Non-standard amino acids in peptide design and protein engineering

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The introduction of non-coded amino acids with well defined stereochemical and functional properties will greatly enhance the scope of protein design and engineering. The present state of methodologies for incorporation of non-coded residues into proteins is examined. The prospects for conformationally constrained amino acid residues are evaluated in the light of peptide structural studies. Templates for secondary-structure nucleation and recent experiences in the incorporation of novel residues into proteins are considered.

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Introduction

Protein engineering using the standard 20 genetically coded amino acids is necessarily restricted by the limited repertoire of residues. Although remarkable structural and functional diversity can be generated by variations in polypeptide primary sequences, the incorporation of non-standard amino acids with well defined stereochemical and functional properties would greatly enhance the scope of protein engineering. The rich literature on the use of an enormously diverse range of unusual residues in designing analogs of biologically active peptides is testimony to the potential usefulness of incorporating side-chain and backbone modifications as a means of modulating the stability and activity of peptide sequences [1,2].

In the area of protein engineering, two immediate goals appear desirable. First, unusual amino acids with defined conformational properties could be used to impose local restrictions on polypeptide chain stereochemistry, thereby conferring stability on specific regions of secondary structure. In *de novo* design approaches, novel residues and templates may be employed to nucleate and stabilize specific secondary structures as a prelude to controlled tertiary organization [3]. Second, residues with versatile chemical functions in their side chains can be used to impart novel binding and catalytic properties. Two requirements critical to the further development of protein engineering are: the development of techniques to incorporate unusual residues into proteins by genetic methods [4,5••], total chemical synthesis [6•,7••] or by semisynthesis using enzyme mediated ligation [8•,9]; and the development of a palette of 'designer residues' which will permit definitive structural control in the process of assembling sequences with predictable folding properties.

Methodologies for unusual residue incorporation

Total chemical synthesis using highly developed solid-phase approaches is undoubtedly the most direct way of introducing novel residues into proteins. However, this method is restricted to relatively short polypeptide chains of ~100 residues or less in length. The present level of technology is exemplified by the recent synthesis of the 99-residue sequence of human immunodeficiency virus (HIV)-1 protease, which incorporates all amino acids of the unnatural D configuration [6•]. A particularly dramatic example of the power of chemical approaches is the introduction of a thioester bond into HIV-1 protease by chemical ligation of the 1–51 fragment (i.e. residues 1–51), which incorporates a carboxy-terminal thiol nucleophile, with the 52–99 fragment, which features an alkyl bromide at its amino terminus [7••]. Solid-phase methodology is, however, less attractive because of low coupling yields in the synthesis of segments containing stereochemically hindered residues, such as α-aminoisobutyric acid. In such situations, enzymatic methods hold greater promise, as illustrated by recent applications to short-peptide antibiotics of the alamethicin family [8•]. Site-specific incorporation of non-coded residues may also be achieved by semi-synthesis, where the chemical synthesis of a short segment is followed by protease-mediated ligation to a longer polypeptide in organic-aqueous solvent mixtures [8•]. This approach is most suitable for modifications at amino- or carboxy-terminal segments. The reg-

Abbreviations

Ac_nc—1-amino-cycloalkane-1-carboxylic acid; **Aib**—α-aminoisobutyric acid; **HIV** human immunodeficiency virus; **Lac**—lactic acid; **Pip**—pipecolic acid.

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ular and routine application of non-standard residues in protein engineering will, however, become a reality only when genetic methods of incorporation become readily accessible. In early work, non-coded amino acids which are largely isosteric with one of the coded residues have been incorporated into proteins following the misacylation of tRNA by the appropriate aminoacyl-tRNA synthetase [10].

The substrate specificity of the tRNA synthetases is strict, however, and only structurally very similar residues can be introduced in this way. Site-specific incorporation is also generally not possible, with the exception of cases where the replaced residue occurs at a lone position in the sequence. For example, L-2-aminohexanoic acid (or norleucine) has been substituted for methionine at position 21 in human epidermal growth factor [11]. Another intriguing report has suggested that the novel residue furanomycin **(1)** [Fig. 1], which is structurally quite distinct from isoleucine, is charged onto $tRNA^{Ile}$ by the isoleucyl-tRNA synthetase and, indeed, incorporated into a β-lactamase precursor in an *in vitro* biosynthetic experiment [12].

Recent approaches which appear to point towards generally applicable procedures involve the use of semi-synthetic aminoacylated suppressor tRNAs [13••,14•,15•] and the incorporation of an appropriate translatable amber codon at the site chosen for mutagenesis [4,5••,14•]. Interestingly, a biochemical precedent for such a strategy does exist. The biosynthetic incorporation of selenocysteine into *E. coli* formate dehydrogenase is directed by a UGA codon and utilizes selenocysteinyl-tRNA and a unique translation factor [16]. Efficient procedures for the chemical acylation of suppressor tRNAs by any amino acid analog are a prime requirement for the success of this method.

Elaborate protection and deprotection strategies and low yields are major limitations in the use of tRNAs acylated with non-coded residues. Procedures developed recently for chemical aminoacylation of tRNAs using a photolabile protecting group (nitroveratryl) have allowed preparation of the aminoacyl tRNAs in high yields [13••]. Chemical strategies may, however, be intrinsically limited in scope because of synthetic difficulties. The high specificity of aminoacyl-tRNA synthetases precludes their use at present, although an engineered, non-discriminating tRNA synthetase presents an attractive possibility for the future.

Chemoenzymatic procedures that permit acylation of the terminal 2',3'-diol of the suppressor tRNA with an amino acid ester using a non-selective lipase constitute another potential strategy [17]. Initial reports have suggested that the suppressor tRNA route can be used to incorporate a wide variety of amino acids into T4 lysozyme (see below) [5••].

Conformationally constrained residues

Stereochemically constrained non-coded residues may have great potential in protein engineering studies once

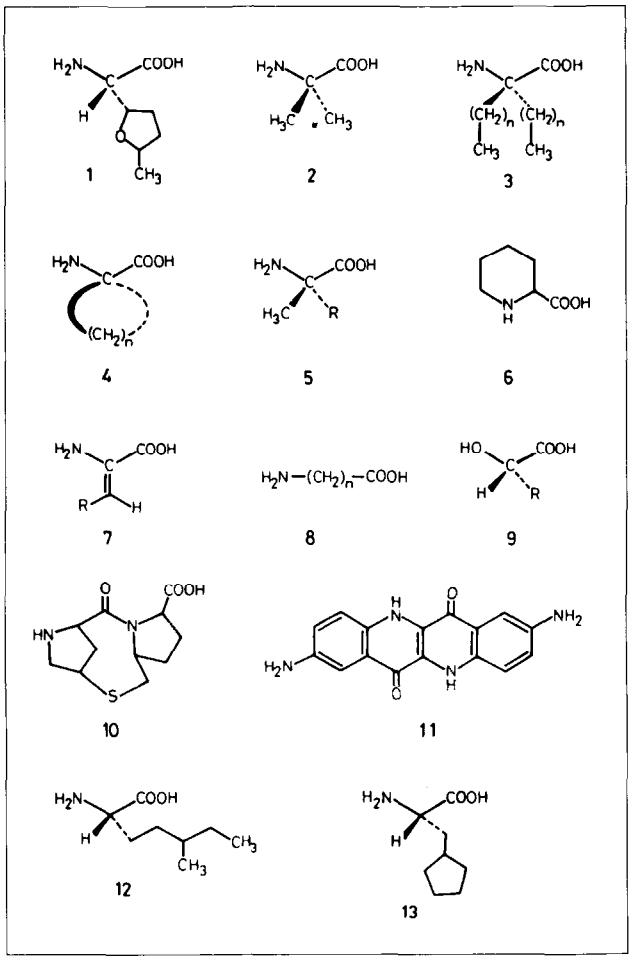

Fig. 1. Structures of some representative non-coded amino acids and templates discussed in the text. **(1)** Furanomycin; **(2)** α-aminoisobutyric acid; **(3)** α,α-di-n-alkylglycines (n = 1, diethylglycine; n = 2, dipropylglycine; **(4)** 1-aminocycloalkane-1-carboxylic acids; **(5)** α-methyl-α-amino acid (R groups can be identical to those found in the coded amino acids); **(6)** pipecolic acid; **(7)** α,β-dehydroamino acids (R = H, dehydroalanine; R = phenyl, Z-α,β-dehydrophenylalanine); **(8)** ω-amino acids (n = 2, β-alanine; n = 3, γ-aminobutyric acid; n = 5 ε-aminocaproic acid); **(9)** α-hydroxy acid; **(10)** helical template; **(11)** template for β-sheets; **(12)** 2-amino-4-methylhexanoic acid; **(13)** amino-3-cyclopentylpropanoic acid.

the problem of incorporation has been overcome. The α,α-dialkylated glycines constitute the most widely studied class of amino acids that introduce backbone rigidity. α-Aminoisobutyric acid **(2)** [Aib; also known as 2-methylalanine] (Fig. 1) is the most extensively investigated residue of this group because of its widespread occurrence in voltage-gated membrane-channel-forming polypeptides of fungal origin [18,19]. The presence of an additional methyl group at the C^{α} atom in Aib greatly restricts the sterically allowed region of conformational space because of van der Waals clashes. Energetically favourable minima are limited to helical regions ($\phi \approx \pm 60 \pm 20°$ and $\psi \approx \pm 30 \pm 20°$) [20]. Experimentally, the Aib residue has been found to be strongly helicogenic and to stabilize 3_{10}- and α-helical conformations in a very large number of synthetic peptides, a feature which has been established conclusively by crystallographic studies (Fig. 2) [21,22•,23].

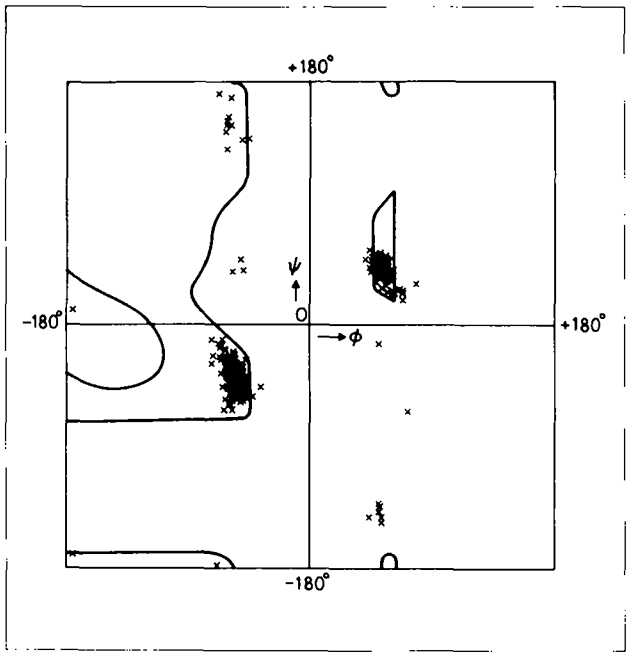

Fig. 2. Crystallographically observed ϕ,ψ values for α-aminoisobutyric acid (Aib) residues in peptide structures. 305 Aib residues from 108 independent crystal structures are represented [24]. In the case of achiral peptides crystallizing in centrosymmetric space groups, the choice of the sign of the dihedral angles is arbitrary. The sterically allowed regions of the Ramachandran map for an L-alanine residue are marked (these are also valid for all coded residues except glycine and proline). Note the extremely intense clustering of residues in right- and left-handed helical ($3_{10}/\alpha$) conformations. Residues lying in non-helical regions are almost always observed at the carboxyl terminus of very short peptides or in cyclic systems.

The use of this residue in a *de novo* approach to the design of helical super-secondary structural motifs has been explored [24,25•]. In this 'Meccano (Lego) Set' approach, prefabricated conformationally rigid helical fragments containing strategically positioned Aib residues are covalently connected by flexible linking segments. The crystallographic characterization of a linked helix structure, employing ε-aminocaproic acid as a connecting element between two helical heptapeptide modules, each of which is stabilized by a single centrally placed Aib residue, is a good illustration of this strategy [26•]. The achiral Aib residue can adopt both right- (α_R) and left-handed (α_L) helical conformations, depending on the context of the sequence. In short oligopeptides, Aib residues at the penultimate position (position n−1 in an n-residue sequence) adopt α_L conformations which result in the formation of a π-turn involving a 6→1 hydrogen bond, leading to helix termination (IL Karle *et al.*, unpublished data). This feature is reminiscent of helix-termination signals in proteins, which generally involve glycine or, less often, asparagine residues in α_L conformations [27]. A pronounced feature of the large number of studies on model Aib peptides is the high degree of local stereochemical rigidity that accompanies the incorporation of this residue into peptide chains. As a consequence, relatively long oligopeptides (up to 16–20 residues in length) crystallize readily and there is generally excellent agreement between the solid-state and solution conformations. In contrast, oligopeptides comprising the 20 coded amino acids only are highly conformationally flexible. Most biologically active peptides (such as bradykinin, angiotensin, endorphins and adrenocorticotropic hormone) have resisted efforts to crystallize them over many years. Definitive conformational information can be deduced only in specific cases where complexes with macromolecules are crystallizable, as illustrated by a recent structure of an angiotensin complex with an anti-idiotypic antibody [28].

The single reported attempt at introducing Aib into proteins was the replacement of Ala82 in T4 lysozyme by Schultz and colleagues [5••]. In the wild-type enzyme, Ala82 has ϕ,ψ values of $-67°$ and $-24°$. The Ala82Aib mutant was shown to be marginally more stable ($\sim 1\,°C$) in thermal denaturation experiments. Although the results are undramatic, this study clearly emphasizes the feasibility of introducing unusual residues into proteins (the terms 'unusual' or 'non-coded' are preferable to the term 'unnatural'). In retrospect, it appears that the choice of Ala82, which is a surface residue and not part of a regular helix, may have contributed to the small observed stabilization. Residues in surface loops may be less important in determining the temperature at which thermal unfolding commences. In general, the introduction of Aib at sites on protein helices might stabilize local secondary structure, with a consequent effect on overall melting temperature. It is also conceivable that refolding processes might be facilitated by the more efficient nucleation of secondary structures.

Other residues that might impose conformational restraints and which have been well characterized in peptides are shown in Fig. 1. The achiral Aib homologs, α,α-dialkylated glycines **(3)**, have been shown to favour fully extended ($\phi \approx \psi \approx 180°$) conformations in homo-oligopeptides, for the cases of diethyl and dipropylglycines [29•]. Nevertheless, this stereochemical preference is not absolute. Energy minima have been defined theoretically in the region of helical conformations ($\phi \approx \pm 50°$, $\psi \approx \pm 50°$). A recent crystal structure of a fully protected homo-tripeptide of α, α-di-n-propylglycine did indeed reveal a type III β-turn conformation [30].

The 1-amino-cycloalkane-1-carboxylic acids **(4)** [Ac_nc, where n is the number of carbon atoms in the cycloalkane ring] are limited almost exclusively to helical conformations in peptides [31]. Ac_nc residues with n = 3–6 have been incorporated at position 82 of T4 lysozyme, but no details of mutant enzyme stabilities were reported [5••]. The high yield preparation of chiral α-methyl-α-amino acids **(5)** by chemoenzymatic procedures [32] promises to make available conformationally restricted analogs of many of the coded amino acids. This, in turn, should provide great scope for the imposition of backbone conformational restraints without major side chain alterations. The introduction of such residues would only involve replacement of the C^α hydrogen with a methyl group. Such mutations should, in general, stabilize helical conformations at the site of replacement.

Pipecolic acid **(6)** [Pip], a proline homolog, has also been shown to be a useful local conformational determi

nant in small peptides. This residue is conformationally distinct from proline because the carboxylic acid occupies an axial position in the cyclohexane ring. Whereas L-proline residues prefer conformations in the helical ($\phi \approx -60°$, $\psi \approx -30°$) and semi-extended, polyproline-like ($\phi \approx -60°$, $\psi \approx 120°$) regions of ϕ,ψ space, theoretical studies have indicated that low positive values of ψ may be preferred for the Pip residue [33]. Indeed, the incorporation of a Pip residue at position 82 of T4 lysozyme resulted in a lowering of the melting temperature by ~2°C, whereas the Ala82Pro mutant is more stable by 2°C [5••]. Although Schultz and co workers attributed this to a larger ϕ angle in Pip, peptide crystal structures [34] suggest that ϕ_{Pip} is indeed close to −60°, a value similar to that for Ala82 in the wild-type enzyme. The observed destabilization may indeed be a consequence of changes in the ψ value at the Pip residue.

α,β-Dehydro residues **(7)** also offer a means of restricting backbone conformations. Dehydroalanine residues have indeed been found in enzymes, albeit very infrequently, and are formed by the post translational dehydration of serine residues [35]. Recent studies of model peptides suggest that β-turns are often stabilized in short sequences, whereas helix formation may be promoted in longer peptides. However, the available experimental data are limited, with most of the studies that have been reported featuring relatively short peptides containing Z-dehydrophenylalanine [36], whereas very few featured investigations of dehydroalanine [37]. Interestingly, the incorporation of dehydroalanine into proteins may be possible by chemical modification of charged $tRNA^{Ser}$ or $tRNA^{Cys}$.

Flexible residues and conformational variability

ω-Amino acids [glycine homologs] **(8)**, which incorporate additional methylene groups between the amino and carboxyl groups (e.g. β-alanine, γ-aminobutyric acid and ε-aminocaproic acid), might be expected to enhance conformational flexibility. Relatively little information is available on their conformational influences in peptides [38]. It is noteworthy that an attempt to introduce β-alanine at position 82 of T4 lysozyme by the suppressor tRNA route was not particularly successful, with less than 5% suppression being observed [5••]. α-Hydroxy acids **(9)**, e.g. lactic acid (Lac) and phenyllactic acid, have been incorporated at position 82 in T4 lysozyme [5••]. The Ala82Lac mutant is thermally destabilized by 3.7°C, a rather surprising result as the Ala82 NH group is only hydrogen bonded to water in the wild-type enzyme. Hydroxy acid residues may be expected to enhance local conformational freedom because they lack the NH group for intramolecular hydrogen bonding. Further, differences in the barriers to rotation about the ester and amide groups may also contribute. The incorporation of α-hydroxyisobutyric acid into proline-containing peptides has been shown to lead to structures analogous to the α-aminoisobutyryl peptide [39]. In this case, however, the hydroxyl residue was at a position uninvolved in intramolecular hydrogen bonding. The introduction of ester bonds into proteins may eventually provide an excellent chemical handle for controlled site-specific cleavage, a feature which may be useful in the processing of expressed fusion proteins to yield desired products.

D-Residues provide an attractive means of stabilizing conformations with positive ϕ values, which are generally adopted in proteins by glycine and, to a lesser extent, by asparagine [27]. Such conformations (α_L) occur in β-turns and are critical in determining folding patterns. Although D-residues are used extensively in the design of analogs of biologically active peptide, particularly with a view to enhancing proteolytic stability, few systematic investigations of their influence on regular secondary structures have been reported. A study of a model 29-residue peptide with a centrally placed D-alanine residue revealed a helix destabilization of 0.95 kcal mol^{-1}, compared with the parent L-alanine peptide [40]. Although two recent reports have described the chemical synthesis of all D analogs of HIV 1 protease [6•] and rubredoxin [41•], the stereochemical consequences of the site-specific introduction of D-residues into proteins have not been explored. Attempts to incorporate D-alanine into β-lactamase [4], T4 lysozyme [5••] and model 16-residue sequences [14•] by the suppressor tRNA route proved unsuccessful. This failure may be a consequence of discrimination at the level of the formation of the complex between aminoacyl tRNA and elongation factor EF-Tu [5••], illustrating the nature of the difficulties that may be encountered in the biosynthetic introduction of non-coded residues.

Templates

A survey of non-standard residues and protein structure would be incomplete without mentioning at least some of the many ingenious templates that have been used to nucleate specific secondary structures in oligopeptides and to assemble super-secondary structural motifs in *de novo* protein-design approaches. Template **10**, which is formally related to a Pro-Pro dipeptide, has two bonds fixed in pyrrolidine rings at ϕ values necessary for helix formation while a $-CH_2-S-$ bridge contains a third rotatable bond. This template nucleates α-helical conformations in oligo-L-alanine sequences [42•,43•]. In another study, diacylaminoepindoleidiones **(11)** have been shown to act as templates for β-sheet formation [44]. In these cases, chimeric molecules were constructed by attaching linear peptide sequences to non-peptide functions bearing appropriately oriented hydrogen-bond donors and acceptors. The studies reported, however, are confined to relatively short sequences.

A fruitful use of templates is the orientation of helical peptides to generate helix bundles mimicking those found in proteins. The covalent attachment of multiple helices to side-chain functions on a rigid cyclic peptide base has been examined in an attempt to prepare template-assembled synthetic proteins [45•]. In addition,

metal-ion-promoted oligomerization of helical peptides bearing a terminal pyridyl or bipyridyl metal-coordinating site has been used to demonstrate self assembly of designed three- and four-helix bundles [46•–48•]. The insertion of templates into native protein sequences appears to be technically forbidding at present, yet templates may be central to *de novo* design strategies using total chemical synthesis.

Prospects for proteins incorporating non-coded residues

Data on the purposeful introduction of non coded residues into proteins in order to address issues of structure and stability are extremely limited. In the initial study of Schultz's group [4], Phe66 in a β lactamase could be replaced by closely related analog residues such as *p*-nitrophenylalanine, *p*-fluorophenylalanine and homophenylalanine, which differ from phenylalanine in electronic properties and to some extent in steric bulk. Interestingly, the *p*-nitro and homo-phenylalanine mutants appeared to be less stable and were degraded proteolytically during purification [4]. Attempts to modify the backbone by the introduction of an ester linkage, an additional methylene group or a D-phenylalanine residue were unsuccessful [4]. A later study of T4 lysozyme demonstrated the successful introduction of an ester linkage (hydroxyacid) but very little or no incorporation of β alanine and D-residues was observed [5••]. This study also established the possibility of incorporating α,α-dialkylated residues and Pip at position 82 of T4 lysozyme [49••], as discussed above.

A recent report has explored the use of non-coded residues to replace Phe133, a buried residue, in T4 lysozyme [49••]. Norvaline, ethylglycine, O-methylserine, S,S-2-amino-4-methyl hexanoic acid **(12)** and S-2-amino-3-cyclopentyl propanoic acid **(13)** were used to examine the cost of stepwise removal of methyl groups, side-chain solvation, the effect of packing density and side-chain conformational entropy on protein stability. These experiments were supported by modelling and molecular dynamics simulations and yielded two interesting 'non-coded' cavity-filling mutants which are stabilized by 1.9 °C and 4.3 °C compared with the wild-type enzyme, which has a melting temperature of 43.5 °C under identical conditions [49••]. Clearly, the availability of a structurally diverse set of amino acids can enhance the scope of protein engineering for stabilization. In particular, amino acids that introduce conformational constraints may be usefully employed in stabilizing specific secondary structural features. The introduction of such residues should also reduce conformational entropy of the unfolded state, so indirectly stabilizing the folded form. At present, Aib and related residues offer a means of reinforcing helical and β-turn backbones. Obligatory β-sheet or extended structure stabilizers are, as yet, less well characterized. Achiral or D-residues may also help in promoting α_L conformations, which are generally attained in proteins only by glycine or asparagine residues.

Rational site-directed mutagenesis, however, requires a clear understanding of the structural role of the residue being replaced and an equally clear definition of the conformational properties of the non-coded replacement. The identification of potential weak sites (Achilles' heels) in protein structures is a pre-requisite for rationally engineering stability. The synthetic development of novel amino acids with a strong preference for well defined regions of backbone (ϕ,ψ) conformational space would increase the tools available to the protein engineer. Novel residues that permit covalent disulfide crosslinks over longer distances than cystine bridges have also been developed [50•]. Although enhancing the repertoire of available residues is a desirable goal, the subtle stereochemical requirements of ribosomal synthesis may lead to unexpected problems in incorporation. For relatively small proteins (< 100 residues), total chemical synthesis may provide an alternative approach, although solid-phase methodology may falter with increasing stereochemical constraints [8•].

Although this review has focussed largely on the effect of non-coded residues on backbone conformation, modification of functional side chains may provide interesting insights into enzyme mechanisms. At present, site-specific changes are restricted to special situations, as illustrated in a recent study of conversion of an active-site arginine in aspartate aminotransferase into homoarginine [51•]. Novel residues and templates are particularly attractive elements in *de novo* design, as they offer a definitive means of controlling the fold of the polypeptide backbone.

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The preparation of all-D rubredoxin was motivated by a desire to grow centrosymmetric crystals of a racemic protein. However, the successful synthesis suggests that site-specific incorporation of D-residues by chemical methods should pose no problems in small proteins.

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See [43•].

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These two papers [42•,43•] describe a novel synthetic template for nucleating peptide helices, which is based on a modification of a Pro-Pro dipeptide.

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A cyclic carrier molecule (decapeptide) bearing four lysine residues is used as a template for the covalent attachment of four putative helical (16-residue) peptides to the lysyl ε-amino groups. Limited circular-dichroism and NMR data demonstrate the helix-promoting effect of the template but do not unambiguously establish a four-helix bundle topology.

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See [48•].

47. • LIEBERMAN M, SASAKI T: **Iron (II) Organises a Synthetic Peptide into Three-helix Bundles.** *J Am Chem Soc* 1991, 113:1470–1471.

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48. • GHADIRI MR, SOARES C, CHOI C: **A Convergent Approach to Protein Design. Metal Ion-assisted Spontaneous Self-assembly of a Polypeptide into a Triple-helix Bundle Protein.** *J Am Chem Soc* 1992, 114:825–831.

This and the two preceding papers [46•,47•] address the common theme of using a metal-coordination site at the terminal ends of helical peptides to promote self assembly into oligomeric bundles. Detailed NMR or crystallographic characterizations of these synthetic 'metalloproteins' remain to be accomplished.

49. •• MENDEL D, ELLMAN JA, CHANG Z, VEENSTRA DL, KOLLMAN P, SCHULTZ PG: **Probing Protein Stability with Unnatural Amino Acids.** *Science* 1992, 256:1798 1802.

This paper is a forerunner of things to come in the area of protein engineering with non-coded residues. T4 lysozyme is the model and position 133 is probed with a variety of replacements to examine packing, solvation and side-chain conformational entropy effects on stability. Two 'non-coded mutants' with marginally higher thermal stabilities are obtained.

50. • JACKSON DY, KING DS, CHMIELEWSKI J, SINGH S, SCHULTZ PG: **General Approach to the Synthesis of Short α-Helical Peptides.** *J Am Chem Soc* 1991, 113:9391–9392.

A higher homolog of cysteine featuring the insertion of three additional methylene groups into the side chain, which is derived by chemical synthesis from lysine, disulfide bridges the i and i + 7 residues in potentially helical peptides (where i represents residue number from the amino terminus). Disulfide crosslinking enhances peptide helicity.

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This homologation of Arg292 is based on an initial site-specific mutation to lysine followed by chemical guanidination with O-methylisourea. Although modification occurs at several lysine residues, guanidinated enzymes often possess the same activity and are stable.

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