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Role of mutual interactions in the chemical and thermal stability of nucleophosmin NPM1 domains

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ABSTRACT

Nucleophosmin (NPM1) is a key factor involved in fundamental biological processes. Mutations involving the NPM1 gene are the most frequent molecular alterations in acute myeloid leukemia. Here we report a biophysical characterization of NPM1 and of its domains in order to gain insights into the role that inter-domain interactions plays in the protein stabilization. Thermal denaturation analyses show that the N-terminal domain is endowed with an exceptional thermal stability, as it does not unfold in the investigated temperature range (20–105 °C). This finding is corroborated by chemical denaturation experiments showing that this domain is not significantly affected by the addition of 8 M urea. These results are consistent with the chaperone function of NPM1. In line with literature data, the other folded domain of the NPM1, a 3-helix bundle domain located at the C-terminus, shows a lower stability. Interestingly, the chemical and thermal stability of this latter domain, which embeds natural mutations related to acute myeloid leukemia, is influenced by the presence of other regions of the protein. Small but significant stabilizations of the C-terminal 3-helix bundle are provided by the adjacent unfolded fragment as well as by the rest of the protein.

1. Introduction

The large majority of proteins are multidomain proteins, as a result of complex gene duplication events [1]. In these proteins, each domain may fulfill its own function independently, or may operate in a concerted manner with its neighbors. For practical reason, most of biochemical and structural studies are concentrated on the analysis of individual domains. Therefore, the mutual influence of individual domains in determining the functional properties of multidomain proteins is an open question in many cases.

Nucleophosmin (NPM1), also known as nucleolar phosphoprotein B23 or numatrin, is a multidomain protein that is implicated in many fundamental biological processes [2,3]. Physiologically, NPM1, despite its nucleolar localization, shuttles constantly across nucleolar, nucleoplasmic and cytoplasmic compartments. This shuttling activity of NPM1 is critical for most of its functions, including regulation of ribosome biogenesis and control of centrosome duplication. Deregulation of NPM1 is implicated in the pathogenesis of several human malignancies. Indeed, NPM1 has been described both as an oncogene and as a tumor suppressor depending on the cell type [4–6]. It is important to note that mutations involving the NPM1 gene are the most frequent molecular alteration in acute myeloid leukemia (AML) accounting for about 60% of cases (i.e. one-third of adult AML) [7].

From the molecular point of view, NPM1 has a modular structure (Fig. 1). The analysis of the protein sequence shows the presence of distinct domains [8]. The N-terminal domain ($NPM1_{N\text{-}Ter}$ residues 1–117) is the oligomerization domain, that plays a crucial role in the molecular chaperone activity of the protein [2]. The high resolution three-dimensional structure of this domain shows that the $NPM1_{N\text{-}Ter}$ pentamer is formed by monomers that fold into an eight-stranded β-barrel with a jellyroll topology [9]. The analysis of the crystal packing has also suggested that $NPM1_{N\text{-}Ter}$ has a propensity to form decamers through the association of two distinct pentamers [9]. These NPM1 oligomers are the structural units that engage in interaction with several proteins including protamines, protamine-like type proteins, APE1/Ref-1, etc. [10,11].

Abbreviations: AML, acute myeloid leukemia; NPM1, Nucleophosmin; NPM1_fl, full length Nucleophosmin; $NPM1_{N\text{-}Ter}$, residues 1–117 of NPM1; $NPM1_{C\text{-}Ter108}$, residues 188–294 of NPM1; $NPM1_{C\text{-}Ter53}$, residues 243–294 of NPM1; $NPM1_{C\text{-}Ter70}$, residues 226–294 of NPM1; $Pep_{225\text{–}243}$, residues 225–243 of NPM1.

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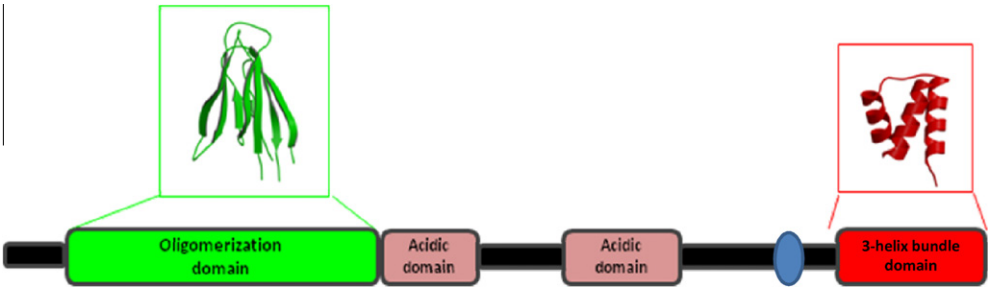

Fig. 1. Schematic organization of NPM1 domains. Models of the folded domains at the N- (residues 9–117) and the C-terminal (residues 243–294) of the protein are reported in the insets. The DNA binding region recently identified by Federici et al. is shown in cyan [13].

The C-terminal region of the protein contains the structural elements responsible for RNA/DNA recognition. NMR investigations have shown that the last 53 C-terminal residues ($NPM1_{C\text{-}Ter53}$) fold in a 3-helix bundle [12]. Interestingly, mutagenesis studies, carried out on the protein region containing last 70 C-terminal residues ($NPM1_{C\text{-}Ter70}$), have shown that the high affinity of NPM1 for DNA is due to the region proceeding the 3-helix bundle (residues 225–243) [13], although it has been recently demonstrated that no direct interaction is established by this fragment with the DNA [14]. The central portion of NPM1 is characterized by the presence of two acid domains (residues 119–133 and 161–188). Finally, the protein contains two nuclear export and two nuclear localization signals.

It is important to note that the interest for this protein is not only dictated by its multiple biological functions. In recent years, this protein, and in particular its C-terminal region $NPM1_{C\text{-}Ter53}$, has been a valuable subject for understanding basic questions related to protein folding and stability [15,16]. In this scenario, we here report an analysis of the thermal and chemical stability of NPM1 full length and some of its domains. Present analyses demonstrate that the N-terminal domain $NPM1_{N\text{-}Ter}$ is endowed with an uncommon thermal stability. Moreover, the completely different secondary structure content of the folded N- and C-terminal domains and the presence of Trp residues exclusively in the C-terminal region of the protein have provided the opportunity to selectively analyze the folding state of different NPM1 domains, and, specifically, to evaluate the stability of the 3-helix bundle in different contexts. Our analysis suggests that the stability of this motif, that embeds mutation sites related to AML, is affected by the rest of the protein.

2. Materials and methods

2.1. Preparation of recombinant NPM1, $NPM1_{N\text{-}Ter}$, and $NPM1_{C\text{-}Ter108}$

NPM1 full-length (NPM1_fl residues 1–294), $NPM1_{N\text{-}Ter}$ (residues 1–117), and $NPM1_{C\text{-}Ter108}$ (residues 188–294) constructs were expressed and purified as HisTag-fusion proteins in *Escherichia coli* BL21(DE3) following or adapting the procedure previously described [11]. The quality of purification was checked by coomassie-stained SDS–polyacrylamide gel electrophoresis (PAGE) analysis. Extensive dialysis against PBS was performed to remove any trace of imidazole from the purified proteins.

2.2. Chemical synthesis of the fragment 225–243 ($Pep_{225–243}$)

The fragment 225–243 (QESFKKQEKTPKTPKGPSS) embedding the K229–K230 motifs, that are essential for the high affinity DNA binding by NPM1, was chemically synthesized. Reagents for peptide synthesis (Fmoc-protected amino acids and resins, activation and deprotection reagents) were from Novabiochem (Laufelfingen, Switzerland) and InBios (Napoli, Italy). Solvents for peptide synthesis and HPLC analyses were from Romil (Dublin, Ireland); reversed phase columns for peptide analysis and the LC–MS system were from ThermoFisher (Milan, Italy). Solid phase peptide syntheses were performed on a fully automated multichannel peptide synthesizer Syro I (Multisynthech, Germany). Preparative RP-HPLC were carried out on a Shimadzu LC-8A, equipped with a SPD-M10 AV detector and with a Phenomenex C18 Jupiter column (50 × 22 mm ID; 10 μm). LC–MS analyses were carried out on a LCQ DECA XP Ion Trap mass spectrometer equipped with a OPTON ESI source, operating at 4.2 kV needle voltage and 320 °C with a complete Surveyor HPLC system, comprised of MS pump, an autosampler and a photo diode array (PDA). Narrow bore 50 × 2 mm C18 BioBasic LC–MS columns were used for these analyses.

The peptide was synthesized employing the solid phase method on a 50 μmol scale following standard Fmoc strategies [17]. Crude product was purified by RP-HPLC applying a linear gradient of 0.1% TFA CH3CN in 0.1% TFA water from 5% to 65% over 12 min using a semi-preparative 2.2 × 5 cm C18 column at a flow rate of 20 mL/min. Peptide purity and identity were confirmed by LC–MS and once purified it was lyophilized and stored at −20 °C until use.

2.3. CD spectroscopy and thermal denaturation

Far-UV CD spectra were recorded on a Jasco J-810 spectropolarimeter (JASCO Corp) in the wavelength interval 195–260 nm. For each spectrum the signal was converted to mean residue ellipticity in units of deg cm^2 $dmol^{-1}$ res^{-1}.

Experiments were performed employing protein concentrations of 16 μM in 50 mM phosphate buffer at pH 7.0, 1 mM DTT using a 0.1 cm path-length quartz cuvette. $Pep_{225–243}$ at 100 μM concentration was tested using the same buffer and identical instrumental experimental conditions.

Thermal denaturation experiments were performed by following the CD signal at 214 nm for $NPM1_{N\text{-}Ter}$ and at 222 nm for NPM1_fl and $NPM1_{C\text{-}Ter108}$.

Data were collected at 0.2 nm resolution, 20 nm/min scan speed, 1.0 nm bandwidth and 4 s response. A Peltier temperature controller was used to set up the temperature of the sample.

2.4. Chemically induced denaturation

A buffer solution containing 50 mM of sodium phosphate (pH 7.0) 1 mM DTT was used in chemical denaturation experiments. Urea was purchased from Sigma and further purified by recrystallization from ethanol/water (1:1) mixtures. Stock solutions of urea were mixed with protein solutions to give a constant final value of the protein concentration (8 μM). The final concentration of denaturant was in the range 0.0–8.0 M. Each sample was incubated overnight. Longer incubation times led to identical spectroscopic signals.

Table 1
Thermodinamic parameters of urea-induced denaturation of NPM1 and of its truncated forms.

	NPM1_fl	$NPM1_{C\text{-}Ter108}$	$NPM1_{C\text{-}Ter70}$*	$NPM1_{C\text{-}Ter53}$*
m Value (kcal mol^{-1} M^{-1})	0.66 ± 0.04	0.99 ± 0.03	0.93 ± 0.16	0.787 ± 0.07
ΔG (kcal mol^{-1})	2.77	3.76	3.49	2.02

* Values are taken from Ref. [13].

Chemical denaturation profiles as a function of urea concentration were followed by fluorescence spectroscopy investigations that were carried out at 25 °C. In order to follow the signal of the Trp residues located in the C-terminal domain, spectra for NPM1_fl and $NPM1_{C\text{-}Ter108}$ were recorded upon excitation at 295 nm. On the other hand, for $NPM1_{N\text{-}Ter,}$ which does not contain Trp residues, an excitation at 280 nm was employed. Spectra were recorded in the 300–400 nm wavelength range using a Varian Cary Eclipse spectrofluorimeter. The determination of the *m* values reported in Table 1 was performed by using the linear extrapolation method (LEM) as described by Pace [18]. ΔG was calculated as $mC_{1/2}$, where $C_{1/2}$ is the concentration of the denaturant at the denaturation midpoint.

3. Results and discussion

3.1. NPM1 overall structure: insights from secondary structure prediction and circular dichroism studies

Previous literature studies have highlighted the modular domain organization of NPM1. Two structured domains at the N- and the C-terminus have been identified. Interestingly these domains are characterized by different types of secondary structure elements (Fig. 1). Indeed, the N-terminal folded domain (∼residues 1–117) assumes essentially a β-structure [9], whereas the C-terminal folded domain (residues 243–294) forms a well defined right handed 3-helix bundle [12].

In order to check the presence of additional structured domains within the protein, we performed a secondary structure prediction session on NPM1 sequence by using the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/). As shown in Fig. S1, the program predicts the occurrence of several β-strands in the 15–120 region of the protein, in agreement with the structural data reported on the N-terminal oligomerization domain. In line with previous observations [13], the server provides a convincing prediction of helical regions that form the C-terminal bundle. These findings proved the reliability of the prediction method employed in this analysis. As shown in Fig. S1, PSIPRED also predicts that the central region of the protein is essentially unfolded, as only minor and sporadic secondary structure elements with a low level of confidence are detected. No secondary structure elements is detected in the region 225–243 (QESFKKQEKTPKTPKGPSS) preceding the three helix bundle, which contains the motif responsible for the high affinity DNA binding. The analysis of the circular dichroism spectrum of $Pep_{225\text{–}243}$ confirms that this fragments is intrinsically unfolded since it presents a single minimum below 200 nm (Fig. 2A). This observation is in line with the very recent finding that this region is disordered in the NMR structure of $NPM1_{C\text{-}Ter70}$ [14].

The circular dichroism spectrum of the full-length protein NPM1_fl registered at 20 °C in the far-UV exhibits a rather sharp minimum centered at 205 nm and a broader one at 222 nm (Fig. 2B). These features are indicative of the presence of both α and β secondary structure elements. A quantitative estimation of the secondary structure content, performed by using the software distributed with the Jasco-810 spectropolarimeter, suggests that the α-helix and the β-sheet contents are 14% and 34%, respectively.

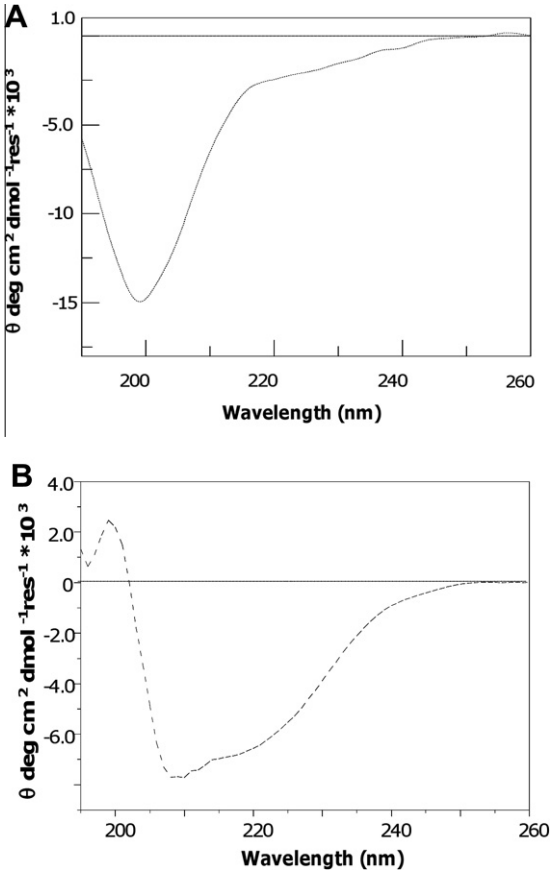

Fig. 2. CD spectra registered at 20 °C of the synthetic $Pep_{225\text{–}243}$ (A) and NPM1_fl (B) in 50 mM phosphate buffer 50 mM (pH = 7).

These values are in line with those derived from the crystallographic structures of the N- and C-terminal domains and by assuming that the central region is unfolded. Under this hypothesis, the number of residues in α-helix and in β-sheet is 45 and 75, respectively. Taking into account the total number of the residues in the protein (294) the α-helix and the β-sheet content is 15% and 26%.

Altogether these results corroborate the notion that the modular structure of NPM1 is characterized by regions endowed with distinct structural properties. Indeed, an unfolded central region separates two folded domains displaying different secondary structure elements. This feature has been here exploited to follow the behavior of a single domain in the context of the entire protein.

3.2. The N-terminal domain of NPM1 is endowed with a remarkable structural stability

In line with literature crystallographic data, the CD spectrum of NPM1 N-terminal domain (residues 1–117 and hereafter denoted as $NPM1_{N\text{-}Ter}$), characterized by the presence of a single minimum at 214 nm, is suggestive of domain assuming a β structure (Fig. 3). The analysis of $NPM1_{N\text{-}Ter}$ CD spectra as a function of temperature clearly indicates that the protein is stable over a wide range of temperatures. Indeed, no significant variations of the spectra are detected in the interval 20–90 °C (Fig. 3A). Moreover, the signal at the minimum (214 nm) does not display any significant variation up to 105 °C (Fig. 3A). The remarkable thermal stability of this domain is in line with the chaperone function of NPM1. Indeed, previous literature studies have unveiled the ability of this protein to protect several proteins from aggregation and to preserve their activity at high temperatures.

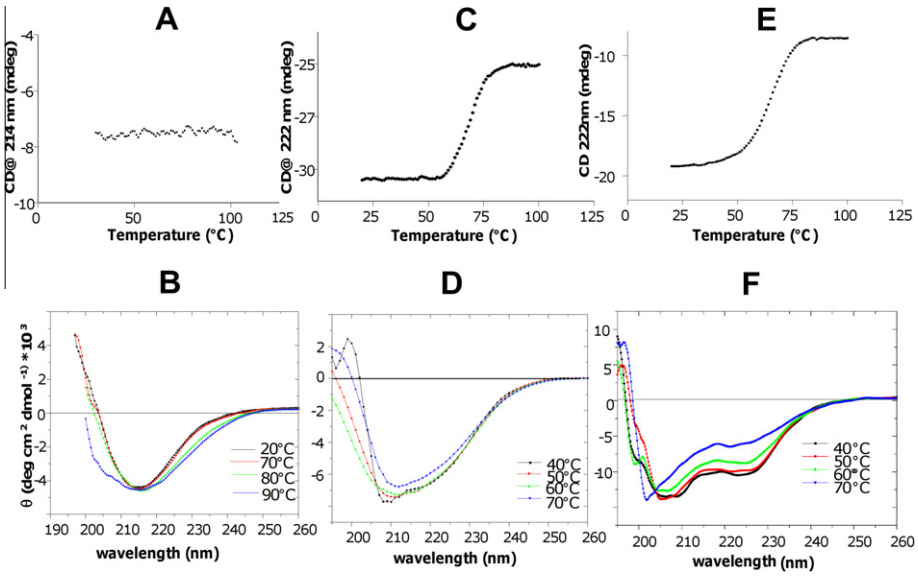

Fig. 3. Thermally induced denaturation of NPM1$_{N\text{-}Ter}$, NPM1_fl, NPM1$_{C\text{-}Ter108}$, in the presence of 50 mM sodium phosphate buffer (pH 7.0) and 1 mM DTT. Panels (A, C, and E) report the evolution of the CD signal as function of temperature for NPM1$_{N\text{-}Ter}$, NPM1_fl, NPM1$_{C\text{-}Ter108}$, respectively. Panels (B, D, and F) report the overlay of CD spectra recorded at different temperatures for NPM1$_{N\text{-}Ter}$, NPM1_fl, NPM1$_{C\text{-}Ter108}$, respectively.

3.3. Thermal stability of full-length NPM1and NPM1$_{C\text{-}Ter108}$

The dependence of NPM1_fl CD spectra with temperature is reported in Fig. 3C. A clear transition is detected at 68.3 °C. Interestingly, the protein presents a significant level of residual secondary structure upon heating (Fig. 3D). Taking into account the high thermal stability of NPM1$_{N\text{-}Ter}$, these data may be interpreted by assuming that the observed transition corresponds to the unfolding of the C-terminal domain of the protein. Literature data carried out on the isolated C-terminal 3-helix bundle NPM1$_{C\text{-}Ter53}$, under the same experimental conditions here employed to characterize the full-length protein, indicate an unfolding temperature below 60 °C (see Fig. 2 of Ref. [16]). Altogether, these observations suggest that the 3-helix bundle is stabilized in the protein context by approximately 10 °C. To identify the structural determinant of this stabilization, we performed thermal denaturation analysis on a domain containing the last 108 C-terminal residues (NPM1$_{C\text{-}Ter108}$) that follows the second acidic region of the protein. Therefore, NPM1$_{C\text{-}Ter108}$ includes, in addition to the 3-helix bundle, residues 223–245 implicated in the high affinity binding of DNA as well as the fragment 188–222. As shown in Fig. 3E, NPM1$_{C\text{-}Ter108}$ presents a Tm of 64.3 °C, a value that is intermediate between those displayed by NPM1$_{C\text{-}Ter53}$ and NPM1_fl. This finding indicates that the presence of both the fragment 188–243 and the N-terminal regions of the protein induce a stabilization of the C-terminal 3-helix bundle.

3.4. Chemical stability of NPM1$_{N\text{-}Ter}$, NPM1_fl, and NPM1$_{C\text{-}Ter108}$

The chemical stability of NPM1 domains was checked by using urea as denaturant. For NPM1$_{N\text{-}Ter}$, upon the addition of urea, spectra were recorded after an excitation at 280 nm to measure the fluorescence signal of the three Tyr residues located in this domain (positions 17, 29, and 67). These denaturation experiments confirmed the uncommon stability of the NPM1$_{N\text{-}Ter}$ domain highlighted by the thermal analyses. Indeed, as shown in Fig. 4A and B, no significant variation of the fluorescence signal was detected after the addition of the denaturant. Moreover, the maximum of the fluorescence signal, located at ~310 nm, suggests that the three Tyr residues are buried in the entire range of urea concentration tested (0–8 M).

The particular distribution of Trp residues, that are exclusively located in the C-terminal region of the protein (positions 288 and 290), allows the evaluation of the folding state of the C-terminal domain in different contexts. Indeed, *ad hoc* excitation of Trp residues (using a radiation with a 295 nm wavelength) could provide information about their local environments independently from the presence of other protein domains. As shown in Fig. 4C, both the intensity and the wavelength of the peak of the fluorescence signal exhibited by NPM1_fl are strongly affected by the addition of the denaturant. The comparison of the spectra of samples containing the protein incubated with either 0 or 8 M urea shows a clear increase of the peak intensity and a shift of its wavelength to higher values. The shift of the wavelength of the maximum from 325 to 355 nm is indicative of a process in which the initially buried Trp residues are fully exposed after the addition of 8 M urea. The analysis of the progression of the increase of the peak intensity upon denaturant addition shows that denaturation midpoint ($C_{1/2}$) occurs at [urea] = 4.2 M (Fig. 4D).

In analogous experiments (Fig. 4E and F), the $C_{1/2}$ exhibited by NPM1$_{C\text{-}Ter108}$ against urea denaturation is 3.8 M. This value is virtually coincident with that found for NPM1$_{C\text{-}Ter70}$ in experiments carried out by following the CD signal at 222 nm [13]. On the other hand, literature data show that NPM1$_{C\text{-}Ter53}$ presents a much lower value for $[urea]_{1/2}$ 2.6 M.

Taken together these findings show that the presence of the fragment 225–243 and the N-terminal regions significantly stabilizes the terminal 3-helix bundle also against chemical denaturants. The similar stability exhibited by NPM1$_{C\text{-}Ter70}$ and NPM1$_{C\text{-}Ter108}$ suggests that the fragment 188–225 does not provide any further contribution to the 3-helix bundle stability.

The similarity of the denaturation process of NPM1$_{C\text{-}Ter108}$ and NPM1$_{C\text{-}Ter70}$ is also confirmed by the analysis of the *m* values and ΔG values reported in Table 1 Notably, NPM1_fl presents a lower *m* value when compared to the truncated forms. Since the *m* value is related to the change in solvent accessible surface area upon denaturation, this observation suggests that the other regions of the protein (the N-terminal domains and/or the central acidic region) influences the denaturated state of the C-terminal domain.

In conclusion, present investigations show that the individual domains of NPM1 are endowed with different stabilities. Indeed, the N-terminal domain is endowed with an extraordinary stability

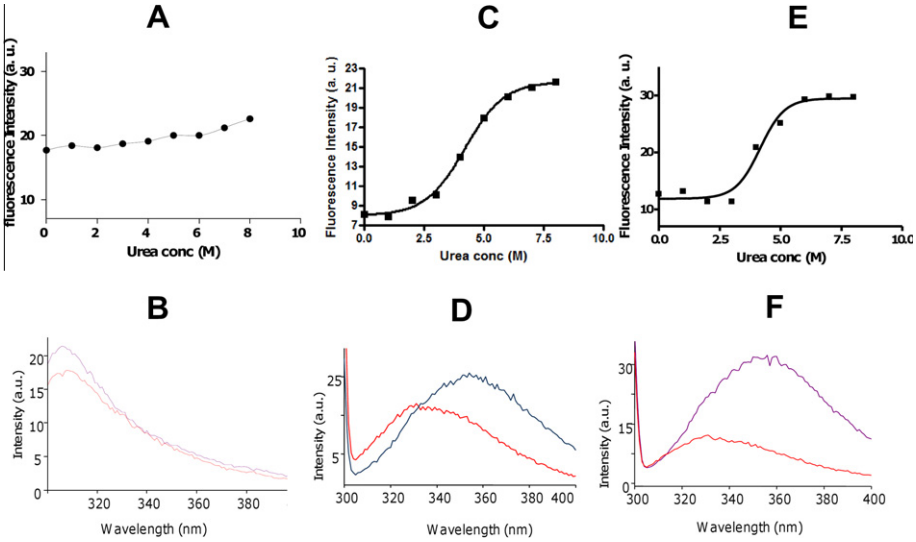

Fig. 4. Effect of urea incubation for for $NPM1_{N\text{-}Ter}$, NPM1_fl, $NPM1_{C\text{-}Ter108}$, followed at pH 7.0 and 25 °C in the presence of 50 mM sodium phosphate buffer (pH 7.0) and 1 mM DTT. (A) Tyr fluorescence upon excitation at 280 nm after the treatment with urea in the concentration range 0–8 M for $NPM1_{N\text{-}Ter}$. (B) Overlay of fluorescence spectra at of $NPM1_{N\text{-}Ter}$ sample untreated (red) or incubated with 8 M urea (blue). Panels (C and E) Trp fluorescence upon excitation at 295 nm after the treatment with urea in the concentration range for NPM1_fl and $NPM1_{C\text{-}Ter108}$, respectively. (D and F) Overlay of the fluorescence spectra at of the protein sample untreated (red) or incubated with 8 M urea (blue) for NPM1_fl, and $NPM1_{C\text{-}Ter108}$, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

against both urea and temperature. These observations are in line with the chaperone role of this domain highlighted by literature reports [3,19,20]. On the other hand, present and literature data indicate that the C-terminal 3-helix bundle, which embeds natural mutations causing severe human diseases, undergoes thermal transitions in the temperature range 55–70 °C, depending on the presence of additional protein regions in the investigated constructs. Particularly significant is the increase of the thermal stability of this domain likely due to the presence of the proximal fragment 225–243. Although present data show that this latter region is intrinsically unfolded and that this fragment is disordered in $NPM1_{C\text{-}Ter70}$ [14], it confers an extra stability to the bundle of about 8 °C. The presence of the remaining regions of the protein confers further stability to the C-terminal domain. Similar results have been obtained by performing chemical denaturation experiments. Present results suggest that the effects of natural mutations linked to severe diseases located in NPM1 C-terminal domain should be evaluated in vitro by considering the entire protein or at least the C-terminal fragment 225–294. On more general basis, the common practice of dissecting multi-domain proteins into small fragments may, in some circumstances, be an oversimplification that can lead to artifactual interpretations of the results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.002.

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