Published in final edited form as:

J Am Chem Soc. 2017 September 27; 139(38): 13292–13295. doi:10.1021/jacs.7b07930.

Helix Propensities of Amino Acid Residues via Thioester Exchange

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Abstract

We describe the use of thioester exchange equilibria to measure the propensities of amino acid residues to participate in helical secondary structure at room temperature in the absence of denaturants. Thermally or chemically induced unfolding has previously been employed to measure α -helix propensities among proteinogenic α -amino acid residues, and quantitative comparison with precedents indicates that the thioester exchange system is reliable for residues that lack side chain charge. This system allows the measurement of α -helix propensities for D- α -amino acid residues and propensities of residues with non-proteinogenic backbones, such as those derived from a β -amino acid, to participate in an α -helix-like secondary structure.

Graphical Abstract

The functional diversity of proteins emerges from structural diversity. Thousands of tertiary folds are known; however, poly-L- α -amino acid backbones appear to favor only a very small number of regular secondary structures, among which the α -helix and β -sheet are dominant. This situation has sparked interest in establishing quantitative scales for secondary structure “propensity” among the proteinogenic amino acid residues,¹ because these propensities must be essential to the relationship between a protein’s sequence and its native folding preference. As synthetic advances provide access to proteins containing unnatural side

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Notes

The authors declare no competing financial interest.

Supporting Information

Experimental details including peptide synthesis, thioester exchange UPLC chromatograms, and comparisons of thioester exchange data with other helix propensity scales. This material is available free of charge via the Internet at <http://pubs.acs.org>.

chains and ultimately push the concepts of secondary and tertiary structure beyond L- α -amino acid-derived subunits,² the challenge of determining subunit secondary structural propensities broadens.

Here we describe an approach that enables the helix propensity of a “guest residue” to be linked quantitatively to a chemical exchange process for which the equilibrium constant can be measured by HPLC or UPLC at room temperature in the absence of denaturants. The resulting α -helix propensity ranking for a set of proteinogenic residues is found to correlate quantitatively with precedents based on more classical experimental designs. In the classical approach, a chemical denaturant is employed to disrupt the folded state, as monitored by circular dichroism or other spectroscopic method, and extrapolation is required to derive thermodynamic parameters in the absence of denaturant.³ Because of the small size of the peptide components required, our approach can be readily employed to evaluate subunits that are not derived from L- α -amino acids, as we illustrate by extension to a D- α -amino acid residue and to β -amino acid residues.

Our measurement strategy is based on thiol-thioester exchange reactions (Figure 1), which rapidly reach equilibrium in aqueous solution at neutral pH.⁴ In properly designed systems, the thiol-thioester exchange equilibrium constant can be related to the stability of the tertiary structure formed by a long thiodepsipeptide, such as **TE-T** in Figure 1 (right side). This experimental design has been useful for examining factors that control the packing of α -helices against one another in coiled coils, which are common tertiary structural motifs in proteins.⁵ Specifically, we have used the thioester exchange strategy to evaluate thermodynamic contributions of side chains at the α -helical interface to coiled-coil stability.⁶ Here we show that varying residues at sites that are distal to the helix-helix interface provides an accurate measure of α -helical propensity for residues bearing side chains that are not charged.

The system used for the current experiments (Figure 2) emerged from a previous study⁷ and was designed based on the heptad repeat pattern that is characteristic of sequences that form coiled coils.⁵ In thiodepsipeptide **TE-T**, Leu and Ile are placed at heptad positions *a* and *d* to drive intramolecular packing of the two helical segments via knobs-into-holes interactions. The exterior faces of both helices, defined by heptad positions *b*, *c* and *f*, feature ionic side chains to discourage intermolecular associations. The *e* and *g* residues are cationic for the thioester peptide helix (designated **TE**), and the *e* and *g* residues are anionic for the thiol peptide helix (designated **T**); this Coulombic complementarity should stabilize the intended parallel coiled-coil tertiary structure via interhelical salt bridges.⁸ Arg or Glu was selected for specific *b*, *c* and *f* positions so as to maximize the number of intrahelical *i,i*+3 and *i,i*+4 salt bridges⁹ and thereby stabilize the individual α -helices and the intramolecular coiled-coil in **TE-T**. The two α -helix-forming components are linked via a flexible Gly-rich segment in **TE-T**. The labile thioester bond is located near the center of this linker.

Factors that affect the stability of an isolated α -helix also affect the stability of tertiary¹⁰ or quaternary¹¹ structures that incorporate that helix. We selected *f* positions as “guest” sites in the **T** segment for evaluation of α -helical propensities because residues in *f* positions should

not contact the helix formed by the **TE** segment upon adoption of the helix-loop-helix tertiary structure by **TE-T**. Therefore, any effect on tertiary stability caused by changing guest residue identity should reflect the impact of the guest on α -helix stability. Two variable sites at *f* positions, bearing identical guest residues, were employed to maximize the resulting effects on stability. Because both sites reside in the thiol peptide, each new measurement requires the synthesis of only one new **T** peptide. The **TE** component is invariant in these studies.

Initial evaluation focused on the version of **T** with Glu residues at both guest sites, which corresponds to a variant from the previously studied system.⁷ Thioester exchange measurements indicated $G_{\text{fold}} = -2.32$ kcal/mol for this **TE-T** variant ($G_{\text{fold}} = -RT \ln(K_{\text{fold}})$; K_{fold} defined in Figure 1; see ref. 4a for calculation of K_{fold} based on K_{ex}). This value did not change significantly when peptide concentration was varied between 25 and 200 μM , which suggests that K_{ex} measured in this range is not influenced by intermolecular interactions (Figure 3a).¹² This concentration-independence is essential if a reliable G_{fold} value is to be derived from the thioester exchange equilibrium constant.^{4,6}

Our experimental design stipulates that tertiary folding is driven by burial of hydrophobic surfaces (*a* and *d* side chains) on each helical segment. This hypothesis was tested by replacing one of the *d* position Leu residues in the **T** component with Asn, the polar side chain of which is not well accommodated within a hydrophobic coiled-coil interface.⁶ If the formation of the predicted hydrophobic interface drives tertiary folding in our system, then the Leu→Asn change should be destabilizing. This prediction was borne out: $G_{\text{fold}} = -0.70$ kcal/mol for the Asn-containing **T** variant with Glu at the back-face guest sites, a decline in tertiary structure stability of ≈ 1.5 kcal/mol relative to the variant containing Leu at the *d* position (Figure 3b).

Having validated the thioester exchange system, we examined several variants in which the **T** component bore identical residues at the two guest sites (Table 1). Precedents^{11,13} suggest that the guests we employed, Ala, Leu, Ser, Val, Thr and Gly, span a wide range of α -helical propensities among proteinogenic residues. Only proline, which is strongly destabilizing, lies outside this range. Ala and Leu are generally judged to have the strongest α -helical propensities among residues with non-ionizable side chains. Ser, Val and Thr have more moderate α -helical propensities, and the flexible Gly residue has a low propensity. For the cases with Ala or Gly residues in the guest sites, we evaluated the controls in which a *d*Leu residue was replaced with Asn. In both cases, large declines in G_{fold} result from this interfacial Leu→Asn modification (Figure 3b). These observations support the conclusion that tertiary folding of **TE-T** requires formation of the expected coiled-coil interface.

α -Helical propensities of proteinogenic residues have been measured based on variations at guest sites in diverse polypeptide hosts,^{11,13–14} and these precedents offer a basis of comparison for the results in Table 1. Several studies have involved synthetic peptides that autonomously adopt α -helical conformations in solution.^{11,13c,15} In other systems, α -helix formation has been linked to adoption of higher-order structure.^{13b,14,15c} For comparisons with our results, we focused on two previous studies. O’Neil and DeGrado designed a host sequence that forms a parallel coiled-coil homodimer with the guest site on the back face of

each helix.¹¹ Fersht *et al.* developed a barnase-based system in which the guest site is a solvent-exposed position on an α -helix within the tertiary structure of the protein.¹⁴ In both systems, thermodynamic comparisons among variants with different guest residues were conducted in the presence of substantial urea concentrations. Thioester exchange measurements, in contrast, are conducted in the absence of denaturants.

α -Helix propensities derived from thioester exchange measurements for the six proteinogenic residues lacking an ionized side chain show a good quantitative correlation with results from O'Neil and DeGrado (Figure 4a).¹¹ A good correlation is observed also with results from Fersht *et al.* (Figure 4b)¹⁴ and with helix propensity scales described by others.¹² However, our system indicates that Glu, a guest residue that bears a charged side chain under the measurement conditions, is substantially more favorable for α -helix formation than is reported by O'Neil and DeGrado¹¹ or by Fersht *et al.*¹⁴ We attribute this difference to the fact that the side chain carboxylates of the guest Glu residues in the thiol peptide can engage in favorable intrahelical Coulombic interactions with nearby Arg residue side chains in the folded state. In contrast, the systems employed by O'Neil and DeGrado¹¹ and by Fersht *et al.*¹⁴ were carefully designed to minimize interactions of guest residue side chains with neighboring sites in the folded state.

Our explanation of Glu behavior is supported by percent helicity predicted with the program AGADIR¹⁶ for the **T** peptides bearing different guest residues (Figure 4c). AGADIR takes into account Coulombic interactions between ionized side chains that are juxtaposed upon helix formation ($i, i+3$ and $i, i+4$ sequence relationships). The calculated percent helicity values for **T** peptides, including the variant with Glu in the guest sites, correlate well with G_{fold} values derived from thioester exchange measurements. Circular dichroism (CD) data for the **T** peptides lend additional support to our explanation for the high G_{fold} value for Glu as guest relative to the experimental scales from O'Neil and DeGrado and from Fersht *et al.* (Figure 4d). The variant of **T** containing Glu displays a very strong minimum at 223 nm, which is characteristic of α -helicity, as expected if intrahelical electrostatic interactions contribute to G_{fold} for this variant.

There is growing interest in the use of site-specifically incorporated D- α -amino acid residues for the design of bioactive peptides and proteins,^{2d} and we therefore used the thioester exchange system to evaluate the propensity of D-Ala to be incorporated into a right-handed α -helix, i.e., an α -helix composed largely of L- α -amino acid residues. The results indicated that D-Ala is substantially more destabilizing to a right-handed α -helix than is Gly, which is qualitatively consistent with previous reports.¹⁷ DeGrado *et al.*^{17a} used a coiled-coil homodimer to address this question and determined that incorporation of a D-Ala residue into an α -helix is 0.18 kcal/mol less favorable than incorporation of a Gly residue. Krause and coworkers^{17c} found that D-Ala was approximately 0.5 kcal/mol less favorable than Gly in an α -helix, using a monomeric model system. The latter value is consistent with our thioester exchange results, which suggest that D-Ala is 0.55 kcal/mol less favorable than Gly in a right-handed α -helix. Given the quantitative correlation between our system and that of O'Neil and DeGrado for L- α -amino acid residue helix propensities in the absence of side chain charge (Figure 4a), the origin of the discrepancy in terms of D-Ala

is unclear. Perhaps the α -helix propensity of D-Ala, and D residues generally, is more sensitive to local environment than are propensities of L residues.

The new system was used to evaluate the propensity of β -amino acid residues to participate in an α -helix-like conformation. These experiments were designed to detect the impact of a side chain on helix stability. β^3 -Homoalanine (β^3 hAla) is the homologue of alanine that contains an additional methylene between the side chain-bearing carbon and the carbonyl carbon, and β -homoglycine (β hGly) has no side chain. Thioester exchange measurements indicate $G_{\text{fold}} = -0.86$ kcal/mol for the **TE-T** variant containing β^3 hAla at the guest positions. When these two β substitutions are combined with a *d* position Leu $\rightarrow$ Asn substitution, $G_{\text{fold}} = +0.16$ kcal/mol. The ≈ 1 kcal/mol destabilization resulting from this Asn substitution suggests that formation of the coiled-coil interface remains the driving force for tertiary folding of **TE-T** after the backbone has been altered via two $\alpha\rightarrow\beta$ modifications. This conclusion is consistent with previous crystallographic demonstrations that peptides containing combinations of α and β^3 residues can adopt an α -helix-like secondary structure.¹⁸ Thioester exchange experiments using the **T** peptide with β hGly at the guest positions gave $G_{\text{fold}} = -0.47$ kcal/mol. Thus, the data suggest an energetic penalty of approximately 0.2 kcal/mol for each β^3 hAla $\rightarrow\beta$ hGly substitution. This trend is consistent with the destabilization of the α -helix that has been generally observed to result from replacement of Ala with Gly.^{11,13–15} Direct comparisons in the context of our thioester exchange system suggest that the beneficial effect of a methyl side chain may be smaller for β residues than for α residues (Figure 5).

The thioester exchange measurement involving β^3 hAla at the guest sites implies that each $\alpha\rightarrow\beta$ substitution causes a conformational destabilization of 0.6 kcal/mol per residue, because Ala at the guest site yielded $G_{\text{fold}} = -2.07$ kcal/mol. We propose that the conformational destabilization associated with $\alpha\rightarrow\beta$ substitution arises largely from the impact of backbone modification (insertion of a methylene unit) on helical stability, since structural evidence shows that β^3 residues are compatible with an α -helix-like secondary structure.^{18c} More specifically, we propose that Ala $\rightarrow\beta^3$ hAla substitution is destabilizing to the helical state because of the additional backbone bond, which has relatively low barriers to rotation, in the β^3 residue. Restriction of rotation upon folding might increase the entropic cost of helix formation for a β^3 hAla residue relative to an Ala residue; however, experiments by Reinert and Horne have shown that $\alpha\rightarrow\beta^3$ substitution in a different tertiary context causes a helix stability penalty that is enthalpic.¹⁹ It is possible that the backbone modification in our system causes subtle changes in the arrangement of side chains projected from the other side of the helix²⁰ (positions *a* and *d*), which could affect the energetics of knobs-into-holes packing at the coiled-coil interface. Such changes in helix-helix packing energy would contribute to G_{fold} . Whether or not the unfavorable 0.6 kcal/mol increment associated with $\alpha\rightarrow\beta$ substitution can be attributed entirely to a change in helical propensity, this measurement provides valuable insight on the energetic consequences of α residue-to- β^3 residue substitution in the context of an α -helix-like conformation.

We have shown that thioester exchange equilibria can provide quantitative measurements of α -helical propensity for α -amino acid residues bearing uncharged side chains under “native” conditions (no denaturant; room temperature). Moreover, we have shown that this

operationally convenient approach can be extended to non-proteinogenic subunits, including D- α -amino residues and β -amino acid residues. There is growing interest in the structures and functions of polypeptide analogues containing subunits with unnatural backbones,^{2,21} and this interest is magnified by the prospect that these unnatural subunits may be brought into the realm of ribosomal incorporation.²² The results described above suggest that thioester exchange offers a versatile approach for analyzing the impact of backbone modification on conformational stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the National Science Foundation (CHE-1565810). The authors thank Dr. Young-Hee Shin for providing small thiol **Y** and Prof. Jay Steinkruger for helpful comments.

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Figure 1.

Outline of thioester exchange experiment. **X** indicates positions of guest residues for which helix propensities can be evaluated. $[TE-T]_u$ and $[TE-T]_f$ indicate folded and unfolded states of the coiled-coil-forming full-length thiodepsipeptide, respectively.

Figure 2.

(a) Helix-wheel diagram of thioester peptide **TE** (with small molecule **Y** as the thiol segment, highlighted in orange) and thiol peptide **T**. (b) Sequence of full-length thiodepsipeptide **T-TE**. Succ = succinyl.

Figure 3.

(a) Thioester exchange-derived G_{fold} at different concentrations of **T** and **TE**. Red circles indicate results from individual experiments. Error bars are $\pm$ standard deviation. “Asn” in parentheses indicates **T** variant with Leu $\rightarrow$ Asn substitution at coiled-coil interfacial residue 13'. (b) G_{fold} measured for **T** variants with various guest residues and their coiled-coil interfacial Leu $\rightarrow$ Asn counterparts.

Figure 4.

(a) Comparison of thioester exchange-derived G_{fold} values (G_{fold} per residue normalized to the value for the variant of **T** containing Gly at guest sites) with helix propensities measured in the coiled-coil model system of O'Neil and DeGrado (G_{pept}).¹¹ The datum for the variant of **T** containing Glu, indicated in red, was omitted from the linear regression. (b) Comparison of G_{fold} with helix propensities measured in the barnase system of Fersht *et al.* (G_{prot}).¹⁴ The datum for the variant of **T** containing Glu, indicated in red, was omitted from the linear regression. (c) Comparison of G_{fold} with AGADIR-predicted helix content for the **T** peptides. (d) Comparison of G_{fold} with per-residue molar ellipticity of **T** peptides at 223 nm (100 μM **T** variant, 50 mM phosphate, pH 7.0, 2 mM TCEP, 20°C).

Figure 5. Thioester exchange-derived G_{fold} for T peptides containing Ala, Gly, $\beta^3\text{hAla}$, or βhGly . Error bars are $\pm$ standard deviation.

Table 1

G_{fold} measured by thioester exchange for thiol peptides containing various guest residues.^a

Guest Residue	G_{fold} (kcal/mol) ^b
Glu	-2.32 ± 0.14
Glu(Asn) ^c	-0.70 ± 0.16
Ala	-2.07 ± 0.12
Ala(Asn) ^c	-0.39 ± 0.04
Leu	-1.91 ± 0.05
Ser	-1.56 ± 0.03
Val	-1.46 ± 0.07
Thr	-1.13 ± 0.10
Gly	-1.06 ± 0.06
Gly(Asn) ^c	$+0.02 \pm 0.12$
D-Ala	$+0.05 \pm 0.12$
$\beta^3\text{hAla}$	-0.86 ± 0.14
$\beta^3\text{hAla(Asn)}^c$	$+0.16 \pm 0.15$
βhGly	-0.47 ± 0.05

^a $G_{\text{fold}} = -RT \ln(K_{\text{fold}})$; K_{fold} is defined in Figure 1. The guest positions are defined in Figure 2. See ref. 4a for calculation of G_{fold} based on K_{ex} . Conditions: 50 μM each **T** and **TE**, 50 mM phosphate, pH 7.0, 2 mM TCEP, room temperature.

^b Mean $\pm$ standard deviation of at least five separate experiments.

^c Leu $\rightarrow$ Asn substitution at coiled-coil interfacial residue 13' of **T**.