Published in final edited form as:

Sci China Chem. 2014 December ; 57(12): 1615–1624. doi:10.1007/s11426-014-5225-5.

Tightening up the structure, lighting up the pathway: Application of molecular constraints and light to manipulate protein folding, self-assembly and function

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Abstract

Chemical cross-linking provides an effective avenue to reduce the conformational entropy of polypeptide chains and hence has become a popular method to induce or force structural formation in peptides and proteins. Recently, other types of molecular constraints, especially photoresponsive linkers and functional groups, have also found increased use in a wide variety of applications. Herein, we provide a concise review of using various forms of molecular strategies to constrain proteins, thereby stabilizing their native states, gaining insight into their folding mechanisms, and/or providing a handle to trigger a conformational process of interest with light. The applications discussed here cover a wide range of topics, ranging from delineating the details of the protein folding energy landscape to controlling protein assembly and function.

Keywords

Protein folding; aggregation; self-assembly; cross-linker; phototrigger; light-activation

1 Introduction

The ability to spontaneously self-assemble, which includes intramolecular folding and intermolecular complexation or association, is a hallmark of biological molecules. Research over the past few decades has resulted in tremendous progress in our understanding of the fundamental principles that govern, for example, how proteins fold and aggregate [1-3]. However, the majority of naturally occurring proteins lack a structural element or motif that can be used as a molecular switch or constraint to direct a self-assembly process of interest towards a specific direction, making it difficult or impossible to precisely control the outcome. Thus, a great deal of effort has been made in recent years to develop new strategies to extrinsically modify protein structures, aiming to manipulate and/or control protein/peptide self-assemblies. In particular, the strategies of using a chemical cross-linker to confine the molecule of interest to a specific conformation and employing a photoswitchable cross-linker or functional group to induce a desired conformational

transition or function with light have gained popularity. This is due to the fact that these methods are not only useful in controlling the structural framework of proteins, but also in helping understand the molecular mechanism of the self-assembly process. Herein, we present a brief review of the recent advances that utilize molecular constraints in peptides and proteins, with an emphasis on discussing the applications of photoresponsive structural cross-linkers and photolabile motifs to explore protein folding, misfolding, and aggregation.

Below, we will first provide a brief account of the available cross-linking methods for constraining polypeptides to particular structures, and then discuss how well-chosen cross-linkers can be used to gain detailed insights into the folding energy landscape of proteins. Next, we will review recent applications of various light-activated moieties, including photoresponsive cross-linkers and photolabile groups, in protein folding and aggregation studies, as well as in creating light-responsive biomaterials. Finally, we will briefly mention how light can be used to control biological functionality.

2 Stapling to fold

Because folding leads to a large decrease in conformational entropy, stable structures can emerge only when the associated enthalpic gain overcomes this energetic penalty. For relatively small systems, however, this requirement may be difficult to achieve. Therefore, various sidechain-to-sidechain cross-linking strategies have been put forth to reduce the conformational entropy of the unfolded state, thus forcing short peptides to fold into α -helices or β -sheets (Figure 1). Such examples include disulfide bond formation [4-10], ring-closing metathesis [11-14], lactam bridge formation [15-19], hydrocarbon bridges [20-23], and hydrazone [24] and oxime linkages [25-27]. In particular, due to the ease of incorporation and natural abundance of cysteines in biological systems, cysteine alkylation [28-30] has become a popular method for incorporating cross-linkers that stabilize α -helical conformations [31, 32]. For example, Jo *et al.* have used this strategy to create stable and potent α -helical peptide inhibitors for calpain [32]. Besides these sidechain-to-sidechain covalent constraints, attempts have also been made to cross-link two backbone atoms together [33-35].

3 Probing the details of the folding energy landscape via cross-linking

The strategy of chemical cross-linking is not only useful for increasing the stability of folded conformations [36], but also is an effective tool to study the mechanisms of protein folding and, in particular, the structure of the folding transition state ensemble [37-42]. For example, Sosnick and coworkers have exploited the metal binding property of histidine and used divalent metal ions (e.g., Zn^{2+} and Co^{2+}) to create an unconventional linker in protein systems of interest via engineered bihistidine sites [43, 44]. Because the apparent strength of this cross-linker depends on the metal ion concentration as well as the local structure, they showed that it is possible to use this cross-linking approach in combination with thermodynamic and kinetic measurements, to determine whether a specific native structural element is formed in the transition state ensemble. Analogous to mutational Φ -value analysis [45], if the increase in protein stability induced by metal ion binding arises exclusively from the increase in the folding rate, the structural element stabilized by the metal-ion linker is

formed in the transition state. Similarly, covalent cross-linkers, such as dichloroacetone structures and disulfide bridges, have also been utilized to elucidate the nature of the folding transition state ensemble [37-39, 41, 42, 46].

Confining a peptide to well-defined conformations enables a folding/unfolding reaction to proceed from a unique position on the folding free energy landscape (see below), potentially allowing for control of the flux of a particular folding pathway or isolation of a specific folding event. In one example, Serrano *et al.* [47] have taken advantage of this strategy and used a cyclic peptide generated by linking its Lys (lysine) and Asp (aspartic acid) sidechains via a lactam bridge to determine the nucleation time of α -helix formation. In another example, we have shown that it is possible to use a cross-linker to eliminate the free energy gap between the unfolded and transition states, thus allowing for assessment of the folding rate on the downhill side of the free energy barrier [48]. Specifically, we employed a well-studied β -hairpin, Trpzip4, as a model to illustrate the feasibility of this approach. As shown (Figure 2), when a sidechain-to-sidechain disulfide bond was introduced to prevent the unfolding of the turn of this β -hairpin, which has been shown to be formed in the transition state [49], infrared temperature-jump (*T*-jump) measurements revealed the existence of two folding pathways; one having a relaxation rate that is almost identical to the wild-type and another relaxing an order of magnitude faster (~ 500 ns). This faster kinetic event, which was not observed for the uncross-linked β -hairpin [50], was attributed to a barrierless folding process. In other words, the disulfide cross-linked state in Figure 2 mimics the folding transition state of β -hairpins and its folding time likely represents an upper limit for folding of β -hairpins on a barrierless energy landscape. Indeed, this time agrees well with that predicted by end-to-end loop-closure kinetics for unstructured polymer chains.

Furthermore, we have recently hypothesized and demonstrated, in a proof of principle study, that it is possible to use a well-chosen cross-linker to help assess the effect of internal friction on protein folding dynamics [51]. Specifically, we showed that when a *m*-xylene cross-linker was introduced in such a manner that it spans the central portion of the single α -helix in the miniprotein Trp-cage 10b, both the folding and unfolding rates of the cross-linked variant (referred to as 4-8-CL-Trp-cage) show a similar decrease (Figure 3). Because in this case the *m*-xylene cross-linker does not alter the protein stability to any appreciable extent, these results were interpreted within the framework of internal friction. In other words, the *m*-xylene acts to increase the roughness of the folding energy landscape, but has no significant effect on the native interactions. Interestingly, our results suggest that a cross-linker of this size can increase the roughness of the Trp-cage 10b folding energy landscape by 0.4-1.0 $k_B T$, depending on the direction of the underlying conformational motion. Considering the fact that naturally occurring proteins are much larger and encounter interactions between neighboring chains more frequently, our findings suggest that internal friction, arising from the excluded volume effect and/or local steric hindrance between sidechains, will have a significant effect on protein conformational and folding dynamics.

4 Triggering protein conformational events with light

Due to the ease and convenience of manipulating light, phototriggering is becoming a more widely used method to initiate protein conformational changes. In comparison to other

initiation methods, phototriggering can potentially offer several advantages, since light-triggered reactions, such as isomerization and cleavage, (1) can take place on ultrafast timescales, (2) can lead to significant changes in backbone geometry, (3) and can provide precise conformational control between equilibrium and non-equilibrium states. While there are a large number of molecules that strongly interact with light, only a small fraction have found use as a phototrigger in protein conformational studies. This is because the photoreaction of an effective trigger must meet several criteria, some of which depend on the nature of the question being investigated: (1) it should be selectively initiated by light that has a wavelength higher than 310 nm, as lower-wavelength light could potentially excite protein backbone and sidechain electronic transitions, (2) it should occur on a timescale that is faster than that of the kinetic event of interest, (3) it should have a sufficiently high quantum yield, and (4) it should ideally produce relatively inert byproducts. In practice, other considerations include the reversibility of the photoreaction, as well as the rate of the reversible reaction [52], and the ease of incorporation into the protein system of interest.

Over the past few decades, a substantial number of molecules have been examined as potential phototriggers. Because of its easy incorporation into peptides and proteins, early attempts have explored the utility of using a disulfide as a phototrigger [53]. While these studies showed that disulfides can be cleaved with ultraviolet light (~ 270 nm) [54, 55], the free radicals formed upon photoexcitation often undergo ultrafast geminate recombination and are also very reactive, making it a less-than-ideal trigger. Recently, several studies have shown that it is possible to remediate these pitfalls. For instance, Kolano *et al.* [54] have shown that it is possible to reduce the degree of geminate recombination after photocleavage by placing a disulfide in a strained geometry. Another alternative, which is currently being pursued in our lab [56], is to break the disulfide bond irreversibly using an electron transfer mechanism [57-60].

Other examples of irreversible phototriggers include tetrazines, hydrazines, and dimethoxybenzoins [61]. In particular, Tucker *et al.* [62] have shown that tetrazines are ideal irreversible phototriggers due to their relative ease of incorporation, their fast timescales of reaction (picoseconds), relatively high photochemical yields ($\sim 22\%$), their excitation wavelengths (355 nm), and their inert byproducts (N_2 and nitriles). As shown (Figure 4), using S,S-tetrazine as an ultrafast phototrigger, which creates a 'kink' in the α -helix investigated, and isotopically labeled residues [63] as structural probes in a two-dimensional infrared spectroscopy (2D IR) experiment, they were able to obtain snapshots along the time course of the photo-induced conformational relaxation process [64].

Photolabile molecules (i.e., photocages) constitute another type of irreversible phototrigger [65-67], and have also been used to trigger protein conformational events [68]. The main idea is to use light to remove a moiety that disrupts native structure formation from the protein of interest, thus initiating folding. The advantage of using photocages, in comparison to photoresponsive cross-linkers, is that they can be localized to a single amino acid sidechain, thus offering greater flexibility. The disadvantage, on the other hand, is that typically more sample is needed and, in some cases, the diffusion of the photoproduct out of the protein matrix can significantly decrease the effective time-resolution of the method.

Examples of photocages that have been used to study protein folding include 4,5-dimethoxy-2-nitrobenzene [69] and 4-(bromomethyl)-6,7-dimethoxycoumarin [70].

Reversible phototriggers have the advantage of using less material and, thus, have received more attention. In particular, azobenzene and its derivatives [71-77] have become the most popular choice. Similar to stilbene [78-80], azobenzene undergoes ultrafast isomerization at the excited electronic state [81, 82]; it is this process (Figure 5), which can effectively change the distance between the two ends of the azobenzene linker, that is used to modulate protein structures in practice. When choosing a specific azobenzene linker, it is important to note that at equilibrium in the dark the moiety is predominantly (>90%) in the *trans* configuration, and the *cis* to *trans* conversion at the ground electronic state occurs on a seconds to minutes timescale, depending on temperature and the functionalization of the azobenzene cross-linker [83]. In protein conformational studies, di-iodoacetamide azobenzene, which can be inserted into proteins via cysteine alkylation [83, 84], is a widely used reversible phototrigger. Upon excitation with 355 nm light, this phototrigger can achieve an isomerization rate of $(1 \text{ ps})^{-1}$ and a yield of ~50% [85], making it ideally suited for studying ultrafast conformational rearrangements of small peptides and/or secondary structural elements. For example, Hamm and coworkers [84] have utilized this strategy to study the mechanism of α -helix formation and found that the coil-to-helix transition is inherently heterogeneous. Despite many successful applications of azobenzene-based cross-linkers, we note that developing reversible phototriggers is still a very active research area. For example, efforts to expand the excitation wavelength of azobenzene derivatives to the visible regime [86] and to identify/design other reversible phototriggers, such as spiropyrans [87] and thioamides [88-90], still continue.

Finally, another type of widely used phototriggering strategy, which has been recently reviewed [91], uses light to induce a change in the solution conditions, such as temperature [92, 93], pH [94-96], or electronic property of the protein [97-99], which consequently alters the folding/unfolding equilibrium of interest and thus induces a conformation relaxation.

5 Manipulating biological assemblies with light

It is now a widely accepted notion that intermolecular self-assembly of polypeptides, which typically leads to formation of highly ordered fibrillar structures that are rich in β -sheet content [100], further decreases the free energy of the system. However, *in vivo* formation of such insoluble amyloid fibrils has been shown to be associated with several degenerative diseases such as Alzheimer's, Parkinson's, and Type II Diabetes [101, 102]. On the other hand, the same process has been actively pursued as a way to generate biological architectures and scaffolds, such as peptide hydro-gels, that are posited to yield many new applications, ranging from regenerative medicine to the delivery of therapeutics [103, 104]. Because of their apparent importance, a wide variety of studies have been conducted to understand the mechanisms of amyloid formation and to find ways to combat this pathological condition. In particular, a recently pursued direction is to use light and phototriggers to manipulate and control the self-assembly process of interest, and also, to reverse the higher order structures thus formed.

For example, azobenzene has been introduced into various aggregation-prone systems to serve as a photoswitch between different aggregation or molecular states. These include the studies of Waldauer *et al.* [105] and Deeg *et al.* [106], which demonstrated reversible switching between aggregated and non-aggregated states of azobenzene-linked amyloid-like peptides, and the study of Doran *et al.* [107], which used an azobenzene-containing A β (1-42) peptide to investigate whether turn nucleation is the rate-limiting step in fibril self-assembly. In addition, Hamil *et al.* [108] have shown that an azobenzene-containing and light-responsive surfactant can inhibit A β (1-40) fibril formation when the azobenzene linkage is in the *trans* form.

Similar to applications in protein folding studies, light-induced decaging also finds novel use in studying the assembly of amyloid structures. For instance, Taniguchi *et al.* [109] applied a coumarin-derived photocage to trigger an intramolecular acyl migration, which converts a nonnative *O*-acyl group to an *N*-acyl group within the backbone of A β (1-42), restoring the native backbone composition and thus initiating aggregation upon illumination. Recently, we demonstrated the feasibility of using Lys-sine(4,5-dimethoxy-2-nitro-benzyloxycarbonyl) (Lys(nvoc)), which produces a native Lys sidechain upon decaging (Figure 6), to disassemble amyloid fibrils [110]. The working principle is that generation of a charged moiety (i.e., Lys) in a predominantly hydrophobic environment, which places the system in an energetically unfavorable position, will cause fibrils to dissociate. In a proof of principle study [110], we showed that this approach is effective in disassembling fibrils formed by a small amyloidogenic peptide wherein one of the native Phe (phenylalanine) residues was replaced by Lys(nvoc). As shown in the AFM images in Figure 6, the modified peptide self-assembles into well-ordered fibrils that can be destroyed by light. Using similar principles, a nitrobenzyl derivative serving as a photolabile linker between a hydrophobic alkyl chain and a small peptide was also efficient in disassembling well-formed fibrils [111, 112].

In addition, we and others have applied such photoswitching strategies to control the integrity of peptide hydrogels since their molecular packing and architectures are similar to those of amyloid fibrils [113]. Because of their ability to swell in water and, perhaps more importantly, their biological compatibility, peptide hydrogels hold potential for numerous biomedical applications such as drug delivery, tissue engineering scaffolds, and cell growth [104]. Since light is an external stimulus that offers precise spatial and temporal control, light-responsive hydrogels are expected to find great use in applications where such controls are required.

In one application, a photoswitch either an azobenzene [114-116] or a spiropyran [117], which links hydrogel-forming dipeptides together, was used to control π - π stacking between sidechains and hydrogen bonding patterns and thus the morphology of the hydrogel with light. Nilsson and coworkers [118] have shown that this strategy is also effective for longer peptides. In another application, He *et al.* [119] designed photodegradable hydrogels by linking a biaryl-substituted tetrazole to a small peptide, which undergoes a rapid intramolecular photoclick reaction that leads to a rearrangement within the ring system and disruption of the hydrogel matrix.

Moreover, Schneider and coworkers [120] have shown that the light-activated release of a nitrobenzyl-cage from a cysteine residue can trigger the self-assembly of the MAX1 amphiphilic β -hairpin peptide, which spontaneously self-assembles into hydrogels in its folded conformation. To demonstrate photoactivity in the opposite direction, we recently showed that modification of two Phe residues in the peptide hydrogel sequence (FKFE)₂ with Lys(nvoc) preserves the ability of the peptide to form a macroscopic hydrogel as shown in the AFM image in Figure 7A. However, these mutations render the formed hydrogel photodegradable in response to light, as indicated in Figure 7B [121]. In a slightly different treatment, Griffin *et al.* [122] utilized an *ortho*-nitrobenzyl cage as a photodegradable linker to tether a cell-adhesive peptide to a macromer-based hydrogel, thus allowing light-induced release. Furthermore, by incorporating photocages to the termini of cell-adhesive peptides derived from fibronectin (RGDS), which are typically covalently secured to a polymeric 3D hydrogel, photorelease of the cage prompts cell patterning and cell migration [123-126].

Indirect approaches, where the light-absorbing molecule is not directly linked to the hydrogel-forming peptide, have also been explored. For example, Raeburn *et al.* [127] employed photoacid generators, molecules that release an acid byproduct upon irradiation, to induce hydrogelation of low pH assembling dipeptide units. Similarly, Messersmith and coworkers [128] have shown that a 16-residue ionic complementary peptide, which gels at high salt concentrations, can be triggered by light when suspending the peptide in a solution of near-IR degradable liposomes containing CaCl₂.

6 Controlling biological function with light

Another highly important and actively pursued area of research is to develop methods that would allow us to regulate or control a biological process of interest, *in vivo* or *in vitro*, with light. While an in-depth summary [67, 129-132] of the recent advances in this field is beyond the scope of this Review, we would like to highlight several examples to underscore the rapid growth of this exciting research field. For instance, photoremovable protecting groups have been used to trigger acquisition of protein functions [133-135], to investigate enzyme binding and inhibition [136-138], protein translocation [139], chemotactic activity [140], peptide mediated lipid degradation [141], phosphopeptide-binding for signaling pathways [142, 143], and to regulate or control the function or activity of oligonucleotides [144, 145], neurotransmitters [146, 147], hormones [148], and signaling molecules [149, 150].

7 Conclusions

In this brief review, we highlighted recent advances in applications of inert and/or photoresponsive molecular constraints to better understand how proteins fold and self-assemble, to control the structural integrity and morphology of high-order biological assemblies, such as fibrils and hydrogels, and to regulate biological activity. While significant progress has been made recently in each and every one of these areas, we believe that further efforts are needed to (1) optimize the photochemical and photophysical properties of existing photoactivatable molecules, including wavelength of excitation, photochemical yield, timescale of photoswitching or photocleavage, and photoproducts, (2)

increase the pool of useful photoresponsive cross-linkers and photocages, (3) improve existing incorporation methods and develop *in vivo* incorporation techniques, and (4) explore new biological and biophysical applications and utilities. For instance, one new application we are currently testing is to take advantage of the mechanical force associated with isomerization of an azobenzene cross-linker to force the folding process of interest to rapidly populate the folding transition state.

Acknowledgments

This work was supported by the National Institutes of Health (GM-065978, AG-039253).

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Figure 1.
Cartoon representation of the effect of a covalent cross-linker or staple on α -helix formation.

Figure 2.

Cartoon illustration of the effect of a disulfide cross-linker on the folding free energy surface of a β -hairpin, where the polypeptide backbone is colored in blue, the hydrogen bonds are colored in green, and the disulfide cross-linker is colored in orange. Our study [48] suggests that it is possible to use an appropriately-placed linker to significantly decrease or even abolish the folding free energy barrier.

Figure 3. Temperature dependence of the folding (blue) and unfolding (red) rate constants of Trp-cage 10b (solid lines) and 4-8-CL-Trp-cage (dashed lines). As indicated in the inset, the latter contains a *m*-xylene cross-linker. This figure was generated based on the results of ref. [51].

Figure 4.

Top: Cartoon illustration of the principle of using light to remove a kink that is introduced by a tetrazine cross-linker in a model α -helix. Bottom: Representative molecular snapshots along the time course of helix reorganization initiated by photoexcitation of the tetrazine chromophore with a 2 ps laser pulse centered at 355 nm. This figure is adapted from ref. [64], with permission from the National Academy of Sciences.

Figure 5.

Top: Isomerization of azobenzene. Bottom: A cartoon representation of a β -hairpin peptide containing an azobenzene phototrigger in the *trans* (left) or *cis* (right) conformation.

Adapted from ref. [71], with permission from John Wiley and Sons, Inc.

Figure 6.

Representative AFM images showing the amyloid fibrils (left panel) formed by a mutant of A β (16-22) (sequence KLVF*FAE, where F* represents Lys(nvoc)) and the effectiveness of using light to disassemble these fibrils (right panel). Adapted from ref. [110], with permission from the American Chemical Society.

Figure 7.

(A) AFM image of the hydrogel formed by a Lys(nvoc) containing peptide (KFE8-C). (B) Infrared spectra of KFE8-C in the amide I' region before and after irradiation with 355 nm light for 90 min, showing the effectiveness of using light to disassemble the underlying β -sheet network of the hydrogel. The amide I' band reports on the carbonyl stretching vibrations of the peptide backbone and is an established reporter of secondary structures. The blue spectrum indicates that KFE8-C assembles into well-ordered anti-parallel β -sheets indicated by the bands centered at 1620 and 1685 cm⁻¹. After irradiation, the secondary structure undergoes a β -sheet to disordered conformational transition, evidenced by the appearance of a single, broad band centered at ~ 1650 cm⁻¹. Adapted from ref. [121], with permission from Elsevier.