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Foldamer Tertiary Structure through Sequence-Guided Protein Backbone Alteration

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CONSPECTUS

The prospect of recreating the complex structural hierarchy of protein folding in synthetic oligomers with backbones that are artificial in covalent structure (“foldamers”) has long fascinated chemists. Foldamers offer complex functions from biostable scaffolds and have found widespread applications in fields from biomedical to materials science. Most precedent has focused on isolated secondary structures or their assemblies. In considering the goal of complex protein-like tertiary folding patterns, a key barrier became apparent. How does one design a backbone with covalent connectivity and a sequence of side-chain functional groups that will support defined intramolecular packing of multiple artificial secondary structures? Two developments were key to overcoming this challenge. First was the recognition of the power of blending α -amino acid residues with monomers differing in backbone connectivity to create “heterogeneous-backbone” foldamers. Second was the finding that replacing some of the natural α -residues in a biological sequence with artificial-backbone variants can result in a mimic that retains both the fold and function of the native sequence and, in some cases, gains advantageous characteristics. Taken together, these precedents lead to a view of a protein as chemical entity having two orthogonal sequences: a sequence of side-chain functional groups and a separate sequence of backbone units displaying those functional groups.

In this Account, we describe our lab’s work over the last ~10 years to leverage the above concept of protein sequence duality in order to develop design principles for constructing heterogeneous-backbone foldamers that adopt complex protein-like tertiary folds. Fundamental to the approach is the utilization of a variety of artificial building blocks (e.g., D- α -residues, C α -Me- α -residues, N-Me- α -residues, β -residues, γ -residues, δ -residues, polymer segments) in concert, replacing a fraction of α -residues in a given prototype sequence. We provide an overview of the state-of-the-art in terms of design principles for choosing substitutions based on consideration of local secondary structure and retention of key side-chain functional groups. We survey high-resolution structures of backbone-modified proteins to illustrate how diverse artificial moieties are accommodated in tertiary fold contexts. We detail efforts to elucidate how backbone alteration impacts folding thermodynamics and describe how such data informs the development of improved design rules.

Graphical Abstract

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Notes

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Collectively, a growing body of results by our lab and others spanning multiple protein systems suggests there is a great deal of plasticity with respect to the backbone chemical structures upon which sequence-encoded tertiary folds can manifest. Moreover, these efforts suggest sequence-guided backbone alteration as a broadly applicable strategy for generating foldamers with complex tertiary folding patterns. We conclude by offering some perspective regarding the near future of this field, in terms of unanswered questions, technological needs, and opportunities for new areas of inquiry.

INTRODUCTION

The intricacy of protein structure belies a remarkable simplicity in covalent connectivity. From an alphabet of just 20 α -amino acids arranged in chains of varying sequence and length, countless complex folds and functions emerge. Nature accomplishes this feat by placing protein covalent structure (primary sequence) at the root of a hierarchy of higher-order structure that results from defined non-covalent intra- and inter-chain contacts. Chemists have long sought to recreate protein-like structural hierarchies in oligomers with backbones that differ from natural α -peptides, termed “foldamers”.¹ Research dating back >20 years has shown diverse artificial backbones can support ordered folds and that foldamers have a vast array of potential applications.^{2–4} Specific examples include catalysis,^{5,6} protein recognition,⁷ small molecule encapsulation,⁸ and self-assembly to form novel abiotic architectures.^{9–11}

An interesting observation in surveying work on foldamers to date is that most precedent involves isolated secondary structures or their assemblies. The paucity of protein-like tertiary folding patterns results from the challenge of designing both a backbone connectivity and a side-chain sequence that will support multiple different secondary structures and their defined packing in a single chain. Two developments were key to overcoming the above barrier. First was the recognition that, while proteins are polymers with homogeneous-backbones and variable side chains, a foldamer can have a “heterogeneous backbone” that blends monomers differing in both backbone connectivity and side-chain identity.¹² Second, when one or more α -residues in a biological sequence are replaced by an artificial backbone unit compatible with the native fold, as detailed further below, the resulting artificial oligomer can mimic the fold and function of the native sequence. Taken together, these ideas lead to a view of a protein as having two orthogonal sequences: a sequence of side chains and a sequence of backbone units displaying those side chains. In this account, we describe our lab’s work leveraging the above concept to develop design principles for constructing heterogeneous-backbone foldamers that adopt complex

protein-like tertiary folds (Figure 1). We review precedent that informed our efforts, survey key results obtained to date, and look to the future of this area of research.

OUR JOURNEY TO TERTIARY STRUCTURE MIMICRY BY ARTIFICIAL BACKBONES

Sequence-Guided Backbone Alteration in Secondary Structures

If protein folding is a language, then secondary structure is the vocabulary of that language. A robust strategy toward tertiary structure mimicry requires artificial versions of individual secondary structures that are compatible with each other (i.e., an artificial helix that can pack against an artificial sheet). At the outset of our work toward tertiary structure mimicry by heterogeneous backbones in 2009, we considered the state of the field with respect to known strategies for recreating common secondary structural elements in artificial backbones derived from natural sequences. Below, we discuss some specific precedents that informed and inspired our approach toward artificial-backbone tertiary structure.

In broad terms, there are three main issues to consider in evaluating a potential residue for use in the modification of a natural peptide or protein backbone: (1) hydrogen bonding – Does the new residue position donors/acceptors in a native-like way? If not, is the altered backbone still compatible with the prototype fold?; (2) conformational propensities – Is the new residue predisposed to a particular fold? Does it differ in rigidity from the native backbone?; (3) side-chain positioning – How does the modified residue position its side chain? If lacking a side chain, is it introduced at a site where the lost side chain is crucial to fold/function? We detail these issues further below in discussion of various specific α -residue replacements (Figure 2).

α -Helices are common mediators of protein-protein interactions, making helix mimicry a subject of intense study.¹³ Backbone engineering of natural helical sequences offers one route to protease-resistant analogues with native-like folds and functions.¹⁴ Among the most widely used building blocks to this end are C_{α} -methylated α -residues such as Aib, acyclic β -residues (e.g., β^3 , β^2), and cyclic β -residues such as ACPC. Incorporation of these monomers at one site per turn of an α -peptide helix leads to heterogeneous backbones that can fold to form quaternary assemblies, including homomeric helix bundles^{15,16} and heteromeric complexes with protein receptors.^{17,18} Encouraged by precedent on helix modification in larger proteins,¹⁹ we reasoned that such units would be viable in a variety of tertiary folds.

Motivated by the utility of β -hairpin model systems for studying β -sheet folding, early work identified monomers, such as D-Pro and Aib, that induce a stable turn in short peptides.^{20,21} These modifications are particularly effective when paired with Gly at the $i+1$ position. Later studies showed the non-canonical amino acid Orn can promote a tight turn when the polypeptide chain is propagated from the side-chain.²² Of note, some of the above modifications as well as other more exotic moieties had been examined in protein tertiary fold contexts.^{23–26}

Among backbone alterations to β -strands examined at the outset of our work, most disrupted hydrogen bonding. One example is the introduction of a methyl group on a backbone amide nitrogen, which was used to engineer a variant of the protein interleukin 8 that retained a native-like fold but was unable to associate via the β -sheet interface found in the native dimer.²⁷ Another established strategy for sheet backbone modification was the replacement of extended peptide segments with aromatic amino acids.²⁸ The resulting monomer can display hydrogen bond donor and acceptor moieties such that one face of the strand is complementary to a native α -peptide, while the other is blocked. Such selective backbone alteration has proved instrumental in revealing structure and assembly mechanisms of amyloidogenic sequences²⁹ and developing inhibitors of amyloid formation.^{30–32} C_{α} , C_{α} -dialkylated residues of increased steric bulk relative to Aib can also promote extended backbone conformations and cap β -sheets.^{33,34}

A drawback to backbone modifications that disrupt hydrogen bonding in a β -strand is incompatibility with incorporation at an internal position of a larger β -sheet. Given the prevalence of such motifs in proteins, we were motivated to evaluate alternative substitution strategies. Our initial efforts focused on β -residues, as these had proved so versatile in helices. Thus, we systematically compared β -residues of varied backbone stereochemistry, substitution pattern, and ring constraint for the ability to promote β -sheet folding at cross-strand positions in a β -hairpin host.³⁵ The results showed that some β -residue classes (e.g., β^3 , $\beta^{2,3}$) supported native-like folds; however, $\alpha \rightarrow \beta$ substitution altered the display of side chains on residues flanking the modification. We went on to establish that the same monomer types could be used in alternate substitution patterns ($\alpha\alpha \rightarrow \beta$, $\alpha\alpha \rightarrow \beta\beta$), resulting in heterogeneous backbone sheets that displayed side chains in a native-like fashion.³⁶ We later showed that the cyclically constrained γ -residue *cis*-1-amino-3-cyclohexanecarboxylic acid (ACC) was a strong sheet stabilizer compatible with placement at internal β -strands.³⁷ Thus, $\alpha \rightarrow \gamma^{cyc}$ substitution at two sites in a host hairpin led to a native-like folded state and a folding free energy superior to the natural backbone.³⁶

The final secondary structure type we considered, prior to attempting modifications in a larger protein, was surface-exposed non-regular loops. We hypothesized these would be most tolerant to modification based on prior work showing incorporation of artificial backbone moieties,³⁸ as well as our own demonstration that a simple polyethylene glycol (PEG) based monomer could replace tripeptide segments in surface-exposed loops from a small protein.³⁹

Backbone Modification in the B1 Domain of Streptococcal Protein G

The precedents outlined above on the mimicry of individual secondary structures by backbones in which one or two α -residues is replaced by some artificial counterpart set the stage to examine combinations of such modifications in larger, more complex tertiary folds (Figure 3). As detailed in the introduction, a key motivation for seeking a general strategy toward tertiary structure mimicry by foldamers is to broaden the scope of folds and functions possible in these agents. In the pursuit of this goal, the selection of an optimal prototype protein was crucial. Desirable characteristics included a compact fold with all the common elements of secondary structure in a chain lacking disulfides and short enough to be

synthetically accessible. Based on these considerations, we selected the B1 domain of *Streptococcal* protein G (GB1). Over the last few years, we have examined the folding behavior of >30 backbone-modified variants of GB1. Collectively, this data set has led to improved understanding of how to design effective secondary structure mimics, an appreciation of the interplay among varied backbone modifications in a protein context, and insights into structural and thermodynamic consequences of backbone alteration. Below, we summarize key findings from our work with the GB1 system (Figure 4).

Reflecting their versatility in secondary structure mimicry, β^3 -residues proved to be well accommodated in different parts of GB1.⁴⁰ X-ray crystal structures revealed native-like folds in a GB1 variant with $\alpha \rightarrow \beta^3$ replacements in every turn of the helix and another with $\alpha \rightarrow \beta^3$ substitutions in each loop. Unfortunately, our earlier efforts to develop design rules for incorporation of β -residues in sheet secondary structure proved futile in GB1. We synthesized several variants with sheet substitution patterns that were well tolerated in hairpins; however, none showed any evidence of cooperative folding. While disappointing, the inability of these sequences to fold offered two valuable lessons: (1) some modifications to a protein backbone, while subtle, can abolish folding entirely and (2) design principles developed in simplified models are not always applicable in more complex environments. Thus, we took a more conservative approach and made $\alpha \rightarrow \text{N-Me-}\alpha$ replacements at the edge strands of the sheet; the resulting GB1 variant was only slightly destabilized relative to the prototype. For the replacement of the two β -turns, we employed Aib and D-Pro, and an X-ray crystal structure confirmed the artificial turns supported a native-like tertiary fold.

At this point, we had shown the loops, helix, sheet, and turns of GB1 could be individually modified without abolishing tertiary folding, although stabilities of heterogeneous-backbone variants were slightly to somewhat compromised.⁴⁰ We next combined the modifications into a single variant. The resulting protein, in which ~20% of the residues in the prototype were replaced, retained a cooperative unfolding transition but was significantly less thermodynamically stable than the native. A published study a decade prior to our efforts had reported the optimization of the folded stability of GB1 through side-chain mutagenesis.⁴¹ Gratifyingly, appending the side-chain sequence of one of the mutants from that study onto the heterogeneous backbone we had developed in our work resulted in a dramatic improvement in resistance to thermal denaturation.⁴⁰ This result confirmed that substantially altered backbone could support a complex fold of high thermodynamic stability. Moreover, it established that side-chain sequence optimizations carried out in native backbone contexts could inform the design of heterogeneous-backbone variants.

Our work to this point had demonstrated a great deal of plasticity with respect to backbone chemical structures upon which the sequence-encoded tertiary fold of GB1 can manifest; however, the results also raised questions. What was the molecular origin of decreased folded stability observed for variants with artificial backbones relative to natural counterparts? Related, was it possible to gain back that lost stability through improved design strategies?

Turning to the first question, we examined the thermodynamic consequences of backbone modification.⁴² $\alpha \rightarrow \beta^3$ substitutions in the helix were the largest source of destabilization in

first-generation variants. We reasoned that the addition of a rotatable bond might lead to a greater entropic penalty to folding. To test this hypothesis, we quantified folding enthalpy and entropy in GB1 and several β^3 -residue containing variants. Counter to expectations, β^3 -residue incorporation consistently reduced the entropic penalty toward folding. This small favorable change is overwhelmed by a significant unfavorable enthalpic effect, leading to a net unfavorable impact on folding free energy. Examination of other parameters describing the folding equilibrium suggested the entropic boost arises from a greater favorable desolvation of a less ordered unfolded ensemble containing β^3 residues. The central role of solvation in protein folding has long been appreciated,⁴³ and these results underscore the importance of considering how backbone alteration affects interactions with water.

We next turned attention to identifying superior strategies for helix backbone modification.⁴⁴ Prior work had shown that β^{cyc} -residue like ACPC stabilize α -helical folds in α/β -peptides,¹⁶ presumably by preorganizing the central bond in a conformation compatible with helical secondary structure. We carried out $\beta^3 \rightarrow \beta^{\text{cyc}}$ substitutions in GB1 in both the helix and loop; crystal structures of several variants revealed that these residues are structurally interchangeable in both contexts.⁴² Consistent with first principles, $\beta^3 \rightarrow \beta^{\text{cyc}}$ substitution has an entropically favorable effect on folding in GB1 that appears to arise from a more compact, native-like unfolded ensemble for the more rigid backbone. Unexpected was the enthalpic penalty from β^{cyc} residue incorporation. The compensating enthalpy/entropy change leads to higher resistance to thermal unfolding; however, the folding free energy is at best comparable after $\beta^3 \rightarrow \beta^{\text{cyc}}$ substitution. We next compared the helix promoting $\text{C}_\alpha\text{-Me-}\alpha$ residue Aib alongside β^{cyc} residues.⁴⁴ Aib was structurally well accommodated in the helix of GB1, and thermodynamic analysis showed it is superior to β^{cyc} residues at stabilizing the folded state.

A drawback of both ACPC and Aib compared to β^3 residues is the loss of a side chain upon replacement of an α -residue, which can prove detrimental to folding and/or function. In an effort to retain native side chains but stabilize the fold, we examined GB1 variants with modified helices bearing β^2 residues, regioisomers of β^3 residues.⁴⁴ Data from a series of proteins suggested β^2 and β^3 residues are identical in terms of fold structure and stability when the side chain is not involved in tertiary contacts. However, when the side chain is involved in key tertiary interactions, one or the other regioisomer is far superior at displaying the side chain in a way that retains fold stability.

Chiral $\text{C}_\alpha\text{-Me-}\alpha$ residues share the favorable rigidifying effect of Aib but also retain a native side chain. Side-by-side comparison showed that these residues are energetically comparable when incorporated at solvent-exposed sites in the GB1 helix; however, chiral $\text{C}_\alpha\text{-Me-}\alpha$ residues are superior when the site of backbone modification engages in side-chain contacts essential to the fold.⁴⁴

Turning to optimization of sheet modification strategies, we later demonstrated that $\alpha \rightarrow \gamma^{\text{cyc}}$ substitutions could be introduced at every strand of the GB1 β -sheet with a minimal effect on fold stability.⁴⁵ Interestingly, even a modification as subtle as thioamide incorporation proved dramatically more destabilizing at an internal strand position.⁴⁶

Through work on GB1, we were able to demonstrate that alterations throughout the backbone of a protein with a complex tertiary structure can yield variants that fold in a native-like fashion. Beyond this, the systematic approach by which the variants were designed and characterized generated valuable information about the thermodynamic implications of individual backbone changes as well as the cumulative effect of combined modifications. One overarching observation was a correlation between energetic penalty to fold stability and fraction of the backbone altered.

Backbone Modification in the Third Zinc Finger of Specificity Factor 1

To probe the generality of design principles developed in GB1 and examine effects of backbone modification on folding energetics in other contexts, we focused on a new prototype. The third finger of specificity protein 1 (Sp1-3) is a Cys₂His₂ zinc finger with a classical $\beta\beta\alpha$ fold held together by a small hydrophobic core and the tetrahedral coordination of a Zn²⁺ ion (Figure 5). One motivation in targeting Sp1-3 for mimicry was the prospect of expanding the scope of heterogeneous-backbone foldamer design to a tertiary fold completely distinct from GB1 that also depends on a precise metal coordination site. From the standpoint of design, Sp1-3 presented additional challenges compared to GB1. First, backbone modifications must be compatible with the positioning of side chains necessary for metal coordination. Second, the design of variants of the isolated domain were made with an eye toward incorporation into larger DNA-binding multi-domain zinc finger proteins; thus, modifications had to also be informed by key interactions that contribute to DNA binding.

With the above criteria in mind, a series of Sp1-3 variants were designed, synthesized, and characterized.⁴⁷ All shared the same modification to the β -sheet: $\alpha \rightarrow \text{N-Me-}\alpha$ substitution at cross-strand positions not hydrogen bonded in the folded state. For the helix, we examined two different backbone modification patterns: (1) $\alpha \rightarrow \beta^3$ replacements at every turn of the helix along the solvent-exposed face and (2) substitution of Aib in place of β^3 -residues in design 1 with side chains not expected to contribute to interaction with DNA. Gratifyingly, Sp1-3 variants with modified helix and hairpin were shown to bind metal with native-like Cys₂His₂ tetrahedral coordination and adopt folds highly homologous to the prototype domain. The heterogeneous-backbone variants also showed metal binding affinities comparable or slightly superior to wild-type Sp1-3. Because folding and metal binding are closely intertwined for Sp1-3, folding energetics can be elucidated by differences in the thermodynamics for metal interaction. Remarkably, analysis of data for Sp1-3 and variants suggested that both heterogeneous-backbone mimics have folded stability superior to the native protein.

From first principles, it would perhaps be reasonable to expect that that a natural side-chain sequence will always fold best on a native protein backbone (beyond cases of isolated, secondary-structure- bolstering substitution). Our results in GB1 supported this assumption. Replacement of ~20% of the residues in the native protein with an artificial counterpart based on first-generation design rules came at an energetic cost to folding of ~8 kcal mol⁻¹. For comparison, the same fraction and types of substitution in Sp1-3 stabilized the folded state by up to ~0.5 kcal mol⁻¹. This drastic difference in the effect of backbone

modification on overall folding free energy in GB1 and Sp1-3 suggests that as backbone alterations become better understood, disadvantageous side effects may be minimized while desired characteristics are improved upon.

Likewise, the striking similarities found in the thermodynamic basis for each system provides data to guide future designs. In both proteins, $\alpha \rightarrow \beta^3$ substitution leads to an entropic boost to folding with a compensating enthalpic penalty, while $\beta^3 \rightarrow \text{Aib}$ replacement stabilizes the fold enthalpically with a corresponding unfavorable entropic effect. The reason similar trends in enthalpy entropy compensation lead to different net outcomes in terms of folding energetics in the two systems is a difference in the relative magnitude of the effects.

Work on Sp1-3 also highlighted limitations in understanding of how to engineer protein backbones. When modifications to the hairpin and helix were combined with alterations to the turn, the resulting variants did not fold or bind metal with measurable affinity. The β -turn in question is flanked by two Cys residues coordinated to metal in the folded state. The inability of any of the turn-modified variants to fold in a native like fashion suggests the artificial turn-inducers examined (D-Pro, Aib, Orn) are unable to properly orient the side chains to support tetrahedral coordination. More broadly, this observation underscores the need for continued refinement of design rules for backbone modification.

Recent Work in Other Systems

In parallel to our own lab's efforts detailed above, work in recent years by other researchers has significantly broadened the scope of chemical backbone alteration in folded protein tertiary structures. Examples include the incorporation of triazole heterocycles as replacements for amides in protein loops, facilitating synthesis by non-native ligation methods.⁴⁸ Supporting their versatility, β -residues of various types have been incorporated into diverse systems, including helices in an engineered enzyme,⁴⁹ villin headpiece,⁵⁰ and a helix-turn-helix motif⁵¹ as well as loops in a vascular endothelial growth factor mimic.⁵² Finally, a recent report showed that loops in a knottin protein can be replaced with extended D- α -residue segments to generate heterochiral variants with native-like folded structures.⁵³

CONCLUSIONS

The growing body of work on the retention of complex folds after alteration to protein backbone connectivity shows that many natural sequences can tolerate, and even benefit from, such modification. Progress notwithstanding, important open questions, needs, and opportunities for the field remain.

One key question is the extent to which sequence-guided backbone modification, already shown feasible in several systems, will be applicable to the full panoply of folds found in nature. In ongoing unpublished efforts, we are testing the validity of design principles in diverse small proteins, sequences both from nature and computationally designed.⁵⁴ Another open question is whether appropriately designed backbones that retain the fold of a given sequence will also retain biological function. The answer to this question in the case of foldamer mimics of smaller peptide secondary structure has been a resounding yes;⁴ however, the data set in tertiary fold contexts remains limited.

One important need in the field is an improvement in how these molecules are made. Chemical synthesis is still the only method able to produce sequence-specific oligoamides that are both large and highly modified. Obtaining material in sufficient quantity and purity to test hypotheses about folding and function is a bottleneck. Challenges related to production also place constraints on establishing the scope of design principles. Backbone modification of the type described in this Account should be applicable to folds of arbitrary size and complexity; however, the limit in length of chains that are synthetically accessible hinder the ability to probe this idea. An exciting development that may prove enabling is ongoing effort to engineer the ribosome to allow biosynthesis of backbone-modified peptides and proteins.^{55–57}

One opportunity for the field is to go beyond the idea of mimicry as the end goal and seek systems where backbone modification can be used to probe fundamental biological processes. One example coming out of our work is the prospect of gaining new insights into the sequence-specific recognition of DNA by zinc finger proteins. High sequence fidelity binding requires precisely tuned thermodynamics that enable zinc finger proteins to rapidly cascade down a DNA strand but bind tightly when a target sequence is encountered.⁵⁸ Backbone modification could make it possible to probe search vs. binding by, for example, rigidifying a local region in a way that allows for tighter binding to a recognition sequence but inhibits the search mode and examining the impact on overall binding affinity, specificity, and kinetics. Another example of a possible application beyond mimicry arises from the observation that backbone alteration can have profound effects on intrinsically unstructured chains. We recently showed dramatic variation in the efficiency of enzymatic hydrolysis of heterogeneous backbones as a function of modification type and position relative to the labile amide.⁵⁹ These results suggest the possibility of applying sequence-guided backbone alteration to rationally modulate substrate degradation rates and/or selectivity for particular protease classes.

It is important to emphasize that systematic protein backbone alteration is only one of an array of methods available to enable foldamer design, and *de novo* construction remains a powerful approach to complex folds, assemblies, and functions.^{2–11} Moreover, the foldamer concept is only one of a growing variety of technologies available to control molecular shape.⁶⁰ Continued efforts to advance the frontier of structural complexity possible in artificial scaffolds promise to broaden the scope of folds possible, shed new light on natural systems, and greatly expand the functional repertoire of such agents.

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Biographies

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Figure 1. Heterogeneous backbones that blend natural α -amino acid residues with various artificial building blocks can adopt protein-like tertiary folds when backbone displayed side-chain sequence encodes such behavior.

Figure 2.
Key to nomenclature for commonly used building blocks in heterogeneous-backbone peptide and protein mimics.

Figure 3.

Overview of design principles for sequence-guided protein backbone alteration. Each panel presents a canonical secondary structure (from PDB 2QMT) alongside select replacement strategies shown viable in tertiary fold contexts.

Figure 4.

Mimicry of GB1 by heterogeneous backbones. (A) Sequence and crystal structure (PDB 2QMT) of wild-type GB1. (B) Some backbone modifications made to GB1, grouped by secondary structure context. All panels are from published crystal structures of GB1 variants (PDB: 5HFY, 5HI1, 4OZB, 4OZC, 4KGR, 4KGS, 4KGT), except data for the PEG-modified loop (from a molecular dynamics simulation) and the γ^{cyc} -modified sheet (from the NMR structure of a hairpin peptide).

Figure 5.

Mimicry of Sp1-3 by heterogeneous backbones. (A) Sequence of Sp1-3 and two variants (R groups, when present, are defined by the single letter code of the corresponding α-residue). (B) NMR structures of Sp1-3 (PDB 1SP1) and one variant (PDB 5US3). (C) Retention of a native-like metal-coordination environment (inset) in the modified backbones is supported by visible absorption spectra with bound Co²⁺. (D) Comparison of folding thermodynamics shows that the modified backbones have stabilities superior to native Sp1-3.