Cross-talk between prion protein and quadruplex-forming nucleic acids: a dynamic complex formation

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ABSTRACT

Prion protein (PrP) is involved in lethal neurodegenerative diseases, and many issues remain unclear about its physio-pathological role. Quadruplex-forming nucleic acids (NAs) have been found to specifically bind to both PrP cellular and pathological isoforms. To clarify the relevance of these interactions, thermodynamic, kinetic and structural studies have been performed, using isothermal titration calorimetry, surface plasmon resonance and circular dichroism methodologies. Three quadruplex-forming sequences, d(TGGGGT), r(GGA GGAGGAGGA), d(GGAGGAGGAGGA), and various forms of PrP were selected for this study. Our results showed that these quadruplexes exhibit a high affinity and specificity toward PrP, with KD values within the range 62–630 nM, and a weaker affinity toward a PrP-β-oligomer, which mimics the pathological isoform. We demonstrated that the NA quadruplex architecture is the structural determinant for the recognition by both PrP isoforms. Furthermore, we spotted both PrP N-terminal and C-terminal domains as the binding regions involved in the interaction with DNA/RNAs, using several PrP truncated forms. Interestingly, a reciprocally induced structure loss was observed upon PrP–NA interaction. Our results allowed to surmise a quadruplex unwinding-activity of PrP, that may have a feedback in vivo.

INTRODUCTION

The concept that a protein can be the causative agent for transmissible diseases challenged the traditional knowledge that transmission results solely from an agent that carries genetic information. This new paradigm was brought by cellular prion protein (PrPc), which is involved in a rare but fatal family of neurodegenerative disorders that affect humans and animals. Prion diseases, in addition to sporadic and inherited forms, may be acquired by transmission of an infectious agent. Accordingly to the ‘protein only’ hypothesis, the infectious agent is a self-perpetuating conformer of prion protein (PrP), namely PrPSc, capable of transmitting and replicating its ‘wicked’ conformation (1,2). Indeed PrPc, which possesses a high α-helix content, is converted in the β-rich insoluble conformer PrPSc. This form acts as a template for PrPc to induce misfolding and then generating more PrPSc. How this conversion occurs is not yet elucidated but all proposed mechanisms include a formation of soluble β-oligomers, that are neurotoxic species. The ‘protein only’ hypothesis excluded the participation of nucleic acids (NAs) in prion propagation. However, several groups have suggested that an additional, as yet unknown, factor—including NA—might initiate or modulate the PrPc to PrPSc conversion (3–5).

Similarly to other proteins associated to neurodegeneration, PrP has been found in the cytosol (6) and in the nucleus as well (7–11). Having a common place to cross-talk, NAs do actually form an interesting group of PrP molecular partners. Indeed, it was shown that PrP can bind small NAs in vitro with nanomolar affinity (3,4,12) and these interactions can lead to conformational changes
of the protein (3,4,12,13), suggesting a physiological role of PrP as an NA-binding protein (14–18). Furthermore, NAs have been used to create de novo synthetic prion and therefore have been proposed as the lacking cofactors that might facilitate the PrP transconformation in vivo (19,20).

Many studies have been devoted to the selection of RNA and/or DNA molecules able to specifically bind to both PrPβ and its β-rich isoform (12,21–25). Moreover, many works have highlighted the relevance of specific features possessed by NAs for a high-affinity binding with PrP. In particular, some of them were shown to contain a shared sequence and fold, i.e. contiguous guanine repeats and quadruplex structures (21,23,25,26).

In a previous work, RNA aptamers against recombinant bovine PrP and its amyloidogenic β isoform were previously reported to form a tetramolecular parallel-quadruplex in genomic regions and recombination hot spot sites (29). It is worth noting that GGA triplet repeats, potentially able to fold into G-quadruplexes, are also widely present in regulatory regions and recombination hot spot sites (29).

The full-length ovine PrP (flPrP) (A136 R154 Q171 variant, 20-234) and the truncated forms, ΔPrP (103–234) and ΔPrP (124–234), were produced in Escherichia coli and purified as previously described (34). The buffer used to desalt the proteins, on a HiPrep desalting column, was MES 15 mM pH 6.0, KCl 70 mM. Final protein concentration was measured by optical density at 280 nm using an extinction coefficient of 58 718 M⁻¹ cm⁻¹ for the flPrP and 180 05 M⁻¹ cm⁻¹ for both the ΔPrP forms, deduced from the aminoacid sequence.

The generation of oligomers from ovine flPrP was performed by dissolving the protein in a solution of HCl 0.1 M, pH 1.0. The solution, at a final concentration of 80 μM, was incubated for 12 h at 25°C. The samples were cooled down to 15°C and eluted with sodium citrate 20 mM, pH 3.4 on a TSK 4000SW (60 cm x 0.78 cm) column using an Akta FPLC chromatography equipment (GE Healthcare). The oligomeric PrP form collected, elutes at 12.5 ml, which corresponds to O1 oligomer (36-mer), accordingly to previous results (35). Homogeneous fractions of the oligomer were collected.

NAs were purchased from PRIMM (Milan) and purified by standard methods. The samples were prepared by dissolving the lyophilized compound in MES 15 mM pH 6.0, KCl 70 mM. The solutions have been annealed by
heating at 95°C for 5 min and slowly cooling to room temperature. The concentration of the dissolved oligonucleotide has been evaluated by UV measurement at 95°C, using molar extinction coefficient of 57 800 M⁻¹ cm⁻¹ for d(TGGGGT), 110 700 M⁻¹ cm⁻¹ for d(CGCGAATTCGCG) and 135 500 M⁻¹ cm⁻¹ for both r(GGAGGAGGAGGA) and d(GGAGGAGGAGGA), calculated by the nearest-neighbor model (36). Native gel electrophoresis was performed to verify the conformational homogeneity of G-quadruplex species (Supplementary Methods and Supplementary Figure S1). In all experiments, R12 and D12 concentration was referred to quadruplex dimers, apart from [d(TGGGGT)]₄ for which concentration was referred to a single tetramolecular quadruplex. The unfolded [d(TGGGGT)]₄, R12 and D12 were obtained by removing K⁺ ions from the solution through dialysis against either MES 15 mM pH 6.0 or MES 15 mM pH 6.0, LiCl 70 mM. Dialysis was performed for 24 h, a time longer with respect to the kinetics of tetramolecular quadruplex-folding/unfolding processes. The unfolded state of dialyzed NAs solutions was assessed by recording CD spectra in the 320–720 nm interval.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were carried out at 25°C using a high-sensitivity CSC-5300 Nano-ITC microcalorimeter from Calorimetry Science Corporation (Lindon, Utah) with a cell volume of 1 ml. Only after reaching the baseline stability, the experiments were performed. In each titration experiment, volumes of 10 µl of a solution containing [d(TGGGGT)]₄, R12 or D12 at a concentration within the range 80–130 µM were injected into a PrP-containing solution in the same buffer (MES 15 mM pH 6.0, KCl 70 mM), using a computer-controlled 250 µl microsyringe. In the case of ITC titration carried out with the unfolded D12, both the aptamer and PrP were in the same buffer MES 15 mM, pH 6.0. All the PrP forms were prepared at a concentration of 16 µM. In order to allow the system to reach the equilibrium, we applied a time interval of 300 or 400 s between each injection. Heat produced by NAs dilution was evaluated by performing a control experiment, titrating each NA into the buffer alone. The heat of interaction (enthalpy change, ΔH) was calculated after correction for the heat of NA dilution. The corrected heat values were plotted as a function of the NA:PrP molar ratio, to estimate the stoichiometry of interaction (n) (37).

Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed using a Biacore T200 system (GE Healthcare, Uppsala, Sweden), at 25°C. flPrP, the truncated PrP forms and the PrP-β oligomer were immobilized on a CM5 (research grade) by the standard amine coupling procedure, using HBS-EP buffer (HEPES 10 mM, NaCl 150 mM, EDTA 3 mM, 0.005% Surfactant P20, pH 7.4) as running buffer. The PrP forms were immobilized through activation of the sensor chip with 60 µl of N-hydroxysuccinimide (NHS) and N-ethyl-N-(dimethylaminopropyl)-carbodiimide (EDC) at 10 µl min⁻¹, followed by 30 µl injection of PrP forms diluted in 10 mM sodium
acetate buffer pH 5.0. Unreacted activated groups were blocked by a 60 μl injection of 1 M ethanolamine at 10 μl min⁻¹. Final PrP immobilized levels were typically between ~3500 and 4300 RU, corresponding to a protein concentration of 35 ± 43 mg ml⁻¹ on the surface layer. Subsequently, [dTGGGT]₄, R12, D12 and Dickerson duplex were injected as analytes at various concentrations (from 50 nM to 50 μM), and using MES 15 mM pH 6.0, KCl 70 mM as running buffer. The binding assays were performed under various conditions: (i) contact time of 60, 120 and 360 s and dissociation time of 180, 240, 300 and 480 s to evaluate the best kinetic model; (ii) flow rate of 30 and 60 μl min⁻¹ to evaluate the contribution of mass transport. Analogously, SPR experiments with the unfolded [dTGGGT]₄, R12 and D12, were carried out injecting the analytes at various concentrations (from 50 nM to 50 μM), at a contact time of 120 s, dissociation time of 300 s and flow rate of 30 μl min⁻¹ and using either MES 15 mM pH 6.0 or MES 15 mM pH 6.0 70 mM LiCl as running buffers.

For all SPR experiments, the chip was regenerated by removing bound analytes using the mobile phase MgCl₂ 0.25 M, injected for 30 s at 30 °C. For ITC experiments with the unfolded [dTGGGT]₄, R12 and D12, we performed ITC and SPR experiments. Various PrP truncated forms, ad hoc designed, were produced to identify the regions involved in the interaction, as well as the forces that drive the complex formation. Indeed, three distinct PrP binding sites have been previously identified for NAs: two lysine clusters (aa 25–34 and 101–110 in ovine PrP) located at the ends of the unstructured N-terminal domain (18,22), and a third site, located in the structured C-terminal domain, in which the amino acids involved are not yet well-established (18,38). Hence, in addition to the wild-type (wt) PrP, we also investigated the interaction with the PrP(103–234) and PrP(124–234) that lack the first and both lysine clusters, respectively (Figure 1D).

Additionally, the binding with the pre-amyloid ovine PrP-β oligomer, that mimics the early stage of PrPSc formation, was investigated by SPR. As ovine PrP produces a mixture of β-rich oligomers, we have selected the largest one, corresponding to 36-mer, which is the only precursor of amyloid-like fibrils (35).

Finally, in order to study the structural modifications that might be induced by the PrP–NA interactions, we performed CD experiments to monitor the changes in the secondary structures either of protein or NAs. To gain more information on the thermal stability of all studied NAs we have measured their melting temperature (Supplementary Figure S2).

**RESULTS AND DISCUSSION**

**Transmission electron microscopy**

TEM images were collected using a Zeiss EM902 (80 kV) microscope. Briefly, 10 μl of the samples were adsorbed onto carbon-coated Formvar grids (Agar Scientific). Then, each grid was washed three times with water, stained with 2% uranyl acetate and finally air-dried. All the samples were in MES 15 mM pH 6.0, KCl 70 mM. Since precipitation occurred as soon as the two partners are mixed, there was no incubation time to wait for.

**Circular dichroism**

Circular dichroism (CD) spectra were recorded with a Jasco J-815 spectropolarimeter equipped with a Peltier type temperature control system (Model PTC-4235). CD measurements (190–320 nm) were carried out in MES 15 mM pH 6.0 KCl 70 mM at 20 °C by using a 0.1 cm optical path length cell. CD titration was performed on the full-length and the truncated PrP forms, keeping constant the concentration of PrP (10 μM), and increasing the NA concentration up to a PrP:NA molar ratio of 1:2. Each solution of the mixture PrP–NA was prepared independently, without successive additions, to avoid complications due to dilution effects within titration experiments, and the spectra were recorded after few minutes upon addition of NAs. CD spectra, recorded with a time constant of 4 s, a 2 nm band width, and a scan rate of 20 nm min⁻¹, were signal-averaged over at least three scans. The baseline was corrected by subtracting the buffer spectrum. Melting curves of all NAs were also recorded (Supplementary Methods).

**Thermodynamic data from ITC titration**

The resulting profiles obtained by ITC titrations of flPrP, ΔPrP(103–234) and ΔPrP(124–234) with [dTGGGT]₄ are shown in Figure 2, as the representative ones. ITC data of all the PrP forms with R12 and D12 are shown in...
Supplementary Figure S3, with the exception of the flPrP-D12 system, since precipitation at the end of ITC experiments precluded any further analysis. In particular, ITC profiles along with the integrated heat data are shown for all the system studied, apart from 
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PrP124–234-R12, for which only the raw ITC profile is shown, since no binding was detected (Supplementary Figure S3C). In all figures, the heat values were plotted as a function of the NA:PrP molar ratio to estimate, from the inflection point, the stoichiometry of interaction. In the plot, both R12 and D12 aptamers were considered to be a quadruplex dimer, while [d(TGGGGT)]4 was considered as a single tetramolecular quadruplex. The data suggested that the stoichiometry relative to the interaction of [d(TGGGGT)]4, R12 and D12 with the truncated PrP forms resulted to be 1:0.5 (ratio of PrP per NA), whereas the stoichiometry obtained for the interaction of [d(TGGGGT)]4 and R12 with flPrP was 1:1. Since during the binding events a concomitant conformational change of reactants occurs (see below), we choose not to fit the ITC data to a model, as no appropriate fitting model is currently available to adequately describe the data (two-step binding reaction model in which ligand binding is coupled to a conformational transition). The values are reported in Table 1 and represent the total, model free, enthalpy change for the process, and indicate that, in all cases, the overall processes are enthalpically favorites.

Table 1. Stoichiometry and enthalpy change for the interaction of flPrP, ΔPrP103–234 and ΔPrP124–234 with [d(TGGGGT)]4, R12 and D12, determined by ITC at 25°C

| PrP-NA interaction | n   | ΔH (kJ mol⁻¹) |
|--------------------|-----|---------------|
| flPrP-[d(TGGGGT)]₄| 1.1 ± 0.1 | -76.4 ± 1.0 |
| ΔPrP103-234-[d(TGGGGT)]₄ | 0.5 ± 0.2 | -40.5 ± 1.0 |
| ΔPrP124-234-[d(TGGGGT)]₄ | 0.5 ± 0.2 | -26.2 ± 1.0 |
| flPrP-R12         | 0.9 ± 0.1 | -81.6 ± 1.0 |
| ΔPrP103-234-R12   | 0.5 ± 0.2 | -68.4 ± 1.0 |
| ΔPrP124-234-R12   | NB     | NB            |
| flPrP-D12         | ND    | ND            |
| ΔPrP103-234-D12   | 0.5 ± 0.2 | -86.2 ± 2.0 |
| ΔPrP124-234-D12   | 0.5 ± 0.2 | -17.6 ± 1.0 |

ND, not determined; NB, no binding detected.

In order to evaluate a possible interaction with single-stranded NAs and to confirm the PrP specificity toward the quadruplex structure, D12 was unfolded by removing K⁺ ions from the solution, and ITC titration was performed using ΔPrP103-234, to avoid difficulties due to precipitation with the full-length protein. The heat changes obtained upon addition of the unfolded single-stranded D12 correspond to the heat of NA dilution (Supplementary Figure S4), demonstrating that PrP does not bind to unfolded RNA/DNA molecules lacking the quadruplex structure.
Kinetic and binding data from SPR experiments

To get insights into the kinetics and the affinity of these interactions, SPR experiments were performed on the same systems used in ITC titrations. In addition, (i) the beta structured PrP oligomer, PrP-β, that mimics the scrapie infective form, has been used to verify whether this pathological-like PrP form participates in the binding; and (ii) the Dickerson duplex has been used to assess the specificity of the PrP toward quadruplex-forming NAs.

SPR sensorgrams obtained for the interaction of flPrP with [d(TGGGGT)]₄ are shown in Figure 3A, as example, whereas SPR data of the other systems are shown in Supplementary Figure S5. Since the kinetic and binding constants were independent of the experimental conditions, SPR reported sensorgrams are those obtained at the lowest contact time, at a dissociation time of 240 s and at the highest flow rate (See Materials and Methods section).

Experimental SPR data were fitted using various models and on the basis of χ² values and CD results (see below), the two-step binding reaction model, that takes into account a conformational change of one or both components upon complex formation, was chosen as the best representative of the studied PrP–NAs interactions. The association (k_{on1}, k_{on2}) and dissociation rate constants (k_{off1}, k_{off2}) of the two reaction steps were derived from the fitting and the binding dissociation constants (K_D) were kinetically determined (Table 2). For the systems studied, the dose response curves were obtained by plotting the maximum SPR signal against NA concentration (Figure 3B). In all cases, the curves reached a final plateau, indicating that all binding sites have been saturated and no aspecific binding has occurred.

In details, k_{on1} values were greater for the interaction of all the NAs with flPrP with respect to the values obtained for the interaction with both ΔPrP_{103-234} and ΔPrP_{124-234}. On the other hand, k_{off1} values are very similar among all the systems, suggesting that the dissociation phase does not depend on the binding of NAs to the unstructured PrP N-terminal domain. Interestingly, the fast dissociation process occurring upon recognition and binding suggests that the formed complexes are dynamic. This is in line with the fact that PrP–NA complexes have never been isolated so far, and NMR studies (18,39) did not result in a well-defined structure of the complex, or in that of the NA within the complex; only some changes in PrP structure were revealed. A reliable hypothesis is that other
Table 2. Kinetic and binding parameters for the interaction of flPrP, ΔPrP103–234, ΔPrP124–234 and PrP-β oligomer with [d(TGGGGT)]₄, R12 and D12, determined by SPR. Data relative to flPrP-Dickerson duplex determined by SPR

| PrP-NA interaction                          | $k_{on}$ (×10⁶ M⁻¹ s⁻¹) | $k_{off}$ (×10⁻¹ s⁻¹) | $k_{on2}$ (×10⁻2 s⁻¹) | $k_{off2}$ (×10⁻³ s⁻¹) | $K_D$ (×10⁻⁶ M)² |
|---------------------------------------------|--------------------------|-----------------------|-----------------------|------------------------|--------------------|
| flPrP-[d(TGGGGT)]₄                          | 38 ± 2                   | 1.6 ± 0.1             | 1.7 ± 0.1             | 2.9 ± 0.2              | 0.63 ± 0.02       |
| ΔPrP₁₀₃–₂₃₄-[d(TGGGGT)]₄                   | 19 ± 2                   | 1.8 ± 0.2             | 1.9 ± 0.1             | 14 ± 1                 | 4.0 ± 0.2          |
| ΔPrP₁₂₄–₂₃₄-[d(TGGGGT)]₄                   | 3.0 ± 0.2                | 1.7 ± 0.1             | 0.74 ± 0.02           | 5.0 ± 0.1              | 18 ± 2             |
| flPrP-R12                                   | 81 ± 3                   | 0.39 ± 0.02           | 6.5 ± 0.1             | 1.14 ± 0.03            | 0.075 ± 0.0002    |
| ΔPrP₁₀₃–₂₃₄-R12                            | 16.9 ± 0.4               | 1.66 ± 0.05           | 5.41 ± 0.06           | 1.17 ± 0.03            | 0.21 ± 0.01       |
| ΔPrP₁₂₄–₂₃₄-R12                            | 27.3 ± 0.4               | 1.22 ± 0.04           | 5.48 ± 0.05           | 1.41 ± 0.04            | 0.22 ± 0.01       |
| flPrP-D12                                   | 79 ± 1                   | 1.6 ± 0.2             | 4.6 ± 0.1             | 1.4 ± 0.1              | 0.062 ± 0.002     |
| ΔPrP₁₀₃–₂₃₄-D12                            | 37 ± 1                   | 1.9 ± 0.1             | 7.7 ± 0.2             | 1.7 ± 0.1              | 0.11 ± 0.02       |
| ΔPrP₁₂₄–₂₃₄-D12                            | 19 ± 4                   | 2.0 ± 0.1             | 13 ± 2                | 1.9 ± 0.1              | 0.42 ± 0.04       |
| PrP-β-[d(TGGGGT)]₄                          | 4.9 ± 0.3                | 5.5 ± 0.1             | 0.72 ± 0.02           | 0.84 ± 0.06            | 2.4 ± 0.3         |
| PrP-β-R12                                   | 6.1 ± 0.3                | 1.5 ± 0.1             | 2.8 ± 0.2             | 4.3 ± 0.1              | 3.2 ± 0.3         |
| PrP-β-D12                                   | 0.21 ± 0.01              | 0.17 ± 0.02           | 0.74 ± 0.02           | 0.41 ± 0.02            | 4.1 ± 0.2         |
| flPrP-Dickerson duplex                      | 3.1 ± 0.3                | 0.20 ± 0.01           | 0.22 ± 0.01           | 7.5 ± 0.3              | 500 ± 0.03        |

²The values of $K_D$ were kinetically determined.

cellular components may participate in a larger and more stable macromolecular complex, *in vivo*.

An increase of the $K_D$ values was observed for the interactions with the truncated PrP forms. Surprisingly, SPR experiments revealed a binding also between R12 and ΔPrP₁₂₄–₂₃₄, in apparent contrast to the results obtained by ITC. The different result obtained by ITC could be explained considering that the enthalpic contribution due to the interactions established between R12 and the structured PrP C-terminal domain may be compensated by the opposite contribution of the PrP unfolding process, not allowing to measure a binding-related heat.

This interpretation is supported by the ITC data obtained for the interaction of ΔPrP₁₂₄–₂₃₄ with [d(TGGGGT)]₄ and D12, that presented the highest enthalpy change values (−26.2 and −17.6 kJ mol⁻¹, respectively).

SPR has proved to be very advantageous also in the study of the interaction between flPrP and D12, for which no information was obtained by means of ITC, due to precipitation. Indeed, using SPR methodology, the binding reaction occurs in a solution-independent manner. The comparison of $K_D$ obtained for both R12 and D12 with flPrP reveals very similar values. Therefore, these findings indicate a high-affinity binding (within the same order of magnitude) of D12 with flPrP, such as found for R12 aptamer. It is worth mentioning that our results are in contrast with previous work in which R12 interaction with bovine PrP resulted to be stronger than the one with D12 (23). However, this difference could be ascribed to the PrP species (bovine and ovine) considered, as well as to the different methodologies used.

We also evaluated the affinity of the quadruplex-forming NAs toward the beta structured PrP-β oligomer (Figure 3C and Supplementary Figure S6). Prior to use, a CD spectrum was recorded to confirm that the oligomer was in the β-rich structure (data not shown). From a kinetic point of view, the association phase of all the NAs with PrP-β is slower compared to the α-rich PrP isoform, whereas a similar dissociation phase is displayed with both PrP isoforms. Specifically, the association rate obtained for the interactions involving [d(TGGGGT)]₄ and R12, are very similar, but the dissociation rate for the PrP-β-[d(TGGGGT)]₄ system is slower compared to the PrP-β-R12 system. Differently, the association and dissociation rates referred to the interaction of PrP-β with R12, are higher than those related to the interaction with D12.

Regarding the binding affinity, all the NAs exhibited a similar decrease in the affinity toward PrP-β oligomeric species with respect to the biologically functional monomeric α-PrP isoform, in agreement with previous data obtained using bovine PrP (23). Indeed, the $K_D$ value obtained for the interaction between [d(TGGGGT)]₄ and PrP-β is 38-fold higher with respect to the interaction between [d(TGGGGT)]₄ and the full-length α-PrP isoform. Similarly, R12 and D12 showed an affinity toward PrP-β over 40-fold and 70-fold lower than for the full-length α-PrP isoform, respectively. This is in line with the diverse molecular complexity of the two PrP isoforms. The α-rich PrP isoform is monomeric, whereas PrP-β is a high molecular mass oligomer of 36-mer. Within this molecular assembly, Lys clusters may be less exposed, resulting in a weaker interaction with NAs.

To confirm the specificity of PrP toward the quadruplex-forming NA, affinity measurements were carried out toward the 12-mer Dickerson duplex (same size and 33% G content); results are listed in Table 2, last row. For the flPrP-[d(CGCGAATTCCGCG)]₂ interaction, $K_D$ is significantly higher. In detail, $K_D$ is 794-, 6667- and 8064-fold higher with respect to [d(TGGGGT)]₄, R12 and D12, respectively, assessing the specificity of flPrP toward the quadruplex over the Dickerson duplex.

Finally, SPR binding assays were performed to evaluate the binding of the flPrP as well as the PrP-β oligomer, with the unfolded single-stranded d(TGGGGT), r(GGAGGA GAGGGA) and d(GGAGGAGGAGGA) sequences (data not shown). Two sets of measurements were carried out with and without Li⁺, because this cation does not affect the NAs quadruplex structure but its effect on PrP features is unknown. No binding was observed in the two sets of measurements, neither with the monomeric PrP forms, in good agreement with ITC data, nor with the PrP-β oligomer. All together our results fully demonstrate the specificity of PrP toward quadruplex
over duplex and single strands. In other words, NA quadruplex architecture is essential for the recognition by both ‘healthy’ and ‘pathological’ PrP isoforms.

Identification of the PrP binding sites. Possible modes of interaction

All the NAs here used interact with flPrP as well as with the truncated PrP forms, suggesting that the interactions involve the three binding sites located along the protein. For the [dTGGGGT]₄, a roughly comparison between the difference of ∆H° relative to the interaction with flPrP and ∆PrP₁₀₃₋₂₃₄ (Δ∆H° = −35.9 kJ mol⁻¹), as well as the difference of ∆H° relative to the interaction with ∆PrP₁₀₃₋₂₃₄ and ∆PrP₁₂₄₋₂₃₄ (Δ∆H° = −14.3 kJ mol⁻¹), suggests a greater number of contacts with the first lysine cluster (25–34) compared to the second lysine cluster and to the C-terminal domain. On the other hand, the comparison of the ∆H° obtained for the interaction of R12 with flPrP and ∆PrP₁₀₃₋₂₃₄ suggests the second lysine cluster (101–110) being the major binding site, since the difference between the enthalpy change values is rather small (∆∆H° = −13.2 kJ mol⁻¹ kcal⁻¹).

It is worth recalling that ITC data showed a binding of R12 with both flPrP and ∆PrP₁₀₃₋₂₃₄, but no binding was detected with ∆PrP₁₂₄₋₂₃₄. Differently, SPR allowed to detect a specific binding also with the PrP C-terminal domain, ∆PrP₁₂₄₋₂₃₄, in contrast with previous data in which the interaction with RNAs was found to involve only the PrP N-terminal domain, without any contribution of the structured C-terminal domain (13,16,39), which instead participates in the interaction with DNAs (18). Therefore, the use of different techniques allowed detecting and characterizing a binding, which could have been neglected, if investigated with a single approach.

Finally, speculations about a major PrP binding site for D12 are restricted since no thermodynamic data were obtained for the interaction with flPrP. However, SPR experiments revealed a high binding affinity with flPrP. Moreover, D12 showed to interact with both PrP truncated forms, even though the major binding sites should be the two lysine clusters located in the N-terminal domain. In fact, the ΔH° value derived for the system ∆PrP₁₂₄₋₂₃₄-D12 is markedly higher than the one obtained for ∆PrP₁₀₃₋₂₃₄-D12, indicating the formation of a considerably lower number of favorable interactions. As result, the selected PrP forms allowed to identify the binding sites on PrP.

Regarding the PrP–NAs binding mode, it is worth recalling that a stoichiometry ratio of 1:0.5 was found for all the interactions with the truncated PrP forms, whereas the interaction of flPrP with [dTGGGGT]₄ and R12, presents a stoichiometry of 1:1. These results indicate that the whole PrP is required to establish the specific binding with the quadruplex-forming sequences. Moreover, the enthalpy change values obtained for all the interactions suggest that, although the PrP structured C-terminal domain participates to the interaction, the two lysine clusters at the N-terminal domain are the major binding sites for the NAs. In particular, we can speculate that the binding mode may involve an interaction of the PrP N-terminal domain with the grooves of the quadruplexes through electrostatic interactions between the positive charges of the two lysine clusters and the negative charges of the phosphate groups, as supported by the highly negative enthalpy values obtained for the systems involving flPrP and ∆PrP₁₀₃₋₂₃₄. On the other hand, the C-terminal domain may cooperate to the binding with further weaker interactions, since the ΔH° values related to the binding of ∆PrP₁₂₄₋₂₃₄ (the structured PrP C-terminal domain) with both [dTGGGGT]₄ and D12 are higher compared to those obtained with flPrP and ∆PrP₁₀₃₋₂₃₄.

Nevertheless, it is worth noting that the diverse affinity obtained for the NA sequences here studied toward PrP indicates that these NAs bind in a diverse mode, likely with a diverse orientation of the regions involved in the binding. Also the differences of ΔH° may reflect a diverse contribution of non-covalent bonds to the complex stability.

Structural analysis of the interaction between PrP and quadruplex-forming DNA/RNA

At a molecular level, upon binding, pronounced structural variations on PrP (3,4,12,13) and on NAs, such as annealing, bending or strand exchange (15,17,40) were previously reported. To evaluate whether PrP and/or the quadruplex-DNA/RNA molecules undergo structural changes upon interaction, CD titration experiments were carried out. In all cases the CD analysis revealed structural alterations of one or both partners, providing an experimental support to a two-steps mechanism for the complex formation, involving a conformational change. For each experiment, it was taken into account that [dTGGGGT]₄, D12 and R12 exhibit a significant CD signal in the wavelength range typical of the secondary structure of proteins (Supplementary Figure S7). Therefore, it was evaluated whether the CD spectrum, corresponding to the PrP–NA complex, overlaps with that of the NA alone.

[dTGGGGT]₄ binding to PrP

CD spectra related to the interaction of flPrP with [dTGGGGT]₄ showed a loss of secondary structure for both complex partners (Figure 4A). Although it was not possible to subtract the CD spectrum of [dTGGGGT]₄ alone from the one corresponding to the complex, the CD signal decrease, observed for the protein, at a PrP: [dTGGGGT]₄ molar ratio of 1:0.2, can be ascribed to an actual loss of protein secondary structure. Indeed, [dTGGGGT]₄ has a low CD signal at 209 nm at this concentration (Supplementary Figure S7A). Interestingly, the CD spectra corresponding to the G-quadruplex, upon interaction with PrP, exhibited a shift of the maximum from 264 nm to ~275 nm, and of the minimum from ~244 nm to 254 nm, consistent with the unstructured single-stranded DNA. A similar behavior was observed for the interaction of ∆PrP₁₀₃₋₂₃₄ with [dTGGGGT]₄. Also in this case, both components lost secondary structure (Figure 4B), although the structural loss of [dTGGGGT]₄ is lower compared to the complete disruption of the G-quadruplex architecture found for the interaction with
flPrP. Finally, the binding with ΔPrP124–234 revealed no structural variations on the G-quadruplex fold of [d(TGGGGT)]₄, whereas the protein partially lost a-helical content (Figure 4C).

**R12 binding to PrP**

Also in this case, the CD structural analysis of the interaction with flPrP showed a loss of secondary structure for both interacting partners (Figure 4D). Analogously to [d(TGGGGT)]₄, the CD signal decrease, observed for the protein, at a PrP:R12 molar ratio of 1:0.2, is directly related to an actual loss of secondary structure of PrP, without the inference of the signal due to the presence of R12 (Supplementary Figure S7B). Moreover, R12 undergoes a complete loss of quadruplex structure, up to an equimolar ratio of R12 relative to flPrP. Interestingly, at a flPrP:R12 molar ratio of 1:2, a CD signal at 265 nm is observed, albeit it is lower compared to the one corresponding to R12 alone at the same concentration. ITC experiments showed a binding stoichiometry for flPrP-R12 of 1:1, thus, the signal observed at a molar ratio 1:2 could be due to unbounded R12 molecules present in solution. For the binding with ΔPrP103–234, both protein and RNA partially lost secondary structure (Figure 4E). Similarly to the results obtained with [d(TGGGGT)]₄, R12 partially lost its quadruplex fold compared to the total crash found for the interaction with flPrP. At an equimolar concentration of R12 relative to ΔPrP103–234, a higher CD signal of R12 is observed. Also in this case, the CD signal observed with a higher NA content with respect

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**Figure 4.** CD structural analysis of PrP interaction with [d(TGGGGT)]₄, R12 and D12. CD spectra recorded at various PrP:NA molar ratios for the interaction of: [d(TGGGGT)]₄ with (A) flPrP, (B) ΔPrP103–234 and (C) ΔPrP124–234; R12 with (D) flPrP, (E) ΔPrP103–234 and (F) ΔPrP124–234; D12 with (G) ΔPrP103–234 and (H) ΔPrP124–234. In all panels the CD spectra of the NAs alone are also shown. In panels (F–H), CD spectra corresponding to the complex obtained by subtracting the CD spectra of the NA alone are shown.
to a molar ratio 1:0.5, which corresponds to the ITC derived stoichiometry ratio, may be due to free R12 molecules in solution. Finally, the intriguing binding between R12 and the truncated ΔPrP124–234 revealed a structural aspect that can explain the apparently contradictory results obtained by ITC and SPR. Indeed, CD spectra revealed no changes in the secondary structure of R12, at any molar ratio, thus, it was possible to subtract the CD spectrum referred to R12 alone from the one corresponding to the complex (Figure 4F). At both molar ratios 1:0.5 and 1:1, the protein showed a partial loss of secondary structure, revealing a structural effect, hence supporting the interaction with R12, detected by SPR.

D12 binding to PrP

Since the addition of D12 to fPrP leads to precipitation, a question has arisen about the nature of this precipitate: might D12 induce a conformational variation of the protein, forming fibril-like structures? To address this question, TEM images were collected for the solution containing fPrP in the absence and in the presence of D12, revealing no aggregates in the D12-free PrP sample (Supplementary Figure S8A), whereas amorphous aggregates were observed for the sample containing both components (Supplementary Figure S8B). Therefore, there was no evidence for a PrP fiber-like aggregate formation induced by D12. Interestingly, the solutions prepared at different PrP:D12 molar ratios showed no longer precipitation after 2 days. The CD spectra recorded for these solutions revealed a recovery of the secondary structure of the protein (Supplementary Figure S9A). On the other hand, D12 showed a complete loss of the quadruplex structure, without recovering of any type of secondary structure (Supplementary Figure S9B). In the case of the interaction between ΔPrP103–234 and D12, CD titration experiments revealed no changes of the secondary structure of D12 (Figure 4G). The resulting CD spectra, obtained by subtraction of the spectrum related to D12 alone from that of the complex, showed a progressive decrease of the α-helical content of ΔPrP103–234. Similar results were obtained for the system ΔPrP124–234-D12, in which no structural changes were observed for D12, whereas a progressive loss of secondary structure can be observed for PrP (Figure 4H).

The CD analysis revealed for the first time an NA quadruplex structure disruption upon interaction with the fPrP. To our knowledge, an unwinding activity exerted by PrP on NAs was never observed before. This new finding shows that NAs may undergo highly diverse structural modifications. Indeed, a PrP chaperoning role in assisting the RNA folding and DNA strand transfer has been postulated (15,17,40). The secondary structure variation, induced upon binding, differs in the various partner pairs investigated. Indeed, [d(TGGGGT)]4 and R12 exhibit a null, partial or complete secondary structure loss upon interaction with the ΔPrP124–234, ΔPrP103–234 and fPrP, respectively. Analogously, D12 does not lose its secondary structure when it interacts with the truncated PrP forms. All these results indicate that the interaction with the PrP C-terminal domain does not affect the structural integrity of the NAs, whereas the charge interactions established with the lysine clusters located in the PrP N-terminal domain are responsible for the disruption of the quadruplex architecture.

Indeed, PrP loses a relatively high percentage of α-helical content in all cases, according to literature data (3,18,39,41), even though for the system ΔPrP124–234–R12, only a slight decrease of the secondary structure was observed. Furthermore, the fPrP binding to D12 revealed the almost completely recovery of the PrP α-helical content after 2 days from the mixing, whereas a complete disruption of D12 quadruplex structure was observed. Similar results were previously reported for the interaction with a synthetic small RNA, in which NMR experiments revealed that PrP recovers most of its original fold after few days from RNA addition (39).

Overall, these findings point to a cross-talk between PrP and quadruplex-forming NAs, in which the unstructured N-terminal and the structured C-terminal domains play distinct functional roles. Indeed, the PrP intrinsically unstructured N-terminal domain induces the unwinding of NAs, and in turn, NAs trigger the unfolding of the PrP structured C-terminal domain.

CONCLUSION

Sequence versus structure specificity

Despite all the efforts to identify a key sequence shared by NAs, no clear evidence has been provided, since PrP is able to bind a wide repertoire of NAs that can be rather different in sequence and structure (21–23,27,42). Recent works have attracted our attention toward quadruplex-forming NAs (21,23,39). Here, we have selected three NA sequences: (i) d(TGGGGT), (ii) r(GGAGGAGGAGGA) and (iii) d(GGAGGAGGAGGA), that possess contiguous guanine repeats in the sequence and adopt a quadruplex architecture (Figure 1A–C). Our results indicate that the quadruplex-forming NA sequences bind to the cellular PrP conformation with high affinity, whereas the Dickerson duplex binds to the PrP with a significant lower affinity and the quadruplex-forming NA sequences do not interact at all when they are unfolded single strands. Therefore the quadruplex fold is an essential feature for the binding to PrP, in agreement with previous works (25,26). Furthermore, we show that the quadruplex architecture is essential for the recognition by the PrP-β oligomer as well, albeit it is worth recalling that the affinity of the quadruplex-forming NAs toward this β-rich PrP conformer is lower with respect to the α-rich PrP isoform. The 3D-structure of NAs, here studied, shares four G-containing planes that can be considered the structural determinant for the molecular recognition. As a general result, any sequence, embodying guanine duplets able to adopt a quadruplex fold, should then be recognized by both PrP isoforms. In conclusion the binding is guided by the NA structure and not by the specific nucleotide sequence.

Biological relevance

Several in vitro and in cellulo studies have been recently carried out about the PrP binding with NAs, looking for
the biological relevance that these interactions may hold inside cell environment. The NA-binding properties of PrP are further supported by the emerging concept that proteins possessing intrinsically disordered and positively charged N-tails may modulate several key aspects of protein–DNA interactions (43). PrP belongs to this class of proteins, able to form dynamic or fuzzy complexes with NAs, and thereby may play multifunctional roles. In particular, it is worth pointing out the possibility of an in vivo binding between PrP\(^{\text{C}}\) and quadruplex-forming NAs.

The occurrence of quadruplex motifs in eukaryotic genomes has been widely observed in regions of biological significance, such as telomeres and promoters of many genes and oncogenes (30,31). Moreover, a subset of messenger RNAs were shown to adopt a G-quadruplex fold and to be specifically recognized by an RNA-binding protein (44). To this regard, many proteins able to bind different G-quadruplex-DNA/RNA, and to promote and stabilize this motif, have been described (45,46). On the contrary, little is known about proteins able to disrupt these NA motifs, also because quadruplexes are exceedingly stable entities that do not spontaneously fall apart (47). However, proteins able to resolve quadruplex-DNA structures were found (48,49). In particular, it was reported that the protein hnRNP A1 can destabilize and unfold the G-quadruplex KRAS promoter, facilitating the hybridization to the complementary strand (50). Analogously to the retroviral nucleocapsid protein NCp7 of HIV-1, PrP accelerates the hybridization of complementary DNA and RNA strands and chaperones the viral synthesis (14,15,17). In summary, PrP was shown to possess NA binding and annealing activities, similarly to NA chaperone proteins, but there was no evidence for an unwinding activity so far.

Our results indicate the formation of dynamic complexes between PrP and quadruplex-forming NAs, that may have a feedback in vivo, supporting a biological interference of PrP on regulation of quadruplex-structured NAs. Very likely, other unidentified cellular partners might participate to form larger assemblies. We showed that the quadruplex architecture of NAs was completely disrupted, upon interaction with flPrP, whereas a partial loss was observed upon interaction with the deleted protein \(\Delta\text{PrP}_{103-234}\), which still has one binding site at the N-terminal domain. Finally, no structural loss was detected upon interaction with the deleted protein \(\Delta\text{PrP}_{124-234}\), which completely lacks the N-terminal domain. These findings are in line with other works in which the chaperoning properties of PrP were charged to the unstructured PrP N-terminal domain (14,15,40), showing that this domain affects the NA folding/unfolding. Therefore, the observed unwinding of the NA quadruplex architecture upon interaction with the PrP N-terminal domain allows us to surmise a resolvase-like activity performed by PrP, as found for other proteins (48). Reciprocally, all the PrP forms herein used underwent a partial loss of secondary structure upon interaction with DNA/RNA molecules, suggesting that the partial unfolding of the protein is triggered by the interaction of NAs specifically with the PrP structured C-terminal domain. It was previously suggested that the establishment of charge interactions between the phosphate groups of NAs with the PrP N-terminal domain may bring the two partners in close proximity, favoring the interaction with the PrP structured C-terminal domain (51). These two binding modes might be part of a functional mechanism in which the interaction of NAs with the PrP N-terminal domain may lead to resolve the NA quadruplex structures, and the interaction with the PrP C-terminal domain may trigger a partial loss/rearrangement of the PrP structure. It is well known that PrP\(^{\text{C}}\) traffics through the cell by endocytosis and recycling to the cell surface (52). Then PrP structure variation would be a signal for the dissociation of the complex, that would bring the protein free to recover its functional structure and to begin a new cell cycle.

These interactions may play a critical role also in the conversion process of PrP toward its pathological isofrom. The most in vogue hypothesis, about the biological role of PrP–NA complexes, concerns the possible catalytic action of NAs in the PrP\(^{\text{C}}\) to PrP\(^{\text{Sc}}\) conversion mechanism. This hypothesis has gained more strength since de novo infectious prions were formed from a minimal set of components, including synthetic NA, by using PMCA technique (19,20). Moreover, many DNA and RNA are able to induce PrP misfolding with the formation of \(\beta\)-rich oligomeric species with amyloidogenic features (41,42) and/or amorphous aggregates (39), depending on the NA used.

Consistently, also the structural analysis performed in the present work suggests that NAs may trigger the conversion of PrP\(^{\text{C}}\) into PrP\(^{\text{Sc}}\). The transformation goes along with a structural modification from an \(\alpha\)-helix to a beta conformation and requires a direct interaction between PrP\(^{\text{C}}\) and PrP\(^{\text{Sc}}\) which acts as a template (2). In all our experiments, NAs induced a PrP \(\alpha\)-helix loss, which is a necessary event in the PrP\(^{\text{C}}\) to PrP\(^{\text{Sc}}\) conversion. Furthermore, we demonstrated that the quadruplex-forming NAs were able to recognize and bind also the PrP-\(\beta\) oligomer, a good model to mimic PrP\(^{\text{Sc}}\). Therefore, taking collectively into account all our findings, we put forward the hypothesis that NAs, with a quadruplex fold, may assist in prion propagation, inducing the PrP misfolding/misfunction. Indeed, quadruplex structures may act as molecular scaffolds that favor the interaction between the healthy PrP\(^{\text{C}}\) and the pathological PrP\(^{\text{Sc}}\) forms, by providing the appropriate spatial orientation. Quadruplex may be rarely encountered by PrP in a much higher concentration of duplex genomic DNA, consistently with the rarity of the prion conversion events.

As a last but not least consideration, the hypothetical physio-pathological role of PrP–NA interactions would depend on the possibility that both PrP\(^{\text{C}}\) and PrP\(^{\text{Sc}}\) isoforms can encounter NAs within the cell. In non-pathogenic conditions, PrP\(^{\text{C}}\) was found in the cytoplasm (6,8–10) of some neurons of the hippocampus, neocortex and thalamus, and was found also in the nuclear lamina of endocrine and neuronal cells, interacting with structural chromatin components (9,10). On the other hand, in pathological conditions, PrP\(^{\text{Sc}}\) has been detected in endocytic compartments, peri-nuclear regions and in the nuclei of prion-infected cells, where it interacts with...
chromatin in vivo (8,11). A common denominator of neurodegenerative disorders due to protein misfolding and aggregation seems to be an aberrant DNA interaction with the misfolded protein able to translocate into the nucleus (7). All these findings further corroborate the in vivo occurrence of intriguing cross-talks with physiopathological consequences relevant to prion.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–9 and Supplementary Methods.

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