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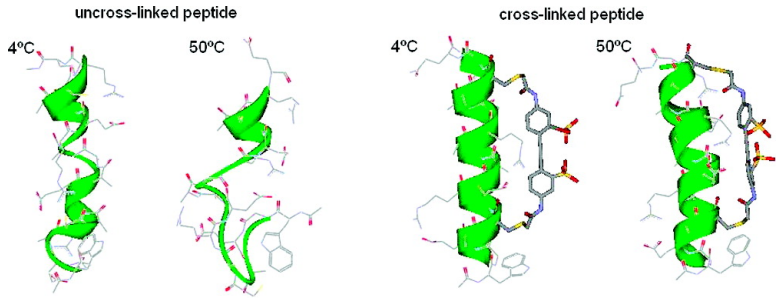

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Stabilization of Folded Peptide and Protein Structures via Distance Matching with a Long, Rigid Cross-Linker

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The functions of peptides and proteins often depend critically on their folded three-dimensional structures. However, the folded states of most proteins are only ≤10 kcal/mol lower in energy than their unfolded states.[1] Short peptide sequences are usually unfolded in aqueous solution, although they adopt specific folded structures when bound to their biological targets. Stabilizing the active folded forms of peptide or proteins is thus important for maintaining or enhancing the functions of these molecules under a wide variety of conditions. Owing to its prevalence as a secondary structural element in proteins, the α-helix has received considerable attention as a target for conformational stabilization. Stabilized helices have been employed as biological agents, for example, against microbial infections,[2] and for targeting tumor suppressor proteins[3] and proteins involved in apoptosis.[4]

Methods developed to stabilize peptide α-helical structures include introduction of α,α-dialkyl amino acids,[5] cross-linking amino acid side chains via disulfide bonds,[6] metal chelates,[7] lactam bridges,[8,9] or via ruthenium-catalyzed ring closing metathesis (RCM) to form cyclic peptides.[10] These methods require peptide synthesis using non-natural amino acids. Recently, Fujimoto et al. introduced acetylenic cross-linking agents that could be introduced via pairs of naturally occurring lysine side chains;[11] however, the degree of helix stabilization was moderate.

In the absence of specific favorable interactions between a cross-linker and the folded state of a peptide or protein, the mechanism of stabilization appears to be primarily the decrease in conformational entropy of the unfolded state caused by an intramolecular covalent linkage.[12,13] We anticipate that maximal stabilization should occur with (i) optimal matching between the cross-linker length and the distance distribution between the two attachment points of the folded peptide or protein, (ii) enhanced rigidity of the cross-linker, and (iii) a long cross-linker that can bridge sites far apart in amino acid sequence. Here we introduce a water-soluble, thiol-reactive cross-linking reagent (EY-CBS) (3,3′-ethyne-1,2-diylbis-{6-[(chloroacetyl)amino]benzenesulfonic acid} (**1**) that can be used to introduce long, rigid bridges into peptides and proteins. We compare its stabilizing effects on helices as well as on a small β-sheet protein with the effects of more flexible linkers, including the commercially available 1,4-di[3′-(2′-pyridyldithio]propionamido]butane (DPDPB).

Figure 1 shows the structure of EY-CBS (**1**), synthesized in four steps (see Supporting Information), together with an analogue EA-CBS (**2**) with a single bond in place of the central triple bond, and the flexible commercial cross-linker DPDPB (**3**). EY-CBS is designed as linear, symmetric, and Cys-reactive to minimize the number of possible conformations of the cross-link with respect to the peptide backbone attachment points. Sulfonate groups are included for water solubility since introduction of a large hydrophobic cross-linker to a protein surface may lead to aggregation or insolubility. Whereas each of these cross-linkers can span a ∼20 Å distance, their end-to-end distance probability distributions as

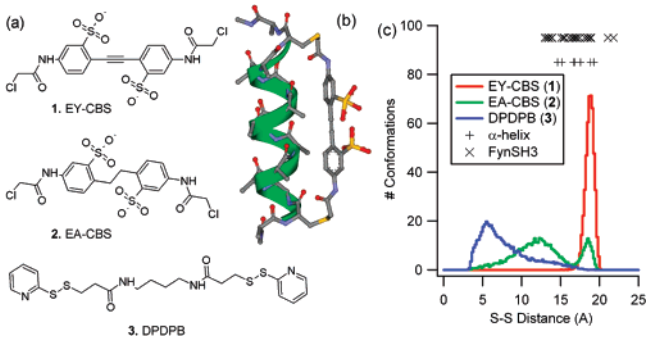

Figure 1. (a) Structures of cross-linkers. (b) Model of helical peptide with Cys residues at *i*, *i*+11 positions cross-linked with **1**. (c) Distance distributions of cross-linkers calculated using Langevin dynamics simulations. Cys−Cys sulfur distances corresponding to different side-chain rotamers in an ideal helix with Cys residues spaced at *i*, *i*+11 and a T14CE33C-FynSH3 mutant are also shown.

calculated using Langevin dynamics[14] vary considerably (Figure 1). EY-CBS gives a narrow distance distribution centered at 18.7 Å, and EA-CBS gives a bimodal distribution due to the three possible rotamers around the central carbon−carbon single bond. DPDPB, although marketed as a cross-linker with a spacer length of 19.9 Å,[15] gives a broad distribution with numerous short conformers.

An end-to-end distance of 18.7 Å closely matches the sulfur-to-sulfur distance in an ideal α-helix with Cys residues spaced at *i*, *i*+11 positions (Figure 1). These positions span three helical turns and are located on the same face of the helix so that they can be cross-linked without steric inference between the cross-linker and protein side chains.

We first applied these cross-linkers to FK11W, a short peptide sequence that has been used as a model helix previously.[16] Cross-linking reactions were carried out in aqueous solution at mildly basic pH and ambient temperature. All the cross-linking reactions proceeded to more than 95% completion when 4 equiv of cross-linker was present. There was no detectable amount of doubly reacted peptide (two cross-linkers attached to one peptide), indicating the cross-linking step, an intramolecular reaction, is much faster than a second intermolecular reaction.

The effect of cross-linking on peptide structure was examined by circular dichroism (CD). At room temperature, the CD spectra of FK11W cross-linked with each of **1**−**3** showed an increase in α-helix as compared with the uncross-linked peptide (Figure 2a). The order in increased helix percentage was **1** > **2** > **3** with the flexible DPDPB cross-linked peptide only slightly more helical than uncross-linked FK11W, much less than the rigid EY-CBS-FK11W. There was no evidence of peptide self-association from 2 to 300 μM as determined by CD.

Next, we examined the response of these cross-linked peptides to thermal melting. The α-helical contents of the peptides were evaluated on the basis of the mean residue ellipticity at 222 nm[16]

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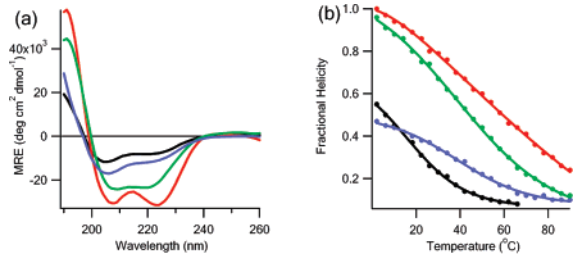

Figure 2. (a) CD spectra of uncross-linked FK11W (black), EY-CBS-FK11W (red), EA-CBS-FK11W (green), DPDPB-FK11W (blue) at 25 °C. (b) Thermal melting curves FK11W (black), EY-CBS-FK11W (red), EA-CBS-FK11W (green), DPDPB-FK11W (blue).

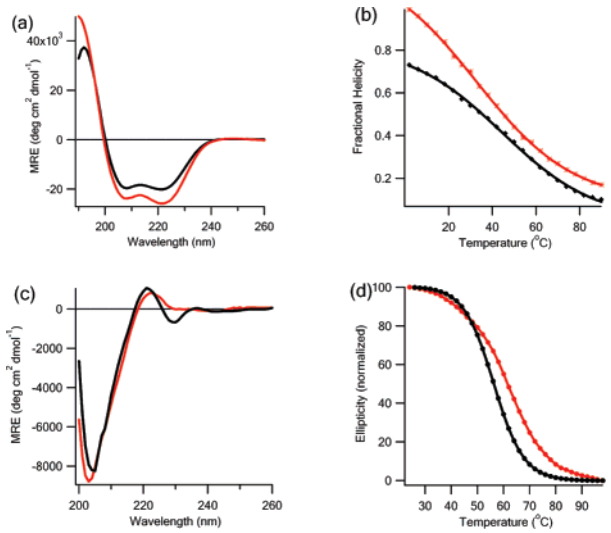

Figure 3. (a) CD spectra of FK22C (black) and EY-CBS-FK22C (red) at 25 °C. (b) Thermal melting curves of FK22C (black) and EY-CBS-FK22C (red). (c) CD spectra of T14CE33C-FynSH3 (black) and EY-CBS-T14CE33C-FynSH3 (red) at 25 °C (d) Thermal melting curves of T14CE33C-FynSH3 (black) and EY-CBS-T14CE33C-FynSH3 (red) at 25 °C

over the temperature range of 2−98 °C (Figure 2b). At 2 °C, the peptide cross-linked with **1** is almost 100% helical. Even at 62 °C, where the uncross-linked FK11W has already completely unfolded, the EY-CBS-FK11W is still 44% helical, more helical than uncross-linked FK11W at 2 °C. At 37 °C, the EY-CBS cross-link increases FK11W helicity from 16 to 71%. Introduction of a single bond in place of the triple bond in an otherwise identical cross-linker decreases its effectiveness at conformational stabilization (Figure 2b). The very flexible DPDPB-FK11W showed only slight stabilization (up to 14%).

To explore the effectiveness of **1** at conformational stabilization of larger, more complex targets, we examined its effects on FK22, a 32-residue α-helical peptide and on the FynSH3 domain, a small β-sheet protein. We expect that the extent of stabilization in these cases will in general be smaller and will depend on the extent to which the cross-linker can influence distinct segments of the protein. Cys residues were installed near the C-terminal end of FK22 with an i, i+11 spacing, thereby targeting about half the total length of the peptide. To identify sites for cross-linking in the FynSH3 domain, we used the program sGAL,[17] which identifies surface-exposed pairs of residues a specified distance apart taking a PDB file as input. This procedure identified two residues T14 and E33 located on two loops of the FynSH3 domain with a spacing that matched the distance of the cross-linker well (Figure 1, Supporting Information Figure S1).

Figure 3 shows CD spectra and thermal melting curves for FK22C and T14CE33C-FynSH3 with and without cross-linkers. Cross-linking of FK22C increased the helix content to almost 100% at 2 °C and enhanced helix content by more than 10% from 30 to 90 °C. At 37 °C, the helicity was increased from 49 to 60%. The CD spectrum of cross-linked T14CE33C-FynSH3 showed little difference with that of uncross-linked FynSH3 at 25 °C, indicating that EY-CBS-T14CE33C-FynSH3 is fully folded at this temperature and that cross-linking does not significantly disturb the native folded state of SH3. The thermal melting curve indicated that the cross-linker enhanced the conformational stability of T14CE33C-FynSH3, causing an increase in the T_m by almost 10 °C, despite the fact that it links flexible loops in the protein rather than more rigid elements of secondary structure.

These results demonstrate the effectiveness of a long rigid cross-linker at conformational stabilization of peptides and proteins. Flexible linkers that can adopt similar overall lengths, even an analogue that differs in flexibility only at one bond, are demonstrably less effective. The strategy is particularly useful for stabilizing α-helical segments with Cys residues at i and i+11 positions but also appears applicable to larger structures.

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Supporting Information Available: Synthesis, ^{1}H NMR, ^{13}C NMR of compounds **1** and **2**. Synthesis of peptides, expression and purification of protein and cross-linking reactions. Modeling of cross-linker distance distributions. This material is available free of charge via the Internet at http://pubs.acs.org.

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