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Using Thioamides To Site-Specifically Interrogate the Dynamics of Hydrogen Bond Formation in β-Sheet Folding

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Supporting Information

ABSTRACT: Thioamides are sterically almost identical to their oxoamide counterparts, but they are weaker hydrogen bond acceptors. Therefore, thioamide amino acids are excellent candidates for perturbing the energetics of backbone−backbone H-bonds in proteins and hence should be useful in elucidating protein folding mechanisms in a site-specific manner. Herein, we validate this approach by applying it to probe the dynamic role of interstrand H-bond formation in the folding kinetics of a well-studied β-hairpin, tryptophan zipper. Our results show that reducing the strength of the peptide's backbone−backbone H-bonds, except the one directly next to the β-turn, does not change the folding rate, suggesting that most native interstrand H-bonds in β-hairpins are formed only after the folding transition state.

Folded (globular) proteins are characterized by two important structural features: a hydrophobic core consisting of well-packed side chains and an intricate network of backbone−backbone hydrogen bonds (hereafter referred to as BB-HBs). The latter exclusively determines the secondary structure content of the protein. Thus, in order to provide a comprehensive understanding of how a protein folds, one needs to determine the order and kinetics of its side-chain packing, as well as the temporal sequence of its BB-HB formation. Because site-directed mutagenesis is relatively easy and straightforward, almost all previous protein folding kinetic studies have relied on ϕ-value analysis[1] through side-chain perturbation to infer the underlying folding mechanism. In comparison, perturbing the energetics of an individual BB-HB is more difficult; as a result, only a few experimental studies[2−6] have been performed, for example, using the technique of amide-to-ester (hereafter referred to as A-to-D) mutation to directly assess the role of BB-HB formation in the folding dynamics of proteins. Since esters are sterically different from amides and replacement of an amide with an ester completely eliminates a BB-HB, an A-to-D mutation could affect molecular packing and thus complicate interpretation of the experimental findings. Herein, we demonstrate an alternative approach, which only reduces the strength of the targeted BB-HBs, for mechanistic studies of protein folding.

Among the existing methods[2−6] for BB-HB mutations, replacing a backbone amide unit with a thioamide represents a distinctly advantageous approach to modulate the strength of targeted BB-HBs because thioamides are not only weaker hydrogen-bond acceptors but also sterically very similar to their oxoamide counterparts,[7] with the carbon−sulfur double bond in a thioamide slightly longer than the carbon−oxygen double bond of an oxoamide. Previous studies[8,9] suggested that an oxoamide-to-thioamide (hereafter referred to as O-to-T) mutation would decrease the protein's stability by about 1.6 kcal/mol, a value that is appropriate for ϕ-value analysis.[10] Furthermore, advances in synthetic chemistry have made it relatively easy to synthesize thioamide amino acids, especially those containing aliphatic side chains,[11−13] making O-to-T mutational studies of protein folding more practical.[9]

The applicability of thioamides in the conformational study of helical proteins has recently been established.[9,10,14] Using β-hairpins as an example, herein we extend the utility of O-to-T mutations to interrogate the dynamic role of BB-HB formation in β-sheet protein folding. While there are a large number of experimental studies on the folding mechanism of β-hairpins,[15−21] a direct assessment of interstrand H-bond formation in the folding transition state of β-hairpins, to the best of our knowledge, has never been done before. Specifically, we chose to study a variant of well-studied β-hairpins, tryptophan zippers (Trpzips), due to the large body of experimental and computational research[22−43] on their folding thermodynamics, kinetics, and mechanisms. As shown (Figure 1), this Trpzip

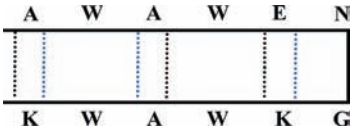

Figure 1. Cartoon representation of the β-hairpin structure of Trpzip-2c with the BB-HBs shown (dotted lines). BB-HBs that are perturbed using O-to-T mutation are shown in blue.

variant (Trpzip-2c following Keiderling and co-workers' sequence:[37] NH_2-AWAWENGKWAWA-$CONH_2$) folds into an antiparallel β-sheet structure that is stabilized by several interactions, including six BB-HBs, among which three are perturbed in the current study by individually substituting Ala1, Ala10, and Glu5 with their thioamide derivatives, i.e., thioalanine (TA) and thioglutamate (TE), and the correspond-

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ing mutants are hereafter referred to as A1/TA, A10/TA, and E5/TE, respectively.

The details of synthesis of Fmoc-thioalanine-nitrobenzotriazole and Fmoc-thioglutamate-nitrobenzotriazole are given in the Supporting Information (SI). The Trpzip-2c peptides were synthesized on a PS3 automated peptide synthesizer (Protein Technologies, Boston, MA) using Fmoc-protocols, except for incorporation of Fmoc-thioalanine-nitrobenzotriazole or Fmoc-thioglutamate-nitrobenzotriazole, which was added manually (0.4 mmol scale) to the deprotected, resin-bound peptide chain in 4 mL of dimethylformamide and coupled for 45 min. Thioamide peptides were cleaved in a modified cocktail of trifluoroacetic acid, water, and triisopropylsilane (14:5:1, v/v). Peptide products were further purified by reverse-phase chromatography and identified by matrix-assisted laser desorption/ionization mass spectroscopy. Trifluoroacetic acid removal and H-D exchange were achieved by multiple rounds of lyophilization.

All peptide samples were prepared in 20 mM phosphate buffer solution (pH 7), and the peptide concentrations were in the range of 30−100 μM for circular dichroism (CD) and 1−2 mM for infrared (IR) measurements. The details of all spectroscopic measurements, including the temperature jump (T-jump) IR setup, have been described elsewhere.[44]

As shown (Figure S1), at 1.0 °C both the wild-type and thioamide mutants of Trpzip-2c show the characteristic far-UV CD spectrum of Trpzips,[22] with a distinctive positive band centered at 227 nm that arises from the excitonic coupling between the B_b transitions of tryptophan residues.[45] In comparison to that of the wild type, however, the mean residue molar ellipticities of the mutants at 227 nm are lower, indicating that the O-to-T mutation in all cases decreases the β-hairpin stability, as expected. Moreover, because the underlying excitonic coupling is sensitive to both the distance and orientation of the tryptophan residues in the folded state, the fact that the 227 nm band only changes its intensity upon O-to-T mutations suggests that thioamide incorporation does not significantly perturb the β-hairpin structure. Following our previous studies,[46,47] we further quantify the unfolding thermodynamics of these Trpzip-2c peptides by globally fitting all the CD thermal melting curves obtained at 227 nm to a two-state model[37] (see SI for details), wherein the folded CD baselines and ΔC_p for unfolding of the mutants are assumed to be the same as those of the wild type. Because the CD thermal unfolding curves of the thioamide mutants lack folded baselines, such a stringent global fitting constraint is necessary to enable us to best estimate the folding/unfolding thermodynamics of the mutants. As indicated (Figure 2 and Table S1), the thermal melting temperature (T_m) of the wild-type Trpzip-2c is quantitatively consistent with that reported by Keiderling and co-workers,[37] whereas those of the thioamide mutants show different degrees of decrease, depending on the position of the BB-HB that is perturbed. It is clear that the energetic destabilization arising from thioamide mutation is greatly reduced when a BB-HB close to the terminal region is perturbed, consistent with several previous studies indicating that the ends of β-hairpins are frayed.[40,48,49]

To determine how these O-to-T mutations change the folding and unfolding rates of the β-hairpin, we further measured the relaxation rates of these peptides in response to a laser-induced T-jump[50] using time-resolved IR spectroscopy.[51] As shown (Figure S2), the relaxation kinetics of these peptides can be well described by a single-exponential function,

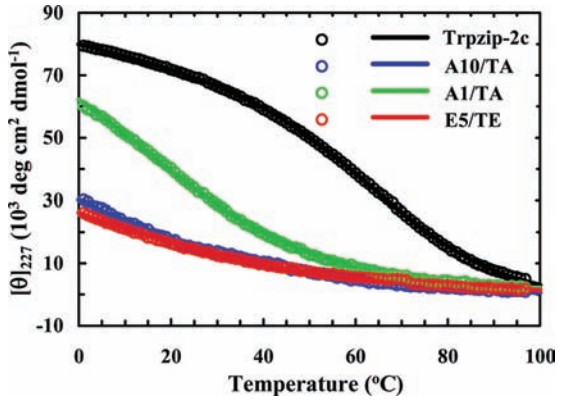

Figure 2. CD thermal melting curves of the Trpzip-2c peptides, as indicated. Smooth lines are global fits of these data to a two-state model discussed in the text.

consistent with previous studies.[52−54] The folding rate constant of wild type Trpzip-2c (Figure 3), determined from the corresponding relaxation rate constant (k_R) and folding thermodynamics, shows a modest dependence on temperature in the temperature range of the experiment. Specifically, the folding rate constant at 25.0 °C is determined to be (3.2 ± 0.7 μs)$^{-1}$, comparable to the (2.5 μs)$^{-1}$ measured for Trpzip2.[52] Since Trpzip2 and Trpzip-2c share the same turn sequence, this result alone provides additional evidence supporting our previous conclusion that the turn sequence plays a key role in determining the folding rate of β-hairpins.[53−55]

As indicated (Figure 3 and Table 1), the folding rates of A1/TA and A10/TA are almost identical, within experimental uncertainty, to that of the wild type. In contrast, their unfolding rates at any given temperature are significantly faster than that of the wild type. Taken together, these results indicate that the reduced stability of these mutants arises almost exclusively from a smaller unfolding free energy barrier. In other words, the corresponding native interstrand H-bonds perturbed by the O-to-T mutation are formed at the downhill side of the folding free energy barrier.

More interestingly, the E5/TE mutant, which is designed to decrease the strength of the first BB-HB next to the turn region of the peptide, has a more dramatic effect on both the relaxation and folding rates of the β-hairpin (Figure 3 and Figure S2). For example (Table 1), at 25 °C its relaxation rate constant is decreased to about (17.5 μs)$^{-1}$, compared to (3.0 μs)$^{-1}$ for the wild type, whereas its folding rate is slowed down by nearly a factor of 32. While this mutation also affects the unfolding rate of the β-hairpin, it leads to an increase in the unfolding rate constant by only a factor of ∼4. Thus, these results together provide strong evidence indicating that the BB-HB immediately next to the turn region stabilizes both the folding transition state and the native state. While such a folding mechanism has been observed in simulations,[33] the current study provides direct experimental insights into the mechanistic detail of BB-HB formation in β-hairpin folding. The comparatively smaller but significant increase in the unfolding rate further suggests that either this BB-HB is not entirely native-like in the transition state ensemble or the corresponding thioamide mutation induces a local distortion to the transition-state conformation.

Thus, taken together, our findings demonstrate that the O-to-T mutation method is a useful technique for site-specifically probing the dynamics of H-bonding formation and indicate that

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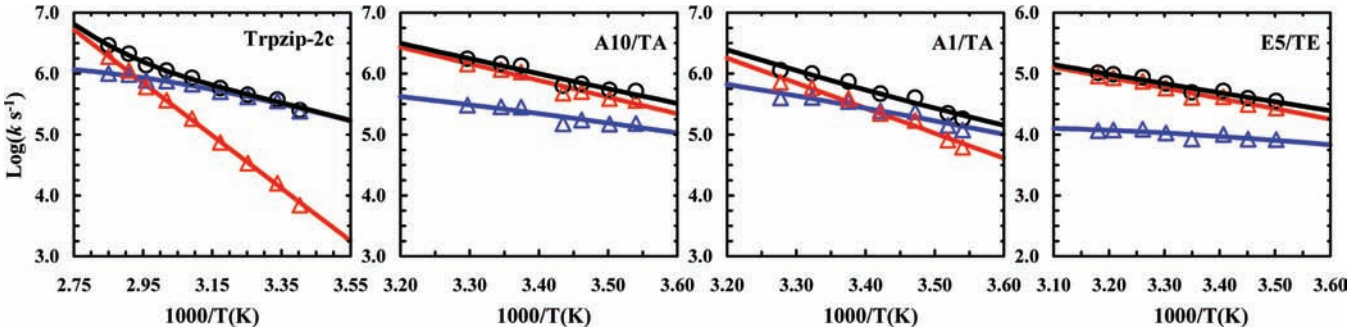

Figure 3. Arrhenius plot of the relaxation (black), folding (blue), and unfolding (red) rate constants of Trpzip-2c and mutants.

Table 1. Relaxation, Folding, and Unfolding Times at 25 °C

peptide	τ_R (μs)	τ_f (μs)	τ_u (μs)
Trpzip-2c	3.0 ± 0.7	3.2 ± 0.7	79.4 ± 18
A10/TA	0.8 ± 0.2	3.8 ± 0.5	1.0 ± 0.5
A1/TA	1.3 ± 0.2	3.0 ± 0.6	2.4 ± 0.6
E5/TE	17.5 ± 2.5	100.6 ± 14	21.2 ± 3.0

for β-hairpins the BB-HBs located beyond the turn region stabilize the folded state only by increasing the unfolding free energy barrier. In other words, they are formed not in but after the folding transition state. On the other hand, the BB-HB directly next to the β-turn is formed in the transition state, potentially acting as a staple to hold the turn region together for further propagation of folding down the two strands. This mechanistic picture is entirely consistent with our previous notions that for β-hairpin folding the turn formation is the rate-limiting step and that the native hydrophobic cluster is only formed at the downhill side of the major folding free energy barrier when folding begins from thermally denatured states.[53−56] It is worth pointing out, however, that the O-to-T mutation strategy described here is insensitive to probing alternative folding pathways that, for example, involve an initial collapsing step arising from side chain−side chain interactions.

In conclusion, we have demonstrated that thioamide mutation provides a site-specific means to interrogate the role of backbone−backbone hydrogen bonds in protein folding dynamics. Application of this method to a model β-hairpin allows us to pinpoint which H-bond is formed in the folding transition state, yielding a conclusion fully supported by several previous studies.[33,53−56] We believe that this method is a useful addition to the existing experimental toolkit used in protein folding studies and will find many important applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Thioamide amino acid synthesis details, CD spectra, and representative *T*-jump traces. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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