Protein Folding

DOI: 10.1002/ange.200904714

Detection and Analysis of Chimeric Tertiary Structures by Backbone Thioester Exchange: Packing of an α Helix against an α/β-Peptide Helix**

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Efforts to extrapolate from well-folded biopolymers, such as proteins, to synthetic oligomers with unnatural backbones that display discrete conformational propensities, or foldamers, have led to many recent advances.[1–3] Early foldamer studies tended to focus on discovery of new secondary structures, a goal that remains important, but recent efforts have expanded to include tertiary and quaternary structure.[4–14] Chimeric examples, in which one portion or partner is composed entirely of α amino acid residues and the other is foldameric, have emerged as particularly interesting systems. Protein analogues in which a small discrete element of secondary structure is replaced by an oligo-β-amino acid ("β-peptide") segment constitute chimeric systems with defined tertiary structure.[15,16] Chimeric quaternary structures have been formed between proteins and helix-forming foldamers, with the latter designed to mimic natural helical binding partners for the protein component.[17–21] The few examples to date in these two categories suggest that it may be possible to create a wide range of chimeric structures based on interweaving of protein and foldamer subunits.

Herein we introduce a versatile experimental strategy for exploring a fundamental type of protein–foldamer packing motif, the association of an α helix with a foldameric helix. This motif is analogous to an antiparallel coiled-coil tertiary structure in a pure α-residue backbone.[22,23] In our system, the foldamer component has a 1:1 α-residue:β-residue pattern in the backbone and should be prone to forming a specific helical secondary structure.[24–29] The data suggest that side chain packing preferences at this chimeric tertiary interface are comparable to those that determine pairing propensities among antiparallel α helices. Information of this type provides a foundation for pursuing important design goals, such as design of new protein-like polymers and development of antagonists of specific protein-protein interactions.

Our experimental system (Figure 1; $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$ and $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$) contains an N-terminal segment intended to form an α helix, a C-terminal α/β segment intended to form a "14/15 helix" (this designation is derived from the internal H-bonding pattern), and a central glycine-rich segment intended to form a loop that enables the two helices to pack against one another. The 14/15-helical conformation available to 1:1 α:β backbones is similar to the α helix formed by pure α backbones in terms overall shape and residue periodicity.[24–26] Both helices have about 3.5 residues per turn and contain backbone C=O(i)···H-N(i+4) H-bonds. The design of our α + α/β system is based on our recent exploration of sequence–stability relationships within an antiparallel coiled-coil tertiary structure (α residues only; exemplified by $\mathbf{N_A}$-$\mathbf{C}$).[30,31] Our approach makes use of backbone thioester exchange (BTE)[30–34] for thermodynamic measurements, which enables rapid exploration of side-chain packing preferences at the helix–helix interface. Conclusions derived from our all-α coiled-coil BTE system correlated with results of bioinformatic analysis of antiparallel coiled-coil structures among proteins,[31] and this correlation validates the BTE approach.

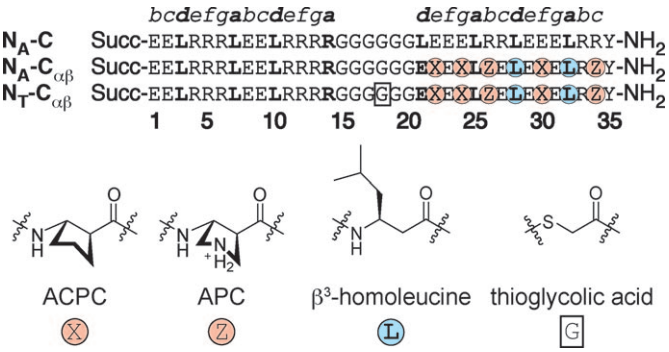

Figure 1. Amino acid sequences of α-peptide antiparallel coiled-coil $\mathbf{N_A}$-$\mathbf{C}$[30] and chimeric (α + α/β)-peptide $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$. α-Amino acids are abbreviated with one-letter codes; β-amino acids: L in blue circle: β^3-homoleucine; X in red circle: ACPC; Z in red circle: APC; G, in rectangle: thioglycolic acid. Residues occupying hydrophobic *a* and *d* heptad positions are shown in boldface type.

Initial evaluation of the chimeric helix–loop–helix design focused on the 35-mers $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$ and $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ (Figure 1), which differ only at a central backbone linkage (conventional peptide bond in $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$, thioester in $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$). The 14 residues closest to the N terminus can form four α-helical turns

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[**] This research was supported by the NIH Grant GM-61238. J.L.P. was supported in part by an NIH Chemical Biology Training Grant (T32 GM008505). We thank Dr. Darrell McCaslin for assistance with AU experiments, and Peptech for providing β^3-amino acids at a discounted price.

Supporting information for this article, including solid-phase peptide synthesis, peptide purification, circular dichroism, analytical ultracentrifugation, and backbone thioester exchange assays, is available on the WWW under http://dx.doi.org/10.1002/anie.200904714.

(Figure 2a). The design of this segment, taken directly from $\mathbf{N_A}$-**C**, was based on the heptad repeat pattern that is characteristic of coiled-coil sequences:[35,36] hydrophobic residues are placed at the *a* and *d* positions within the *abcdefg*

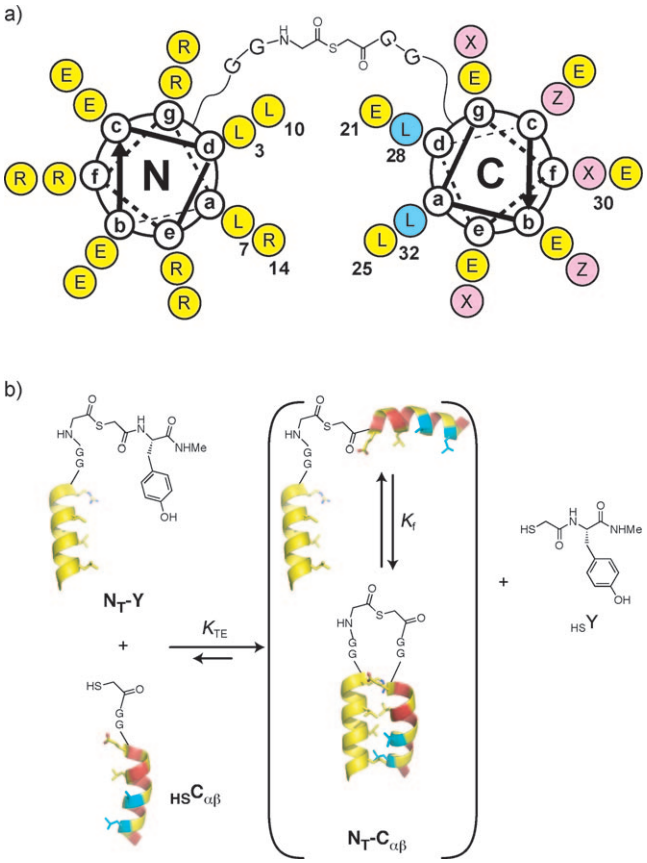

Figure 2. a) Helical wheel representation of the antiparallel coiled-coil structural model for $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$. α-Amino acids: yellow; β^3-amino acids: blue; cyclic β-amino acids: red, X = ACPC, Z = APC. b) Thioester exchange process for $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$.

heptad pattern. Leu residues occur at three of these positions in $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$ and $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ because Leu is the most common residue at coiled-coil interface positions. Arg was placed at the fourth of the *a*/*d* positions because, in refining the all-α design, we previously found that Leu at this position encouraged intermolecular assembly.[30] Residues at other heptad positions were chosen to promote intrahelical and interhelical salt bridge formation.[37] The 14 residues closest to the C-termini of $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$ and $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ feature a 1:1 α:β backbone pattern. The sequence in this region is based on the sequence in the C-terminal region of all-α design $\mathbf{N_A}$-**C**. Five of the seven β residues in $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$ and $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ have a cyclic constraint (ACPC or APC) that is known to promote 14/15-helical secondary structure among α/β peptides.[38] The other two β residues are β^3-homoleucine (β^3hLeu28 and β^3hLeu32) because the side chains from these residues are intended to form part of the hydrophobic core of the chimeric coiled coil tertiary structure. Also intended to contribute to this core are α-Leu25 and the side chain methylene groups of α-Glu21. In the all-α prototype $\mathbf{N_A}$-**C**, position 21 is Leu, but preliminary work on the chimeric design revealed that Leu at position 21 encourages undesired self-association.[39]

The central six Gly residues in $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$ are intended to form a flexible interhelical loop. In $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$, the fourth of these Gly residues is replaced with an α-thioglycolic acid (tG) residue, which results in a single thioester bond at the center of the sequence. Upon introduction of a thiol (tyrosine-derived thiol $_{HS}$**Y** is employed here), this thioester group participates in thiol–thioester exchange, a process that detaches the α-helical and 14/15-helical segments from one another and precludes tertiary helix–helix packing (Figure 2b). The equilibrium constant (K_{BTE}) for this thiol–thioester exchange provides quantitative insight on the favorability of packing the N-terminal α helix against the C-terminal 14/15 helix.[30–34]

Analytical ultracentrifugation (AU) of $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$ at 100 or 200 μM in 50 mM phosphate (pH 7) indicated that this chimeric polypeptide is monomeric in this concentration range.[39] Analogue $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ could not be analyzed by AU, a process that requires several days, because of slow thioester hydrolysis, but we assume that the self-association propensities of $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$ and $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ are very similar. As all BTE studies with $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ were carried out in the AU concentration range or below, we interpret the BTE results strictly in terms of intramolecular interactions. $\mathbf{N_A}$-$\mathbf{C_{\alpha\beta}}$ and $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ display very similar CD signatures, as expected (see the Supporting Information, Figure S3). Both show a strong minimum at 206 nm and a shoulder at about 222 nm. This signature is consistent with the additive contributions from an α helix (minima at 208 nm and 222 nm) and an α/β-peptide helix (minimum at ca. 206 nm).[21] Helicity in the N- and C-terminal segments of $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ has been encouraged by the incorporation of many side-chain ion-pairing possibilities,[30] and CD analysis of fragments corresponding to the N-terminal or C-terminal halves of $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ indicates extensive helicity in both cases.[39]

BTE analysis of our chimeric coiled-coil design was undertaken by mixing $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ with small thiol $_{HS}$**Y** in pH 7 aqueous buffer. HPLC showed that rapid thiol–thioester exchange occurred, thus generating $\mathbf{N_T}$-**Y** and $_{HS}\mathbf{C_{\alpha\beta}}$. By starting the exchange with a mixture of $\mathbf{N_T}$-**Y** and $_{HS}\mathbf{C_{\alpha\beta}}$, we established that equilibrium is reached within 40 min, with $K_{BTE} = 6.5$.[39] The parameter of greatest interest to us is K_F, the equilibrium constant for folding of $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$; in the folded state, the α and 14/15 helices pack against one another, and in the unfolded state the two helical segments are not in contact (although they probably retain substantial helicity). As previously discussed, $K_{TE} \approx (K_F + 1)$ if the full-length thioester does not self-associate, as in this case, and if the tyrosine side chain does not engage in energetically significant packing interactions with the rest of the molecule in $\mathbf{N_T}$-**Y**, as has been shown previously.[30] Thus, the BTE analysis of $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ suggests that $\Delta G_F = -1.1$ kcal mol^{-1}; that is, that intramolecular packing of the α and 14/15 helices is favored by 1.1 kcal mol^{-1} relative to a lack of such packing interaction. This value is comparable to $\Delta G_F = -1.4$ kcal mol^{-1} deduced for the analogous all-α system (the thioester corresponding to $\mathbf{N_A}$-**C**).[30]

To determine whether ΔG_F deduced for $\mathbf{N_T\text{-}C_{\alpha\beta}}$ corresponds to a specific intramolecular helix–helix packing interaction analogous to an antiparallel coiled-coil, we mutated each of the Leu or β^3hLeu side chains individually to Asn or β^3hAsn. Our design hypothesis is that the six isobutyl side chains in $\mathbf{N_T\text{-}C_{\alpha\beta}}$ would form the hydrophobic core of a helix–loop–helix tertiary structure (Figure 2); therefore, we would predict that changing any of these side chains to the approximately isosteric but hydrophilic Asn/β^3hAsn side-chain should destabilize the intended tertiary structure.[30] As shown in Table 1, each of the six mutations caused ΔG_F to become substantially more positive, in accord with our design hypothesis. The least destabilizing mutation involved one of the hydrophobic side chains nearest the open end of the intended helix–loop–helix conformation (β^3hLeu32). Hydrophobic→hydrophilic mutations at the four "inner" core positions led to about 1 kcal mol^{-1} destabilization.

Table 1: ΔG_F for chimeric ($\alpha+\alpha/\beta$)-peptide $\mathbf{N_T\text{-}C_{\alpha\beta}}$ and hydrophobic core mutants.[a]

Thioester	ΔG_f [kcal mol^{-1}][b]
$\mathbf{N_T\text{-}C_{\alpha\beta}}$	−1.1
Leu3→Asn	−0.2
Leu7→Asn	0.1
Leu10→Asn	−0.1
Leu25→Asn	−0.2
β^3hLeu28→β^3hAsn	0.0
β^3hLeu32→β^3hAsn	−0.4
ACPC30→β^3hLeu	−0.8
ACPC30→β^3hAsn	−0.9

[a] Determined by backbone thioester exchange. Measurements made at 25 °C in 50 mM phosphate (pH 7). [b] Estimated uncertainty of $\Delta G_f \approx \pm$ 0.1 kcal mol^{-1}.

As a control, we mutated ACPC30 to either β^3hLeu or β^3hAsn. In the expected chimeric tertiary structure, ACPC30 should reside on the outward-facing side of the 14/15 helix and therefore interact with solvent rather than participate in the hydrophobic core. The observation that hydrophobic β^3hLeu and hydrophilic β^3hAsn are energetically equivalent at this position suggests that the large destabilizations from Leu/β^3hLeu→Asn/β^3hAsn mutations at *a*/*d* positions do not arise from a difference in intrinsic helix propensity between Leu/β^3hLeu and Asn/β^3hAsn but rather from the unfavorability of burying a hydrophilic primary amide group at the helix–helix tertiary interface.

Bioinformatic analysis of coiled-coils in the protein structure database[31,35,36] and extensive complementary studies with coiled-coil model systems in solution[30,31,40–42] have established preferences among the amino acid side chains that occur at positions *a* and *d* of the α-residue heptad repeat; that is, at the hydrophobic interface of a coiled-coil. We examined mutations at core positions in $\mathbf{N_T\text{-}C_{\alpha\beta}}$ to determine whether comparable side chain preferences are manifested at the tertiary interface formed between an α helix and a 14/15 helix (Table 2). We chose core positions that are centrally located within the helical segments for these experiments: Leu7 in the α helix and β^3hLeu28 in the 14/15 helix. The trend at Leu7 is typical for coiled-coils, with Asn highly destabilizing at this position, Ala moderately destabilizing, and Ile and Val comparable to Leu. The trend at β^3hLeu28 is similar, except that β^3hVal is somewhat destabilizing relative to β^3hLeu. These results suggest that the tertiary interface formed between the α helix and the 14/15 helix features an intimate interdigitation of side chains that is comparable to the knobs-into-holes pattern observed in coiled-coils (pure α backbone).[43] At position 30, which should reside on the solvated surface of the chimeric tertiary structure, there is little or no distinction among the five β^3-homoamino acid residues we examined, which suggests that all five have similar intrinsic helical propensities.

Table 2: Thermodynamic consequences of mutations at selected hydrophobic-core and solvent-exposed residues in chimeric ($\alpha+\alpha/\beta$)-peptide $\mathbf{N_T\text{-}C_{\alpha\beta}}$.[a]

Position in $\mathbf{N_T\text{-}C_{\alpha\beta}}$	Mutation	$\Delta(\Delta G_F)$ [kcal mol^{-1}][b]
Leu7		
	Asn	+1.2
	Ala	+0.7
	Val	+0.1
	Ile	−0.2
β^3hLeu28		
	β^3hAsn	+1.1
	β^3hAla	+0.7
	β^3hVal	+0.4
	β^3hIle	+0.1
	ACPC	+0.4
ACPC30		
	β^3hAsn	+0.2
	β^3hAla	+0.5
	β^3hVal	+0.3
	β^3hIle	+0.4
	β^3hLeu	+0.3

[a] Measurements made at 25 °C in 50 mM phosphate (pH 7). [b] Estimated uncertainty of $\Delta(\Delta G_f) \approx \pm 0.1$ kcal mol^{-1}.

We used BTE to examine the contribution of β-residue cyclic constraints, such as those found in ACPC and APC, to overall conformational stability. Previous studies of short α/β peptides provided qualitative evidence that the helical propensity of acyclic β residues is lower than that of ACPC.[38] Similar conclusions have been suggested by more recent work with longer α/β peptides containing 25–33% β residues that self-associate to form homomeric helix bundles.[14,21] The BTE approach offers quantitative insight. The data for ACPC30 mutations in Table 2 suggest that the backbone constraint in a single β residue contributes about 0.3 kcal mol^{-1} to global conformational stability of the chimeric tertiary structure. An interesting contrast is observed at core position 28, where replacing a flexible hydrophobic β^3hLeu residue with a rigid hydrophobic ACPC residue causes a 0.4 kcal mol^{-1} loss of global conformational stability. Presumably the non-backbone carbons of the ACPC residue do not pack efficiently at the interhelical interface in this case. All five of the cyclically constrained β residues in $\mathbf{N_T\text{-}C_{\alpha\beta}}$ are intended to occupy non-core positions in the helix–loop–helix tertiary structure. When these five constrained residues were simultaneously replaced with analogous flexible β residues ($3\times$ACPC→β^3hLeu and $2\times$APC→β^3hArg), $\Delta G_F = +1.1$ kcal mol^{-1}; that is, the total

decrease in conformational stability is 2.2 kcal mol^{-1}. Thus, the average effect is about 0.4 kcal mol^{-1} per cyclic→acyclic modification, which is consistent with the results obtained for the five different cyclic→acyclic replacements evaluated at position 30 (Table 2).

We have shown that the BTE method offers a useful way to detect and probe tertiary contacts between conventional peptide and foldamer segments. The position of the thiol–thioester exchange equilibrium shown in Figure 2b (parent sequence), which reveals a preference for $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ and small thiol $_{\mathbf{HS}}\mathbf{Y}$ relative to the alternative pair, provides strong evidence that the α and α/β peptide segments of full-length thioester $\mathbf{N_T}$-$\mathbf{C_{\alpha\beta}}$ engage in a stabilizing intramolecular interaction in aqueous solution. The impact of Leu→Asn and β^3hLeu→β^3hAsn replacements (Table 1) supports our design hypothesis regarding the nonpolar side chains that should comprise the hydrophobic core of the helix–loop–helix tertiary structure. Nonpolar side chains from the α-peptide and α/β-peptide segments appear to experience similar packing environments in the core, as judged by the impact of mutations at a representative site in each segment (Table 2). This experimental design has allowed us to make the first quantitative assessment of the difference between a cyclic β-amino acid residue and an acyclic analogue in terms of folding thermodynamics. As expected, the cyclic constraint enhances conformational stability (ΔG), presumably because this constraint diminishes the conformational entropy loss associated with folding. This initial study suggests that the BTE method will be generally useful for exploring conformational preferences of foldamers and of hybrid structures containing foldameric subunits.

Received: August 24, 2009
Published online: December 3, 2009

Keywords: biophysics · foldamers · peptides · protein folding · thioesters

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