Protein Folding

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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.

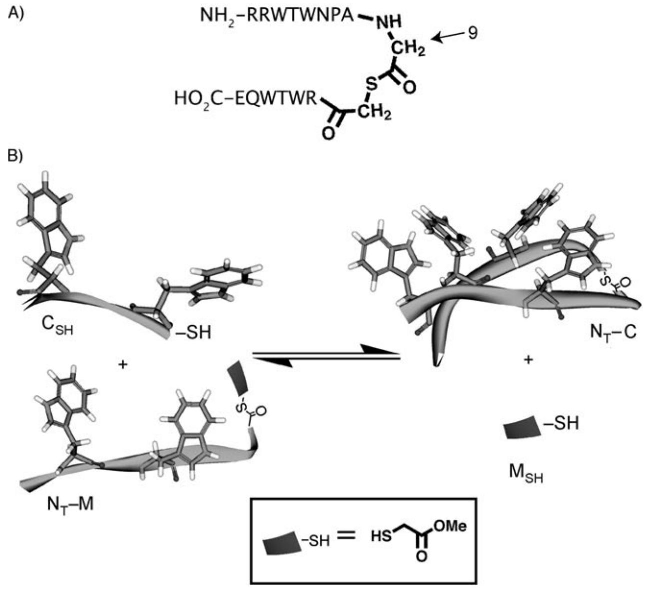

Figure 1. A) Sequence of N_T-C (for N_T-C′, residue 9 is Thr, rather than Gly as in N_T-C). B) Thioester exchange process for N_T-C. The thiol–thioester pair on the left is comprised of the C- (top) and N-terminal (bottom) segments C_{SH} and N_T-M, respectively, while the pair on the right contains the full-length thiodepsipeptide (N_T-C) and a small thiol (M_{SH}) [for N_T-M′, residue 9 is Thr, rather than Gly as in N_T-M].

1 5 10 15
HP5W4: H_2N-KKWTWNPATGKWTWQE-CO_2H
N_T-C′: H_2N-RRWTWNPATXRWTWQE-CO_2H
N_T-C: H_2N-RRWTWNPAGXRWTWQE-CO_2H
N_A-C: H_2N-RRWTWNPAGGRWTWQE-CO_2H

N_T-M′: H_2N-RRWTWNPATX-OMe
N_T-M: H_2N-RRWTWNPAGX-OMe

C_{SH}: HS-GRWTWQE-CO_2H
$C_{SH/Q\text{-}Ac}$: HS-GRWTWQQ-CO_2H
$C_{SH/E\text{-}Am}$: HS-GRWTWQE-$CONH_2$
$C_{SH/Q\text{-}Am}$: HS-GRWTWQQ-$CONH_2$
$C_{SH/R\text{-}Am}$: HS-GRWTWQR-$CONH_2$

N_A-$C_{Q\text{-}Ac}$: H_2N-RRWTWNPAGGRWTWQQ-CO_2H
N_A-$C_{E\text{-}Am}$: H_2N-RRWTWNPAGGRWTWQE-$CONH_2$
N_A-$C_{Q\text{-}Am}$: H_2N-RRWTWNPAGGRWTWQQ-$CONH_2$
N_A-$C_{R\text{-}Am}$: H_2N-RRWTWNPAGGRWTWQR-$CONH_2$

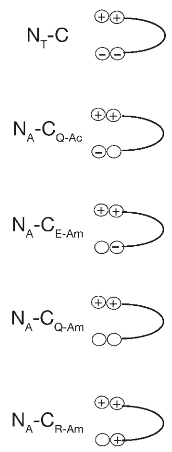

Figure 2. Sequences of peptides and thiodepsipeptides. Right: schematic representations illustrating the juxtaposition of terminal polar groups in the β-hairpin conformations of several sequences. X = -$SCH_2C(O)$-.

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 thiodepsipeptide such as N_T-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 N_T-M′—which corresponds to the N-terminal portion of N_T-C′—with thiol C_{SH}—which corresponds to the C-terminal portion of N_T-C′—equilibrium was reached within 40 minutes, with $K_{BTE} \approx 1$.[13] We have previously shown that $K_{fold} = K_{BTE} - 1$ if the thioester bonds in the two thiodepsipeptides are energetically equivalent,[11] as we expect for N_T-M′ and N_T-C′. Therefore, the observation that $K_{BTE} \approx 1$ suggests that the β-hairpin conformation of N_T-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 N_T-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 thiodepsipeptide N_T-C. We found $K_{BTE} = 5.0$ for the equilibration of N_T-M and C_{SH} with N_T-C and M_{SH}, which suggests that the β-hairpin conformation of this thiodepsipeptide is stabilized by 0.8 kcal mol^{-1} (ΔG) relative to the unfolded state. The value of K_{BTE} was constant over an N_T-C concentration range of 0.05 to 0.5 mM,[13] which suggests that intermolecular interactions involving N_T-C, N_T-M, or C_{SH} are not energetically significant and probably do not occur under these conditions.

The ability of N_T-C to adopt a β-hairpin conformation in aqueous solution is crucial for our experimental approach. We compared thiodepsipeptide N_T-C with peptide N_A-C, in which the thioester bond has been replaced with an amide bond. (Relative to HP5W4, N_A-C has four mutations: 3 × Lys→Arg and Thr9→Gly.) Two-dimensional NMR spectroscopy was used to determine whether the folding patterns of N_A-C and N_T-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 N_A-C and N_T-C. Numerous NOE interactions between protons on non-adjacent residues were observed for N_A-C in ROESY measurements (ROESY experiments were not successful for thioester N_T-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 N_A-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 N_A-C allowed us to examine the folding of thiodepsipeptide N_T-C in more detail, by comparing the chemical shifts of the α proton ($\delta C_\alpha H$) of each residue in the two sequences. $\delta C_\alpha H$ values are influenced in characteristic ways by local secondary structure.[5f,14] Relative to "random-coil" values, the $\delta C_\alpha H$ 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 $\Delta\delta C_\alpha H$ data [$\Delta\delta C_\alpha H = \delta C_\alpha H(\text{observed}) - \delta C_\alpha H(\text{random coil})$] for HP5W4, N_A-C, and N_T-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 $\delta C_\alpha H$ values. The glaring exception is the strongly upfield-shifted $\delta C_\alpha H$ value for Trp 5 (in all three molecules), which presumably reflects the juxtaposition of this proton and the aromatic side chain of Trp 12 in the β-hairpin conformations. The general similarity between the

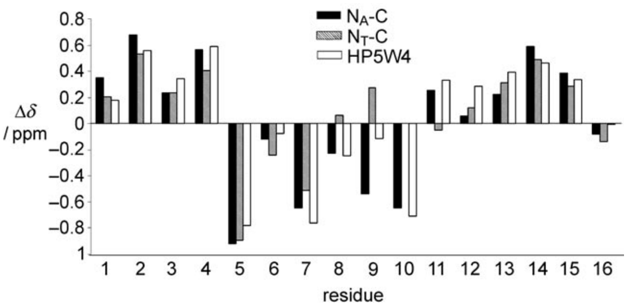

Figure 3. $\Delta\delta C_\alpha H$ data [$\delta C_\alpha H$(observed)$-\delta C_\alpha H$(random coil)] for each residue in N$_A$-C, N$_T$-C, and HP5W4.

$\Delta\delta C_\alpha H$ values observed for thiodepsipeptide N$_T$-C and those for the two peptides (except in the region near the thioester bond) supports the conclusion that N$_T$-C adopts a β-hairpin conformation similar to those of peptides HP5W4 and N$_A$-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 measureable 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 N$_A$-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 N$_T$-M with thiol C$_{SH}$, C$_{SH/Q\text{-}Ac}$, C$_{SH/E\text{-}Am}$, C$_{SH/Q\text{-}Am}$, or C$_{SH/R\text{-}Am}$ (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 N$_A$-C, N$_A$-C$_{Q\text{-}Ac}$, N$_A$-C$_{E\text{-}Am}$, N$_A$-C$_{Q\text{-}Am}$, and N$_A$-C$_{R\text{-}Am}$. 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 (ΔG_{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 N$_A$-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 thiodepsipeptides mirrors the trend in β-hairpin stabilities for analogous peptides.

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

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

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 thiodepsipeptides 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 N$_A$-C and N$_T$-C by using standard methods, which allowed the assignment of all chemical shifts. ROESY data were also collected on N$_A$-C to determine the folded structure. Details are provided in the Supporting Information.

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