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ARTICLE

Light Controlled Reversible Michael Addition of Cysteine: A New Tool for Dynamic Site-Specific Labeling of Proteins

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Cysteine-based Michael addition is a widely employed strategy for covalent conjugation of proteins, peptides, and drugs. The covalent reaction is irreversible in most cases, leading to a lack of control over the process. Utilizing spectroscopic analyses along with X-ray crystallographic studies, we demonstrate Michael addition of an engineered cysteine residue in human Cellular Retinol Binding Protein II (hCRBPII) with a coumarin analog that creates a non-fluorescent complex. UV-illumination reverses the conjugation, yielding a fluorescent species, presumably through a *retro*-Michael process. This series of events can be repeated between a bound and non-bound form of the cysteine reversibly, resulting in the ON-OFF control of fluorescence. The details of the mechanism of photoswitching was illuminated by recapitulation of the process in light irradiated single crystals, confirming the mechanism at atomic resolution.

Introduction

Site-specific protein labelling by synthetic fluorescent dyes is essential for studying protein function and localization in living cells.[1-2] Live cell imaging is commonly accomplished by fusing the protein of interest (POI) with a tag, often a protein, which can then be covalently labelled with a fluorochrome. Cysteine-based thiol conjugation has been widely used due to its relatively high nucleophilicity and low natural occurrence in protein sequences.[3] Commonly used approaches for cysteine modifications are transthioesterification (e.g. PYP), S-alkylation (e.g. SNAP, CLIP), and Michael addition (e.g. TMP-acrylamide, α,β-unsaturated dyes, maleimide containing dyes).[4-14] Utilizing these chemical approaches, an array of fluorescent molecules such as, BODIPY,[9] Fluorescein,[6] Cyanine,[4] and Coumarin[5, 7-8] based dyes have been conjugated with protein targets. Most systems, however, exhibit irreversible binding, where the fluorescent labels permanently bind with the protein tags. Ideally, a more versatile system would toggle on-demand between non-fluorescent (OFF) and fluorescent (ON) states using an external stimulus. In this context, stimuli-responsive labels offer enhanced spatial and temporal resolutions, and hence provide an additional level of control for the system of interest.[15-31]

Light offers several advantages over other forms of stimuli as it can be delivered with high spatiotemporal precision through control of wavelength, intensity, and irradiation time.[32-33] In particular, photoswitchable synthetic dyes provide a unique opportunity to achieve selective ON/OFF control of fluorescence. One such example is the photoswitchability of carbocyanine dyes (Cy5) with thiols, the association/dissociation complex of which can be toggled between a fluorescent (ON) and a dark (OFF) state (Figure 1a).[34-35] These systems can be cycled hundreds of times, yielding thousands of detected photons per switching cycle. Dempsey *et. al*. unveiled that under illumination of red light, Cy5 reacts covalently with externally added thiol via Michael addition to form a non-fluorescent adduct (OFF state). This can be reversed by illuminating the adduct with ultraviolet light. The latter observation piqued our interest to investigate the potential light-controlled reversible covalent chemistry between a fluorescent dye and a judiciously placed cysteine residue inside a protein host.

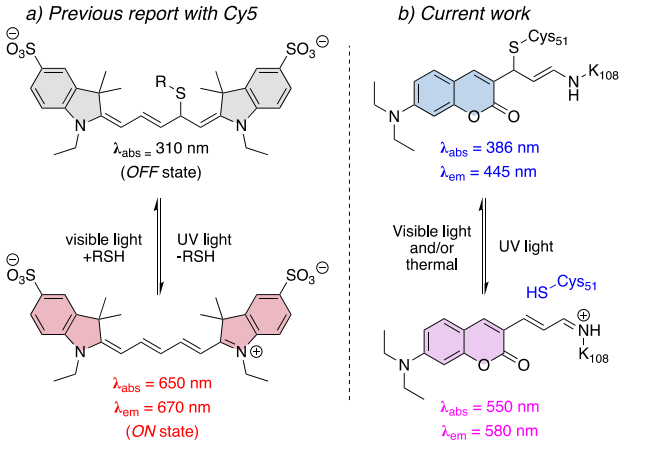

Figure 1. a) Light-controlled reversible Michael addition of thiols with a Cy5 photoswitch. b) Reversible site-specific conjugation of cysteine in hCRBPII with the **CM1V** fluorophore.

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Recent studies have highlighted the value of a reversibly photoswitchable fluorescent system for live cell super-resolution microscopies (e.g. RESOLFT, psSIM, STORM, PALM), information storage, and optical control of protein activity.[36-37] Herein, we report a light-controlled reversible site-specific labelling of human Cellular Retinol Binding Protein II (hCRBPII) with a fluorescent ligand **CM1V** (Figure 1b). The attractive features of this labelling include a fast rate of reaction and the low equivalence of ligand for quantitative functionalization. We demonstrate labelling of rationally engineered hCRBPII with **CM1V** leads to the formation of a complex that emits primarily in the blue region of the visible spectrum (denoted as OFF state in terms of red fluorescence). Photoactivation of this complex initiates a *retro*-Michael process that liberates the thiol, reestablishing the extended conjugation of the polyene, and consequently turns the fluorescence ON in the red wavelength regime. The well-behaved nature of the complex yields a photoswitchable system that is capable of reversible addition of the active-site cysteine residue over several cycles. Also, **CM1V** is a fluorogenic molecule, i.e.; it becomes fluorescent upon binding with the protein host and forming an iminium with an active-site Lys residue. This is in contrast to the cyanine system depicted in Figure 1a, which is always fluorescent (Figure S1). We have further characterized both the bound and non-bound forms using X-ray crystallography. The crystal structures unequivocally revealed the photochemical states at atomic resolution, clearly demonstrating the switchability of the cysteine linkage.

Results and discussion

Choice of fluorophore for photochemically reversible C-S bond formation

To initiate our investigation, we required a platform that would support photoinduced reversibility of the carbon-sulfur bond. **CM1V**, a fluorescent dye belonging to the Coumarin family, was selected because of its conjugated polyene backbone and strong intramolecular charge transfer (ICT) properties. It is also a bifunctional fluorescent molecule because of its ability to react either at the electrophilic double bond or the aldehyde functional group (Figure 2a). ICT dyes are hypersensitive to the changes in the local environment, displaying polarity dependent spectral shift. Michael addition to the polyene can disrupt the continuity of the conjugation and result in the blue shift of absorption and emission.[38] In fact, various dyes structurally analogous to **CM1V** are used as sensor for thiols (e.g. cysteine, homocysteine, and glutathione) based on this optical change. Thus, we surmised the suitability of **CM1V** as a ligand for labelling hCRBPII.[39-44]

The compound was synthesized following previously reported procedures.[45] It was envisaged that a cysteine residue can be engineered in the binding cavity of hCRBPII to promote Michael addition with the electrophilic double bond**.** The proposed Michael addition would disrupt π-conjugation, resulting in large spectroscopic changes. Further, similar to cyanine dyes, we presumed photoactivation of the complex

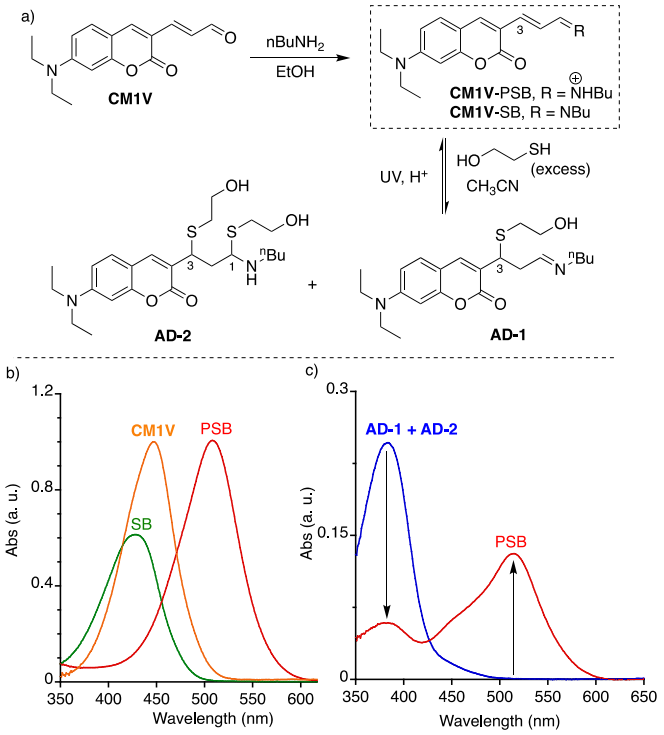

Figure 2. a) Structure of **CM1V** contains two reactive sites, an aldehyde and electrophilic double bond. Reaction of the aldehyde with an amine form either **CM1V**-SB or **CM1V**-PSB. Excess addition of *β*-mercaptoethanol (*β*-ME) to **CM1V**-SB/PSB leads to the covalent complexes **AD-1** and **AD-2**, which can be reverted to the starting material with UV irradiation; b) Absorption spectra of **CM1V**-aldehyde, SB and PSB in acetonitrile; c) Photoirradiation (UV, BP300-400nm) of **AD-1** and **AD-2** complexes (blue spectrum) cleaves the thiol, regenerating **CM1V**-PSB (redshifted in the

would drive the reverse reaction through disruption of the thioether linkage. This putatively reversible reaction would set the stage for the desired light-controlled dynamic covalent labelling. Additionally, a lysine residue appropriately oriented relative to the 'active' cysteine, can form the requisite imine/iminium with the fluorophore's aldehyde. This secondary contact can function as an anchor for the ligand, maintaining the proximity of the ligand to the active cystine residue required for dynamic covalent bonding.

Prior to *in vitro* studies, the photochemical reversibility of the C-S bond with **CM1V** was examined in organic solvents. *β*-Mercaptoethanol (*β*-ME) was used as a surrogate for the active site cysteine residue. The requisite imine (**CM1V**-SB, Schiff base, obtained from the reaction of **CM1V** and *n*-butylamine) was used to mimic the imine/iminium formed with the engineered lysine residue in the binding pocket of hCRBPII (Figures 2a, 2b, and S1). Addition of excess *β*-ME to **CM1V**-SB leads to the formation of thioether linkages at both C_1 and C_3. ^{1}H-NMR analysis confirmed the production of the mixture of the mono (**AD-1**) and the bis (**AD-2)** adducts (Figure S2).

Controlling the stoichiometry of *β*-ME to obtain the mono-addition product was not fruitful, presumably because of competing reactivities at C_1 and C_3. In addition, the reaction was significantly slower with addition of 1 equivalent of thiol, requiring excess to drive the transformation to completion. Nevertheless, as depicted in Figure 2c, the resultant thioether-

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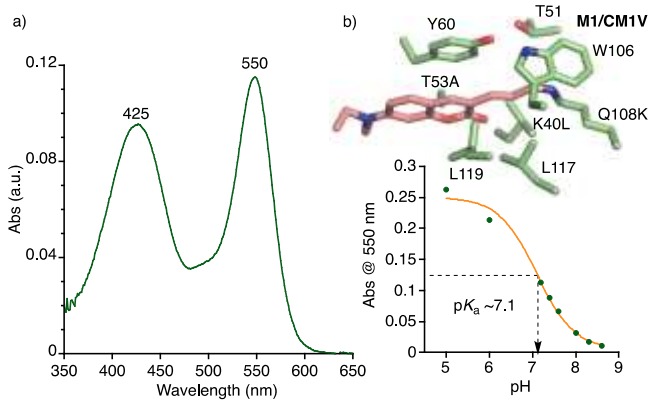

Figure 3. a) Absorption spectrum of **M1/CM1V** exhibits the formation of SB (425 nm) and PSB (550 nm) at physiological pH. The pK_a of **M1/CM1V** iminium is 7.1, thus explaining the presence of SB and PSB at physiological pH; b) Crystal structure of the complex, showing the conjugation of the fluorophore with the protein through an imine bond. The highlighted residues were targeted to incorporate the cysteine required for the dynamic covalent conjugation.

CM1V adduct leads to a truncation in conjugation, which exhibits a blue-shifted absorption (λ_{abs} = 390 nm) in comparison to **CM1V**-SB (λ_{abs} = 432 nm) and **CM1V**-PSB (protonated Schiff base, λ_{abs} = 510 nm) as expected. The Michael addition reaction was tested with both **CM1V**-SB and **CM1V**-PSB, independently. Surprisingly, fully conjugated **CM1V**-PSB required an excess of *β*-ME as observed from UV-vis analysis (Figure S3). This contrasts with the expected reactivity since the PSB is a better nucleophile acceptor and thus should possess higher reactivity as compared to the corresponding SB. We postulate that this is due to the diminished reactivity of *β*-ME in the acidic environment (depressed nucleophilicity), which was required to form the PSB. It is noteworthy that the use of excess *β*-ME (>10^3 equiv.) is required for the optimal photoswitching of cyanine dyes in a basic environment.[34] Nonetheless, the room temperature stable thioether adducts photochemically revert back to the parent **CM1V**-PSB with UV irradiation (BP300-400 nm). The resulting PSB absorbs in the visible region with a significant red shift (>120 nm) from the initial complexes **AD-1** and **AD-2**. This demonstrates a photoactivable system, as it displays changes in spectral properties upon exposure to light. Although 1,2 and 1,4 attack of **CM1V**-SB/**CM1V**-PSB with *β*-ME is observed in solution, we surmised selective Michael addition (C_3 attack) inside an appropriate protein cavity is possible via the incorporation of a single cysteine residue.

Formation of cysteine linkage in hCRBPII/CM1V

Our next efforts were directed towards the design of a protein host capable of recapitulating the results observed with **CM1V**-SB/**CM1V**-PSB and *β*-ME in acetonitrile. hCRBPII belongs to the intracellular Lipid Binding Protein (iLBP) family and has high tolerance to mutations without affecting its structure.[46-48] Our previous reports have demonstrated the ability of engineered hCRBPII to bind a myriad of fluorescent dyes.[49-53] Furthermore, we have shown that the optical properties of these complexes can be tuned by changing the amino acid residues in the binding cavity. Hence, for this study, we chose hCRBPII as a suitable protein host to incorporate an active site cysteine residue as the nucleophile for the Michael addition with a suitable fluorescent ligand.

First, an appropriate hCRBPII mutant was required to bind and react with **CM1V** to form the requisite SB/PSB. Our previous efforts in engineering hCRBPII proved the 108 position is ideally suited for the active site Lys residue (Q108K), while K40L, T53A, R58L, Q38F, and Q4F mutations were required to facilitate and stabilize the formation of the SB/PSB (Figure S4).[48-52] Incubation of **M1** (Q108K:K40L:T53A:R58L:Q38F:Q4F) with **CM1V** led to the formation of PSB (λ_{abs} = 550 nm) and SB (λ_{abs} = 425 nm) at pH 7.2 (Figure 3a). The assignment of the peaks was based on the observed absorptions of the **CM1V** SB/PSB complex formed with *n*-butylamine in acetonitrile (Figure 2b). It is worth noting that the PSB formed with **M1** shows a clear bathochromic shift (~40 nm) as compared to that in acetonitrile, highlighting the effect of the protein environment on the optical property of the ICT dye. The PSB is highly fluorescent with an emission maximum deep into the visible spectrum (λ_{em} = 590 nm, Figure S5). The presence of both species at physiological pH suggests a pK_a of the iminium close to the pH of the solution. In fact, the pK_a of the **M1**/**CM1V** complex was measured to be 7.1 (Figures 3b and S6) Note that an additional control element available with hCRBPII is the ability to alter the pK_a of the fluorophore/protein complex with appropriate mutations of residues that line the binding pocket.[49]

The crystal structure of the **M1**/**CM1V** complex shows the orientation of the ligand inside the binding pocket and suggests

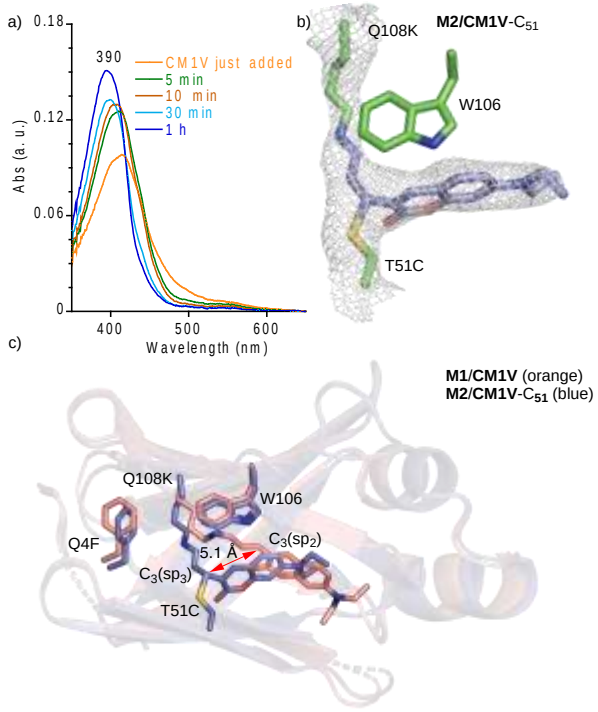

Figure 4. a) Time course study of **M2/CM1V** complexation during the first hour. Blue shift of absorption signals the reduction in conjugation. b) X-ray crystal structure of the **M2/CM1V**-C_{51}, showing the formation of the cysteine linkage. c) Overlay of the crystal structures **M1/CM1V** (in orange) and **M2/CM1V**-C_{51} (in blue) depicting the movement of the fluorophore inside the binding cavity of hCRBPII.

amino acid, suitably oriented for nucleophilic attack when altered to cysteine. As depicted in Figure 3b, **M1** binds **CM1V** to form a *cis*-iminium. Stabilization of the iminium arises from the π-cation interaction of Trp106, positioned 3.6 Å away. The structure provided the opportunity to explore residues within 10 Å of C_3 for incorporation of the necessary cysteine. Residues Leu117, Leu119, Tyr106, Tyr60, Lys40, Thr51, and Thr53 were mutated to cysteine separately in the **M1** mutant. The resulting proteins were conjugated with **CM1V.** Among these mutants T51C exhibited the fastest rate of binding with complete conversion to the product within 6 hours. Hence, T51C mutants of **M1** were used for further studies. Shown in Figure 4a, the UV-vis time-course spectra indicate the diminution of the free aldehyde with increasing adduct formation (λ_{max} = 395 nm) upon incubation of **CM1V** with **M2** (Q108K:K40L:**T51C**:T53A:R58L:Q38F:Q4F) at room temperature. The time-dependent blue shift in absorption is indicative of the reduction in conjugation, putatively as a result of the Michael addition of the cysteine residue at position 51 with the polyene. The adduct is stable for a day at room temperature.

The 1.69 Å resolution crystal structure of the **M2/CM1V**-C_{51} complex showed clear electron density consistent with the formation of a thioether linkage via the Michael addition of T51C to C_3 of the bound ligand (Figure 4b). The overlap of the **M1/CM1V** and **M2/CM1V**-C_{51} structures reveal a large conformational change of the fluorophore upon covalent attachment (Figure 4c). In the new binding orientation, the ligand is forced deeper into the cavity (C_3 has moved by 5.1 Å). Additionally, the chromophore adopts a more twisted conformation to accommodate dual binding with Lys108 and Cys51. As expected, thioether formation changes the hybridization of C_3 ($sp^2 \rightarrow sp^3$), short-circuiting conjugation of the coumarin unit and the imine that results in the observed blue-shifted absorption. It is worth noting the absence of C-S linkage with C_1, unlike that observed with **CM1V** and *β*-ME in acetonitrile (**AD-2**, Figure 2a).

To probe the sequence of transformations (imine formation vs. Michael addition), complexation of **M2** with **CM1V** was followed spectroscopically at 4 °C to reduce the rate of reaction. The time course study demonstrates the concomitant decrease in the absorption of free **CM1V** (460 nm), with the appearance of an intermediate peak at 415 nm (Figure S7). The absorption maximum of this intermediate complex matches well with the **M1/CM1V**-SB that was incapable of Michael addition (lacking the reactive Cys residue). Hence, SB formation (reaction of Lys108 with the aldehyde to generate the imine) presumably is followed by the Michael addition. Likely, this first step helps to orient the olefin such that the cysteine residue in close proximity can engage in the requisite Michael addition, even in the absence of forming the more reactive iminium. Note that only the active-site Lys residue (Q108K) is capable of forming an imine during the time of incubation, although there are 13 other Lys residues in the protein. This was verified upon incubation of **CM1V** with **Q108A**:K40L:T51C:T53A:R58L:Q38F:Q4F, an hCRBPII mutant which is devoid of the active site Lys residue. The complex did not form a SB/PSB with **CM1V** as apparent by the lack of significant spectral change with time and acidification of the medium (Figure S8).

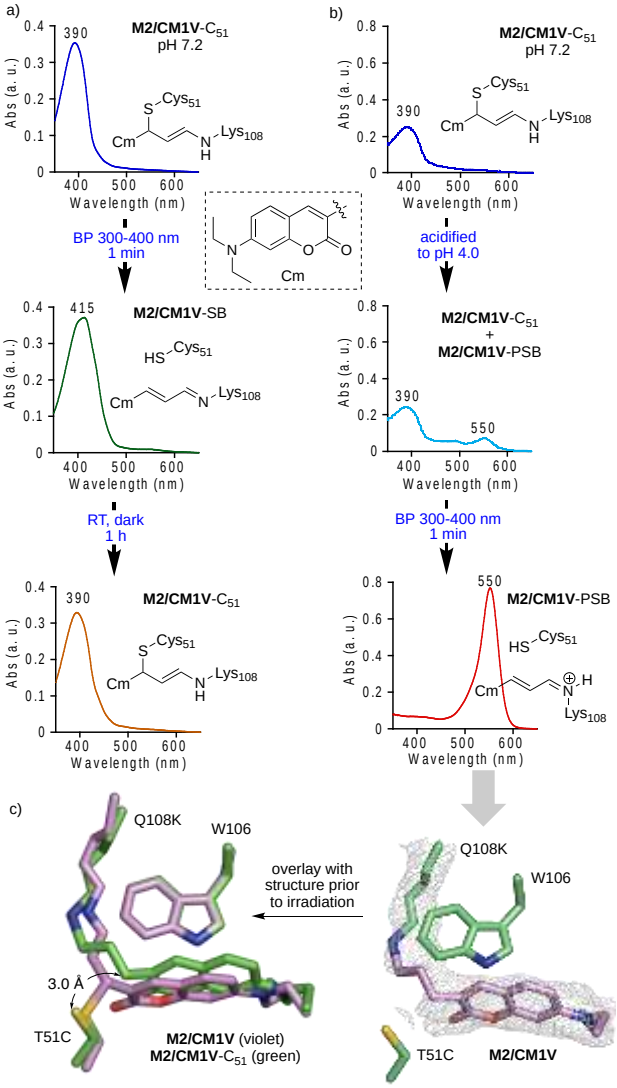

Figure 5. a) UV photoirradiation of the **M2/CM1V**-C_{51} complex (in blue) at pH 7.2 leads to the formation of **CM1V**-SB (in green), which reverts to the initial complex within 1 h (in orange); b) Acidification of the **M2/CM1V**-C_{51} complex (blue), presumably leads to the protonation of the imine as the major populated constituent (cyan). UV photoirradiation of the latter solution generates **CM1V**-PSB (red) as evident by the large change in absorption; c) Crystal structure of the **M2/CM1V-**PSB reveals the release of Cys51 residue after photoirradiation. Its overlay with the structure obtained before photoirradiation illustrates the movement of the chromophore.

Photoactivation of hCRBPII/CM1V through dissociation of cysteine linkage

Having established that **CM1V** undergoes facile Michael addition with Cys51 of **M2**, we next investigated the photodissociation of the thioether linkage which is similar to that observed with the model compound in acetonitrile. The protein-fluorophore complex (**M2/CM1V**-C_{51}) was illuminated with UV light (BP300-400 nm) for 1 minute. As illustrated in Figure 5a, the photoirradiation results in shifting of the absorption from 390 nm to 415 nm, indicating the formation of the fully conjugated SB. This was the first indication that the photodissociation of the C-S bond is possible inside the protein

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cavity. The bond cleavage is reversible, as the fully conjugated SB, formed after illumination, thermally reverted to the initial state within an hour.

Photoirradiation of the **M2/CM1V**-C_{51} complex incubated in an acidic buffer (pH = 4.0) promotes the cleavage of the thioether linkage, leading to the formation of the fully conjugated PSB as apparent by its red-shifted absorption (λ_{abs} = 550 nm, Figure 5b). In contrast to the SB, the newly generated PSB does not convert back via Michael addition. Although surprising, since the PSB is a more electrophilic system, the lack of reactivity may be related to an unproductive conformation of the fluorophore in its protonated state. We have previously observed large conformational changes of bound ligands upon protonation of the imine.[52, 54-55] It is also possible that under acidic conditions, the nucleophilicity of Cys51 is attenuated to a degree that hinders addition to the polyene. In fact, this was observed in our earlier studies where *β*-ME was less reactive

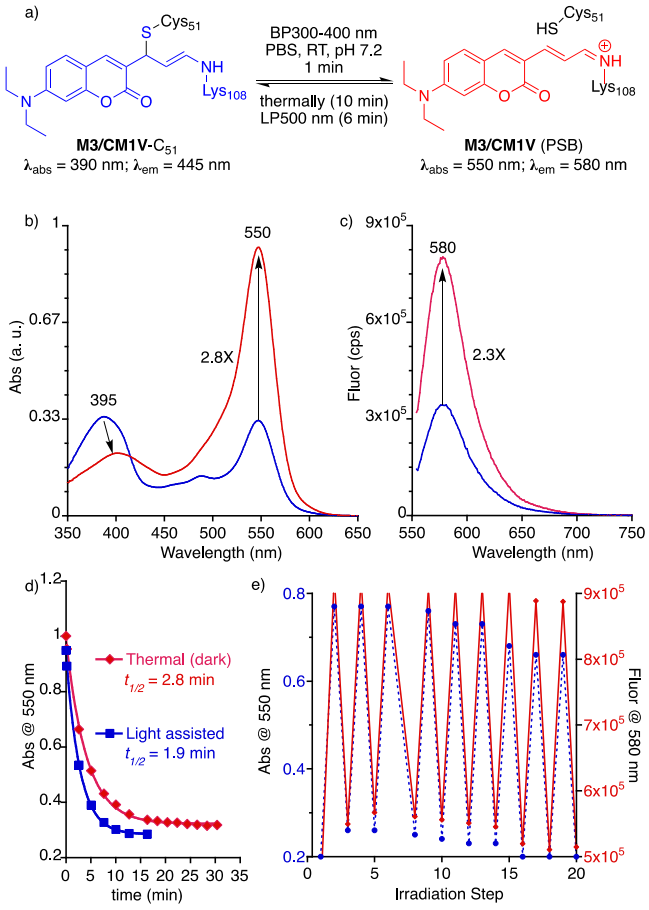

Figure 6. a) Photoswitching of **M3/CM1V**-C_{51} at physiological pH illustrates the reversibility of the cysteine linkage. Unlike photo-induced cleavage of the C-S bond of **M2/CM1V**-C_{51} in acidic conditions, the **M3/CM1V** PSB reverts to the initial cysteine bound complex (**M3/CM1V**-C_{51}); b,c) Photoirradiation (BP300-400 nm) of **M3/CM1V**-C_{51} leads to a 2.8x increase in absorption, along with a 2.3x increase in the fluorescence (excited at the PSB absorption, 550 nm; d) Decay of the PSB as observed via its absorption through thermal (red) and continuous irradiation (LP500 nm, blue); e) Iterative Michael and *retro*-Michael addition of Cys51 with the **M3/CM1V** complex is observed with sequential photoirradiation (BP300-400 nm for 1 min, and LP500 nm for 5 min).The change of absorption is denoted by the dashed blue line, while the change in emission is highlighted with the solid red line.

with the **CM1V**-PSB, presumably due to the increased acidity of the solution that was required to generate the PSB.

In an effort to unravel the mechanism of photoswitching, crystals of the **M2/CM1V**-C_{51} were incubated in pH 7.2 and 4.0 buffers, irradiated with UV light (380 nm) for 5 minutes and subsequently frozen in liquid nitrogen within 30 seconds of irradiation. The electron density of photoirradiated crystals incubated at pH 7.2 showed no evidence of bond cleavage (Figure S9). This is either because the cleavage in the solid state does not occur, or that the rebinding of the thiolate with the photo-dissociated ligand in the crystal is fast and thus cannot be trapped. The results from irradiation of crystals incubated at pH of 4.0 support the latter supposition. Crystals photoirradiated under acidic conditions yield structures lacking electron density for the thioether linkage, clearly demonstrating the dissociation of C-S bond (Figure 5c). The red coloring of the crystal indicates the presence of the anticipated chromophore as a PSB (red-shifted). The observed conformational change of the chromophore following photoirradiation is demonstrated in the overlayed crystal structures of bound and unbound forms. Despite the proximity between the reactive Cys51 and the C_3 of the unbound **CM1V** (3.0 Å, Figure 5c), **M2/CM1V**-C_{51} acts as a photoactivable system, as UV exposure switches the OFF state ($\lambda_{abs/em}$ = 390/450 nm) to the ON state ($\lambda_{abs/em}$ = 550/580 nm, see Figure S10 for fluorescence spectra).

Photoactivation of hCRBPII/CM1V to form PSB at pH 7.2

The **M2/CM1V**-C_{51} complex provided proof-of-principle that an active site thiol (Cys51) can engage in a Michael addition reaction, and that *retro*-Michael reaction can be triggered with the appropriate wavelength of light. Nonetheless, a system with practical use would benefit from a large change in absorption upon 'ON/OFF' transition, especially at a physiologically relevant pH. As mentioned earlier, under photoirradiation **M2/CM1V** complex forms a blue-shifted imine at pH 7.2 due to the low pK_a (pK_a = 5.2, Figure S11). To generate a system that would lead to a large shift in absorption, the ground state complex should support the formation of a PSB at physiological pH. Additionally, we would expect an accelerated rate for the Michael addition with the more electrophilic polyene that terminates with the iminium. Note that this scenario is distinctly different from the PSB generated with the **M2/CM1V** complex (Figure 5b), where the thiol seemed unreactive to engage in the Michael addition. In the case above, the PSB was generated by acidifying the medium, potentially attenuating the reactivity of the cysteine residue, while in this case, we desire a protein with a higher iminium pK_a such that it is protonated at physiological pH (Figure 6a).

The **M2/CM1V**-C_{51} crystal structure suggests that Phe4 is well situated to increase the pK_a of the SB if appropriately mutated (Figure 4c). As predicted, re-introduction of the wild-type Gln4 to generate **M3** (Q108K:K40L:T51C:T53A:R58L:Q38F:**Q4**) led to a protein host capable of generating the PSB upon incubation of **CM1V** at physiological pH. The putative PSB absorbs at 550 nm, which over time reduced in intensity, presumably as a result of Cys51

Michael addition that interrupts the fully conjugated system (Figure S12). The mature **M3/CM1V**-C_{51} complex exhibits two peaks (550 nm and 390 nm), apparently due to incomplete conversion to the Michael adduct. Irradiation of the **M3/CM1V**-C_{51} complex with UV light (BP 300-400 nm) for 1 minute led to a substantial increase in the PSB absorption (550 nm), likely because of the *retro*-Michael reaction that liberates the fully conjugated PSB complex. During this process, the absorption was increased by 2.8-fold, while the fluorescence observed at 580 nm was enhanced by more than 2-fold (Figure 6b). The system turns 'OFF' within 10 minutes in the dark as evident by the decay in the PSB absorption with a half-life ($t_{1/2}$) of 2.8 minutes at room temperature (Figure 6c). The decay could be accelerated by irradiation with visible light (LP500 nm) with an apparent half-life of 1.9 minutes. It is important to note that no temperature change was observed during the photoirradiation process, thus the increased rate is solely because of light exposure.

Crystals of the **M3/CM1V** complex were informative in understanding the system (Figure 7a). They were deeply red colored (Figure S13), consistent with the presence of a PSB. Efforts at conversion between the PSB and the covalently bonded cysteine adduct at pH 7.2, however, were unsuccessful in the crystalline form. The PSB is in the *cis* iminium conformation, making a cation-π interaction with Trp106, similar to that seen in the **M1** variant. The distance between the Cys51 sulfur atom and C_3 of **CM1V** is 7.0 Å, too far for a covalent bond to form. This leads to the conclusion that the spectroscopically observed mixture (550 and 390 nm absorptions) in the resting state of the **M3/CM1V** complex are due to the presence of two conformations, which ultimately have a bearing on the reactivity of the conjugated polyene with the active-site Cys residue. One conformation is competent for photoswitching between a visible light absorbing conjugated form and the covalently bound Cys51 UV absorbing form, while the other is in a conformation consistent with a stable conjugated PSB. The form of the **M3/CM1V** complex that crystallizes is clearly the non-interconverting permanently conjugated PSB, absorbing at 550 nm. The conformation that interconverts is presumably similar to that seen in the **M2/CM1V** structure, where the imine points to residue 4 (in this case Phe4) in the visible light absorbing PSB form (Figure 7b).

Iterative ON/OFF switching, critical for certain imaging applications, was conducted to probe the stability and durability of the complex. As depicted in Figure 6e the conversion between the cysteine bound (OFF state) and non-bound form (ON state) was performed repeatedly using UV (BP 300-400 nm) and visible light (LP500 nm) irradiation. The **M3/CM1V**-C_{51} complex showed good fatigue resistance, with minimal loss in fluorescence signal upon each ON/OFF cycle (insignificant loss up to twenty cycles). This indicates a robust photoswitching process where the cysteine residue adds reversibly to the fluorophore. Analogous to **M2/CM1V**-C_{51}, the switching of **M3/CM1V**-C_{51} does not occur at acidic pH as the photoirradiation leads to the formation of a stable PSB (Figure S14). However, at physiological pH, Cys51 rebinds after photodissociation in both cases. Note that the photoirradiation

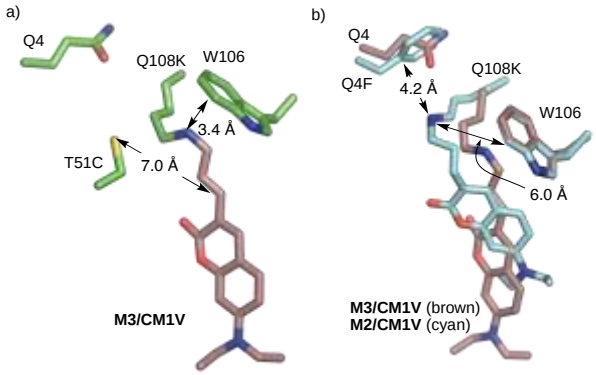

Figure 7. a) X-Ray crystal structure of **M3/CM1V** representing the non-interconverting PSB where the reactive cysteine is far away from the C_3 of the ligand. b) Overlay of **M3/CM1V** and UV-irradiated **M2/CM1V** crystal structures, depicting the role of Q4 in forming a PSB which is

of **M2/CM1V**-C_{51} leads to a SB whereas **M3/CM1V**-C_{51} generates a PSB at the same pH (Figures 5a and 6b). In total, the data suggests that cysteine addition is dependent on the pH of the solution regardless of the reactivity of the imine. Thus, the lack of photoswitching at acidic pH is most likely the result of attenuated reactivity of Cys51 even in the presence of the highly reactive iminium. Nonetheless, the reactivity of the cysteine can be tuned in the protein through rational mutagenesis of nearby residues. Hence, unlike Cy5/thiol, this photoswitchable complex requires a near stoichiometric addition of **CM1V** with hCRBPII. Moreover, the switchability is maintained in presence of oxygen, and thus can be useful for imaging in aerobic conditions.

Conclusions

To the best of our knowledge, the hCRBPII/**CM1V**-C_{51} complex illustrates the first example of a reversible site-specific labelling of a protein with a fluorophore that employs light induced dynamic covalent chemistry of a cysteine residue. The regulation of the protein microenvironment by rational mutagenesis yields a photoactivatable protein/fluorophore complex with large shift in absorption at physiological pH. Moreover, the conjugation can be cycled between cysteine bound and non-bound forms, repetitively, making it a photoswitchable system. Structural evidence indicates a process involving a Michael type addition reaction of a nucleophilic cysteine residing in the binding pocket of the host protein with an electrophilic double bond. The relatively weaker carbon-sulfur bond is broken under photoirradiation to liberate the cysteine residue and the fully conjugated polyene through a *retro*-Michael process. This approach utilizes the reactivity of an α,β-unsaturated aldehyde-based dye, and thus opens the door for expanding the methodology by exploring a broad range of intramolecular charge transfer (ICT) dyes with a similar functional handle for imaging purposes.

Author Contributions

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The bulk of the writing was the work of SM, JHG, and BB. The mutations and photophysical measurements were performed by SM, STN, and TEK. Crystallography was performed by CB and NE under the supervision of JHG. Synthetic efforts were the work of SM and DC. CV, JHG, and BB oversaw the supervision of the overall project.

Conflicts of interest

There are no conflicts to declare.

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