Tuning the Continuum of Structural States in the Native Ensemble of a Regulatory Protein

Abhishek Narayan and Athi N. Naganathan*

Department of Biotechnology, Bhupat & Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai 600036, India

Supporting Information

ABSTRACT: The mesoscale nature of proteins allows for an efficient coupling between environmental cues and conformational changes, enabling their function as molecular transducers. Delineating the precise structural origins of such a connection and the expected spectroscopic response has, however, been challenging. In this work, we perform a combination of urea–temperature double perturbation experiments and theoretical modeling to probe the conformational landscape of Cnu, a natural thermosensor protein. We observe unique ensemble signatures that point to a continuum of conformational substates in the native ensemble and that respond intrinsically to perturbations upon monitoring secondary and tertiary structures, distances between an intrinsic FRET pair, and hydrodynamic volumes. Binding assays further reveal a weakening of the Cnu functional complex with temperature, highlighting the molecular origins of signal transduction critical for pathogenic response in enterobacteriaceae.

**Supporting Information**

It is well established that proteins sample a variety of functionally relevant conformations in their native ensemble. The flexibility arises from the weak noncovalent nature of the stabilizing interactions, the large degree of freedom associated with the main chain, and the finite sizes of protein molecules. The resultant mesoscopic nature of proteins translates to large surface-area-to-volume ratios, thus contributing to specific interactions with the solvent molecules and large solvent-coupled fluctuations even when in thermodynamic equilibrium. Solvent properties can, therefore, be tuned either by temperature or by adding cosolvents (urea or guanidinium hydrochloride) to modulate these interactions and hence perturb the folding landscape of proteins. Perturbation experiments have therefore contributed immensely to the understanding of protein properties, particularly in two-state-like systems. In such proteins, adding cosolvents tunes the relative macroscopic populations of the folded and unfolded states, resulting in distinct sigmoidal-like unfolding curves. However, it has generally been challenging to extract or interpret the origin of signals beyond a simple two-state equilibrium because of the complexity intrinsic to such analysis. In fact, double-perturbation experiments involving cosolvents and temperature reveal distinct signal dependencies in globally downhill and incipient downhill folders, arising from the differences in the structural features of ensembles that are populated in response to one perturbation and that are tuned by another. Such an intrinsically tunable landscape allows for proteins to act as molecular transducers or rheostats; that is, they couple the changes in ambient conditions to their conformational changes that in turn can determine the functional response.

In this regard, it was recently identified that the four-helix bundle protein Cnu (Figure 1a), a single gene product, displays thermosensor-like properties that are critical for efficient pathogenic response in enterobacteriaceae that commonly infect human gastrointestinal tracts. Global spectroscopic, site-specific NMR experiments, hydrodynamic measurements, theoretical modeling, and simulations indicate that the Cnu native ensemble is best described by an array of conformational states that are in dynamic equilibrium with one another in a single broad native well. If this is indeed the case, then solvent modulations with chemical denaturants together with thermal perturbations should result in nontrivial effects on the folding landscape. Moreover, the cosolvent- or temperature-dependent spectroscopic signatures are expected to be different from conventional observations. To explore these issues in detail, we monitor the response of the native ensemble of Cnu to perturbations by urea and temperature with far- and near-UV circular dichroism (CD), fluorescence (specifically, tryptophan resonance energy transfer), hydrodynamic measurements, simulations and also perform binding studies.

We first probe the features of Cnu folding landscape with a variant of the statistical mechanical Wako–Saitó–Muñoz–Eaton (WSME) model. Using identical parameters as a previous study, a chemical denaturant dependence is introduced following the linear free-energy relation commonly observed in experiments (see the Supporting Information) and as employed before. Such a perturbation reveals that the native ensemble of Cnu can be coarsely divided into two subensembles, N and N* (Figure 1b). Importantly, their properties vary as a function of both temperature and cosolvent...
component that reports on spectral changes (Figure 2d). The temperature at which N


temperature at different urea concentrations. (f) Apparent melting (Figure 2b,c). SVD (singular-value decomposition) analysis of 1 and 3 M urea in Figure 2a). Remarkably, the signals at 290 nm, clearly indicating that distinct ensembles are populated at these urea concentrations (for example, compare the spectra at 0 and 3 M urea in Figure 2a). The resulting apparent T_m, measured as the temperature at which the signals crossover in signs, should follow a linear trend with urea (Figure 1f).

Cnu has one tryptophan in the fourth helix and five tyrosines that are distributed throughout the structure. In proteins rich in aromatic residues, near-UV CD spectral analysis can provide detailed structural information, as they are sensitive to the tertiary packing environment of tyrosine and tryptophan.17 The near-UV CD spectral signatures of Cnu at four different urea concentrations (0 to 3 M) reveal distinct amplitudes for the overall- and relative-spectral bands (265, 270, 280, and 290 nm), clearly indicating that distinct ensembles are populated at these urea concentrations (for example, compare the spectra at 1 and 3 M urea in Figure 2a). Remarkably, the signals at 290 and 280 nm follow the exact same trend predicted by the WSME model, suggesting that they probe the overall population of the native ensemble and that of N*, respectively (Figure 2b,c). SVD (singular-value decomposition) analysis of the temperature—wavelength spectra reveals an anticorrelation between the bands of tyrosine and tryptophan in the second component that reports on spectral changes (Figure 2d). The amplitude of this component decreases linearly with temperature and changes sign (positive to negative) at specific temperatures as a function of urea (Figure 2e). This observation is also in accordance with the predictions of the WSME model that points to this dependence to be originating from the differences in the populations of N and N* (Figure 1e).

The urea-dependent far-UV CD signal at 222 nm again displays a pattern that has not been reported in any protein system: the signal intensity increases (becomes more negative) with the urea concentration, reaches a plateau, and then decreases in intensity in a sigmoidal fashion (Figure 2f and Supporting Information Figure S1). The position of the minima moves toward lower urea concentrations and concomitantly decreases in magnitude, suggestive of a malleable native ensemble. What could contribute to this unique dependence? Careful analysis of the spectral features of far-UV CD bands in proteins has shown that tyrosine exhibits a strong positive band when in a helical conformation.18 The fact that Cnu has five tyrosines and that the signal intensity increases with urea suggests that some tyrosines populate nonhelical conformations even at 298 K, despite the overall structure appearing to be folded. As the temperature is increased, the probability of the unfolded ensemble increases, thus resulting in a decrease in the signal intensity. In other words, the observed rollover in far-UV CD signals at 222 nm arises from a delicate balance between these two features.

Figure 1. WSME model predictions. (a) Structure of Cnu highlighting the various aromatic residues. Note that W67 is in the fourth helix while Y40 is in the third helix. (b) Expected changes in the 1D free-energy profile (in k J mol\(^{-1}\)) and the corresponding populations as a function of urea at 278 K. (c–e) Predicted changes in the population of the folded ensemble, N*, and the relative population as a function of temperature at various urea concentrations. (f) Apparent melting temperature at different urea concentrations measured as the temperature at which N* starts to dominate over N (arrow in panel e).

Figure 2. Unique solvent sensitivity of Cnu. Panels a–e follow the same color code as panel a. Lines in panels b–f are shown to guide the eye. (a–c) Near-UV CD spectra at 298 K in mean residue ellipticity (MRE) units of deg cm\(^2\) dmol\(^{-1}\) and normalized unfolding curves at 290 and 280 nm at different urea concentrations. (d) Spectral signatures of the two significant components from an SVD of the urea—temperature near-UV CD data. (e) Amplitude of the second component as a function of temperature and urea. Note that the temperatures at which the amplitudes change sign (vertical lines) are urea-dependent (arrow in panel e). (f) Far-UV CD monitored changes in secondary structure as a function of urea at representative temperatures. The dashed line signals the molar ellipticity at 0 M and 278 K.
Circular dichroism experiments highlight the native ensemble of Cnu to be changing both its secondary and tertiary structure with solvent perturbations in a distinct manner. The structural changes are more probable at the C-terminal helix due to its weak packing and the large conformational flexibility of the loop connecting the third and fourth helices. We therefore expect the relative distances between W67 (located in the fourth helix) and Y40 (in the third helix; Figure 1a) to increase with perturbation magnitude within the native ensemble, that is, in the pretransition region where there is only a minimal population of the unfolded state.

The quantum yield (QY), as estimated by exciting the protein at 274 nm, increases with urea concentration, reaches a plateau, and then decreases sigmoidally, mirroring far-UV CD observations (Figure 3a). The apparent chemical midpoint is estimated to be ~5.3 M at 298 K from a first-derivative analysis of the QY data. To understand the possible structural changes that contribute to this unique observation, we perform a global SVD of the raw temperature/urea-wavelength fluorescence data. The spectral deconvolution results in two significant components, U1 and U2, from SVD of the urea–temperature fluorescence data. (c) Temperature dependence of the amplitude of the second component at select urea concentrations of 0 (blue), 1 (green), 3 (orange), 5 (magenta), 5.3 (red), and 6 M (black). Note that the temperatures at which the amplitudes change sign (vertical lines) are urea-dependent (arrow in panel c). (d) Effective spectral change at 0 M urea, obtained by multiplying U2 with V2, is shown as a representative example to highlight the changes. Inset plots the temperatures at which the signs change at different urea concentrations (from panel c).

Figure 3. Fluorescence monitored structural changes in the native ensemble. Lines in panels a and c are shown to guide the eye. (a) Quantum yield (QY) of the protein upon excitation at 274 nm as a function of urea at select temperatures. (b) Spectral signatures of the two significant components, U1 and U2, from SVD of the urea–temperature fluorescence data. (c) Temperature dependence of the amplitude of the second component at select urea concentrations of 0 (blue), 1 (green), 3 (orange), 5 (magenta), 5.3 (red), and 6 M (black). Note that the temperatures at which the amplitudes change sign (vertical lines) are urea-dependent (arrow in panel c). (d) Effective spectral change at 0 M urea, obtained by multiplying U2 with V2, is shown as a representative example to highlight the changes. Inset plots the temperatures at which the signs change at different urea concentrations (from panel c).

The sensitivity of Cnu to solvent conditions, a feature of protein molecular rheostats, raises questions on the biological necessity for a tunable native ensemble.21,22 Because the enterobacteriaceae family predominantly infects human hosts, the constant body temperature of 310 K becomes a major thermodynamic variable. Microbiological–biochemical
experiments have also shown that a complex between Cnu and H-NS represses pathogenic response at low temperatures (∼280 K) while promoting the expression of toxins at higher temperatures (∼310 K; the body temperature of humans).23 It is the molecular patch formed by the helices 3 and 4 of Cnu that is responsible for binding with H-NS.24,25 Because experiments and simulations point to a continuous increase in the distances between these two helices with changing solvent conditions, it points to a simple mechanism by which the binding affinity can be regulated: the binding interface should be well formed at 280 K, thus promoting complex formation while the interface should be destabilized at high temperatures (Figure S3b). To test for this experimentally, we monitored the change in tryptophan fluorescence anisotropy upon titration with H-NS1−59 at select temperatures. (d) Apparent dissociation constants from fits (line in panel c) assuming a 1:1 binding equilibrium.

Figure 4. Structural swelling and functional significance. (a) Changes in the Stokes radius as monitored by analytical size-exclusion chromatography (aSEC) at 278 K and at various urea concentrations (0 M is in blue while the rest are in gray bars). The horizontal cyan and black lines signal the protein dimensions as measured by analytical ultra centrifugation at 278 and 298 K, respectively. The horizontal dashed line indicates the expected dimension of a molten-globular conformation of Cnu from size-scaling arguments. (b) Distribution of Cα−Cα distances between Y40 in the third helix and W67 in the fourth helix from all-atom MD simulations at different temperatures. (c) Changes in tryptophan anisotropy upon titration with H-NS1−59 at select temperatures. (d) Apparent dissociation constants from fits (line in panel c) assuming a 1:1 binding equilibrium.

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.7b00475. Methods, the far-UV CD double-perturbation data, amplitudes of the fluorescence second component compared against experimental QY measures, Y40−W67 distances from coarse-grained simulations, and the unfolding curve of H-NS1−59 (PDF)

ACCESS SHEET

Corresponding Author

E-mail: athi@iitm.ac.in.

ORCID

Athi N. Naganathan: 0000-0002-1655-7802

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was funded by the grants BT/06/IYBA/2012-14 from the Department of Biotechnology (DBT) and IA/1/15/1/501837 from the Wellcome Trust/DBT India Alliance Intermediate Fellowship to A.N.N. We acknowledge the FIST facility sponsored by the Department of Science and Technology (DST), India at the IITM.

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