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Review Article

Let there be light: how to use photoswitchable cross-linker to reprogram proteins

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Azobenzene is a photo-isomerizing molecule whose end-to-end distance changes upon external illumination. When combined with site-specific reactive groups, it can be used as molecular tweezers to remote-control the structure and function of protein targets. The present study gives a brief overview over the rational design strategies that use an azobenzene-based photoswitchable cross-linker to engineer ON/OFF switches into functional proteins or to reprogram proteins for novel functions. The re-engineered proteins may be used as remote controls for cellular pathways, as light-gated drug delivery platforms or as light-powered machinery of synthetic cells and micro-scaled factories.

Introduction

Re-engineering of biological materials to control cellular and molecular functions via external stimuli is of great interest in the field of Synthetic Biology and might have useful applications, e.g. in medicine for targeted drug delivery and in industry for the development of 'smart' materials. Light is an excellent stimulus for such approaches as it can be applied with a high degree of spatio-temporal control. Light control of biological function has been achieved in the field of optogenetics by the heterologous expression of light-gated ion channels in neurons [1]. In other approaches, genetically encoded light-sensitive domains of photoreceptors are fused to other proteins to control the activation of cellular pathways [2,3]. Nanotechnology aims at gaining control over the structure of matter on the nanometer scale. A breakthrough in the field has been the synthesis of nanometer-scaled molecular motors that are driven by light and that are even able to move micrometer-scaled objects [4,5]. The topic of this review, the re-engineering of protein targets with an azobenzene-based photoswitchable cross-linker, lies at the intersection of both fields. It uses synthetic, atom-scaled, light-switchable molecular tweezers (azobenzenes) to control the conformational state and/or the function of biological nanomachines (proteins) [6].

Molecular properties of azobenzene

The fundamental basis of the 'molecular tweezer' engineering strategy lies in the photo-physical properties of the azobenzene group. It exists in two isomerization states with different end-to-end distances (Figure 1, change in distance ∼3.5 Å) [6]: a thermally stable, elongated *trans* isomer and a metastable, short *cis* isomer [7,8]. Both isomerization states have different absorption spectra in the UV–Vis region with specific bands characteristic for each isomer. The *trans* state has a strong $\pi \rightarrow \pi^*$ band at 320 nm, whereas the *cis* state has a slightly weaker $n \rightarrow \pi^*$ band ∼440 nm [9]. The absorption of a photon triggers an isomerization of the azobenzene group around the central N–N double bond, which makes it possible to selectively depopulate either the *trans* or *cis* isomer by illumination at a wavelength that is specific for this isomerization state (Figure 1). Therefore, azobenzenes can be utilized as a bi-stable molecular spacer that is switchable between two different lengths with light of two different wavelengths.

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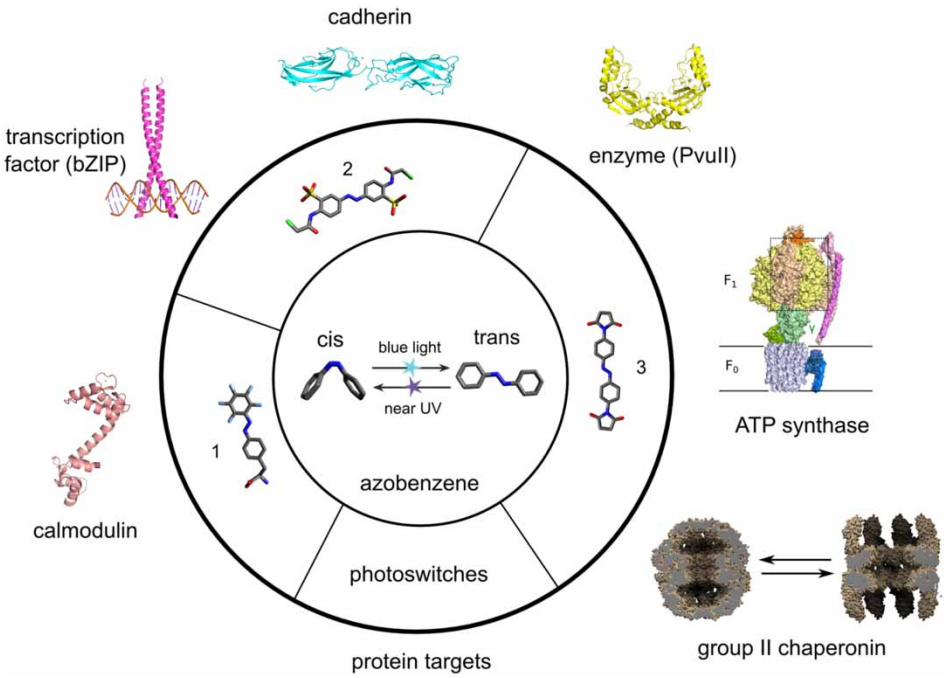

Figure 1. Molecular structure of azobenzene-based photoswitchable cross-linkers and their protein targets.
1: F-PSCaa is a pentafluoro azobenzene-based photoswitchable click amino acid. F-PSCaa reacts with a nearby cysteine within the protein generating an azo bridge *in situ* [13]. Owing to an enhanced n → π* band of the *trans* isomer that is slightly red-shifted in comparison with the one of the *cis* state, PSCaa can be switched between both isomers with green (540 nm) and blue (405 nm) light. 2: BCBCA, 3,3′-bis(sulfonato)-4,4′-bis(chloroacetamido)azobenzene; an azobenzene cross-linker with two thiol-reactive chloroacetamide groups on both ends for cross-linking of two cysteine residues. The sulfonate groups increase the water solubility of the molecule [35]. 3: ABDM, azobenzene-dimaleimide is an azobenzene cross-linker with two thiol-reactive maleimide groups [14].

Spectral tuning

The azobenzene group can been modified to enable the photoswitching between the *cis* and *trans* isomer with visible light [10–12]. Examples of this approach are the modification of the benzene rings with methoxy groups [10] or fluorine (Figure 1, photoswitch 1) [13].

Engineering goals

Control function vs. creating novel functions

Most azobenzene re-engineering projects have the goal to use light to control the native function of a protein (Figure 2A). An example would be modulating the catalytic activity of an enzyme by adding an external ON/OFF switch [19]. In this case, the photoswitch is used to perturb the functional state of the protein.

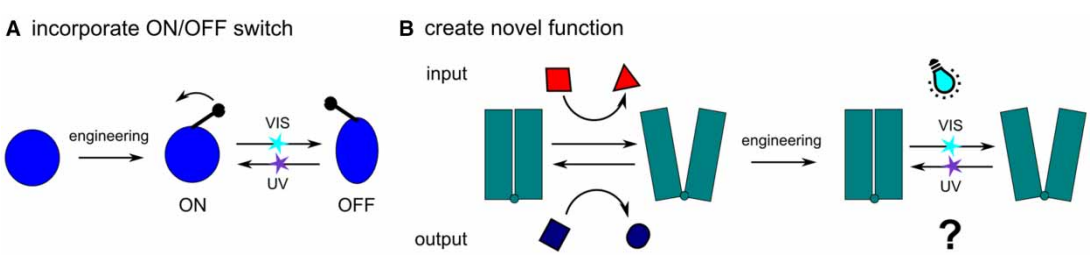

Figure 2. Engineering goals.

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There is also the possibility to completely repurpose a protein. By re-engineering the group II chaperonin Mm-cpn, which is an ATP-gated protein folding machine, we exchanged the 'fuel' of the machine and altered its function (Figure 2B). In particular, we exploited the structural shape transition of the protein complex during its functional cycle and created a light-gated nanocage for the capture and release of non-native cargos [23].

Design strategy

Site-directed cross-linking

To conjugate the azobenzene with a protein target, reactive groups are added to the benzene rings. Examples are thiol-reactive groups like maleimides [14], iodoacetamides [15] or bromides [16] that form covalent bonds with solvent-exposed cysteine residues that are introduced into the amino acid sequence of a protein by site-directed mutagenesis. The isomerization state of the photoswitch controls the distance of the cross-linked amino acids. This approach has been used to fold/unfold helical peptides [15], coiled coils [17] and small protein domains [18]. More recently, it has been applied to control the activity of a restriction enzyme [19], the calcium-binding affinity of cadherin [20], the function of an ATP-gated ion channel [21,22], a protein-conducting pore [16], the conformational state of a group II chaperonin [23] and the ATP hydrolysis activity of F_1 ATPase [24]. In most of the cases, the azobenzene is used to perturb the functional state of a protein (Figure 3A); however, this strategy can also be used to switch between two functionally relevant conformational states (Figure 3B) [23].

Protein targets

The protein targets of azobenzene re-engineering/modifications are very diverse with respect to their size and function. Their sizes range from 8-residue peptides [25] up to an 1 MDa protein complex [23]. Below I will introduce a few instructive examples of functions that have been controlled.

Transcription factors (bZIP)

Woolley et al. [17] used an azobenzene cross-link to reversibly stabilize/destabilize the helical structure of the coiled-coil region of GNC-bZIP which modified its DNA-binding affinity (Figure 1). A similar strategy was used later to inhibit the transcription factor AP-1 in living cells [26].

Cadherin

Ritterson et al. [20] used an azobenzene cross-linker to modify the Ca^{+}-binding pocket of the cell adhesion protein E-cadherin (Figure 1). The authors reported a reversible 18-fold change in Ca^{2+}-binding affinity that was linked to cadherin dimerization.

Restriction enzyme (PvuII)

Schierling et al. used a combination of two azobenzene cross-links per monomer to control the enzymatic activity of the homodimeric restriction enzyme PvuII (Figure 1). In this way, the DNA cleavage activity could be modulated reversibly by a factor of up to 16 by illumination with either blue or near-UV light [19].

Group II chaperonin (Mm-cpn)

We used an azobenzene cross-linker to reversibly switch the group II chaperonin Mm-cpn between two functionally relevant conformational states: a doughnut-shaped open state and a spherical closed state (Figure 1)

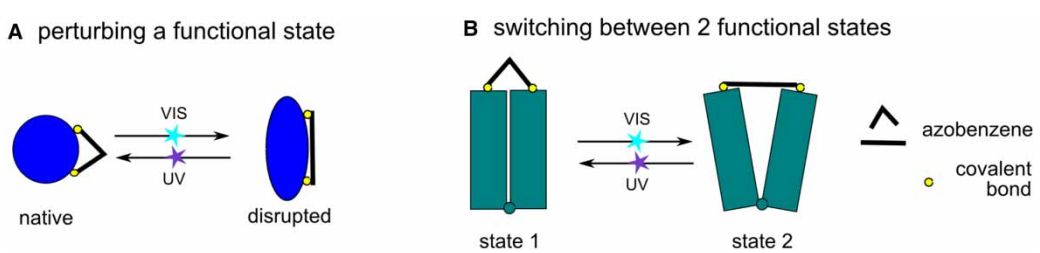

Figure 3. Design strategies for azobenzene re-engineering of protein targets.
Figure adapted from ref. [36].

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[23]. Using published atomic models for both states, we screened for positions in the structure of two neighboring monomers in the chaperonin barrel whose distance in the closed and open state matches the lengths of the azobenzene cross-linker in its *cis* and *trans* isomerization state, respectively. By introducing cysteines in these positions and cross-linking them with azobenzene-dimaleimide (Figure 1, molecule 3), we were able to engineer a chaperonin that switches between the open and closed state in response to illumination with blue and near-UV light. The re-engineering effectively exchanged the energy source for the conformational cycle of the chaperonin from ATP hydrolysis to light absorption. We, furthermore, showed that the device can be utilized as a light-gated nanocage for the transient capture and release of non-native cargos.

ATP synthase

With an engineering approach similar to the one applied to Mm-cpn, I engineered azobenzene cross-linking sites between the α and β subunits of the F_1 subcomplex of *Escherichia coli* F_0F_1 ATP synthase (Figure 1). In this way, it was possible to perturb the conformational state of the nucleotide-binding pocket at the interface of the α and β domain and control the ATP hydrolysis activity of the rotary motor reversibly with light.

In vivo experiments

Most azobenzene re-engineering of proteins has been done *in vitro* as thiol-reactive groups cannot easily be used to specifically modify a protein target inside a cell because of the reducing environment of the cytosol and the presence of solvent-exposed cysteines in off-target proteins. However, some groups have developed work-arounds for this problem.

Incorporation of proteins previously modified with azobenzene *in vitro* into living cells and organisms

Zhang et al. [26] designed a system in which the transcription factor AP-1 is inhibited in a light-dependent manner in HEK293T cells transfected with an azobenzene cross-linked peptide. Beharry et al. [27] introduced an azobenzene cross-linked fluorescent peptide into live zebrafish via microinjection and showed that the fluorescence brightness of the peptide could be modulated by photoswitching the azobenzene group for up to 2 days.

Artificial amino acids

Artificial amino acids harboring an azobenzene group were introduced by Bose et al. [28]. Hoppmann et al. developed azobenzene-bearing amino acids that are able to form cross-links with neighboring cysteine residues *in vivo* [13,29]. To demonstrate the viability of this engineering approach, an azobenzene cross-link was introduced into a functionally important helix of calmodulin *in vivo* (Figure 1). In an *in vitro* experiment, the binding of a peptide ligand to the cross-linked calmodulin could be controlled with light.

Computational approaches

Molecular dynamics simulations

Computational simulations of the structural response of biomolecules to the photo-isomerization of a conjugated azobenzene group have first been performed for small peptides. Using a combination of molecular dynamics (MD) simulations and ultrafast UV–Vis absorption spectroscopy, Sporlein et al. [30] found a match between simulated and experimentally derived reaction kinetics. For an azobenzene cross-linked PDZ domain, Buchli et al. [31] used a similar approach combining MD simulations and time-resolved IR spectroscopy to investigate the dynamic coupling of the photo-isomerization of the azobenzene group and the structural response of the protein backbone. Furthermore, MD simulations were used to model the structural perturbations caused by an azobenzene cross-linker conjugated to an ATP-gated ion channel [21].

Structure prediction

Beharry et al. [32] applied a combination of *in vitro* experiments and Go-type coarse-grained explicit-chain models of protein folding to investigate the influence of the length and stiffness of an azobenzene cross-linker on the conformational equilibrium of an SH3 domain. They found that a longer, but somewhat flexible, cross-linker is less destabilizing to the folded state than a shorter, more rigid cross-linker.

For the 1 MDa chaperonin complex, we used an all-atom Monte Carlo energy minimization strategy to investigate the structural response of the protein to a distance constraint mimicking the light-induced length

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change of the azobenzene cross-linker [33]. The simulations suggested that rigid body motions of the protein subdomains couple the azobenzene length change to rearrangements of the nucleotide-binding pocket of the protein that results in destabilization of the closed state of the protein complex. We tested the model *in vitro* by designing a mutant for which the orientation of the two protein subdomains forming the nucleotide-binding domain is directly controlled by the cross-linker.

Future directions

In recent years, azobenzene cross-linking has been proved to be a powerful tool for the re-engineering of protein function. In the following, I will highlight only two of many promising future applications of this technology.

Investigation of the allosteric coupling of proteins

Azobenzene cross-linkers are site-specific and fully reversible photoswitchable molecular tweezers that attach to engineered cysteine residues in protein targets. In response to illumination, the azobenzene cross-linker triggers a defined, reversible and temporally controllable perturbation to the protein structure. This makes azobenzene re-engineering the ideal tool to investigate the allosteric coupling of proteins whose molecular mechanism is not yet understood. By screening different attachment sites for the cross-linker on the surface of the protein, it might be possible to map the long-range interactions between the cross-linker attachment site and the active site of a protein (Figure 4A). In that way, models for the allosteric coupling of proteins can be tested rigorously, and novel allosteric sites and possible drug target sites may be discovered. This method is especially powerful in combination with *in silico* experiments that simulate the structural perturbation of the protein. The simulations can be used to predict promising cross-linking sites in an *in silico* screen or to develop models for the allosteric coupling of a protein if an allosteric site is already known (e.g. from an *in vitro* azobenzene cross-linking screen).

Engineering of light-powered molecular machines

So far, it has been shown that, with azobenzene modification, it is possible to unfold protein domains, switch between distinct conformational states of protein complexes and regulate protein function. The next engineering challenge would be to use the energy of the azobenzene photo-isomerization to power a molecular machine that performs measurable work against the thermodynamic equilibrium. Hugel et al. [34] estimated the quantum efficiency of the energy transduction from photon energy into mechanical work for the UV light-induced *trans* → *cis* isomerization of azobenzene to be ~0.1 (as measured by single-molecule atomic force spectroscopy of an azobenzene-bearing polymer). This translates into an available mechanical energy of 27 kJ/mol per isomerization event, which is in the same order of magnitude as the most common energy source of

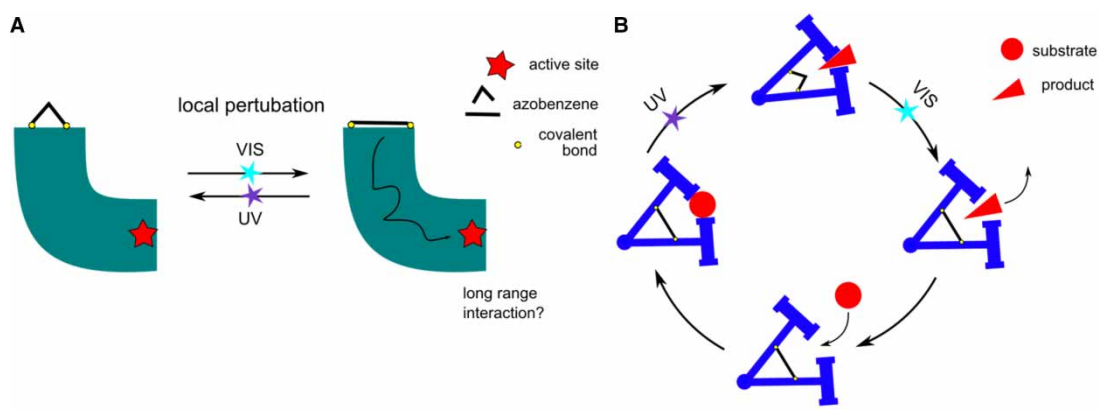

Figure 4. Future directions.

(**A**) Investigating the allosteric coupling of proteins with azobenzene cross-linkers that trigger temporally controllable site-specific and reversible perturbations to the protein structure. (**B**) Engineering light-powered molecular machines that perform quantifiable work against the thermodynamic equilibrium.

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protein-based biological machines: ATP hydrolysis (30 kJ/mol). This energy may be harnessed by an azobenzene-modified protein to actively transport a cargo, or to synthesize an energy-rich compound (Figure 4B). Possible protein targets for this engineering strategy are motor proteins, membrane-bound pumps or synthesis machines like ATP synthase. Once successfully re-engineered, these light-driven protein-based nanomachines might have interesting applications, for example, as energy suppliers for synthetic cells, as light-responsive 'smart' materials or as synthetic micro-scaled factories based on protein parts that can be precisely controlled by engineers.

Abbreviations

MD, molecular dynamics.

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Competing Interests

The Author declares that there are no competing interests associated with this manuscript.

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