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Thermally versus Chemically Denatured Protein States

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Supporting Information

ABSTRACT: Protein unfolding thermodynamic parameters are conventionally extracted from equilibrium thermal and chemical denaturation experiments. Despite decades of work, the degree of structure and the compactness of denatured states populated in these experiments are still open questions. Here, building on previous works, we show that thermally and chemically denatured protein states are distinct from the viewpoint of far-ultraviolet circular dichroism experiments that report on the local conformational status of peptide bonds. The differences identified are independent of protein length, structural class, or experimental conditions, highlighting the presence of two sub-ensembles within the denatured states. The sub-ensembles, U_T and U_D for thermally induced and denaturant-induced unfolded states, respectively, can exclusively exchange populations as a function of temperature at high chemical denaturant concentrations. Empirical analysis suggests that chemically denatured states are ∼50% more expanded than the thermally denatured chains of the same protein. Our observations hint that the strength of protein backbone−backbone interactions and identity versus backbone−solvent/co-solvent interactions determine the conformational distributions. We discuss the implications for protein folding mechanisms, the heterogeneity in relaxation rates, and folding diffusion coefficients.

Denatured protein states are perhaps the most challenging to experimentally characterize given the vast conformational space that is sampled and the extreme conditions employed to populate them. While it is well established that a folded protein can be denatured by different perturbations (temperature, chemical denaturants, pH, force, pressure, and mutations), there has always been a question about the nature of ensembles that are populated.[1−4] This has been further renewed due to the explosion of research in intrinsically disordered proteins (IDPs), their functional significance, their dimensions, and the advantages and limitations of experimental techniques used to characterize them.[5−7]

Of particular interest are the differences in conformational preferences of thermally and chemically denatured protein states. The simplest spectroscopic technique used to study denaturation is far-ultraviolet (far-UV) circular dichroism (hereafter termed CD) that interrogates the local conformational preference of peptide bonds.[8] Earlier studies have pointed to differences in the magnitude of the CD signal between thermally and chemically denatured states on specific proteins.[9−20] Here, we extend these works by collecting CD data from the literature, show that the differences observed previously are robust to structure−sequence changes, and explain their implications.

To illustrate, Figure 1A plots the thermal denaturation data of Cnu, a bacterial nanosensor of environmental conditions.[21,22] The unfolding curve is apparently two-state-like with a thermally denatured ensemble exhibiting a CD signal of −5000 deg cm^2 dmol^{-1} [mean residue ellipticity (MRE) units] at 222 nm. A urea-induced unfolding experiment at a constant temperature, on the other hand, reveals a similar two-state-like transition but with a denatured state displaying a CD signal of −2000 deg cm^2 dmol^{-1} (Figure 1B). As an additional example, we show the thermal and chemical unfolding curve of PurR, a member of the LacI DNA-binding domain family. Clear differences in the CD signals of the denatured states are also apparent in these data (Figure 1C,D), consistent with earlier works.[9−20]

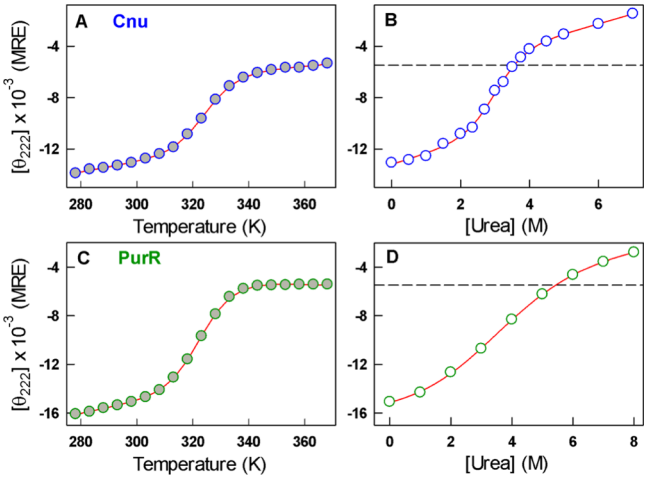

Figure 1. Thermal (filled circles, panels A and C) and chemical unfolding (empty circles and at 298 K, panels B and D) curves of Cnu at pH 5.0 (blue) and PurR at pH 7.0 (green) monitored by far-UV CD at 222 nm and reported in MRE units (degrees square centimeters per decimole). Dashed lines in panels B and D represent the highest-temperature far-UV CD signal. Red curves are fits to two-state models.

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The differences reported above could have multiple origins: errors in concentration measurement, effect of aromatics,[23] and even nontrivial intrinsic sequence effects arising from preferential interaction with the chemical denaturants.[24,25] To identify potential reasons for this deviation, we generated a database of far-UV CD data from thermal and chemical denaturation experiments that are reported in absolute units (MRE units) (see the Supporting Information). CD signals reported in absolute units are independent of protein concentration, protein length, and slight differences in sample path length and thus serve as a common ground for comparison across varied proteins and research laboratories. The database consists of 31 proteins of predominantly the α-helical or α/β structural type, with protein lengths spanning 29−150 residues. A majority of the data are at pH 7 and 298 K and include both urea-based (∼47%) and GuHCl-based (∼53%) denaturation.

A comparison of the far-UV CD signal at 222 nm for all the proteins between the highest temperature (in the absence of denaturant) and highest denaturant concentration (at a fixed temperature) reveals a consistent difference between the two, as noted in Figure 1, with a mean absolute difference of ∼3100 deg cm^2 dmol^{-1} (Figure 2). This suggests that thermally and chemically denatured protein states are fundamentally different and that the differences cannot be ascribed to protein type, structural class, nature of the denaturant, or temperature. The CD spectral shapes are distinct and reversible (see Figures S1 and S2 for select proteins), indicating that the differences cannot be attributed to errors in the estimation of concentration. Because far-UV CD is sensitive to the peptide bond conformations and the extent of alignment of amide transition dipoles (specifically that of the amide carbonyl at 222 nm), these differences can be taken as strong evidence that the two perturbants (chemical denaturants and temperature) contribute to different conformational sub-ensembles within the denatured state.

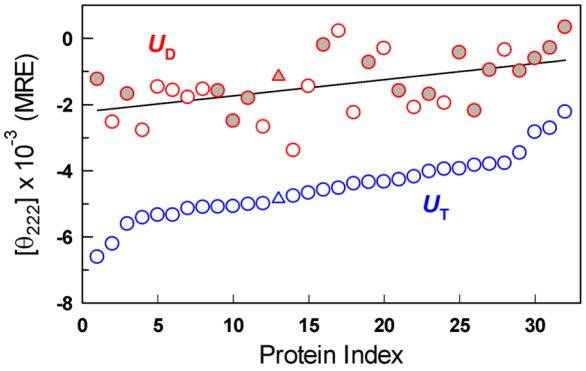

Figure 2. Far-UV CD signal at 222 nm of thermally (U_T) and chemically denatured states (U_D at ∼298 K) for the protein database used in this work (Table S1). Filled and empty red circles represent signals of proteins denatured at high concentrations of GuHCl and urea, respectively. The triangle represents the signal at 230 nm for protein index 13.

Can the sub-ensembles, U_T and U_D for thermally and chemically denatured states, respectively, exclusively exchange populations within the denatured state? Thermal perturbations of urea or GuHCl-denatured states of folded proteins reveal a continuous and reversible increase in the intensity of the CD signal at 222 nm (Figure 3A and Figures S3 and S4).[10,11,13−15,17−20] Even IDPs exhibit a tendency where the signal at 222 nm displays a negative slope with temperature (Figure 3B).[26−29] Interestingly, the CD signal range and slopes (34.7 ± 9.4 deg cm^2 dmol^{-1} K^{-1}) are very similar despite the large differences in sequence. The CD signals at high temperatures and in the absence of denaturant (U_T) agree very well with the CD signals at high denaturant concentration and high temperatures ($U_{D,\,High\ T}$) with a mean absolute difference of just ∼550 deg cm^2 dmol^{-1} (Figure 3C) for a small database of proteins (Table S2). This agreement is evidence that the two sub-ensembles can indeed exclusively exchange populations with the temperature effect trumping the denaturant-induced conformational distribution.

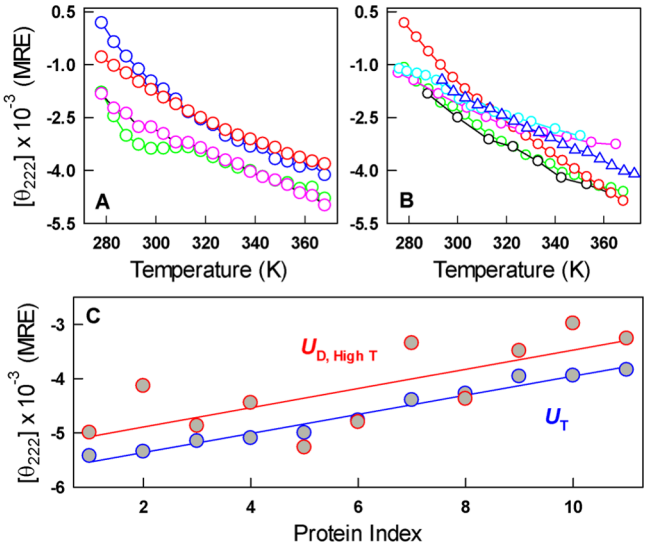

Figure 3. (A) Transitions within the unfolded ensemble observed by far-UV CD in our lab for the folded proteins Cnu at pH 5 and 8, 7 M urea (blue and green), PDD F43W at pH 3, 7 M GuHCl (red) and PurR at pH 7, and 8 M urea (magenta). (B) Transitions within the IDP ensembles (in the absence of denaturant) of RVCaB (blue), α-synuclein (magenta), the phosphodiesterase γ-subunit (cyan), Caldesmon (black), a disordered peptide C-pep (green), and the IDP CytR at 7 M urea as a reference (red). The latter two data sets were generated in our lab. (C) Comparison of far-UV CD signals of folded proteins at high temperatures (U_T, in the absence of denaturant) and under high-denaturant, high-temperature conditions ($U_{D,\,High\ T}$).

Numerous studies highlight that denatured states of folded proteins and IDPs expand with an increase in denaturant concentration while collapsing to compact globules at higher temperatures in the absence of denaturants.[28−37] Thus, it is likely that the thermally and chemically denatured states (Figure 2) represent the expanded and collapsed conformations, respectively, that are often identified in simulations[38,39] and have been reported in prior small angle X-ray scattering and CD studies.[9,12,16,17] From a purely structural view of the CD signals and employing the Uversky−Fink empirical relation between the relative CD signals and hydrodynamic volumes characterized by Stokes radii (R_S),[40] we arrive at a simple expression for the relative dimensions of U_T and U_D given the CD signals in Figure 2 (Table S1):

$$(R_S^{U_T}/R_S^{U_D})^3 \simeq (\theta_{222}^{U_D}/\theta_{222}^{U_T})$$

$$R_S^{U_D} \simeq 1.5 R_S^{U_T}$$

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This relation signifies that chemically denatured states at 298 K are on average ∼50% more expanded than thermally denatured states (in the absence of denaturant), potentially due to specific interactions of the denaturant with the protein backbone.[41−43] The caricature of U_D employed here (as a single sub-ensemble) is an approximation, as the dimensions of chemically denatured states themselves change with denaturant in a continuous[30,31,36,44] or cooperative manner.[19]

What specific structural features in the denatured ensemble could contribute to the observed differences? CD experiments point to an enhancement of the propensity of left-handed polyproline II-like local conformations (PPII) in homopolymeric peptides, denatured proteins, and IDPs with increasing urea and GuHCl concentrations.[45−49] Temperature, on the other hand, has an opposite effect on the PPII conformational bias.[50] However, a comparison of far-UV CD spectra from select proteins studied in our lab does not show the signature appearance of a peak in the CD spectrum between 220 and 230 nm (Figure S1), though the trends follow earlier observations.[50] NMR experiments clearly demonstrate that an increase in temperature does not promote helical structure acquisition in IDPs or disordered proteins.[28] Interestingly, the temperature dependence of the CD signal of the urea-unfolded state of LacI DBD is similar to that of the short fragments generated using extensive digestion of the protein by proteinase K under native conditions.[13] These experiments raise questions about employing a structural view of CD signals at high temperatures and high denaturant concentrations. Resolving these conflicts, with experiments and simulations, could provide a better understanding of the denatured state characteristics.

From a folding mechanistic perspective, the two sub-ensembles could exchange populations in a direction orthogonal to the reaction coordinate for folding (Figure 4A). In such a scenario, kinetic experiments starting from U_D will be characterized by a first step in which the unfolded population equilibrates with U_T, after which the folding reaction proceeds. Second, U_D and U_T could equilibrate with each other and the folded state (three-state triangular mechanism), while a third scenario is one in which folding proceeds strictly from either of the ensembles depending on the perturbation. Temperature-jump (T-jump) experiments on the villin headpiece reveal an order of magnitude difference in relaxation rates when the folding reaction is initiated from U_T or U_D, with the latter being slower,[51] the only such evidence available to date.

If the sub-ensembles are populated along the reaction coordinate to folding, then the rates could again be different if the folding is initiated from U_T or U_D (Figure 4B). In such cases, even proteins with smaller folding thermodynamic barriers could display slow or multiple relaxation rates, as the dynamic term (i.e., the folding diffusion coefficient) itself could be quite slow due to the initial pre-equilibration between the two sub-ensembles apart from the expected rough landscape. This is more likely in proteins rich in α-helices whose structural features are dominated by local interactions and as shown experimentally and theoretically in Barstar[52,53] and bACBP.[54,55]

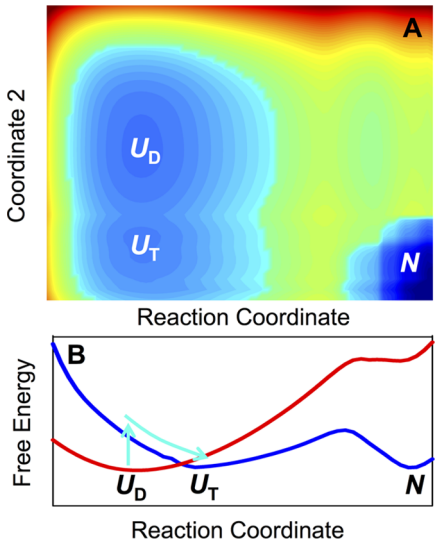

Figure 4. (A) Folding free energy surface with multiple minima corresponding to denatured (U_D and U_T) and folded (N) states at non-zero denaturant concentrations. Coordinate 2 could represent any conformational feature that distinguishes the two denatured state sub-ensembles. (B) One-dimensional (1D) free energy profiles under stabilizing (blue) and destabilizing (red) conditions. The arrows represent the downhill-like conformational redistribution that occurs on switching to conditions favorable for folding. Panel B is not a projection of panel A onto a 1D coordinate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.9b00089.

Tables S1 and S2, Figures S1−S4, and additional references (PDF)

Accession Codes

Cnu, P64467; Hha, P0ACE3; PurR, P0ACP7; and PDD, P11961.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CD, circular dichroism; PPII, polyproline II; MRE, mean residue ellipticity; R_S, Stokes radius; IDP, intrinsically disordered protein.

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