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End-Capped α -Helices as Modulators of Protein Function

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Abstract

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, the largest class of protein secondary structures, play fundamental roles in a multitude of highly specific protein-protein and protein-nucleic acids interactions. Herein, we describe the potential of a helix nucleation strategy to afford modulators of protein-protein interactions.

Introduction

α -Helices are critical elements of biomolecular recognition as reflected by the fact that 62% of protein-protein complexes in the PDB feature a helical interface [1]. Mimicry of the interacting helical elements provides a potentially useful means for the modulation of protein-protein interactions [2,3]. However, this strategy is challenging in large part because the organization of peptides into defined three dimensional structure is energetically demanding [4] and the simple excision of short peptide sequences from a parent protein leads to a loss of organized secondary structure [5]. Maintenance of defined structure is important for molecular recognition and proteolytic stability *in vivo*.

The unstructured nature of short peptide sequences in solution can be explained by helix-coil transition models, which propose a two-stage process for helix formation: nucleation followed by propagation [6,7]. Nucleation involves organization of the first three amino acids into a helical turn and is the most energetically demanding step. This is reflected by experimental estimates of the nucleation factor, σ , which, when expressed as an equilibrium constant are in the order of 10^{-3} to 10^{-4} [8,9]. With such low σ values, helix stability tends to be strongly reliant on chain length and, in the absence of specific stabilizing interactions short synthetic peptides are essentially unstructured in solution.

Structural features can be introduced into peptides in order to nucleate and stabilize α -helical structures. These include helix capping [10,11], non-natural amino acid substitutions [12,13], side-chain constraints [14–20] and hydrogen bond surrogates (HBS) [21,22]. Table 1 summarizes some of the helix stabilization technologies that have been used to modulate protein-biomolecule interactions [23]. This review will focus on strategies that incorporate modifications at the N-terminus of peptide chains.

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N-Terminal End-Cap Strategies

Templates

Helical templates attempt to mimic the first turn of a helix, providing at least two hydrogen bond acceptors. Kemp and co-workers synthesized template **1** to mimic the conformation of proline residues commonly found as *N*-terminal motifs in protein α -helices [27,28]. The pyrrolidine rings have a fixed ϕ value near -60° , which is close to the ideal helix value. A 3,5'-thiomethylene linkage was incorporated for rigidity and to impart a ψ angle of -47° . Structural studies revealed that the template adopted three major conformations in solution, but its ability to adopt the desired helix nucleating structure was heavily dependent on solvent and appended peptide length.

Müller *et al.* utilized the cage compound **2** with the rigid structure displaying three carbonyl groups [29]. Stereochemistry of the template was important. Only the enantiomer depicted in Figure 1 increased helicity in an appended peptide chain when compared to same peptide with a Boc group at the *N*-terminus.

Austin *et al.* designed an alternative *N*-terminal α -helical template based on hexahydroindol-4-one 3,6-diacid, **3** [30]. Like the templates discussed above, **3** was designed to mimic the three consecutive carbonyls at the *N*-terminus of a peptide helix. The authors synthesized the *S,S*-, *R,S*-, *S,R*- and *R,R*-stereoisomers and found that the *S,S*-isomer was most effective in inducing helicity in an appended peptide chain. Other requirements for helix induction were a 9-*O*-ester linkage, as opposed to an amide that resulted in 3_{10} -helical conformation of the template.

The *N*-cap templating strategies discussed above are elegant demonstration of the helix nucleation principle but are limited in scope because they require multi-step syntheses and a large hydrophobic appendage at the *N*-terminus that may interfere with binding interactions.

Hydrogen Bond Surrogates

The hydrogen bond surrogate (HBS) helices build on the success of the templating strategy for helix nucleation. In HBS modified peptides, a single *N*-terminal *i* and *i*+4 hydrogen bond is substituted with a covalent linkage [21,22]. The modification results in σ values of ~ 1 [31] and amino acid side-chains remain available for molecular recognition purposes. Introduction of the covalent bond creates a 13-membered ring, which mimics the single turn of an α -helix.

The covalent linkage can be introduced in the form of a carbon-carbon bond *via* a ring closing metathesis (RCM) reaction (Figure 2). Theoretically, any covalent linkage could be used (N-Y-X=C in Figure 2) but the carbon-carbon linkage is preferable because it provides chemical stability in biological settings. The key advantage of the HBS approach is that it stabilizes helical conformation without blocking any molecular recognition surface of the target helix. HBS peptides have been shown to successfully modulate protein-biomolecule interactions in cell-free and cell culture assays.

HBS Synthesis

The synthetic strategy for HBS peptides utilizes a Grubbs ring closing metathesis reaction [22]. One of the olefin coupling partners is installed by appending 4-pentenoic acid to the *N*-terminal amino acid residue, whilst the other olefin is an *N*-allyl group incorporated at the *i* +4 position. Optimized procedures afford efficient synthesis of HBS helices [32,33].

Structure and stability of HBS α -helices

Extensive NMR and circular dichroism spectroscopies were used to examine the structure and stability of HBS α -helices; the conformation of these compounds is also supported by an X-ray crystal structure. These studies demonstrate that the HBS approach affords stable short α -helices from biologically relevant sequences.

Characterization by Circular Dichroism Spectroscopy

For a canonical α -helix, the CD spectrum displays double minima at 208 and 222 nm and a maximum near 190 nm. The CD spectra for HBS helices display these hallmark features. It is interesting to note that when the 13-member *trans* alkene macrocycle was reduced *via* hydrogenation to the saturated form, the CD spectra were almost identical to those of the *trans* alkene parent molecule [31]. This is an indication that the rigidity and stereochemistry provided by the double bond are not necessarily required to produce stable α -helices. When an additional methylene unit is incorporated into the N-terminal macrocycle to produce a 14-member ring, CD indicates a less helical structure [31].

Variable temperature CD studies were used to assess the thermal stability of the helical structures [34]. Remarkably, HBS helices were shown to maintain 60–70% of their room temperature helicities at 85 °C.

Characterization by NMR Spectroscopy

A canonical α -helix is characterized by hydrogen bonds between the C=O of the i^{th} amino acid and the NH of the $i+4^{th}$ amino acid of a peptide chain, which results in a right-handed helix with an average 3.6 residues per turn. The N-C α and C α -C=O dihedral angles, referred to as the phi (ϕ) and psi (ψ) angles, are generally set very close to -47° and -57° , respectively.

A peptide folded in the α -helical conformation would be expected to provide sequential NN (i and $i+1$) NOEs, and medium range NOEs, including, $d\alpha N(i, i+3)$, $d\alpha N(i, i+4)$ and $d\alpha\beta(i, i+3)$. All of these major crosspeaks expected from a stable α -helix are observed from HBS helices [34].

Structural constraints from NMR studies were used to generate three dimensional solution state structures by a simulated annealing and energy minimization protocol. The final structures exhibit a hydrogen bonding network along the backbone in an i and $i+4$ configuration consistent with a well-defined α -helix [34]. H/D exchange and variable temperature experiments provide a measure of the stability of intramolecular hydrogen-bonding within a helix. Results indicate that HBS helices composed of seven to fourteen residues contain highly stable hydrogen-bonding networks with minimal C-terminal fraying.

Characterization by X-ray Crystallography

X-ray crystal structure analysis of an HBS α -helix at 1.15 Å resolution (Figure 3) shows that the helix superimposes well onto a model of an idealized α -helix, supporting the hypothesis that stable short helices can be accessed by the HBS strategy [35]. All i and $i+4$ C=O and NH hydrogen bonding partners in the HBS helix fall within distances and angles expected for a fully hydrogen-bonded short α -helix (Figure 3b). 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 of sequence AcQVARQLAEIY-NH₂ (Figure 3c). The X-ray crystal structure provides explicit support for our hypothesis that replacement of the hydrogen bond between the i and $i+4$ residues at the N-terminus of a short peptide with a carbon-carbon bond results in a highly stable constrained α -helix. Importantly, the

crystal structure shows that the alkene-based macrocycle faithfully reproduces the conformation of a pre-nucleated α -turn.

In summary, the biophysical studies show that the HBS approach is a valid means to nucleate stable α -helical peptides. However, perhaps a more significant aspect of HBS technology is its application in modulating protein-protein interactions. The following section will discuss, with reference to specific examples, the use of HBS peptides in biological systems.

Biological Applications

HBS helices have proven to be remarkably effective tools for the regulation of protein interactions. Significantly, HBS helices outperform their unconstrained counterparts in cell culture studies reflecting their enhanced metabolic stability and cellular uptake properties. The selected examples below highlight the potential of HBS helices as inhibitors of protein-protein interactions.

An HIV fusion inhibitor [36]

Entry of HIV-1 into its target cells to establish an infection is mediated by viral envelope glycoprotein (Env) and cell surface receptors (CD4 and a coreceptor, such as CXCR4 or CCR5). The mature Env complex is a trimer, with three gp120 glycoproteins associated non-covalently with three membrane-anchored gp41 subunits. Binding of gp120/gp41 to cellular receptors triggers a series of conformational changes in gp41 that ultimately leads to formation of a postfusion trimer-of-hairpins structure and membrane fusion. The core of the postfusion trimer-of-hairpins structure is a bundle of six α -helices: three N-peptide helices form an interior, parallel coiled-coil trimer, while three C-peptide helices pack in an antiparallel manner into hydrophobic grooves on the coiled-coil surface. Peptides and synthetic molecules that bind to the N-terminal hydrophobic pocket and inhibit the formation of the six-helix bundle would be expected to inhibit gp41-mediated HIV fusion. Several HBS helices and unconstrained peptide derivatives that mimic a key region in the C-peptide helix were tested to identify a sequence that inhibited HIV fusion in cell culture. Both the constrained and unconstrained derivatives of the optimized sequence bound the N-terminal three helix bundle with high affinities (submicromolar to nanomolar) in a fluorescence anisotropy assay but only the HBS helix analog inhibited the fusion in cell culture.

Modulation of HIF-1 α p300/CBP interaction [37]

Transcription of hypoxia inducible genes is important for cancer growth and metastasis. Regulation of the transcription process is mediated by binding of the CH1 region of co-activator p300 (or CREB binding protein) and the C-terminal trans-activation domain (C-TAD₇₈₆₋₈₂₆) of hypoxia inducible factor 1 α (HIF-1 α). Structural analysis shows that two helical domains of HIF-1 α C-TAD are important for recognition of p300/CBP and are therefore potential structures on which to base synthetic mimics to inhibit the interaction. Interference with this interaction is intended to down regulate expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR2, which are involved in angiogenesis. HBS helix mimics of the CH1 domain of HIF-1 α have been shown to downregulate transcription of VEGF gene in HeLa cells under hypoxic conditions. Cell viability assays revealed HBS helices to be less cytotoxic than chemotin, a known inhibitor of the interactions between HIF-1 α and p300/CBP.

Inhibition of the p53-HDM2 Interaction [38]

A critical system preventing the development of human breast tumors is provided by the interaction between p53 protein and regulatory factors such as HDM2 [39]. The so-called ‘guardian of the genome’, p53 is a key factor mediating the cellular response to oncogenic stress (e.g., high expression levels of proto-oncogenes) and DNA damage. Overexpression of the p53 antagonist hDM2 occurs in one-third of benign breast lesions and two-thirds of malignant lesions. HDM2 overexpression results in reduced p53 protein levels, allowing incipient breast cancer cells to evade cellular mechanisms that would normally cause them to undergo apoptosis or cell cycle arrest. Development of synthetic ligands for HDM2 – long considered to be an “undruggable target” – has been a significant challenge for bioorganic and medicinal chemists. The p53 activation domain (AD), residues 17–31, targets a deep hydrophobic pocket of HDM2. The crystal structure of this complex shows that a small segment of the p53 N-terminus adopts an alpha helical structure which binds in a hydrophobic cleft of HDM2 [40]. An HBS helix that mimics the p53 activation domain targets HDM2 with high affinity, whereas the negative control HBS failed to bind this protein.

A central question for the field of peptide mimicry centers on the specificity of the native domain for its natural target. HBS helices have demonstrated outstanding specificity for their cognate receptor. A cell free split-protein fluorescence based assay was used to determine the selectivity of the optimized HBS helix for HDM2 in comparison to other peptide helix accommodating proteins. The p53 mimic showed a high preference for HDM2 as compared to Bcl-2, Bcl-w, p300 and BFL [38].

Conclusions

The HBS approach provides an *N*-terminal cap for helix nucleation to produce stable α -helical peptides with high affinities for protein targets. The potential of HBS helices has been demonstrated with inhibitors designed to modulate HIV fusion, transcription of hypoxia inducible genes and p53/hDM2 interactions. Due to the rational design principles, ease of synthesis and reliable reproduction of protein α -helices, it is expected that HBS peptides will be useful probes for answering important biological questions that involve protein-biomolecule interactions.

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Figure 1.

Rigid N-Cap templates with carbonyl groups ordered to nucleate the first turn of an α -helix.

Figure 2. Nucleation of short α -helices by replacement of an N-terminal i and $i+4$ hydrogen bond ($C=O\cdots H-N$) with a covalent link ($C=X-Y-N$). The hydrogen bond surrogate-based (HBS) α -helices contain a carbon-carbon bond derived from a ring-closing metathesis reaction.

Figure 3.

(a) Crystal structure of the HBS α-helix with electron density map superimposed onto the refined molecular model. (b) Putative *i* and *i*+4 hydrogen bonds (magenta lines) in crystal structure-derived molecular model of HBS helix. (c) Overlay of crystal structure and a model of an idealized α-helix.

Table 1Methods for Stabilizing α -Helical Peptides for Biological Applications

Technology				
	Hydrogen Bond Surrogate	Hydrocarbon stapling	Lactam bridges	Triazole bridge
Pros	<ul style="list-style-type: none"> Shows promise in a range of biological systems. Side-chains unaltered for molecular recognition Constrained helices shown to be cell permeable. 	<ul style="list-style-type: none"> Most extensively used side-chain cross-linked mimetic in biological systems. Constrained helices shown to be cell permeable. 	<ul style="list-style-type: none"> Easily synthesized; transition metal catalysis not required. 	<ul style="list-style-type: none"> Easily synthesized.
Cons	<ul style="list-style-type: none"> Most appropriate for helices of 2–4 turns. 	<ul style="list-style-type: none"> Cross-linked side-chains unavailable for molecular recognition Requires transition metal catalyst 	<ul style="list-style-type: none"> Side-chains unavailable for molecular recognition. Constraint not stable to metabolic degradation. 	<ul style="list-style-type: none"> Cross-linked side-chains unavailable for molecular recognition.
Refs	[22]	[24]	[25]	[26]