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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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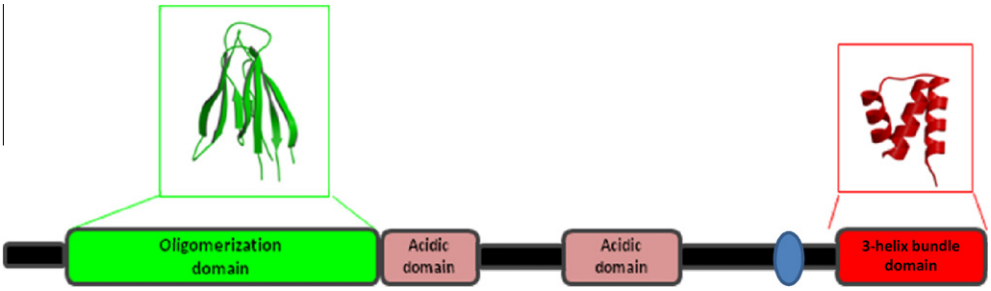

Fig. 1. Schematic organization of NPM1 domains. Models of the folded domains at the N- (residues 9–117) and the C-terminal (residues 243–294) of the protein are reported in the insets. The DNA binding region recently identified by Federici et al. is shown in cyan [13].

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.