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Enhanced prion protein stability coupled to DNA recognition and milieu acidification

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ABSTRACT

The prion protein (PrP) is the major agent involved in the transmissible spongiform encephalopathies (TSEs). Nucleic acids have been reported to bind PrP with high affinity, although the physiopathological roles for recognition are still not clear. In this work we investigate the stability of a soluble, 1:1 complex formed between an 18 base-pair DNA fragment and the full-length murine recombinant prion protein (mrPrP). DNA confers a gain in mrPrP stability against urea and guanidinium denaturation, which is enhanced at lower pHs and in moderate concentrations of NaCl. We discuss the cooperative folding transition coupled to DNA binding and acidification in terms of the possible cellular scenarios found during complex internalization and degradation.

1. Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a group of rare, neurodegenerative and fatal pathologies that can be infectious, sporadic or inherited [1]. They are related to modifications in the prion protein (PrP), an extracellular, GPI-anchored protein, which is constitutively expressed in a normal cellular form (PrP^C) in all mammals, with a very wide tissue distribution [1,2]. Several functions have been attributed to PrP^C, including neuroprotection and recognition of copper, heparin, laminin and nucleic acids [3–7]. The onset of the disease is triggered by the conversion of PrP^C, rich in α-helices, into PrP^{Sc} (prion scrapie), a PrP pathological isoform, rich in β-sheets [1]. Although the elucidation of the PrP^C physiological function and of its conversion mechanism into the scrapie isoform has been under investigation for decades, there are several questions that remain to be answered [2,8,9].

TSEs are described as "protein-only" diseases, and PrP is thought to be the only agent necessary to trigger these pathologies [10,11]. It has been shown in a cell-free system, that PrP^{Sc} is capable to convert the cellular PrP into the abnormal isoform [12]. The finding that PrP knock-out mice are resistant to infection with PrP^{Sc} undeniably assures that endogenous PrP is necessary for the onset of the disease [11]. However, several evidences indicate that an additional unknown factor might participate in the PrP^C to PrP^{Sc} conversion by acting as a catalyst [13–16]. This unidentified molecule would act by lowering the energetic barrier that prevents the conversion between both isoforms [14,17]. Currently, there are several candidates to play such a role, such as cellular adhesion molecules, sulfated glycans and nucleic acids (NAs), among others [18].

The scrapie isoform has already been found in the nucleus associated with the DNA [19], as well as in the cytoplasm [20]. PrP can interact with NAs both *in vitro* [14,21–23] and *ex vivo* [19,24], showing some structural and sequence selectivity. Depending on the PrP:NA stoichiometric ratio, the interaction between PrP and nucleic acids can lead to formation of a β-sheet-rich, amyloid-like particle, partially resistant to proteinase-K digestion [14,15,25–27], or to a soluble, 1:1 complex [7]. Currently, the concept that the infective species is free from NAs [1,28] and the idea that this same class of macromolecules (nucleic acids) might participate as molecular chaperones or as cellular partners in the establishment of a functional complex are both well-accepted [8,29,30].

Several works have reported the physicochemical features of aggregated PrP:NA assemblies [14,15,23,25–27,31,32]. However, the stability of both PrP^C and the DNA in soluble complex has not yet been described. We have recently obtained for the first time a soluble, 1:1 complex of recombinant murine PrP (mrPrP) and DNA, as characterized by static light scattering, SAXS and NMR, among other techniques [7]. After a transient aggregation event induced by nucleic acid binding by the prion protein, a solution consisting of a soluble, 1:1 DNA:PrP complex is obtained. This complex can be obtained for both the full-length prion protein and the globular domain (comprising amino acids 120 to 231), in a broad pH range [7,32]. In this work, we

Abbreviations: PrP, prion protein; PrP^{Sc}, prion scrapie; mrPrP, recombinant murine prion protein; PrP^C, cellular prion protein; TSEs, transmissible spongiform encephalopathies; GdmCl, guanidinium chloride; NA, nucleic acid.

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evaluate the chemical stability of the soluble 1:1 complex, showing that binding of DNA to mrPrP provides a gain in prion protein stability, which is enhanced at acidic pHs and in isotonic ionic strength.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade. Urea and guanidine hydrochloride stock solutions were checked for concentration by refractive index measurement [33].

2.2. Construction, expression and purification of mrPrP

The full-length mrPrP was cloned into the pRSET plasmid (kindly provided by Prof. R. Brentani and Prof. Vilma R. Martins) and was expressed in *Escherichia coli* and purified by high-affinity column refolding as described elsewhere [14,34].

2.3. Synthetic oligonucleotide

The synthetic, HPLC-purified, single-stranded oligonucleotides, were purchased from Integrated DNA Technologies (Coralville, IA, USA). Concentrations were calculated from extinction coefficients at 260 nm [14,35]. The double-stranded 18 bp oligonucleotides were prepared by mixing equimolar amounts of single-stranded oligonucleotide 5′ GTA ACCG AAAT CGGT TGA 3′ and its complementary strand in 10 mM Tris–Cl, 50 mM NaCl, 1 mM EDTA, pH 7.8, heating them up to 90 °C and slowly cooling to 25 °C. Annealed oligonucleotides were checked for complete annealing by native polyacrylamide gel electrophoresis and stored at −20 °C. This DNA sequence was selected since it can be recognized by mrPrP with high affinity, in the nanomolar range [7,14].

2.4. Equilibrium unfolding experiments

All experiments were performed at 22 °C, in 10 mM Tris (tris (hydroxymethyl)aminomethane) or sodium acetate, adjusted to the desired pH with HCl, containing the indicated amount of NaCl. The mrPrP:DNA complex was prepared for each experiment by mixing mrPrP with equimolar amounts of DNA and incubating the sample at 22 °C for three days. Equilibrium unfolding was performed by incubating mrPrP (2 μM) or mrPrP:DNA (2 μM) in the indicated denaturant and buffer conditions and was allowed to equilibrate for at least 60 min before each measurement. No further significant changes were observed at longer incubation times, indicating that equilibrium was reached.

The use of spectroscopic techniques in the investigation of PrP conformational transition coupled to DNA binding is limited, especially for circular dichroism, since DNA binding leads to a substantial decrease in ellipticity [14], which is not susceptible to further changes as a function of urea concentration or increase in temperature (not shown). In opposition, fluorescence spectroscopy exhibits an overall sensitivity for the global conformational changes the protein would present. Excitation at 280 nm allows monitoring the intrinsic fluorescence of PrP tryptophan and tyrosine residues, both from the C-terminus (residues 121 to 230, comprising 1 Trp and 10 Tyr) and N-terminus (residues 23 to 120, 7 Trp and 2 Tyr).

Fluorescence measurements were performed in a Jasco FP-6300 spectrofluorimeter (Jasco Corporation, Tokyo, Japan), with excitation set at 280 nm and emission collected from 300 to 420 nm, at a scanning rate of 100 nm/min and response 8 s, datapitch 0.5 nm, and excitation and emission slits of 2.5 nm. Fluorescence spectra were quantified by the center of spectral mass $\langle\lambda_{obs}\rangle$ according to Eq. (1):

$$\langle\lambda_{obs}\rangle = \left(\sum \lambda_i * F_i\right) / \sum F_i \quad (1)$$

where F_i is the fluorescence emitted at wavelength λ_i, and the summation is carried out over the range cited above. All spectra were subtracted from the respective buffer spectrum and collected with two accumulations each. All experiments were performed at least in triplicate, with different protein and DNA batches.

3. Results and discussion

mrPrP is able to bind, with high affinity, a wide variety of nucleic acids [7,8,14,21–23,32]. Despite the amount of information currently available, both the structural and sequence consensus for recognition are still unknown. We have previously demonstrated that mrPrP can form a stable, 1:1 soluble complex, with a short, 18 base-pair double-stranded DNA, which differs punctually from the free mrPrP [7]. However, the biophysical implications of this complex formation have not yet been reported.

The murine rPrP has eight tryptophan and twelve tyrosine residues [36]. This is an interesting feature, since intrinsic fluorescence spectroscopy allows a direct assessment of overall conformational transition in the mrPrP tertiary structure [37]. mrPrP fluorescence emission has a spectral center of mass of about 346 nm at pH 7.5, and it is not significantly affected by a decrease in milieu acidity, displaying a spectral center of mass of about 345 nm at pH 4.0 (Fig. 1A). These data corroborate previous NMR studies of the recombinant human PrP globular domain ($hrPrP^{121-230}$), which indicate an absence of large changes in the global conformation as a function of pH [38], although its stability is shown to be largely decreased upon acidification [39]. In contrast with the data obtained in the absence of DNA, binding to DNA by mrPrP leads to a blue shift in its fluorescence emission at low pH, with spectral center of mass of 341 nm at pH 4.0 both in the absence (Fig. 1A) and in the presence (Fig. 1B) of 6 M urea, which is indicative of structural rearrangement of mrPrP when in complex with DNA.

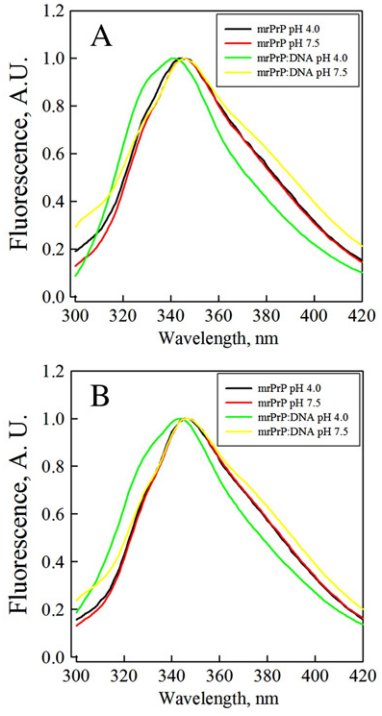

Fig. 1. Effect of DNA binding on mrPrP conformation. mrPrP (at 2.0 μM) and mrPrP:DNA (at 2.0 μM) emission fluorescence spectra were recorded at pH 7.5 (red and yellow lines) and 4.0 (black and green lines), in the absence (black and red lines) or presence (green and yellow lines) of DNA. Data are shown as mrPrP normalized fluorescence intensity spectra without urea (A) and in the presence of 6.0 M urea (B). Details in Materials and methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In order to determine the implications of DNA binding in the overall stability of mrPrP, we conducted equilibrium unfolding measurements using urea and guanidinium chloride (GdmCl) as denaturants. As PrP^C is known to transit through different cellular environments [2,8,9,40], we also probed its stability at a compatible range of pH and salinity. Both urea (Figs. 2A and 3A) and GdmCl (Figs. 2C and 3C) induced a progressive increase in the spectral center of mass of mrPrP intrinsic fluorescence, for the pH range of 4.0 to 7.5 and up to 150 mM NaCl, indicating that a denaturation process had occurred, as previously described elsewhere [17,41]. The observed shift seen in the unfolding was limited, from about 356 nm to 358 nm. However, seven out of eight aromatic residues are located in the N-terminal, unstructured portion of the mrPrP that is exposed to the aqueous milieu. Thus, a more dramatic spectral shift is not likely to occur, since the limiting value of free Trp in water is about 358 nm (data not shown).

We have observed that the 1:1 mrPrP:DNA complex behaves significantly different when compared with the free mrPrP in the presence of denaturants and at acidic pH. The primary effect of acidification (Fig. 2B and D) was to decrease the initial value of the spectral center of mass for the mrPrP:DNA complex, an event indicative of substantial structural change. The pronounced blue shift that follows the decrease in pH seems to be an inherent feature of the DNA-bound mrPrP (Fig. 2B and D), since this effect is not observed for mrPrP alone (Fig. 2A and C).

Variations in pH did not manifest in an increase in the steepness of the mrPrP:DNA complex unfolding isotherms. Instead, incubation with increasing amount of urea (Fig. 2B) or GdmCl (Fig. 2D) resulted in small, linear shifts in the spectral center of mass (lower than 1 nm). A significant spectral shift was observed for the mrPrP:DNA complex only at very high urea and GdmCl concentrations, revealing an effective enhancement of mrPrP stability, with a gain in tertiary structure upon DNA-binding into a dissimilar structure that is resistant to unfolding.

In the free mPrP unfolding isotherms induced by GdmCl (Fig. 2C), we observed a biphasic behavior, at the investigated pH range (4.0 to 7.5). With increasing GdmCl concentration up to 3 M a red shift in fluorescence emission is observed, indicating a protein unfolding process. Further increase in the GdmCl concentration led to a significantly progressive decrease in the spectral center of mass. Similar behavior was not observed in the urea unfolding isotherms (Fig. 2A). We interpret this blue shift effect of the intrinsic fluorescence emission as the refolding of some mrPrP regions, which is

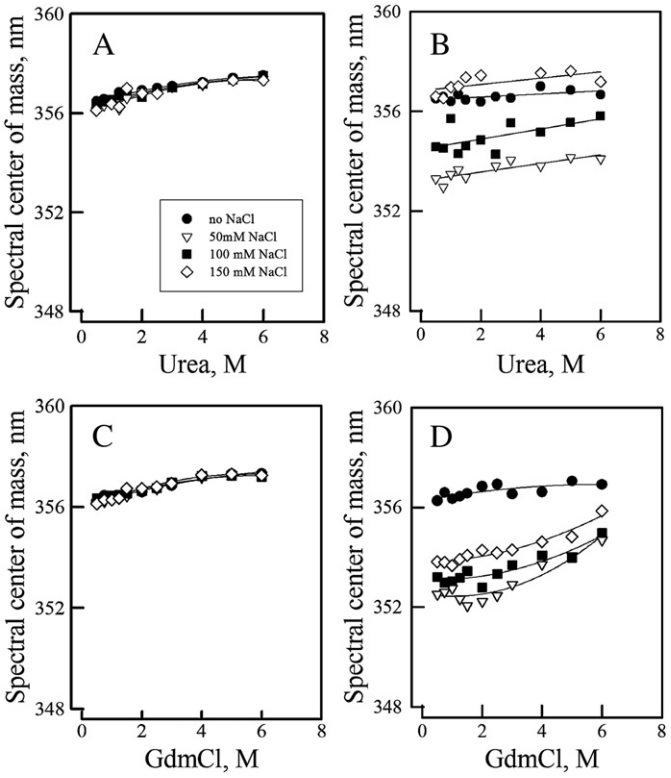

Fig. 3. Salt effect on mrPrP and soluble mrPrP:DNA stability. Urea (A and B) and GdmCl (C and D) unfolding isotherms of mrPrP (A and C) and mrPrP:DNA (B and D) in the absence (●) or in the presence of (▲) 50 mM NaCl; (■) 100 mM NaCl; (♦) 150 mM NaCl. Experiments were performed in 10 mM Tris HCl pH 7.5, 22 °C. Details in Materials and methods.

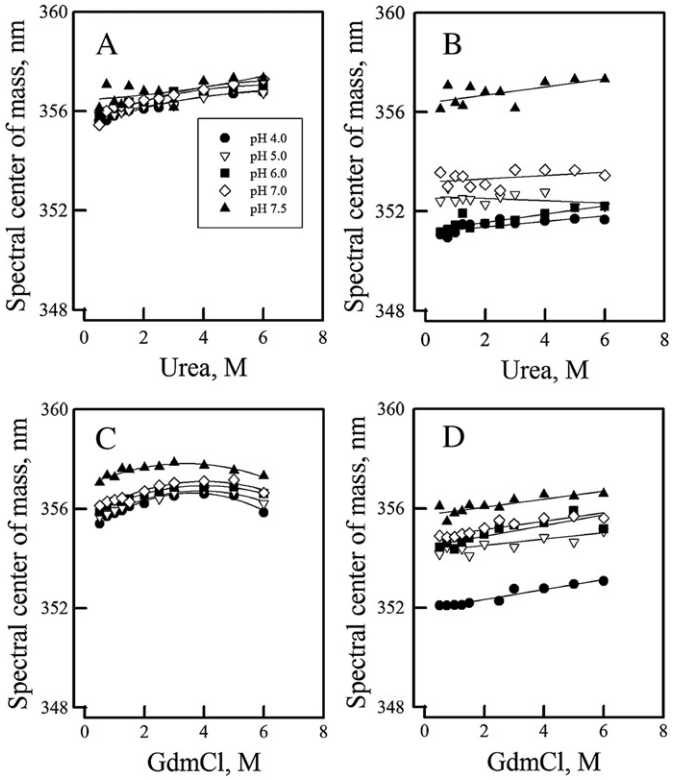

Fig. 2. pH effect on mrPrP and soluble mrPrP:DNA stability. Urea (A and B) and GdmCl (C and D) unfolding isotherms of mrPrP (A and C) and mrPrP:DNA (B and D) at pH (●) 4.0; (▼) 5.0; (■) 6.0; (♦) 7.0; (▲) 7.5, in 150 mM NaCl. Experiments performed at 22 °C, in 150 mM NaCl, 10 mM Tris/sodium acetate buffer adjusted to the desired pH with HCl. Details in Materials and methods.

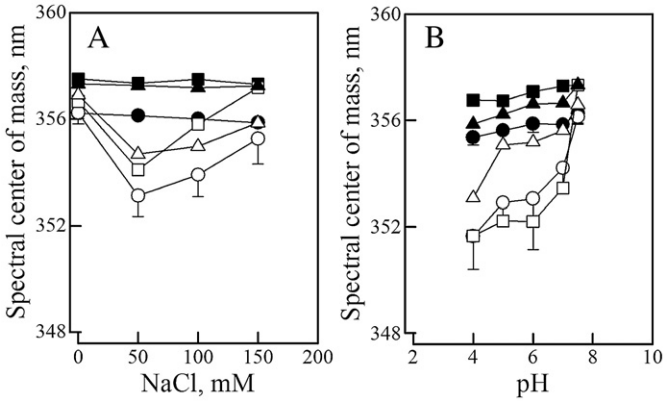

Fig. 4. Salt and pH effect on the free mrPrP and soluble mrPrP:DNA folding. A) Data from Figs. 2 and 3 were replotted as (A) NaCl and (B) pH isotherms at different denaturant concentrations. Closed symbols, free mrPrP; open symbols, mrPrP:DNA complex, in the absence of denaturant (circles) or in the presence of 6 M urea (squares) or GdmCl (triangles).

likely to be induced by the ionic character of GdmCl solutions at high concentrations.

In order to address the hypothesis of ionic strength effects over mrPrP stability, we conducted urea and GdmCl denaturation experiments at varying NaCl concentrations (Fig. 3). At pH 7.5 decreasing NaCl concentrations did not influence the unfolding curves of free mrPrP by urea (Fig. 3A) or GdmCl (Fig. 3C). In contrast, a decrease in the spectral center of mass was observed for mrPrP:DNA, becoming more pronounced at 50 mM NaCl for both urea (Fig. 3B) and GdmCl (Fig. 3D). In the absence of NaCl (Fig. 3B and D, closed circles), the mPrP:DNA unfolding curves are shifted to a higher spectral center of mass, indicating a dissimilar behavior for mrPrP when complexed to DNA.

Although the mrPrP exhibits a gain in tertiary structure in the presence of DNA as indicated by blue shift in fluorescence spectra, we were not able to calculate stability parameters from these mrPrP:DNA denaturation isotherms (Figs. 2 and 3). Instead, when these results are plotted as NaCl (Fig. 4A) and pH (Fig. 4B) curves, their overall effect over mrPrP conformation can be better evaluated.

Increasing NaCl concentration lead to an increase in stability of the mrPrP:DNA complex, which is reverted by NaCl concentrations higher than 50 mM. In contrast, incubation with increasing NaCl concentrations exert minor or no detectable effects when in the presence of 6 M urea (closed triangles) or GdmCl (closed squares). Similar behavior was observed for the free human recombinant PrP [41], but we could not detect perceptible effects with the free mrPrP (Figs. 3 and 4A), maybe due to differences in PrP^C species susceptibility to ionic strength variations.

When the urea and GdmCl denaturation curves at varying pH are plotted as pH curves, a cooperative unfolding for the mrPrP:DNA complex can be noticed. As the pH is risen from 4.0 to 7.5, an unfolding transition between folded mrPrP:DNA to unfolded mrPrP occurs, spanning through 2 pH units as typically observed for protein:ligand interactions [42].

The onset of TSEs is connected with the accumulation of insoluble proteic deposits in the cytosol and in the extracellular milieu [1]. Although PrP^C is anchored to the cellular membrane and therefore experiences a neutral milieu, its degradation pathway possesses a stage of lysosomal internalization [42], where pH drops gradually down to about 5.0 [43] favoring free PrP unfolding [39] and further degradation. Some PrP molecules constitutively cycle between the plasma membrane and an acidic endosome [44], from which most of the PrP^C molecules are recycled back to the cell surface without modifications [40]. In addition, not only free PrP but also both nucleic acids and PrP:DNA complex can traffic through a lysosomal stage [45–48].

We observed that the soluble, 1:1 mrPrP:DNA complex displays an enhanced stability compared to mrPrP. Moreover, at acidic milieus the mrPrP:DNA complex becomes even more stable and displays an apparent gain in tertiary structure. Collectively, and based on DNA and rPrP:DNA complexes internalization results cited above, our data suggest that the gain in stability due to DNA recognition would minimize lysosomal degradation of PrP. Such features bring new horizons in the understanding of prion biology, since NAs might be considered as functional partners in the prion protein physiology and biology [8]. Further biophysical and cellular investigation should be conducted in order to address these issues.

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