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Contemporary Strategies for the Stabilization of Peptides in the α -Helical Conformation

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Summary of recent advances

Herein we review contemporary synthetic and protein design strategies to stabilize the α -helical motif in short peptides and miniature proteins. Advances in organometallic catalyst design, specifically for the olefin metathesis reaction, enable the use of hydrocarbon bridges to either crosslink side chains of specific residues or mimic intramolecular hydrogen bonds with carbon-carbon bonds. The resulting hydrocarbon-stapled and hydrogen bond surrogate α -helices provide unique synthetic ligands for targeting biomolecules. In the protein design realm, several classes of miniature proteins that display stable helical domains have been engineered and manipulated with powerful in vitro selection technologies to yield libraries of sequences that retain their helical folds. Rational re-design of these scaffolds provide distinctive reagents for the modulation of protein-protein interactions.

Introduction

Examination of complexes of proteins with other biomolecules reveals that proteins tend to interact with partners via folded sub-domains, in which the backbone possesses secondary structure. α -Helices constitute the largest class of protein secondary structures, and play a major role in mediating protein-protein interactions[1,2]. Significantly, the average length of helical domains in proteins is rather small and spans two to three helical turns (or eight to twelve residues)[3]. These complexes suggest that it may be possible to develop short helices that potentially participate in selective interactions with biomolecules. However, peptides rarely retain their conformation once excised from the protein; much of their ability to specifically bind their intended targets is lost because they adopt an ensemble of shapes rather than the biologically relevant one. The proteolytic instability of peptides is an additional factor that limits their utility as reagents in molecular biology and drug discovery. In principle, stabilization of peptides in the helical structure should not only reduce their conformational heterogeneity but also substantially increase their resistance to proteases as these enzymes typically bind their substrates in the extended conformation[4]. The proteolytic stability of the helix should thus be directly proportional to its conformational stability. The chemical biology community has focused much of its attention on studying different approaches to either stabilize the α -helical conformation in peptides or mimic this domain with nonnatural scaffolds [5].

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Figure 1 illustrates the different approaches that have been adopted either to stabilize or mimic an α -helix, with the overall aim of endowing peptidic and nonpeptidic oligomers with conformational rigidity, proteolytic stability, and the desired array of protein-like functionality. These approaches can be divided into three general categories: helix stabilization, helical foldamers and helical surface mimetics. Helix stabilizing methods based on side chain crosslinks and hydrogen-bond surrogates preorganize amino acid residues and initiate helix formation; miniproteins that display helical domains would also be part of this category. Helical foldamers, such as beta-peptides and peptoids, are composed of amino acid analogs and are capable of adopting conformations similar to those found in natural proteins. Helical surface mimetics utilize conformationally restricted scaffolds with attached functional groups that resemble the *i*, *i*+4, *i*+7 pattern of side-chain positioning along the face of an α -helix. Excellent recent reviews of foldamers and helix surface mimetics are available in the literature[5–11]. In this report we focus on general strategies for stabilizing peptides composed of α -amino acids in the α -helical conformation.

Side Chain Crosslinked α -Helices

The α -helix features 3.6 residues per complete turn, which places the *i*, *i*+4, *i*+7, and *i*+11 side chains on the same face of the folded structure. The classical strategy to stabilize the α -helical conformation in peptides employs covalent bonds between the *i* and *i*+4 or *i* and *i*+7 side chain groups. Earliest side chain crosslinks utilized lactam, disulfide and metal-mediated bridges [12–21]. Helices containing lactam-bridges and disulfide links have successfully targeted their intended receptors[17,22–26]. Schafmeister and Verdine recently described hydrocarbon bridged[27]. side chain crosslinked helices obtained from an olefin metathesis reaction[28]. These researchers carefully examined the linker length and stereochemistry to arrive at the optimal design. The hydrocarbon stapled helices were subsequently shown to target antiapoptotic proteins hDM2 and Bcl-2 in cell culture and animal models[29,30]. The hydrocarbon linker was employed because it was expected to be chemically more stable than linkers built from amide or disulfide bonds; but, significantly, these hydrocarbon stapled helices have also shown an increased tendency to penetrate the cell membrane possibly due to the lipophilic nature of the linker. Debnath and coworkers also discovered that the hydrocarbon stapled helices can penetrate cells and target HIV-1 capsid assembly[31].

The lactam-bridged and hydrocarbon stapled helices feature flexible crosslinks. Entropic considerations would suggest that rigid linkers might afford more stable helices. Two groups have recently studied the effect of linker flexibility on helix stability. Woolley and coworkers found that a rigid aromatic linker that matches the distance between the *i* and *i*+11 side chains provides much greater stability than a flexible linker[32]. Fujimoto, et al. performed a detailed examination of various flexible and rigid linkers, and crosslinking positions on the helix[33]. They hypothesized and demonstrated that rigid linkers that are shorter than the target helix pitch lead to more stable helices. These interesting findings may lead to reevaluation of linker lengths in side chain crosslinked helices.

Hydrogen Bond Surrogate Derived α -Helices

The α -helix is characterized by a 13-membered intramolecular hydrogen bond between the C=O of the *i*th and the NH of the *i*+4th amino acid residues. The helix-coil transition theory in peptides suggests that the energetically demanding organization of three consecutive amino acids into the helical orientation, which leads to the intramolecular hydrogen bond between the *i* and *i*+4 residues, is the rate-determining step[34]. Preorganization of amino acid residues in an α -turn is expected to overwhelm the intrinsic nucleation propensities and initiate helix formation. Accordingly, one strategy for the stabilization of α -helices involves replacement of one of the main chain intramolecular hydrogen bonds with a covalent linkage. Cabezas and Satterthwait have proposed a hydrazone bridge as a mimic for the hydrogen bond[35], while

Arora and coworkers utilized a carbon-carbon bond prepared by a ring closing metathesis reaction (Figure 2a)[36,37]. This RCM-derived hydrogen bond surrogate (HBS) approach stabilizes the α -helical conformation in short (7-12 residue) peptide sequences, which has been a challenge for the field. The high resolution (1.15 Å) crystal structure of a short HBS α -helix shows that the RCM-based macrocycle faithfully reproduces the conformation of a canonical α -helix (Figure 2b)[35]. All i and $i+4$ C=O and NH hydrogen bonding partners fall within distances and angles expected for a fully hydrogen-bonded short α -helix (Figure 2c). The backbone conformation of the HBS α -helix in the crystal structure superimposes with an RMS difference of 0.75 Å onto the backbone conformation of a model α -helix (Figure 2d), supporting the hypothesis that stable short helices can be accessed by the HBS strategy.

HBS α -helices show a remarkable degree of thermal stability as the constrained peptide retains 60–70% of its room temperature helicity at 85 °C. Although HBS helices are conformationally stable at high temperatures, they can be denatured with concentrated guanidinium chloride. The thermal stability and the broad thermal denaturation curves displayed by HBS helices are consistent with the theoretical predictions for a nucleated helix. The nucleation constant, σ , refers to the organization of three consecutive amino acid residues in an α -turn and is typically very low (10^{-3} – 10^{-4}) in unconstrained peptides, disfavoring helix formation[38,39]. The intent behind the hydrogen-bond surrogate approach was to afford pre-nucleated helices such that σ would be ~ 1 . Estimates of σ in HBS helices obtained from the Zimm-Bragg model by comparing theoretical denaturation curves as a function of different nucleation constants with the experimental denaturation curve for HBS helices suggests that the nucleation constant is close to unity in HBS helices[40].

HBS helices can target gp41-mediated HIV-1 fusion in cell culture, which highlights the potential of these artificial helices as inhibitors of chosen protein-protein interactions in complex settings[41]. An attractive feature of the main chain hydrogen bond surrogate strategy is that placement of the crosslink on the inside of the helix does not block solvent-exposed molecular recognition surfaces of the molecule, as compared to the side chain crosslinking strategies. This feature potentially allows HBS helices to target tight binding pockets on proteins[42]. As expected from their conformational stability, HBS helices are significantly more resistant to proteases than their unconstrained counterparts[41,42].

Miniature Proteins that Display α -Helical Domains

The α -helix design strategies described above applied synthetic constraints to stabilize this domain in short peptides. Typical α -helical domains within proteins are stabilized through noncovalent contacts within protein tertiary structures. Engineered miniature proteins that display stable, solvent-exposed helical scaffolds present appealing opportunities for the design of ligands for specific targeting of chosen biomolecular receptors. The intrinsic advantage offered by these miniproteins is that they are readily amenable to diversification by in vitro biological combinatorial library generation and selection technologies[43]. Several classes of such miniproteins have been engineered and optimized for target receptors[44]. Figure 3 shows miniproteins protein Z, zinc fingers, Trp-cage, and avian pancreatic polypeptide (aPP) that display helical domains.

The Schepartz lab successfully utilized the aPP scaffold for the presentation of an α -helical domain that can be diversified through the phage display technology[45]. The structure of pancreatic fold proteins feature an N-terminal polyproline II helix that interacts with a C-terminal α -helix through hydrophobic interactions (Figure 3a). In a series of experiments, these researchers identified high affinity miniprotein ligands for a diverse assortment of proteins and DNA targets[46,47]. The inefficient cellular uptake is a major obstacle limiting the potential of these miniprotein scaffolds as leads in drug discovery. However, continuing research efforts may eventually improve our understanding of conditions required for efficient cellular uptake.

In recent studies, Schepartz and coworkers have shown that judicious placement of cationic residues provides aPP analogs that are cell permeable while maintaining their conformational stability[48].

The Trp-cage is a 20-residue C-terminal sequence of extendin-4, and contains a 9-residue α -helix followed by short 3_{10} -turn and a 5-residue polyproline II helix (Figure 3b)[49]. This miniprotein has a well-defined tertiary structure that features a central tryptophan residue from the α -helix encased by several hydrophobic residues as the interface between the α - and PPII helices. This hydrophobic cluster endows the protein with significant structural stability. Importantly, the residues that do not participate in the hydrophobic interface may be randomized to provide libraries of miniproteins displaying short α -helices[50].

Conclusions

A fundamental limitation of current drug development efforts centers on the inability of traditional pharmaceuticals to target spatially extended protein interfaces. The majority of modern pharmaceuticals are small molecules that target enzymes or protein receptors with defined pockets. However, in general these small molecules cannot target protein-protein interactions involving large contact areas with the required specificity. α -Helices comprise the largest class of protein secondary structures and play an integral role in the interaction of proteins with their binding partners. One major goal of the chemical biology community has been to develop efficient means of mimicking this conformation in peptidic and non-peptidic oligomers, such that the resulting product resists proteolytic degradation, penetrates cell membranes, and arrays functional groups over a larger surface area as compared to traditional pharmaceuticals. The recent advances in design of stabilized helices, helical foldamers, and helix surface mimetics will allow the community to test basic principles of biomolecular recognition in complex settings, while providing new reagents for molecular biology and drug discovery.

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Figure 1.

Stabilized helices and nonnatural helix mimetics: several strategies that stabilize the α -helical conformation in peptides or mimic this domain with nonnatural scaffolds have been described. Recent advances include β -peptide helices, terphenyl helix-mimetics, mini-proteins, peptoid helices, side-chain crosslinked α -helices, and the hydrogen bond surrogate (HBS) derived α -helices. Green circles represent amino acid side chain functionality.

Figure 2.

(a) Nucleation of short α -helices by replacement of an N-terminal i and $i+4$ hydrogen bond with a covalent bond. The hydrogen bond surrogate-based (HBS) α -helices contain a carbon-carbon bond derived from a ring-closing metathesis reaction. (b) Crystal structure of the HBS α -helix with electron density map superimposed onto the refined molecular model. (c) Putative i and $i+4$ hydrogen bonds (magenta lines) in crystal structure-derived molecular model of HBS helix. (d) Overlay of crystal structure and a model of an idealized α -helix.

Figure 3. Miniature proteins that display stable helical folds: (a) avian pancreatic protein (PDB code: 1ppt), (b) Trp cage (PDB code: 112y), (c) zinc finger protein (PDB code: 1a11), and (d) Z domain of staphylococcal protein A (PDB code: 2b88).