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De novo design: backbone conformational constraints in nucleating helices and β-hairpins

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Abstract: A modular approach to synthetic protein design is being developed using conformationally constrained amino acid as stereochemical directors of polypeptide chain folding. An overview of studies aimed at constructing peptide helices using α,α-dialkyated residues and β-hairpins using D-Pro as a turn nucleator is presented. The construction of helix-helix motifs and three- and four-stranded structures has been achieved using non-protein amino acids to stabilize specific elements of secondary structures.

Abbreviations: Acp, ε-aminocaproic acid; Aib, α-aminoisobutyric acid; Dbg, α,α-di-*n*-butylglycine; Deg, α,α-diethylglycine; Dpg, α,α-di-*n*-propylglycine; HPLC, high-performance liquid chromatography; L-LAC, L-lactic acid; TASP, template-assembled synthetic protein.

The design of synthetic sequences that adopt folded secondary and tertiary structures is a firm test of our understanding of the principles that govern polypeptide chain folding. Most *de novo* design strategies are based on knowledge derived from the growing body of protein crystal structure data, which provides key information on the propensities of specific amino acids and short sequence segments to favor particular secondary structures. Further assembly of supersecondary and tertiary structural motifs, such as helical bundles and β-sheet 'sandwiches', relies on patterning hydrophobic residues so as to exploit solvent forces in achieving compaction (1–4). Spatial organization of secondary structure elements may also be achieved by covalently clamping designed peptides to a scaffold, as in the case of template-assisted synthetic proteins (TASPs; 5). Metal ions which can contribute substantially to energetics

by interacting with suitably positioned liganding side chains have also been advanced as structure-organizing templates (6, 7). A conceptually distinct strategy that has been developed in our laboratory is based on the construction of stereochemically rigid modules of defined structure using backbone conformational restraints provided by the incorporation of α,α-dialkylated residues, most notably α-aminoisobutyric acid (Aib) (8–10). Backbone constraints imposed by insertion of D-residues, specifically D-Pro, have also been explored (11). Assembly of sequences containing multiple elements of secondary structure is being approached using linking segments, which are conformationally restricted to adopt 'irregular' torsion angles, and by the use of nonprotein residues such as ω-amino acids (12) and the hydroxy acid, lactic acid.

3_{10}-/α-Helices

The ability of Aib and related α,α-dialkylglycines to nucleate and stablize helical structures in oligopeptides is extremely well documented (9, 10, 13, 14). The stereochemical rigidity of Aib-containing sequences, promotes crystallinity even in relatively long peptides, permitting high resolution (< 1.0 Å) characterization of helical conformations, by X-ray diffraction (10, 15). The stabilization of structures containing as many as two to three helical turns may be achieved by the incorporation of a single nucleating Aib residue (16). While there has been some debate on the precise nature of the helical conformation (3_{10}/α) formed under different conditions (17), it must be stressed that conformational variability is widespread even in the crystalline state (18). Quite often mixed helical structures have been observed for Aib-containing oligopeptides and several examples illustrate variations of stereochemical detail in polymorphic crystal forms (19, 20). However, the most important point to emerge from these studies is that incorporation of Aib and related residues permits the rational design of cylindrical, helical structures, which are particularly soluble and stable in apolar solvents that compete ineffectively for hydrogen-bonding sites on the peptide backbone.

Fully Extended Structures

A particularly interesting feature of the higher α,α-dialkylated glycine residues (α,α-diethylglycine, Deg; α,α-di-*n*-propylglycine, Dpg and α,α-di-*n*-butylglycine, Dbg) is their tendency to adopt fully extended C_5 ($\phi \sim 180^\circ$, $\psi \sim 180^\circ$) conformations. This property was first noted by Toniolo, Benedetti & Hardy in their studies of oligomers of Deg (21, 22). Theoretical calculations reveal that in these higher dialkylglycines the minima in the helical ($\phi \sim \pm 60^\circ$, $\psi \sim \pm 30^\circ$) and fully extended ($\phi \sim 180^\circ$, $\psi \sim 180^\circ$) regions of conformational space are energetically comparable (23), in contrast to Aib, where the minima in the helical region are substantially deeper (9). Subsequent experimental studies on synthetic peptides containing Dpg and Dbg residues reveal that while conformations may be dependent on sequence content, the choice is limited to C_5 or helical conformations (24, 25). The possibility that fully extended φ,ψ-values can be stabilized is of importance in designing stereochemically well-defined linking segments, which may be used to connect prefabricated secondary structure modules.

Helix–Linker–Helix Motifs

Initial attempts to design linked helix motifs by connecting canonical seven-residue helical segments using linking sequences, with a tendency to break continuous helix formation, e.g. Gly-Pro and D-Phe-Pro, were unsuccessful (26, 27). In these instances the two Aib residues positioned at the center of the N- and C-terminal segments were sufficient to propagate a helical conformation throughout the length of model 16 residue peptides, despite a central, potentially helix-breaking segment. It then became apparent that rational termination of designed helical segments would require a greater insight into the stereochemistry of termination signals in proteins (28) or the introduction of nonprotein residues that are structurally incapable of helix propogation. With this end in view we examined the use of ω-amino acids (12), particularly ε-aminocaproic acid (Acp), D-Pro and L-lactic acid (L-Lac). A series of 15 residue peptides containing a central Acp linker yielded NMR evidence, in organic solvents, for distinct helical segments, although interhelix orientation could not be determined conclusively (29). The crystal structure of a 15-residue peptide, Boc-Val-Ala-Leu-Aib-Val-Ala-Leu-Acp-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe, revealed an extended arrangement of two helical segments; a conformation that may be a consequence of crystal packing, with the staggered helical segments forming continuous hydrogen-bonded chains with near neighbors in the crystal lattice (30). Limited evidence for compact structures was obtained from comparative analysis of high-performance liquid chromatography (HPLC) retention times on a C_{18} column for a series of helices of varying length (31). Interestingly, despite reversal of helix sense of the C-terminal segment using a seven-residue sequence

incorporating D-amino acids, a very similar arrangement of the two helices was obtained in crystals (32). Direct fusion of two helices of opposite chiral sense in the peptide Boc-Val-Ala-Leu-Aib-Val-Ala-Leu-D-Val-D-Ala-D-Leu-Aib-D-Val-D-Ala-D-Leu-OMe, yielded a novel ambidextrous molecule in which an N-terminal, seven-residue, right-handed helix terminates in a Schellman motif, induced by the D-residue at position 8 adopting an α_L conformation. This feature then propogates a left-handed helix through the C-terminal segment (33). Recently determined crystal structures of helices incorporating L-Lac residues, suggest that hydrogen bond interruption may, in certain sequence contexts, terminate peptide helix propogation (I.L. Karle, personal communication). The control of linking segment stereochemistry has also been attempted using Gly and Dpg residues for promoting nonhelical conformations (24). Thus far, ambiguous characterization of structures of helix–linker–helix motifs containing such segments has not been achieved.

β-Hairpins

The *de novo* design of all β-sheet proteins has been beset with problems associated with aggregation and lack of solubility (34, 35). The simplest target motif is the β-hairpin, which consists of two antiparallel strands linked by a β-turn of appropriate stereochemistry. Illuminating analyses of β-hairpins in proteins revealed that for appropriate strand registry and interstrand hydrogen bonding, type I′ and II′ β-turns were most often preferred (36). The stereochemical features of the short segments linking two antiparallel strands have been clearly delineated in a subsequent analysis, which used a large database of high-resolution protein structures (37). The requirement of type I′/II′ β-turns as the fulcrum in hairpins places stringent stereochemical restraints on the backbone conformational angles of the $i + 1/i + 2$ residues of the β-turn. The φ-values must be positive ($\sim+60^\circ$) at both residues in type I′ β-turns, while in the type II′ turns the φ-value at residue $i + 2$ must be $\sim+60^\circ$. In proteins, the only residues that adopt positive φ-values with great facility are Gly (achiral) and Asn, which has a high propensity for α_L conformations (38, 39). It is these residues that are most frequently found in the β-turns at the center of hairpins (37, 40). The requirement of a positive φ-value restricted to $\sim+60^\circ \pm 20^\circ$ is easily achieved in the D-Pro residue, where φ is constrained in the pyrollidine ring. Several recent β-hairpin design efforts have used suitably positioned D-Pro-XXX segments to nucleate type I′/II′ β-turns (11, 41–44). A β-hairpin structure has been established in crystals for an apolar octapeptide, Boc-Leu-Val-Val-D-Pro-Gly-Leu-Val-Val-OMe (45). NMR studies in solution establish conformational differences between octapeptides with central L-Pro-Gly and D-Pro-Gly segments, reiterating the importance of β-turn stereochemistry in hairpin nucleation (46). While Asn-Gly segments have been used to design water-soluble β-hairpins (47, 48), the work of Satnger & Gellmann (49) points to the superiority of D-Pro-Gly in promoting hairpin nucleation. Success in β-hairpin construction has stimulated the design of longer sequences, with recent studies establishing three-stranded β-sheet (or multiple β-hairpin) structures in both polar, water soluble and apolar, organic solvent soluble peptides (50–53). In all these cases aggregation has not been a major impediment. Figure 1 illustrates a four-stranded β-sheet structure determined by 500 MHz ^{1}HNMR in a 26-residue synthetic peptide which contains three internal D-Pro-Gly segments. The four-stranded structure is stable in methanol and 50% methanol/H_2O, but the backbone is significantly solvated in water, resulting in a loss of critical cross-strand interactions, although the three β-turns remain intact (54). Sequence choices in the design of β-sheet structures are clouded by the absence of strong cross-strand residue correlations (37). Covalent reinforcement by disulfide bridging across strands may be an attractive possibility.

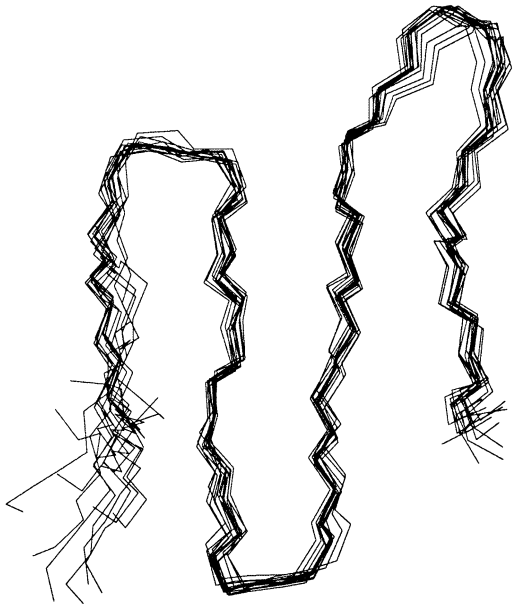

Figure 1. Four-stranded β-sheet structure in a synthetic 26-residue peptide (β-4,R-G-T-I-K-^{D}P-G-I-T-F-A-^{D}P-A-T-V-L-F-A-V-^{D}P-G-K-T-L-Y-R). Superposition of 15 structures of the peptide obtained from simulated annealing calculations using NOE-derived distance restraints. Only backbone atoms were used for superposition. (mean global backbone RMSD: 1.2 Å) (54).

The design of both helices and β-hairpins using backbone conformational constraints as a nucleating feature has been achieved. Assembly of these preformed modules in a 'Meccano (Lego) set' approach to larger protein like structures, requires elaborate stereochemical control over linking peptide segments. Alternatively, designed attractive interactions between the modules involving side chains, metal ligation or covalent bridging may result in complex, but predetermined, tertiary arrangements in synthetic structures. In such approaches, only limited emphasis is placed on solvophobic interactions in generating compact polypeptide folds, in marked contrast with strategies which derive their conceptual framework from available knowledge of protein folding in aqueous solutions. Exploiting nonstandard amino acids to impose backbone conformational constraints may bridge the gap between diverse strategies for *de novo* design of well-defined polypeptide structures; approaches which are more 'biochemical' in relying on protein-like sequences on the one hand and strategies which are more close to 'organic chemistry' on the other, with the latter often being based on completely synthetic templates that permit organization of pendant polypeptide chains.

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