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Calorimetric Evidence for a Two-State Unfolding of the β-Hairpin Peptide Trpzip4

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β-Hairpins are antiparallel β-strands connected by a reverse turn and represent the smallest isolated unit of β-sheet structure. The discovery of water-soluble monomeric β-hairpins[1] has made them attractive models for the experimental and theoretical studies of β-sheet stability and propensity, independent of tertiary structural context. To determine the effect of loop length, β-sheet forming propensity, formation of cross-strand interactions, and types of interactions on stability host−guest β-hairpin peptide models have been used.[2,3] Even though β-hairpins represent relatively simple β-sheet structures, the detailed mechanism of their folding remains unclear.

One of the first β-hairpins, the tryptophan zipper (trpzip) family of peptides, was designed by Cochran et al.[4] based on the protein GB1 domain β-hairpin. The structures of several trpzips were solved by NMR, and it was found that the β-hairpin structure was highly populated in each case.[4] The trpzips were found to be highly soluble in aqueous solutions, maintained their monomeric state, and showed reversible thermal denaturation when circular dichroism (CD) spectroscopy was used to monitor unfolding.[3−6] Remarkably, the thermodynamic parameters of the trpzips were found to be similar to those of larger proteins when compared on a per-residue basis.[4] A subsequent investigation into the thermal stability of selected trpzip β-hairpins used CD and infrared (IR) spectroscopy and showed that each method yielded different thermodynamic parameters.[5] For example, the transition temperature, T_m, for trpzip4 was found to be ∼70 °C when CD was used to monitor temperature-induced transition, compared to ∼65 °C when IR spectroscopy was used. It is widely accepted that if there is superimposition of the thermal transition profiles, measured using different methods that monitor different structural elements (e.g., far-UV−CD and IR spectroscopy monitor secondary structure and near-UV−CD monitors tertiary structure), then the unfolding reaction is considered to be a two-state.[7] Thus, from comparison of the results obtained from CD and IR[4,5] spectroscopies, it appears that there is little evidence supporting the two-state model used to fit the thermal unfolding data, which could be a reason for the discrepancy between the derived thermodynamic parameters.

Differential scanning calorimetry (DSC) is the method of choice to determine the mode of protein unfolding because it can simultaneously and directly estimate the two enthalpies, namely, the experimental calorimetric enthalpy, ΔH_{cal}, and the fitted enthalpy, ΔH_{fit}, calculated according to a two-state model.[8,9] When the ΔH_{fit} and ΔH_{cal} enthalpies are the same, the transition is closely approximated by a two-state model where only native and unfolded molecules are significantly populated in the transition region. A nonequality of these two enthalpies implies either that the reaction is not monomolecular or that intermediate states are present.[10] In this study, we used DSC to monitor the unfolding of trpzip4 and complemented these experiments with the near- and far-UV−CD measurements.

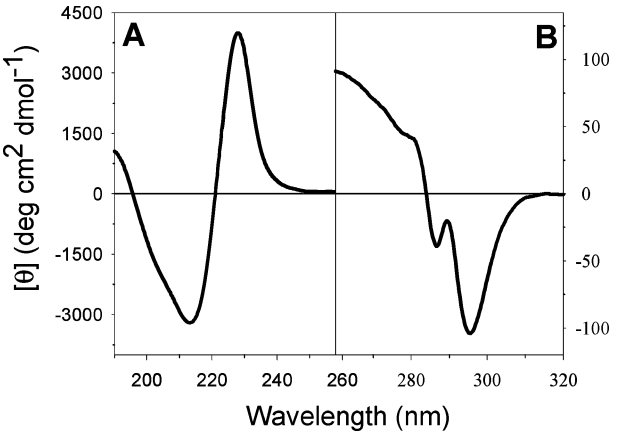

Figure 1. (A) Far-UV−CD spectrum of folded trpzip4 at 2 °C showing the maximum and minimum at 228 and 213 nm, respectively. (B) Near-UV−CD spectrum of trpzip4 at 2 °C showing the minimum at 295 nm.

Trpzip4 was synthesized with a C-terminal amide using standard Fmoc chemistry (trpzip4 amino acid sequence: GEWTWDDAT-KTWTWTE-NH_2). The peptide was purified by reverse-phase HPLC on a C18 column with an acetonitrile gradient in the presence of 0.05% TFA. After HPLC, fractions containing the peptide were pooled, lyophilized, and resuspended in deionized water, and the process was repeated to remove residual TFA. The identity and purity of the sample was confirmed by MALDI-TOF (data not shown). Trpzip4 concentration was determined spectroscopically from the UV absorbance at 280 nm using the extinction coefficient $\epsilon_{280,0.1\%} = 11.41$ o.u. All experiments were performed in 20 mM Na phosphate, pH 7.0. Sample preparation for CD and DSC was performed as described in ref 11 Trpzip4 concentrations were in the range of ∼1.5 mg/mL for DSC and 0.1 mg/mL for CD experiments.

Folded trpzip4 shows a unique far-UV−CD spectrum (Figure 1A) with a maximum at 228 nm and a minimum at 213 nm due to exciton coupling between the Trp residues.[4] Measuring the unfolding at these wavelengths gives a measure of tertiary structure as the disruption of cross-strand Trp−Trp interactions would result in uncoupling and a loss in CD signal at these wavelengths. However, the β-sheet structure of trpzip4 also contributes to the CD signal at 213 nm because it falls within the same wavelength range as the β-sheet far-UV−CD signal.[12] The near-UV−CD spectrum of trpzip4 (Figure 1B), unique for different proteins/peptides, has a minimum at 295 nm and shows that the environment of the Trp residues is chiral, which is indicative of a folded structure.[13]

Thus, to complement the DSC data, CD spectroscopy was used to monitor the thermal unfolding of trpzip4 at 213, 228, and 295

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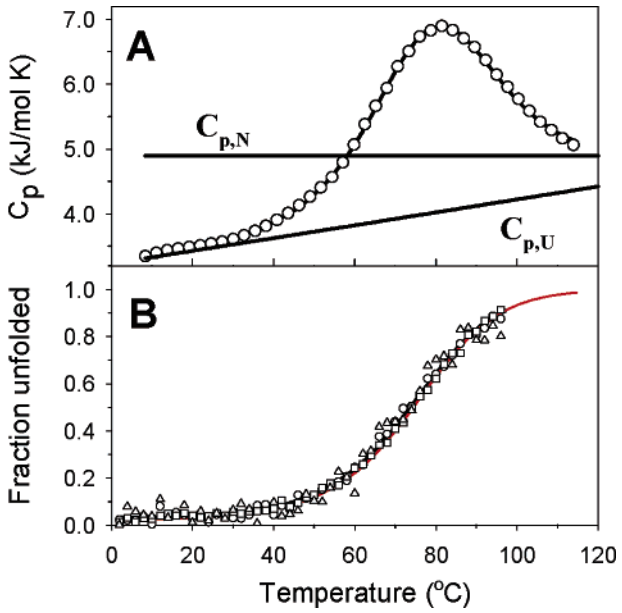

Figure 2. (A) Thermal unfolding profile of trpzip4 monitored by DSC (○) with the data fitted to a two-state unfolding model shown in a thin solid line. The solid lines represent the native and unfolded baselines. (B) Fraction unfolded for CD data fitted to a global two-state unfolding model (solid black line) and DSC data fitted to a two-state unfolding model (solid red line). The open symbols represent the fraction unfolded for the CD data at 213 nm (○), 228 nm (□) and 295 nm (△). For clarity, only every tenth point is shown.

nm. Pre- and post-thermal unfolding CD spectra were found to be the same at 2 °C, indicating that thermal unfolding was reversible. Rescanning the DSC sample yielded a superimposable heat capacity profile relative to the first, again indicating that the thermal unfolding of trpzip4 was reversible (data not shown). Figure 2A shows the DSC data fitted to a two-state unfolding model as well as the folded and unfolded baselines.

It appears that trpzip4 is not fully unfolded even at the highest accessible temperature for DSC (~115 °C). For a two-state unfolding process, the transition temperature (T_m), enthalpy of unfolding (ΔH), and the heat capacity change upon unfolding (ΔC_p) adequately describe the thermodynamic parameters of unfolding in the entire temperature range.[8] Analysis of the DSC profiles determined T_m, ΔH_{cal}, ΔH_{fit}, and ΔC_P to be 75 ± 1 °C, 89 ± 2 kJ/mol, 94 ± 2 kJ/mol, and 1.4 ± 0.2 kJ/mol K, respectively. The CD data, at all three wavelengths, were globally fitted to a two-state unfolding model, and the ΔC_P was kept constant at 1.4 kJ/mol K. From the analysis of the CD unfolding profiles, the T_m and ΔH_{fit} were found to be 74 ± 1 °C and 91 ± 1 kJ/mol, respectively and are within error of the calorimetrically determined values. Figure 2B shows the quality of a two-state fit of the CD and DSC data. Importantly, the calorimetrically determined ΔH_{cal} and ΔH_{fit} were found to be very similar, which arguably indicates that the unfolding of trpzip4 is a two-state process. Thus, the previously observed variation in the thermodynamic parameters using CD and IR spectroscopy is probably related to the inherent difficulty in defining the unfolded state baseline, particularly in experiments with the upper temperature limit of only 75 °C.[14]

Unlike the cooperative thermal unfolding observed for trpzip4, the β-hairpin derived from protein GB1 does not show the same degree of cooperativity upon unfolding.[15] Trpzip4 is a triple variant of the protein GB1 β-hairpin where Trp 5, 12, and 14 in trpzip4 substitute Tyr, Phe, and Val, respectively. The four Trp residues in trpzip4 have been shown by NMR to interdigitate and form the Trp-zipper motif where the non-hydrogen-bonded cross-strand pairs of Trp residues interact.[4] It is thus possible that the Trp-zipper motif could be responsible for the additional cooperativity observed for trpzip4. In comparison, the α-helical peptides show non two-state behavior upon thermal unfolding.[11] The structural basis for this difference could originate from the differences in the hydrogen-bonding patterns between these two most common elements of secondary structure. In the β-hairpin formed by trpzip4, the hydrogen-bonding interactions are separated in sequence space and are further strengthened by the interactions between the Trp side chains. This is in contrast to the localized hydrogen bonding ($i \rightarrow i + 4$) observed for α-helices and relatively weak side-chain to side-chain interactions.[16] The lack in cooperativity of α-helix unfolding could manifest itself on a larger scale as residual structure in the denatured states and a lack of two-state unfolding behavior observed for some all-α-helical proteins where molten globules, for example, are readily populated.[17] Conversely, it is possible that stable β-hairpins, or β-sheets, could be responsible for the two-state equilibrium and kinetic unfolding of all β-proteins.[18]

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Supporting Information Available: Far-UV−CD spectra for trpzip4 at different temperatures showing two isodichroic points. This material is available free of charge via the Internet at http://pubs.acs.org.

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