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Hierarchy of local structural and dynamics perturbations due to subdenaturing urea in the native state ensemble of DLC8 dimer

P.M. Krishna Mohan [1,2], Swagata Chakraborty [1], Ramakrishna V. Hosur *

Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai 400 005, India

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ABSTRACT

Local structural and dynamic modulations due to small environmental perturbations reflect the adaptability of the protein to different interactors. We have investigated here the preferential local perturbations in Dynein light chain protein (DLC8), a cargo adapter, by sub-denaturing urea concentrations. Equilibrium unfolding experiments by optical spectroscopic methods indicated a two state like unfolding of DLC8 dimer, with the transition mid-point occurring around 8.6 M urea. NMR studies identified the β3 and β4 strands, N-, C- terminal regions, loops connecting β1 to α1, α1 to α2 and β3 to β4 as the soft targets of urea perturbation and thus indicated potential unfolding initiation sites. Native-state hydrogen exchange studies suggested the unfolding to traverse from the edges towards the centre of the secondary structural elements. At 6 M urea the whole protein chain acts like a cooperative unit. These observations are expected to have important implications for the protein's multiple functions.

1. Introduction

DLC8, the smallest (10.3 kDa, 89 residues) light chain of the dynein motor complex is a highly conserved protein that is found in all organisms [1,2]. DLC8 interacts with proteins of diverse biological functions [3–8]. The conserved nature, ubiquitous expression, multiple biochemical forms, and numerous cellular targets of DLC8 strongly suggest that the protein might be playing a conserved cellular function in multiple protein complexes [1,2,9] . DLC8 exists as a homo dimer at physiological pH and a monomer at pH 3 [10,11]. However, DLC8 is functional only in its dimeric form. The DLC8 dimer consists of two α-helices (α1, residues 15–31; α2, residues 35–50) and 5 β-strands (β1, residues 6–11; β2, residues 54–59; β3, residues 62–67; β4, residues 72–78 and β5, residues 81–87) [2]. DLC8 is involved in cargo trafficking across the cell. In this process it encompasses small folding/unfolding events accompanied with small structural/dynamic fluctuations in order to facilitate loading/unloading of cargo molecules. pH dependent native energy landscape analysis and target binding studies on DLC8 suggested small environmental perturbations can efficiently modulate target binding efficacies by regulating the dynamics of the dimer [12,13]. In view of this it is important to monitor the structural and motional behavior of the DLC8 in presence of various agents that cause differential local perturbations in the protein. These slight perturbations create ensemble of protein conformations which inter-convert amongst themselves and are important from the functional point of view of the protein.

Stability and unfolding features of DLC8 protein both in its dimeric and monomeric forms have been studied extensively using denaturants, guanidine hydrochloride and urea [10,14–19]. These denaturants interact differently with the folded protein; guanidine being positively charged leads to efficient hydrophobic collapse via electrostatic screening while urea is neutral and is a milder structure perturbing agent. Being a chaotropic agent, urea has been thought to disrupt hydrophobic interactions responsible for the globular structure of proteins [20–24]. However, recent studies on urea interaction with amino acids indicated that urea has preference for a favorable interaction at polar amide surface, located mostly on the peptide backbone [25,26]. Moreover, transfer model analysis by Auton et al. [27] clearly showed that urea's favorable interaction with peptide backbone is the driving force for urea-induced denaturation, with nonpolar group-urea interactions playing little or no role in the process. All these results strongly suggest that hydrogen bonds between urea and the protein backbone, contribute significantly to the overall energetics of urea denaturation [25–27].

There are numerous effects of urea that can influence protein function, such as solute-induced attenuation of hydrophobic/electrostatic interactions which are important between substrate and protein

Abbreviations: HSQC, Hetero nuclear single quantum coherence; TOCSY, Total correlation spectroscopy; HX, Hydrogen exchange; NHX, Native state hydrogen exchange; NOE, Nuclear overhauser effect; CPMG, Carr Purcell Meiboom Gill; TALOS, Torsion angle likelihood obtained from shift and sequence similarity; CD, Circular dichroism; GdnHCl, Guanidine Hydrochloride; aa, amino acids; DTT, Dithiothreitol.

* Corresponding author. Tel.: +91 22 2278 2488; fax: +91 22 2280 4610.
E-mail address: hosur@tifr.res.in (R.V. Hosur).

[1] Both authors contributed equally to this work.

[2] Present address: Department of Chemistry and Chemical Biology, Rutgers University, 610 Taylor Road, Piscataway, NJ, 08854, USA.

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[28]. Moreover urea is present in the renal cells of many organisms. Thus, in view of the possible implications of urea interactions to protein stability and function, we explored here the preferential local perturbations by sub-denaturing urea concentrations in native DLC8. These, in some sense, also provide a simplistic identification of the initiation sites of DLC8 unfolding.

2. Materials and methods

2.1. Protein expression and purification

DLC8 was expressed and purified as described elsewhere [11,13].

2.2. Optical spectroscopy

2.2.1. Circular dichroism and fluorescence spectroscopy

Far-UV CD spectra were recorded at pH 7 on a JASCO model J-810 spectropolarimeter using a 1 nm bandwidth at 27 °C. The global unfolding of protein was monitored at 222 nm. Steady-state fluorescence emission spectra were recorded with $\lambda_{ex} = 295$ nm on a Spex Fluorolog-dM3000F spectrofluorimeter using a 1 cm path-length cuvette with a band pass of 1.5 nm for both excitation and emission. For all the experiments the protein concentration is 20 μM as a dimer (20 mM Tris, 200 mM NaCl, 2 mM DTT, pH 7). Denaturation profiles of DLC8 were measured as described by Chatterjee et al. [14] at 27 °C (also see Supplementary material).

2.2.2. Data analysis

The analysis of optical unfolding data of DLC8 is complicated because DLC8 is a dimer. Often the intermediate is not clearly identified by the optical techniques and the model D⟺2U, described as a two-state process can be used, with a concentration dependent second order dissociation/unfolding approximation.

The spectroscopic signal measured from the CD/fluorescence has been used to calculate fraction unfolded (monomeric protein) using Eq. (1) and the equilibrium constant was determined using concentration dependent second order dissociation Eq. (2).

$$f_u = \frac{S_d - S_i}{S_d - S_u} \tag{1}$$

$$K = \frac{[U]^2}{[D]} = \frac{2Pf_u^2}{1-f_u} \tag{2}$$

Where f_u is the apparent fraction unfolded, S_d is the spectroscopic signal associated with the pure dimer, S_u is the signal of the unfolded, S_i is the signal at varying concentrations of the denaturant, K is the equilibrium constant for unfolding, and P is the protein concentration.

The free energies at various concentrations of urea (ΔG_i) have been calculated using Eq. (3) and the free energy ΔG_i is calculated as $\Delta G_i = -RT \ln K_i$, (K_i obtained from Eq. (2)).

$$\Delta G_i = \Delta G - m[\text{Urea}] \tag{3}$$

The free energy change ΔG was determined by a fit for points in the transition region of the denaturation curve using a linear fitting. In the Eq. (3), ΔG is the intercept of the line which provides the free energy of denaturation or unfolding at 0 M urea and m is the slope of the line given by a plot of ΔG_i vs [Urea] [29–34].

2.3. NMR spectroscopy

2.3.1. NMR data acquisition, processing and analysis

For NMR studies the protein purified as described above was concentrated to ~1.0–1.5 mM. Tris buffer (20 mM Tris, 200 mM NaCl, 2 mM DTT, pH 7.0) was used for all the experiments. All NMR experiments were performed at 27 °C either on Bruker Avance 800 MHz or on Varian Unity-plus 600 MHz NMR spectrometer. ^{1}H chemical shifts were calibrated relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). All the triple resonance experiments required were recorded as discussed earlier [13,14]. Native state hydrogen exchange experiments (NHX) were carried out under the sub-denaturing conditions at various urea concentrations (2, 4 and 6 M) as discussed by Mohan et al. [18] Transverse relaxation rates (R_2) were measured with the following CPMG delays: 10, 30, 50, 70, 90, 110, 130, 150, 170 and 190 ms. Longitudinal relaxation rates (R_1) were measured with the following inversion recovery delays: 10, 50, 120, 220, 350, 500, 700 and 900 ms. For steady state heteronuclear (^{1}H–^{15}N) NOE experiments proton saturation time of 2.5 s and relaxation delay of 2.5 s were used. A relaxation delay of 2.5 s was used in the experiment without proton saturation.

All the data were processed using FELIX 2002. Residue specific cumulative chemical shift changes (ΔCS) at native state (0 M urea) and at different concentrations of urea (1 M, 2 M, 4 M and 6 M) were calculated as described earlier [16]. The most probable (ϕ,ψ) values were obtained from H^{α}, C^{α}, C^{β}, CO and ^{15}N chemical shifts by using the TALOS algorithm [35] for the urea perturbed states (2 M and 4 M Urea) as well for the native protein (0 M urea) for the purpose of comparison as described by Chatterjee et al. [14] and are listed in S1-Table (Supplementary material). The TALOS output file also provides the range of phi and psi deviations (δphi and δpsi). In order to calculate the significance of Δ (ϕ,ψ) values between two states we defined two new parameters given by

$$S_{phi} = |\Delta\phi| \sqrt{(k_{1,phi}^2 + k_{2,phi}^2)} \tag{4}$$

$$S_{psi} = |\Delta\psi| \sqrt{(k_{1,psi}^2 + k_{2,psi}^2)} \tag{5}$$

where, $k_{1,\text{phi}}$ and $k_{2,\text{phi}}$ are the ratios $\delta(\text{phi}_1)/\text{phi}_1$ and $\delta(\text{phi}_2)/\text{phi}_2$; $k_{1,\text{psi}}$ and $k_{2,\text{psi}}$ are the ratios $\delta(\text{psi}_1)/\text{psi}_1$ and $\delta(\text{psi}_2)/\text{psi}_2$ respectively and the suffices 1 and 2 indicate two different urea concentrations.

The R_1 and R_2 values were extracted by fitting the peak intensities to the equation, $I(t) = B \exp(-R_{1,2}t)$. Steady state ^{1}H–^{15}N NOE was calculated as a ratio of intensities of the peaks with and without proton saturation. The errors in the NOEs were obtained as described by Farrow et al. [36]. Reduced spectral density analysis was carried out for calculation of spectral densities as described by Chatterjee et al. [14]. For hydrogen exchange studies a series of HSQC spectra were recorded at regular intervals of time for around 12 h. These decays were fitted to single exponential functions to obtain the first order rate constants, k_{obs} and hence the protection factors as described elsewhere [18]. The highly protected non decaying residues are given a constant value of log (PF) = 9. The initial HSQC spectrum was recorded 8 min (dead time) after adding D_2O (S1-Figure, Supplementary material).

2.3.2. Resonance assignment of urea dependent HSQC spectra

The backbone resonance assignment of the DLC8 dimer (0 M urea) at pH 7, 27 °C was reported earlier [11,13]. We assigned the resonances in the HSQC spectra up to 6 M urea, in order to throw light on the urea induced perturbations to the protein. As the spectra were almost similar we were able to assign most of the resonances using direct transfer from the 0 M spectrum. However, in case of ambiguities, the resonances were confirmed with experiments such as CBCA(CO)NH and CBCANH. The number of peaks assigned in the HSQC spectra at 2 M, 4 M and 6 M urea are 88, 85 and 77 respectively.

3. Results and discussion

3.1. Urea induced equilibrium unfolding of DLC8 dimer

Equilibrium denaturation experiments using circular dichroism and steady state fluorescence provide a convenient way to understand