

Perspective

β -Peptides as inhibitors of protein–protein interactions

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Received 23 August 2004; accepted 8 September 2004

Available online 28 October 2004

Abstract—We became interested several years ago in exploring whether 14-helical β -peptide foldamers could bind protein surfaces and inhibit protein–protein interactions, and if so, whether their affinities and specificities would compare favorably with those of natural or miniature proteins. This exploration was complicated initially by the absence of a suitable β -peptide scaffold, one that possessed a well-defined 14-helical structure in water and tolerated the diverse sequence variation required to generate high-affinity protein surface ligands. In this perspective, we describe our approach to the design of adaptable β -peptide scaffolds with high levels of 14-helix structure in water, track the subsequent development of 14-helical β -peptide protein–protein interaction inhibitors, and examine the potential of this strategy for targeting other therapeutically important proteins.

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1. Introduction

Nature uses a finite number of chemical building blocks to generate molecules with stunningly diverse molecular functions. The key to this economical but powerful strategy is the construction of linear chains that fold independently into complex three-dimensional structures. Indeed, proteins assembled from natural α -amino acids are among the most potent and diverse ligands for cellular macromolecules, especially other proteins that contain large, shallow binding surfaces.¹ Despite the attractiveness of well-folded proteins as ligands for protein surfaces *in vitro* and as research tools *in vivo*, their widespread use as therapeutics is limited currently by low cell permeability, high proteolytic sensitivity, and poor pharmacokinetics.² By contrast, β -peptides—a quintessential example of the class of molecules known as non-natural folding oligomers, or foldamers,^{3,4}—consist of linear chains of β -amino acids, and are thus virtually invulnerable to proteases.^{5,6} Early results suggest β -peptides have favorable pharmacodynamics.⁷ Moreover, β -peptides can fold into stable secondary structures without the need for tertiary interactions,^{3,4} allowing an extended, variable, protein-binding surface to be pre-

sented by a relatively short oligomer. This feature may translate into potent ligands with favorable cell permeability and tunable pharmacodynamics. For these reasons we became interested in exploring whether β -peptide foldamers could bind protein surfaces and inhibit protein–protein interactions, and if so, how their affinities and specificities would compare with those of natural⁸ or miniature proteins.^{9–16} In this perspective, we describe our approach to the design of a general β -peptide scaffold possessing high levels of 14-helix structure in water, track the development of 14-helical β -peptide protein–protein interaction inhibitors based on this scaffold, and examine the potential of this strategy for targeting other therapeutically important protein targets.

Early work in the field, most notably by Seebach,⁶ Gellman,^{17,18} and their co-workers, demonstrated that β -peptide foldamers can adopt a variety of ‘protein-like’ helical secondary structures in organic solvents such as methanol (Fig. 1A).¹⁹ β -peptide helices are named for the number of atoms in the ring closed by a helix-specific hydrogen bond, and include the 10-helix, the 10/12-helix, the 12-helix, and the 14-helix (Fig. 1A). In general, the helical structure preferred by a β -peptide oligomer is dictated by the substitution pattern of the constituent β -amino acids: acyclic, monosubstituted residues (β^2 - and β^3 -residues, Fig. 1B) tend to fold into 14-helices, or 10/12 helices if patterned as alternating β^2/β^3

Keywords: Foldamer; Helix; Proteomics; Protein recognition.

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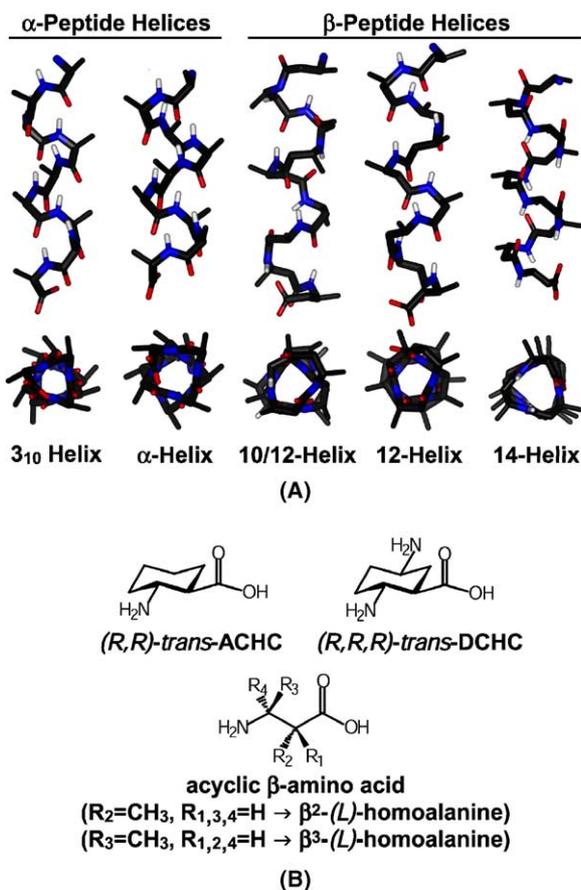


Figure 1. (A) Comparison of secondary structures formed by α - and β -amino acid oligomers. Carbon atoms are shown in black, nitrogen atoms in blue, oxygen atoms in red, and amide hydrogen atoms in white. Other hydrogen atoms are omitted for clarity. (B) β -amino acid monomers that promote the formation of unique helices.

residues.^{6,20–24} Cyclopentyl and cyclohexyl ring constraints promote formation of a 12-helix and a 14-helix, respectively.^{17,18} Thus, control over preferred helical secondary structure can be achieved via judicious choice of substitution pattern.

We were intrigued by the structural relationship between the α -helix and the 14-helix, and in particular by the close superposition of side chains located at positions i , $i+4$, and $i+7$ on the α -helix with those at positions i , $i+3$, and $i+6$ on the 14-helix. This relationship suggested that it might be possible to present a short α -helical functional epitope on a well-folded 14-helix, in much the same way as we have presented such epitopes on the well-folded α -helix of pancreatic fold polypeptides.^{9,11–16} When we began this work, however, there were only two examples of β^3 -peptides that possessed appreciable 14-helix structure in water.^{25,26} Both molecules contained pairs of oppositely charged side chains positioned three residues apart, in perfect position to form stabilizing intra-molecular salt bridges. Indeed, addition of high salt or extremes of pH abolished 14-helix structure in these molecules, demonstrating convincingly that inter-residue electrostatic interactions could stabilize a β^3 -peptide 14-helix in water. These molecules represented a breakthrough in

β -peptide design, but their long-term utility as scaffolds for the design of protein surface ligands was limited by their requirements for intra-molecular salt bridges on two of the three 14-helix faces. Thus our first task was to identify a complementary strategy for stabilizing a β^3 -peptide 14-helix in water that would permit variation of at least two full 14-helix faces. With this adaptable β -peptide scaffold in hand we could then try to reconstitute an α -helical functional epitope by incorporation of β -amino acids bearing the appropriate proteinogenic side chains.

2. A general strategy for the stabilization of β^3 -peptide 14-helices in water

A primary consideration in de novo protein design is the α -helix macrodipole, which results in partial positive charge at the N-terminus and partial negative charge at the C-terminus.^{32–35} It is well known that α -helix stability can be enhanced significantly by neutralizing this macrodipole. Neutralization can be achieved by introducing negatively charged side chains near the N-terminus and/or positively charged side chains near the C-terminus,³² or by neutralizing charges associated with free N- and C-termini.³³ Because of its unique hydrogen-bonding pattern, the 14-helix macrodipole is oriented in the direction opposite that of an α -helix, with partial positive charge at the C-terminus and partial negative charge at the N-terminus.¹⁹ This orientation predicts that 14-helix structure should be stabilized by introducing positively charged side chains near the N-terminus and negatively charged side chains near the C-terminus, and by preserving the charge associated with free termini.

To test these predictions, we asked whether 14-helix structure in the previously reported β^3 -heptapeptide **S1**²⁶ could be enhanced by switching the relative orientation of two side chains to better alleviate the overall 14-helix macrodipole while retaining the number of potential intra-molecular salt bridges (as in **S2**, Fig. 2). Indeed, this simple sequence change doubled the extent of 14-helix structure in water as judged by CD (Fig. 2B).³⁶ We next designed a β^3 -undecapeptide that contained only one face of stabilizing salt bridges (β -peptide **1**, Fig. 2),³⁶ and refined the scaffold to yield β^3 -undecapeptide **2**, which possessed roughly 50% 14-helix structure in aqueous solution (Fig. 2).³⁷ CD spectroscopy (Fig. 2B) and NMR measurements^{36,38} confirmed the presence of significant 14-helix content in β -peptides **1** and a variant of scaffold **2**.^{36,38,51} Both molecules possess a complex β^3 -homoglutamate/ β^3 -homoornithine salt bridge on one face, three β^3 -homoalanine residues on a second face, and primarily β^3 -homovaline on the third face.

3. 14-Helical β -peptide scaffold **2** is amenable to a variety of substitutions

Our next step was to determine the extent to which 14-helix structure is retained when proteinogenic side

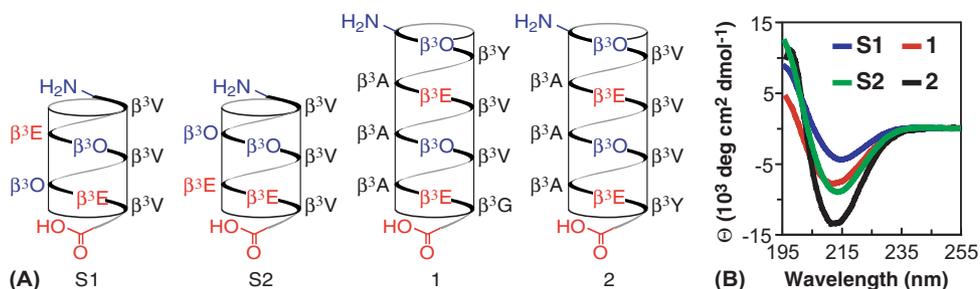


Figure 2. Helical net diagrams (A) and circular dichroism (CD) spectra (B) of β^3 -peptides with significant 14-helix stability in water. Residues are abbreviated β^3X , where X denotes the common single-letter abbreviation of the analogous α -amino acid. CD spectra are plotted in units of mean residue ellipticity, and were obtained at 25°C from samples containing 100 μM β -peptide in 1 mM sodium phosphate/citrate/borate buffer (pH 7.0). Note the relative intensities of minima near 214 nm; while circular dichroism spectra of β -peptides must be interpreted with care,²⁷ these values are commonly used to estimate relative 14-helix content in a series of analogous peptides.^{19,28–31}

chains are substituted within β^3 -undecapeptide **2**. To explore this question in a systematic way, we prepared 27 analogs of **2**, each substituted individually at one of three positions with a different β^3 -amino acid. The nine β^3 -amino acids chosen for this ‘host–guest’ study represent a wide and diverse set of proteinogenic side chains. Each β^3 -peptide was characterized by circular dichroism, and results were correlated to computational and Monte Carlo simulation analyses performed on β^3 -amino acids and β^3 -oligomers.³⁷ These host–guest studies demonstrated that β^3 -undecapeptide **2** retains its well-folded structure when presenting a wide range of proteinogenic side chains, including those that predominate at protein–protein interfaces.^{8,39} In addition, our results verified the importance of macrodipole stabilization for maintaining 14-helix structure, provided comprehensive evidence that β^3 -amino acids branched at the first side chain carbon are 14-helix-stabilizing, and suggested a novel role for side chain hydrogen bonding as an additional stabilizing force in β^3 -peptides containing β^3 -homoserine or β^3 -homothreonine. Notably, the 14-helix propensities of β^3 -amino acids differed starkly from the α -helix propensities of analogous α -amino acids, suggesting that 14-helix folding is governed by radically different biophysical forces than is α -helix folding (Fig. 3).

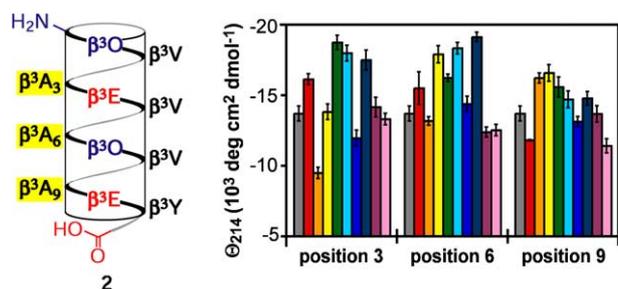


Figure 3. Relationship between side chain structure and 14-helix stability among 27 analogs of β -peptide **2** in which the β^3 -homoalanine residue at position 3, 6, or 9 is replaced by β^3 -homolysine (red), β^3 -homoglutamic acid (orange), β^3 -homoserine (yellow), β^3 -homothreonine (green), β^3 -homoisoleucine (cyan), β^3 -homoleucine (blue), β^3 -homovaline (navy), β^3 -homophenylalanine (purple), or β^3 -homotryptophan (pink). Data is plotted as mean residue ellipticity (Θ) at 214 nm for each β -peptide studied.

4. Incorporating function into our 14-helical β -peptide scaffold

Having demonstrated the adaptability of β^3 -peptide scaffold **2**, we sought to use it to inhibit a discrete protein–protein interaction. We were encouraged by earlier work of Seebach, who demonstrated that β -peptide hairpins could bind somatostatin receptors with high affinity and specificity,^{40,41} and by work of Seebach and co-workers,⁴² DeGrado and co-workers,^{43,44} and Gellman and co-workers,^{45–47} who demonstrated that amphipathic β -peptides could perform a variety of functions including inhibition of cholesterol and fat uptake,⁴² potent antibacterial activity,^{43–46} and RNA binding.⁴⁷ We chose the complex between hDM2 and p53 as a first target⁴⁸ because of the established importance of p53 as a transcriptional activator critical for stress-induced cell cycle arrest and apoptosis.⁴⁹ In the absence of stress, hDM2 down-regulates p53 activity by sequestering p53’s activation domain (p53AD), exporting p53 from the nucleus, and directly ubiquitinating p53.⁵⁰ Cancerous cells often overexpress hDM2, resulting in a loss of the cell’s primary response to stress and leading to unchecked cell growth.^{49,50} The interface between p53 and hDM2 is exceptionally well-characterized,⁴⁸ aiding our design of potential 14-helical inhibitors. Three residues (F19, W23, L26) projecting from a short α -helix on p53AD form a functional epitope recognized by hDM2.⁴⁸ Structural modeling indicated this functional epitope could be recapitulated if the side chains of F19, W23, and L26 were presented at successive positions three residues apart on β^3 -peptide scaffold **2**. The designed β -peptide, **β 53-1** (Fig. 4A), was analyzed by CD and NMR to verify its 14-helix structure.³⁸ Using fluorescence polarization, **β 53-1** was shown to bind directly to hDM2 with an equilibrium dissociation constant (K_d) between 368 and 583 nM, only 1.6–2.5-fold lower in affinity than an α -peptide derived from p53AD (Fig. 4B). Further, **β 53-1** displaces a peptide derived from p53 from hDM2 with an IC_{50} of $94.5 \pm 4.4 \mu\text{M}$, but does not inhibit complexation of a different, unrelated peptide–protein pair.³⁸ Using **β 53-1** variants and various controls, we demonstrated that the observed binding affinity and specificity were dependent on the presence and relative spatial

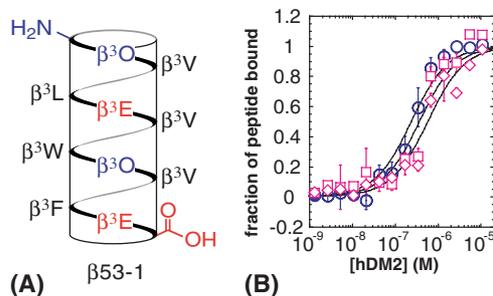


Figure 4. (A) Helical net illustration of the sequence of $\beta 53-1$. (B) Plots illustrating the fraction of fluorescein-labeled p53AD₁₅₋₃₁ (p53AD^{Flu}, blue circles), $\beta 53-1$ labeled on its N-terminus (^{Flu} $\beta 53-1$, pink squares), or $\beta 53-1$ labeled on its C-terminus ($\beta 53-1^{\text{Flu}}$, pink diamonds) bound as a function of hDM2 concentration as measured by fluorescence polarization. Labeled peptides were incubated at a concentration of 25 nM with various concentrations of hDM2₁₋₁₈₈ at room temperature until equilibrium was reached.

orientation of the β^3 -homophenylalanine, β^3 -homotryptophan, and β^3 -homoleucine residues, in accord with the original design rationale. $\beta 53-1$ represents the first helical β -peptide that binds a discrete macromolecular target with high affinity and specificity, and exemplifies our strategy for the development of folded, functional non-natural oligomers.

To further explore the interplay between structure and function involved in $\beta 53-1$ -hDM2 complexation, and to assist in the future design and refinement of functional 14-helices, we solved the NMR solution structure of $\beta 53-1$ in CD₃OH.⁵¹ Using a torsional dynamics program⁵² that we reparameterized to operate on β -peptides, we applied 151 ROESY-derived upper distance limits to simulated annealing simulations starting from 100 random torsional configurations. The resulting 20 lowest-energy structures are shown in Figure 5. They show no violated constraints, an average backbone RMSD to mean of 0.17 ± 0.07 Å, and an average heavy atom RMSD to mean of 0.60 ± 0.10 Å. These values indicate a very well-folded structure, even at the termini where some ‘fraying’ might be expected. The complex salt bridge we had originally designed in scaffolds 1 and 2 is evident in molecular detail, and even in solution $\beta 53-1$ appears to fully recapitulate the p53AD functional epitope. Intriguingly, the β^3 -homovaline residues, which

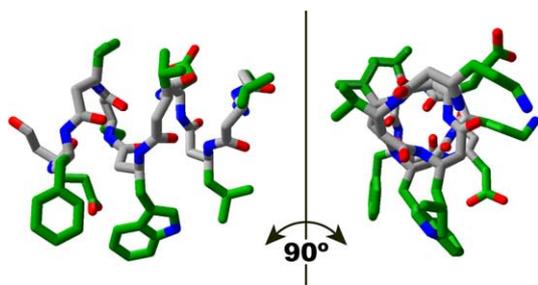


Figure 5. NMR solution structure of $\beta 53-1$ in CD₃OH at 10 °C,⁵¹ viewed from the side and down the helical axis. Models shown represent the mean of 20 lowest-energy structures. Backbone carbons are shown in gray, nitrogens in blue, oxygens in red, and side chain carbons in green. Hydrogen atoms have been omitted for clarity.

were shown to be 14-helix-promoting in the host–guest analysis, pack against one another along one helical face, shielding their isopropyl groups and the helix backbone from solvent. Future structural work will explore the hDM2-bound conformation of $\beta 53-1$, demonstrating at the atomic level how β -peptide 14-helices can be designed to recognize protein targets.

5. The future of β -peptides

Our results suggest that the β -peptide field is now poised to make significant contributions to chemical biology. Our strategy for the design of well-folded β -peptide 14-helices generates relatively small molecules possessing a broad binding surface and nearly unlimited chemical diversity—molecules with great potential as protein–protein interaction inhibitors. Using a combination of rational design and well-established high-throughput combinatorial methods,^{53–55} or perhaps evolution,⁵⁶ it may soon be possible to quickly generate small, folded β -peptide ligands for some fraction of the 75% of the human proteome currently considered ‘undruggable’.⁵⁷ As this approach is applied to more targets and tested for in vivo efficacy, we will evaluate the potential of functionalized β -peptides as biological tools and therapeutics. For example, we are currently targeting proteins in the Bcl-2 family that help regulate apoptosis. β -peptides that bind to Bcl-2 family members would be extremely useful as tools to control programmed cell death and even as potential cancer drugs. We have also designed ligands that target the HIV membrane fusion protein gp41. β -Peptides that bind in gp41’s hydrophobic pocket could inhibit membrane fusion and represent a cost-effective alternative to the current α -peptide-based fusion inhibitor Fuseon™. Designing specific β -peptide ligands for these targets would complement our success with hDM2, reaffirming our design strategy and demonstrating the broad applicability of our approach. Protein–protein interactions are notoriously difficult to target; our water-stable foldamer scaffold may provide a general platform for controlling crucial interactions with high potency and specificity.

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