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The folding pathway of a functionally competent C-terminal domain of nucleophosmin: Protein stability and denatured state residual structure

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ABSTRACT

Nucleophosmin (NPM1) is a nucleolar protein implicated in ribosome biogenesis, centrosome duplication and cell cycle control; the *NPM1* gene is the most frequent target for mutations in Acute Myeloid Leukemia. Mutations map to the C-terminal domain of the protein and cause its unfolding, loss of DNA binding properties and aberrant cellular localization. Here we investigate the folding pathway and denatured state properties of a NPM1 C-terminal domain construct encompassing the last 70 residues in the reference sequence. This construct is more stable than the previously characterized domain, which consisted of the last 53 residues. Data reveal that, similarly to what was discovered for the shorter construct, also the 70-residue construct of NPM1 displays a detectable residual structure in its denatured state. The higher stability of the latter domain allows us to conclude that the denatured state is robust to changes in solvent composition and that it consists of a discrete state in equilibrium with the expanded fully unfolded conformation. This observation, which might appear as a technicality, is in fact of general importance for the understanding of the folding of proteins. The implications of our results are discussed in the context of previous works on single domain helical proteins.

1. Introduction

Nuclophosmin (NPM1) is a ubiquitously expressed nucleolar protein, involved in several cellular processes encompassing chaperone activity of nucleic acids and proteins, ribosome biogenesis, cell cycle regulation and response to stress stimuli [1,2]. NPM1 recently gained considerable attention because naturally occurring mutations in the *NPM1* gene have been reported as the most frequent genetic lesion in Acute Myeloid Leukemia (AML) [3]. Insertion or duplication of a few bases at the last gene exon results in the expression of a protein that is four residues longer at the C-terminal domain and has a different sequence in the last seven residues. The NPM1 mutated at the C-terminal domain is destabilized [4], and thereby DNA binding and nucleolar localization of the protein are lost [3,5]. The loss of nucleolar functions in the mutated protein and its association with destabilization of the C-terminal domain demanded an investigation of the folding of NPM1 in an attempt to unveil the molecular basis of malignant transformation [6].

Understanding the folding of a protein demands an extensive description of the pathway leading from the unfolded to the native functional state [7]. To address this problem, it is of critical importance to probe the structural properties of the starting species in the reaction, i.e. the denatured state [8]. Several studies on single domain proteins have demonstrated that whilst the denatured state in the presence of high concentrations of denaturants is an expanded random coil (the so-called unfolded state), under physiological conditions it may retain some residual structure that may be involved in driving folding [6,9–14]. It is therefore critical to distinguish between the denatured state under physiological conditions, classically referred as the *D* state and the random coil expanded unfolded state *U*, which is generally populated in the presence of high concentrations of denaturant. The inter-conversion between *U* and *D* upon denaturant dilution is generally very fast and tends to elude experimental characterization. Thus, very little is known about the mechanisms whereby the residual structure in denatured state is formed, and it is still under debate whether such transition occurs via a continuum of gradually folded states (downhill, second order transition) [15] or via partitioning between discrete states (barrier limited, first order transition) [16].

We have previously characterized the folding pathway of the 53 residues construct of the C-terminal domain of NPM1 (NPM1-C53), comprising the terminal three-helix bundle; we discovered that folding proceeds *via* a compact denatured state whose structural features have been unveiled by site-directed mutagenesis [6,17]. In this work, we have addresses the crucial problem of the folding mechamism of a bigger construct of NPM1 comprising 70 residues (NPM1-C70), i.e. additional 17 residues at the N-terminus of the

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domain. As demonstrated by high resolution NMR and surface plasmon resonance experiments these additional 17 residues are critical for the DNA binding properties of NPM1, although they appear to be unstructured even in the 1:1 complex with G-quadruplex DNA [18,19].

While the longer construct appears to fold *via* an overall mechanism similar to NPM1-C53 [6,17], its higher thermodynamic stability [20] allowed us to probe the properties of its denatured state over a much wider range of stabilities. Thus, we addressed the folding equilibrium and kinetics in the presence of different concentrations of NaCl in order to modulate stability. As described below, analysis of the folding co-operativity highlights a clear dependence on protein stability of the change in solvent accessible surface area upon unfolding; a feature that is diagnostic of the presence of residual structure in the denatured state [21]. Thanks to the increased stability of NPM1-C70, when we assessed folding co-operativity as a function of protein stability, we observed that the change in solvent-accessible surface area approaches a plateau when the free energy of unfolding exceeds ~4.0 kcal mol^{-1}. This finding suggests that the denatured protein is a discrete state, in equilibrium with the fully random unfolded conformation, and thus its structure is not completely malleable to changes in experimental conditions.

2. Materials and methods

2.1. Cloning and purification

NPM1-C70 was expressed and purified as previously described [18]. SDS–PAGE electrophoresis and mass spectrometry were used to confirm that the protein was of the expected mass and to evaluate its purity. All reagents were purchased from Sigma (Sigma–Aldrich, St. Louis, MO) and were of analytical grade.

2.2. Fluorescence

Fluorescence emission spectra of NPM1 were recorded between 300 and 400 nm with an excitation wavelength of 280 nm at a concentration of 10 μM, using a Fluoromax spectrofluorimeter (Jobin Yvon, New Jersey) in a 1 × 0.4 cm quartz cuvette (Hellma).

2.3. Stopped-flow measurements

Single mixing kinetic folding experiments were carried out on a SX18-MV stopped-flow instrument (Applied Photophysics, Leatherhead, UK); the excitation wavelength was 280 nm and the fluorescence emission was measured using a 320 nm cut-off glass filter. In all experiments, performed at 298 K, refolding and unfolding were initiated by an 11-fold dilution of the denatured or the native protein with the appropriate buffer. Final protein concentrations were typically 1 μM. The observed kinetics were always independent on protein concentration, as expected for a monomolecular reaction without effects due to transient aggregation [22].

2.4. Data analysis

Equilibrium experiments. Assuming a standard two-state model, the urea-induced denaturation transitions were fitted to the equation:

$$\Delta G_d = m_{D-N} \cdot (D - D_{1/2}) \quad (1)$$

where ΔG_d is the free energy of folding at a concentration D of denaturant, m_{D-N} is the slope of the transition (proportional to the increase in solvent-accessible surface area on going from the native to the denatured state) and $D_{1/2}$ is the midpoint of the denaturation transition. An equation that takes into account the pre- and post-transition baselines was used to fit the observed unfolding transition [23].

Kinetic experiments. Analysis of observed time courses was performed by non-linear least-squares fitting of single exponential time courses using the fitting procedures provided in the Applied Photophysics software. The chevron plots were fitted by numerical analysis based on a two-state model following the equation:

$$k_{obs} = k_F + k_U \quad (2)$$

where k_F and k_U represent the folding and unfolding rate constants, respectively. The logarithm of each microscopic rate constant was assumed to vary linearly with denaturant concentration [24], following the equation:

$$k_{obs} = k_F \exp(-m_F[\text{urea}]) + k_U \exp(m_U[\text{urea}]) \quad (3)$$

By following the two state assumption [25], the total m upon unfolding was calculated as follows:

$$m_{D-N} = m_F + m_U \quad (4)$$

3. Results

3.1. Equilibrium unfolding of NPM1-C70

The equilibrium unfolding denaturation of NPM1-C70, monitored by intrinsic fluorescence recorded at pH 7.2 and 10 °C, is reported in Fig. 1. The denaturation process displays a simple sigmoidal profile consistent with a two state mechanism. The unfolding free energy derived from a two state analysis is 3.2 ± 0.2 kcal mol^{-1}, with a m_{D-N} value of 0.82 ± 0.04 kcal mol^{-1} M^{-1}. Interestingly, the magnitude of the m_{D-N} value is the same as that previously observed for NPM1-C53, which was 0.84 ± 0.08 kcal mol^{-1} M^{-1}. Because it has been theoretically

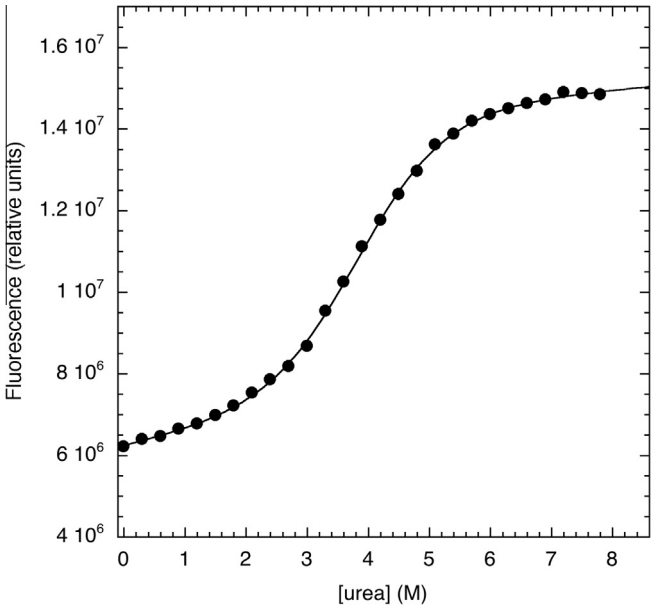

Fig. 1. Equilibrium unfolding of NPM1-C70. The urea-induced denaturation, measured at pH 7.2 in 50 mM sodium phosphate buffer and 10 °C, was followed by fluorescence at different wavelengths (1 μM protein concentration). The line represents the best fit to a two-state model. Thermodynamic analysis of the observed transition yielded an apparent unfolding free energy of 3.2 ± 0.2 kcal mol^{-1}, displaying an m_{D-N} value of 0.82 ± 0.04 kcal mol^{-1} M^{-1}.

demonstrated [26] and empirically observed [27], that the m_{D-N} value can be correlated with the change in solvent accessible surface area upon unfolding, we conclude that the additional 17 residues in the NPM1-C70 are largely unstructured, in agreement with NMR spectroscopy [19]. On the other hand, since the NPM1-C70 construct appears to be over-stabilized by 1.3 ± 0.2 kcal mol^{-1}, we conclude that the N-terminus, whilst largely unstructured, is involved in establishing favorable interactions in the native state.

3.2. Folding kinetics: evidence for a two-state mechanism

In an effort to describe quantitatively the folding mechanism of NPM1-C70, we carried out extensive kinetic experiments. Thus, the folding and unfolding kinetics were investigated at several pH values, from 3.0 to 8.5, and in the presence of increasing concentration of NaCl (up to 1 M), to increase the thermodynamic stability of the protein. Under all investigated conditions, the folding and unfolding time courses were consistent with a single exponential decay, suggesting two-state folding. A semi logarithmic plot of the observed rate constants as a function of denaturant concentration (chevron plot), measured at pH 7.2 and 10 °C, is reported in Fig. 2. It is evident that the observed kinetics is consistent with a V-state chevron, a hallmark of two-state folding [24]. Furthermore, the thermodynamic parameters are consistent with those calculated from equilibrium experiments, yielding a kinetic m_{D-N} value of 0.88 ± 0.04 kcal mol^{-1} M^{-1} and a change in free energy upon unfolding of 3.3 ± 0.1 kcal mol^{-1}. These observations strongly suggest that, in analogy to what previously observed for NPM1-C53, also NPM1-C70 follows a two-state folding mechanism.

The chevron plots of NPM1-C70 measured at pH 7.2 and 10 °C in the presence of increasing concentrations of NaCl from 0 to 1 M (see Fig. 2) show that salt has a pronounced effect on (i) the folding rate constant, (as mirrored by the left arm of the chevron plot that increases at higher concentrations of NaCl), and (ii) the unfolding rate constant (as inferred from the right arm of the chevron, which decreases with increasing concentrations of NaCl). Remarkably, however, the slopes of the folding and unfolding arms appear to be unaffected by salt, suggesting the folding of NPM1-C70 to be highly robust to changes in experimental conditions. A quantitative analysis of the dependence of the folding and unfolding m values and their implications for the folding mechanism of NPM1-C70 is described below.

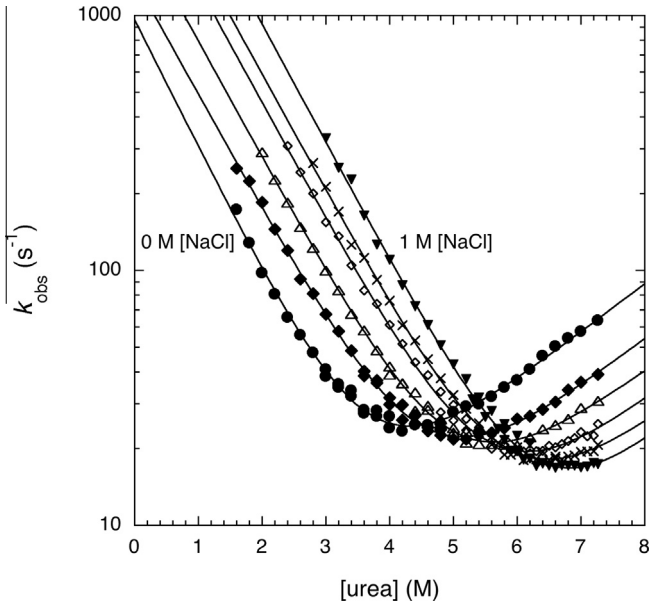

Fig. 2. Folding kinetics of NPM1-C70 at pH 7.2 and 10 °C. The different chevron plots were measured in the presence of 0.0 (●), 0.2 (♦), 0.4 (△), 0.6 (◇), 0.8 (×) and 1.0 (▼) M sodium chloride. The lines are the best fit to a two state chevron plot as formalized in Eq. (3). It is evident that, whilst both the folding and unfolding rate constants are affected by addition of salt, the slopes of the folding and unfolding arms appear essentially independent on ionic strength changes.

3.3. Dissecting the properties of the denatured state of NPM1: dependence of folding parameters on protein stability

A thermodynamic cycle describing the unfolding of a protein upon addition of denaturant implies that the unfolding free energy change calculated in the presence and in the absence of denaturant equals the change in free energy of transfer of the native and the denatured states from water to denaturant, i.e. $\Delta G^{\text{denaturant}}_{N\to D} - \Delta G^{\text{water}}_{N\to D} = \Delta G_{\text{transfer},D} - \Delta G_{\text{transfer},N}$ [26]. Thus, it was postulated, and empirically observed [27], that the slope of the dependence of the change in free energy between two states, the so-called m value, is correlated to the change in solvent accessible surface area of the two states. Consequently, the analysis of the dependence of the equilibrium and kinetic m values is a powerful test to analyze under different experimental conditions the overall structural properties of folding intermediates, as well as denatured and transition states [21].

In an effort to analyze in depth the folding of NPM1-C70 we determined the dependence of the m_F, m_U and m_{D-N} value on protein stability. The calculated values from data under different experimental conditions are reported in Fig. 3. The dependence of m_{D-N} clearly parallels the dependence of m_F, while m_U is essentially insensitive to relative protein stability. Indeed, it appears that both m_F and m_{D-N} level off to a plateau value when the free energy of unfolding is higher than ~4.0 kcal mol^{-1}. This feature suggests that the change in accessible surface area between (i) the dena-

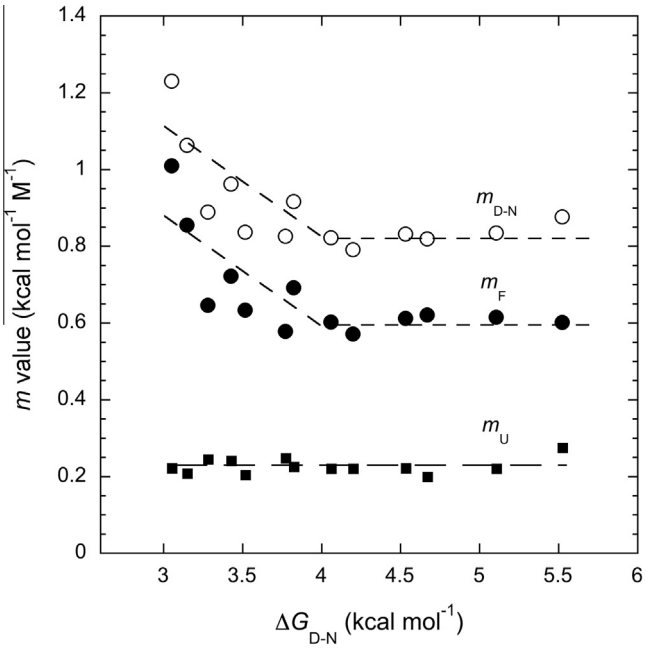

Fig. 3. Dependance of m_F (●), m_U (■) and m_{D-N} (○) on protein stability. Data were obtained at 10 °C and at pH 3.0, 4.0, 4.7, 7.2 and 8.5 by measuring individual chevron plots at variable protein stability modulated by pH and NaCl, following the approach employed for NPM1-C53 [6]. Both m_F and m_{D-N} display a complex dependence on protein stability but tend to level off at a value of ΔG_{D-N} higher than ~4 kcal mol^{-1}; on the other hand, m_U appears to be independent on protein stability. The implications of such complex dependence are discussed in the text. Broken lines have been drawn to guide the eye.

tured state and the transition state (m_F) and (ii) the native and the denatured state (m_{D-N}), are constant above a threshold of protein stability. Thus, above $\sim$4.0 kcal mol^{-1} (Fig. 3), the folding of NPM1-C70 follows a two-state mechanism, and although its denatured state retains some degree of residual structure, it is structurally robust to changes in experimental conditions.

4. Discussion

The folding pathway of some single domain proteins is dictated by the residual structure in their denatured states [6,9–14]. A typical test to detect this elusive structural feature lies in studying the correlation between the experimental m values and the overall ΔG_{D-N} of a protein. Following Tanford [26], the m value is proportional to the difference in solvent accessible surface area between two states; in going from D to N the total change is defined as m_{D-N}. From kinetic experiments, it is possible to calculate m_F, reflecting the change in accessible surface area in going from D to the transition state, and m_U, reflecting the change between N and the transition state. If the denatured state of the protein retains some residual structure, the dependence of m_F on protein stability parallels the dependence of m_{D-N}, whilst the m_U value is independent [21]. In the case of NPM1-C53, we could detect this pattern [6] and track down by site-directed mutagenesis the structural contacts present in the denatured state [17]; however, because of the limited thermodynamic stability, only a relatively narrow window along the stability scale could be explored. Thanks to the greater folding stability of NPM1-C70, in this work, we could demonstrate that, when the free energy of unfolding is higher than $\sim$4.0 kcal mol^{-1}, both m_F and m_{D-N} follow the same trend and reach a plateau value (Fig. 3). Therefore above this threshold, the denatured state, while retaining some detectable residual structure, appears robust to changes in experimental conditions, and may therefore by formally considered a discrete state along the folding reaction pathway.

The transition between two different states of a protein may occur *via* two alternative scenarios, generally referred to as first- and second-order phase transitions [28]. In the case of a first-order transition, there is an abrupt and discontinuous change in structural features and a genuine thermodynamic barrier separates the initial and final states. On the other hand, in the case of second order transitions, the structure undergoes a glassy continuous structural rearrangement with no transfer of heat from the system. Thus, while the first-order transition postulates a statistical distribution of molecules between two different discrete thermodynamic wells, in the case of second-order transitions there is a continuum of states between the initial and final phases. The question whether protein folding reactions may proceed *via* the former or the latter scenario is gaining importance [29,30]. An experimental method to address the structure of intermediates and denatured states has been introduced by Fersht and co-workers [9,12]. In fact, by specifically destabilizing the native state without altering the structure of intermediates along the folding pathway(s), it is in theory possible to preferentially populate such intermediate state(s) at equilibrium and also under "physiological" conditions. By using this approach, for example, it was possible to isolate the denatured state of En-HD at physiological conditions (namely by producing the L16A variant) and to characterize its structural [12] and thermodynamic properties [9]. In this context, it is of interest to compare the observed behavior for the denatured state of En-HD to that of NPM1-C70. In the case of En-HD, calorimetry showed that the D state melts its residual structure with no endothermic barrier in the D to U transition [9]; furthermore, residue level NMR monitored denaturation clearly revealed a lack of co-operativity [9], strongly indicating that D unfolds following a second order transition, characterized by a continuum of states. Conversely, in the case of NPM1-C70 we clearly observed that, when stabilized, the denatured state is characterized by a discrete value of m, implying for this species a constant accessible surface area. This behavior suggests that, contrary to what observed for En-HD, the denatured state of NPM1-C70 represents a separate thermodynamic state, which may be robust to changes in experimental conditions and separated from the fully unfolded state by a genuine thermodynamic barrier.

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