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Minireview

Design of proteins with photocontrollable structure and function and their applications

Brankica JANKOVIĆ

University of Zurich, Department of Chemistry, Zurich, Switzerland

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Summary. Nature employs very sophisticated strategies of using light to control certain biological processes such as vision or photosynthesis. If we borrow that concept from Nature and make it universally applicable to a wide variety of proteins, we would be able to control many aspects of life on a molecular level. One of the goals is to design proteins to be specifically photoresponsive, so that light can be used to trigger the conformational changes which are subsequently followed by real-time spectroscopy. Such a combination of tools enabled the studies of interesting biochemical phenomena such as protein binding mechanisms and allostery. More precisely, the design of a protein-peptide interaction that can be light-controlled, enabled the detailed kinetics investigation of their binding mechanisms. Furthermore, the design of a photoswitchable allosteric proteins made it possible to directly investigate the speed of allosteric signaling within the single domains. Many other proof-of-concept studies and possible applications of photocontrollable proteins will be discussed.

Keywords: azobenzene, genetic code expansion, optogenetics, photoswitch, protein design.

INTRODUCTION

Proteins are key players in virtually any biological process. In order to study complex biological processes within living cells, a high level of protein control in space and time is required (Redchuk et al. 2020). Light evolved as one of Nature's unprecedented strategies to control important biological processes in a highly specific manner. Employing Nature's strategy and trying to "teach" proteins how to feel light represents an attractive field of modern biochemistry. Due to the many favorable and advantageous aspects that photocontrol can bring, numerous approaches of designing photoresponsive molecules have been developed (Hoorens and Szymanski 2018).

The main advantages of light as an external stimulus are its high spatial and temporal resolution (Hunter 2016). Homeostasis within a cell or organism relies on a perfect coordination of different processes in time, as well as their proper arrangement in space. Especially for cell biology studies, it becomes evident that the precise regulation of processes is a prerequisite to deriving conclusions and learning about cellular mechanisms (Fan and Lin 2015). DNA knock-out or RNA interference based gene silencing approaches are a valuable knob of control (Negi et al. 2022). However, these processes are slow and do not provide sufficient time resolution for investigating cellular processes (Fan and Lin 2015). This is where the light stimulus comes into play. Furthermore, available light sources make it possible to focus light on very limited parts of the tissue down to even subcellular precision (Mathony and Niopek 2021). If the photoresponsive drug is designed to be activated by light only in certain parts of the body, this type of control can be easily achieved. Together

E-mail: brankica.jankovic@chem.uzh.ch

with being non-invasive, easily applicable and cost-efficient, all these characteristics make light a preferable and attractive stimulus with wide potential applications (Fan and Lin 2015).

Therefore, the concept of designing photoresponsive molecules attracted the attention of numerous fields, such as pharmacology (Bamberg et al. 2018), material science (Yager and Barrett 2006), neuroscience (Bamberg et al. 2018), and fundamental research (Dagliyan and Hahn 2019). Regarding the molecular constructs and their design, there are several levels where the photocontrol was successfully achieved: starting with metal ions and small molecules, to peptides, proteins and nucleic acids. The photocontrol has been performed *in vitro*, i.e. on the molecular level, but also on the cellular, and on the organism level (Ankenbruck et al. 2018).

In this minireview, the focus will be on the progress in the development of photorespoinsive proteins and peptides, and their potential applications in fundamental research, as well as in industry. Design principles concerning photocontrollable proteins developed so far include three main branches: optogenetics, optochemistry (chemical modifications of proteins), and genetic code expansion (incorporation of unnatural amino acids) and are schematically illustrated in Fig. 1.

STRATEGIES FOR THE DESIGN OF PHOTOCONTROLLABLE PROTEINS

Proteins are biomolecules centered at the core of every biological process in living systems. A large majority of proteins are not naturally photoresponsive, meaning that they can not be controlled by light. By being able to use light as an external stimulus to control protein structure and function, in theory any biological process could be regulated with large precision. This brings another dimension in probing complex processes within the cellular environment and ultimately drives the creation of novel medicine and drug delivery systems (Ankenbruck et al. 2018; Bamberg et al. 2018).

Originally motivated by different final goals, optogenetics and optochemistry complement one another in their advantages and disadvantages and the choice of the specific approach will largely depend on the purpose and the level of studies – *in vitro* molecular level, cellular or organism level (Kneuttinger 2022). The third and newest genetic code expansion strategy fills existing gaps of the other two approaches, by providing a relatively robust principle for the implementation of a light-controllable element in expressed proteins (Courtney and Deiters 2018).

OPTOCHEMISTRY

The optochemical approach of designing photocontrollable proteins considers a combination of chemical modification and light (Kneuttinger 2022). It was traditionally based on a photocleavage of a caging group that protects the functional groups of *e.g.* enzymes or removes the sterical hindrance (Klán 2013). These approaches are irreversible. To make the light-control reversible by applying different wavelengths of light, the most common approach is to incorporate azobenzenes (Hoorens and Szymanski 2018). It requires a chemical modification of either expressed proteins or synthesized peptides or protein fragments (Courtney and Deiters 2018). The focus of this section will be on the use of azobenzene compounds to switch the protein or peptide structure.

Azobenzene is a small organic molecule that isomerizes around its central N=N double bond. Thereby, when the light of the specific wavelength is shine on this molecule, it changes the configuration from the stable, dark-adapted *trans* state to the less stable *cis* state. The process is reversible, as the molecule relaxes back to its dark-adapted state thermally, or by illumination with a different wavelength. By *trans*-to-*cis* isomerization the molecule changes its geometry from the

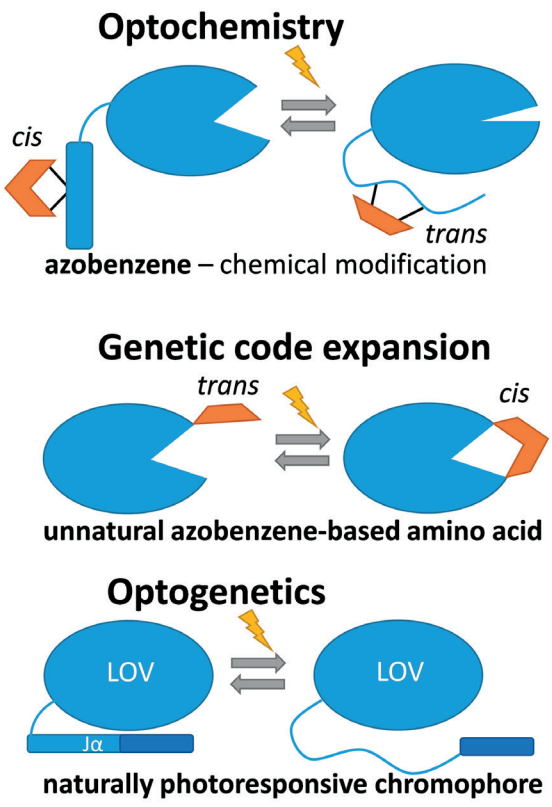

Fig. 1. Schematical representation of three different design strategies of photocontrollable proteins. LOV – Light, oxygen, voltage domain from *Avena sativa* (oat). Jα – C-terminal extension of the LOV domain that perturbs with blue light. Proteins are presented as blue entities, while the photoswitchable moieties are highlighted in orange. LOV domain has its natural photoresponsive chromophore incorporated into its structure.

more elongated to the shorter state (Fig. 2A) (Woolley 2005). The wavelengths of light that azobenzene-based compounds absorb in either their *cis* or their *trans* state are highly dependent on the substituents of benzene rings. Therefore, its spectral characteristics are tunable (Dong et al. 2017). The same is true for the time-scale of thermal relaxation – it strongly depends on the derivatization and changes once the molecule is incorporated into the peptide or protein structure. Tunability is one of the appreciated features of these molecules, as it allows for adjusting the properties to the specific requirements. Namely, going towards the far-infrared or red region of visible light allows for enhanced penetrability through a biological matrix such as layers of cells, tissues or skin (Dong et al. 2017). Non-invasiveness is also achieved once moved from the UV region. Back-relaxation time is an important parameter to be considered depending on the design. E.g., if the photosensitive drug is injected into the bloodstream, the timing of its activation/deactivation should be considered relative to the speed of its clearance (Samanta et al. 2013; Dong et al. 2017).

Towards the direction of applying azobenzene modified proteins and peptides to living systems, studies of azobenzene biocompatibility are already performed. They have shown the feasibility of azobenzene modified peptide incorporation into zebrafish, showing its toxicity profile, resistance to reducing environment and proteolysis as well as cell permeability (Beharry et al. 2011; Dong et al. 2017). The most commonly used azobenzene derivative for biomolecules is the water-soluble version (with charged sulfonate groups), made to be more compatible with an aqueous environment (Zhang et al. 2003).

Before applying azobenzenes to proteins, first studies used azobenzenes to switch the helical conformation of short peptides. Woolley group performed groundbreaking research in this field and they derived the concept of bifunctional azobenzene crosslinking to the isolated helical peptides (Flint et al. 2002). A relatively simple linking reaction via a pair of cysteine residues yielded the photoswitchable peptides in which the perturbation of the helical structure was demonstrated. Cysteine spacings of four and seven residues (encompassing approximately one or two turns of an α-helix, respectively) have been shown to bring the largest difference in helicity between the *cis* and *trans* state of the photoswitch (James et al. 2001; Flint et al. 2002; Zhang et al. 2003; Woolley 2005). Transferring this knowledge and applying it to larger proteins is a non-trivial task. It is usually based on the design of several constructs and their further characterization or screening for the desired effect (Zhang et al. 2009; Schierling et al. 2010). Several possibilities for incorporating the photoswitch to the protein structure consider its anchoring to the helical element (with the analogy to already described peptide helices) (Bozovic et al. 2021), extended β-strands (Zhang et al. 2009), loops (Schierling et al. 2010) or simply between two different parts of the protein to perturb their relative distances (e.g. opening and closing of the binding groove) (Buchli et al. 2013).

The Hamm group creates photoswitchable proteins and peptides with an ultimate goal based on fundamental biophysics. The aim is to investigate the intriguing phenomena – protein interaction mechanisms and allostery in real time (Bozovic et al. 2022). Applying ultrafast time-resolved spectroscopy (primarily infrared spectroscopy) to proteins requires that they can be promptly triggered to a certain conformational change that is subsequently followed in real time (Bozovic et al. 2022). Azobenzene-based engineering of proteins and peptides enables an ultra-fast trigger, since it isomerizes on the time scale of hundreds of picoseconds (Hamm et al. 1997). This is fast enough relative to the time scales of protein conformational changes, allostery and (un) binding of peptides from the protein. Therefore, azobenzene photoswitching fulfills the requirements to be coupled to ultra-fast infrared spectroscopy (Bozovic et al. 2022).

The previous principles of switching short peptides are applied to either helical or extended β-strand peptides or larger proteins. Different approaches performed by Hamm group are illustrated in Fig. 2B. In efforts to explore protein dynamics and allostery small allosteric protein domains – PDZ domains were employed. These domains are commonly

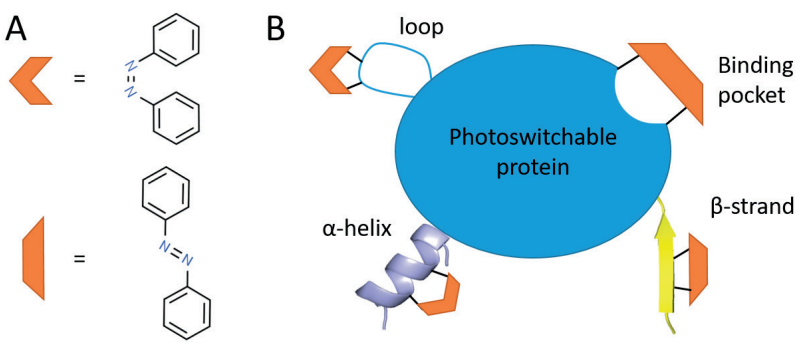

Fig. 2. A, Structure of azobenzene in its *cis* vs. *trans* configuration; **B**, Possible design strategies for engineering a photoswitchable protein via azobenzene-based optochemistry of various (secondary) structural elements.

used systems to study allostery (Gautier et al. 2018).

In the case of the PDZ2 domain, the Hamm group first designed the modified domain by mutating a pair of residues on two sides of the domain's binding groove. The azobenzene bridged the binding groove and thereby enabled mimicking the opening and closing of the binding groove by switching the azobenzene's configuration from *cis* to *trans* (Buchli et al. 2013).

In the follow-up study on the same protein domain, the goal was to keep the domain intact, yet to trigger its dynamics by light. In this case, photoswitching was achieved by designing a photoswitchable extended β-strand peptide binder. Namely, the natural peptide of the PDZ2 domain was chemically modified with crosslinked azobenzene with the spacing for its anchoring such that it preserves the extended β-strand in the elongated *trans* state of the photoswitch, but perturbs it in the *cis* state (Bozovic et al. 2020). Photoswitching the PDZ2 domain shed light on the dynamics and allostery of this protein in real-time (Bozovic et al. 2020, 2022).

The PDZ3 domain has an important structural and functional difference compared to PDZ2 domains. Belonging to the same family of protein domains, all PDZ domains share the same canonical fold, with the exception of the PDZ3 which in addition has an extra helix, shown to be the allosteric element. Petit et al. have shown that the cleavage of this helix which is located on the opposite side of the binding pocket, reduces the binding affinity 21 times (Petit et al. 2009). Therefore, the PDZ3 domain with a photoswitchable helix was designed by applying the principle of helix perturbation via crosslinking the azobenzene spaced by 7 residues. The helix perturbation upon photoswitching was demonstrated and this system was used to study and determine the speed of allosteric signaling by time-resolved infrared spectroscopy (Bozovic et al. 2021).

Protein interactions were investigated on the very well characterized model for protein-peptide binding mechanisms – Ribonuclease S. This is a non-covalent complex of S-protein and S-peptide derived from the enzyme Ribonuclease A. Therefore, five different photoswitchable peptides were synthesized (with various spacings for the azobenzene anchoring, as well as with an additional mutation). The S-peptide fragment of this non-covalent complex (20 amino acid residues) is disordered in isolation, while it orders in an α-helix structure when complexed to the S-protein. By switching the helicity to various extents, a range of effects was observed depending on the peptide: from almost no effect on binding to complete unbinding in the extreme case (Jankovic et al. 2019). Such construct enabled describing the whole sequence of events happening upon peptide unbinding from the protein (spanning a huge time scale range from picoseconds to milliseconds) using transient infrared spectroscopy (Jankovic et al. 2021b). It also unraveled the intrinsic dynamics of speed-limiting peptide unbinding (happening in hundreds of microseconds) by transient fluorescence (Jankovic et al. 2021a). The time-resolved findings brought the conclusion that the binding mechanism is a combination of induced fit and conformational selection, observed from the perspective of protein or peptide, respectively (Jankovic et al. 2021b).

Finally, to study intrinsically disordered peptide-protein interaction, MCL-1 protein and BIM peptide were investigated as known apoptosis-related interaction partners (Heckmeier et al. 2022). Similar principles of photoswitching were employed to dissect the dynamics of intrinsically disordered peptide (BIM) to the apoptosis regulator (protein MCL-1). This is another example of perturbing a stable long helix and triggering thereby the dynamic response of the partner protein. In this case, it was possible to elucidate the mechanism of the intrinsically disordered interaction based on a single experiment (Heckmeier et al. 2022). For a more detailed overview on these time-resolved findings and their interpretation, the readers are referred to the recent review (Bozovic et al. 2022).

Many other interesting examples of azobenzene-based photoswitching of proteins include the modulation of enzymatic activity (Schierling et al. 2010; Liu et al. 2017), binding affinity (Mart and Allemann 2016), molecular motor function (Hoersch 2016), ion-channels (Dagliyan and Hahn 2019), and many others. One of the most recent examples includes proteolysis targeting chimeras - PROTACs photoswitching (Negi et al. 2022). This already fascinating strategy of controlling the degradation of proteins of interest has been further improved by providing an additional knob of control – light switching. Namely, PROTACs are conjugated protein systems that consist of three parts: part A that binds the protein of interest, part B as a linker and part 3 as an E3 ligase binding protein. After binding the protein of interest to part A, E3 ligase labels it with ubiquitin which represents the signal for targeting for the proteasomal degradation. Azobenzene incorporation directly into their structure enables temporal and spatial control over target degradation and shows promising results towards deriving specific PROTACs precision medicine (Negi et al. 2022).

OPTOGENETICS

The basics of the optogenetics field started to develop early in the 70s, with the discovery of channel rhodopsins – naturally light-responsive proteins. They were the first proteins used to design light-responsive neuronal cells. Today, optogenetics can be more generally defined as using various natural photoresponsive proteins or protein domains to design a fusion product on a genetic level. Numerous proteins

of plant or microorganism origin are discovered that can feel the light of specific wavelengths and exert structural and functional changes as a consequence of light absorption. These proteins or protein domains represent a versatile tool, i.e. "pieces of the puzzle" to be combined by rational design with other proteins and/or domains with a goal of transferring the effect of light on otherwise non-responsive molecules (Courtney and Deiters 2018; Dagliyan and Hahn 2019; Kneuttinger 2022). Since the modification is performed on the genetic level, such a construct offers a great advantage of being expressed inside cells. Namely, proteins are gaining increasing popularity as highly specific and efficient medicines, but there is a large obstacle in the way how they can be administrated to living cells. Therefore, the possibility of expressing them *in vivo* is a great plus (Möglich et al. 2009; Redchuk et al. 2020).

Although optogenetics originally emerged as a tool in neurosciences, it became widely used for the targeted and precise control over biological processes in various cells, tissues or organisms. Nowadays, one of the most popular design elements used in optogenetic studies is the LOV domain of plant phototropin (Dagliyan et al. 2019). Homologous domains were found in microorganisms, and they are involved in phototropism. It absorbs blue light by its flavin chromophore which then forms a covalent bond with a nearby cysteine residue. The most interesting feature of this domain is a C-terminal Jα helical extension whose α-helical character changes with illumination by blue light. This is the main design/fusion element used in optogenetics to transfer the huge conformational change to an effector protein. Namely, in one state this helix is folded and anchored to the rest of the domain, while it detaches from it in the other state (Kneuttinger 2022). LOV domains were primarily used for the photocontrol of allostery (Strickland et al. 2008; Dagliyan et al. 2019; Mathony and Niopek 2021), but also for the direct sterical hindrance of the active or binding site of an enzyme or protein in general (Möglich et al. 2009; Kneuttinger 2022).

In the study by Allemann and colleagues, the LOV domain Jα helix was combined with the helical BH3 domain of the pro-apoptotic protein BID. This helical peptide was anchored together with the main Jα to the core of the LOV domain in the dark state. Upon illumination with the blue light, the target peptide becomes exposed and free for interaction with its partner, anti-apoptotic protein BCL-XL. This study provided a basis for the possible *in vivo* photocontrol of apoptosis, providing a valuable tool in cancer research (Mart et al. 2016).

GENETIC CODE EXPANSION

The genetic code expansion technique relies on designing the pair of orthogonal tRNA and aminoacyl tRNA synthetase that recognizes an unnatural amino acid and incorporates it into the growing protein chain during translation upon recognizing the amber stop codon. This innovative methodology provides site-specific incorporation of unnatural amino acids (Chin 2014). This is how the natural repertoire of functionalities in proteins is further expanded if not available by 20 conventional proteinogenic amino acids. Most commonly, the genetic code expansion concept introduced photocaged groups making control of many enzymatic activities possible by light (Courtney and Deiters 2018). Successful designs report photodecaging of various residues: lysine, cysteine, tyrosine and serine. One of the most prominent examples is the incorporation of the caged lysine into a fusion construct of nuclear localization sequence of p53 tumor suppressor protein and EGFP. Illumination of this fusion protein expressed in cell culture leads to a photocontrol of nuclear localization (Gautier et al. 2010; Riggsbee and Deiters 2010).

Similar to the previously mentioned caging groups' installations into the proteins by optochemistry approach, this method yields irreversible photocontrol. However, one step forward in this technology enabled the incorporation of photoswitchable amino acid that is functional and reactive with a nearby inserted cysteine residue (Hoppmann et al. 2014; Hoppmann et al. 2015). This tool avoids the necessity for bulky fusion constructs and chromophores (used in optogenetics), and provides the site-specific bridging of the azobenzene *in vivo*. Therefore, it combines the advantages of both previous approaches (optogenetics and optochemistry). As a relatively new and largely unexplored tool, it is yet to be discovered how its versatility could be incorporated to solve important questions of modern biochemistry.

APPLICATIONS

Possible applications of molecular photocontrol span the whole range all the way from fundamental science down to applied research, such as producing functional materials (Yager and Barrett 2006), diving ultimately into drug discovery (Zhang and Cohen 2017).

Fundamental research uses all these tools to specifically regulate either the proteins themselves or biological processes in which they are the key players. Optochemistry is used in conjunction with fast time-resolved spectroscopy techniques (Buchli et al. 2013; Bozovic et al. 2020, 2022; Heckmeier et al. 2022), for the modulation of enzymatic activity (Schierling et al. 2010; Hoersch 2016), as well as for controlling protein binding (Jankovic et al. 2019). Genetic code expansion was so far primarily focused on controlling the activity of enzymes or designing photoswitchable allostery in proteins (Chin 2014; Courtney and Deiters 2018; Kneuttinger 2022). Optogenetics is mainly investigating cel-

lular processes like cell signaling and metabolic pathways (Fan and Lin 2015). All these branches of science brought unprecedented precision in studying dynamic processes and brought invaluable conclusions about time-dependent changes in biomolecules (Courtney and Deiters 2018; Redchuk et al. 2020).

The applications of optogenetics are by far the most advanced, as they are used on different hierarchical layers of life. Starting from remote control of cell movement (Wu et al. 2009), through specific neuronal excitation enabling the control of many processes such as locomotion (Feng et al. 2020), all the way down to restoring partial vision in a blind patient (Sahel et al. 2021). To no surprise, optogenetics was selected as a "Method of the Year" by the Nature Methods journal in 2010 (MY2010 2011).

CONCLUSION

Fascinating results achieved in the field of protein photocontrol show an enormous scope of possibilities. Although recognized and appreciated by the scientific community, the concept of designing photoresponsive proteins and other molecules remains a highly demanding and non-trivial task. This is largely due to the lack of a universal or easily transferable strategy that would unify the existing efforts applied so far to diverse model systems. This is not surprising having in mind that the field is still in its infancy and there are many challenges yet to be addressed. If all the existing ways of photocontrol start to converge together, and the novel approaches join them on the way, this would help in filling the gaps, so that many more branches of science, medicine and industry can finally see the light of photoswitches.

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