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In Situ Monitoring of Backbone Thioester Exchange by ^{19}F NMR

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Backbone thioester exchange (BTE) is a new technique for assessing the stability of folding patterns adopted by polypeptides.[1–5] Implementation of this method requires the replacement of a single backbone amide group in the polypeptide of interest with a thioester; this replacement is an approximately isostructural substitution.[6,7] Combining the backbone-modified polypeptide analogue (a thiodepsipeptide) with a small thiol in pH 7 aqueous buffer initiates a thiol–thioester exchange reaction.[8] The equilibrium constant for this exchange is strongly influenced by the stability of the folded conformation adopted by the full-length thiodepsipeptide. We have shown that BTE enables examination of sequence–stability relationships for a variety of small folding motifs under native conditions including β -hairpins, coiled coils and a small protein, the chicken villin headpiece subdomain.[1–5] BTE analysis is complementary to traditional methods of assessing protein conformational stability, in which the folding pattern is disrupted with heat or chemical additives.[9] The traditional approaches require extrapolation to obtain folding parameters that apply under native conditions, while BTE enables direct measurement under native conditions. BTE has provided new insights on common protein folding motifs[1,3–5] and on contributions of unnatural side chains to tertiary structural stability,[2] but continued development is necessary to maximize the utility of this new tool.

Here we report that quantitative BTE analysis can be conducted in situ by using ^{19}F NMR if the thiols and thioesters are appropriately labeled. Previous BTE analyses have relied upon HPLC to separate and quantify the populations of the thiol and thioester species after equilibrium has been attained.[1–5] We show that ^{19}F NMR offers an alternative method for determining the thiol–thioester exchange equilibrium constant (K_{BTE}). In addition, we find that ^{19}F NMR offers a very simple way to estimate folded state stability for the full-length thiodepsipeptide even before chemical equilibrium has been achieved, if sufficient calibration data are available. Thus, BTE by ^{19}F NMR enables rapid screening of sets of peptide variants to identify sequences that confer the greatest conformational stability.

To investigate the feasibility of BTE analysis by ^{19}F NMR we used an antiparallel coiled-coil system previously developed in our laboratory (Scheme 1).[3,5] This system contains two 14-residue segments that are intended to form α -helices and an intervening glycine-rich segment that contains the thioester. The α -helical segments feature heptad repeat motifs that are typical

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of coiled-coil-forming sequences, with hydrophobic residues, such as Leu, at the first and fourth positions (positions *a* and *d* in an *abcdefg* heptad).[10,11] The other residues in the α -helical segments are mostly Glu or Arg and are arranged to allow maximal formation of intra- and interhelical salt bridges and thereby stabilize the desired helix-loop-helix tertiary structure. [12] Packing of the α -helical segments against one another is driven by dehydration of the nonpolar side chains that form the core of the antiparallel coiled coil. Shape complementarity among the buried side chains is an important determinant of tertiary structural stability, as shown by previous HPLC-monitored BTE studies involving mutations at core positions (a_1' , d' , a_2' in the C-terminal helix and *a* in the N-terminal helix).[3,5] Sequence–stability relationships manifested in this model system were found to agree with those observed in a completely independent model system and with those detected by using proteomic analysis of antiparallel coiled coils in the protein structure database.[5] These correlations establish our model system as a useful tool for analyzing the factors that control the stability of natural coiled coils.

^{19}F NMR has previously been used to study folding processes in proteins and nucleic acids, [13] but two features of our use of ^{19}F NMR to assess BTE equilibria distinguish our work from these important precedents. First, determination of K_{BTE} by this method involves monitoring not simply a conformational equilibrium but rather a chemical equilibrium that is coupled to the conformational equilibrium of interest. Second, in previous applications of ^{19}F NMR to study biopolymer folding, the fluorous tag has been placed near the center of the folded unit, which maximizes the impact of folding on $\delta^{19}\text{F}$. [13b,d,e,i,j] Our goals, however, require placement of the fluorous tag at the *periphery* of the folding unit. We seek to use BTE to understand the ways in which amino acid sequence governs helix pairing preferences among antiparallel coiled coils in natural proteins, and previous work has shown that fluorinated side chains placed in the hydrophobic core of a coiled coil (*a* and/or *d* sequence positions) can exert a profound and unnatural impact on stability,[14] which discourages such placement in our system. When we began this work it was not obvious that peripheral positioning of fluorous tags would confer sufficient chemical shift sensitivity to enable measurement of K_{BTE} via ^{19}F NMR.

Preliminary studies indicated that the thiol–thioester equilibrium for the parent sequence ($a = a_1' = d' = a_2' = \text{Leu}$) could be readily monitored with ^{19}F NMR in cases in which the “small thiol” component ($\text{H}_\text{S}\text{F}$ in Scheme 1) contains a *p*- CF_3 -anilide group and the C-terminal residue of the thiol-peptide and full-length thiodepsipeptide ($\text{H}_\text{S}\text{Cf}$ and $\text{N}_\text{T}\text{-Cf}$ in Scheme 1) is 3,5-difluorophenylalanine (f_2Phe). ^{19}F resonance sensitivity is maximized by the use of tags that contain multiple fluorine atoms. Resonance separation is promoted by pairing a tag that has fluorine on an sp^3 carbon (resonances around -110 ppm) with a tag that has fluorine on an sp^2 carbon (resonances around -62 ppm). Each tag will experience two different molecular environments in a thiol–thioester exchange, and the crucial question is whether the ^{19}F resonances from these two environments are sufficiently resolved. As seen in Figure 1, the four species in the thiol–thioester exchange equilibrium give rise to four distinct ^{19}F resonances that display good signal-to-noise ratios at concentrations typically employed for BTE measurements (total thiol and thioester concentrations ~ 0.1 – 0.2 mM). The CF_3 singlet arising from small thiol $\text{H}_\text{S}\text{F}$ is 0.097 ppm upfield of the CF_3 singlet arising from $\text{N}_\text{T}\text{-F}$, which is the thioester formed from reaction of the full-length thiodepsipeptide $\text{N}_\text{T}\text{-Cf}$ with $\text{H}_\text{S}\text{F}$. The multiplet arising from f_2Phe in full-length thiodepsipeptide $\text{N}_\text{T}\text{-Cf}$ is 0.25 ppm downfield of the f_2F multiplet arising from $\text{H}_\text{S}\text{Cf}$.

We integrated the ^{19}F resonances for the parent system at equilibrium to determine K_{BTE} ($[\text{N}_\text{T}\text{-Cf}][\text{H}_\text{S}\text{F}]/[\text{N}_\text{T}\text{-F}][\text{H}_\text{S}\text{Cf}]$), which enables quantitative comparison with the analogous measurement made previously by using HPLC. The molecules used for the HPLC studies[3] differ slightly from those employed for ^{19}F NMR analysis. The “small thiol” component in the

HPLC study (designated $_{HS}Y[3]$) was formed by acylating tyrosine-*N*-methyl amide (rather than *p*-CF₃-aniline) with thioglycolic acid, and the C-terminal residue on the C-terminal helical segment was tyrosine (rather than f₂Phe). We anticipated that these differences would have little effect on K_{BTE} , because the fluorine tags are placed at the periphery of the coiled-coil hydrophobic core, and the values determined by ¹⁹F NMR and HPLC did indeed prove to be similar. ¹⁹F NMR analysis produced $K_{BTE} = 8.9$, which corresponds to $\Delta G_{Fold} = -1.2$ kcal mol⁻¹; we previously deduced $\Delta G_{Fold} = -1.4 \pm 0.1$ kcal mol⁻¹ from HPLC data for the closely related system.[3]

In addition to using ¹⁹F NMR to *measure* K_{BTE} , we became interested in the possibility that $\delta^{19}F$ for N_T-Cf could be used to *estimate* K_{BTE} and thereby streamline the screening of a large set of sequence variants to identify those sequences that confer the highest conformational stability. The single ¹⁹F resonance observed for full-length thiodepsipeptide N_T-Cf represents a population-weighted average of contributions from unfolded and folded conformations, which interconvert rapidly on the NMR time scale. $\delta^{19}F$ arising from the unfolded state of N_T-Cf is presumably very similar to $\delta^{19}F$ measured for $_{HS}Cf$. We postulate that $\Delta\delta^{19}F$ for N_T-Cf vs. $_{HS}Cf$ ($\Delta\delta^{19}F = \delta^{19}F(N_T-Cf) - \delta^{19}F(_{HS}Cf)$) reflects the contribution to $\delta^{19}F$ of N_T-Cf from the folded state, in which the local environment of the f₂Phe side chain is influenced by the proximity of the N-terminal segment. We were gratified by the size of $\Delta\delta^{19}F$ for the f₂Phe tag (0.25 ppm); it was not obvious at the outset that $\delta^{19}F$ would be sufficiently sensitive to the presence of the other helical segment, because f₂Phe is located at a terminal position. These preliminary observations raised the possibility that $\Delta\delta^{19}F$ for the C-terminal f₂Phe side chain could provide a basis for rapid estimation of ΔG_{Fold} among a large set of sequence variants, to identify the most stable examples for more thorough characterization. This possibility is attractive because it would not be necessary to wait for the system to reach equilibrium (~40 min), and because the $\Delta\delta^{19}F$ measurement can be made in <5 min.[15]

The hypothesis that $\Delta\delta^{19}F(f_2Phe)$ provides a basis for estimating ΔG_{Fold} was tested by measuring $\Delta\delta^{19}F(f_2Phe)$ for 25 variants of the antiparallel coiled-coil design in which core positions *a* and *d'* were independently varied among Leu, Ile, Val, Ala and Asn ($a_1'=a_2'=Leu$ in each case; $a=d'=Leu$ is the parent sequence discussed above). The *a* and *d'* side chains pack next to one another upon antiparallel coiled coil formation; we have referred to this type of pairing as “lateral” because a line connecting the two side chains is roughly perpendicular to the long axes of the helices.[3,5] Previously we used BTE monitored by HPLC to measure ΔG_{Fold} for slightly different versions of these 25 variants (containing Tyr, rather than fluorinated tags, to allow HPLC detection; ΔG_{Fold} range = +0.8 to -1.7 kcal mol⁻¹). Figure 2 shows that there is a reasonably good linear correlation ($R^2=0.93$) between $\Delta\delta^{19}F(f_2Phe)$ and ΔG_{Fold} measured by using HPLC for Tyr-containing systems.[15]

The correlation in Figure 2 encouraged us to use $\Delta\delta^{19}F(f_2Phe)$ measurements to try to identify the most stable coiled coils among a new set of sequence variants. In this set we explored packing interactions involving “vertical” contacts at the coiled-coil interface, that is, contacts for which a line between neighboring side chains is roughly parallel to the long axes of the helices. We recently used BTE monitored by HPLC to demonstrate that vertical interactions can exert a significant effect on antiparallel coiled-coil stability.[5] That study involved vertical “triads” formed by the $a_1'-a-a_2'$ residue cluster in our model system (Figure 3) and focused on distinctions between Leu-X-Leu and Ile-X-Ile triads. Data from the model system indicated that heterotriads Leu-Ile-Leu and Ile-Leu-Ile are preferred relative to the isomeric homotriads Leu-Leu-Leu and Ile-Ile-Ile; proteomic analysis revealed the same preference among antiparallel coiled coils in the Protein Data Bank. A set of 27 $a_1'-a-a_2'$ vertical triads can be generated from the residues Leu, Ile and Val. Six of the possibilities were examined in our previous work (those with $a_1'=a_2'=Leu$ or $a_1'=a_2'=Ile$). We used the $\Delta\delta^{19}F$ approach to screen 15 previously unexplored triads from this set. As indicated in Table 1, $\Delta\delta^{19}F(f_2Phe)$ varied

between 0.09 and 0.35 ppm (see also Table S4). The correlation between $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$ and ΔG_{Fold} (Figure 2) suggests that the most favorable $\text{a}_1'-\text{a}-\text{a}_2'$ vertical triad among the new set is Ile-Leu-Leu (largest $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$), the next most stable is Ile-Ile-Leu, and the least stable is Val-Val-Val (see also Figure S4).

To test the validity of the $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$ -based estimates of ΔG_{Fold} among the new sequences, we selected four of the 15 new sequences for full-fledged BTE equilibrium analysis by ^{19}F NMR and, independently, by HPLC (the HPLC analysis involving Tyr-bearing peptides). [16] This subset included the two vertical triads with the largest $\Delta\delta^{19}\text{F}$ values, the one with the smallest $\Delta\delta^{19}\text{F}$ value and one with an intermediate $\Delta\delta^{19}\text{F}$ value (Table 1). Comparisons among the estimated ΔG_{Fold} values based on $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$ analysis and the measured ΔG_{Fold} values based on BTE/HPLC or BTE/ ^{19}F NMR indicate reasonably good correlation among these three approaches. For the two BTE measurements (two rightmost columns in Table 1), ΔG_{Fold} values are within experimental error for three of the four sequences, and for the fourth sequence the two ΔG_{Fold} values are similar. The $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$ -based estimates of ΔG_{Fold} (middle column in Table 1) suggest a clear order of stability among the four sequences examined, and this order is matched in the ΔG_{Fold} measurements from BTE/HPLC. A similar order is found in the ΔG_{Fold} measurements from BTE/ ^{19}F NMR, but in this case the two most stable coiled coils are not distinguishable. Overall, the correlations in Table 1 indicate that $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$ data can be used for rapid identification of the most stable coiled coils among a set of previously uncharacterized sequences, based on comparison with a benchmark data set.[17] In addition, the results in Table 1 provide evidence that in situ measurement of K_{BTE} with ^{19}F NMR gives results that are comparable to those obtained with HPLC analysis.

Our findings significantly expand the utility of the BTE technique for evaluating conformational stability of polypeptides, because in situ analysis with ^{19}F NMR is complementary in useful ways to HPLC analysis, and modern instruments enable rapid and sensitive ^{19}F NMR spectroscopy on a routine basis. One advantage of the ^{19}F NMR approach is illustrated here: differences in conformational stability can be estimated based on $\Delta\delta^{19}\text{F}$ for the full-length thiopeptide vs. a fragment, even when the fluorine tag is placed at a peripheral position (to minimize the contribution of the tag to the driving force for folding). Thus, if a correlation between ΔG_{Fold} and $\Delta\delta^{19}\text{F}$ is observed among a set of sequence variants, as we find in the antiparallel coiled-coil system discussed above, then this correlation can serve as a basis for rapid identification of the most stably folded among a set of new sequences in the same family. In our example, this screening approach enabled us to identify new vertical triads at the antiparallel coiled-coil interface that confer the highest conformational stability we have measured to date. In addition, the use of ^{19}F NMR is likely to have advantages relative to HPLC for measurement of K_{BTE} and ΔG_{Fold} in many systems, because we have found that it is sometimes challenging to achieve the necessary resolution of analytes with HPLC.

Experimental Section

Peptide synthesis

Details of peptide solid-phase synthesis and purification of peptides can be found in the Supporting Information.

Backbone thioester exchange (BTE) assays

NMR assays were initiated by mixing approximately equal portions of C-terminal thiol peptide $_{\text{HS}}\text{Cf}$ and the desired thioester (typically 0.1–0.2 mM). Assays were conducted in buffer (5:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$ phosphate buffer, pH 7, 42 mM) and TCEP (1.7 mM). For full BTE experiments, a 75 min equilibration time was included prior to measurement. For $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$ -based measurements, NMR data were acquired immediately after mixing. A detailed description of

NMR methods can be found in the Supporting Information. Details of BTE assays using HPLC can be found in the refs. [3]–[5].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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15. See the Supporting Information.
16. The HPLC-based BTE equilibrium analyses were conducted as described previously (refs 3 and 5); each thiol and thioester contained a Tyr residue and K_{BTE} was measured by determining the relative populations of the four components by using HPLC after equilibration was complete. The ^{19}F NMR-based BTE equilibrium analyses were conducted with fluorine-labeled components; K_{BTE} was measured by determining the relative populations of the four components by using integration of ^{19}F resonances after equilibration was complete. In contrast to this latter approach, $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$ -based ΔG_{Fold} estimates are obtained simply by comparing $\delta^{19}\text{F}(\text{f}_2\text{Phe})$ for the full-length thiodepsipeptide and the C-terminal peptide thiol. It is not necessary that equilibrium be attained for this estimation.
17. HPLC was used to measure ΔG_{Fold} for all 15 new sequences and comparison of these measured ΔG_{Fold} values with ΔG_{Fold} estimated based on $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$ shows that the ^{19}F NMR-based estimation method did indeed correctly identify the most and least stable coiled coils within this set. Details may be found in the Supporting Information.

Figure 1. ^{19}F and $^{19}\text{F}\{^1\text{H}\}$ NMR data. A) ^{19}F resonances observed for $\text{N}_\text{T}\text{-F}$ and HSF when HSF is mixed with a nonfluorine-labeled peptide thioester.[15] B) $^{19}\text{F}\{^1\text{H}\}$ resonances observed for $\text{N}_\text{T}\text{-Cf}$ and HSCf when HSCf is mixed with a nonfluorine-labeled thioester.[15] C) ^{19}F resonances observed for the BTE mixture depicted in Scheme 1B.

Figure 2.

Correlation of $\Delta\delta(\delta^{19}\text{F} [\text{N}_\text{T}\text{-Cf}] - \delta^{19}\text{F} [\text{H}_\text{S}\text{Cf}])$ with ΔG_{Fold} previously determined by HPLC for various a - d' combinations. The ΔG_{Fold} values were determined with a system[3] similar to that shown in Scheme 1 (see the main text for a description of the modifications necessary for HPLC measurement). Residues $a_1'=a_2'=\text{Leu}$.

Figure 3. Partial helical net diagram for the intramolecular coiled-coil inter-face in N_T-Cf. The N-terminal segment is shown with solid lines, the C-terminal segment is shown with dashed lines and varied positions $a/a_1'/a_2'$ are indicated by dashed and filled circles.

A) Top: sequence of N_T-Cf; Succ = N-terminal succinyl group, Z = 3,5-difluorophenylalanine. Below are the sequences of N_T-F (middle) and H_SCf (bottom). Residues *a/a'*/*d'/a₂'* correspond to mutation sites. B) Thioester exchange process for N_T-Cf. The thioesterthiol pair on the left comprises N-terminal segment N_T-F, and C-terminal segment H_SCf, while the pair on the right contains the full-length coiled-coil N_T-Cf, and a small thiol H_SF.

Table 1

Comparison of ΔG_{Fold} estimated from $\Delta\delta^{19}\text{F}$ ($\delta^{19}\text{F}[\text{N}_\text{T}\text{-Cf}] - \delta^{19}\text{F}[\text{H}_\text{S}\text{Cf}]$) analysis, ΔG_{Fold} measured by using BTE monitored by ^{19}F NMR, and ΔG_{Fold} measured by using BTE by HPLC.

$\mathbf{a_1'-a_2'}$	$\Delta\delta$	$\Delta G_{\text{Fold est}}^{[a]}$ [kcal mol ⁻¹]	$\Delta G_{\text{Fold}}^{19\text{F NMR}}$ [kcal mol ⁻¹]	$\Delta G_{\text{Fold HPLC}}$ [kcal mol ⁻¹]
Ile-Leu-Leu	0.348	-2.1	-2.12 ± 0.06	-2.48 ± 0.10
Ile-Ile-Leu	0.305	-1.7	-2.05 ± 0.04	-2.08 ± 0.02
Leu-Val-Ile	0.220	-1.0	-1.04 ± 0.01	-1.08 ± 0.04
Val-Val-Val	0.089	0.08	-0.28 ± 0.07	-0.44 ± 0.09

^[a] $\Delta G_{\text{Fold est}}$ was estimated from $\Delta\delta^{19}\text{F}(\text{f}_2\text{Phe})$ using the correlation curve in Figure 2.