An NMR and molecular dynamics investigation of the avian prion hexarepeat conformational features in solution

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Abstract

The prion protein is a copper binding glycoprotein that in mammals can misfold into a pathogenic isoform leading to prion diseases, as opposed, surprisingly, to avians. The avian prion N-terminal tandem repeat is richer in prolines than the mammal one, and understanding their effect on conformation is of great biological importance. Here we succeeded in investigating the conformations of a single avian hexarepeat by means of NMR and molecular dynamics techniques. We found a high flexibility and a strong conformational dependence on pH: local turns are present at acidic and neutral pH, while unordered regions dominate at basic conditions.

1. Introduction

The prion protein (PrP C) is a cell surface glycosyl-phosphatidylinositol (GPI) anchored glycoprotein [1,2], whose biological function has not yet been identified, even if it has been established that in mammals its conformational change gives rise to the β-sheet rich and pathogenic isoform PrPSc [1,2]. Avian species also express prion protein, but no evidence of neurodegenerative disorders have been reported among them [3–5] and chicken prion protein (ChPrP C) has initially been described to induce acetylcholine-receptor activity [4]. This protein shares about 33% of primary sequence identity with the mammalian one and some essential features are conserved [3]. In particular, both proteins possess: (i) multiple N-glycosylated sites; (ii) an amino-terminal signal sequence that is removed in the mature protein; (iii) a carboxy-terminal signal that is eliminated when the mature protein is linked to GPI, and (iv) an N-terminal domain featured by tandem amino acid repeats (PHNPGY in avians, PHGGGWGQ in mammals) followed by a highly conserved hydrophobic core [3,4]. Different from mammalian PrP C, the digestion of ChPrP C with trypsin or proteinase K produces peptide fragments stable to further proteolysis. One of these fragments comprises the 49–129 sequence, consisting of a large part of the N-terminal domain [6]. This resistance to proteolysis may suggest a compact domain of the proline/glycine rich N-terminus, although the hexarepeat amino acid sequence seems to show no tendency towards a particular structured conformation [6]. More recently, NMR results showed that the ChPrP C globular domain contains three α-helices and two short antiparallel β-sheets, with a spatial arrangement similar to that observed in mammalian PrP C [7]. The N-terminal domain results to be flexible and unstructured and the 50–73 residues, encompassing part of the tandem hexarepeat sequence, have not yet been individually assigned by NMR [7].
Besides the wide conformational freedom of the N-terminal backbone, the significant content of proline residues may in principle bring about further complications in the structure determination of this part of protein, due to the presence of an equilibrium between cis and trans form in each Xxx-Pro peptide bond, even if the stabilization of a preferred isomer may be assisted by other residue side chains, particularly histidine and tyrosine.

In native and folded proteins the Xxx-Pro bond exists essentially in either cis or trans form because the interactions with neighbouring groups favours one of them, but an equilibrium exists in unfolded proteins. In small peptides containing proline residues, the two forms are almost isoenergetic and are both present, with an observed increase of trans isomer going from polar to non-polar solvents [8–10]. In solution, polyproline peptides have been shown to exist predominantly in the PPII conformation, a left-handed helix in which all the residues are in the trans form. Also short peptides not containing proline are supposed to partially adopt this conformation, but it seems quite sure that protein domains having an intra-chain PXXP motif fold into a PPII helix. One of these motifs has been identified in mammalian as well as avian prion proteins and it corresponds to the region 101–104 in mouse PrP C and 107–111 in chicken PrP C [11]. In this context, MD studies performed at various pH provide precious information on the different conformational states populated in the 10–100 ns time scale, as well as helping the interpretation of NMR results in the same conditions, when available.

With the long-term purpose of clarifying the role of the repeat regions in prion protein biological function, here we use both techniques in a pH and solvent dependent study concerning the conformation of the single hexarepeat PHNPGY (1-HexaPY), with both N- and C-termini blocked by acetylation and amidation, respectively. This peptide, which is replicated seven times in the 53–94 region of the chicken prion protein, represents a starting model system for future studies concerning longer portions of avian hexarepeat and human octarepeat sequences.

2. Materials and methods

2.1. NMR measurements

The peptide under study was synthesized as previously reported [12]. All the experiments were carried out at 500 MHz on a VARIAN INOVA UNITY PLUS located at the Department of Chemical Sciences, Catania (Italy), and on a VARIAN UNITY 500 spectrometer, located at the Department of Environmental Sciences, Caserta (Italy). Spectra were processed using the VARIAN VnmrJ and XEASY [13] software. Sample solutions (5 mM) were prepared in TFE-d3/H2O (80/20 v/v) and H2O/D2O (90/10 v/v). NMR spectra for the three-dimensional structure determination were collected at 300 K and referenced to external TMS (δ = 0 ppm); the pH of the aqueous solution was adjusted to 4.2. The dependence of the amide chemical shifts on the temperature was observed in the range 300–311 K. Furthermore, for all the solvent systems the possible occurrence of aggregation was verified by analyzing the cross peak patterns in ROESY spectra, recorded at a peptide concentration of 0.8 mM. Deuterated D2O (99.9% relative isotopic abundance) and TFE-d3 (99%) were purchased from Cambridge Isotope Laboratories. Mono (1D) and two dimensional (2D) spectra were accumulated with a spectral width of 6000 Hz in H2O/D2O and 4800 Hz in TFE/H2O (80/20 v/v). 2D experiments DQF COSY, TOCSY, ROESY and NOESY [14] were recorded in the phase sensitive mode using the States–Haberkorn method. Water suppression was achieved by DPFGSE sequence [15]. TOCSY, NOESY and ROESY spectra were acquired with mixing time of 70, 250 and 150 ms, respectively. Typically, 64 transients of 4 K data points were collected for each of the 256 increments; the data were zero filled to 1 K in δ1. Squared shifted sine-bell functions were applied in both dimensions prior to Fourier transformation and baseline correction.

2.2. Structure calculations

Due to unfavourable correlation time, NOESY experiments were scarcely informative and not suitable for structure calculations. Therefore distance restraints for structure calculations were derived from the cross-peak intensities in ROESY spectra, recorded in H2O/D2O and TFE/H2O (80/20 v/v). The ROESY cross peaks were manually integrated using the XEASY software [13] and converted to upper distance constraints according to an inverse sixth power peak volume-to-distance relationship for the backbone and to an inverse fourth power function for side chains, by using the CALIBA module of the CYANA program [16]. Distance constraints together with the obtained scalar coupling constants were then used by the GRIDSEARCH module, implemented in CYANA, to generate a set of allowable dihedral angles. Structure calculations, which used the torsion angle dynamics protocol of CYANA, were then started from 100 randomized conformers. The 20 conformers with the lowest CYANA target function were further refined by means of unrestrained energy minimization in vacuo, using the GROMOS 96 [17] force field (FF) within the program SPDBV viewer [18]. Several cycles of steepest descent were repeated until the energy difference between two successive steps was less than 10−3 kJ mol−1. The structure analysis has been performed with the program MOLMOL [19]. Consistently with suggestions from a recent work [7] we also minimized the conformers in a water shell and in a box with TFE/H2O 80/20 with a conjugate gradient method and tolerance of 10−3 kJ mol−1, using ORAC 4.0 program [20] and the AMBER94 FF [21].

2.3. Molecular dynamics

All the simulations were run in water using ORAC 4.0 [20] and the AMBER94 FF [21]; a cubic sample containing one
1-HexaPY chain and 1184 water molecules with periodic boundary conditions (PBC) was studied in the isothermal-isobaric ensemble (NPT, $P = 1$ atm, $T = 300$ K); temperature was controlled with a Nose-Hoover thermostat [22] and the SPC model [23] was used for water. The ESP charges of deprotonated tyrosine, not available in the FF, were calculated at HF/6-31G* level for the N-acetyl- (t)-tyrosinate amide after geometry optimization, following the AMBER charge fitting philosophy. An r-RESPA multiple time-step algorithm with a potential subdivision specifically tuned for proteins [20] was used for integrating the equations of motion, using a time step of 10 fs. Due to its partial double bond character, the torsional barrier around the peptide bond Xxx-Pro is not thermally overcome [24] in the 1–100 ns time scale, typical of MD simulations. Consequently, the different isomers, originated from the Asn-Pro and Ac-Pro $\omega$ torsion angles (N- and C-termi ned were capped with acetyl and amide groups, respectively), were simulated separately. For each isomer, three different protonation states were studied to mimic the different pH conditions. Taking into consideration the $pK_a$ of histidine and tyrosine (6.34 and 9.77, respectively [12]), we can assume in our simulations that at acidic pH, the histidine is protonated and tyrosine is in its neutral form (labelled HY), while at neutral pH both histidine and tyrosine in their neutral states (labelled neutral). The total simulation time was about 100 ns for each of the twelve simulations of the four isomers in three different protonation states. Each run was equilibrated for at least 30 ns to avoid a starting configuration bias and, after equilibration, we checked that both volume and total energy fluctuated around their average value, without systematic drifts. The trajectory analysis was performed on 73 ns-long production runs, with configurations stored every 5 ps. To assess if the force field was able to predict PPII conformations, we also carried out a 30 ns simulation of 9-mer poly-l proline in the $\alpha$-helix form (NPT, $P = 1$ atm, $T = 300$ K) using a cubic box with 2123 explicit water molecules and PBC. The PPII structure ($\phi = -75^\circ$, $\psi = 145^\circ$) [25] was retained for the whole simulation time, with the exception of the C-terminal proline, whose average $\psi$ angles resulted in equilibrium between $-30^\circ$ and $150^\circ$ values.

During the 1-Hexap simulations, the presence of hydrogen bonds between carbonyl oxygens and amide hydrogens belonging to different amino acids was estimated following the DSSP criteria [26]. To investigate their persistence we also calculated the variation of a given O–H distance and the angle between the C-O and N-H vectors ($\tau$) as a function of time, on one hand to understand the orientation of the two atoms involved in the hydrogen bond and on the other to calculate the $\tau$ angle/O–H distance/energy surface so as to decide if a hydrogen bond was present. The hydrogen bond energy used in this work is purely electrostatic and it is expressed as $E_{HB} = u_{q1q2} - 1/r_{CN} + 1/r_{CH} - 1/r_{OH} + 1/r_{NO}$ [26], where $q_1$ and $q_2$ are respectively 0.42e and 0.20e, with the unit electron charge, $u = 332$ the conversion factor from $e^2$/Å to kcal/mol and $r_{CN}$, $r_{CH}$, $r_{OH}$ and $r_{NO}$ the inter-residue distances in Å; a hydrogen bond is considered to be present when $E_{HB} \leq -0.5$ kcal/mol, $\tau \leq 60^\circ$ and $r_{OH} \leq 3$ Å.

3. Results and discussion

3.1. NMR analysis and structure determination

The conformational behaviour in solution of the 1-HexaPY hexapeptide was studied by proton NMR spectroscopy in two different solvents: water (H$_2$O/D$_2$O 90/10), because of its biological relevance and trifluoroethanol (TFE/H$_2$O 80/20 v/v). The TFE/H$_2$O 80/20 mixture, often used as a membrane-mimicking solvent (see, e.g. [27]) and as a hydrogen bond promoting solvent, here can provide indications on the possible folded states of the peptide, which may not appear in water, and of the conformation of the heptapeptide region when inserted in a biological membrane. Identification of the complete spin systems in both solvents was readily accomplished by the homonuclear $J$-correlated 2D techniques TOCSY and DQFCOSY. Since these were not sufficient to determine their position in the sequence, sequential NOE connectivities between backbone protons were employed to unambiguously assign all the amino acids and their position in the peptide chain. Complete chemical shift assignments in H$_2$O/D$_2$O and TFE/H$_2$O (80/20 v/v) are reported in Tables 1 and 2, respectively. $^3J_{HH}$ and $^3J_{CHCH}$ coupling constants and temperature coefficients are reported in Table 3.

A key feature of 1-HexaPY is the presence of two proline residues in the sequence; the first one is located at the beginning of the chain, the other at a central position in the Ac-Pro–His–Asn–Pro–Gly–Tyr–NH$_2$ sequence: consequently Pro$^3$ plays a major role in determining the distribution of conformers in solution. Starting with the TFE/H$_2$O solvent, we find that the hexapeptide shows a well resolved 1D $^1$H spectrum, characterized by a good dispersion of the proton resonances and a predominant set of sharp peaks for amide protons. Its features indicate the existence of two main families of conformers: a dominant one representing 95% of the population, and a minor one covering less than 5%. Two observable sets of resonances, presumably due to the cis–trans isomers about the Xxx-Pro bond and with relative populations 83% and 17%, are clearly present also in the proton spectrum of 1-HexaPY in H$_2$O/D$_2$O. The hexapeptide assumes a trans conformation for the Ac-Pro and Asn-Pro peptide bonds in TFE/H$_2$O, as indicated by the strong NOEs between the H$^\alpha$ of Pro$^3$ and Ac, and between the H$^\alpha$ of Pro$^4$ and H$^\beta$ of Asn$^3$; also in H$_2$O/D$_2$O, both the Xxx-Pro bonds of the major conformer family assume a trans conformation as confirmed by the presence of two NOE contacts between...
the Hβ of Pro1 and Ac, and between the Hδ of Pro4 and Hα of Asn3 (see Tables A and B of Supplementary data). The presence of a less populated conformer group is presumably due to a cis arrangement of the Asn3-Pro4 peptide bond since an Ac-Pro1 NOE contact is not observed in the ROESY spectrum and, moreover, the Hα resonance of Asn3 of the minor conformer is obscured by the water signal. All amide protons present a linear and negative dependence of their chemical shifts on temperature; the backbone amide protons of residues His2 and Tyr6 in TFE/H2O and Tyr6 in H2O/D2O show low temperature gradients in both solutions (Table 3). In particular, the value obtained for the Tyr6 was the lowest and identical in both solvent systems indicating that this amide proton has a good tendency to be solvent shielded likely by its aromatic side chain, as the up-field shifted chemical shift of Tyr6 amide proton also suggests. The scalar coupling constants, $J_{\text{NH}-\text{CH}}$ for residues His2, Asn3 and Tyr6 show values in the range 6–8 Hz (Table 3), not evidencing the formation of a canonical folded conformation. On the basis of $J_{\text{CH-CH}}$ coupling constants and $\gamma$CH–$\beta$CH, NH–$\beta$CH NOEs, the preferred conformations of the side chains were identified [28] as a trans arrangement for all the amino acids except for Asn3 which, only in TFE/H2O, appears to prefer a gauche conformation. These data were confirmed by an analysis of rotamer populations [29] reported in Table C of the Supplementary data. After a careful analysis of the ROESY spectra, 50 proton–proton

**Table 1**

Proton chemical shifts (ppm) for the major conformer family (a) and the minor conformer family (b) of 1-HexaPY at 300 K in H2O/D2O (90/10 v/v).

<table>
<thead>
<tr>
<th>AA</th>
<th>NH</th>
<th>$\alpha$CH</th>
<th>$\beta$CH</th>
<th>$\gamma$CH</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro4</td>
<td>/</td>
<td>4.41</td>
<td>3.91/3.84</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Asn3</td>
<td></td>
<td>3.95</td>
<td>3.24/3.13</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Pro4</td>
<td>/</td>
<td>4.41</td>
<td>3.61/3.52</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>His2</td>
<td></td>
<td>4.95</td>
<td>3.20/3.14</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Asn3</td>
<td>4.92</td>
<td>4.41</td>
<td>3.21/3.15</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Pro4</td>
<td>/</td>
<td>4.41</td>
<td>3.05/2.95</td>
<td>/</td>
<td></td>
</tr>
</tbody>
</table>

The values for the Acetyl and CONH2 groups are 2.10 ppm and 7.55/7.05 ppm (a), 2.10 ppm and 7.55/7.05 ppm (b).

**Table 2**

Proton chemical shifts (ppm) for 1-HexaPY at 300 K in TFE/H2O (80/20 v/v).

<table>
<thead>
<tr>
<th>AA</th>
<th>NH</th>
<th>$\alpha$CH</th>
<th>$\beta$CH</th>
<th>$\gamma$CH</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro4</td>
<td>/</td>
<td>4.30</td>
<td>2.18/1.88</td>
<td>1.96</td>
<td>$\delta$CH3.45</td>
</tr>
<tr>
<td>Asn3</td>
<td></td>
<td>3.95</td>
<td>2.81/2.65</td>
<td>/</td>
<td>$\delta$NH7.60/6.93</td>
</tr>
<tr>
<td>Pro4</td>
<td>/</td>
<td>4.41</td>
<td>3.25/3.15</td>
<td>/</td>
<td>$\delta$CH3.55</td>
</tr>
<tr>
<td>Asn3</td>
<td>4.99</td>
<td>4.41</td>
<td>3.08/2.95</td>
<td>/</td>
<td>$\delta$CH3.55</td>
</tr>
</tbody>
</table>

The values for the Acetyl and CONH2 groups are 2.07 ppm and 7.14/6.54 ppm, respectively.

**Table 3**

Temperature coefficients (ppb/K) and $J$ coupling constants (Hz) for 1-HexaPY in TFE/H2O (80/20 v/v) and in H2O/D2O (90/10 v/v).

<table>
<thead>
<tr>
<th>AA</th>
<th>TFE/H2O (80/20)</th>
<th>H2O/D2O (90/10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta\delta/\Delta T$</td>
<td>$J_{\text{NH-CH}}$</td>
</tr>
<tr>
<td>Pro4</td>
<td>–</td>
<td>3.7</td>
</tr>
<tr>
<td>Asn3</td>
<td>–</td>
<td>5.8</td>
</tr>
<tr>
<td>Gly</td>
<td>–</td>
<td>5.8/5.8</td>
</tr>
<tr>
<td>Tyr</td>
<td>–</td>
<td>7.4</td>
</tr>
<tr>
<td>CONH2</td>
<td>–/– 6.2</td>
<td>–/– 6.2</td>
</tr>
</tbody>
</table>

In H2O/D2O data are reported only for the major conformer family.
NOE cross peaks were assigned and integrated for 1-HexaPY in TFE/H₂O (80/20 v/v), and 37 in H₂O/D₂O, for the principal conformer family. A list of the relevant distances derived from the integration of the ROESEY peaks is reported in Tables A and B of the Supplementary data; it should be noted that in both solvents a correlation between the NH of Gly and the NH of Tyr, corresponding to a calculated distance of about 3 Å, is clearly observed. Furthermore, only in TFE/H₂O a medium range NOE value could be assigned as a weak correlation between the Pro CH₃ and the Tyr amide proton. Four δNH·H and ten δCαδCH₃ coupling constants were extracted from the ¹H monodimensional spectra of 1-HexaPY either in TFE/H₂O (80/20 v/v) and H₂O/D₂O (Table 3). Stereospecific assignments for His (βCH₂ protons) and Asn (βCH₂ and δCH₂ protons) of 1-HexaPY in TFE/H₂O (80/20 v/v) were derived from the input data, using the GRID-SEARCH module of CYANA software. The final input files for the structure calculation contained 26 meaningful distance constraints (16 intraresidue and 10 short-range) and 13 angle constraints for the 1-HexaPY in H₂O/D₂O, and concerning the study in TFE/H₂O (80/20 v/v), 26 meaningful distance constraints (15 intraresidue, 10 short- and 1 medium-range) and 23 angle constraints. These constraints were then used to generate a total of 100 structures and among them the 20 structures with the lowest target function values were selected and energy minimized. The best 20 CYANA conformers of 1-HexaPY in H₂O/D₂O (target function 0.77) and TFE/H₂O (80/20 v/v) (target function 1.05) were then minimized in vacuo and in a solvent shell; a stick drawing of the representative structures is reported in Fig. 1 of the Supplementary data. In TFE/H₂O (80/20 v/v), the NMR structure of 1-HexaPY is well defined in a water shell at acidic pH: disordered trans–trans (a) and turn-like (b). 1-HexaPY trans–trans (c) isomer obtained from MD simulations in the H’Y form sampled every 2.5 ns. 1-HexaPY trans–trans MD isomer (c) shows the turn conformer, underlined also in the NMR structures (b) (top: N-terminus, bottom: C-terminus).

The NMR study discussed above gives a fairly clear picture on the averaged conformation adopted by the 1-HexaPY peptide chain. However, for technical difficulties the study was limited to acidic pH values, thus allowing only a partial interpretation of other available data, such as the CD spectra, previously reported by some of us [12], that while Pro, His, Asn, Gly, Pro, Asn, Gly, Tyr, and Pro angles only reflect a propensity towards this elongated conformation.

The NMR structure in H₂O/D₂O is reasonably defined (RMSD = 0.43 in the 1-4 region; RMSD = 0.59 in the region 1–5) and elements of PPII conformation are present (Fig. 1a and b), which include the Asn and Pro residues; the presence of Gly causes the break of this secondary structure and allows Tyr to experience an higher conformational freedom.

### 3.2. Molecular dynamics simulations

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<table>
<thead>
<tr>
<th>Table 4</th>
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<tbody>
<tr>
<td>Average dihedral angles obtained from the 20 NMR structures minimized in solvent for 1-HexaPY in water (a) and in TFE/H₂O 80/20 (b)</td>
</tr>
<tr>
<td>AA</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>(a)</td>
</tr>
<tr>
<td>His</td>
</tr>
<tr>
<td>Asn</td>
</tr>
<tr>
<td>Pro</td>
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<tr>
<td>Gly</td>
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<tr>
<td>Tyr</td>
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<tr>
<td>(b)</td>
</tr>
<tr>
<td>His</td>
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<tr>
<td>Asn</td>
</tr>
<tr>
<td>Pro</td>
</tr>
<tr>
<td>Gly</td>
</tr>
<tr>
<td>Tyr</td>
</tr>
</tbody>
</table>

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Fig. 1. From left to right: 1-HexaPY trans–trans conformers obtained from CYANA structures minimized in a water shell at acidic pH: disordered (a) and turn-like (b). 1-HexaPY trans–trans (c) isomer obtained from MD simulations in the H’Y form sampled every 2.5 ns. 1-HexaPY trans–trans MD isomer (c) shows the turn conformer, underlined also in the NMR structures (b) (top: N-terminus, bottom: C-terminus).
cover a wide range of pH values. In order to get a more complete understanding at molecular level and in particular to further investigate the pH dependence of 1-HexaPY conformational states in aqueous solution, a fully atomistic molecular dynamics (MD) study was then carried out in water.

It is known that the \textit{trans} conformation of proline tends to stabilize type I $\beta$ turn ($\phi_{i+1} = -60^\circ$, $\psi_{i+1} = -30^\circ$, $\phi_{i+2} = -90^\circ$, $\psi_{i+2} = 0^\circ$) [30] and PPII structures ($\phi = -75^\circ$, $\psi = 145^\circ$) [25]; conversely the \textit{cis} conformation of proline stabilizes a special type of turn, devoid of intrachain hydrogen bonds, called VI[31] ($\phi_{i+1} = -60^\circ$, $\psi_{i+1} = 120^\circ$, $\phi_{i+2} = -120^\circ$, $\psi_{i+2} = 0^\circ$, VIa1 $\phi_{i+1} = -120^\circ$, $\psi_{i+1} = 120^\circ$, $\phi_{i+2} = -60^\circ$, $\psi_{i+2} = 0^\circ$, VIa2 $\phi_{i+1} = -135^\circ$, $\psi_{i+1} = 135^\circ$, $\phi_{i+2} = -75^\circ$, $\psi_{i+2} = 160^\circ$). To claim the presence of such conformers, the existence of consecutive amino acid residues with the typical angles is required, thus we calculated the percentage of type I $\beta$ turn and PPII considering both 2, 3, 4 consecutive residues (see Table D of the Supplementary data), using the standard tolerance interval of $\pm 30^\circ$ for all the dihedrals. During the MD simulations, no more than two consecutive residues with type I $\beta$ turn $\phi$ and $\psi$ dihedrals were found, indicating a high conformational flexibility of the hexarepeat. The time evolution of the conformers (Fig. 2), calculated taking into account only two residues, proves the fast flipping of backbone dihedrals, showing also a prevalence of angles typical of $\beta$ turn and unordered structures.

The analysis of $i - i + 3$ hydrogen (H) bonds and of intrapeptide contacts (evaluated as $C_{\alpha i} - C_{\alpha i+3}$ distances inferior to 7 Å [32]), revealed the pivotal role of asparagine and of the conformation of prolines on the peptide structure: in fact, H-bonds and contacts always involve asparagine, despite its tendency to adopt PPII dihedrals registered both by NMR and MD, and they exist only in regions containing \textit{trans} prolines (cf H-bond percentages in Table 6). In particular we found a NPGY $\beta$ turn fulfilling the DSSP criteria [26], and a second turn region was detected, albeit in lower extent, in the AcPHN sequence. The existence of turn structures is indicated also by $C_{\alpha i} - C_{\alpha i+3}$ distances, quite large for PHNP and HNPG segments, and short in the NPGY C-terminal sequence (Table E of the Supplementary data). Summarising, the peptide shows a tendency to adopt $\beta$ turn conformations, which on the other hand exist only in a fraction of the trajectory and quickly interconvert with unordered ones.

Fig. 2. Time evolution of 1-HexaPY conformers for each of the four isomers (\textit{trans}–\textit{trans} (a), \textit{cis}–\textit{trans} (b), \textit{trans}–\textit{cis} (c), \textit{cis}–\textit{cis} (d)) in the three different protonation states obtained from MD simulations. The calculation is based on at least two consecutive residues with the characteristic dihedral angles concerning type I $\beta$ turn and three consecutive residues for PPII.
the involved proline is in trans adopted mainly when tyrosine is not deprotonated and turn conformation in the NPGY and Ac-PHN regions is minimized in water and the MD (/Fig. 3. Superposition of the backbone dihedrals of the 20 NMR structures (9,77 [12], see Table D of the Supplementary data ). The pH-dependent features. We notice first that the Ramachandran maps of all the isomeric forms, calculated from the simulations, present strong variations as a function of pH, the most striking feature being the presence of distinct regions of local minima (see Tables F–H of the Supplementary data). For an easier comparison with the NMR results in water, in Fig. 3 we report the experimental dihedrals and the MD distribution obtained by the trans–trans simulation at acidic pH. The two maps show a nice agreement, in particular in highlighting that the Asn3 and Pro4 residues assume polyproline II dihedral.

Besides unordered structures, type I β turn results to be the principal conformation at pH lower than tyrosine pKa (9.77 [12], see Table D of the Supplementary data). The turn conformation in the NPGY and Ac-PhN regions is adopted mainly when tyrosine is not deprotonated and the involved proline is in trans conformation, as in the trans–trans isomer (Figs. 1b, c and 4a, b ) and in lower amounts, in the cis–trans isomer at neutral and acidic pH conditions. The occurrence of such turn conformations decreases as pH increases, thus shifting the equilibrium toward less ordered structures. The conformational change with pH is likely driven by the interaction between the phenolate oxygen of tyrosine (available only at basic pH) and the side chain amide hydrogen of asparagine. This interaction determines a tilt of the glycine residue, breaking the turn structure (Fig. 4a and b) in favour of unordered ones (Fig. 4c). The conformational change therefore can be also tracked following the variations of glycine (φ,ψ) distribution angles minima from −97.1 in the H+Y and in the HY form, to 137,−15 in the HY+ form (see Tables F–H of the Supplementary data), and more evidently from the distribution of Cα+Cα+3 distances in the NPGY region (Fig. 5). This result agrees with the CD spectra blue shift for the minimum around 205 nm at pH 10 and 298 K previously reported [12], and explains the driving force of the phenomenon.

The presence of the NPGY turn at acidic pH, visible also in the NMR minimized structures (both in vacuum and in water, Fig. 1b), is compatible with the ROESY correlation between the NH of Gly5 and the NH of Tyr6. As a final test of the agreement between the MD and the NMR structures at acidic pH we report in Table 7 the average

![Fig. 3. Superposition of the backbone dihedrals of the 20 NMR structures minimized in water and the MD (φ,ψ) distributions of the 1-HexaPY obtained by the trans–trans simulations at acidic pH.](image)

![Fig. 4. 1-HexaPY trans–trans typical conformations in the H+Y [a], HY [b] and HY+ [c] form obtained from MD simulations. It is worth to note the short distance between the phenolate oxygen of tyrosine and the amide side chain hydrogen of asparagine residue (blue line) only in the HY+ form, where tyrosine is deprotonated. In the H+Y and HY forms, instead, a turn structure is stabilized by an intra-chain ASN=C=TYR NH H-bond (yellow line). The orange line shows the distance between the NH of Gly5 and the NH of Tyr6, corresponding to a calculated distance of about 3 Å.](image)

![Fig. 5. Cα+Cα+3 distance distribution concerning the NPGY region of 1-HexaPY trans–trans in the three different protonation states. It can be noticed the short distance in the HY+ and HY forms and the larger distance distribution in the HY− form.](image)
dihedrals emerged for the Asn3 and Pro 4 residues. The further increased to 95–5. From the analysis of the best avian prion hexarepeat Ac-PHNPGY-NH2. NMR tions to the investigation of the conformational behaviour 4. Conclusions Group b shows the lowest RMSDs, confirming again the with respect to the MD configurations sampled each 2 ns. in two groups (disordered and turn as in Fig. 1 a and b) backbone RMSD values of the 20 NMR structures divided in two groups (disordered and turn as in Fig. 1a and b) with respect to the MD configurations sampled each 2 ns. Group b shows the lowest RMSDs, confirming again the presence of the turn conformation in the NPGY sequence.

4. Conclusions

In this study we have applied NMR and MD simulations to the investigation of the conformational behaviour of avian prion hexarepeat Ac-PHNPGY-NH2. NMR experiments indicated that the trans–trans isomer is the predominant species in H2O/D2O at pH 4.2 (83%), followed presumably by the trans–cis one (17%), while in the less polar TFE/H2O environment the relative ratio is further increased to 95–5. From the analysis of the best 20 NMR structures minimized in vacuum and water, an averaged conformation with characteristic polyproline II dihedrals emerged for the Asn2 and Pro6 residues. The presence of such dihedrals inside a small region of the peptide is not related to a more extended sequence, and the local conformation does not propagate along the backbone, which is found instead to have more frequently either an unordered or a turn-like structure in the NPGY region (Fig. 1a and b). MD simulations of the trans–trans isomer in water succeeded in explaining this behaviour on the basis of the hydrogen bond distribution, hard to detect from NMR investigations of peptides. In particular, we found an i–i + 3 turn structure in the NPGY region at acidic and physiological pH with Asn1 and Pro6 angles typical of polyproline II. Moreover, MD calculations were performed for all four isomers in water at acidic, neutral and basic pH conditions, showing in all the cases a fast interconversion among the accessible conformations. An interesting dependence of the peptide shape on the tyrosine residue deprotonation was also detected (Figs. 4 and 5), mainly for the trans–trans isomer. The deprotonation causes a shortening of the distance between the phenolate oxygen of tyrosine and the amide side chain hydrogens of asparagine thus tilting the glycine residue. This appears to be the driving force for the increase of unordered structures at basic pH explaining the relative blue shift in the CD spectrum [12].

The presence of turns inside the hexarepeat fragment has also been suggested by secondary structure prediction algorithms [33], and it is believed to play a crucial role in the endocytosis processes, as a turn conformation is adopted by the NPXY internalization signal of the LDL receptor [34,35]. The present combined NMR and MD study provides a further evidence of turn conformations in hexarepeats and represents a first step in understanding the structural features of the N-terminal region of the avian prion protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cplett.2007.05.046.

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