Design of Folded Peptides†

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I. Introduction

The construction of complex protein folds relies on the precise conversion of a linear polypeptide chain into a compact 3-dimensional structure. The interplay of forces that link sequence and folding is intricate and yet to be firmly elucidated. Examination of protein 3-dimensional structures suggests that complex tertiary folds and quaternary associations can be deconstructed into a limited number of secondary structural elements, such as strands, helices, and turns, which are assembled using loosely structured loops (Figure 1). The stability of a specific fold is determined by tertiary interactions between residues which are distant in sequence. De novo design of existing or novel protein folds demands a thorough understanding of the rules that underlie protein structure and stability.

The recurring theme in many design strategies is the use of amino acid propensities to adopt various secondary structures. The 'inherent property' of such a derived set of amino acids is then arranged to maximize hydrophobic interactions to form a compact core, and patterns of ionic interactions and hydrogen bonds are utilized to further stabilize the structure. An alternate approach to the design of rigid secondary structures is to exploit the constraints imposed by backbone conformations. Such a strategy dictates the use of amino acids with restricted access to conformational space such as α-aminoisobutyric acid (Aib) and its higher homologues, proline enantiomers, and a variety of synthetically designed residues.

The prevalent themes of 'residue patterning' and 'stereochemical restraints' have often been extrapolated to the design of supersecondary structures. Amphipathic molecules that preferentially aggregate into controlled assemblies, introduction of appropriately positioned liganding groups for metal-mediated assembly, introduction of disulfide bonds that permit covalent tethering of individual modules, and covalent assembly of polypeptide chains on a suitable template (template-assisted synthetic protein, TASP) are strategies that have been investigated.

Here, we attempt to review the various approaches used to construct isolated secondary structural modules and to assemble them into compact tertiary structures with defined folds and at times, function. Approaches that emphasize stereochemical control over polypeptide chain folding are considered in detail.

II. Construction of Secondary Structural Modules

A. Secondary Structure Propensities and Patterning

1. Helices

Several groups have made remarkable progress in the de novo design of isolated α helices using the 20 naturally occurring α-amino acids. Systematic studies have resulted in the formulation of a set of rules for the construction of stable helices.[1,2] Strategies used in such design include (a) use of residues with large helix-forming propensities such as leucine, glutamic acid, or lysine[3,4] or stretches of alanine

† Dedicated to the memory of Professor G. N. Ramachandran.

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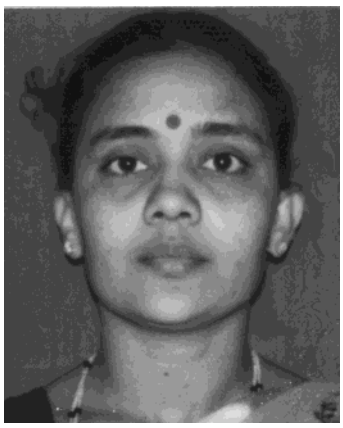

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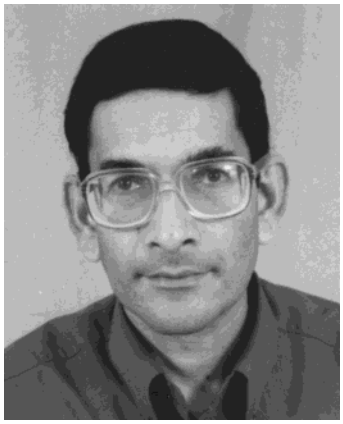

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residues,[5] (b) use of appropriate capping groups to remove terminal charges, preventing unfavorable charge interactions with the helix dipole,[6−8] (c) use of polar and charged amino acids to introduce stabilizing hydrogen bonds or ionic interactions between residues separated by one helical turn,[9−12] (d) use of hydrophobic (aliphatic and aromatic) van der Waals interactions between residues separated by a turn of the helix,[13,14] and (e) use of aromatic−charge or aromatic−sulfur interactions.[15,16] The spatial proximity of residues placed at positions i and i+4 in an α helix has been further exploited to stabilize helices by the incorporation of metal ligands at these positions. Both natural[17] and unnatural aminodiacetic acid-based amino acids[18,19] that can serve as metal ligands have been incorporated into peptides and their helicities assayed using CD. An extreme example of the utilization of side chain interactions between residues across a single helical turn to 'lock' a desired conformation is the construction of covalent linkages between such residues. In one such example, a lactam bridge was introduced between lysine and aspartic acid residues at positions i and i+4 in a parathyroid hormone-related protein.[20a] This cyclic analogue was found to be 5−10-fold more potent than the linear, parent sequence in PTH receptor binding assays. Covalent helix stabilization has been elegantly achieved by Grubbs and co-workers using ruthenium-catalyzed ring-closing metathesis (RCM) of *O*-allyl serine residues to introduce olefinic cross-links between i/i+4[20b] and i/i+7[20c] positions in a helical sequence. In analogous studies, i/i+7 positions in a helix were linked using disulfide bridges[20d] and alkyldiyl tethers between glutamine residues.[20e]

In addition to factors responsible for helix initiation and termination, the existence of specific helix termination signals ("stereochemical punctuation marks") has been recognized and many attempts have been made to understand their conformational characteristics via analyses of helical structures in protein structural databases and construction of such motifs in synthetic sequences.[21−29a] The Schellman motif, which involves helix termination by reversal of the signs of the backbone torsion (ϕ,ψ) angles, most frequently involving achiral residues or Asn, has been most widely studied.[22,26,27,29]

2. β-Turn and Hairpin Nucleation

The construction of a stable β-turn is a prerequisite for β-hairpin formation. The analysis of β-hairpins in proteins by Sibanda and Thornton[30,31] reveals the specific requirements of turn stereochemistry for nucleating β-hairpins. Subsequent analyses have examined amino acid conformational propensities in small connecting loops found in β-hairpins.[32,33] While such analyses aid in the design of turn sequences, residue propensity considerations are not easily adapted to the design of β-strands. Unlike helices, where local conformational effects and amino acids not further apart than 3−4 residues play a deciding role in dictating structural stability, the β-sheet can be viewed as largely a tertiary structure, with complex geometry and interactions between residues far apart in primary sequence. This implies that a scale for the intrinsic sheet-forming propensities of residues is not as easily obtained as for the α-helices, as 'context-dependent propensities' emerge. However, the recent past has seen the appearance of β-sheet model systems that allow the assessment of factors

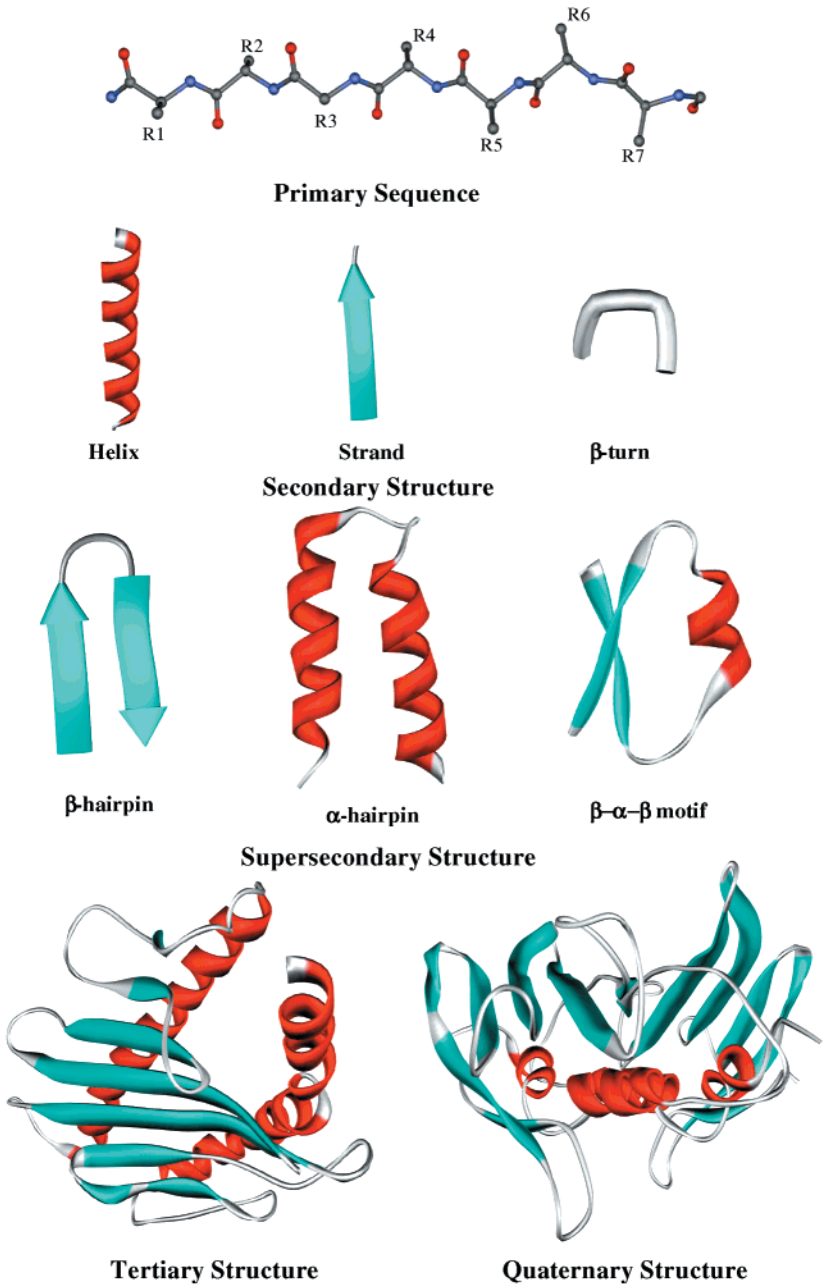

Figure 1. Various levels of structural organization observed in protein structures.

involved in sheet formation and stability.[34] These model systems, a consensus zinc-finger peptide,[35] and the B1 domain of streptococcal protein G[36−38] utilize host−guest methods as previously applied to helical systems[4,39,40] and help construct a hierarchy of β-sheet 'propensities' for the 20 naturally occurring amino acids.[35−38] Again, the observation that the energy difference between the best and least β-sheet-forming residues varies with the experimental system[41] reiterates the fact that simple 'residue propensities' cannot be used in the design of β-strands in β-hairpins. In addition, investigations of side chain−side chain interactions in the context of β-sheet formation[42−44] and analysis of cross-strand residue pairs in antiparallel sheets do not reveal any strong preference.[33,45] Such studies imply that design of β-hairpins does not benefit greatly from the kind of residue patterning adopted in the design of α-helices. While β-branched residues, such Thr or Ile, and aromatic residues such as Phe can be viewed as 'β-sheet formers', the problem of β-hairpin formation is easier solved by the design of stable β-turns, whose conformation serves to form the first hydrogen bond of the β-hairpin and helps stitch together subsequent residues that register in hydrogen-bonding positions. We, therefore, turn to an alternate approach to fold control, namely, conformational control, which arises from the observation that certain protein, unnatural and synthetically designed amino acids have restricted conformational freedom.

B. Conformational Control

The diversity of polypeptide chain folds arises because of the multiple conformations that are energetically accessible at each amino acid residue. The two degrees of conformational freedom N−C$^{\alpha}$ (ϕ) and C$^{\alpha}$−CO (ψ), available at every residue, result in approximately 9 (3^2) stable local conformations. For

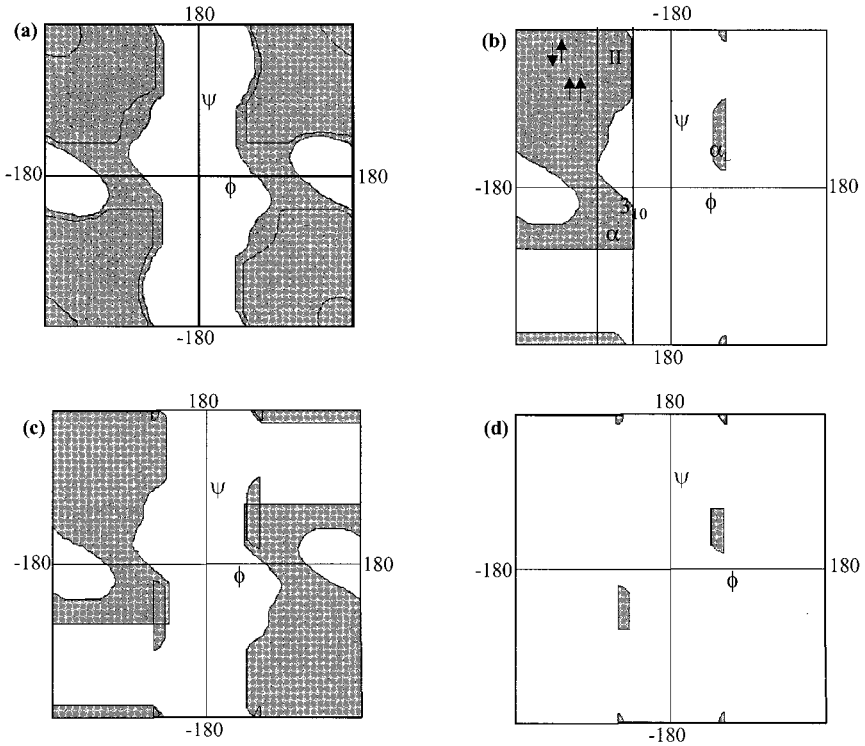

Figure 2. Ramachandran maps showing (a) the sterically allowed regions (shaded) for glycine and (b) the allowed regions for L-alanine (the regions corresponding to important regular structures are marked α (α-helix), 3_{10} (3_{10}helix), ⇈ (parallel β-sheet), ⇅ (antiparallel β-sheet), and II (polyproline)). (c) The superposition of the allowed regions for L-alanine and D-alanine. (d) The allowed regions common to both L- and D-alanine, which constitute the allowed region for the achiral Aib residue. Note that these regions correspond to an extremely limited region of ϕ,ψ space encompassing both the classical 3_{10} and α-helical conformations.

even a small polypeptide chain of 50 residues, the number of conformations that need to be considered escalates to nearly 10.[50] Ramachandran and coworkers,[46−48] at the University of Madras, recognized in the early 1960s that all possible values of ϕ and ψ are not stereochemically allowed, due to forbidding van der Waals clashes involving the backbone atoms and the C^β atoms of the side chains. Thus, the conformational space accessible to the protein amino acids (glycine being the notable exception) is restricted to approximately one-third of the total structural space.

1. Helix Nucleation

Parts a and b of Figure 2 illustrate the Ramachandran maps delineating the allowed regions of ϕ,ψ space for glycine and L-alanine. Clearly, the substitution of a hydrogen atom at the C^α atom by a methyl group results in a remarkable reduction of available conformational space. Extending the argument, Ramachandran and Chandrasekaran[49] and Marshall and Bosshard[50] independently concluded that the dialkylated residue, α-aminoisobutyric acid (Aib, α,α-dimethylglycine or α-methylalanine, abbreviated as 'Mea' in the early literature), should function as an extremely conformationally restricted residue, with allowed regions restricted largely to the right- and left-handed helical regions of conformational space. The conformational map for Aib may be derived by superposing the Ramachandran maps for L- and D-Ala (Figure 2c) and considering the region accessible to both enantiomers (Figure 2d). The first crystal structures of Aib-containing peptides determined at Bangalore[51−53] reveal the pronounced tendency of this residue to nucleate β-turn and 3_{10} helical structures even in small oligopeptides. The extraordinary ability of Aib-containing peptides to adopt conformationally defined structures resulted in a very large number of crystal structure determinations of synthetic sequences by laboratories across the world.[54−66] The flood of crystal structure determinations of Aib-containing peptides has been facilitated by the pronounced tendency of apolar, helical peptides to crystallize, presumably as a consequence of the intrinsic conformational rigidity of these sequences and the facility with which cylindrical structures pack into crystalline lattices.[67]

The large body of conformational work on Aib-containing peptides has been reviewed previously.[55,67−70] The most important stereochemical lessons for purposes of design of folded structures are as follows.

(1) The Aib residue is almost invariably restricted to ϕ,ψ values of −60° (±20°) and −30° (±20°), right-handed 3_{10} or α-helix (α_R), and to the enantiomeric position in ϕ,ψ space (+60° (±20°) and +30° (±20°), α_L). Although there has been considerable discussion in the literature on the factors that determine the precise helical conformations, 3_{10} or α-helix, and the possibility of observing interconversions,[71,72] we do not believe this is critically important for purposes of design. It should be noted that 3_{10} and α-helical structures differ only marginally in their Ramachandran angles (α-helix, $\phi \approx -57°$ and $\psi \approx -47°$; 3_{10} helix, $\phi \approx -50°$ and $\psi \approx -30°$). Facile interconversion between these conformations may be anticipated at the level of a single residue. In long helical constructs,

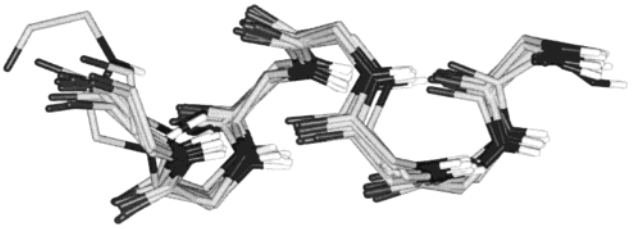

Figure 3. Superposition of 15 structures containing the heptapeptide segment, -Val-Ala-Leu-Aib-Val-Ala-Leu-, all of which are helical from residues 1−6, demonstrating the intrinsic robustness of the helical fold in diverse sequences.

the barrier to interconversion may be raised by the demands of cooperativity. The two structures differ in their hydrogen-bonding patterns, but careful examination of helices in peptides and proteins often reveal mixed 3_{10} and α-helical structures.[71,73] It is also pertinent to note that there are several examples of peptide helices that contain two molecules in the crystallographic asymmetric unit which reveal differences in the nature of the precise hydrogen-bonding patterns, emphasizing that energetic differences are small.[67,74]

(2) Helical conformations in oligopeptides can be nucleated by the presence of a single Aib residue in sequences varying in length from 7 to 9 residues,[75,76] resulting in highly soluble peptides that dissolve in a variety of nonpolar solvents.[77−79] Thus, the design of helical modules requires only infrequent use of the conformationally directing residue. Figure 3 shows the superposition of 15 structures containing the heptapeptide segment, -Val-Ala-Leu-Aib-Val-Ala-Leu-, all of which are helical from residue 1−6.

(3) Nonhelical conformations of Aib residues are extremely rare and have thus far been observed only in cyclic peptides or in amino acids or dipeptide derivatives.[54] (The total number of structures containing Aib in the Cambridge Structural Data Base as of October 1999 is 165, providing examples of as many as 498 independent residues.)

The success in constructing helical structures with Aib residues has spawned a large number of investigations on the conformational properties of higher α,α-dialkyl-related amino acids (Figure 4). Three kinds of residues have been investigated extensively.[69] (1) Achiral dialkyl glycine bearing linear alkyl chains such as diethylglycine (Deg), dipropylglycine (Dpg), and dibutylglycine (Dbg).[69] (2) Cyclic, achiral dialkylglycine bearing cycloalkane side chains such as 1-aminocycloalkane-1-carboxylic acid (Ac_nc, where n = number of atoms in the cycloalkane). Thus far studies on residues having $n = 3-12$ have been reported.[69,80,81] A particularly noteworthy synthetic residue is the amino acid 2,2,6,6-tetramethylpiperidin-1-oxyl-4-amino-4-carboxylic acid (TOAC), which not only incorporates the requisite backbone conformational constraint but also has a sensitive electron spin resonance probe in the form of a nitroxide moiety.[82]

Chiral α,α-dialkylated residues are in fact structurally derived from the protein amino acids by replacing the C^α hydrogen with a methyl group (e.g., α-methyl phenylalanine, α-methyl valine). The accessibility of these residues by enzymatic resolution

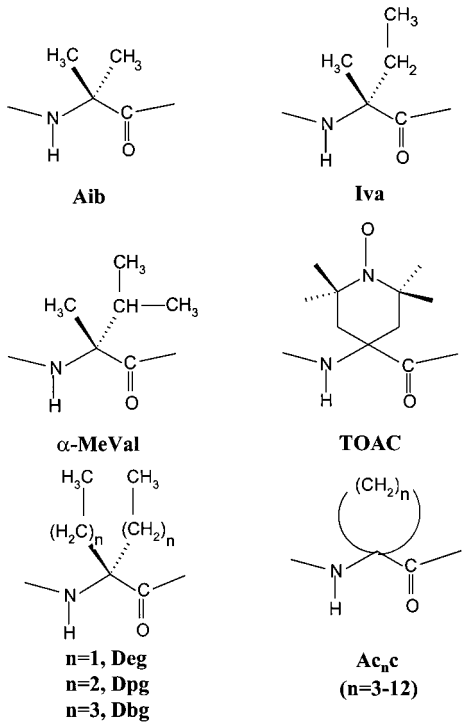

Figure 4. Chemical structures of some representative α,α-dialkylglycines.

procedures provides control over the helical twist sense in designed peptides.[83,84] (It may be noted that isovaline (α-methyl-α-ethylglycine) is present in a variety of fungal peptides such as alamethicin,[85,86] zervamicin,[87,88] and antiamboebin.[89−91] Isovaline, along with Aib, has also been found in meteorites, and both residues have been ascribed an extraterrestrial origin.[92,93]) Among other amino acid residues whose structural rigidity has been exploited in the construction of stable secondary structures are the α,β-dehydroamino acids, notably dehydrophenylalanine, which has been shown to stabilize 3_{10} helical conformations.[94−96] Crisma et al.[97] studied polymers ($n = 1-6$) of the dehydroalanine residue and reported them to adopt fully extended conformations (2.0_5-helix), forming 'flat peptides'.

2. Design of β-Turns and Hairpins

The β-turn is the simplest defined loop structure with conformational characteristics determined by residues at two positions (i+1, i+2). The conformations adopted by β-turns have been reviewed extensively by Smith and Pease[98] and Rose et al.[99] Type I and III β-turns can also be considered as a single turn of a 3_{10} helix, stabilized by a 4→1 hydrogen bond. Consequently, these two turns can be readily constructed using amino acids which stabilize helical conformations. For example, the helix-nucleating Aib residue has often been used in the construction of type III β-turns.[51,58,100]

The ^{L}Pro residue in which the $N-C^\alpha$ torsion angle ϕ is restricted to −60° (±20°) is the most conformationally restricted of the proteinogenic amino acids. As a consequence, the local conformations of ^{L}Pro are largely limited to $\psi \approx -30°$ (±20°) [α_R] or $\psi \approx +120°$ (±30°) (polyproline conformation). Figure 5a shows

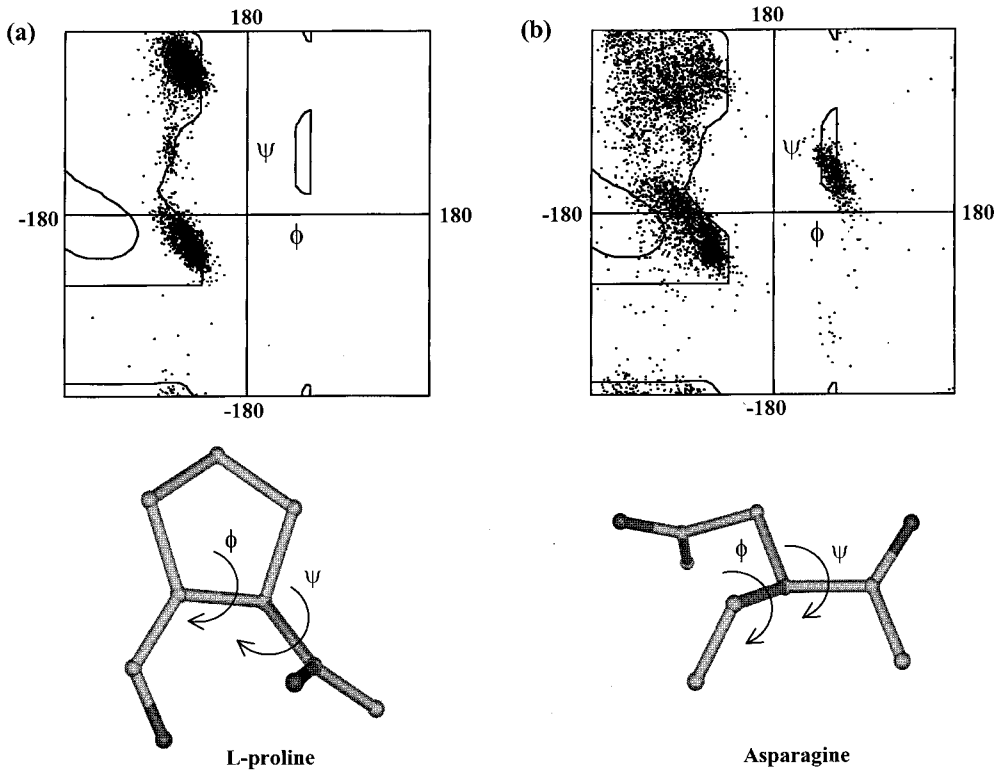

Figure 5. Crystallographically observed ϕ,ψ values of (a) L-Pro and (b) L-Asn residues from 538 independent protein crystal structures. The proteins in the data set used to generate this figure were derived from the Protein Data Bank[101] using a resolution cutoff of 2 Å and a sequence homology cutoff of 40% and contained 4995 proline and 5503 asparagine residues. Note the significant cluster of positive ϕ values lying in the left-handed helical (α_L) region for Asn.

the distribution of 4996 proline residues in high-resolution protein structures deposited in the Protein Data Bank.[101] Clearly, two clusters of observed conformations characterized by distinct ψ values are observed. The most commonly occurring conformations for ^{L}Pro are $\phi = -60°$ ($\pm 20°$), $\psi = -30°$ ($\pm 20°$) and $\phi = -60°$ ($\pm 20°$), $\psi = +120°$ ($\pm 20°$), which are compatible with the i+1 position of type I/III and type II turns, respectively. Both ^{L}Pro-^{L}Xxx (which have high propensity to adopt type I and II β-turns) and ^{L}Pro-^{D}Xxx sequences (which specifically nucleate type II turns) have been used in synthetic peptides to stabilize β-turn conformations.[102−103] As in the case of isolated helices, covalent cross-links have also been applied to the stabilization of β-turns. Both disulfide bridges[104a] and olefinic cross-links[104b] (derived from ring-closing metathesis, RCM, of allylglycines) have resulted in stable tetrapeptide β-turns.

More recently, the peptide motif Asn-Pro-Gly-Asp (which has a high propensity for the formation of type I turns) has been used by Blanco et al.[105] to construct a β-hairpin (derived from a natural β-hairpin spanning residues 15−23 in Tendamistat) with appreciable folding in aqueous solution. This motif was also utilized to modify the turn region of two more naturally derived β-hairpins with the intention of forcing their folding in isolation.[106,107] However, in both cases, the isolated segments formed three residue turns (Pro-Gly-Asp), demonstrating that ^{L}Pro does not provide adequate control of turn stereochemistry, necessary for stable hairpin design.

The recognition by Janet Thornton and her colleagues[108] that the nucleating β-turns in protein β-hairpins are predominantly type I′ or type II′ β-turns suggested that synthetic design of such structures should focus on ensuring appropriate turn stereochemistry.[33] Examination of the accessible regions of conformational space for ^{D}Pro (Figure 6) makes evident that the favored conformational angles for ^{D}Pro ($\phi \approx +60°$, $\psi \approx +30°$ and $\phi \approx +60°$, $\psi \approx -120°$) are also compatible with the requirements of the i+1 residue in type I′ and type II′ β-turns, respectively. Thus, ^{D}Pro-Xxx sequences may be anticipated to provide turn segments which can nucleate β-hairpins in synthetic peptides. The systematic use of ^{D}Pro-Xxx sequences in the construction of β-hairpins has been developed in our laboratory and independently by Sam Gellman at the University of Wisconsin.[109,110] Crystalline β-hairpins have been characterized by X-ray diffraction in octapeptide sequences.[111,112] NMR spectroscopy[112−114] also provides a convenient and unambiguous characterization of hairpin structures because of the larger dispersion of $C^{\alpha}H$ and NH chemical shifts as compared to helices. The observation of cross-strand NOEs between C^{α} and nitrogen protons that are distant in sequence is again an easily recognizable feature.

Studies comparing ^{D}Pro-Xxx- and ^{L}Pro-Xxx-containing sequences have shown that ^{D}Pro-Xxx is superior to its enantiomeric isomer in nucleating hairpin formation;[113,114] indeed, sequences with ^{L}Pro-Xxx turns reveal little evidence for hairpin structures.

The use of Aib and D-residues to facilitate β-turn conformation has been reviewed earlier.[54,99,102] The use of a central Aib-Gly segment in the peptide Boc-Leu-Val-Val-Aib-Gly-Leu-Val-Val-OMe provides an

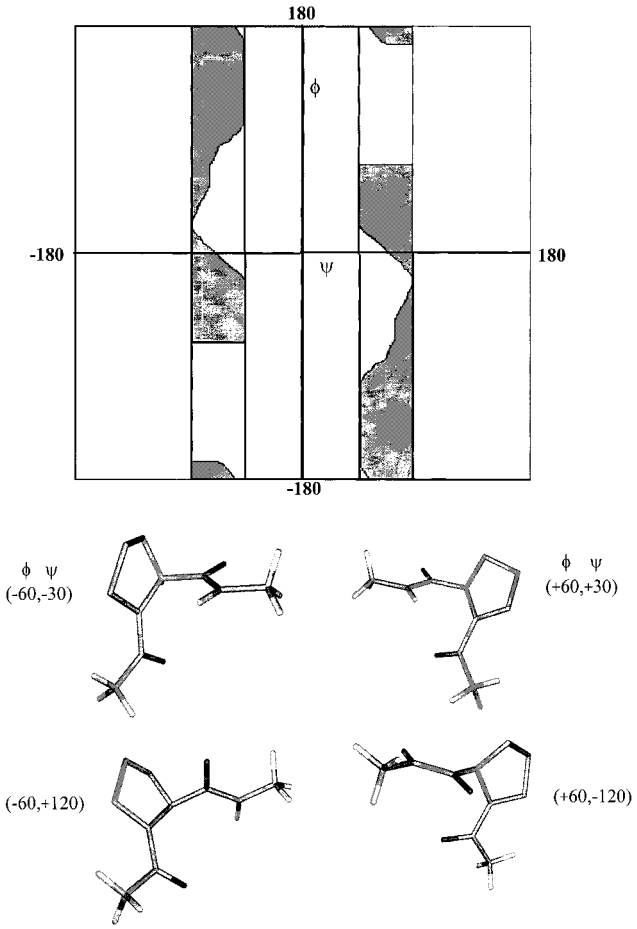

Figure 6. Allowed regions of Ramachandran space for L-proline and D-proline. Also shown below are conformations generated by using the ideal values of ϕ,ψ for L-Pro, corresponding to the i+1 position in Type I (left top) and Type II (left bottom) β-turns, in the structure of Ac-L-Pro-NHMe. The corresponding conformations for the Type I′ and II′ structures in the enantiomer Ac-D-Pro-NHMe are shown on the right.

example of design where a solvent-dependent transformation from a β-hairpin to a helical structure has been studied.[115] The ability of LD-segments to nucleate turns was theoretically recognized by Ramachandran and co-workers[116] and has subsequently been widely exploited for designing synthetic sequences.[117] However, in mixed chiral sequences, the conformational restraints are not overwhelming. The ^{D}Pro-Ser unit has also been used by Imperiali and co-workers to nucleate a tight β-turn in a designed $\beta\beta\alpha$ fold.[118]

Among the 19 non-glycine protein amino acids, asparagine has the highest propensity to occur in the α_L region of conformational space, with positive values of ϕ, and as such has been termed the 'least chiral' of the amino acids by Richardson.[8] Figure 5b shows the distribution of 5503 Asn residues in high-resolution protein structures deposited in the Protein Data Bank,[101] clearly revealing the ability of Asn to adopt positive values of ϕ analogous to ^{D}Pro. Translating such analyses into design strategies, investigators have constructed Asn-Gly-containing turn segments to successfully nucleate β-hairpin formation.[119−121] Comparative studies on sequences which contain ^{D}Pro-Gly and Asn-Gly as turn sequences have revealed that the former has a considerably greater

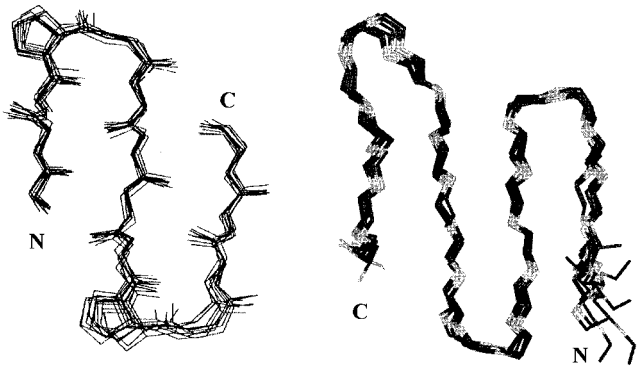

Figure 7. Superposition of 10 NMR-derived structures for de novo designed β-sheets. (a) Three-stranded β-sheet in a 14 residue peptide and (b) four-stranded β-sheet in a 26 residue peptide, calculated using molecular dynamics calculations using NOE-derived restraints. Note these structures are stable in organic solvents.

tendency to nucleate hairpins.[122] The formation of stable β-hairpin structures has recently been reviewed in detail.[109,123]

3. Design of Multiple-Stranded Sheets

The successful design of β-hairpins using ^{D}Pro-Xxx sequences suggests that periodic insertion of these sequences into a polypeptide sequence should facilitate repeated chain reversal providing ready access to synthetic multiple-stranded sheets. Figure 7 illustrates the NMR-derived structure of synthetic peptides designed to form three- and four-stranded β-sheets.[124,125a] Recently, this principle has been extended to a five-stranded structure in a 34-residue peptide.[125b] In all cases, the complete definition of the solution conformation was realized by observation of all relevant interstrand NOEs. The D-proline residue has successfully been utilized by other groups as well in the construction of multiple-stranded β-sheets. Notably, the ^{D}Pro-Xxx motif has been used by Schenck and Gellman[126] to construct three-stranded β-sheets that fold in water. Indeed, the employment of D-residues to construct β-sheets preceded the systematic analysis of ^{D}Pro-Xxx motifs as β-hairpin nucleators during the construction of the betabellins by Richardson, Erickson, and co-workers.[127,128]

The observation that Asn-Gly could nucleate β-hairpins has also been extended into the construction of three-stranded β-sheets by Sharman and Searle[129,130] and Kortemme et al.,[131] wherein two consecutive Asn-Gly motifs were used to engender chain reversal in two separate synthetic sequences that folded into three-stranded β-sheets in 50% water/methanol mixtures and 100% water, respectively. Such three-stranded β-sheets have been applied to the study of the factors governing β-sheet folding and to assay the role of cooperativity along individual hairpins constituting a sheet and across strands of a β-sheet.[126,129,130,132] This aspect, again, has been recently reviewed in detail.[109,133]

C. Use of Templates

The earlier sections discussed the role of specific amino acids in the nucleation of secondary structural modules. This function may also be performed by

synthetic templates. Molecular templates may be used in two distinct ways in generating folded, synthetic polypeptides. First, the designed templates may provide a preorganized folding nucleus which can position hydrogen-bonding groups in a manner that permits propagation of a regular secondary structure. Second, templates may be used to organize prefabricated modules of secondary structure in order to generate compact tertiary folds. An exhaustive review of templates that induce α-helical, *β*-sheet, and loop conformations has appeared.[134] In this section, we consider templates used for nucleating secondary structures.

The peptide α-helix is a structure that is intrinsically unfavorable in an entropic sense with respect to an ensemble of unordered conformations. Nevertheless, peptide α-helices are stable presumably because of the overriding favorable contributions to the free energy of folding from nonbonded and hydrogen-bonding interactions in the helical state.[135,136] In principle, peptide helices may be nucleated at the N-terminus, C-terminus, or the center of the peptide. A 'nucleus' would imply a local segment of structure in which Ramachandran angles are restricted to helical values, providing a seed for the crystallization of cooperative interactions. In proteins, Pro residues are often found at the N-termini of helices.[8,26] In an α-helix, the first three residues at the N-terminus do not contribute an NH group to the helical hydrogen-bonding scheme. As a consequence, positioning of Pro at these positions does not interfere with hydrogen bonding and provides the added advantage that ϕ L-Pro ~60° is ideally suited for accommodation into 3_{10} or α-helical conformations. A study of the model peptide Piv-L-Pro-L-Pro-NHMe in organic solvents[137] suggested that incipient 3_{10} helix formation is facilitated by the presence of contiguous Pro residues. However, such a structure is not sufficiently constrained to enforce helical folding in aqueous media. Kemp and co-workers developed an extremely elegant solution for the problem of designing helical templates by constructing a conformationally restricted analogue of an Ac-Pro-Pro sequence (Figure 8) which would also hydrogen bond with the N-terminal residues in the attached peptide segment and prevent fraying of helical ends. Using this template, these authors have shown stable helix formation in aqueous media for peptides 5−11 residues in length.[138] This model has permitted independent evaluation of helix propensities for the Ala residue.[139] In a recent study, N-templated helical modules provided interesting circular dichroism data, which raise important issues on the use of CD ellipticities at 222 nm as a quantitative index of peptide helicity.[140] In yet another study, Bartlett and co-workers[141] utilized a bicyclic diacid as a template to reduce N-terminal fraying of helices and termed it a peptide 'aglet'. An ester of the diacid has been shown, by circular dichroism and 2-dimensional NMR spectroscopy, to induce significant helicity in a hexapeptide segment. Gani and co-workers addressed the same issue and attempted to fashion macrocyclic templates[142−144] with appropriately positioned carbonyl groups to function as hydrogen-bond acceptors

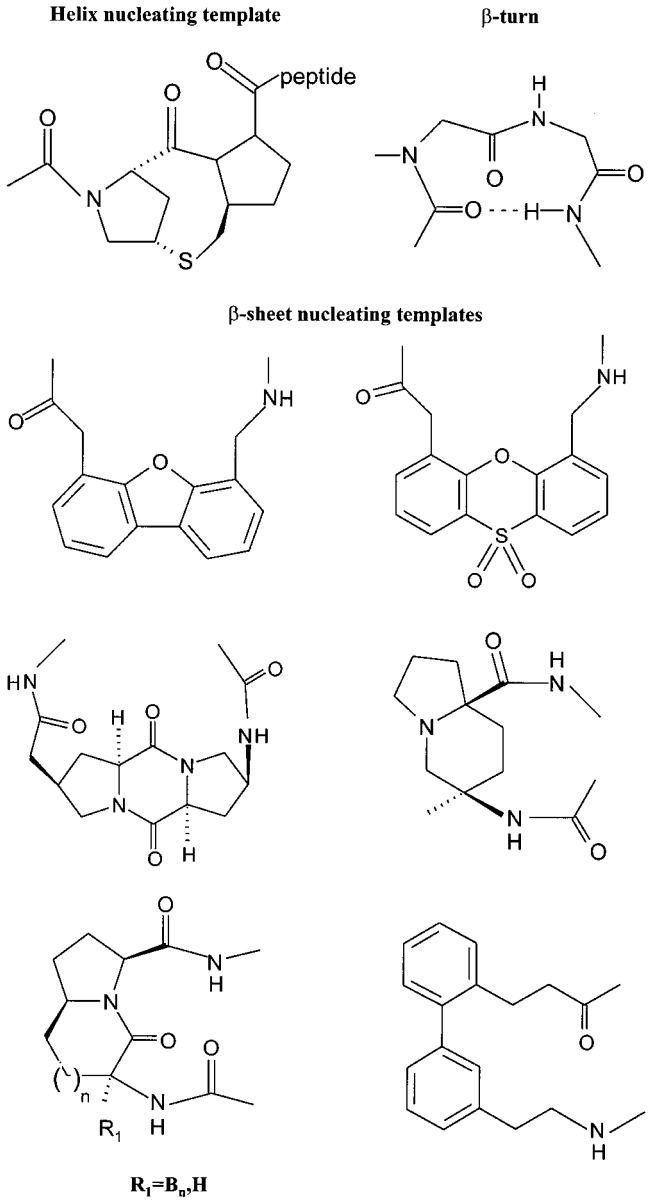

Figure 8. Representative structures of a helix nucleating template designed by Kemp et al.[139] (top left) and a *β*-turn (top right). Also shown are some representative *β*-hairpin nucleating templates.[36,146,276−279]

from N-terminal residues of attached peptides. While the 12-membered macrocycle derived from *N*-[(2*S*)-2-chloropropionyl]-(2*S*)-Pro-(2*R*)-Ala-(2*S*,4*S*)-4-thio-Pro-OMe[144] had the required all-trans conformation (*ttt* form) expected to align the carboxylic groups in the template in an α-helical conformation, this alignment was not observed. Attempts to induce the required orientation of carboxyl groups by attaching mono- and dipeptide units proved unsuccessful. However, the thioether macrocyle based on (2*R*)-*N*-propionyl-(2*S*)-Pro-(2*R*)-Ala-(2*S*)-Pro bearing a positively charged tetraalkylammonium ion near the 'C-terminal end' yielded a minor conformer invested with the requisite α-helical alignment of hydrogen-bond acceptors. Thus far, application of these templates to nucleate helices in specific sequences has not been reported.

Unlike helices, *β*-hairpins lend themselves to the template-based approach in peptide design. In pro-

teins, hairpin structures are quite often nucleated by tight two-residue loops, β-turns. In the β-turn conformation, sharp chain reversal is achieved with the nucleus being stabilized by a 10-atom 4→1 hydrogen bond (Figure 8). Several templates for designing β-hairpin peptidomimetics have been synthetically constructed and their utility demonstrated in model sequences. Figure 8 shows a representative set of templates illustrating the structural principle employed in ensuring polypeptide chain reversal. In all cases, the templates provide two reactive functional handles which can be used to attach N- and C-terminal polypeptide chain segments. The dimensions of the template are chosen such that the pendant antiparallel and parallel peptide chains can be brought into hydrogen-bonding registry. Several topical reviews that have appeared recently illustrate the principles behind the design and application of β-sheet templates.[135,145−147] The area of β-turn mimics has also been extensively reviewed.[135,146,148] Most template-based β-hairpin peptides have been restricted to relatively small model sequences, generally at the level of tetrapeptides. Two notable examples have been reported by Kelly and co-workers.[149,150] Dibenzofuran-based templates[149] have been used to attach two tripeptide arms to yield a hairpin structure, which demonstrates that hydrophobic cluster formation involving interactions between the aromatic template and the attached nonpolar residue is necessary for β-hairpin formation. The 2,3′-substituted biphenyl-based amino acid, [3′-(2-aminoethyl)-2-biphenyl]propionic acid, has also been investigated as a β-hairpin nucleator.[150] Once again, tripeptide segments have been added to the two functional sites of the template yielding a heptapeptide, which demonstrated the presence of a hydrophobic cluster. Extension of the chains to yield a 13-residue peptide results in the formation of a β-hairpin structure which then aggregates. In these cases, the hydrogen-bonded hydrophobic cluster involving the template and the preceding and succeeding hydrophobic amino acid appears to act as a folding nucleus for the hairpin function.

III. Assembly of Secondary Structures

The lessons learned from the successful construction of secondary structural elements can be applied to the design of supersecondary structures. Construction of peptide modules at a higher level of complexity involves the design of oligomeric secondary structural elements whose assembly leading to a compact fold can be achieved by exploiting a variety of strategies (Figure 9) which are discussed in detail below. Design of such aggregates can at times even serve to stabilize the basic structural unit, which might be unstructured in the nonaggregated state. The designed modules for subsequent assembly can be from isolated structural units or tethered to form a single polypeptide chain as in the case of naturally occurring proteins. It is important to note that while the latter option is desirable in the context of de novo protein design, it is difficult to control and has been achieved only in a limited number of cases.[151]

Table 1 lists a representative set of water-soluble supersecondary structures that have been characterized in detail by X-ray crystallography or NMR techniques.

A. Assembly Driven by Solvent Forces

1. Helical Bundles

Construction of polypeptides which self-associate into a predetermined topography can be achieved using amphiphilic molecules, whose aggregation is occasioned by manipulation of solvent conditions. Control over such aggregation phenomena, in terms of directionality of association and association number, can be designed into synthetic molecules by the introduction of favorable interactions such as burial of hydrophobic surfaces and formation of ion pairs, hydrogen bonds, or specific aromatic interactions between monomer units. This approach relies on the extensive understanding of the forces that determine tertiary structure formation in proteins.[152]

Helices associate to form α-helical hairpins, coiled-coils, or helical bundles, and such associations involve interactions between seven-residue geometric repeats which constitute the individual helices of the bundles. The leucine zipper from the transcription activator GCN4 is a naturally occurring dimeric coiled-coil which associates via such heptads.[153] Manipulation of the residues of the hydrophobic core of GCN4 (which contains an polar asparagine residue involved in important interhelical interactions) has been used to construct coiled-coils with varying aggregation states, one of which has been characterized by X-ray diffraction (Table 1) and shown to be a parallel tetramer.[154,155] Such heptad repeats have also been used by Hodges and co-workers, as well as other groups, to design two-stranded coiled-coil structures.[156] Osterhout and co-workers reported the construction of an α-helical hairpin peptide (αtα) that was amenable to detailed NMR characterization.[157] Although this peptide was not designed to have heptad repeats, the amino acids at the interface of the helices were similar to those occurring in naturally occurring coiled-coils, and 2-dimensional NMR experiments suggest the presence of desired tertiary structure. DeGrado and co-workers attempted the design of a 16-residue helical peptide whose arrangement of leucine residues would permit association into a four-helix bundle. The crystal structure of a 12- residue fragment from this peptide[158] indicated that the association of the peptide was more complex than expected from the design. The structure of the same peptide crystallized at a different pH showed the formation of antiparallel four-helix bundles, as anticipated by design.[159] Further attempts by DeGrado and co-workers, based on the designs of Hodges and co-workers, resulted in a 29-residue peptide, coil-Ser, whose crystal structure revealed the formation of trimeric coiled-coils.[160] The formation of coiled-coils and the factors controlling their association number and stability have been reviewed in detail.[161]

Stroud and co-workers described the formation of a four-helix bundle whose repeating helical unit was

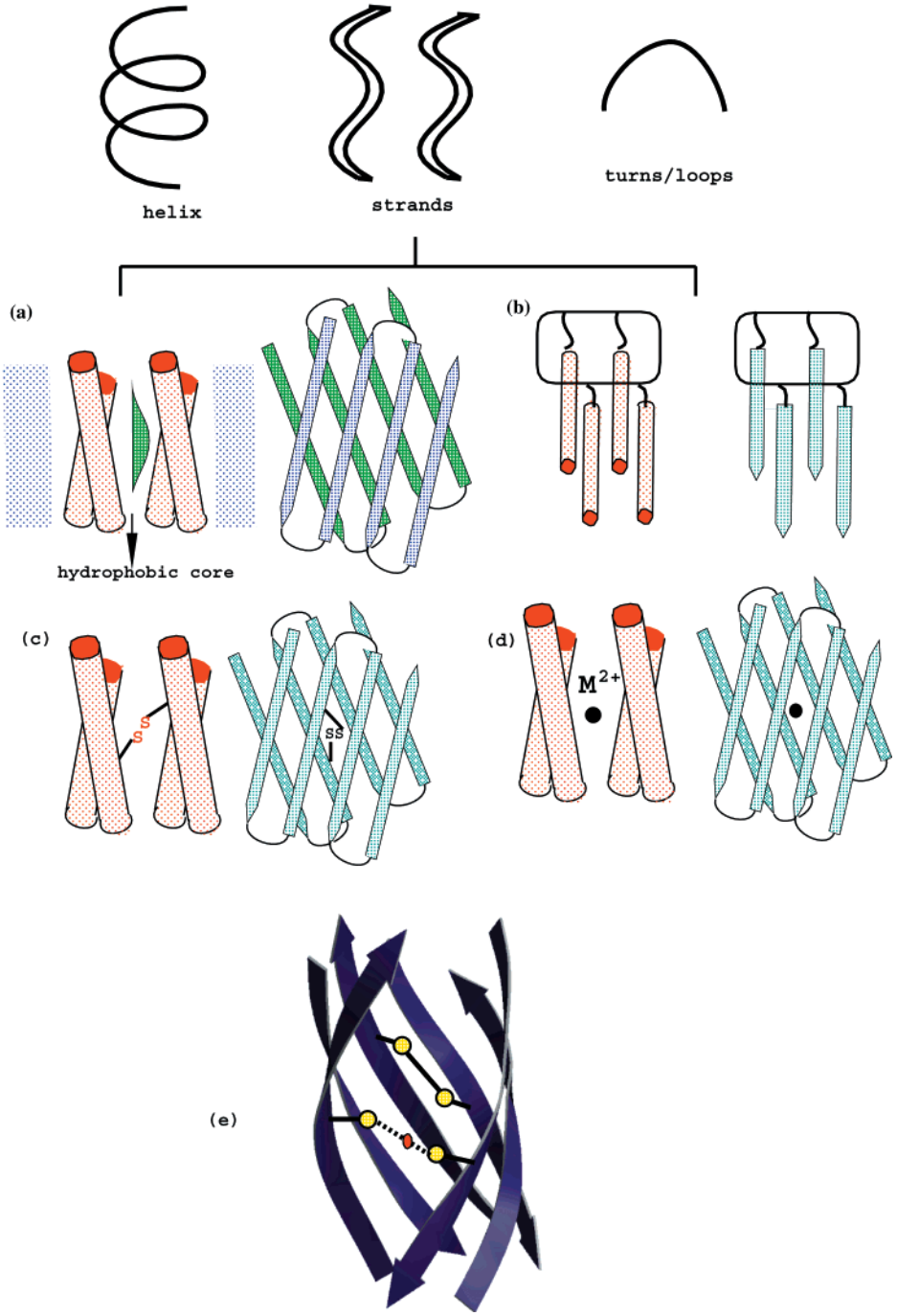

Figure 9. Schematic representation of the multiple techniques by which secondary structural elements can be assembled, namely, (a) patterning of residues to form a stable, hydrophobic core (assembly driven by solvent forces), (b) template-assisted protein folding (TASP), (c) association of structural units via disulfide links, and (d) metal-mediated assembly. (e) A strategy whereby isolated β-sheets could be assembled to form β-barrels using either disulfide bridging (yellow) or metal-ion-mediated assembly (red).

designed to solubilize membrane proteins.[162] The 'peptidergent', while not designed based on a naturally occurring heptad motif, nevertheless has appropriately positioned alanine and leucine residues in the hydrophobic core of the bundle. Another remarkable attempt to design a three-helix bundle (Figure 10a) that would be amenable to detailed structural analysis by NMR has been reported by Walsh et al.[151a] The residue redundancy which results in a homomeric helical bundle has been eschewed by designing the association of three different helical segments which have been incorporated into a single polypeptide chain to form a complex 73-residue peptide. In very recent reports, Eisenberg and coworkers achieved the assembly of a domain-swapped three-helix bundle[151b] and demonstrated that modulation of monomer topology can control formation of discrete domain-swapped dimers or open oligomeric structures.

Some attempts to construct coiled-coils that deviate from naturally observed parallel, left-handed superhelical conformations have been spectacularly successful. Oakley and Kim have achieved coiled-coils with heterodimeric, antiparallel helix association[163]

Table 1. Representative List of Water-Soluble Supersecondary Structures

molecule	technique	resolution	ref
α-helix (α-1 at low pH; **1AL1**)[a] (13 residues)	X-ray crystallography	2.7 Å	158
α-helix (α-1 near neutral pH; **1BYZ**)[a] (13 residues)	X-ray crystallography	0.9 Å	159
four-helix bundle (Peptidergent; single polypeptide chain; **4HB1**) (108 amino acids)	X-ray crystallography	3.0 Å	162
triple-stranded coiled-coil (Coil-Ser; **1COS**) (31 residues/chain)	X-ray crystallography	2.1 Å	160
triple-stranded coiled-coil (Coil-Vald; **1COI**) (31 residues/chain)	X-ray crystallography	2.1 Å	283
trimeric coiled-coil (**1BB1**) (36 residues/chain)	X-ray crystallography	1.8 Å	284
trimeric coiled-coil (**1GCM**) (34 residues/chain)	X-ray crystallography	1.8 Å	285
tetrameric coiled-coil (**1GCL**) (34 residues/chain)	X-ray crystallography	2.1 Å	155
right-handed, tetrameric coiled-coil (**1RH4**) (35 residues/chain)	X-ray crystallography	1.9 Å	164
four-helix bundle with a diiron-binding center (association of two helix−loop−helix motifs) (Due Ferro 1; **1EC5**) (50 residues/chain)	X-ray crystallography	2.5 Å	256
helical hairpin (αtα; **1ABZ**) (40 residues)	NMR	23 structures	157
helical hairpin(α-2D; **1QP6**) (35 residues)	NMR	16 structures	286
ββα motif (**1FSD**) (28 residues)	NMR	41 structures	191
ββα motif (**1PSV**) (28 residues)	NMR	32 structures	192
three-helix bundle (single polypeptide chain; **2A3D**) (73 residues)	NMR		151

[a] These are single helices, but the discussion of crystal state aggregation may be relevant to supersecondary structure design.

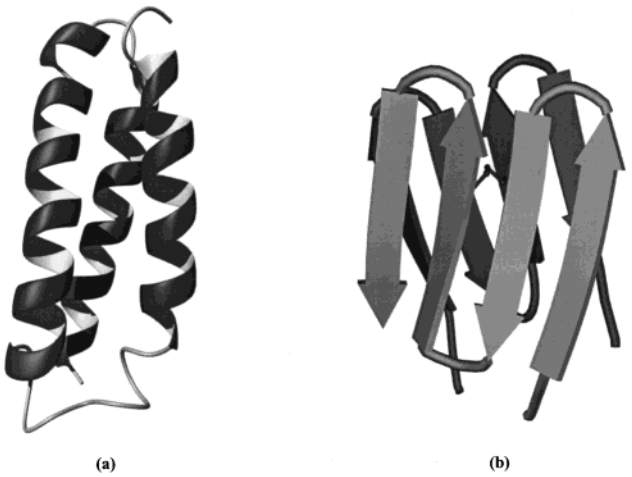

Figure 10. Ribbon diagrams showing (a) the three-helix bundle fold in a designed, 73-residue peptide (the peptide structure determination was performed using high-resolution NMR in aqueous solutions (PDB code, **2A3D**)[151]) and (b) a theoretical structural model for Betadoublet, a 33-residue peptide designed to dimerize into a β-sandwich structure tethered by an intermolecular disulfide bond (in black) (PDB code, **1BTD**).[176]

by utilizing a stabilizing buried, polar interaction between the two heterodimers that would form only when helix association occurs in an antiparallel fashion. Further work from the laboratories of Alber and Kim resulted in the formation of coiled-coils with a reversal of superhelical twist. Harbury et al.[164] describe the de novo design of a family of α-helical bundle proteins with right-handed superhelical twist by the use of a 11-residue repeat as opposed to the naturally observed heptad repeats. Two-, three-, and four-helix bundles have been constructed, and the crystal structure of a four-helix bundle has been studied in detail.[164] Micklatcher and Chmielewsli[165] recently reviewed the design of helical proteins in detail.

A minimalist approach to inducing folding in de novo designed polypeptides uses the concept of binary patterning of residues.[166] The appropriate positioning of polar and nonpolar amino acids, irrespective of their actual identity, is utilized to drive hydrophobic collapse to form a compact fold.[167] Hecht and co-workers described a combinatorial approach to arrive at a large family of designed four-helix bundle proteins[166] which differ in actual placement of amino acids but share a common pattern of polar and hydrophobic residues. While these proteins have been shown to form collapsed α-helical structures, later attempts have been more successful in constructing proteins with more 'nativelike' properties, such as cooperative denaturation, significant dispersion in NMR spectra, and the presence of amide protons which are resistant to ready hydrogen exchange.[168,169]

The construction of tertiary structures, such as helix−helix motifs, in completely apolar sequences has been approached by linking preformed helical motifs, often Aib-containing sequences, by linking segments. In cases where flexible linking segments have been used, extended arrangements of helical modules have been observed in crystals.[170] Limited evidence for folded compact arrangement of helices in two-helix structures has been presented.[171] An interesting recent example describes the characterization of an antiparallel helix arrangement in a two-helix peptide containing dehydrophenylalanine residues. A flexible Gly_4 linker and aromatic−aromatic interactions appear to favor the close-packed arrangement.[96] The effect of linker segment length on the formation of compact helix−loop−helix motifs has been assayed by Suzuki and Fujii[172] and a loop consisting of seven glycine residues (corresponding to a length of 25 Å) suggested to be optimal for fold stabilization.

2. β-Sheet Assembly

β-Sheets assemble to form β-sandwich or β-barrel folds (Figure 11). It might be rationalized that the intrinsic nature of β-sheet structures to adopt right-handed twists and to aggregate noncovalently might be profitably controlled to form sandwich structures. The investigations on the family of betabellins have been a systematic though not completely successful study toward this end by the Richardsons, Erickson, and co-workers.[173,174] The target fold in these studies was an antiparallel β-sandwich. A variety of design strategies have been successively considered, and the residues comprising the β-sheets have been refined

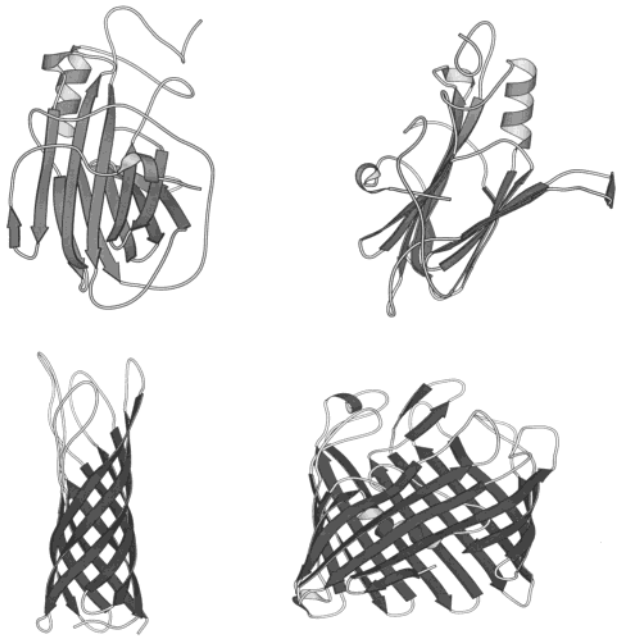

Figure 11. Representative structures of a β-sandwich protein (top left and right) illustrating association of two β-sheets (a β-sandwich domain from the structure of a chemokine inhibitor, VCCI, from cowpox virus, PDB code **1CQ3**).[280] The structures at the bottom illustrate the association of end strands in closed β-barrel structures. (Bottom left) Transmembrane domain of outer membrane protein A (PDB code, **1BXW**).[281] (Bottom right) Outer membrane protein F (PDB code, **2OMF**).[282]

over repeated cycles of design and synthesis. As mentioned previously, one of the design strategies involved the use of D-residues to construct tight type I′ and II′ turns.[127,128] This was also an example of negative design, wherein the formation of tight turns would counteract the formation of Greek keys. The intrinsic twist of the type I′ and II′ turns also favors the right-handed twist of β-sheets that is most frequently observed in protein structures.[175]

The patterning of residues over the strands to create amphipathic β-sheets whose association would be driven by solvent forces was supplemented in some of the betabellins (as well as in betadoublet,[176] a variant composed purely of naturally occurring amino acids) by a disulfide bond in the core of the sandwich.[128,177] One such betabellin, betabellin 14D,[128] demonstrated folding into a β-sheet structure in the dimeric form (as monitored by circular dichroism), though the monomer was largely unstructured. The work on betabellins and betadoublet[176] highlights many of the difficulties that are likely to be encountered in de novo design. The focus of the study has been the improvement of the solubility and total β-content in successive molecules, rendering them finally amenable to structural elucidation. Betabellin 12 has been marginally successful in this light, with interpretable NMR spectra in dimethyl sulfoxide.[127] However, the absence of long-range nuclear Overhauser effects precluded detailed structural analysis. Figure 10b shows a theoretical model constructed for betadoublet, illustrating the desired conformation. It is pertinent to note that while disulfide bonding of the constituent β-sheets of a desired β-sandwich is a convenient technique which has seen some success,[128,176−177] steric constraints imposed by the centrally positioned disulfide bond greatly limit the choice of residues in the inner face of the β-sheets. Such a disulfide bond, while serving as a highly efficient tether, might compromise the overall integrity of the β-structure.

In yet another attempt to construct β-sandwiches, Mayo and co-workers modeled synthetic peptides on the sequence of the β-sheet domain of the α-chemokines.[178−180] The NMR analysis in one such study was complicated by the surprising observation of multiple resonances for many residues, which was interpreted as arising from two conformationally distinct β-sheet dimers that undergo slow exchange over the NMR time scale. Pulsed field gradient NMR experiments have established the presence of tetramers, which have been rationalized as being due to the association of two β-sheet dimers. A curious example of a protein that contains a large, solvent-exposed β-sheet is the outer surface protein A (OspA) from *Borrelia burgdoferi*.[181,182] Koide, Engelman, and co-workers[183] engineered this protein to yield stable, five- and seven-stranded β-sheets in solution and attributed the stability of the extended sheet to hydrophobic interactions between the sheet edges and flanking globular domains. They further speculate that burial of edge surfaces, causing stabilization, may be important in the design of β-sheet domains. As a corollary, it is tempting to interpret the substantial stability associated with β-barrel structures as a result of the absence of hydration-sensitive edge strands. Such an argument, coupled with the observed difficulty in assembling β-sheets into β-sandwiches with well-defined cores, would imply that design of sheet structures that associate in the requisite geometry for barrel formation would be synthetically more accessible.

In principle, a multistranded β-sheet constructed by the device of using DPro-Xxx sequences should automatically fold into a closed β-sheet stabilized by cooperative hydrogen bonding and facilitated by the intrinsic twist of the β-sheet. The analysis of β-barrel geometry and protein β-barrels[185,186] (Figure 11) helps understand the requirements of barrel formation: the minimum number of strands required for strand closure, the registering of strands in a barrel (which is essential in the correct positioning of putative interacting residues), and the packing of the barrel interior, which is a consequence of barrel size and geometry and in turn dictates barrel stability. The formation of perfect barrels (as seen in the porin sequences[187]) necessitates the presence of either a large number of strands or fewer strands with a large number of residues in each strand. The observation that a minimum of eight strands and large, flexible loops are required for the formation of natural β-barrels implies the need of polypeptides of large size, which is synthetically formidable. While recently established chemical ligation techniques[188,189] have been successfully employed to construct large polypeptides, the technology is still sufficiently sophisticated to preclude widespread application. As a consequence, a feasible approach toward the construction of β-sandwiches and β-barrels is the assembly of β-sheets on metal templates or by disulfide bridging.

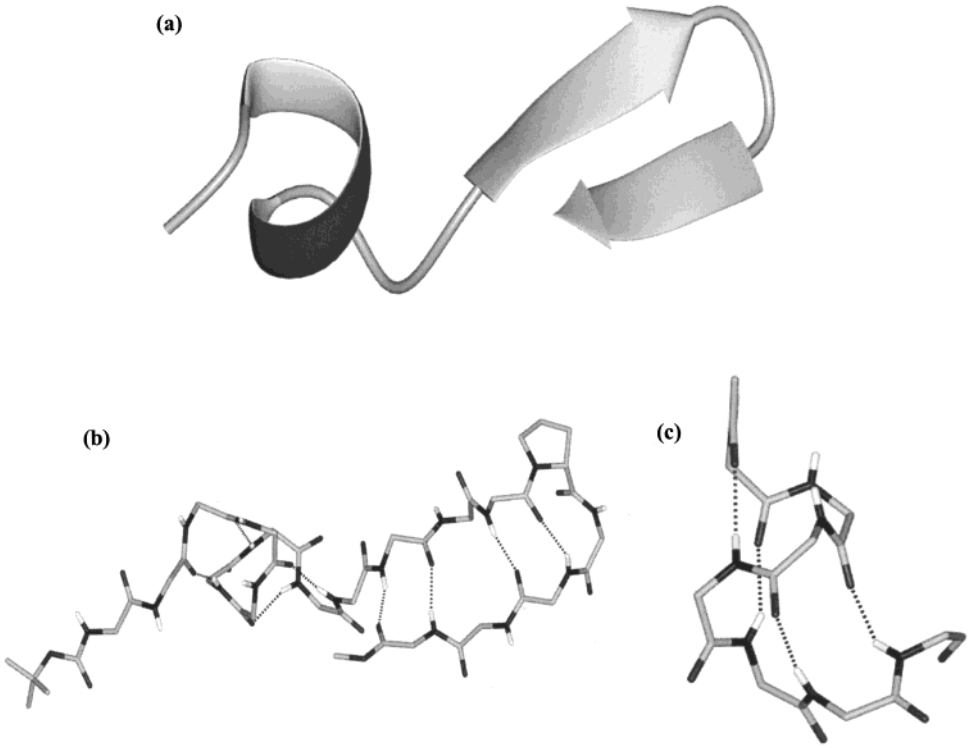

Figure 12. Structure of a 17-residue peptide[194] showing (a) the ribbon representation of the N-terminal helix linked to a C-terminal β-hairpin module, (b) molecular structure showing hydrogen bonds, and (c) the portion of the molecule involved in the formation of the Schellman motif.

The novel use of a disulfide bridge to tether edge strands of β-sheets might help synthetically create large sheets, whose folding would be driven by hydrophobic forces. In addition, simultaneous use of two linking disulfide bonds (as shown in Figure 9e) might aid in the forced formation of β-barrels.

3. α/β Mixed Assemblies

The assembling of mixed α/β structures has also attracted considerable attention. A popular approach in protein design has been to examine the properties of the fold of choice and to attempt an adaptation of such a fold to synthetic strategies. The design of a zinc-finger motif has been attempted by two groups. Imperiali and co-workers[118,190] followed an iterative protocol of rational design to arrive at a $\beta\beta\alpha$ fold that resembled the zinc-finger motif but could fold in the absence of metal ions. Mayo and co-workers[191,192] used computational techniques to optimize the core of a zinc-finger motif and designed an $\beta\beta\alpha$ fold with a novel sequence, which was characterized using NMR spectroscopy. An alternate approach is the Meccano (or Lego) set approach,[70,193] wherein individually constructed, rigid, secondary structural modules are assembled to form a novel structure. This approach has been successfully utilized in the design of a synthetic peptide containing helical and hairpin segments.[194] The crystal structure (Figure 12) of this peptide reveals several interesting features, including the presence of a Schellman motif as a helix-termination signal, and suggests that modular assembly of novel folds are viable approaches to protein design. Moe and co-workers[195,196] also attempted to design a mixed α/β structure wherein a single β-strand packs against an α-helix. While detailed structural characterization was not possible, the authors performed hydrogen/deuterium exchange NMR experiments and ascribed their observations to the existence of folded structures. Attempts have also been made to test the design rationale by mutation of certain key residues. Analogous to the betabellins, the design of a family of α/β-barrel proteins (octarellins) has been attempted by Martial and co-workers.[197−199] Analysis of the α/β-barrel geometry, packing of residues in the interior of the barrel, and barrel symmetry was performed to arrive at a synthetic sequence. Preliminary structural investigations (CD and infrared spectroscopy) reveal the presence of both α and β structures in the expected ratios.

While many attempts to synthesize polypeptides that fold into compact domains have been successful, design of folded polypeptides that collapse into the required fold solely on the basis of sequence is still a challenge. Many approaches have been made to induce folding and assembly in designed peptides, as discussed in the subsequent sections.

B. Template-Assisted Protein Folding (TASP)

The forced association of isolated peptide modules was elegantly achieved by Mutter and colleagues[200] by covalently tethering peptide chains on a template (Figure 9). This concept was exploited in a cyclic peptide template system to study aspects of helix formation and association,[201,202] to construct models for helical bundles functioning as ion-channels,[203,204] and to mimic conformational epitopes which can subsequently be used in the raising of antibodies.[205] The use of various templates by Mutter and co-workers in the creation of novel folds has been extensively reviewed.[206]

A variation on the same theme was the construction of a collagen-like triple-helical module[207] on the template *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid (known as Kemps's triacid[208]). Detailed NMR characterization of the compound revealed the success of the design strategy.

More recent examples of novel templates and template-assisted peptide folding and assembly comes from the laboratory of Darshan Ranganathan at Hyderabad. Norbornene[209] and pyridine dicarbonyl[210] units have been used as templates for the synthesis and assembly of oligopeptides. These template-bound oligopeptides have been studied in their crystalline and solution states and found to adopt a curious range of conformations (3_{10}helices,[209] helices with both screw senses,[210] sheetlike structures[209]) depending on the chain length of the peptide units under study.

C. Use of Disulfide Bridges as Templates

An alternate approach to the template-mediated association of peptide chains is the use of disulfide bridges to tether peptide modules and induce local folding. This strategy was utilized in the classical design of some members of the betabellin family: betadoublet[176] and betabellins 14D[128] and 15D.[177] The structure and stability of the designed protein dimers have been analyzed using circular dichroism and infrared spectroscopy.[128,176] The metal-binding capacity of betabellin 15D has been studied using mass spectrometry and used as a reporter of folded structure; the metal-binding histidine clusters would presumably be formed only on correct folding of the protein.[177] An example of disulfide-directed folding in a single, polypeptide chain of designed sequence is that of Felix.[211] However, both the betabellins and Felix have not proved amenable to detailed structural analysis. Kuroda et al.[212] constructed a helical hairpin (ALIN) linked via a disulfide bond, which has been characterized using 2-dimensional NMR techniques. Observation of interhelical NOEs and a well characterized hydrophobic core establish the success of disulfide linkage as a design strategy. A similar strategy has been adopted by Marti et al.[213] to construct a heterodimeric, two-stranded coiled-coil based on the sequence of the leucine zipper domain in the transcription activator GCN4. Fold stabilization has been achieved by using a combination of electrostatic interactions and disulfide tethering of helical segments.

D. Use of Metals as Templates

A less coercive approach to assembled proteins involves metal-mediated association of synthetic modules, whereby strong metal−peptide interactions would drive assembly. Such an approach necessitates the presence of a metal ligand in each interacting subunit and further dictates that such complexes be exchange-inert. Ghadiri and co-workers[214,215] utilized a 2,2′-bipyridyl group, which has a strong affinity for transition metals, and a ruthenium(II) ion, whose complexes are exchange-resistant, to selectively construct triple-helical[214] and four-helix bundles,[215] as revealed by gel filtration and mass spectrometry. Both synthetic peptide assemblies exhibit cooperativity in their denaturation by guanidium hydrochloride, which is a feature associated with compactly folded structures. It must be noted that the factors controlling aggregation number are still a property of the helix sequence, within the scope of the coordination number of the metal template. Further, the metal template only serves to facilitate subunit association and must be augmented by other stabilizing interchain interactions.

More recently, protein amino acids which can function as metal ligands have been incorporated into the monomeric units of putative helical bundle proteins. Triple-stranded helical assemblies have been constructed using both histidine[216] and cysteine[217] as metal ligands. Suzuki et al.[216] showed the induction of helical structure in unstructured peptides containing His on complexation with Ni^{2+}. The formation of metal complexes and consequent structure induction was pH dependent. All six His residues equally bind Ni^{2+} ions, as deduced from metal-ion titrations monitored by NMR spectroscopy. Li et al.[217] show cadmium binding to Cys residues in a triple-helical bundle by both ^{113}Cd NMR and ^{1}H−^{113}Cd heteronuclear multiple quantum correlation spectroscopy.

IV. Unnatural Peptide Backbones

No discussion of synthetically designed, folded peptides will be complete without a reference to the many engineering attempts that are currently underway to design oligomeric and polymeric molecules that mimic the structural properties of biopolymers. The eventual hope of these studies is that they will result in the design of new heteropolymeric structures which may reveal novel structural and functional properties not observed in nature.[218] The most dramatic progress has been made in developing β-amino acids as potentially versatile residues for constructing novel folded peptides.[219,220]

Chiral β-amino acids (Figure 13a) are now readily derived from the corresponding α-amino acids by Arndt−Eistert homologation.[221] In the β residues there are three degrees of torsional freedom N−C$^{\beta}$(ϕ), C$^{\beta}$−C$^{\alpha}$(θ), C$^{\alpha}$−CO (ψ) as compared to the two degrees noted in α-amino acids. Although the presence of an additional torsional variable might be expected to considerably enhance the structural space of these residues, the fact that θ is limited to values of ±60°-(gauche) and 180° (trans) simplifies consideration of possible conformation. In cyclic β-amino acid structures such as *trans*-2-aminocyclohexanecarboxylic acid and *trans*-2-aminocyclopentanecarboxylic acid, the θ values are constrained to gauche conformations. Extensive work in the laboratories of Seebach and Gellman focused on the structural properties of oligopeptide sequences derived from chiral acyclic and cyclic β-amino acids. These groups characterized new folding patterns in which the direction of intramolecular hydrogen bonding is reversed, resulting in the formation of novel helical structures.[222−224] The facility with which β-peptide oligomers adopt highly ordered structures has been mildly surprising, as the

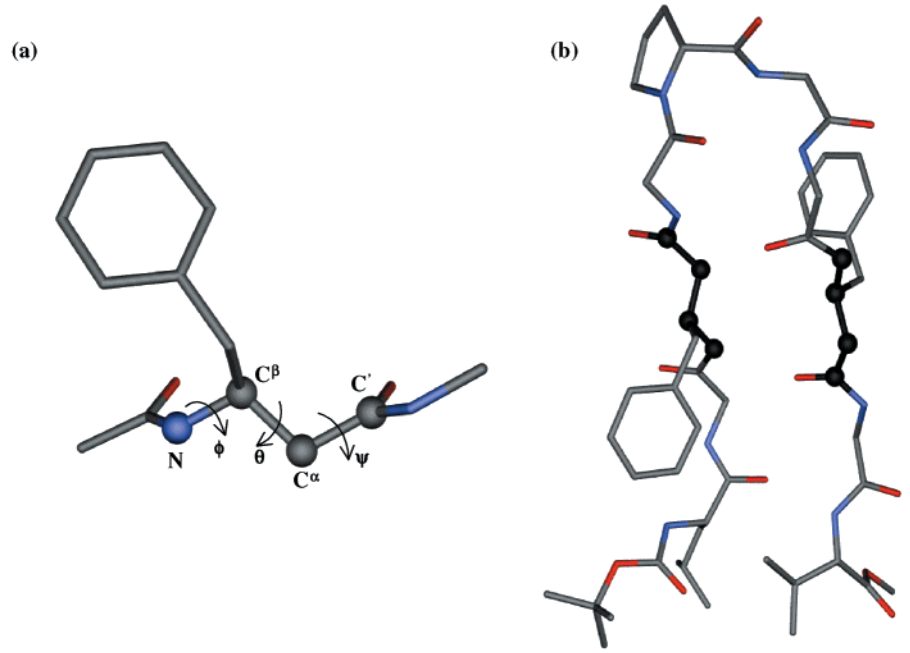

Figure 13. (a) β-Amino acid residue illustrating the degrees of torsional freedom. (b) Molecular structure of a 10-residue β-hairpin peptide containing both α- and β-amino acids. The β-amino acid residue (β-Phe) in the strands is highlighted.[233]

additional torsional variable in the β-residue might have been expected to result in a tendency to form less ordered structures than their α-amino acid counterparts.[225] The extensive recent literature on β-peptides has been the subject of reviews.[220,225,226]

Considerable recent effort has also been directed toward understanding the folding properties of constrained oligomers of the higher ω-amino acids.[227−229] β-Amino acid residues may be introduced into regular structures formed by α-amino acid sequences without significant disruption of the folding patterns[230] (Figure 14a). It should be noted with each α-residue, three atoms contribute to the polypeptide backbone, while each β-residue contributes 4 atoms. In principle, therefore, a tetrapeptide segment of α-amino acids can be grossly mimicked by a tri-β-amino acid sequence. Such segments may be referred to as homomorphic sequences, a concept that can be extended to the higher ω-amino acids and to sequences which contain different types of ω-amino acids.[230] Thus, a β-Ala-γ-Abu (β-alanyl-γ-aminobutyric acid) sequence (nine backbone atoms) will be homomorphic with a $(Gly)_3$ segment (Figure 14b).

As a consequence, it is possible to consider the construction of hybrid sequences which contain both α- and ω-amino acids which adopt well-defined folded structures. The insertion of a $-NH-(CH_2)_2-CO-NH-(CH_2)_3-CO-$ segment (note that the term β-Ala has been widely used in the literature[231] for a residue which should be really called β-Gly) into oligopeptide helices has been shown by crystallography to cause almost no disruption of helical folding (Figure 14a).[230a] In the 8- and 11-residue peptide helices, the additional methylene groups are comfortably accommodated without significant distortion of the overall helical structure. ω-Amino acids can be inserted in the β-turn nucleus of β-hairpins as demonstrated in the octapeptide Boc-Leu-Val-Val-^{D}Pro-δAva-Leu-Val-Val-OMe.[232] More recently, β-residues have also been

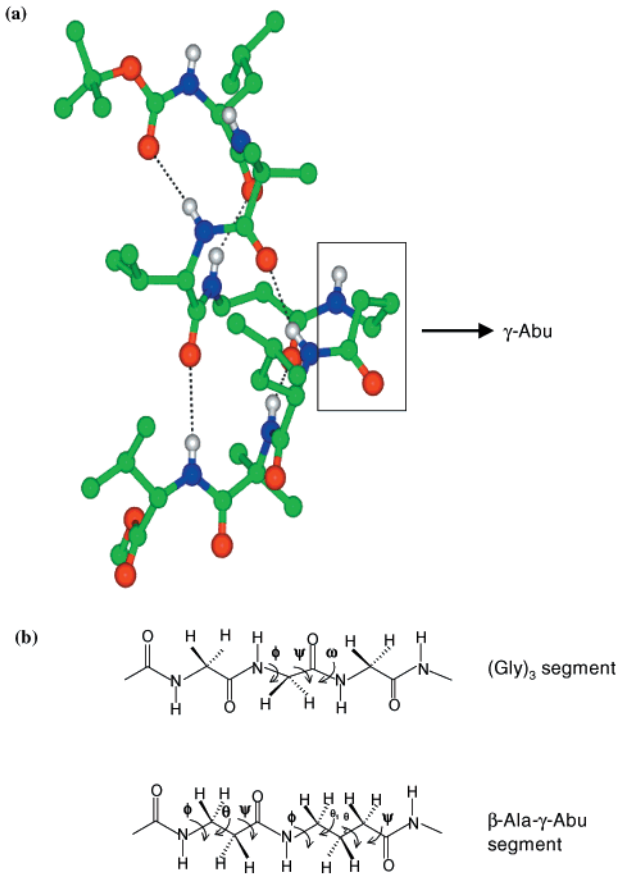

Figure 14. (a) Molecular structure of an 8-residue helical peptide[230a] containing a β-Ala-γ-Abu segment. The γ-Abu residue is boxed: β-Ala, $-NH-(CH_2)_2-CO$; γ-Abu, $-NH-(CH_2)_3-CO-$. (b) The homomorphic segments $(Gly)_3$ and β-Ala-γ-Abu.

inserted into the facing strand positions of an octapeptide[233] whose structure in crystals (Figure 13b) reveals appropriate hydrogen-bonding registry. The formation of a mixed α/β-tetrapeptide turn with a

central di-β-peptide segment flanked by two α-amino acids has also been reported.[234] The selective use of β-residues permits side chains to adopt orientations distinctly different from that possible for α-residues in regular secondary structures. For example, the facing residues on the antiparallel strands of the β-hairpin have the side chains pointing in the same direction in the case of α-amino acids and in opposite directions in the case of β-amino acids. By extension, within an extended β-strand conformation adjacent residues have their side chains facing in the same direction in the case of β-residues while they project on opposite faces in the case of α-residues.

V. Solvent Effects and Peptide Design

Any approach to peptide design must consider the competing effects of enthalpic and entropic factors in directing folding and in the stabilization of specific structural motifs. In naturally occurring proteins there is a delicate balance between these two contributions to the overall free energy of folding, the folded structures being only marginally stabilized over an ensemble of conformations.[235] While favorable enthalpic interactions such as van der Waals forces, hydrogen bonds, and a variety of weak electrostatic interactions contribute to folding,[152] the driving force for compaction appears to be a consequence of solvent forces, the hydrophobic effect promoting the burial of nonpolar side chains from an aqueous environment.[236] Polypeptide chain entropy necessarily favors unordered structures, and it is this contribution that must be overcome by compensating interactions. In the case of designed, synthetic peptides, two distinct groups can be considered.

(1) Peptides whose sequences are based on the 20 naturally occurring amino acids and in which patterning of polar and nonpolar residues is achieved on a suitable secondary structural template. In these cases, assembly into super secondary structures is driven by hydrophobic effects in aqueous solvents in a manner similar to that in native proteins. Also, the stability of the isolated secondary structures is sometimes compromised by solvation of the peptide backbone with consequent disruption of the intramolecular hydrogen bonds. Indeed, many studies have revealed only marginal stability for isolated helices[2,237] and β-sheets[133] in aqueous systems. Many studies of isolated secondary structures in synthetic peptides have employed cosolvents, particularly 2,2,2-trifluoroethanol (TFE), hexafluoro-2-propanol (HFIP), and hexafluoroacetone (HFA), to enhance secondary structure.[238] While a detailed mechanism of structure stabilization by these additives remains a matter of debate,[238] there is a general consensus that dehydration (desiccation) of the vicinity of the peptide backbone may in fact enhance intramolecular hydrogen-bonding contributions in these systems.

(2) Nonpolar sequences which contain stereochemically constrained amino acids that act as strong nucleators of local secondary structure. The large body of work that has used Aib and related dialkylated amino acids for helix stabilization[55,67−70] has demonstrated that introduction of local conformational constraints, determined primarily by nonbonded interactions, can effectively nucleate secondary structure. In these cases, propagation of helical structure is governed by the energetic advantage of cooperative hydrogen-bond formation. In most cases, the demonstration of structure formation has been achieved in organic solvents, which compete poorly for backbone hydrogen-bonding sites.[55,67−70]

Structure formation has also been repeatedly demonstrated in the crystalline state under diverse conditions of packing, suggesting that both peptide helices and β-hairpins are robust enough to avoid conformational distortions due to packing forces.[54−67,111−112] The use of backbone conformational constraints in water-soluble sequences has also revealed enhanced structure-forming ability in designed peptides.[2,109,123,133] Clearly, the synthesis of both these approaches will eventually provide a powerful strategy for the construction of complex designed folds.

VI. Breathing Life into Designed Peptides

Much of the work in the area of peptide design focuses primarily on mimicking secondary structures and tertiary folds in proteins. Designed peptides may be used as agonists and antagonists of biologically active peptides in pharmacological applications.[239,240] For example, amphipathic helices in which the polar face is predominantly basic have been shown to exhibit potent antimicrobial activity, presumably because of their ability to disrupt membrane structures.[241−243] Interesting recent examples of β-peptides with antibiotic activity have been reported,[244,245] as has been a β-peptide analogue of somatostatin.[246] Such an application of designed peptides has been reviewed elsewhere.[247] We also do not consider many applications of structurally defined peptides for creating novel antigens in approaches to vaccines and the sophisticated attempts at constructing various tubular structures which function as transbilayer channels in membranes.[248−250]

Several attempts to engineer functional sites into designed peptides have been reported, particularly aimed at binding metal ions or prosthetic groups.[251] The design of binding sites for metal ions is conceptually simple, requiring merely appropriate positioning of liganding groups in 3-dimensional space. The strength of metal-ion interaction can, in principle, suitably deform synthetically derived structures to achieve binding. However, due to lack of detailed understanding of the numerous factors affecting fold nucleation and stability, hitherto designed metal-binding sites have largely been based on known metal-binding motifs in protein structures, such as those seen in zinc fingers and the metal-binding segments of carbonic anhydrase. Hence, the $(His)_x$-$(Cys)_x$ themes have often been stressed in the introduction of metal-binding sites into peptide and protein folds. Again, the folds that have been explored in detail from the perspective of peptide design, such as helical bundles and to some extent β-sheets, serve as potential hosts for the grafting of such sites. Notably, His_3[252] and His_2Cys_2[253] Zn^{2+} binding sites have been introduced into a designed four-helix bundle α_4 and the His_3 site into minibody, a designed, six-stranded β-sheet protein.[254] Klemba et al.[255] dis-

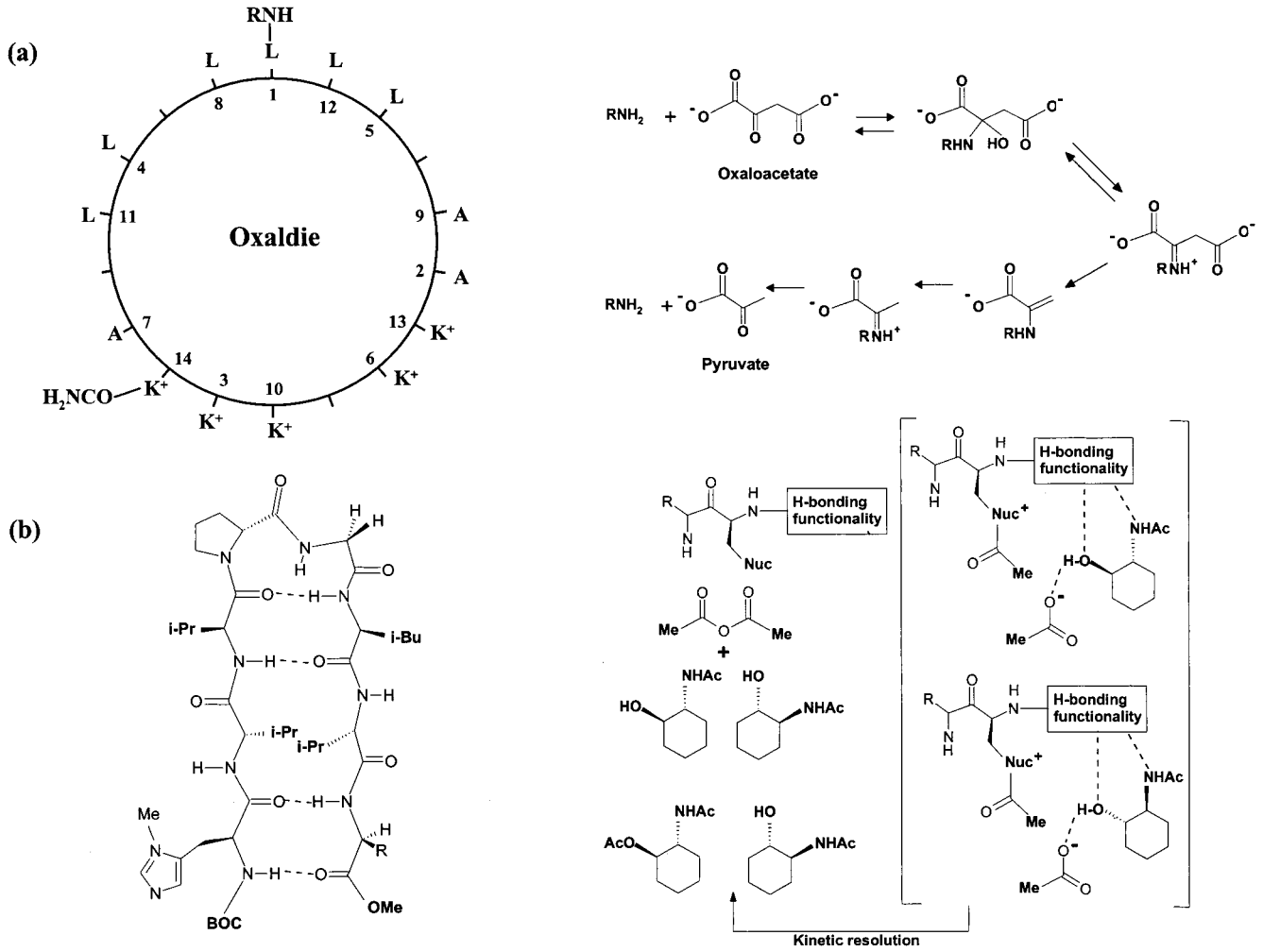

Figure 15. (a) De novo designed peptides (Oxaldie 1 and 2) with decarboxylase activity (from Johnsson et al.).[259] The sequences of the two peptides are shown on the left on a helical wheel diagram (R = H, oxaldie 1; R = CH_3CO, oxaldie 2). Also shown on the right is the reaction scheme for the decarboxylation of oxaloacetate that is catalyzed by the 'oxaldie' peptides (represented as RNH_2 in the scheme). (b) Histidine-containing β-hairpins that catalyze enatioselective acylation reactions.[269] The structure of the most efficient catalytic β-hairpin is illustrated on the left, while the reaction scheme for the acyl transfer between anhydrides and secondary alcohols is charted on the right.

cussed the construction of a $His_3Cys\ Zn^{2+}$ binding site in the B1 domain of Streptococcal protein G. More recently, dinuclear metal centers have been introduced into four-helix bundle proteins by Lombardi et al.[256] and characterized by X-ray crystallography. The strong and specific affinity of soft metal ions such as mercury and cadmium for sulfur has been exploited in the design of triple-helical bundles that bind these metals in a novel trigonal geometry.[217] The engineering of metal-binding sites in proteins has been reviewed by Lu and Valentine.[257]

The introduction of function into designed molecules which involves not only binding but also catalytic activity is a more formidable task. While many attempts have been reported in the literature, we briefly mention only representative examples of structurally defined peptides with catalytic potential. The use of designed scaffolds to position catalytic sites has also attracted attention.[1a,247,258]

'Oxaldie 1' (Figure 15a), a 14-residue amphipathic helix that catalyzes the decarboxylation of oxaloacetate when aggregated to form bundle-like structures was one of the earliest examples of a de novo designed functional peptide.[259] The rate enhancement was a consequence of Schiff base formation between the substrate and the N-terminus of the peptide, which had a depressed pK_a. The ability of histidine to participate in acid−base catalysis, with concomitant modification of catalytic activity by residues such as Arg, Gln, and Lys, has been exploited by Baltzer to construct a series of helix−loop−helix systems that dimerize into four-helix bundles and enhance the hydrolysis and transesterification of activated esters.[258,260−263] The ability of peptides to function as ligases has been studied in a series of important investigations by Ghadiri and co-workers.[264−267]

We have been involved in the design of a family of helical peptides that can serve as model systems in the study of the reaction mechanisms and intermediates characterizing the early stages of the protein glycation reaction. The reaction of glucose with the ϵ-amino groups of lysine residues in proteins, with the concomitant formation of a Schiff base, is a consequence of prolonged or increased exposure to glucose, relevant to situations in diabetes and aging disorders. The Schiff base undergoes rearrangement to form a ketoamine product (the Amadori rearrangement), which, after a series of oxidative rear-

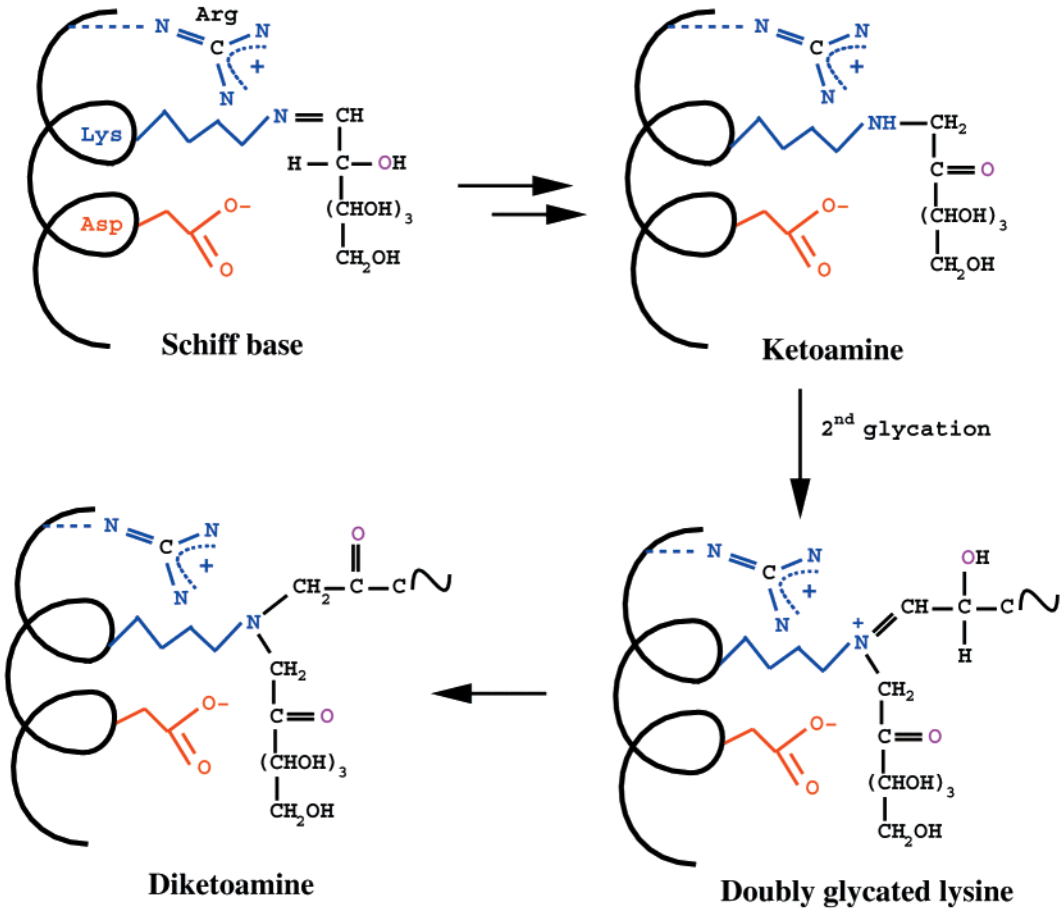

Figure 16. Helical peptide models for investigating catalysis of the Amadori rearrangement. Peptides contain a central lysine (residue 6) and an Asp residue at position 10, which lie on the same and opposite faces of a regular helix. The mechanism of the Amadori rearrangement on Lys6 is shown as catalyzed by Asp10. Asp10 was replaced by a histidine residue in an analogous peptide. The effect of a proximal arginine residue (present in yet another member of the family under study) on the reaction occurring on Lys6 is also highlighted.[268]

rangements, results in the formation of extensive protein cross-links and other advanced glycation endproducts (AGEs), which have been implicated in various complications associated with diabetes and aging. The committing step in protein glycation is the formation of the ketoamine product from reversibly formed Schiff base. We have utilized helical peptides (Figure 16) bearing a lysine residue, the site of glycation, and other potentially catalytic residues implicated in the rearrangement reaction (such as aspartic acid and histidine) to assay their relative roles in Schiff base formation and the Amadori rearrangement. The reaction site lysine and the catalysts are brought into spatial juxtaposition by means of the helix periodicity, which dictates that residues at i and i+4 positions are close together but residues at i and i+2 positions are far apart. The results demonstrate that catalysis of the Amadori rearrangement by a proximal Asp residue may be important in determining the rate of irreversible glycation.[268]

In another interesting example of designing function into rigid peptide templates, Jarvo et al.[269] utilized octa- and tetrapeptide segments as structural modules bearing modified histidine residues (*N*-alkylimidazoles: (*S*)-β-imidazoylalanine and π(Me)-histidine) as nucleophilic catalysts, which catalyze the enantioselective acylation of secondary alcohols (Figure 15b). The property of tight turns to bring into proximity the ends of synthetic peptides containing them has been exploited to graft the catalytic residues onto one end and a hydrogen-bonding functionality onto the other. The authors investigated the effect of a variety of turn sequences and report a correlation between sequences with better turn-forming potential and enhanced substrate selectivity. Interestingly, a covalently rigidified hairpin peptide (a cyclic peptide) showed less enantioselectivity as compared to its acyclic analogue, suggesting that analogous to the active site of protein enzymes a degree of flexibility is required for optimum substrate recognition and selectivity.

In a more recent trend, the application of peptides in the construction of biomaterials for use in a variety of fields has been explored.[247] Peptide motifs have found application in tissue engineering,[270,271] in the formation of synthetic polymers that promote cell−cell interactions and tissue growth. Peptides, especially β-sheets, have been found to form 'tapes', which have interesting mechanical properties.[272] An intriguing recent development is the use of amphiphilic β-strand peptides containing alternating $(\text{Phe-Glu})_n$ sequences and flanking Pro residues to form well-characterized, self-assembled sheetlike monolayer structures at air−water interfaces.[273] Helical, Aib-containing peptides which assemble at air−water

interfaces and form vesicular structures have also been investigated.[274] Ordered peptides appear to hold considerable promise in the design of nanostructured materials.

VII. Conclusions and Perspectives

The design of folded polypeptide structures from 'first principles' provides a stern test of our understanding of the principles of polypeptide chain folding. Synthetic approaches to protein design have largely followed a modular approach in which elements of preformed secondary structures, like helices and hairpins, are first constructed followed by subsequent assembly into compact structures. Two distinct approaches to the design of secondary structures have proved fruitful. One approach has relied on exploiting the secondary structure propensities of amino acids derived from analysis of protein crystal structures. The other has followed the route of controlling the stereochemistry of chain folding by introducing backbone constraints in the form of nonprotein amino acids or synthetic nucleating templates. The assembly of supersecondary structures has largely used solvent forces as the major determinant in facilitating formation of compact structures. Attempts to engineer control over the stereochemistry of linking segments have not yet proved generally applicable. The construction of multistranded β-sheets has progressed rapidly in the past few years, largely as a result of the recognition that turn nucleation of the appropriate stereochemistry is a major determinant of hairpin formation. Significant progress has been made in the construction of helical bundles, and the rational design of β-sandwiches and β-barrels is imminent. The expansion of the amino acid repertoire by the induction of β-amino acids and higher homologues promises to rapidly expand the diversity of conformationally well-defined peptide structures. Synthetic approaches to protein design have thus far advanced at a moderate pace because of the difficulties of controlling all the complex interactions that collectively determine the detailed structures of globular proteins. Nevertheless, the field may be poised for a change from an activity which may conservatively be termed as 'molecular carpentry' to one which may be more legitimately and optimistically be called 'molecular engineering'. The ability to design a wide variety of polypeptide scaffolds may prove important not only in mimicking natural proteins, but also in the designing of new biomaterials. Some years ago, in reviewing the area of de novo design, *Chemical & Engineering News* carried the following comment[275] "I feel that we won't know how to design proteins from first principles, or even second principles, in my scientific lifetime. I'm not a pessimist; I am respectful of protein complexity". There is probably reason to be slightly more optimistic.

VIII. Acknowledgments

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