

Thermodynamic Analysis of β -Sheet Secondary Structure by Backbone Thioester Exchange**

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Understanding the factors responsible for the stability of common protein secondary structures has been a long-standing goal. Analysis of β sheets has lagged behind the study of α helices because the development of suitable model systems has been more challenging for the former than for the latter.^[1] Over the past decade, however, rules for the design of β sheets that fold autonomously in water have been elucidated, with a particular emphasis on two-stranded antiparallel β sheets (“ β hairpins”), which represent a minimum increment of this secondary structure.^[2,3] These model systems have proved useful for evaluating the contributions of factors such as side-chain–side-chain interactions,^[4] interstrand linker composition,^[5] strand length,^[6] and strand number to the stability of β sheets.^[7] As an outgrowth of this work, β hairpins have emerged as excellent platforms for the evaluation of noncovalent interactions that do not necessarily occur naturally in β sheets.^[8] β Hairpins and hairpin-like molecules stabilized by cyclization are also attractive systems for biomedical applications and fundamental studies.^[9]

The use of designed peptides to probe the origins of β -sheet stability, or to study noncovalent attractions between moieties that become spatially juxtaposed upon folding, requires the ability to determine the extent of β -sheet folding in solution. If only two conformational states are populated, unfolded and β sheet, then determining the population of these two states gives the folding equilibrium constant (K_{fold}), which provides insight into the stability of the folded state ($\Delta G_{\text{fold}} = -RT \ln K_{\text{fold}}$). For proteins that adopt defined tertiary structures, conformational stability is often assessed by using heat or a chemical denaturant to disrupt the folded state while monitoring the extent of folding by a conformationally sensitive spectroscopic probe (for example, circular dichroism). This approach is convenient because globular proteins are typically completely folded near room temperature and in the absence of denaturant (“native conditions”), which establishes the spectroscopic signature of the folded state,

and because it is often straightforward to identify the spectroscopic signature of a fully unfolded state generated at high temperature or high denaturant concentration.^[10] In contrast, most of the autonomously folding β -sheet model systems described to date cannot be driven by changing the conditions to the limiting states. Therefore, identifying the spectroscopic signatures for the fully unfolded and fully folded states of β -sheet model systems has frequently required the preparation and characterization of distinct reference peptides or the implementation of elaborate data analysis techniques.^[4–8]

We have recently developed a new approach for studying the conformational stability of small proteins,^[11] and here we describe the extension of this approach to β hairpins. This method involves polypeptide analogues in which one backbone amide group has been replaced by a thioester (namely, thiodepsipeptides). The conformational stability is assessed by monitoring the equilibrium constant for a thiol–thioester exchange reaction that causes the full-length thiodepsipeptide to be reversibly cleaved, which precludes adoption of a native-like fold. The “backbone thioester exchange” (BTE) measurements can be conducted under native conditions, and it is not necessary for the full-length molecule to be completely folded under these conditions. Our previous BTE studies have focused on polypeptides that adopt a discrete tertiary structure.^[11] Here we show that the BTE method can be extended to a secondary structure model system (Figure 1).

Our implementation of BTE began with a designed β hairpin designated HP5W4 (Figure 2) that was developed by Andersen and co-workers.^[5f,12] The sequence of HP5W4 incorporates key design features introduced originally by Cochran et al. in their development of the “tryptophan zipper” peptides.^[2d]

The turn segment of a β hairpin appears to be an optimal region for the amide-to-thioester modification that is required for implementation of BTE. Thioesters are poorer hydrogen-bonding partners than are secondary amides, and our previous efforts have therefore involved replacement of the backbone amide groups that do not form intramolecular hydrogen bonds in the folded state so as to minimize the conformational destabilization that might result from the modification. The optimized β hairpin turn segment of Andersen and co-workers,^[5f] Asn-Pro-Ala-Thr-Gly-Arg, seemed to offer a particularly attractive site for thioester insertion at the Thr–Gly bond. Our first thiodepsipeptide design (N_T -C') contains a thioester linkage at this position as well as three conservative side-chain modifications compared to HP5W4: all three Lys residues were mutated to Arg. These mutations were made to avoid the possibility of acylation of

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

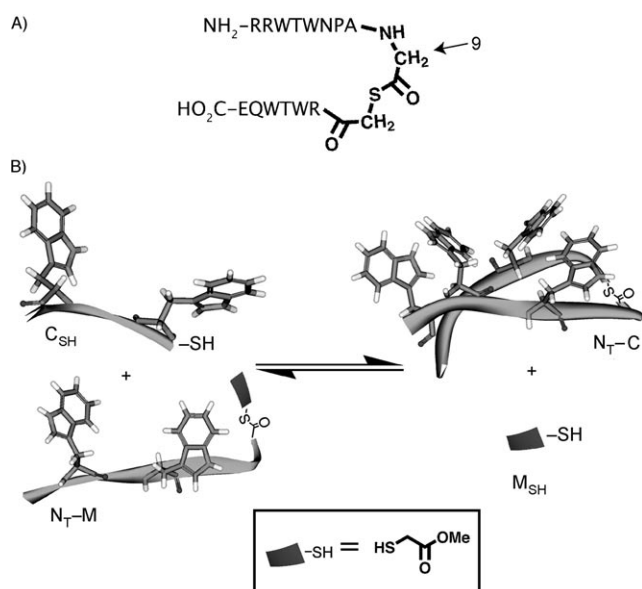


Figure 1. A) Sequence of $\text{N}_\text{T}\text{-C}$ (for $\text{N}_\text{T}\text{-C}'$, residue 9 is Thr, rather than Gly as in $\text{N}_\text{T}\text{-C}$). B) Thioester exchange process for $\text{N}_\text{T}\text{-C}$. The thiol–thioester pair on the left is comprised of the C- (top) and N-terminal (bottom) segments C_SH and $\text{N}_\text{T}\text{-M}$, respectively, while the pair on the right contains the full-length thiopeptide ($\text{N}_\text{T}\text{-C}$) and a small thiol (M_SH) [for $\text{N}_\text{T}\text{-M}'$, residue 9 is Thr, rather than Gly as in $\text{N}_\text{T}\text{-M}$].

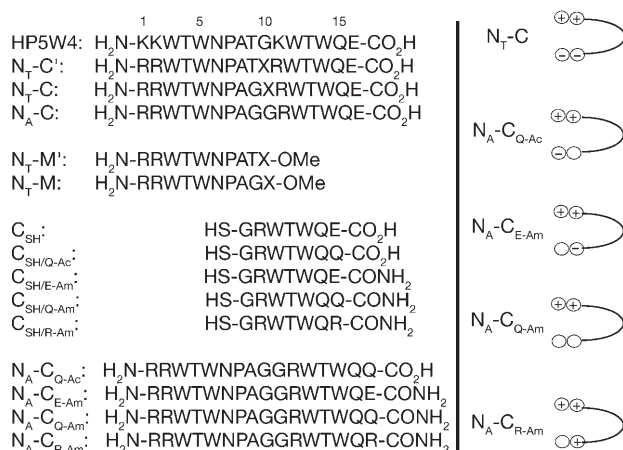


Figure 2. Sequences of peptides and thiopeptides. Right: schematic representations illustrating the juxtaposition of terminal polar groups in the β -hairpin conformations of several sequences. $\text{X} = \text{-SCH}_2\text{C(O)-}$.

the Lys side chain by a thioester, an irreversible reaction that could interfere with the thiol–thioester exchange process.

Thiol–thioester exchange occurs rapidly in aqueous solution at neutral pH, and the equilibrium constant (K_BTE) should provide insight into noncovalent attraction between the β -strand segments in the β -hairpin conformation of a thiopeptide such as $\text{N}_\text{T}\text{-C}'$ (Figure 1).^[11] The exchange reaction can be followed quantitatively by HPLC. When thiol–thioester exchange was initiated in pH 7 buffer by mixing thioester $\text{N}_\text{T}\text{-M}'$ —which corresponds to the N-terminal portion of $\text{N}_\text{T}\text{-C}'$ —with thiol C_SH —which corresponds to the C-terminal portion of $\text{N}_\text{T}\text{-C}'$ —equilibrium was reached within

40 minutes, with $K_\text{BTE} \approx 1$.^[13] We have previously shown that $K_\text{fold} = K_\text{BTE} - 1$ if the thioester bonds in the two thiopeptides are energetically equivalent,^[11] as we expect for $\text{N}_\text{T}\text{-M}'$ and $\text{N}_\text{T}\text{-C}'$. Therefore, the observation that $K_\text{BTE} \approx 1$ suggests that the β -hairpin conformation of $\text{N}_\text{T}\text{-C}'$ is quite unfavorable relative to the unfolded state. Since the linker region of HP5W4 was optimized for β -hairpin stability,^[5f] the behavior of $\text{N}_\text{T}\text{-C}'$ led us to wonder whether this linker must adopt a highly specific turn conformation to enable β -hairpin folding; local conformational rigidity might render the linker segment intolerant to the replacement of an amide group by a thioester. To test this hypothesis we changed Thr9 to Gly, thereby generating thiopeptide $\text{N}_\text{T}\text{-C}$. We found $K_\text{BTE} = 5.0$ for the equilibration of $\text{N}_\text{T}\text{-M}$ and C_SH with $\text{N}_\text{T}\text{-C}$ and M_SH , which suggests that the β -hairpin conformation of this thiopeptide is stabilized by $0.8 \text{ kcal mol}^{-1}$ (ΔG) relative to the unfolded state. The value of K_BTE was constant over an $\text{N}_\text{T}\text{-C}$ concentration range of 0.05 to 0.5 mM,^[13] which suggests that intermolecular interactions involving $\text{N}_\text{T}\text{-C}$, $\text{N}_\text{T}\text{-M}$, or C_SH are not energetically significant and probably do not occur under these conditions.

The ability of $\text{N}_\text{T}\text{-C}$ to adopt a β -hairpin conformation in aqueous solution is crucial for our experimental approach. We compared thiopeptide $\text{N}_\text{T}\text{-C}$ with peptide $\text{N}_\text{A}\text{-C}$, in which the thioester bond has been replaced with an amide bond. (Relative to HP5W4, $\text{N}_\text{A}\text{-C}$ has four mutations: $3 \times \text{Lys} \rightarrow \text{Arg}$ and Thr9 $\rightarrow$ Gly.) Two-dimensional NMR spectroscopy was used to determine whether the folding patterns of $\text{N}_\text{A}\text{-C}$ and $\text{N}_\text{T}\text{-C}$ are consistent with those of previously reported β -hairpin molecules. A combination of COSY and TOCSY measurements allowed assignment of all the proton resonances from $\text{N}_\text{A}\text{-C}$ and $\text{N}_\text{T}\text{-C}$. Numerous NOE interactions between protons on non-adjacent residues were observed for $\text{N}_\text{A}\text{-C}$ in ROESY measurements (ROESY experiments were not successful for thioester $\text{N}_\text{T}\text{-C}$ because the thioester slowly hydrolyzes during the 24 h required for data acquisition). All of the observed medium- and long-range NOE interactions are consistent with $\text{N}_\text{A}\text{-C}$ having a β -hairpin conformation that is comparable to the conformations previously deduced from the NOE data for HP5W4 and other members of the Trp zipper peptide family.^[2d,5f]

The high-resolution conformational analysis of peptide $\text{N}_\text{A}\text{-C}$ allowed us to examine the folding of thiopeptide $\text{N}_\text{T}\text{-C}$ in more detail, by comparing the chemical shifts of the α proton ($\delta\text{C}_\alpha\text{H}$) of each residue in the two sequences. $\delta\text{C}_\alpha\text{H}$ values are influenced in characteristic ways by local secondary structure.^[5f,14] Relative to “random-coil” values, the $\delta\text{C}_\alpha\text{H}$ values for residues in a β -sheet secondary structure are shifted downfield (a shift in the opposite direction is observed for α -helical residues). Figure 3 compares $\Delta\delta\text{C}_\alpha\text{H}$ data [$\Delta\delta\text{C}_\alpha\text{H} = \delta\text{C}_\alpha\text{H}(\text{observed}) - \delta\text{C}_\alpha\text{H}(\text{random coil})$] for HP5W4, $\text{N}_\text{A}\text{-C}$, and $\text{N}_\text{T}\text{-C}$.^[15] Nearly all the residues in the strand regions of the three sequences (Arg/Lys1-Trp5 and Arg/Lys11-Gln15) show the expected downfield shifts relative to the random-coil $\delta\text{C}_\alpha\text{H}$ values. The glaring exception is the strongly upfield-shifted $\delta\text{C}_\alpha\text{H}$ value for Trp5 (in all three molecules), which presumably reflects the juxtaposition of this proton and the aromatic side chain of Trp12 in the β -hairpin conformations. The general similarity between the

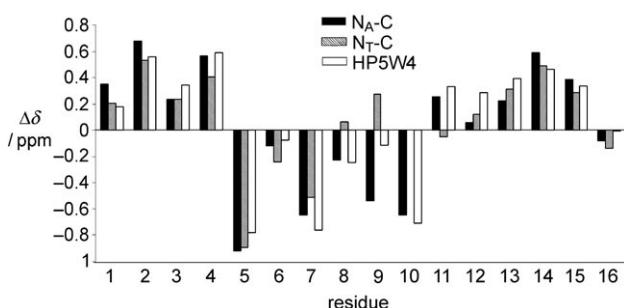


Figure 3. $\Delta\delta_{\text{C}\alpha\text{H}}$ data [$\delta_{\text{C}\alpha\text{H}}(\text{observed}) - \delta_{\text{C}\alpha\text{H}}(\text{random coil})$] for each residue in $\text{N}_\text{A}\text{-C}$, $\text{N}_\text{T}\text{-C}$, and HP5W4.

$\Delta\delta_{\text{C}\alpha\text{H}}$ values observed for thiopeptide $\text{N}_\text{T}\text{-C}$ and those for the two peptides (except in the region near the thioester bond) supports the conclusion that $\text{N}_\text{T}\text{-C}$ adopts a β -hairpin conformation similar to those of peptides HP5W4 and $\text{N}_\text{A}\text{-C}$, as established by NOE analysis.

Electrostatic interactions between opposite charges at the termini of a peptide have been shown by Searle and co-workers to stabilize the β -hairpin conformation of a designed sequence.^[16] Andersen and co-workers built upon these findings in their development of HP5W4, in which a pair of basic residues at the N terminus (Lys) and an acidic residue at the C terminus (Glu) interact to stabilize the folded form.^[5f,12] As these precedents clearly establish that Coulombic interactions between terminal groups can exert small but measurable effects on the conformational stability of the β hairpin, we evaluated the impact of varying the charge at the C terminus on the K_{BTE} value; this comparison was intended to reveal whether BTE can be used to probe relationships between sequence and β -hairpin folding preference. Parallel studies were undertaken with a peptide series based on $\text{N}_\text{A}\text{-C}$ to determine whether BTE and more traditional approaches would yield similar conclusions regarding the importance of Coulombic forces in β -hairpin stability. The BTE studies involved pairing thioester $\text{N}_\text{T}\text{-M}$ with thiol C_{SH} , $\text{C}_{\text{SH}/\text{Q-Ac}}$, $\text{C}_{\text{SH}/\text{E-Am}}$, $\text{C}_{\text{SH}/\text{Q-Am}}$, or $\text{C}_{\text{SH}/\text{R-Am}}$ (Table 1). Within this thiol series, the charge at the C terminus changes incrementally from -2 to $+1$, while the charge at the N terminus is constant at $+2$. Analogous comparisons were made with peptides $\text{N}_\text{A}\text{-C}$, $\text{N}_\text{A}\text{-C}_{\text{Q-Ac}}$, $\text{N}_\text{A}\text{-C}_{\text{E-Am}}$, $\text{N}_\text{A}\text{-C}_{\text{Q-Am}}$, and $\text{N}_\text{A}\text{-C}_{\text{R-Am}}$. The results of this analysis indicate that the terminal charges have a significant impact on the stability of the β -hairpin conformation. Amidation of either the side chain of Glu16 or the C-

terminal carboxy group decreases the net C-terminal charge by 1, which reduces the stability of the β -hairpin by 0.3 and 0.5 kcal mol⁻¹, respectively (Table 1, entries 2 and 3). Amidation of both of the carboxy groups at the C terminus results in a loss of 0.6 kcal mol⁻¹, an increment corresponding to approximately 75 % of the total hairpin stability. Placing Arg at the C terminus (Table 1, entry 5) introduces a charge-charge repulsion at the termini, which results in the least stable β -hairpin conformation in the series ($\Delta G_{\text{BTE}} = +0.2$ kcal mol⁻¹). The ΔG_{BTE} value measured for each thiol/thioester combination was compared with the melting temperature (T_{m}) of the corresponding peptide (Table 1); the T_{m} values are only approximate for mutants of $\text{N}_\text{A}\text{-C}$ because the complete folding transitions were not observed over the accessible temperature range. The patterns are comparable within these two sets of measurements, which suggests that the trend in conformational stabilities measured by BTE for thiopeptides mirrors the trend in β -hairpin stabilities for analogous peptides.

We have shown that the BTE method can be used to examine the relationship between sequence and conformational stability in the formation of a β hairpin. Achieving this goal required modification of a previously optimized inter-strand loop to increase the flexibility. Replacing a backbone amide group within the loop by a thioester causes a decrease in the β -hairpin stability, but the structure of the folded conformation does not appear to change. Despite the drop in conformational stability, BTE-derived information on the thermodynamics of β -hairpin folding of thiopeptides appears to reflect trends among analogous peptides. The feasibility of BTE analysis for β hairpins is a significant finding, because ΔG_{fold} values can be measured under native conditions, without the necessity of reference compounds for the fully unfolded and fully folded states. Thus, BTE should be a useful tool for analyzing the forces that control β -sheet folding preferences.

Experimental Section

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

Backbone thioester exchange assays: Assays were typically initiated by mixing approximately equal portions of a C-terminal thiol peptide and an N-terminal thioester (namely, from the left side of the equilibrium shown in Figure 1). Most assays were conducted with initial peptide concentrations of 0.1 mM and 50 mM buffer (phosphate, pH 7). Each BTE assay was allowed to equilibrate for about 40 min before HPLC analysis. Details are provided in the Supporting Information.

NMR spectroscopy: COSY and TOCSY NMR measurements were made on $\text{N}_\text{A}\text{-C}$ and $\text{N}_\text{T}\text{-C}$ by using standard methods, which allowed the assignment of all chemical shifts. ROESY data were also collected on $\text{N}_\text{A}\text{-C}$ to determine the folded structure. Details are provided in the Supporting Information.

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Table 1: Comparison of thermodynamic data obtained for hairpin thioesters and the corresponding all-amide polypeptides.

Entry	Thiol ^[a]	Thioester	$\Delta G_{\text{BTE}}^{\text{[b]}}$ [kcal mol ⁻¹]	Amide peptide	$T_{\text{m}}^{\text{[c]}}$ [°C]
1	$\text{C}_{\text{SH}} (-2)$	$\text{N}_\text{T}\text{-M}$	-0.8	$\text{N}_\text{A}\text{-C}$	49
2	$\text{C}_{\text{SH}/\text{Q-Ac}} (-1)$	$\text{N}_\text{T}\text{-M}$	-0.5	$\text{N}_\text{A}\text{-C}_{\text{Q-Ac}}$	37
3	$\text{C}_{\text{SH}/\text{E-Am}} (-1)$	$\text{N}_\text{T}\text{-M}$	-0.3	$\text{N}_\text{A}\text{-C}_{\text{E-Am}}$	35
4	$\text{C}_{\text{SH}/\text{Q-Am}} (0)$	$\text{N}_\text{T}\text{-M}$	-0.2	$\text{N}_\text{A}\text{-C}_{\text{Q-Am}}$	24
5	$\text{C}_{\text{SH}/\text{R-Am}} (+1)$	$\text{N}_\text{T}\text{-M}$	$+0.2$	$\text{N}_\text{A}\text{-C}_{\text{R-Am}}$	20

[a] The charge on the C terminus is shown in parentheses. [b] Uncertainty about ± 0.1 kcal mol⁻¹. [c] T_{m} values are approximate for entries 2–5.^[15]

Keywords: β hairpin · biophysics · peptides · protein folding · thioesters

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