interrogation of the unusual 7GP-03 could reveal the basis for its unusual energy landscape and lead to its potential physiological function and signaling pathway.

METHODS

Strains and plasmids

Synechococcus sp. PCC 7002 was obtained from the Pasteur Culture Collection (PCC). It was kept as DMSO-cryopreserved stocks used as inoculum for working cultures. The pET21c-GPR-EGFP vector was provided by Dr Ross Eaglesfield (University of Glasgow). The pACYC-ho1-pcyA vector was a gift from Prof. Kai Hong Zhao (Huazhong Agricultural University). pACYC-ho1-pcyA provides the machinery for *E. coli* cells to produce the phycocyanobilin (PCB) chromophore which self-ligates into CBCRs.

Molecular cloning

Genomic (gDNA) was extracted from 250 µl of cell pellet of *Synechococcus* sp. PCC 7002 grown in liquid culture (true OD_{730} = 1-3) according was to the method for cyanobacterial gDNA extraction from Tamagnini et al. (1997).

7GP-03 without the endogenous STOP codon was amplified from *Synechococcus* sp. PCC 7002 gDNA by PCR using the 7GP-03-FULL-F6/R4 primers and Phusion DNA polymerase (NEB). The pET21c-GPR-EGFP vector was PCR-linearized with pET-F/R and pre-digested with HindIII-HF and XhoI (NEB). The insert was ligated to the vector in a 3:1 ratio (insert:vector) using T4 ligase (NEB). This introduced C-terminal 6xHis tag to 7GP-03. The pET-7GP-03-6His vector was transformed into and selected from One Shot Top 10 competent cells (Invitrogen). Plasmid minipreps were done using the QIAprep Spin Miniprep Kit (QUIAGEN).

Site-Dircted Mutagenesis (SDM) was performed by subjecting the pET-7GP-03-6His vector prep to PCR with 7GP-03-C73S/A-F/R primers. 1 uL of DpnI (NEB) was added directly to the PCR reactions for 1 hr at 37°C, followed by repeating this step once more. The product was assessed on agarose gel electrophoresis and transformed into TOP10 competent cells as described above, followed by plasmid miniprep with QIAprep Spin Miniprep Kit (QUIAGEN).

Plasmid minipreps were sequenced by first being PCR-amplified using the T7-Prom-F/T7-Term-R primers and cleaned up using the ExoSAP-IT PCR Product Cleanup kit (Thermo Fisher, 78200). Sanger sequencing was then carried out by SourceBioscience for verification.

All commercial materials were used according to the manufacturers' instructions.

Expression and purification of recombinant protein

Recombinant 7GP-03 protein was made by first co-transforming pET-7GP-03-6His and pACYC-ho1-pcyA into competent BL21 Star DE3 cells (Invitrogen) and selecting transformants with the relevant antibiotics. Two 500 mL cultures of LB medium were then inoculated with the double-transformed cells in 2L conical flasks in the presence of the relevant antibiotic selection. These were grown at 37°C and 180 rpm until OD_{600} = 0.6. The cultures were then cooled to 20°C and induced with 0.5 mM IPTG overnight. The pellets were harvested on the following day and suspended in ice-cold 50 mM Tris-Cl (pH 8.0), 500 mM NaCl, 10-20 mM imidazole, 10% (v/v) glycerol, 0.1%(v/v) Triton X-100 in addition to DNase (0.04 u/mL) and protease inhibitor cocktail. The cells were then lysed on ice using a sonicator and centrifuged at 4 °C and 16 000 x g for 20 min. The supernatant was loaded onto a pre-equilibrated Ni-affinity column. The 3 mL resin was then washed with 200 mL 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 10-20 mM imidazole, 10% (v/v) Triton X-100. The protein was subsequently eluted with 8 mL 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 200 mM imidazole, 10% (v/v) glycerol. The selected fractions were pooled together and dialyzed twice at 4oC overnight using a 5000 MW cutoff tubing in 5L 50 mM Tris-Cl (pH 8.0), 150 mM NaCl. The protein was concentrated to 2 mL. Successful generation of recombinant 7GP-03 was verified with Zn^{2+} fluorescence and Western blot (Suppl. Figure 1).

SDS-PAGE, Western blot, Zinc assay

Protein samples were mixed 1:1 (v/v) with 100 mM Tris-Cl (pH 6.8), 4% (w/v) Sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 3 mg/mL bromophenol blue. The samples were then boiled for 15 min before being subjected to SDS-PAGE using 15% (v/v) acrylamide, 375 mM Tris-Cl (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate (APS) at 140 V (DC) for 1-2 hours. The bands were visualized by soaking into Coomassie blue, followed by de-staining with dH2O. Gels for Western blotting/Zinc assays were not stained. Instead, the bands were transferred onto a nitrocellulose membrane using the dry blotting method for 1 h at 10 V (DC). The membrane was hen incubated with blocking solution containing 5% (w/v) milk powder in TBST containing 137 mM NaCl, 2.7 mM KCl, 19 mM Tris-Cl (pH 7.4), 0.1% (v/v) Tween 20. After 1 h the membrane was aspirated and a solution of monoclonal α-6xHis antibody (Cell Signalling, 2366) was prepared according to the manufacturer's instructions, suspended in blocking solution. The antibody was incubated with the membrane at 4°C overnight. The membrane was then washed three times with blocking solution before being incubated with an α-mouse IgG conjugated to horseradish peroxidase (HRP) (Cell Signalling, 7076) in the same way. The membrane was then washed three times with TBST and the bands were visualized using a chemiluminescent kit for HRP (Thermo Scientific, 34075) according to the manufacturer's instructions. Zinc assays were done according to the method in Kuwasaki *et al*. (2019). This involves submerging the nitrocellulose membranes in a solution of $ZnCl_2$ for 30 min, followed by visualization under UV light.

Spectrometry

Absorbance spectra were taken on a Perkin Elmer Lambda 40 UV/Vis spectrometer. The slit size was set to 0.5 nm. The scanning speed used was 960 nm/min. The UV lamp change was at 326 nm. The resolution was 1 nm. A quartz cuvette was used for all measurements. The protein concentration for most experiments was 0.5 mg/mL. Light treatments were done by adjusting the distance to the halogen lamp (after adding the colour filter) so that the intensity at the level of the sample was always 1000 µmol m^2 s^{-1}.

Data analysis

Peak deconstruction was done by importing the spectral data into Igor Pro 8.4 (WaveMetrics) and using the binomial smoothing function (set to 100), followed by the Multipeak Fittin 2 algorithm.

REFERENCES

Agostoni, M., Koestler, B.J., Waters, C.M., Williams, B.L., and Montgomery, B.L. (2013). Occurrence of cyclic di-GMP-modulating output domains in cyanobacteria: An illuminating perspective. MBio *4*.

Agostoni, M., Waters, C.M., and Montgomery, B.L. (2016). Regulation of biofilm formation and cellular buoyancy through modulating intracellular cyclic di-GMP levels in engineered cyanobacteria. Biotechnol. Bioeng. *113*, 311–319.

Aravind, L., and Ponting, C.P. (1997). The GAF domain: An evolutionary link between diverse phototransducing proteins. Trends Biochem. Sci. *22*, 458–459.

Bezy, R.P., and Kehoe, D.M. (2010). Functional characterization of a cyanobacterial OmpR/PhoB class transcription factor binding site controlling light color responses. J. Bacteriol. *192*, 5923–5933.

Bhaya, D. (2016). In the limelight: Photoreceptors in cyanobacteria. MBio *7*.

Burgie, E.S., and Vierstra, R.D. (2014). Phytochromes: An Atomic Perspective on Photoactivation and Signaling. Plant Cell Online *26*, 4568–4583.

Chen, Y., Zhang, J., Luo, J., Tu, J.M., Zeng, X.L., Xie, J., Zhou, M., Zhao, J.Q., Scheer, H., and Zhao, K.H. (2012). Photophysical diversity of two novel cyanobacteriochromes with phycocyanobilin chromophores: Photochemistry and dark reversion kinetics. FEBS J. *279*, 40–54.

Dow, J.M., Fouhy, Y., Lucey, J.F., and Ryan, R.P. (2006). The HD-GYP domain, cyclic Di-GMP signaling, and bacterial virulence to plants. Mol. Plant-Microbe Interact. *19*, 1378–1384.

Enomoto, G., Hirose, Y., Narikawa, R., and Ikeuchi, M. (2012). Thiol-based photocycle of the blue and teal light-sensing cyanobacteriochrome Tlr1999. Biochemistry *51*, 3050–3058.

Fushimi, K., and Narikawa, R. (2019). Cyanobacteriochromes: photoreceptors covering the entire UV-to-visible spectrum. Curr. Opin. Struct. Biol. *57*, 39–46.

Fushimi, K., Rockwell, N.C., Enomoto, G., Ni-Ni-Win, Martin, S.S., Gan, F., Bryant, D.A., Ikeuchi, M., Lagarias, J.C., and Narikawa, R. (2016). Cyanobacteriochrome Photoreceptors Lacking the Canonical Cys Residue. Biochemistry *55*, 6981–6995.

Fushimi, K., Hasegawa, M., Ito, T., Rockwell, N.C., Enomoto, G., -Win, N.-N., Lagarias, J.C., Ikeuchi, M., and Narikawa, R. (2020). Evolution-inspired design of multicolored photoswitches from a single cyanobacteriochrome scaffold. Proc. Natl. Acad. Sci. 202004273.

Galperin, M.Y., Nikolskaya, A.N., and Koonin, E. V. (2001). Novel domains of the prokaryotic two-component signal transduction systems. FEMS Microbiol. Lett. *203*, 11–21.

Gurd, F.R.N. (1972). [34 a] Carboxymethylation. Methods Enzymol. *25*, 424–438.

Hardman, S.J.O., Heyes, D.J., Sazanovich, I. V., and Scrutton, N.S. (2020). Photocycle of Cyanobacteriochrome TePixJ. Biochemistry *59*, 2909–2915.

Hirose, Y., Narikawa, R., Katayama, M., and Ikeuchi, M. (2010). Cyanobacteriochrome CcaS regulates phycoerythrin accumulation in Nostoc punctiforme, a group II chromatic adapter. Proc. Natl. Acad. Sci. U. S. A. *107*, 8854–8859.

Hirose, Y., Rockwell, N.C., Nishiyama, K., Narikawa, R., Ukaji, Y., Inomata, K., Lagarias, J.C., and Ikeuchi, M. (2013a). Green/red cyanobacteriochromes regulate complementary chromatic acclimation via a protochromic photocycle. Proc. Natl. Acad. Sci. *110*, 4974–4979.

Hirose, Y., Rockwell, N.C., Nishiyama, K., Narikawa, R., Ukaji, Y., Inomata, K., Lagarias, J.C., and Ikeuchi, M. (2013b). Green/red cyanobacteriochromes regulate complementary chromatic acclimation via a protochromic photocycle. Proc. Natl. Acad. Sci. U. S. A. *110*, 4974–4979.

Ho, Y.S.J., Burden, L.M., and Hurley, J.H. (2000). Structure of the GAF domain, a ubiquitous signaling motif and a new class of cyclic GMP receptor. EMBO J. *19*, 5288–5299.

Ikeuchi, M., and Ishizuka, T. (2008). Cyanobacteriochromes: A new superfamily of tetrapyrrole-binding photoreceptors in cyanobacteria. In Photochemical and Photobiological Sciences, pp. 1159–1167.

Ishizuka, T., Kamiya, A., Suzuki, H., Narikawa, R., Noguchi, T., Kohchi, T., Inomata, K., and Ikeuchi, M. (2011). The cyanobacteriochrome, TePixJ, isomerizes its own chromophore by converting phycocyanobilin to phycoviolobilin. Biochemistry *50*, 953–961.

Kawano, Y., Saotome, T., Ochiai, Y., Katayama, M., Narikawa, R., and Ikeuchi, M. (2011). Cellulose accumulation and a cellulose synthase gene are responsible for cell aggregation in the cyanobacterium Thermosynechococcus vulcanus RKN. Plant Cell Physiol. *52*, 957–966.

Kehoe, D.M., and Gutu, A. (2006). Responding to Color: The Regulation of Complementary Chromatic Adaptation. Annu. Rev. Plant Biol. *57*, 127–150.

Kuwasaki, Y., Miyake, K., Fushimi, K., Takeda, Y., Ueda, Y., Nakajima, T., Ikeuchi, M., Sato, M., and Narikawa, R. (2019). Protein engineering of dual-cys cyanobacteriochrome am1_1186g2 for biliverdin incorporation and far-red/blue reversible photoconversion. Int. J. Mol. Sci. *20*.

Narikawa, R., Enomoto, G., Ni-Ni-Win, Fushimi, K., and Ikeuchi, M. (2014). A new type of Dual-Cys cyanobacteriochrome GAF domain found in cyanobacterium Acaryochloris marina, which has an unusual red/blue reversible photoconversion cycle. Biochemistry *53*, 5051–5059.

Rockwell, N.C., and Lagarias, J.C. (2010). A brief history of phytochromes. ChemPhysChem *11*, 1172–1180.

Rockwell, N.C., Martin, S.S., Feoktistova, K., and Lagarias, J.C. (2011). Diverse two-cysteine photocycles in phytochromes and cyanobacteriochromes. Proc. Natl. Acad. Sci. *108*, 11854–11859.

Rockwell, N.C., Martin, S.S., Gulevich, A.G., and Lagarias, J.C. (2012). Phycoviolobilin Formation and Spectral Tuning in the DXCF Cyanobacteriochrome Subfamily. Biochemistry *51*, 1449–1463.

Rockwell, N.C., Ohlendorf, R., and Möglich, A. (2013a). Cyanobacteriochromes in full color and three dimensions. Proc. Natl. Acad. Sci. U. S. A. *110*, 806–807.

Rockwell, N.C., Ohlendorf, R., and Moglich, A. (2013b). Cyanobacteriochromes in full color and three dimensions. Proc. Natl. Acad. Sci. *110*, 806–807.

Rockwell, N.C., Martin, S.S., and Lagarias, J.C. (2015). Identification of DXCF cyanobacteriochrome lineages with predictable photocycles. Photochem. Photobiol. Sci. *14*, 929–941.

Romling, U., Galperin, M.Y., and Gomelsky, M. (2013). Cyclic di-GMP: the First 25 Years of a Universal

Bacterial Second Messenger. Microbiol. Mol. Biol. Rev. *77*, 1–52.

Schmitz, O., Katayama, M., Williams, S.B., Kondo, T., and Golden, S.S. (2000). CikA, a bacteriophytochrome that resets the cyanobacterial circadian clock. Science (80-.). *289*, 765–768.

Schultz, J.E. (2009). Structural and biochemical aspects of tandem GAF domains. Handb. Exp. Pharmacol. *191*, 93–109.

van Thor, J.J., Mackeen, M., Kuprov, I., Dwek, R.A., and Wormald, M.R. (2006). Chromophore structure in the photocycle of the cyanobacterial phytochrome Cph1. Biophys. J. *91*, 1811–1822.

Yoshihara, S., and Ikeuchi, M. (2004). Phototactic motility in the unicellular cyanobacterium Synechocystis sp. PCC 6803. Photochem. Photobiol. Sci. *3*, 512–518.

SUPPLEMENTARY

Recombinant protein purification

SDS-PAGE, Zinc assay and Western blot gels from the purification of 7GP-03 are shown in Supplementary Figure 1.

Light sources and colour filters

The emission spectrum of the halogen-tungsten light source and the transmission spectra of the colour filters are shown in Supplementary Figure 2.

Primer sequences

7GP-03-FULL-F6:	5'- GTATAAGCTTATGTTTGATCGAAGTCTTAATCGAG-3'
7GP-03-FULL-R4:	5'- GATCCTCGAGGTCCATGAGTTGTAAATCACC-3'
7GP-03-C73S-F:	5'-GGGGCCGATGATAGTTTTAATGGTG-3'
7GP-03-C73S-R:	5'-CACCATTAAAACTATCATCGGCCCC-3'
7GP-03-C73A-F:	5'-GGGGCCGATGATGCTTTTAATGGTG-3'
7GP-03-C73A-R:	5'-CACCATTAAAAGCATCATCGGCCCC-3'
pET-F:	5'-GTTCCTCGAGCACCACCACCACC-3'
pET-R:	5'-GGTAAAGCTTTATGTATATCTCCTTCTTAAAGTTAAAC-3'
T7-Prom-F:	5'-TAATACGACTCACTATAGGG-3'
T7-Term-R:	5'-GCTAGTTATTGCTCAGCGG-3'

Peak deconstruction

Peak deconstruction analysis was carried out for the spectroscopy data obtained in this work.

Supplementary Figure 3 shows the deconstructed peaks from Figure 3.

Supplementary Figure 4 shows the deconstructed peaks from Figure 4.

Supplementary Figure 5 shows the deconstructed peaks from Figure 5.

Supplementary Figure 6 shows the deconstructed peaks from Figure 6.

Supplementary Figure 7 shows the deconstructed peaks from Figure 7.

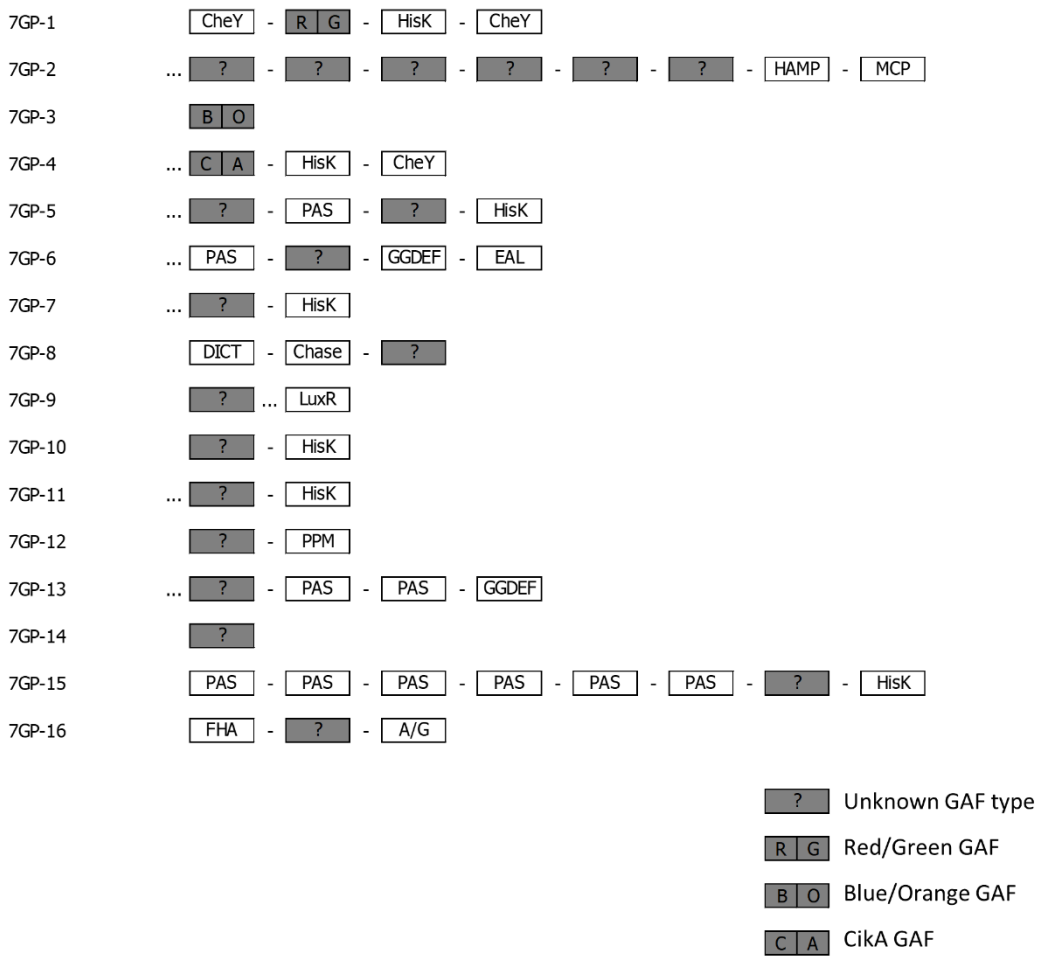
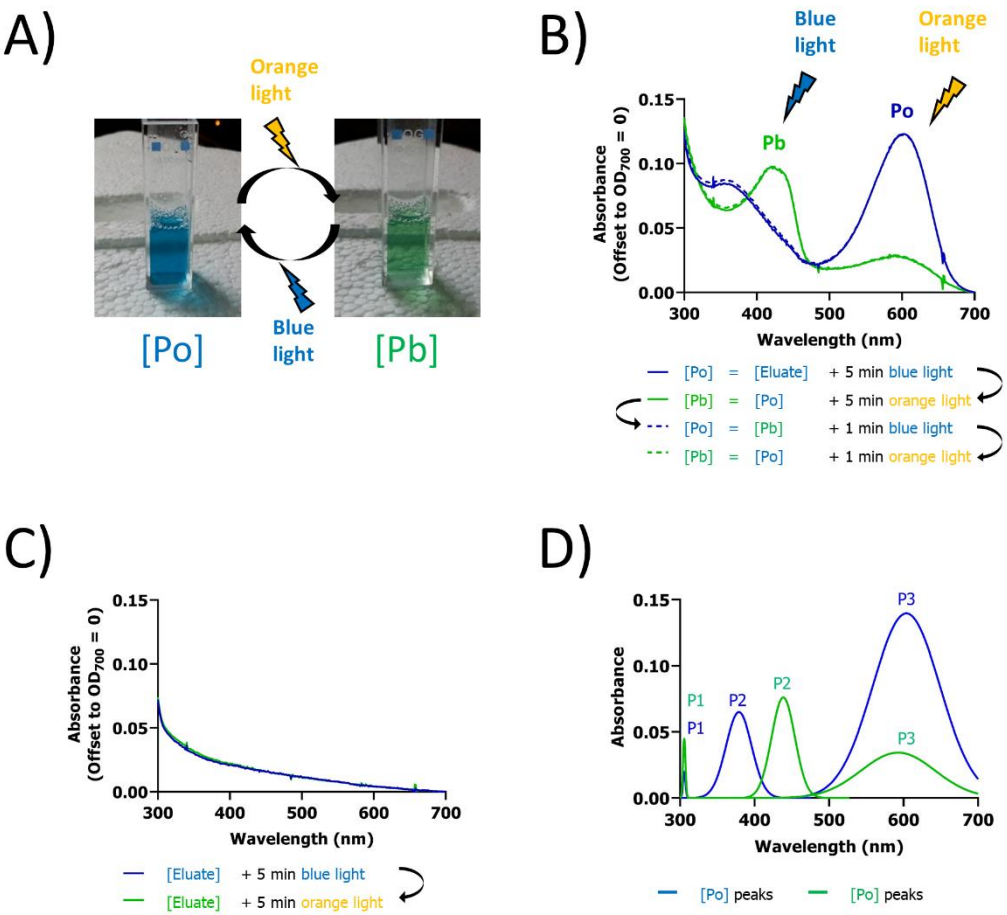


FIGURE 1: **Three CBCR GAF domains are predicted in *Synechococcus* sp. PCC 7002**

A BLAST-P analysis was performed against the genome of *Synechococcus* sp. PCC 7002 using the TePixJg sequence as template. For each of the genes listed, the predicted domain structure of the encoded protein is shown from N-terminus (left) to C terminus (right). GAF domains are marked as dark grey rectangles. The predicted GAF domain family, if known, is marked with two letters: RG, BO, and CA. Regions with unrecognized domain signature are marked as "…". The naming of other domains follows InterPro convention. A/G: Adenylyl cyclase class-3/4/guanylyl cyclase domain. dCach: dCache_1 domain. HOMO: Homodimerization domain. MHY: MHYT domain. S/T-K: Serine/Threonine Kinase domain.



E)

	Peak	Location (nm)	Height	Width	Area
[Po]	P1	305.7	0.02	2.33	0.08
	P2	378.93	0.06	24.38	2.81
	P3	603.78	0.14	63.78	15.79
[Pb]	P1	305.66	0.04	2.31	0.18
	P2	438.61	0.07	22.14	2.99
	P3	592.67	0.03	70.04	4.26

FIGURE 2: **Recombinant 7GP-03 with PCB absorbs orange or blue spectral light**

Absorbance states of recombinant 7GP-03. **A:** A blue solution (Po) was the default state under standard light conditions, converting to a green solution (Pb) upon exposure to orange light. Absorbance spectra were recorded after illuminating the protein solution with a halogen-tungsten lamp through colour filters removing wavelengths in the blue spectrum ('orange' light) or removing longer wavelengths ('blue' light). Emission spectra of the light treatments are detailed in Supplemental Fig. 2. **B:** Absorbance spectra of 7GP-03 co-expressed with the chromophore phycocyanobilin (PCB). The

sample was illuminated for 5 minutes with blue light (blue line). The same sample was immediately illuminated with orange light (green line). The last sample was also illuminated with the blue light again but for the shorter time of 1 min (dashed lines) and with a orange light again for 1 min. The protein states producing the absorption spectra shown were called Po (blue lines) and Pb (green lines). **C:** Absorbance spectra of 7GP-03 without PCB. **D:** Composite peaks (P1-3) predicted from the spectra shown in B). Po peaks are shown in blue. Pb peaks are shown in green. **E:** Composite peak data from D).

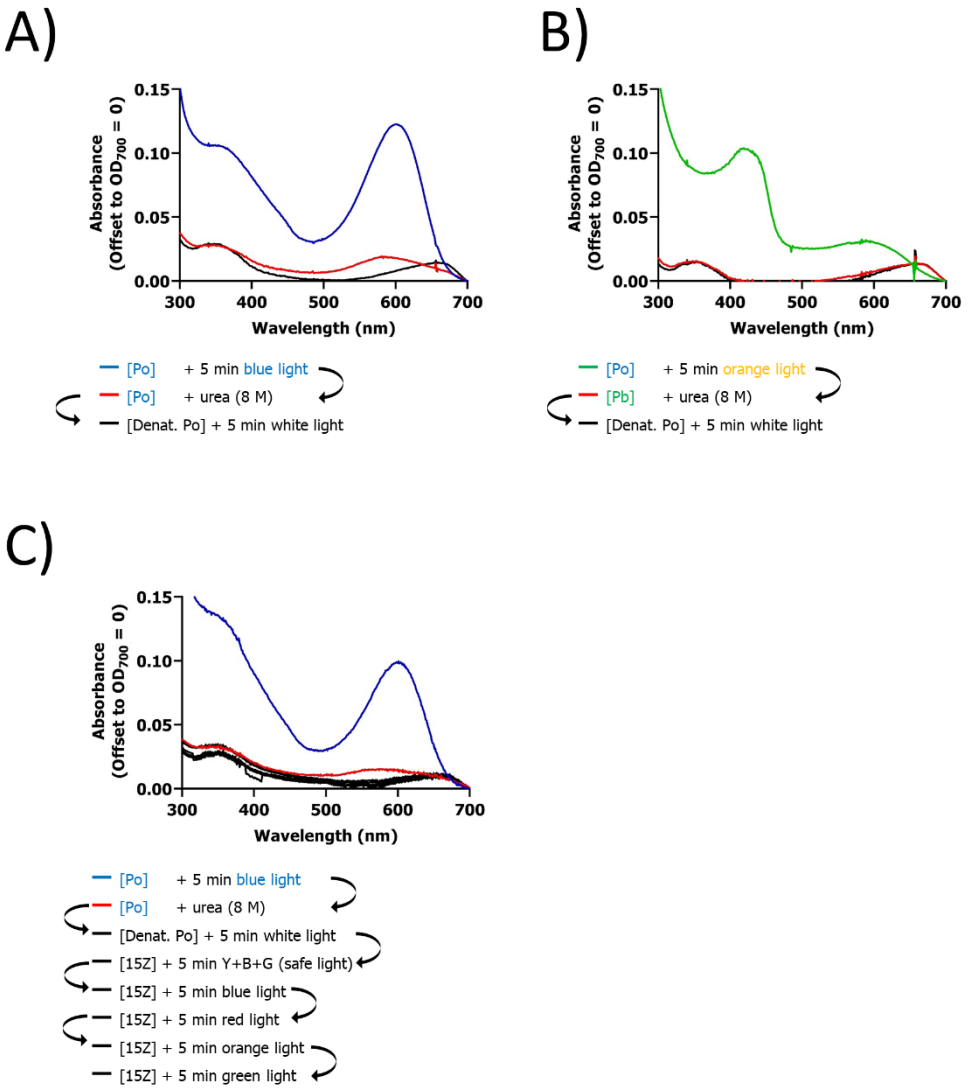


FIGURE 3: **The Po/Pb states of** 7GP-03 **correspond to the 15E/15Z isomers of phycocyanobilin**

Recombinant 7GP-03 was expressed in the presence of PCB and purified as a blue solution under standard light conditions. Aliquots of the solution were exposed for 5 minutes to 'blue' or 'orange' light to convert all protein to Po (blue line) or Pb (green line), respectively. A, B: Absorbance spectra of 7GP-03 in Po state (A) or Pb state (B) before (blue/green lines) and after (red line) addition of urea at a final concentration 8 M. A third absorbance spectrum (black line) was measured after illuminating the urea-treated samples for 5 minutes with white light. C: As in A with additional light treatments applied after white light illumination of the denatured protein (grey lines).

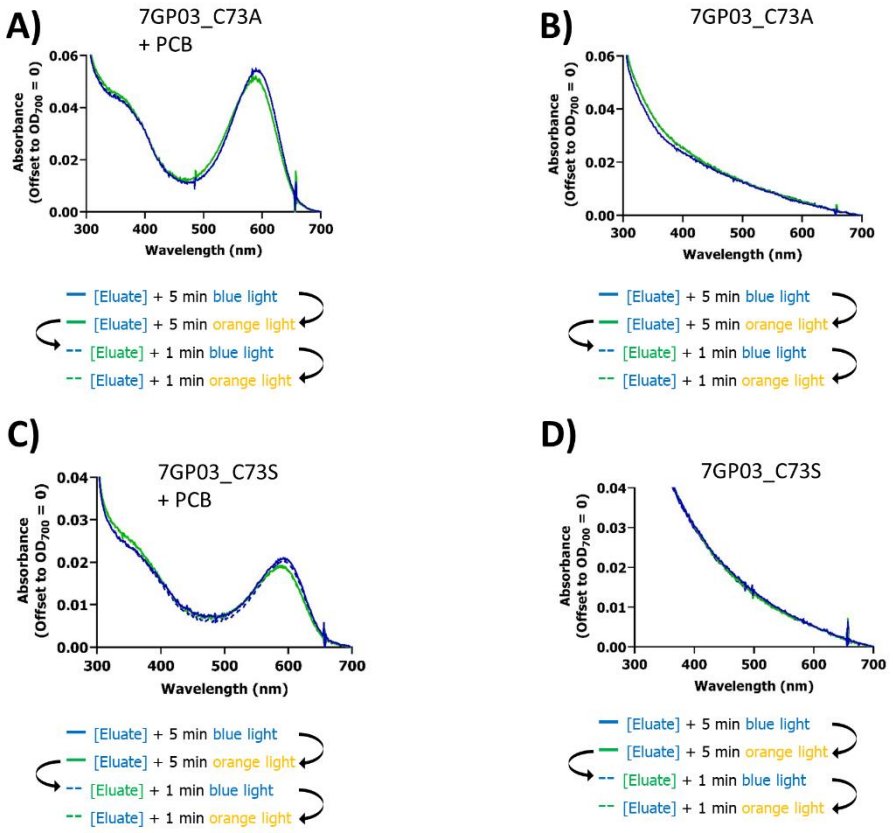


FIGURE 4: **Cys73 is essential for the photocycle**

Absorbance spectra of mutant versions of 7GP-03 in which cysteine 73 was replaced with alanine (7GP-03_C73A) or serine (7GP-03_C73S) by site-directed mutagenesis. The recombinant proteins were expressed in the presence or absence of PCB and purified under standard light conditions. Absorbance spectroscopy was carried out after treating the protein with 'blue' light (blue lines) or 'orange' light (green lines) for 1 minute (dashed blue and dashed green lines) or 5 minutes (blue and green lines). A, B: Absorbance spectra of 7GP-03_C73A with PCB co-expressed (A) or not (B). C, D: Absorbance spectra of 7GP-03_C73S with PCB co-expressed (C) or not (D).

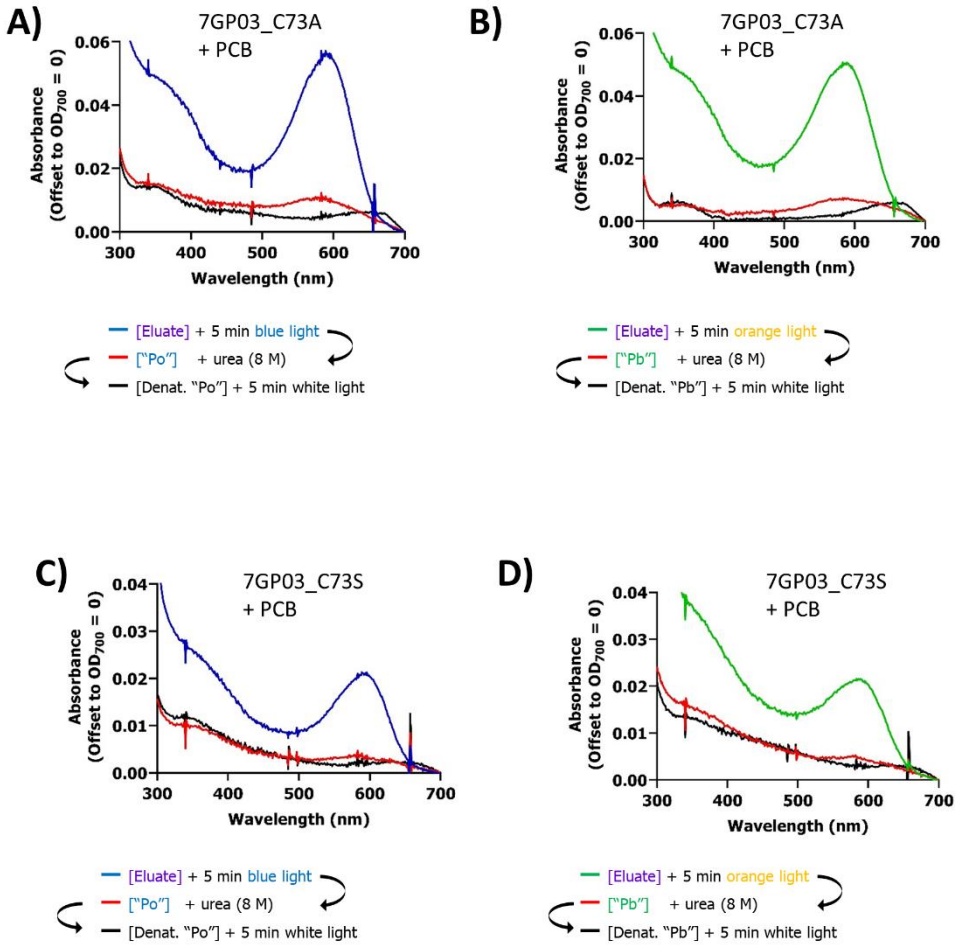


FIGURE 5: **The role of Cys 73 in the photocycle precedes 15E-15Z isomerization**

Absorbance spectra of denatured mutant versions of 7GP-03 replacing cysteine in position 73 with alanine (7GP-03_C73A) or serine (7GP-03_C73S) were generated by site-directed mutagenesis, the recombinant proteins were expressed in the presence or absence of PCB and purified under standard light conditions. Aliquots of the solution were exposed for 5 minutes to 'blue' or 'orange' light to try and convert all protein to Po (blue line) or Pb (green line), respectively. A, B: Absorbance spectra of 7GP-03_C73A in "Po" state (A) or "Pb" state (B) before (blue/green lines) and after (red line) addition of urea at a final concentration 8 M. A third absorbance spectrum (black line) was measured after illuminating the urea-treated samples for 5 minutes with white light. C, D: Absorbance spectra of 7GP-03_C73S in "Po" state (A) or "Pb" state (B) before (blue/green lines) and after (red line) addition of urea at a final concentration 8 M. A third absorbance spectrum (black line) was measured after illuminating the urea-treated samples for 5 minutes with white light.

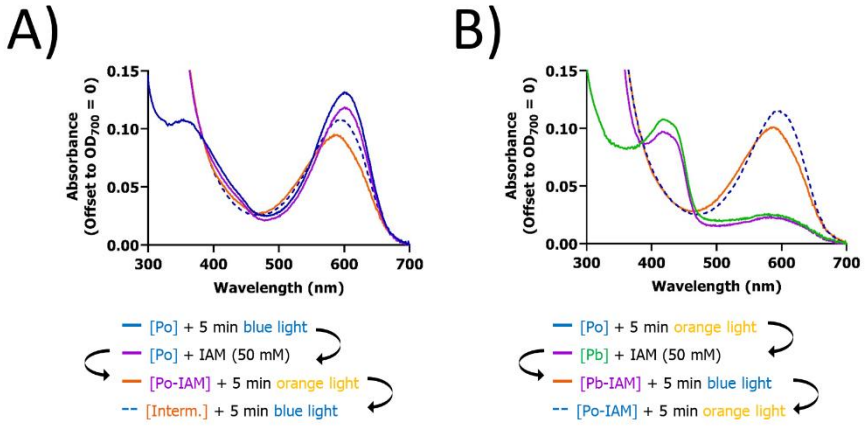


FIGURE 6: **A thiol bridge is likely formed between Cys73 and C10 of PCB in the Po to Pb transition**

Absorbance spectra of 7GP-03 after treatment with iodoacetamide (IAM). Recombinant 7GP-03 was expressed in the presence of PCB and purified as a blue solution under standard light conditions. Aliquots of the solution were exposed for 5 minutes to 'blue' or 'orange' light to convert all protein to Po (A, blue line) or Pb (B, green line), respectively. IAM was then added to a final concentration of 50 mM, immediately followed by an absorbance scan (purple line in A and B. Next, the samples were exposed to 5 min of 'orange'(A) or 'blue' light (B) to drive the protein into the opposite state, and absorbance spectra were measured (orange line in A, dashed blue line in B). Finally, the protein was motivated back to its original state by applying either 'blue' (A) or 'orange' light (B), and absorbance spectra were measured (dashed blue line in A, orange line in B).

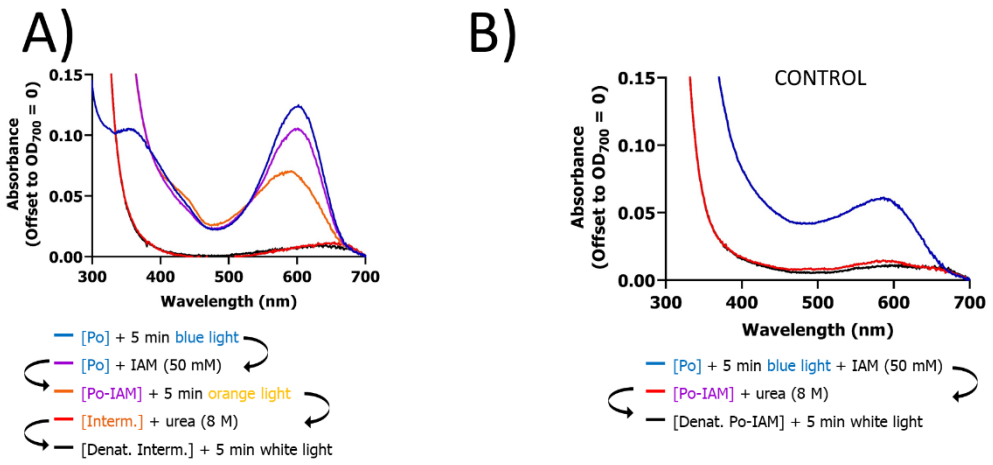


FIGURE 7: **The presence of Cys 73 alone is insufficient to drive the photocycle**

Absorbance spectra of denatured IAM-treated 7GP-03. Recombinant 7GP-03 was expressed in the presence of PCB and purified as a blue solution under standard light conditions. An aliquot of the protein was exposed to 5 min 'blue' light to take it completely to Po. A: Absorbance spectra of protein before (blue line) and after (purple line) addition of IAM to a final concentration of 50 mM. The IAM- treated sample was then illuminated for 5 min with 'orange' light to take it to the intermediate state (orange line). Next, urea was added to a final concentration of 8 M, immediately followed by another absorbance scan(red line). The final absorbance scan was carried out after illuminating the denatured protein +PCB for 5 min with white light (black line). B: Control absorbance spectra of IAM treated 7GP-03 in the Po state (blue line) exposed to urea (red line) and white light (black line) using the same concentration and duration as in A.

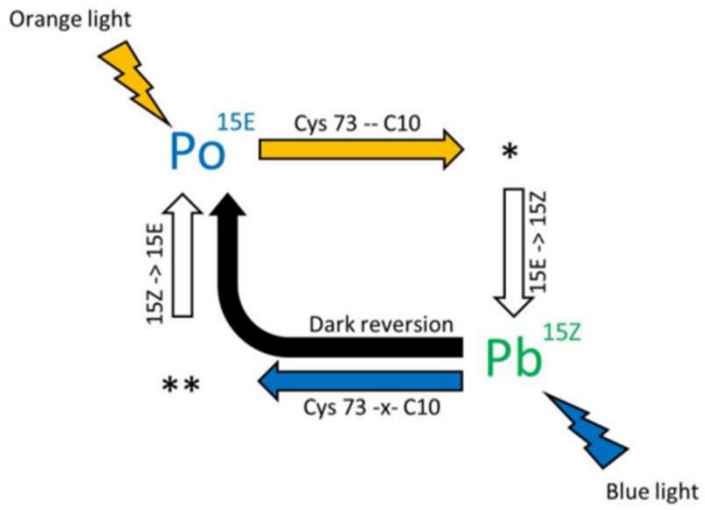
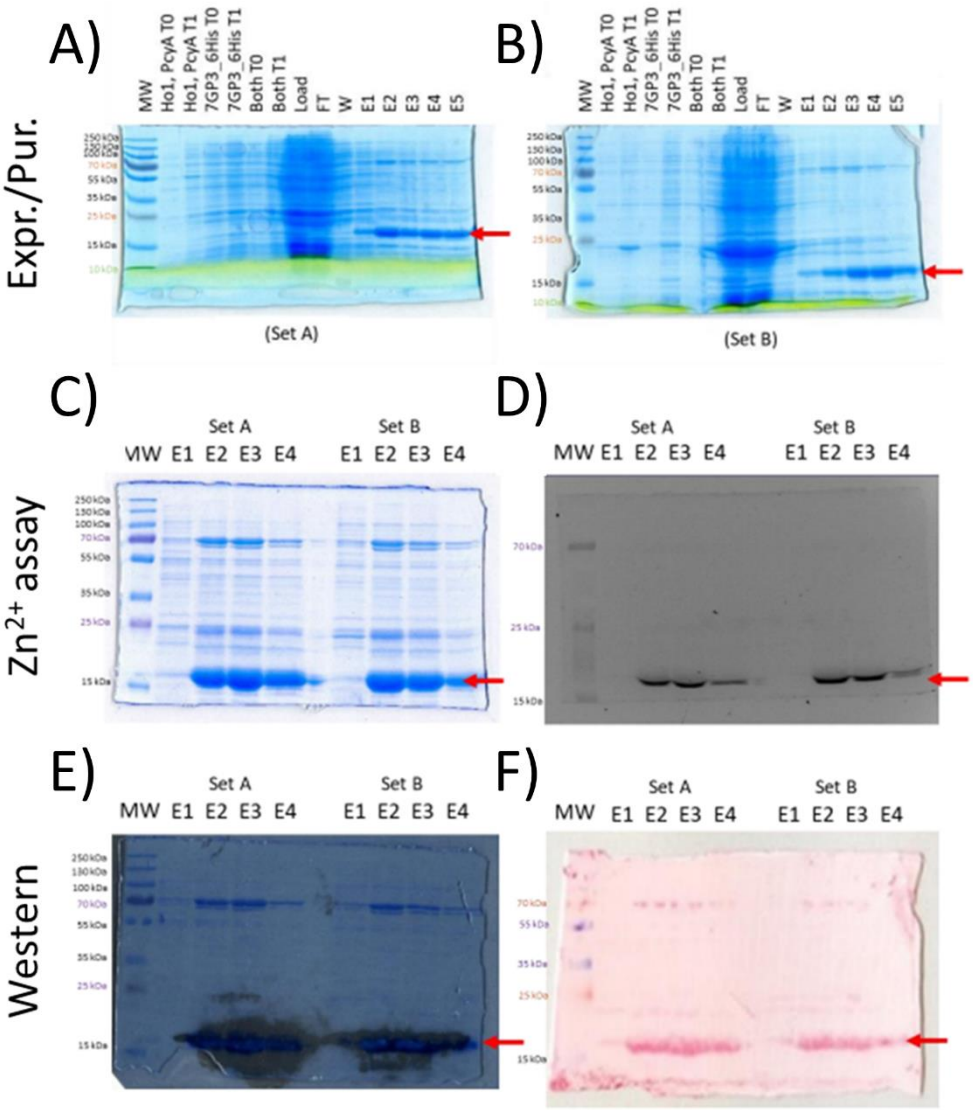


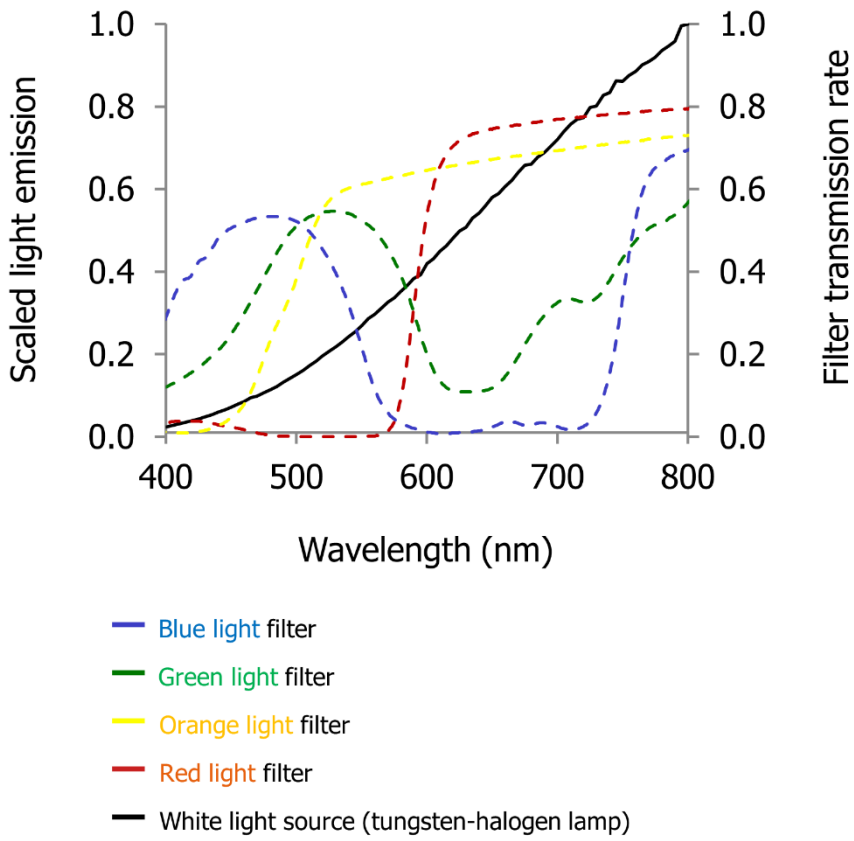
FIGURE 8: **Proposed model of the 7GP-03 photocycle**

The photocycle of 7GP-03 has been proposed to go through Po -> * -> Pb -> ** -> Po. Two of those states have been observed experimentally: Po, Pb. The two three hypothesized states are shown with * and **. The * intermediate state is probably very short-lived and contains a covalent link between Cys 73 and C10 of PCB. The bilin is still 15E. The ** state probably contains 15Z bilin but without a covalent link at C10.



SUPPL. FIG. 1: **Expression and purification of recombinant Synechococcus 7GP-03_6His**

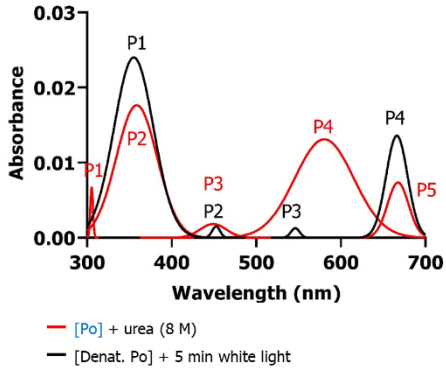
E. coli BL21 Star (DE3) competent cells were co-transformed with pACYC_ho1_pcyA (Set A) or pPcyA (Set B) and pET-R_7GP-03-6His. Recombinant proteins were then expressed and purified using Ni^{2+} -affinity chromatography. A, B: SDS-PAGE gels of protein samples from the two independent transformants (Set A and Set B, respectively) taken at the various points during the purification process and stained with Coomassie blue. C: Separate SDS-PAGE gel of the elution fractions stained with Coomassie blue. D: Zinc fluorescence assay of the elution fractions. E: Overlay of a Western blot assay of the elution fractions. Western blotting was performed using antibodies specific for the 6xHis tag on the protein and visualized using HRP. F: Ponceau stain of the Western blot membrane shown in E. MW: molecular weight marker, Ho1, PcyA T0: transformed without pET-R_7GP-03-6His (before induction), Ho1, PcyA T1: transformed without pET-R_7GP-03-6His (induced for 16 hours), 7GP-03-6His T0: transformed only with pET-R_7GP03-6His (before induction), 7GP-03_6His T1: transformed only with pET-R_7GP-03-6His (induced for 16 hours), Both T0: transformed with both plasmids from the set (before induction), Both T1: transformed with both plasmids from the set (induced for 16 hours), Load: supernatant from cell lysate (before adding to affinity column), FT: supernatant from cell lysate (after flowing through the affinity column), W: column flowthrough after applying wash buffer, E1-E5: eluates in 2 mL increments after applying the elution buffer. For further details see Methods.



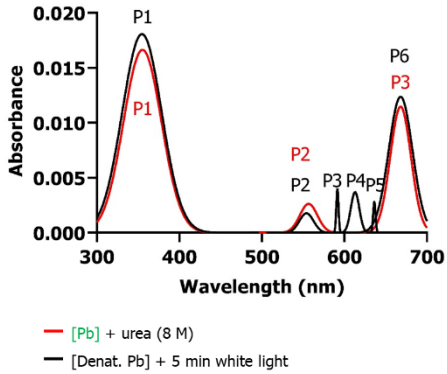
SUPPL. FIG. 2: **Spectra of colour treatments**

The tansmission (%T) spectra of four colour filters were recorded using an absorbance spectrometer. Lines are coloured and labelled according to the largest wavelength band the filter transmits, namely blue [370:560 nm], green [420:620 nm], orange [430:∞), red [570:∞). The emission spectrum of the tungsten-halogen white light lamp is also measured and shown in black.

A)



B)



C)

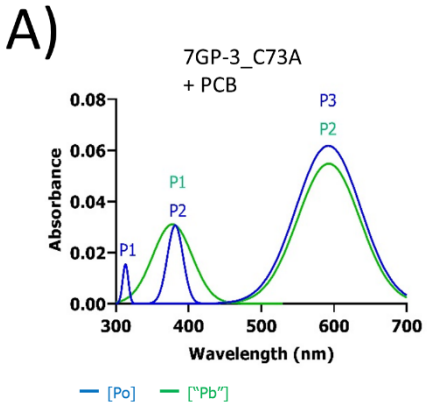
	Peak	Location (nm)	Height	Width	Area
Denat. [Po]	P1	305.38	0.01	2.2	0.03
	P2	358.69	0.02	34.18	1.07
	P3	449.11	0	21.65	0.07
	P4	580.41	0.01	49.17	1.14
	P5	667.61	0.01	17.58	0.23
Denat. [Po] + white light	P1	355.2	0.02	33.16	1.41
	P2	452.47	0	6.54	0.02
	P3	546.18	0	7.25	0.02
	P4	666.22	0.01	17.99	0.43

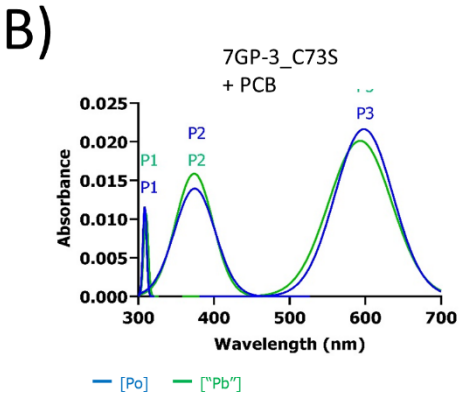
D)

	Peak	Location (nm)	Height	Width	Area
Denat. [Pb]	P1	355.26	0.02	32.62	0.96
	P2	556.6	0	15.02	0.07
	P3	668.07	0.01	17.75	0.36
Denat. [Pb] + white light	P1	354.61	0.02	33.87	1.09
	P2	553.98	0	12.13	0.04
	P3	591.5	0	1.93	0.01
	P4	612.87	0	7.28	0.05
	P5	696.27	0	2.06	0.01
	P6	668.08	0.01	20.36	0.45

SUPPL. FIG. 3: **Peak deconstruction for Figure 3**

Recombinant 7GP-03 was expressed in the presence of PCB and purified as a blue solution under standard light conditions. Aliquots of the solution were exposed for 5 minutes to 'blue' or 'orange' light to convert all protein to Po (blue line) or Pb (green line), respectively. Urea was then added to the samples, followed by white light illumination for 5 min. A and B: Peak deconstruction of the absorbance spectra from the denatured samples in Figure 3a and b, respectively. The spectra taken immediately after urea denaturation are shown in red, the spectra taken after white light illumination are shown in black. Peak deconstruction was performed as described in the Methods.





C)

	Peak	Location (nm)	Height	Width	Area
[Po]	P1	313.29	0.02	5.43	0.15
	P2	381.62	0.03	15.2	0.82
	P3	592.31	0.06	62.86	6.89
["Pb"]	P1	377.82	0.03	38.17	2.1
	P2	592.83	0.05	60.59	5.88

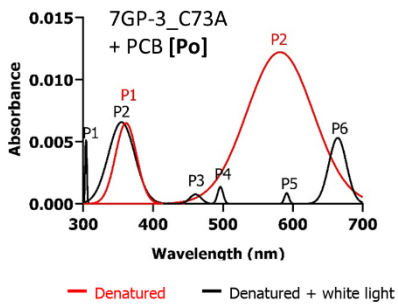
D)

	Peak	Location (nm)	Height	Width	Area
[Po]	P1	308.5	0.01	3.47	5.78
	P2	374.61	0.01	38.02	63.31
	P3	598.22	0.02	54.37	90.54
["Pb"]	P1	310.15	0.01	4.14	0.08
	P2	373.9	0.02	34.87	0.98
	P3	593.33	0.02	58.87	2.1

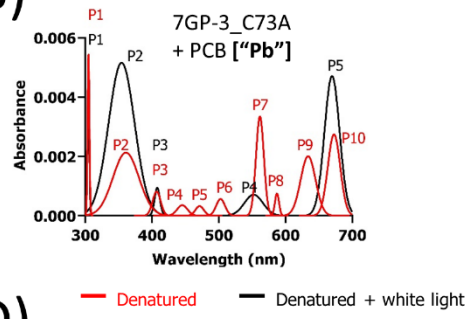
SUPPL. FIG. 4: Peak deconstruction from Figure 4

Mutant versions of 7GP-03 replacing cysteine in position 73 with alanine (7GP-03_C73A) or serine (7GP-03_C73S) were generated by site-directed mutagenesis, the recombinant proteins were expressed In the presence or absence of PCB and purified under standard light conditions. Absorbance spectroscopy was then carried out after treating the protein with 'blue' light (blue lines) or 'orange' light for 1 minute or 5 minutes. A, B: The results of peak deconstruction of the 7GP-03_C73A and 7GP-03_C73S spectra from Figure 4. C, D: the data from A) and B), respectively.

A)



B)



C)

Den. [Po]

Den. [Po] + white light

Peak	Location (nm)	Height	Width	Area
P1	361.37	0.01	21.21	0.24
P2	581.85	0.01	67.04	1.45
P1	304.12	0.01	1.68	0.02
P2	354.97	0.01	26.55	0.31
P3	460.87	0	11.59	0.02
P4	496.46	0	5.49	0.01
P5	591.42	0	4.54	0.01
P6	664.42	0.01	17.82	0.17

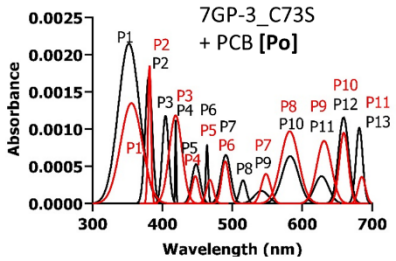
D)

Den. [Pb]

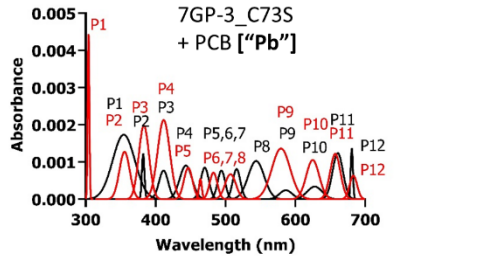
Den. [Pb] + white light

Peak	Location (nm)	Height	Width	Area
P1	304.74	0.01	1.93	0.02
P2	360.83	0	27.5	0.1
P3	406.85	0	8.54	0.01
P4	445.29	0	10.92	0.01
P5	471.23	0	8.83	0.01
P6	502.69	0	9.23	0.01
P7	561.67	0	8.29	0.05
P8	587.19	0	4.31	0.01
P9	633.9	0	15.75	0.06
P10	672.73	0	13.09	0.06
P1	304.58	0	1.87	0.02
P2	354.32	0.01	27.56	0.25
P3	407.79	0	5.62	0.01
P4	552.26	0	19.62	0.02
P5	669.76	0	15.96	0.13

E)



F)



G)

Den. [Po]

Den. [Po] + white light

Peak	Location (nm)	Height	Width	Area
P1	355.71	0	21.842	0.05
P2	381.64	0	3.01	0.01
P3	418.95	0	12.92	0.03
P4	447.2	0	6.8	0
P5	467.93	0	7	0
P6	490	0	7.37	0.01
P7	548.18	0	9.33	0.01
P8	582.16	0	18.09	0.03
P9	631.32	0	14.83	0.2
P10	659.56	0	9.03	0.02
P11	685.34	0	7.32	0
P1	352.12	0	21.36	0.08
P2	380.46	0	7.07	0.02
P3	404.44	0	6.47	0.01
P4	419.04	0	1.06	0
P5	448.07	0	8.34	0.01
P6	463.83	0	2.35	0
P7	490.7	0	9.67	0.01
P8	515.35	0	6.66	0
P9	541.58	0	14.16	0
P10	582.95	0	16.75	0.02
P11	627.77	0	14.23	0.01
P12	659.24	0	10.33	0.02
P13	681.74	0	6.79	0.01

H)

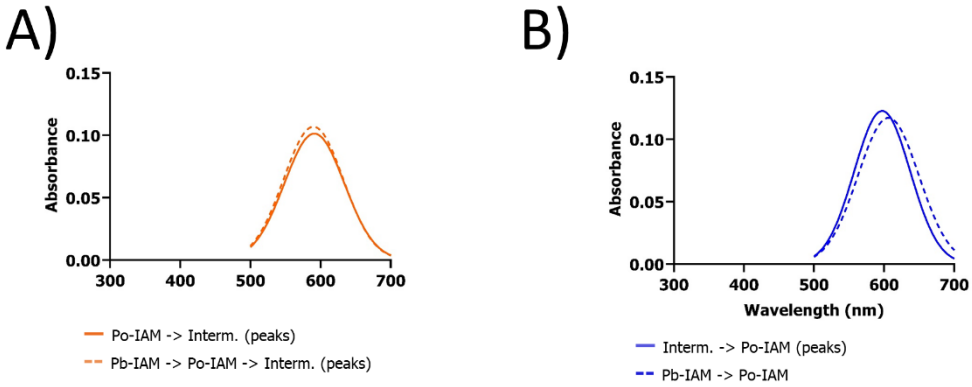
Den. [Pb]

Den. [Pb] + white light

Peak	Location (nm)	Height	Width	Area
P1	304.09	0	1.67	0.01
P2	355.65	0	10.43	0.02
P3	383.31	0	10.31	0.04
P4	411.66	0	12.32	0.05
P5	446.68	0	8.7	0.01
P6	464.15	0	2.94	0
P7	482.88	0	7.76	0.01
P8	507.23	0	10.92	0.01
P9	579.55	0	18.5	0.04
P10	624.93	0	13.04	0.02
P11	657.2	0	10.4	0.02
P12	683.09	0	7.65	0.01
P1	354.52	0	22.33	0.07
P2	382.28	0	2.56	0.01
P3	411.47	0	9.3	0.01
P4	443.43	0	12.59	0.02
P5	470.43	0	8.3	0.01
P6	494.24	0	7.58	0.01
P7	515.99	0	7.72	0.01
P8	543.98	0	16.11	0.03
P9	586.49	0	13	0.01
P10	627.8	0	15.32	0.01
P11	660.75	0	10.48	0.02
P12	680.86	0	2.24	0.01

SUPPL. FIG. 5: Peak deconstruction from Figure 5

Mutant versions of 7GP-03 replacing cysteine in position 73 with alanine (7GP-03_C73A) or serine (7GP-03_C73S) were generated by site-directed mutagenesis, the recombinant proteins were expressed in the presence or absence of PCB and purified under standard light conditions. Aliquots of the solution were exposed for 5 minutes to 'blue' or 'orange' light to try and convert all protein to Po or Pb, respectively. A, B: Absorbance spectra deconstruction of 7GP-03_C73A in "Po" state (A) or "Pb" state (B) after addition of 8 M urea (red) and immediately after white light illumination (black). C, D: the peak deconstruction data from A) and B), respectively. E, F: Absorbance spectra deconstruction of 7GP-03_C73A in "Po" state (A) or "Pb" state (B) after addition of 8 M urea (red) and immediately after white light illumination (black). G, H: the peak deconstruction data from E) and F), respectively.



C)

	Peak	Location (nm)	Height	Width	Area
Po-IAM -> Interm.	P1	592.27	0.1	60.5	10.88
Pb-IAM -> Po-IAM -> Interm.	P1	589.96	0.11	60.19	11.41

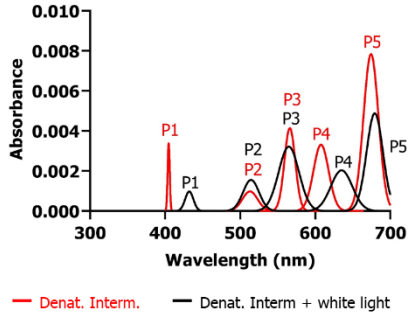
D)

	Peak	Location (nm)	Height	Width	Area
Interm. -> Po-IAM	P1	597.44	0.12	56.55	12.31
Pb-IAM -> Po-IAM	P1	606.36	0.12	61.43	12.77

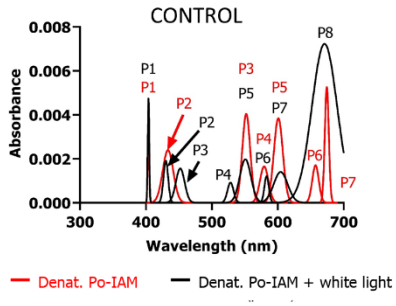
SUPPL. FIG. 6: **Peak deconstruction from Figure 6**

Recombinant 7GP-03 was expressed in the presence of PCB and purified as a blue solution under standard light conditions. Aliquots of the solution were exposed for 5 minutes to 'blue' or 'yellow' light to convert all protein to Po or Pb, respectively. IAM was then added to a final concentration of 50 mM. Next, the samples were exposed to 5 min of 'orange' or 'blue' light to drive the protein into the opposite state. Finally, the protein was motivated back to its original state by applying either 'blue' or 'orange' light. A: the deconstructed peaks of the IAM-blocked Po -> Pb conversion (Interm.). The continuous line shows the Po -> Pb direction. The dashed line shows the Pb -> Po -> Pb direction. B: The deconstructed peaks of the opposite direction are shown. The continuous line shows the Interm. -> Po-IAM direction. The dashed line shows the Pb-IAM -> Po-IAM direction. C, D: the data of the deconstructed peaks from A) and B), respectively.

A)



B)



C)

	Peak	Location (nm)	Height	Width	Area
Denat. Interm.	P1	404.78	0	1.95	0.01
	P2	513.07	0	14.29	0.02
	P3	566.17	0	10.164	0.07
	P4	607.75	0	14.35	0.08
	P5	674.32	0.01	14.08	0.2
Denat. Interm. + white light	P1	432.17	0	7.74	0.01
	P2	514.49	0	15.02	0.04
	P3	564.81	0	19.15	0.11
	P4	635.11	0	20.23	0.07
	P5	679.3	0	15.41	0.13

D)

	Peak	Location (nm)	Height	Width	Area
Denat. Po-IAM	P1	403.89	0	1.59	0.01
	P2	433.48	0	11.28	0.05
	P3	552.15	0	8.44	0.06
	P4	579.33	0	9.1	0.03
	P5	600.82	0	9.57	0.07
	P6	657.2	0	6.98	0.07
	P7	674.32	0	4.25	0.02
Denat. Po-IAM + white light	P1	403.93	0	1.6	0.01
	P2	430.18	0	4.98	0.02
	P3	452.07	0	9.29	0.03
	P4	528.28	0	6.52	0.01
	P5	550.78	0	11.86	0.04
	P6	583.02	0	4.73	0.01
	P7	604.34	0	14.94	0.04
	P8	670.85	0	27.05	0.35

SUPPL. FIG. 7: **Peak deconstruction from Figure 7**

Recombinant 7GP-03 was expressed in the presence of PCB and purified as a blue solution under standard light conditions. An aliquot of the protein was exposed to 5 min 'blue' light to take it completely to Po. IAM was then added to a final concentration of 50 mM. The IAM- treated sample was then illuminated for 5 min with 'orange' light to push the protein in the Po -> Pb direction. Next, urea was added to a final concentration of 8 M, followed by white light treatment for 5 min. A: The deconstructed peaks from Figure 7, where red indicates the peaks immediately after urea-based denaturation and black indicates the peaks after white light illumination. C, D: the data of the deconstructed peaks from A) and B), respectively.