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A Simple Thermodynamic Test To Discriminate between Two-State and Downhill Folding

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One of the main targets in designing protein structures de novo has been to achieve proteins that fold in a two-state manner.[1−3] The implicit assumption is that single domain natural proteins are intrinsically two-state systems.[4] Such a premise might require reevaluation after the recent discovery of downhill protein folding.[5] Downhill folding proteins have only one thermodynamic state with properties that change gradually from folded to unfolded-like as protein stability decreases. It is quite possible that this behavior is found in other proteins, since downhill proteins could work as molecular rheostats.[5] Thus, it is imperative to define simple empirical tests to discriminate between two-state and downhill folding. The original procedure[5,6] involves obtaining data with many spectroscopic techniques and calorimetry. This approach is often not practical, especially for researchers interested in high-throughput design strategies or in investigating mutational effects.

Here we introduce a different strategy. The idea is to combine a structural probe sensitive to the local backbone conformation and two different denaturing procedures. The "local" probe reports the weighted average conformation of all the residues in the protein, being sensitive to any changes in structure. The coupling between two denaturing procedures allows investigating the properties of the ensemble. For a two-state reaction, the combined effect of chemical denaturants and temperature is determined by simple Maxwell relations.[7] In a downhill reaction, even small changes in free energy result in structural shifts in the ensemble, which should translate into modified sensitivity to the denaturing agents.

As the first step, we explored the potential of this approach in a modified version of the elementary statistical mechanical model proposed by Zwanzig.[8] This model produces one-dimensional free-energy profiles as a function of the order parameter S (i.e., number of residues with native conformation) and has the advantage of accommodating all possible folding scenarios.[6] This is easily achieved by defining the interaction energy as third-order polynomials of S (Supporting Information). Figure 1 shows calculations with the model on the effect of adding increasing amounts of the chemical denaturant urea to the thermal unfolding transitions monitored by a local probe (i.e., the average value of S) of two-state, downhill, and an intermediate case. The insets show the probability distribution as a function of temperature for 0 M urea concentration. With the exception of the three coefficients for the interaction energy polynomial, all of the thermodynamic parameters are identical for the three calculations, and in accordance with typical values for proteins (Supporting Information).

The calculations reveal that for a two-state-like process the apparent thermal unfolding transitions are highly sigmoidal. This is the case even when the transition is monitored by a "local" probe with a signal that changes gradually with the degree of structure. Addition of urea lowers the midpoint temperature, but does not change the pre-transition region until cold denaturation becomes detectable. At increasing urea concentrations, the T_{max} (i.e., temperature at which there is a maximum in the "native" signal) increases slightly because of the decreases in folding enthalpy induced by urea.[7] Downhill folding transitions are also sigmoidal but less pronounced and with significant changes in the pre- and post-transition regions. An important implication is that the observation of a sigmoidal transition is not sufficient indication of two-stateness. Addition of urea to a downhill folding protein results in downshifts of the apparent "native" signal, even in conditions of high stability. Furthermore, T_{max} increases much more drastically because the ensemble becomes progressively less nativelike (Supporting Information). Remarkably, these properties are to a lesser extent already observable in the intermediate case (Figure 1C), in which low barriers emerge near the transition midpoint, resulting in a small population of molecules with intermediate values of S (inset Figure 1C, Supporting Information).

To investigate whether these theoretical results are applicable to real proteins, we studied the equilibrium folding transition of the small helical protein BBL as a function of temperature and urea. BBL was previously characterized as a downhill folder by the more complicated procedure.[5] As local probe we measure far-UV circular dichroism (CD) spectra in the range 210−250 nm. By recording complete spectra, we expect to set real structural changes apart from phenomenological shifts in signal induced by either temperature or urea (Supporting Information). The data are analyzed by singular value decomposition (SVD) to identify the spectral changes as a function of the two denaturing agents. In Figure 2A, we show the CD spectrum accounting for the global changes in the data, as derived from the SVD procedure. Figure 2B displays the fraction of this spectrum present in each condition.

The results indicate that all of the observed changes in CD signal correspond to a decrease in α-helix structure. The data in BBL show all the trademarks of the downhill scenario. There are steep pre-transition slopes induced by both temperature and urea, and the apparent T_{max} changes sharply in the range 1.5−4 M urea (Figure 2B). Global fitting of these data with a phenomenological two-state model is also shown in Figure 2B. In this fitting, the changes in α-helix structure observed before and after the main transition are treated as phenomenological baselines of the folded and unfolded states. With such baselines, the two-state global fit reproduces the general trends in the data, but fails to account for the changes in transition slopes and apparent T_{max}. This is so even though the excess enthalpy introduced by urea is an adjustable parameter. Individual fits to data at each urea concentration reproduce the data well, but render heat capacity changes that vary sharply with urea (Supporting Information), stressing the importance of performing a global fit. Furthermore, the fitted phenomenological baselines reveal that the α-helical contents of the assumed native and unfolded states cross at temperatures between 340 and 360 K (Figure 2C). This indicates that the "native" state becomes increasingly disordered by temperature or urea. For example, at

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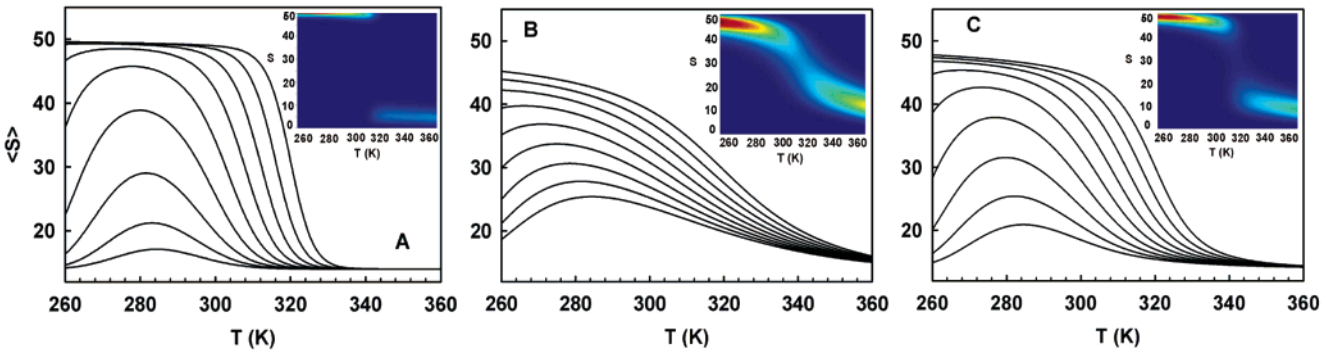

Figure 1. Theoretical temperature−urea denaturation behavior calculated for 50 residue proteins with two-state (A), downhill (B), and intermediate (C) unfolding transitions. Urea concentrations range from 0 M (top) to 4 M (bottom) in 0.5 M intervals. Insets show the probability distribution as a function of S (number of native residues) at 0 M urea. S goes from 0 (fully unfolded) to 50 (fully folded), and $\langle S \rangle$ is the observable value of S (weighted average).

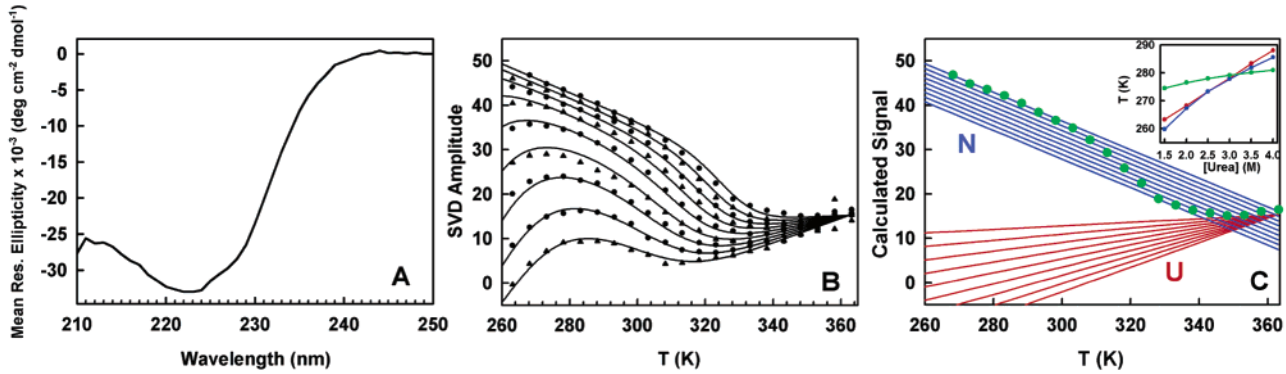

Figure 2. Equilibrium thermal denaturation of BBL in 0−4 M urea (0.5 M intervals) monitored by far-UV CD. (A) SVD component showing the CD spectrum of the conformational changes of BBL upon unfolding. (B) (Circles and triangles) Amplitude (×100) of the SVD component as a function of temperature and urea. (lines) Fit to a global two-state model with phenomenological baselines. (C) Changes in α-helix signal of the native and unfolded states as produced by the global two-state fit. Experimental data at 0 M urea are shown as green dots for comparison. (inset) T_{max} as a function of urea. (red) Experimental, (green) T_H from two-state fit, and (blue) phenomenological T_{max} from two-state fit.

340 K the "native" state in 4 M urea has the same α-helix content than the unfolded state without urea. Pre- and post-transition structural changes could arise from conformational shifts in two ensembles separated by a barrier.[7] However, the barrier ensures that the two ensembles remain independent, and therefore, it is incompatible with baselines crossing within the experimental range.

At urea concentrations between 1.5 and 4 M, the thermal transition goes through a maximum in the accessible temperature range, and thus T_{max} can be estimated directly (inset to Figure 2C). In principle, for a two-state system the T_{max} should agree with the temperature at which $\Delta H = 0$ (T_H). However, T_H calculated from the two-state fit increases only ∼6 K in this range of urea, in great contrast with the ∼25 K increase observed for the experimental T_{max}. The phenomenological baselines are able to partly compensate for this discrepancy by skewing the calculated two-state curves (inset to Figure 2C), but in this case the temperature of maximal signal loses its physical meaning. In contrast, similar experiments have shown that the marginally stable DNA binding domain of lac repressor is compatible with two-state folding.[7]

We find that this simple thermodynamic test can detect the inherent conformational complexity of downhill folding. Because the test only involves standard methods in protein chemistry, it should be very useful in determining the thermodynamic folding properties of small domains and de novo designed proteins. Our results suggest that the test might be sensitive enough to detect the transition from two-state to downhill folding. This is of particular relevance for investigations of mutational effects in very fast folding proteins.[9]

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Supporting Information Available: Materials, methods, and description of the theoretical model and parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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