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Authors
Ritterson, Ryan S
Hoersch, Daniel
Barlow, Kyle A
et al.

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Design of Light-Controlled Protein Conformations and Functions

Ryan S. Ritterson, **Daniel Hoersch**, **Kyle A. Barlow**, and **Tanja Kortemme**

Abstract

In recent years, interest in controlling protein function with light has increased. Light offers a number of unique advantages over other methods, including spatial and temporal control and high selectivity. Here, we describe a general protocol for engineering a protein to be controllable with light via reaction with an exogenously introduced photoisomerizable small molecule and illustrate our protocol with two examples from the literature: the engineering of the calcium affinity of the cell–cell adhesion protein cadherin, which is an example of a protein that switches from a native to a disrupted state, and the engineering of the opening and closing of the chaperonin Mm-cpn, an example of a switch between two functional states. This protocol guides the user from considering which proteins may be most amenable to this type of engineering, to considerations of how and where to make the desired changes, to the assays required to test for functionality.

Keywords

Photoswitches; Computational protein design; Light-modulatable proteins; Protein engineering

1 Introduction

There has been considerable interest in light-based control of protein functions [1], and successful applications include light modulation of neuronal ion channels [2], light-switchable cell adhesion proteins [3], and light-controlled protein machines [4]. Light-based methods offer titratable, precise spatial, and temporal regulation that has been demonstrated in vitro [5], in cell culture [6, 7], and in whole animals [8]. Most examples of light-based control fall into one of two categories: (a) those that are genetically encoded using fusions with a light-sensitive protein borrowed from nature [6] and (b) those created via targeted insertion of amino acids into a protein sequence and subsequent reaction with them of an exogenously introduced photoisomerizable small molecule, typically azobenzene based [9]. Azobenzene and its derivatives undergo a reversible *cis–trans* isomerization upon illumination with either near-ultraviolet or visible light, leading to a change in end-to-end distance of ~18 Å in the *trans* state to a ~5–12 Å distribution in the *cis* state; this change in molecular shape can then be coupled to changes in protein function.

In this chapter, we describe two related methods for designing category (b) molecules (Fig. 1). The first method was applied to engineer light control of the group II chaperonin Mm-cpn [4] and illustrates the design of protein photoswitches that reversibly change between two known protein conformations. The described design method is useful when the target

protein has two known functional conformations (e.g., chaperonins, nuclear hormone receptors, many small molecule binders), and the researcher would like to maintain both of them. (Note that, due to the two-state nature of azobenzene-based molecules, directing proteins into three or more conformations using light alone would require additional engineering.) The second method was applied to engineer light control of the cell adhesion protein cadherin [3] and designed a protein photoswitch that reversibility changes from a native to a disrupted conformation. Such designs are useful when a target protein has one functional conformation of interest to be disrupted, and the conformation in the disrupted state need not be specified in detail. We also include basic protocols for conjugating the small molecule to the protein, as well as for measuring the extent to which the protein is conjugated and switchable.

In general, the considerations for both design protocols share much in common. Most importantly, the target protein must be suitable for cross-linking with a cysteine-reactive small molecule at defined sites, which requires elimination of all or most native cysteines and introduction of two nonnative cysteines to use as conjugation sites for cross-linking. In addition, the distance of the side-chain sulfur atoms of the engineered cysteines in the conformation(s) of the target to be maintained must match the end-to-end distance of the chosen azobenzene molecule in its *trans* and/or *cis* states, and the side-chain sulfur atoms of the engineered cysteines must be solvent accessible for cross-linking. The requirement of ensuring geometric compatibility necessitates experimentally determined structures or high-quality models for the protein conformations to be maintained. In addition, as initial characterizations of the photoswitches are carried out in vitro, protein targets should be amenable to protein purification and, ideally, be stable.

Where the protocols differ is in the detailed constraints necessary to satisfy. With two protein target states, one must find an interatomic distance between a pair of residues in the target such that in one state the distance is only compatible with one isomer of the chromophore and the second state only compatible with the other isomer of the small molecule; illumination should then lead to selective destabilization of one of the two protein states and hence reversible interconversion between them. Two states provide the advantage that the desired structures of both illumination states are known, increasing the probability that successful conjugation will produce a functional photoswitch. However, because of the additional geometric constraints, it is likely that only a small number of suitable cross-linking sites will be available, reducing the likelihood of finding one compatible with protein stability, structure, and function.

In comparison, with one functional state, one only needs to find a pair of attachment sites compatible with one isomer of the chromophore. Illumination should alter this distance and thus disrupt the conformation and function of the protein target. Because the geometric constraints need only be satisfied in one conformation, there are likely many more possible attachments sites. As the conformation of the illuminated state is not known, however, it is more likely that chromophore illumination may not result in the desired change in protein function. For example, if the local conformation of the protein is too flexible, the change in structure of the chromophore may be accommodated without a significant change in protein structure [9, 10].

2 Materials

1. Azobenzene chromophore suitable for cross-linking cysteine residues. For Mm-cpn we used azobenzene–dimaleimide (ABDM), and for cadherin we used 3,3′-bis(sulfonato)-4,4′-bis(chloroacetamido)azobenzene (BSBCA).
2. Structure(s) or high-quality models of protein of interest.
3. Academic license of the molecular modeling and design program Rosetta [11].
4. A protein structural viewer (such as PyMOL) capable of measuring distances, or script to compute distances from coordinates.
5. Supplies required to purify the target protein.
6. An assay for target functionality. Mm-cpn, 4 % native polyacrylamide gel (PAGE); cadherin, surface plasmon resonance (SPR).
7. UV–Vis spectrophotometer to determine the switching efficiency of the chromophore.
8. An assay to determine the efficiency of chromophore conjugation/cross-linking. Mm-cpn, 4–20 % SDS-PAGE gel; cadherin, mass spectrometer that is capable of detecting 1 Da changes in whole proteins, such as a Waters LCT Premier.
9. Illumination sources for ultraviolet and visible light to switch the chromophore between the *cis* and *trans* isomerization states. Mm-cpn and cadherin: high-power LEDs with emission wavelength of 365 nm (1 W, Advancemart) and 455 nm (3 W, SparkFun).

3 Methods

3.1 Computational Design of Protein Photoswitches

In this section, we detail the strategies and techniques to computationally design protein photoswitches with the goal of producing a ranked-ordered list of pairs of cysteine mutations to introduce into the target protein. At certain points in the method, the procedures bifurcate into parallel methods, depending on whether the target protein of interest has two conformations to maintain and switch between or a single conformation to disrupt.

3.1.1 Mutational Robustness—In order to preserve target structure and stability, locations must be identified within the target structure where a pair of cysteines can replace the native residues with minimal disruption to the overall fold. One strategy to identify the safest positions for these mutations is to estimate the folding free energy contribution of each native side chain. This energy is commonly estimated by independently mutating each residue to alanine, as alanine reduces the side chain to a single methyl group. This procedure, “alanine scanning,” can be performed experimentally or computationally, which has provided rich data for the development of robustly tested computational protocols, including within Rosetta [12, 13]. Alanine scanning can be run within Rosetta using the

RosettaScripts XML scripting interface. Detailed instructions are available within the RosettaScripts documentation [14].

We use computational alanine scanning within Rosetta to identify positions amenable to mutation to cysteine by only allowing mutations at positions where mutation to alanine is predicted not to destabilize the protein significantly (energy increase of less than 1 Rosetta energy units (approximately 1 kcal/mol); all positions that had a decrease in energy were accepted).

3.1.2 Distance Matching

1. To shift an equilibrium between two defined conformational states of a protein, select cross-linking positions for which the following criteria are satisfied:
   - **(a)** The expected distance of the side-chain sulfur atoms of the engineered cysteines in one conformational state matches the end-to-end distance of the chosen chromophore in its *trans* state, and the other conformational state matches the length of the *cis* state (positive design) (*see* **Note 1**).
   - **(b)** Each isomerization state is only compatible with one of the two conformational states of the protein and not the other (negative design).
2. To shift an equilibrium between one functional and one nonfunctional state (or ensemble) of a protein, select cross-linking positions for which the following criteria are satisfied:
   - **(a)** The expected distance of the side-chain sulfur atoms of the engineered cysteines in the functional, known state matches the end-to-end distance of the chromophore in either the *trans* or *cis* states.
   - **(b)** The expected distance of the side-chain sulfur atoms of the engineered cysteines after isomerization is incompatible with the functional state, leading to a disrupting conformation change in the protein. This can be accomplished, e.g., by disrupting secondary structure or distorting conformations of functional loops.

3.1.3 Solvent Accessibility—In order for the azobenzene chromophore to react efficiently with the target, the residues chosen as cross-linking sites must be solvent accessible. Using PyMOL [15] (or another method of the users' choice), identify all residues that are buried (have a solvent accessible surface area below a given threshold) and remove them from the list of possible mutations.

[1]Keep in mind that the end-to end distance distribution of the *cis* isomerization state is significantly broader than the one of the *trans* state (i.e., more rigid due to the planar extended π electron system) [9].

3.1.4 Steric Clashes (Visual Inspection)—Finally, residues to be cross-linked must be pointing toward each other, and the chromophore must be sterically compatible with the protein structure (e.g., a line drawn between the C_β atoms should not penetrate the protein). Generally, this process is easily done by visual inspection; in our experience, the vast majority of potential cross-link pairs are obviously sterically incompatible, leaving only a few pairs for consideration.

3.1.5 Protocol for the Structure-Based Design of a Photoswitchable Mm-cpn—The following two sections describe the specific parameters of the general protocol that we used for engineering photoswitchable Mm-cpn (Subheading 3.1.5) or cadherin (Subheading 3.1.6):

1. Using the PDB structures of Mm-cpn in the open and closed conformation (identifiers 3IYE and 3IYF, cryo-EM structures, and 3KFB and 3KFK, X-ray structures), calculate the expected distances between sulfur atoms for every possible pair of cysteine mutations in neighboring subunits of Mm-cpn as well as the expected accessible surface area of the sulfur. To create the models of the cysteine mutants and to do the calculations, use PyMOL or software of your choice.
2. Screen for residue pairs with an expected sulfur distance of 5–14 Å in the closed and 16.6–19.5 Å in the open state and a minimum expected surface accessible area for the sulfur atoms of 8 $Å^2$ (10 % of the maximum surface-exposed area of the sulfur atom in a deprotonated cysteine residue).
3. Keep residue pairs which satisfy the selection criteria in both sets of structures (3IYE/3IYF and 3KFB/3KFK).
4. Exclude residue pairs with an intra-monomer (Mm-cpn is a homooligomer of 16 subunits) distance smaller than 19.5 Å to avoid off-target cross-linking.
5. Visually inspect the list of possible cross-linking sites for residue pairs for which there is enough unoccupied space between the attachment sites to accommodate the chromophore ABDM, and choose promising cross-linking sites for in vitro testing.

3.1.6 Protocol for the Structure-Based Design of Photoswitchable Cadherin

1. Using Rosetta and the PDB structures of cadherin (identifiers 1FF5, 1EDH, 2O72, and 1Q1P) with the methodology described in Subheading 3.1.1, computationally mutate all residues in the protein to alanine, and record the predicted change in protein stability. Eliminate all residues with predicted change in stability >1 Rosetta energy unit.
2. Eliminate all residues that directly bind calcium ions.
3. For the residues that remain, compute the C_β–C_β distance between all possible remaining pairs. Eliminate all pairs whose distances do not fall

into the range 17–20 Å. After this step, the number of potential cross-linking pairs was reduced to approximately 1500.

4. Eliminate all pairs that do not have at least one cross-linking site (C_β atom) within 20 Å of a calcium ion.

5. Eliminate all pairs that do not have solvent accessible surface area (SASA) of both cross-linking sites >30 $Å^2$. After this step, the number of potential pairs was reduced to approximately 300.

6. Visually inspect the remaining pairs by drawing a line between the cross-linking sites. Eliminate all pairs whose line intersects with the protein structure, by using the surface representation in PyMOL. This step reduced the number of possible pairs to approximately 30.

7. Using experimenter judgement, select a subset of pairs that meet the criteria of the study. We chose ten pairs based on a desire to have a diverse set of potential cross-linking sites.

3.2 Protein Engineering

After selecting the potential cross-linking sites via computational design, the next stage is to express and test the selected pairs experimentally. This method describes the removal of native cysteines and the addition of the cross-linking cysteines.

3.2.1 Elimination of Native Cysteines—The chromophores we used (ABDM and BSBCA) are cysteine reactive. As native cysteines in the protein target may also be reactive and give undesired side products, native cysteines should be removed, if possible, prior to mutation to cysteine residues at the cross-linking sites (*see* **Note 2**).

1. Using a suitable cloning method (e.g., Gibson assembly, site-directed mutagenesis), mutate a single native cysteine residue in the protein-coding sequence to an alternate amino acid (*see* **Note 3**).

2. Express and purify the mutated protein using a method appropriate for the specific protein, and then test the change in stability of the protein after the mutation using a method of choice (*see* **Note 4**).

3. If the protein has a specific function to be maintained, test changes in protein functionality after the mutation using a method appropriate to the specific function.

[2]Deeply buried cysteines in the native protein may not be reactive and could be maintained. However, proteins often have some flexibility and can transiently expose buried positions. As a result, we recommend attempting to mutate all native cysteines and adding back those that cannot be mutated to an alternate residue without compromising protein stability or function.

[3]We recommend using serine as a replacement for surface-exposed cysteine and alanine for buried cysteines. In theory, all cysteines could be removed in one step. Sequential mutation, although time consuming, allows one to identify any particularly troublesome cysteines that may have to be added back later.

[4]There are a multitude of protein expression and purification methods, and the choice of a particular method is outside the scope of this chapter.

4. If **steps 1–3** result in a satisfactory outcome, repeat **steps 1–3** for an additional cysteine residue, continuing until all possible cysteines have been removed.

5. If **steps 1–3** do not result in a stable or functional protein, replace that cysteine by a different residue (repeat **steps 1–3**) or maintain the cysteine and repeat **steps 1–3** for the next cysteine in series (*see* **Note 5**).

3.2.2 Addition of Cross-Linking Cysteines—After all possible native cysteines have been removed, mutations to nonnative cysteines can be made.

1. Using a suitable cloning method (e.g., Gibson assembly, site-directed mutagenesis), mutate both native residues at the targeting cross-linking sites in the protein-coding sequence to cysteine.

2. Express and purify the mutated protein using a method of choice appropriate for the specific protein, and then test the change in stability of the protein after the mutation using a method of choice (*see* **Note 6**).

3. If the protein has a specific function to be maintained, test changes in protein functionality after the mutation using a method appropriate to the specific function.

4. If **steps 1–3** result in a satisfactory outcome, keep the potential pair. If not, eliminate it from future consideration.

3.3 Conjugating Protein with Small Molecule

In this section, we detail how to cross-link the azobenzene-based chromophore to the target protein, with the goal of optimizing the percentage of cross-linked and folded protein. We describe details of the important parameters controlling the outcome of the reaction, including how they may change based on the particular chromophore chosen.

3.3.1 Choice of Chromophore and Reactive Group (ABDM vs. BSBCA)

1. Chromophore absorption spectrum. Azobenzene cross-linkers with have been recently developed to enable the user to choose between a wide variety of wavelengths to switch the isomer equilibrium of the chromophore [9, 16–18]. The two chromophores used in the methods described in this chapter have the following absorption properties: The absorption peak of the **π**–**π*** transition of the *trans* state of ABDM is at 342 nm, and the long wavelength n–**π*** band of the *cis* state used for selective *cis*–*trans* isomerization is at 440 nm [19]. For BSBCA, the **π**–**π*** band is shifted to 363 nm and the *cis* n–**π*** band is at 450 nm [20].

[5]It is possible that surface-exposed cysteines distant from the intended cross-linking sites, even if they are labeled with chromophore, will not cross-link the protein and thus may not affect function. However, the presence of those additional labeled cysteines complicates measurement of protein concentration, conjugatability, and switchability.

[6]When purifying and handling cysteine-containing proteins, maintain reducing agent (e.g., DTT, TCEP) wherever possible to avoid oxidation/disulfide bond formation of cysteine residues. Note, however, that thiol-based reducing agents can interfere with chromophore conjugation.

2. Reactive groups. Maleimide, the reactive group of ABDM, reacts fast and specifically with thiols at a pH between 6.5 and 7.5 but is unstable in water. Proteins can be cross-linked at incubation times of less than 1 h at RT and at fairly low concentrations of protein and cross-linker (e.g., *see* Subheading 3.3.3). This strategy may be advisable for the conjugation of sensitive target proteins or for the conjugation of metastable protein states.

Chloroacetamide, the reactive group of BSBCA, is also specific toward thiols, but is stable in water, and its reactivity is considerably lower than that of maleimides. This makes incubation times of several hours, high chromophore concentrations, optimized buffer conditions, and elevated incubation temperatures necessary to achieve satisfactory conjugation efficiency (*see* Subheading 3.3.4).

3. Chromophore solubility and bistability of azobenzene isomerization states. ABDM is not very soluble in aqueous solutions in its *trans* isomerization state. Therefore, it is advisable to cross-link a protein with ABDM in the *cis* state after pre-illumination with UV light. An advantage of ABDM, however, is the high bistability of its two isomers. The *cis* isomerization state is stable for several hours due to a low rate of the thermal *cis* to *trans* isomerization [4, 19].

BSBCA is designed to be highly soluble in water due to the addition of sulfonate groups to the aromatic rings of the azobenzene. The rate of thermal *cis* to *trans* isomerization at room temperature is approximately 20 min, though this can be considerably longer when conjugated to protein [3, 20].

3.3.2 Reaction Conditions—*See* Burns et al. [20] for a comprehensive overview of conjugation reaction conditions with BSBCA. For ABDM refer to [4, 19, 21] or the protocol below.

3.3.3 Protocol for Conjugating Mm-cpn with ABDM

1. Dilute purified Mm-cpn to 500 μl at a concentration of 0.25 μM Mm-cpn (complex concentration) in Buffer A (20 mM HEPES pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 10 % glycerol).
2. Bias the conformational equilibrium of Mm-cpn toward the closed state via addition of $ADPAlF_X$ (a phosphate analogue which binds to hydrolyzed ATP after phosphate release) by adding 1 mM ATP, 6 mM $Al(NO_3)_3$, and 25 mM NaF to the solution (buffer A+, pH 7.0), and incubate the sample for 20 min at 43 °C [22].
3. Dissolve ABDM in dimethylformamide (DMF) to a concentration of 1.2 mM. Prior to cross-linking, illuminate ABDM for 1.5 min using the UV LED. UV illumination results in an accumulation of ~75 % *cis* isomer in the solution (estimated by analyzing the absorption spectrum of the sample [19]).
4. Add ABDM to the Mm-cpn solution at a ratio of 1 μl ABDM solution per 50 μl protein solution and shield the sample from background

illumination. Quench the reaction after 40 min incubation time by adding dithiothreitol (DTT) to a concentration of 2 mM.

3.3.4 Protocol for Conjugating Cadherin with BSBCA

1. Dilute purified cadherin (protocol described in Ritterson et al. [3]) to a final concentration of 160 μM in 25 mM Tris–HCl pH 8.5, 400 mM NaCl, 1 mM EDTA, 3 mM KCl, 3 mM Tris(2-carboxyethyl)phosphine (TCEP), 500 μM BSBCA.
2. Place reactions at 25 ° C in the dark for 72 h.
3. Desalt excess chromophore using a HiPrep 26/10 (GE) column (*see* **Note 7**) into 25 mM Tris–HCl pH 8.5, 400 mM NaCl, 1 mM EDTA, 3 mM KCl, 3 mM TCEP.

3.4 Measuring Protein Conjugatability

In this section, we describe methodologies for measuring the fraction of total protein conjugated, with the goal of providing the researcher insight into which parameters of the reaction may need optimization and information about which cysteine pairs conjugate most completely. As in other sections, the method splits into parallel methods, based on the target structure of interest (cadherin monomer versus Mm-cpn chaperonin protein complex). Generally, a wide range of potential methods are possible, and the particular method chosen will depend on the protein target of interest.

3.4.1 Measuring Cross-Linking Ratio for ABDM-Mm-cpn with an SDS-PAGE Gel

1. Remove 20 μl of the sample cross-linked in Subheading 3.3.3, and analyze it on a 4–20 % gradient SDS-PAGE gel. Formation of covalently linked Mm-cpn multimers after subunit cross-linking by ABDM leads to multimer bands which can be easily distinguished from the 60 kDa band of the Mm-cpn monomer (*see* Fig. 2 in ref. [4]).
2. Estimate the cross-linking stoichiometry of the sample defined as the fraction of cross-links to possible cross-linking sites in the protein ensemble by calculating the sum of the relative intensities of the multimer bands (band intensity divided by the sum of the band intensities for all multimers) weighted by their ratio of cross-links to subunits using, e.g., the ImageJ [23] software package.

3.4.2 Estimating Conjugatability for Cadherin Using Mass Spectrometry—This example method for photoswitchable cadherin assumes one has the results of a conjugation reaction on hand (from Subheading 3.3.4) and wishes to know to what extent the reaction completed. Buffers are provided in the original work [3].

[7]BSBCA tends to migrate slowly in common chromatography media and can be difficult to elute, especially in the presence of salt. It can be removed by washing the column thoroughly and repeatedly with pure water.

1. Estimate the protein concentration using A_{280}.
2. Dilute protein to an estimated 1 μM concentration in pure water (*see* **Note 8**).
3. Inject the conjugated sample into the mass spectrometer, observing a peak at 23,813 Da for unconjugated protein and 24,266 Da for conjugated (*see* **Note 9**).
4. Estimate the fraction of protein conjugated by calculating the peak areas for each subspecies and dividing the area of the conjugated peak by the sum of the areas of all subspecies. Potential conjugatabilities range widely, from 0 to 100 % depending on the cysteine pair (*see* **Note 10**).

3.5 Measuring Chromophore Switchability/Rate of Thermal Cis–Trans Back Reaction

In this section, we describe a method to determine the extent to which the chromophore in a cross-linked system undergoes isomerization upon illumination, without describing whether that isomerization causes a functional change in protein structure or state. We also provide a method to measure the half-life of the *cis* isomerization state, so that a researcher may determine which photoswitchable candidates are most promising to test in functional assays.

3.5.1 Illumination Techniques—The *cis–trans* isomer equilibrium of azobenzene-based chromophores can typically be switched in the direction of the *cis* state by exciting the **π**–**π*** band of the *trans* state in the near UV. To switch the equilibrium in the direction of the *trans* state, excite the n–**π*** band of the *cis* isomer with blue (or green) light [9]. We recommend the use of high-power LEDs for illumination as they are widely available, relatively inexpensive, portable, and intense enough to isomerize protein in bulk within seconds to minutes. Lasers can also be used for illumination, particularly in microscope and other applications where high spatial precision is desirable. Keep isomerized protein in the dark to the extent possible to prevent undesirable isomerization due to ambient light.

3.5.2 UV–Vis Spectroscopy—Prior to assessing the extent to which illumination modulates protein function, we recommend determining whether the chromophore conjugated to the protein is photoisomerizable by illuminating *trans*-relaxed protein with UV light. The *trans* states of the azobenzene-based chromophores used in this protocol have a characteristic near-UV absorption peak of the **π**–**π*** transition, and, upon illumination at that wavelength, the peak amplitude decreases as the small molecule isomerizes into the *cis* state.

1. Measure extinction coefficient $\boldsymbol{\varepsilon}_{trans}$ of the unconjugated protein for the peak wavelength of the **π**–**π*** transition of the chromophore (ABDM: 342 nm; BSBCA 363 nm) using protein at a known concentration (if the

[8]The presence of salts leads to adduct formation and the appearance of side peaks in the instrument, obfuscating the results. Cadherin is stable for hours in pure water without any salt; the stability of other proteins may vary.
[9]For BSBCA, the conjugated, cross-linked protein will appear at +453 Da relative to unconjugated protein. In our hands, we never observed single-linked protein or protein conjugated to two single-linked chromophores. This is likely due to the much faster intramolecular reaction rate of the single-linked protein to the remaining cysteine compared to side reactions. If native cysteines in the protein were required to be maintained, however, reaction to them by chromophore will result in the appearance of additional peaks.
[10]We assume the ionizability of the cross-linked protein is the same as the uncross-linked for the purposes of computing cross-linked fraction.

protein has no cofactor bound that absorbs light at that wavelength, ε should be approximately zero) (*see* **Note 11**).

2. Compute ε_{trans} for the conjugated protein using the sum of the extinction coefficients of the free chromophore in the *trans* state and the unconjugated protein.

3. Measure the absorption spectrum of the conjugated protein prior to illumination (*see* **Note 12**).

4. Illuminate the protein at the absorbance maximum (ABDM: 342 nm; BSBCA 363 nm) using a method of choice, and remeasure the absorption spectrum of the protein every 2 min of illumination time. Cease illumination once the absorption of the π–π* band reaches a minimum.

5. Estimate the fraction of protein that photoswitches using the following equation:

$$Frac = \frac{\varepsilon_{\text{peak},trans} - \varepsilon_{\text{peak},mix}}{\varepsilon_{\text{peak},trans} - R^{*}\varepsilon_{\text{peak},trans}}$$

where *R* is the *cis–trans* extinction coefficient ratio for the π–π* band of the chromophore; *peak* refers to the wavelength of the chromophore-specific absorption maximum; ε*trans* is the measured extinction coefficient for the thermodynamically equilibrated, 100 % *trans* state; and ε_{mix} is the measured extinction coefficient for the photostable, UV-illuminated state containing a mix of *cis* and *trans* (**Notes 12** and **13**).

3.5.3 Measuring Bistability/Relaxation Rate

6. Measure the absorbance of the conjugated protein at the peak wavelength of the π–π* transition of the chromophore (ABDM: 342 nm; BSBCA 363 nm) prior to illumination.

7. Using the same methods as in Subheading 3.5.2, illuminate the protein to photostability.

8. Measure the absorbance of the conjugated protein at the peak wavelength of the π–π* transition of the chromophore (ABDM: 342 nm; BSBCA 363 nm) immediately following illumination (time zero, t_0).

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[11] Different chromophores have different extinction coefficients. If protein concentration is to be measured using A_{280}, ε_{280} for the chromophore can be measured using pure chromophore of known concentration, and the conjugated protein concentration can be calculated using $\varepsilon_{280,conjugate} = \varepsilon_{280,chromophore} + \varepsilon_{280,unconjugated\ protein}$. This assumes the extinction coefficient of the small molecule does not change during conjugation; this assumption can be checked by comparing band intensities of unconjugated and unconjugated proteins at the same nominal concentrations with an alternate assay (e.g., Bradford or SDS-PAGE).

[12] A pure population of *trans* protein can be obtained by first illuminating protein with visible light, followed by keeping protein in the dark for an extended period of time (e.g., overnight).

[13] Computing R relies on knowing ε_{peak} for the *cis* chromophore, which may be difficult to obtain, as *cis* chromophore may be difficult to isolate for measurement. A previous study used an *R* value of 0.541 for computing protein concentrations, based on measurements of BSBCA chromophore isomers separated by HPLC [3].

9. Keep the conjugated protein in the dark. Every 5 min (or a time of the experimenter's choosing), remeasure the absorbance of the sample.

10. Repeat **step 3** until the protein relaxes back to the unilluminated state. The half-life ($t_{1/2}$ of the illuminated state) is the time point at which the absorbance of the sample is halfway between the absorbances measured in **steps 1** and **2**.

3.6 Structural/Functional Assay

The particular method chosen for assaying whether photoswitching induces a structural or functional change will depend on the target protein. Here, we provide an example of a native gel assay used to determine changes in conformation upon illumination.

3.6.1 Native Gel Assay to Probe the Light-Induced Conformational Switching of ABDM-Mm- cpn

1. Use the cross-linked samples from Subheading 3.3.3.

2. To switch azobenzene between the *cis* and the *trans* isomerization states, expose the cross-linked Mm-cpn sample to alternating illumination for 20s with the blue LED (*cis* → *trans* isomerization) or for 90s with the UV LED (*trans* → *cis* isomerization). For this the sample is pipetted in a 200 μl PCR tube without a cap and placed in a PCR tube rack. Illuminate from the top by placing the LED as close to sample as possible (in our case in a distance of ~1 cm) to maximize light exposure (*see* **Note 14**).

3. Illuminate the sample alternately with blue and UV light. After each illumination step, remove 20 μl of the sample for structural characterization.

4. Load the samples on a 4 % native PAGE gel and run it for 30 min at 160 V.

5. Stain and destain the gel with Coomassie blue and take a picture of the gel. You can observe the light-induced switching between the closed and open conformations of Mm-cpn via a clear distinct band shift between both conformations on the gel (*see* Fig. 2 in ref. [4]).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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[14] UV light is absorbed by conventional glass and plastic.

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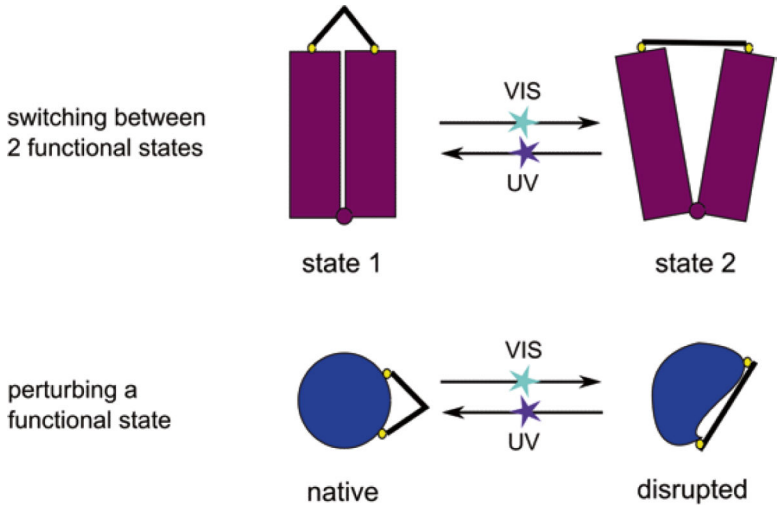

Fig. 1.

Cartoon representation of two strategies for controlling protein function with light. In the first (*top*), the protein is switched between two defined conformations. In the second (*bottom*), the protein is switched between a functional state and a “disrupted” state, in which, for example, the conformation of an active or ligand-binding site is destabilized