

Photocontrolling Peptide α Helices

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ABSTRACT

Reversible optical control of protein structure and function offers the possibility of probing and manipulating individual proteins within the complex environment of a living cell. As a first step toward creating artificial photocontrolled proteins, we have designed and synthesized reversible, photocontrolled peptide α helices. Here, I attempt to summarize the lessons learned from that endeavor.

Introduction

A major goal of current research in chemical biology is the development of tools that enable *in situ* analysis of complex living systems. An example is the development of voltage-sensitive fluorescent dyes that enable the simultaneous monitoring of electrical activity in multiple neurons.¹ Simultaneous monitoring of multiple neurons can lead to fundamental new insights into the functioning of these systems. Optical methods, such as fluorescence imaging, are attractive for tackling complex systems because they can be employed effectively with intact living cells. Because the wavelength, intensity, and polarization of light can all be controlled with exquisite temporal and spatial resolution, very precise measurements can be made on individual cells or parts of cells.

Inspired by successes in the optical imaging of complex systems, we and others have embarked on the development of methods for *manipulating* complex systems *in situ* using light.^{2–4} Central to the success of such an endeavor is the creation of suitable photosensitive molecules that respond to light in known ways. Caged compounds, in which a light pulse is used to trigger removal of a chemical blocking group from a bioactive compound, have already had an impact⁵. *Reversible* photocontrol of bioactive molecules could enable even more sophisticated sorts of studies; for instance, the control of neuronal firing patterns might be controlled by reversible switching of ion-channel proteins.⁴ Proteins are natural targets for designed photocontrol because of their myriad

roles as biological catalysts and information traffickers. Direct photocontrol of gene expression using photosensitive nucleotide derivatives is also being pursued.^{6,7}

Design of Synthetic Photoresponsive Peptides

Initial work on the design of synthetic photoresponsive peptide systems focused on derivatizing synthetic peptide polymers with chromophores such as azobenzene and spiropyran.^{8,9} A variety of chromophores have been considered for use in photoswitching contexts; an excellent overview has been provided by Willner.² Azobenzene, in particular, has been a popular choice because its isomerization is not particularly environment-sensitive, occurs with high quantum yield in the range of 330–450 nm (where proteins are transparent), and is very fatigue-resistant.¹⁰ Additionally, isomerization of azobenzene involves a substantially larger conformational change than some other classes of reversible photoswitches (e.g., the fulgides).² Remarkable effects have been observed with azobenzene-modified peptides including photoinduced helix–coil transitions, helix sense reversal, aggregation/disaggregation, and photomechanical effects.⁸ Many of these effects can be understood in terms of changes in solvation and/or chromophore–polymer or chromophore–chromophore interactions upon isomerization. Indeed, there is now rich literature of azobenzene-induced effects on the material properties of a variety of polymers.¹¹

Natural proteins, in contrast to most polymers, typically adopt a single dominant conformation in their bioactive state. In principle, one might use *cis*–*trans* isomerization of azobenzene to directly affect the bioactive conformation of a protein. This was first attempted in a nonspecific manner (e.g., an amine-reactive azobenzene derivative was mixed with a protein resulting in complex labeling patterns of multiple lysine residues).¹² Subsequently, site-specific incorporation of chromophores near an enzyme active site via fragment complementation¹³ or, more recently, via nonnatural amino acid mutagenesis techniques^{14,15} also led to observable effects. In general, however, the effects of simple attachment of photoactive chromophores to proteins have been difficult to interpret in structural terms and are often not large. For instance, incorporation of phenylazophenylalanine at position 11 in RNase S, directly adjacent to a catalytic residue, led to small effects on enzyme activity distributed among K_M and k_{cat} .¹³ More vexing, from an engineering perspective, is the finding that when effects of photoisomerization on activity are substantial, reversibility is often compromised.^{15,16}

Recognizing that coupling of the isomerization event to protein conformational changes is vital for effective photocontrol and that conformational flexibility is likely to dampen effects of isomerization. Chmielewski and particularly Moroder have investigated a series of cyclic peptides bearing azobenzene units in their backbones.^{17–19} Here, direct conformational effects on the peptide back-

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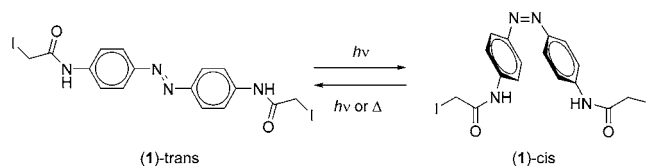
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bone were observed and could be understood in structural terms. Whereas *cis*-azobenzenes permitted regular β -turn-type conformations, trans isomers resulted in an ensemble of “frustrated” unfolded conformations.

Because we wished to focus on the development of a general switching strategy that could be ported to a variety of proteins, we wished for a means of introducing a switch into a natural protein that would have similarly strong and defined conformational effects. Backbone introduction would be difficult; therefore, we opted instead for the design of side-chain reactive reagents that could be introduced after protein synthesis.

Design of a Photoisomerizable Intramolecular Cross-Linker

Our first design aimed at trying to control helix content in a simple peptide system.²⁰ The helix was chosen because of its widespread occurrence in proteins but also because of the availability of well-characterized model systems for studying helix–coil transitions and the enormous body of knowledge surrounding the physical chemistry of helix formation and stability.²¹ β sheets and β turns, while also widespread, are less well-represented by model systems, and often, aggregation is problematic in designed β -sheet systems.²² Because we wanted a design that was simple and easy to employ, we focused on thiol-reactive photoisomerizable reagents. Cys residues can be readily introduced into proteins by site-directed mutagenesis, and the wide usage of thiol-reactive iodoacetamides, maleimides, and methanethiosulfonate (MTS)-type reagents attests to their ease of use. We therefore designed the iodoacetamide-modified azobenzene linker shown below.



Several criteria were involved. First, we wished to have the minimum number of single bonds between the N=N double bond that isomerizes and the peptide backbone so that the conformational changes would not be dissipated before affecting the peptide secondary structure (this is another reason for preferring Cys over Lys-reactive reagents). Second, a symmetrical structure was desired so that only one species would be formed upon reaction with a protein. Maleimides would lead to a mixture of isomers; therefore, iodoacetamides and MTS reagents were preferred. *Para*-substituted rather than *meta*- or *ortho*-substituted azobenzenes have symmetry that simplifies conformational analysis (more on this later). Interestingly, related reagents were introduced many years ago as biochemical cross-linkers but seem never to have been used as agents for photocontrol.²³

Our approach was then to choose one of the model peptides used to study the helix–coil transition²¹ and introduce Cys residues at appropriate sites. We were aiming for a Cys spacing that matched the linker length

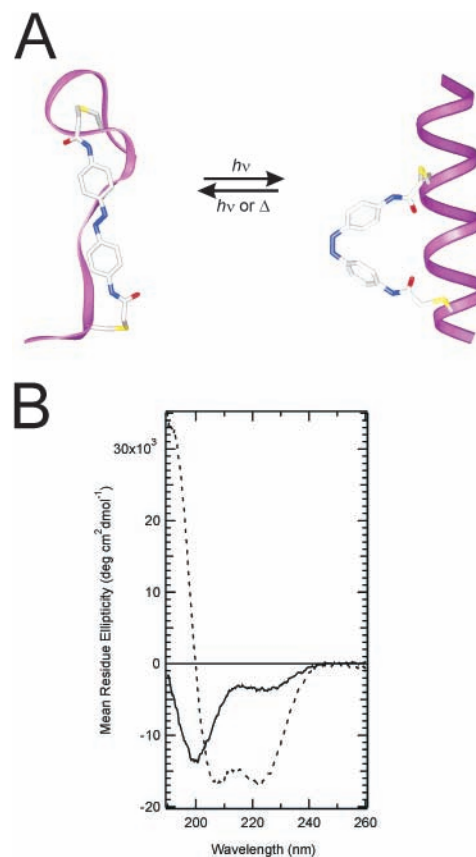


FIGURE 1. (A) JRK peptide (acetyl-EACARVAibAACEAAARQ-amide) cross-linked between Cys residues spaced $i, i + 7$. The peptide backbone is shown as a ribbon with the linker and Cys side chains shown as sticks colored by element type. Photoisomerization from trans to cis induces a substantial increase in peptide helix content. Thermal- or photoisomerization from cis to trans returns the peptide to its disordered form. Photoswitching can occur many hundreds of times without fatigue; however, some cleavage of C–S bonds has been observed after prolonged irradiation at high temperatures and high intensities. (B) CD spectra of trans (—) and cis (---) JRK-X.

in one conformation but not in the other. Using a simple computational approach,²⁰ we settled on an $i, i + 7$ spacing as being compatible with a cis conformation and less compatible with a trans conformation of the linker. We made this peptide, cross-linked it, and found a substantial increase in helix content that appeared after trans to cis isomerization of the linker²⁰ (Figure 1).

The fact that this occurred is rather remarkable in hindsight because the size of the effect depends on helical propensity (see below), which was not carefully designed, and the modeling was based on a rather poor computational description of the azo unit. To obtain realistic computed geometries and energies of the linker, it turns out that one must use a fairly high level of theory.²⁴ Our initial models suggested that a trans conformation of the cross-linker attached at $i, i + 7$ spaced Cys residues might not destabilize a helical conformation unless there was substantial steric clash between the linker and underlying side chains at positions $i + 3$ and $i + 4$. For this reason, Aib (α -aminoisobutyric acid) and Val were placed at these positions. The pro-R methyl group of Aib projects toward

the aromatic rings of the linker in simple models. The choice of Aib was a good one but for unanticipated reasons. Later work showed that steric interactions between the linker and the underlying residues were not important for photoswitching, but Aib did prevent severe aggregation of the noncross-linked peptide.²⁵

Effects of Photoisomerization of the Linker on Helix Content

Considerable effort has now been advanced in understanding how photoisomerization of this and other intramolecular cross-linkers drive changes in helix content. NMR analysis shows that the linker is mobile so that backbone conformational change does not seem to be driven by high-affinity, noncovalent interactions between the linker and the peptide, for instance, between the *cis* form of the linker and the underlying residues. This conclusion is supported by the finding that varying these amino acids does not have a substantial effect on the conformational transition (e.g., Ala, Ala in place of Val, Aib).²⁶

Previous studies on the effects of (nonphotoisomerizable) intramolecular cross-links on helix-coil transitions and on protein folding in general have started by considering the reduction in entropy of the unfolded state induced by the linker.^{27,28} For the helix-coil transition in particular, cross-links are often described in terms of increasing the probability of helix nucleation. However, this sort of analysis does not easily lead to an understanding of what aspects of the linker (e.g., length or flexibility) are important for producing the observed transition. Such understanding is important for the rational application of the cross-linker as well as in the design of improved cross-linkers.

Therefore, in an effort to understand in a quantitative manner the observed change in helix content, we first developed a procedure for calculating the expected distribution of Cys-Cys distances in noncross-linked peptides.²⁹ For a particular peptide sequence and set of experimental conditions, the AGADIR method³⁰ predicts overall helix content in close agreement with the experiment and also predicts the distribution of helix lengths. We then used FOLDTRAJ³¹ to produce a large ensemble of realistic all-atom peptide structures with overall helix content matching the experiment and with the AGADIR-predicted distribution of helix lengths. Because all atoms are represented in the structures generated by FOLDTRAJ, a histogram showing the distributions of distances between Cys sulfur atoms can readily be produced. The distribution of S-S distances predicted for the noncross-linked JRK peptide with an $i, i + 7$ Cys spacing and overall 25% helix content is shown in Figure 2.

Despite the relatively low overall helix content, the distribution is sharply peaked at the Cys-Cys (S-S) distance expected for a standard α -helical conformation (~ 10.8 Å). Note that peptides with very low total helix content ($\sim 5\%$) show a broad distribution with a maximum at longer S-S distances (Figure 2, \cdots). Covalent attach-

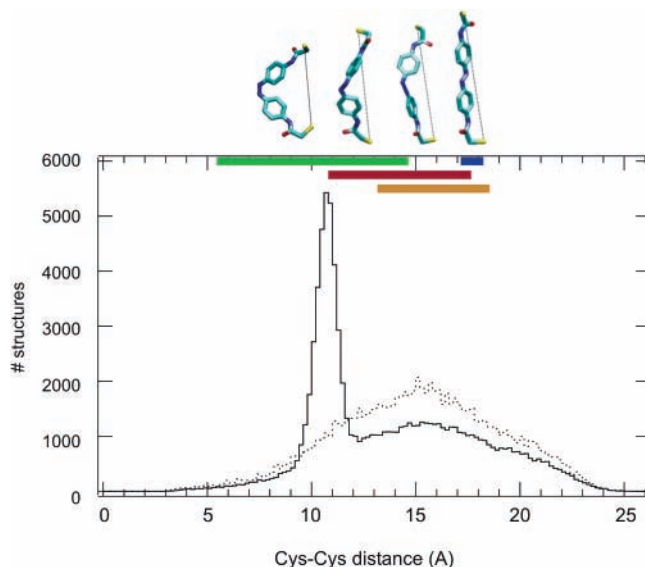


FIGURE 2. Histogram showing the distribution of Cys-Cys (S-S) distances (0.2 Å intervals) expected for the noncross-linked JRK peptide under the conditions of the CD experiment [overall helix content, 25% (—)]. The dotted line shows the expected distribution for noncross-linked JRK, where the overall helix content is 5% (e.g., at high temperature). The total number of structures was $\sim 100\,000$ in each case. The distance range allowed by a *cis* cross-linker is indicated by the green line, and the distance range allowed by a *trans* cross-linker is indicated by a blue line. The distance range allowed by a linker in the inversion transition-state structure is shown with an orange line, and the distance range allowed by a rotational transition state is shown with a red line. Some representative linker structures are shown above.

ment of an azobenzene cross-linker must alter this distribution. We made the simple assumption that the linker does not allow S-S distances outside a particular range, with the allowed ranges being different for *cis* and *trans* conformations of the linker, but does not otherwise greatly distort the distribution. To estimate what these allowed ranges are, we performed systematic conformational searches by rotating each single bond of each isomer of the linker. The range of S-S distances was found to be between 6 and 14.6 Å for the *cis* form (green line in Figure 2) and between 17.1 and 18.7 Å for the *trans* form (blue line in Figure 2). Distances were also calculated for inversion and rotation transition-state structures of the linker and will be discussed below. We did not try to rank the relative energies of these forms because their energy differences are small. Our intent was simply to examine the range of S-S distances that would be available to the isolated cross-linkers under typical experimental conditions. When these ranges are superimposed upon the distribution of S-S distances for noncross-linked JRK peptide (Figure 2), it is immediately evident that the *cis* range encompasses the spike in the distribution at 10.8 Å, whereas the *trans* range does not. If one then calculates the helix content associated with each of these subsets of peptide structures, one finds 48% helix for the *cis* range and 2% helix for the *trans* range. This result is a direct consequence of the greater likelihood of a long helix occurring near the 10.8 Å peak in the distribution.

Remarkably, this simple analysis describes semiquantitatively the nature of the observed effect; i.e., trans to cis isomerization of the covalently attached linker causes a very substantial increase in helix content (2% predicted versus 11% experimental for the trans conformation; 47% predicted versus 60% experimental for the cis form). There are a variety of possible reasons for the inexactness of these predictions. First, the ranges of S–S distances calculated are only approximate and do not take into account the bond and torsion angles required to make the covalent attachment of the peptide to the linker. In addition, no account is taken of possible steric clashes between the linker and the peptide. The fact that this procedure works implies that linker attachment does, in fact, not alter the conformational distribution greatly beyond the restrictions that it places on S–S distances. Thus, the linker may be viewed as simply “corralling” the intrinsic conformational dynamics (folding/unfolding processes) of the peptide. This view is supported by recent kinetic measurements using time-resolved ORD and IR techniques in which linker isomerization is observed to occur within a few picoseconds (similar to an unmodified azobenzene molecule),³² whereas peptide folding/unfolding occurs on the 100 ns to 1 μ s scale (similar to an unperturbed peptide helix).^{33,34}

A useful feature of this analysis is that it provides a straightforward means for thinking about how the linker structure affects the peptide conformational transition (for this and other linkers). This same analysis leads to simple predictions for the effect of cross-linkers attached to Cys residues with spacings other than $i, i + 7$ (next section). For instance, it suggests that if the conformation of the cis linker were more restrictive (i.e., the linker structure less flexible), so that only distances between 10 and 12 Å were permitted, even greater helix content for the cis cross-linked peptide might result. It also implies that for peptides with near zero helix content, i.e., pure random coil, cis–trans isomerization of an attached linker would have little effect on helix content because the helix content of both cis- and trans-allowed S–S ranges would be low. Consistent with this prediction, the difference in helix content between cis and trans cross-linked JRK peptides is observed to decrease with increasing temperature.²⁰

Alternative Spacings

We also studied peptides with an $i, i + 4$ Cys spacing in detail and found that these behave similarly to $i, i + 7$ peptides.³⁵ However, when the azobenzene cross-linker was used to cross-link Cys residues spaced at $i, i + 11$ (a peptide sequence designated FK-11), behavior that is the reverse of the JRK ($i, i + 7$ Cys) or FK-4 ($i, i + 4$ Cys) cases was observed. At equilibrium in the dark, the FK-11-X cross-linked peptide is almost completely helical. Irradiation to produce the cis isomer now destabilizes the helix (Figure 3).

This effect can be understood in the same way as just described for the $i, i + 11$ case. A noncross-linked peptide with Cys-residues spaced $i, i + 11$ has a broad distribution

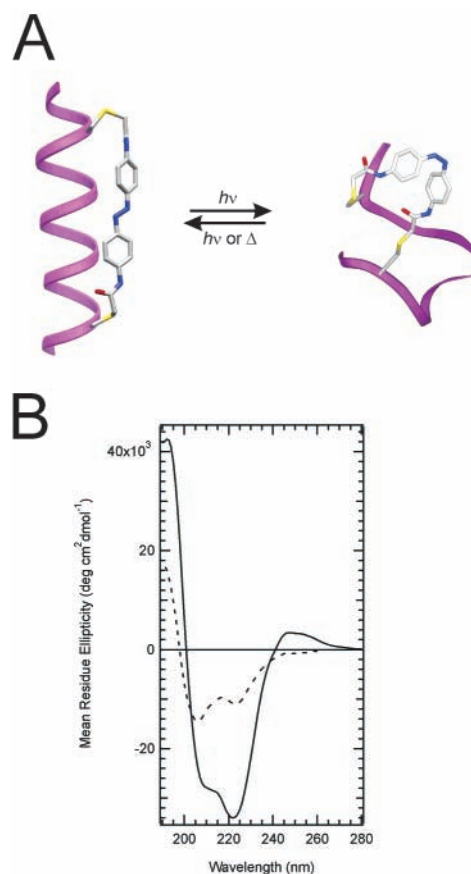


FIGURE 3. (A) FK-11 peptide (acetyl-EACAREAAAREAACRQ-amide) cross-linked between Cys residues spaced $i, i + 11$. Photoisomerization from trans to cis induced a substantial decrease in peptide helix content. Thermal- or photoisomerization from cis to trans returns the peptide to its helical form. (B) CD spectra of trans (—) and cis (---) FK-11-X.

of S–S distances centered at 21 Å with a spike at 17.4 Å, the helix distance (Figure 4). This distance is ideally suited to a trans cross-linker, whereas a cis cross-linker is too short to span this distance and instead forces a subset of unfolded conformations on the peptide.

Being able to choose whether the dark-adapted cross-linker stabilizes a helix ($i, i + 11$) or destabilizes it ($i, i + 4$; $i, i + 7$) enables greater control of the target system because the dark-adapted state of azobenzene is $\gg 99\%$ trans isomer,³⁶ whereas the percentage of cis isomer that can be achieved upon irradiation is typically 70–90% depending on the system. This fact sets intrinsic limits on the extent of the photocontrol of protein activity that may be possible using azobenzene. If the trans form is active, a ~ 5 -fold change in the concentration of the active species is possible (e.g., from $\sim 100\%$ trans in the dark to 20% trans upon irradiation). However, if the cis form is the active form, a much larger change in the concentration of the active species is possible (e.g., from $\ll 1\%$ in the dark to $\sim 80\%$ upon irradiation).³⁷ To maximize photo-switching, one would likely want to have the dark-adapted state as the inactive state of the system. This may correspond to having the trans form of the cross-linker either stabilize a helix or destabilize a helix depending on the structure of the target system.

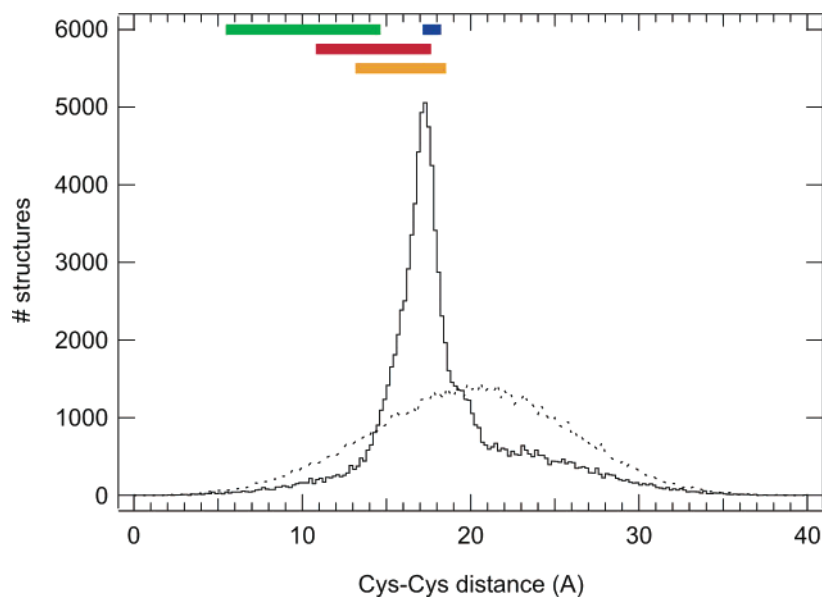


FIGURE 4. Histogram showing the distribution of Cys–Cys (S–S) distances expected for the noncross-linked FK-11 peptide under the conditions of the CD experiment [overall helix content, $\sim 50\%$ (—)]. The expected distribution for $<5\%$ helix content is shown as a dotted line. Cross-linker distance ranges are indicated as described for Figure 2.

Note that, although irradiation at wavelengths >400 nm promotes formation of the trans isomer, the absorption spectra of most azobenzene derivatives is such that this procedure still leaves substantial amounts ($\sim 10\%$) of the cis isomer. For complete ($\geq 99\%$) conversion to the trans isomer, thermal isomerization would seem preferable.

Reversibility: Cross-Linker Structure and Reisomerization

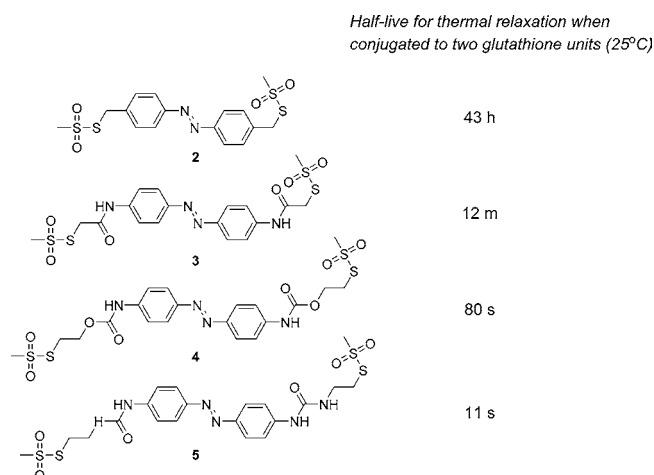
A vital feature of a photochemical switch, as opposed to a trigger, is reversibility. The conformational change (and accompanying activity change) must revert to the off-state reliably. In numerous instances, loss of reversibility is observed when azobenzene is incorporated into a protein or other target molecule.^{15,16,38} If we assume that the off state will be the trans isomer, then maintaining reversibility implies maintaining reasonable rates of cis to trans isomerization. Cis–trans isomerization can occur photochemically or thermally, but it is the thermal process that will be the focus here.

If the cis form of the cross-linker is significantly stabilized through its interaction with a target molecule and if this stabilization is removed in the transition state for cis–trans thermal isomerization, then a higher barrier will inevitably result and reversibility will be compromised. Thus, part of the design process must consider how this transition state will be affected by its attachment to the target molecule. Doing this is complicated by the lack of consensus as to whether azobenzene isomerizes via an inversion mechanism or via a rotation mechanism under a given set of conditions.^{39–42} Nevertheless, for the peptides that we are considering here, one can address this question by considering how the intrinsic conformational preferences of the peptide [e.g., JRK-7 (Figure 2)] fit with the geometrical requirements of either a rotation transition state or an inversion transition state (the orange and

red lines respectively in Figures 2 and 4). Let us take as an example the process of a cross-linked JRK-7 peptide going from cis to trans via thermal isomerization through an inversion transition state. Cis JRK-X peptides that have S–S distances that are also compatible with an inversion transition state will be able to undergo isomerization normally, i.e., almost as if the peptide were not present. These peptides are those in Figure 2 with S–S distances that fall in the overlap region between the green and orange lines. The rest of the cis peptides must first undergo some sort of conformational rearrangement, more complicated than simple rotation about a few single bonds at the linker attachment point, to be able to undergo isomerization. The extent to which reversibility is compromised is thus dependent on whether the intrinsic conformational preferences of the target molecule include conformations that are compatible with a transition state for isomerization and also, potentially, how rapidly conformational equilibrium is established in the cis state. In the present case, conformational equilibrium is established rapidly relative to the half-life from thermal isomerization (~ 12 min at 25°C). Thus, the extent to which a peptide inhibits thermal cis–trans isomerization is determined by the intrinsic preference of the peptide to be in an inversion-competent conformation (overlap between the orange and green lines) versus anywhere in the cis-allowed region (green line). For JRK-X, the ratios of these two populations is about 1:5.5. That is, only about 15% of the cis peptides are inversion-competent at any specific time. This adds ~ 1 kcal/mol to the apparent activation energy for thermal isomerization.⁴³ For FK-11, about 35% of the cis peptides are inversion-competent, leading to distinctly smaller effects on barrier to thermal isomerization.^{35,43}

Rates of thermal cis–trans isomerization can also be manipulated by altering the chemical structure of the

linker. To increase the rate of thermal reversion, we designed derivatives in which enhanced delocalization of the *para* amino group lone-pair electrons would be possible. High reactivity and selectivity toward Cys residues was maintained by introducing methanethiosulfonyl groups.⁴⁴ These groups also permit attachment of the cross-linker to peptides and proteins via disulfide linkages that can be subsequently cleaved, if desired, using reducing agents. When used to cross-link the FK-11 peptide described above, these linkers enabled photocontrol of peptide helical content in a manner similar to the original iodoacetamide-based cross-linker but with a large range of thermal stabilities observed for the cis forms from 11 s to 43 h at 25 °C.



The value of the half-life for thermal isomerization is important for determining the practical usefulness of such photochemical switches. For instance, if structural studies are intended with the cis form, a much longer half-life than 12 min may be desired. Alternatively, if a pulsed conformational change is desired as part of a biochemical switch, then rapid return to the trans state would be preferable. Additionally, a rapid thermal relaxation to the trans form ensures virtually complete conversion to the trans isomer in short times.

Applications and Expected Limits on the Process

At present, the use of azobenzene-based cross-linkers appears to be a fairly robust strategy for the photocontrol of peptide helix content, at least for monomeric peptides in aqueous solution. The next step will be to understand the requirements for photocontrol of peptides and proteins in which a change in helix content affects function. These are necessarily more complex than the simple monomeric peptides described here. In the long-term, however, we hope that this strategy may prove applicable to the reversible photocontrol of protein function in heterogeneous environments such as living cells.

In seeking to apply this strategy for photocontrol to more complex functional peptides and proteins, it is worthwhile to reflect on the possible pitfalls. Loss of reversibility is one possible pitfall, and how it may be

avoided has been discussed above. Another possible pitfall is that isomerization is reversible but has little or no effect on conformation or function. This may well occur if the linker was attached to a sequence with little or no intrinsic helical propensity. For random-coil peptides with no helical propensity, the cross-linkers may be expected to simply cause redistribution in the conformational ensemble as can be seen from Figures 2 and 4.

One may wonder about the possibility that a peptide sequence has such a strong helical propensity that coupling to the linker leads to a substantial change in the relative stabilities of the cis and trans forms of azobenzene. It seems that for most peptides and proteins this would be an unlikely scenario because the difference in thermodynamic stability between cis and trans forms of (unmodified) azobenzene is about 12 kcal/mol,³⁶ a substantial amount of energy relative to typical free energies of folding for proteins.⁴⁵ Nevertheless, as the stability of the target protein increases, one might expect the process of trans–cis photoisomerization to become more difficult. To clarify this point, let us consider a process of trans–cis photoisomerization when the trans linker is attached to a very stable helix (i.e., like the FK-11-X case but with a much more stable helix). Absorption at 370 nm deposits ~75 kcal/mol of energy in the molecule. This amount of energy would be more than sufficient to drive isomerization, but of course, it can also be lost as heat. The critical feature is how the attached peptide or protein affects the shape of the excited-state surfaces of the chromophore. Typical quantum yields for the isomerization of unmodified azobenzene are 0.1 for trans–cis and 0.4 for cis–trans after π – π^* absorption.⁴³ If the quantum yield dropped to 0.001 for trans–cis isomerization, for example, as a consequence of a new barrier being introduced into the excited state surface (because of coupling of the linker to a rigid helix), then switching would be greatly impaired.

Quantum yields for FK-11-X have been measured and are similar to or even greater than unmodified azobenzene values.⁴³ This may be because the peptide does not have a very strong intrinsic conformational preference, or it may be a consequence of the linker geometry itself. Note that the allowed ranges for both the rotation and inversion transition-state linker structures overlap the trans-allowed range (Figures 2 and 4). Thus, single-bond rotations in the linker could allow a substantial part of the isomerization to occur rapidly with no immediate effect on the peptide. The situation is very reminiscent of the situation with the natural light-sensitive protein photoactive yellow protein in which very rapid (subpicosecond) isomerization of the *p*-hydroxycinnamic acid chromophore occurs via movement of the carbonyl group of the thioester linkage, a movement that requires relatively little movement of protein atoms.⁴⁶ The implication, of course, is that linkers with less flexibility might exhibit low photoisomerization quantum yields when attached to stable proteins. The requirements for efficient isomerization thus, to some extent, run contrary to those for maximizing the effect of isomerization on protein conformation.

Summary

The long-term goal of this work is to learn how to make light-sensitive proteins so that we might be able to understand the roles of particular proteins in living systems in a much more integrated manner than is now possible. The fact that nature has evolved reversible, photocontrolled proteins means that it is clearly not impossible to do so. I have attempted here to provide an account of our attempts to learn how to reliably control helix content in peptides in a reversible manner, a first step toward that longer term goal. Through a rather fortuitous series of choices, we managed to create simple peptide systems where photocontrol of helix content appears to occur in much the same way that photocontrol of conformation and activity occurs in natural light-sensitive proteins.⁴⁶ The chromophore and the manner of its attachment to the protein are such that isomerization couples naturally to an intrinsic conformational change in the peptide/protein. The challenge now will be to try to preserve the same features in more complex-designed photocontrolled proteins where conformational changes are linked to changes in a biochemical activity.

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