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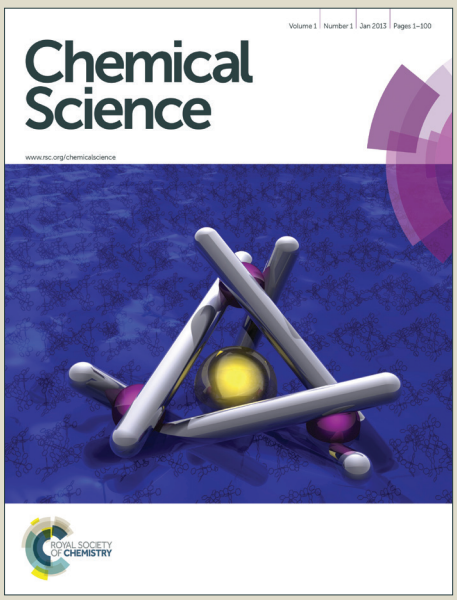

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EDGE ARTICLE

Folding Thermodynamics of Protein-Like Oligomers with Heterogeneous Backbones

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The thermodynamics of protein folding are dictated by a complex interplay of interatomic interactions and physical forces. A variety of unnatural protein-like oligomers have the capacity to manifest defined folding patterns. While the energetics of folding in natural proteins is well studied, little is known about the forces that govern folding in modified backbones. Here, we explore the thermodynamic consequences of backbone alteration on protein folding, focusing on two types of chemical changes made in different structural contexts of a compact tertiary fold. Our results reveal a surprising favorable impact on folding entropy that accompanies modifications that increase disorder in the ensemble of unfolded states, due to differences in the solvation of natural and unnatural backbones.

Introduction

Although proteins are made up exclusively of α-amino acids, numerous unnatural oligoamides are capable of manifesting ordered folds with interesting biological activities. This idea is well illustrated by the development of unnatural-backbone oligomers with defined folding patterns ("foldamers").[1-7] Compared to the wealth of structural information that has accumulated on diverse non-biological oligomers, little is known about fundamental differences in folding thermodynamics between proteins and their unnatural counterparts. Alterations can be made to a protein backbone without abolishing its ability to fold;[8-17] however, few reports have examined how such modifications influence folding thermodynamics beyond free energy.[18-21] Addressing this gap in knowledge offers potential benefits both fundamental, by providing new perspective on the folding energetics of natural biomolecules, and applied, by aiding in the design of protein mimetics with increasingly sophisticated folds and functions.

We have recently demonstrated that helix, loop, sheet, and turn elements of a protein with a compact tertiary fold can be simultaneously modified to create heterogeneous backbones with native-like folding behavior.[22] In that work, a subset of α-residues in a bacterial protein were replaced with an assortment of unnatural building blocks, including D-α-amino acids, C_α-methyl-α-amino acids, *N*-methyl-α-amino acids, and β-amino acids. While structurally well accommodated into the folded state, these alterations consistently lowered resistance of the tertiary fold to temperature-induced denaturation. Here we explore the thermodynamic basis for that destabilization for one type of α-residue replacement, β-amino acids, and show how that destabilization can be partially mitigated by backbone rigidification (Figure 1).

As backbone-homologated analogues of α-residues, β-residues have found widespread use in foldamer design.[1-7] β^3-Residues bearing side chains based on natural amino acids have been used as replacements for α-residues in sequences that form helix[23,24] and sheet[25,26] secondary structures as well as more complex folds.[22,27] A key structural difference between β-residues and α-residues is an additional rotatable bond in the backbone, which results in increased conformational flexibility. Pioneering early studies on β-peptide oligomers showed that incorporating C_α and C_β atoms into a ring to generate cyclic (β^{cyc}) residues can restrict this conformational freedom and, in turn, influence folded structure.[28] More recent work has shown

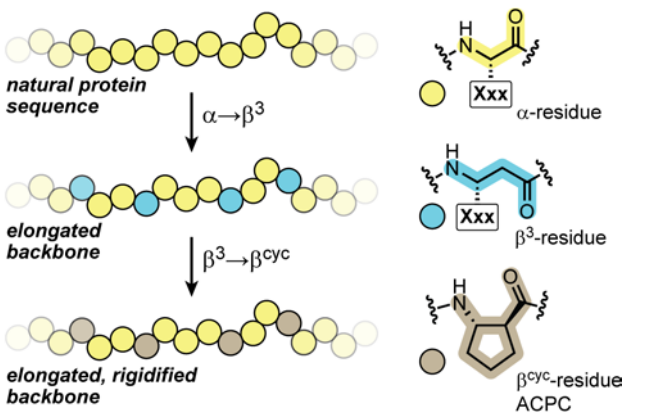

Fig. 1 The use of β-residues (β^3 and β^{cyc}) in sequence-guided protein backbone alteration. The goal in the present work is to determine the thermodynamic impact of $\alpha\rightarrow\beta^3$ and $\beta^3\rightarrow\beta^{cyc}$ substitution on folding in a protein tertiary structure context.

a particular class of cyclic β-residue, exemplified by (*S*,*S*)-ACPC (Figure 1), can promote α-helix-like folds when it replaces β^3-residues in heterogeneous α/β-peptide backbones based on natural sequences.[24,29,30]

Despite their well-studied folding propensities, little is known about the thermodynamic consequences of using β^3-residues as replacements for α-residues on the stability of the folded state. Moreover, although the ability of β^{cyc}-residues to promote folded structure has been seen in many systems, the energetic basis for that stabilization has not been established experimentally. In an effort to address the above open questions, we report here the synthesis, structural characterization, and biophysical analysis of a series of backbone-modified variants of a bacterial protein with a compact tertiary fold. The resulting data provide insights into the thermodynamic impact of $\alpha\rightarrow\beta^3$ and $\beta^3\rightarrow\beta^{cyc}$ residue substitutions on protein folding.

Results and Discussion

Protein Design and Synthesis. We used the B1 domain of protein G from *Streptococcus* bacteria (GB1, **1**)[31] as a host sequence to explore the thermodynamic consequences of backbone alteration by α→β-residue substitution (Figure 2). This 56-residue protein adopts a compact tertiary fold consisting of an α-helix packed against a four-stranded β-sheet with two tight turns and two short loops. We recently reported that heterogeneous-backbone analogues of GB1 (**1**) containing assorted unnatural α-residue replacements can maintain the sequence-encoded tertiary folding behavior of the parent protein.[22] In that work, sequence-guided $\alpha\rightarrow\beta^3$ residue replacement (where α-residues are replaced by β^3 analogues bearing the same side chain) proved effective for modifying the loops (protein **2**) and helix (protein **3**) of GB1. Crystal structures of **2** and **3** revealed tertiary folds essentially identical to wild type; however, simple thermal denaturation measurements indicated the fold was destabilized.[22] Our first aim in the work reported here was to gain insight into the thermodynamic basis of that altered stability. The approach to this goal began with the design and synthesis of several new variants of GB1 incorporating β-amino acids.

In GB1 analogue **3**, four α-residues in the helix were replaced by β^3-residues in an αααβαααβ repeat that places the extra atoms in the backbone opposite the hydrophobic core in the folded state.[23] Inspired by observations that many different α/β residue patterns can support helical folding from the same parent side-chain sequence,[24] we designed and synthesized GB1 variant **4** with an αααβ residue repeat along the helix. Collectively, proteins **2**-**4** provide a series of molecules to systematically examine the thermodynamic consequences of $\alpha\rightarrow\beta^3$ residue replacement in loop and helix secondary structure contexts.

In order to investigate the thermodynamic effect of backbone preorganization by cyclic β-residues, we designed a series of GB1 analogues (**5**-**8**) bearing $\beta^3\rightarrow\beta^{cyc}$ substitutions to protein **3**. In variants **5** and **6**, two of the four β^3-residues from **3** are replaced with ACPC (**X**). Protein **7** combines the modifications from **5** and **6** into a single chain in which all four turns of the helix are rigidified. In the published crystal structures of **2** and **3**, we found that the β^3-residues adopted a similar backbone conformation in both helix and loop contexts.[22] Thus, we hypothesized that ACPC would also be effective for replacement of the β^3-residues in the loops of **2**. Accordingly, we synthesized protein **8**, which combines the optimal helix substitution pattern from **5**-**7** (*vide infra*) with one cyclic β-residue in each loop of the protein.

Crystal Structures of GB1 and Variants. Crystal structures of GB1 (**1**)[32] and heterogeneous-backbone analogues **2** and **3** have been previously determined.[22] We grew crystals of **4**, **5**, and **8** by hanging drop vapor diffusion, and solved their structures to 2.2, 1.8, and 2.3 Å resolution, respectively (Figure 3). The collective set of structures shows that α→β residue replacement is well accommodated in both the helix and loops of GB1. Helix-modified variants **3** and **4** show identical overall folded structures and backbone hydrogen-bonding as wild-type GB1 (**1**), regardless of the pattern of β-residue incorporation. The conformation about the central C_α–C_β bond of the β^3-residues in **2**-**4** (78°±7°) is close to that typically found in the *trans*-substituted 5-membered ring of ACPC.[24,28-30] Thus, the $\beta^3\rightarrow\beta^{cyc}$ substitutions in **5** and **8** lead to backbones structurally identical to corresponding regions of unconstrained analogues **2** and **3** (Figure 3B).

Thermodynamic Analysis of Folding Equilibria. In order to compare the folding thermodynamics among wild-type GB1

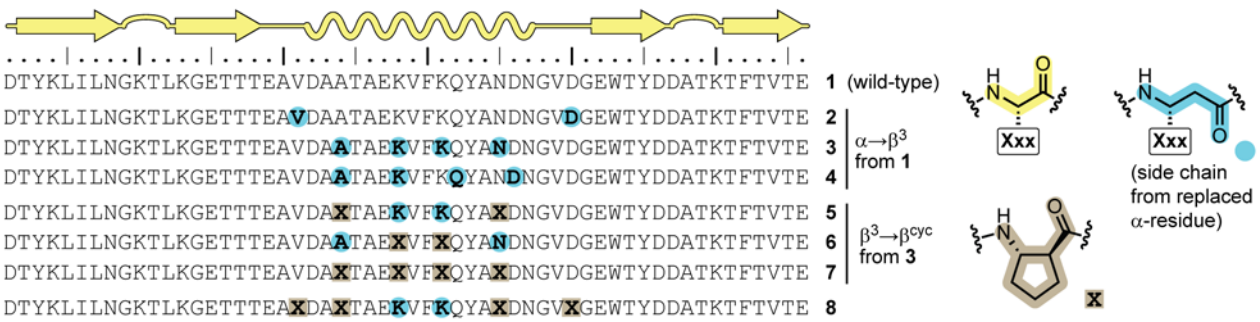

Fig. 2 Sequence and secondary structure map of protein GB1 (**1**) and backbone-modified analogues **2**-**8**. The single letter code denoting side chains displayed on β^3-residues (cyan) is identical to that for natural α-residues (yellow).

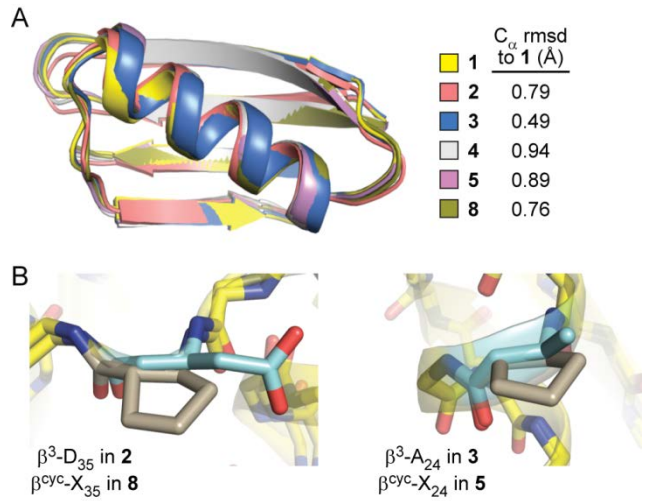

Fig. 3 X-ray crystal structures of wild-type GB1 (**1**) and analogues **2**-**5** and **8** [PDB codes 2QMT (**1**), 4KGS (**2**), 4KGR (**3**), 4OZA (**4**), 4OZB (**5**), 4OZC (**8**)]. (A) Tertiary structure overlays with calculated C□rmsd vs. **1**. (B) Close-up view of ACPC (**X**) as a replacement for β^3-residues in loop (left) and helix (right) contexts.

(**1**) and heterogeneous-backbone analogues **2-8**, we carried out a global analysis of the effect of temperature and chemical denaturant on stability.[33] In this method, applied recently to probe the thermodynamic effect of side chain fluorination,[34] a circular dichroism signature of the folded state is monitored as a function of temperature for parallel samples containing guanidinium chloride at a range of concentrations. The resulting three-dimensional surface (Figure 4) is fit to the Gibbs-Helmholtz equation to obtain the changes to free energy ($\Delta G°$), enthalpy ($\Delta H°$), entropy ($\Delta S°$), and heat capacity (ΔC_p) accompanying the unfolding process as well as the dependence of the folding free energy on denaturant concentration (*m*). We performed the above experiment for proteins **1-8** (Figure 4, Figure S2) and tabulated the resulting thermodynamic parameters (Table S1). Supporting the validity of the

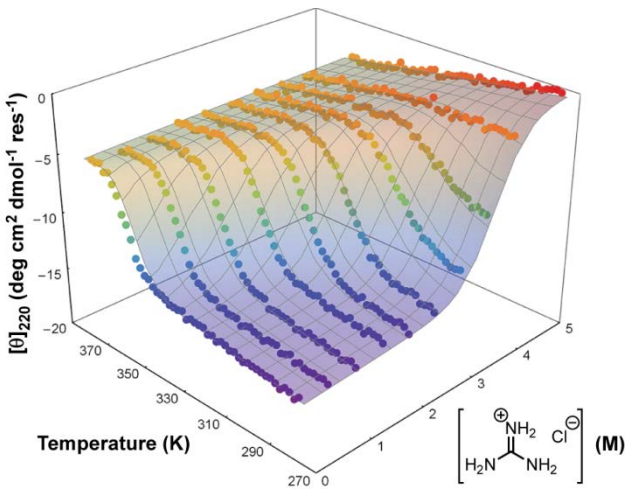

Fig. 4 CD signature of protein **1** at 220 nm as a function of temperature and chemical denaturant. Raw data (points) are fit (surface) to extract thermodynamic parameters for the folding equilibrium.

measurements, values obtained for wild-type GB1 (**1**) showed good agreement with previously reported data.[18,35,36]

For most proteins that adopt unimolecular tertiary folds, formation of the folded state in physiological conditions is enthalpically favored and entropically opposed.[37] The same is true for GB1 (**1**) and all the backbone-modified analogues we examined (**2-8**); however, a number of interesting trends were observed in how the magnitude of these values changed with backbone alteration. Below, we analyze each thermodynamic parameter separately, with a focus on how the values change with backbone elongation by $\alpha \rightarrow \beta^3$ substitution (**2-4** vs. **1**) and backbone rigidification by $\beta^3 \rightarrow \beta^{cyc}$ substitution (**5-7** vs. **3**).

Impact of Backbone Alteration on Folding Enthalpy ($\Delta H°$). Replacement of α-residues in GB1 with β-residues, whether acyclic or cyclic, was detrimental to the enthalpy of folding (Figure 5). Based on data for **2-4** vs. **1**, the magnitude of the change in $\Delta H°$ per $\alpha \rightarrow \beta^3$ replacement depended on structural context. The per-residue $\Delta\Delta H°$ accompanying $\alpha \rightarrow \beta^3$ substitution was much smaller for helix variant **3** compared to closely related protein **4**. Data for **5-7**, analogues of **3** containing $\beta^3 \rightarrow \beta^{cyc}$ substitutions, suggests the impact of ACPC incorporation on folding enthalpy varies considerably with placement in sequence. $\beta^3 \rightarrow \beta^{cyc}$ modifications are at best enthalpically neutral (**5**) and at worst quite detrimental (**6**, **7**). The loss of the two charged β^3-Lys side chains in **3** from the replacement of these residues with ACPC in **6** and **7** led to a large and unfavorable $\Delta\Delta H°$.

One possible origin of the unfavorable change to $\Delta H°$ that accompanied $\alpha \rightarrow \beta$ residue replacement in **2-8** vs. **1** is a change in surface salt bridges upon backbone modification. For example, the side chains of Glu_{27} and Lys_{31} are in close proximity in the wild-type protein. This contact is maintained in the crystal structures of variants **2**, **3**, and **8** but is not observed for variants **4** and **5**. Another potential contributor to lost folding enthalpy is a change in local backbone stereoelectronic interactions upon $\alpha \rightarrow \beta$ residue replacement. It has been suggested that partial donation of backbone carbonyl oxygen n_p electrons into the empty π^* orbital of the carbonyl in the

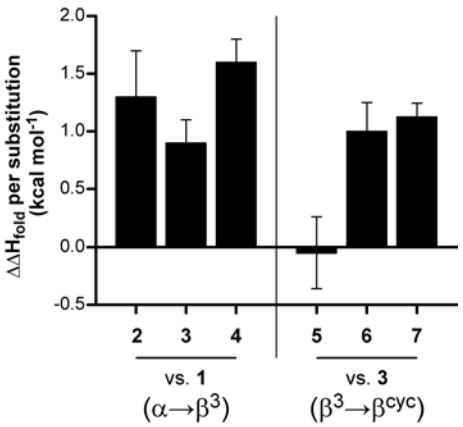

Fig. 5 Summary of changes to folding enthalpy at 25 °C accompanying $\alpha \rightarrow \beta^3$ substitution (**2-4** vs. **1**) and $\beta^3 \rightarrow \beta^{cyc}$ substitution (**5-7** vs. **3**). Each bar is normalized based on the number of backbone alterations.

subsequent residue can stabilize certain peptide conformations, including the α-helix.[38] Due to their extended backbones, β-residues in mixed α/β-peptide helices can act as donors but not acceptors in sequential $n\rightarrow\pi^*$ interactions (due to the ~1 Å longer distance between neighboring carbonyls in an αβ segment).[39] While an intriguing hypothesis, the role of altered backbone orbital interactions in the observed folding energetics is difficult to probe experimentally with the present data set.

Impact of Backbone Alteration on Folding Entropy (ΔS°). Like most proteins, the folding of GB1 is entropically opposed. Based on the additional backbone rotatable bond introduced with each $\alpha\rightarrow\beta^3$ replacement, it would be reasonable to predict an accompanying increase in the magnitude of ΔS° due to greater backbone disorder in the unfolded state lost upon adopting the tertiary fold. Contrary to this expectation, $\alpha\rightarrow\beta^3$ replacements in **2**-**4** consistently *stabilized* the tertiary structure through a favorable decrease in the magnitude of ΔS° relative to wild-type GB1 (Figure 6). One possible explanation for this observation is a less ordered fold in the heterogeneous backbones vs. the natural protein; however, the absence of significant local deviations in B-factor around the β^3-residues in the crystal structures of **2**-**4** argues against it (Figure S3).

The above result pointed away from differences in the folded state as being responsible for the change to folding entropy accompanying $\alpha\rightarrow\beta^3$ residue replacement and led us to consider how the unfolded state might change with backbone alteration. A denatured protein does not exist as a simple random coil but rather as a complex mixture of partially folded states, referred to as the "denatured ensemble."[40] Although challenging to probe experimentally, the molecular details of unfolded proteins are crucial to interpreting their folding thermodynamics, since the denatured ensemble is the reference state against which all energetic changes accompanying folding are measured.

A significant factor in the entropy of protein folding is the expulsion of ordered water solvating the unfolded state to disordered bulk upon burial of backbone and side chain surface area.[37] As detailed above, β^3-residues are expected to increase the backbone entropy of the unfolded state and thereby increase the entropic penalty for folding. What is perhaps less obvious is that a denatured ensemble with less residual folded structure may contribute favorably to folding entropy by creating a larger solvent-exposed surface (ASA), a greater magnitude change in this surface area (ΔASA) accompanying folding, and more possibilities for entropically favorable desolvation. Our results for backbone modification in GB1 suggest that altered solvation of the unfolded ensemble resulting from $\alpha\rightarrow\beta^3$ substitution more than outweighs any unfavorable contribution to folding entropy that results from the more flexible backbone. This hypothesis is further supported by other aspects of the data (*vide infra*). It should be noted that an alternative hypothesis exists involving enhanced rigidity observed in covalently-nucleated helices containing β^3-residues.[41]

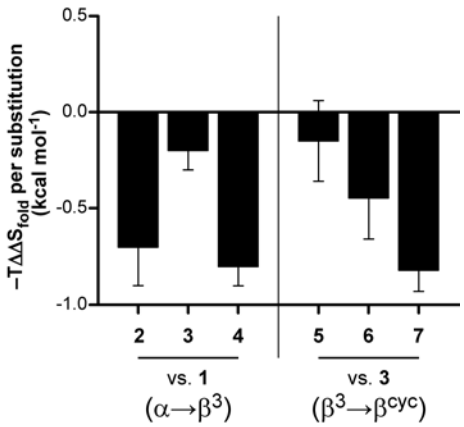

Fig. 6 Summary of changes to folding entropy at 25 °C accompanying $\alpha\rightarrow\beta^3$ substitution (**2**-**4** vs. **1**) and $\beta^3\rightarrow\beta^{cyc}$ substitution (**5**-**7** vs. **3**). Each bar is normalized based on the number of backbone alterations.

Where the impact of $\alpha\rightarrow\beta^3$ residue replacement on folding entropy was somewhat surprising, the consequences of backbone preorganization by $\beta^3\rightarrow\beta^{cyc}$ modification followed more closely with expectation. Substitution of the β^3-residues in **3** with the cyclic β-residue ACPC in variants **5**-**7** led to a consistently favorable reduction to the entropic penalty for folding (Figure 6). When only two of the four β^3-residues are replaced, the magnitude of the change in ΔS° depends on sequence position. The rigidified residues have the largest effect when located at adjacent turns in the center of the helix (**6**) compared to several turns apart at the termini (**5**). Placing cyclic residues in all four turns of the helix maximizes the per-residue change (**7**). Although favorable desolvation of the hydrophobic ACPC residue may contribute to its observed impact on folding entropy, we ascribe the effect primarily to backbone preorganization, as detailed below.

Impact of Backbone Alteration on Solvation. Protein folding is typically accompanied by a positive change to the heat capacity of the sample (ΔC_p). This change results from differences in the interaction of water with the folded vs. unfolded state and leads to a temperature dependence of ΔH° and ΔS°.[42,43] The magnitude of ΔC_p depends on many factors, including solvent accessible surface area,[44] backbone conformational entropy, and the presence of residual structure in the denatured ensemble.[40] Based on the data for proteins **1**-**4**, $\alpha\rightarrow\beta^3$ substitutions had at most a modest effect on ΔC_p (Figure 7A); the magnitude was slightly smaller in variant **3** but identical to wild-type for **2** and **4**. In contrast, replacement of β^3-residues in **3** with cyclic β-residues in **5**-**7** consistently lowered the observed ΔC_p.

In addition to ΔC_p, the dependence of the folding free energy on denaturant concentration (*m*) is also related to protein solvation.[44] Different patterns of $\alpha\rightarrow\beta^3$ replacement in the helix of wild-type GB1 (**1**) to generate variants **3** and **4** were accompanied by identical increases to *m* (Figure 7B). In contrast, incorporation of β^3-residues into the loops (**2**) had no effect. Substitution of β^3-residues in **3** with ACPC in **5**-**7** led to a consistent decrease in *m*.

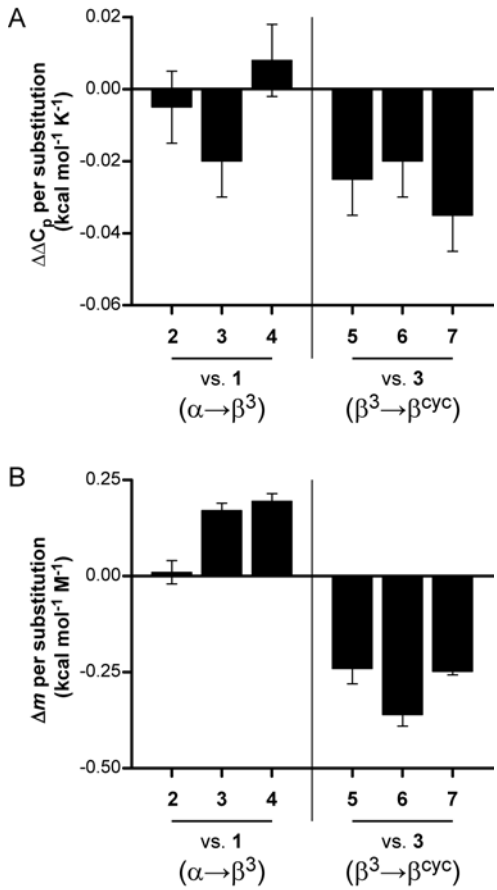

Fig. 7 Summary of changes to ΔC_p (A) and *m* (B) accompanying $\alpha \rightarrow \beta^3$ substitution (**2**-**4** vs. **1**) and $\beta^3 \rightarrow \beta^{cyc}$ substitution (**5**-**7** vs. **3**). Each bar is normalized based on the number of backbone alterations.

Both ΔC_p and *m* are known to show strong correlation with the change in solvent accessible surface area (ΔASA) that accompanies folding.[44] The fact that $\beta^3 \rightarrow \beta^{cyc}$ replacements lower the magnitude of both ΔC_p and *m* is consistent with the proposed mechanism of structure promotion by cyclic β-residues. A preorganized backbone is likely to have a more compact denatured ensemble than a flexible analogue and therefore a smaller ΔASA of folding. The fact that $\alpha \rightarrow \beta^3$ substitution generally increases the magnitude of *m* is consistent with the more flexible backbone creating a more disordered denatured ensemble and accompanying increase to ΔASA for folding. This effect is only apparent when the β^3-residues are placed in the α-helix (**3**,**4**) and is not observed when the loops are modified (**2**) – presumably because the loop segments of the wild-type protein are already highly disordered in the denatured ensemble. One somewhat puzzling outlier in the above analysis is the negligible effect of $\alpha \rightarrow \beta^3$ modification on ΔC_p. This likely reflects the complex array of factors besides ΔASA that influence the heat capacity change of folding.[42,43]

Conclusions. In summary, we have reported here the synthesis, structural characterization, and biophysical analysis of a series of backbone-modified analogues of a bacterial protein with a compact tertiary fold. These efforts focused on replacement of α-residues in the native sequence with two classes of β-amino acid building blocks: β^3 and β^{cyc}. Acyclic β^3-residues are well accommodated as α-residue replacements in the tertiary fold, and the cyclically constrained β^{cyc}-residue ACPC is an effective replacement for β^3-residues in both loop and helix contexts. Analysis of changes to folding thermodynamics as a function of backbone composition reveals some clear trends.

In general, replacing α-residues with β^3-residues is enthalpically unfavorable and entropically favorable to the thermodynamic stability of the tertiary fold. The change in enthalpy may be partially attributed to the loss of stabilizing $n \rightarrow \pi^*$ interactions for β-residues incorporated into helical secondary structures; however, additional factors also appear to contribute. The favorable effect on folding entropy observed upon $\alpha \rightarrow \beta^3$ modification, although not predicted by simple analysis of backbone entropy, is reasonable when the role of water in the folding process is considered. Specifically, backbone modifications that lead to reduced residual folded structure in the denatured ensemble also create more entropically favorable desolvation upon folding. The importance of solvation is further underscored by effects of backbone elongation on heat capacity and sensitivity to chemical denaturant.

With regards to backbone preorganization through incorporation of constrained β^{cyc} residues in place of acyclic β^3 counterparts, our results provide concrete experimental evidence supporting the hypothesis that the rigidified building blocks lower the entropic cost of folding. This gain is largely offset, however, by a compensating enthalpic penalty in sequences where charged side chains are lost as a result of $\beta^3 \rightarrow \beta^{cyc}$ substitution. Overall, the results we report here provide new insights into the folding thermodynamics of oligomers containing β-amino acids and will aid in their ongoing use in the design of protein-mimetic oligomers with increasingly complex folds and functions.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental methods, Figures S1-S3, and Tables S1-S3. See DOI: 10.1039/b000000x/

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