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Unusual Cold Denaturation of a Small Protein Domain

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Supporting Information

ABSTRACT: A thermal unfolding study of the 45-residue α-helical domain UBA(2) using circular dichroism is presented. The protein is highly thermostable and exhibits a clear cold unfolding transition with the onset near 290 K without denaturant. Cold denaturation in proteins is rarely observed in general and is quite unique among small helical protein domains. The cold unfolding was further investigated in urea solutions, and a simple thermodynamic model was used to fit all thermal and urea unfolding data. The resulting thermodynamic parameters are compared to those of other small protein domains. Possible origins of the unusual cold unfolding of UBA(2) are discussed.

Cold denaturation is widely believed to be a general property of globular proteins;[1] however, it is rarely experimentally observed as in most proteins it would require temperatures well below the freezing point of water. The thermodynamic and structural origins of the cold denaturation, along with its biological significance, remain controversial.[2] Obviously, to fully understand the principles that govern the stability of protein structures and their folding, it is necessary to elucidate the mechanism of unfolding at low temperatures as well.

In recent years, a number of small, autonomously folding protein domains have been discovered, which became very attractive models for studying the fundamental mechanism of protein folding.[3] Their small size and often extremely fast folding allow for the direct comparison of experiments with all-atom folding simulations.[4] Identification of additional small protein domains, which closely mimic the properties of larger globular proteins, is therefore important for uncovering the complex details of protein folding by a combination of experiments and simulations.

Here, we present a study of the thermal stability of a 45-residue ubiquitin associated domain UBA(2). This domain is found at the C-terminus of the DNA nucleotide excision-repair protein HHR23A and consists of three helices surrounding a hydrophobic core[5] (Figure 1a). The globular three-helix structure UBA(2) closely resembles some of the most widely studied ultrafast folding proteins.[3,4] The UBA(2) domain was chemically synthesized, purified, and its thermal denaturation studied by circular dichroism (CD). For details of the synthesis and experimental methods, see Supporting Information (SI).

Figure 1b shows the CD spectra at three different temperatures: 273 K (blue), 288 K (green), and 358 K (red). The more intense negative CD signal indicates higher helicity

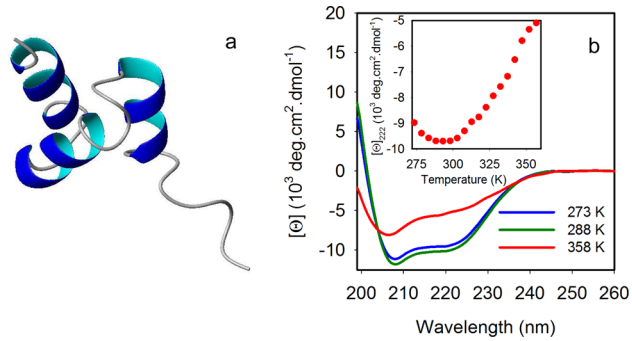

Figure 1. (a) NMR structure of the UBA(2) domain of HHR23A (PDB 1DV0). (b) Mean residue ellipticity of the UBA(2) at three different temperatures as obtained by CD spectroscopy showing the onset of cold denaturation (see Supporting Information for experimental details). Inset: the ellipticity at 222 nm shows not only denaturation upon heating but also the onset of unfolding in the cold.

at 288 K than at 273 K, suggesting the onset of cold denaturation. Above 288 K, the (negative) ellipticity increases again due to thermal unfolding. The temperature profile of the CD signal at 222 nm is shown in the inset, highlighting the loss of the α-helical structure at both the high and low temperatures.

To further investigate the unfolding in the cold and to provide additional data for thermodynamic modeling, the thermal unfolding experiments were carried out in ~1 M and ~2 M urea. The addition of denaturant allowed even lower temperatures (263 K) to be reached. Urea unfolding, spanning the full concentration range (0 to 8 M), was also measured at 298 K.

Figure 2 shows the analysis of thermal and denaturant unfolding data with the two-state thermodynamic model. The model assumes the change in heat capacity upon unfolding (ΔC_P) to be temperature independent, which leads to the standard free energy expression: $\Delta G(T) = ((T_m - T)/T_m)\Delta H(T_m) + (T - T_m)\Delta C_P - T\Delta C_P \ln(T/T_m)$, where T_m is the unfolding midpoint temperature. We note that although calorimetry is the most direct method for determination of the thermodynamic parameters, it has been shown that ΔC_P can be reliably measured by optical spectroscopy as well.[6] The urea dependence of the free energy is assumed to be linear ($\Delta G(\text{urea}) = \Delta G(0) - m[\text{urea}]$), where m is temperature independent. Spectral data are expressed as linear combinations of the contributions of the folded and unfolded state. The CD

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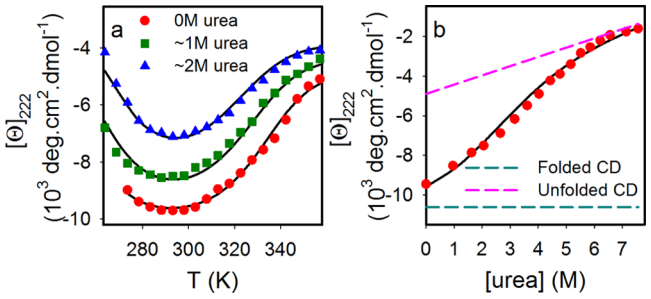

Figure 2. Folding thermodynamics of the UBA(2) domain studied by CD using thermal and denaturant unfolding. The data was analyzed by a two-state thermodynamic model. (a) CD spectral data of thermal denaturation at 222 nm. Red circles are experimental data without the addition of urea, green squares are experimental data in the presence of 1.2 M urea, blue triangles are experimental data in the presence of 2.3 M urea. Solid black lines are obtained by the two-state model. (b) CD spectral data of urea denaturation at 222 nm measured at 298 K. Red circles are experimental data points, the black curve is obtained by the two-state model, and the baseline of the folded state is assumed to be urea independent, while a linear dependency is assumed for the unfolded state. The resulting thermodynamic parameters are $\Delta H(T_m)$ = 16(±2) kcal·mol^{-1}; T_m = 329(±2) K; ΔC_P = 450(±50) cal·mol^{-1}·K^{-1}, m = 420(±40) cal·mol^{-1}·M^{-1}. From these thermodynamic parameters, the melting temperature in the cold is predicted as T'_m= 263(±2) K in the absence of denaturant.

signal of the folded state is assumed independent of urea concentration, while the urea dependence of the CD signal of the unfolded state is approximated by a linear function for simplicity (the thermal denaturation data in Figure 2a suggest a transition-like, sigmoidal relationship). Additional details of the modeling are given in Supporting Information. As seen from Figure 2, this simple model fits all the experimental data remarkably well.

In addition to CD spectroscopy, thermal and denaturant unfolding were monitored by fluorescence of the single intrinsic Tyr residue (Supporting Information). Unfortunately, the strong temperature dependence of the Tyr fluorescence, which requires additional adjustable parameters, makes impossible the independent extraction of the thermodynamic data. However, the same two-state thermodynamic model that fit the CD data was consistent with the fluorescence (Supporting Information).

The ΔC_P, determined from the thermal and cold denaturation curves can be related to the change of the protein solvent accessible surface area (ΔSASA) upon unfolding. Several empirical equations have been developed for the estimation of ΔC_P upon unfolding, based on the ΔSASA of polar and nonpolar groups. We used ProtSA[7] to calculate the polar and nonpolar ΔSASA and compared the estimates of four empirical equations[8] with the experimental ΔC_P of UBA(2). As shown in Figure 3a, the equations of Makhatadze and Privalov (MP) and of Murphy and Freire (MF) overestimate the experimental value, the equation of Myers et al. (MPS) gives a slightly smaller value, while the equation of Spolar et al. (SLR) predicts a value within the experimental error. In Figure 3b, we also compare the m-value calculated from ΔSASA according to Wafer et al.[9] with the experimental value (Figure 2). Good overall agreement inspires confidence in the ΔSASA values determined by ProtSA for proteins of similar size and structure to UBA(2), as well as in the empirical formulas, in particular the SLR,[8c] for estimation of the ΔC_P. The empirical equations

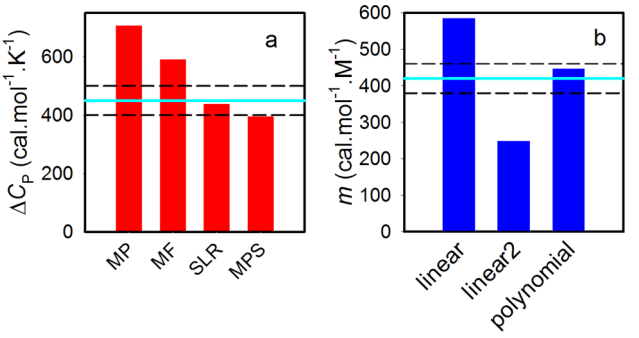

Figure 3. Experimental and calculated ΔC_P and m-values of UBA(2). (a) Comparison of the experimental value of ΔC_P (cyan line; error is indicated by dashed lines) and calculated values using different methods (abbreviations are explained in the text). (b) Experimentally determined m-value (cyan line; error is indicated by dashed lines) compared to calculated values obtained from empirical equations which are based on linear regression (linear), linear regression forced through the origin (linear2), and polynomial regression (polynomial).

used for calculating ΔC_P and m as well as ΔSASA values can be found in Supporting Information.

The pronounced cold unfolding of UBA(2), not seen in other similar helical domains, raises a question: what causes this particular protein to cold denature? As we show in Supporting Information, the cold denaturation temperature can be related to the ratio $\Delta H(T_m)/\Delta C_P$: the greater this ratio, the lower the cold unfolding temperature midpoint. This implies that to observe cold denaturation, the protein has to have a large ΔC_P and/or a small $\Delta H(T_m)$.

In Figure 4, we compare the values of ΔC_P (Figure 4a) and $\Delta H(T_m)$ (Figure 4b), normalized for the number of residues,

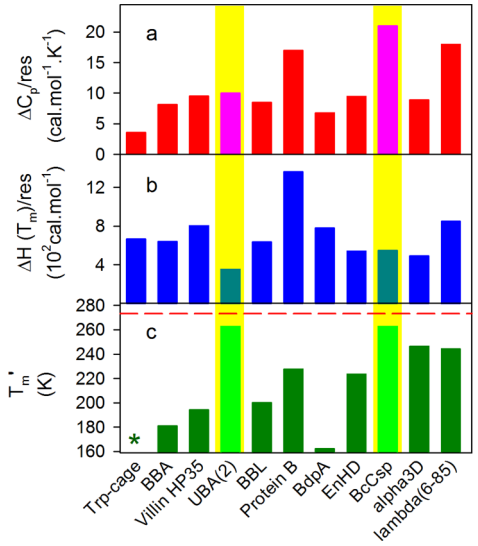

Figure 4. Comparison of ΔC_P and $\Delta H(T_m)$ values per residue and estimated midpoint temperatures in the cold (T'_m) for 11 small proteins. The highlighted regions correspond to the proteins that show cold denaturation (UBA(2) and BcCsp). (a) ΔC_P values per residue. Experimental values were used where available (see Table S4, Supporting Information). For villin HP-35, the calculated value is displayed, as explained above. (b) Experimental $\Delta H(T_m)$ values per residue (see Table S4, Supporting Information). (c) Estimated T'_m values obtained from the temperature dependence of the unfolding free energy (see Supporting Information). The T'_m of Trp-cage (*) is not shown because of its significantly lower value (64 K).

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for several small α-helical proteins, also including a 66-residue double mutant of BcCsp protein, which has been found to cold denature.[10] Experimental ΔC_P values were used where available; otherwise, estimates were obtained from ΔSASA and the SLR method tested above (Figure 3). We note that for HP-35, strongly temperature dependent ΔC_p was measured:[11] this dependence would predict observable cold denaturation for HP-35 similar to that for UBA(2) (see Supporting Information). However, HP-35 was not observed to unfold in the cold even in the presence of high concentrations of denaturant.[12] We therefore used a ΔSASA/SLR estimate for a ΔC_P value for this protein, which does not result in cold denaturation (Supporting Information Figure S3).

It is evident from Figure 4a that ΔC_P (per residue) for UBA(2) is not significantly higher than that for other small helical proteins. The large ΔC_P therefore cannot be responsible for the unusual cold denaturation for this protein. By contrast, UBA(2) has the smallest $\Delta H(T_m)$ (per residue). It must be primarily the small enthalpy change, rather than the heat capacity, which causes the observable cold denaturation of this small domain at relatively high temperatures.

Since the largest contribution to ΔC_P is burial of the hydrophobic groups (or nonpolar ΔSASA),[8] it appears that the cold denaturation cannot be attributed to the hydrophobic effect. The origin of a much smaller unfolding enthalpy for the UBA(2) is not entirely clear. We can only speculate that it may be related to an apparently large amount of residual helical structure in the thermally unfolded state (note the CD and ellipticity values in Figures 1 and 2, and their decrease with the addition of denaturant). Also note that the other cold-denaturing protein, the BcCsp mutant, has both very large ΔC_P and relatively small $\Delta H(T_m)$ values.

Finally, in Figure 4c we compare the estimated midpoints of cold denaturation for the selected proteins. Aside from UBA(2) and BcCsp, the highest predicted cold denaturation midpoints are for the *de novo* designed α_3D and the λ-repressor, around 245 K. However, these proteins are considerably larger than UBA(2), with 73 and 80 residues, respectively. The latter was, in fact, found to cold-denature in the presence of 2 M GndHCl.[13] Protein B is next with the predicted T'_m at 228K, followed by the engrailed homeodomain at 224 K.

In conclusion, we found that the structure of a small 45-residue protein domain, UBA(2), is highly thermostable, but even more importantly, it shows the onset of cold denaturation above 273 K in the absence of denaturants. Cold denaturation at observable temperatures has, to our knowledge, not yet been observed for similar, very small α-helical protein domains even at high denaturant concentrations. Discovery of this rare property for such a small protein may open up the possibility of combining experiment and all-atom folding simulations to shed new light onto the still controversial issue of cold denaturation. Furthermore, T-jump experiments, which became the most widely used technique for studying ultrafast folding kinetics in small helical domains can be used to trigger overall folding from the cold denatured states.[14] As shown by Xu et al.,[15] such experiments also provide important insights into the difference between heat and cold denaturation, and can also help address another controversial issue as to whether unfolding is the reverse of folding.[16] Therefore, the UBA(2) domain presents a valuable model system to study protein folding in general and cold denaturation in particular.

■ ASSOCIATED CONTENT

Supporting Information

Description of peptide synthesis, spectroscopy, and data analysis. 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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