Influence of pH on NMR Structure and Stability of the Human Prion Protein Globular Domain*

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The NMR structure of the globular domain of the human prion protein (hPrP) with residues 121–230 at pH 7.0 shows the same global fold as the previously published structure determined at pH 4.5. It contains three α-helices, comprising residues 144–156, 174–194, and 200–228, and a short anti-parallel β-sheet, comprising residues 128–131 and 161–164. There are strictly, slickly, localized, conformational changes at neutral pH when compared with acidic solution conditions: helix α1 is elongated at the C-terminal end with residues 153–156 forming a 310-helix, and the population of helical structure in the C-terminal two turns of helix α2 is increased. The protonation of His155 and His192 presumably contributes to these structural changes. Thermal unfolding monitored by far UV CD indicates that hPrP-(121–230) is significantly more stable at neutral pH. Measurements of amide proton protection factors map local differences in protein stability within residues 154–157 at the C-terminal end of helix α1 and residues 161–164 of β-strand 2. These two segments appear to form a separate domain that at acidic pH has a larger tendency to unfold than the overall protein structure. This domain could provide a “starting point” for pH-induced unfolding and thus may be implicated in endosome PrPc to PrPSc conformational transition resulting in transmissible spongiform encephalopathies.

The prion protein (PrP), a predominantly synaptic protein present in all higher organisms (1–4), constitutes a major component of the infectious agent (prion) that causes transmissible spongiform encephalopathies (5, 6). The normal cellular isoform of the protein, PrPc, is soluble and protease-sensitive, whereas the disease-associated β-sheet-rich form, PrPSc, is insoluble, partially resistant to protease digestion (7, 8), and thought to propagate by converting PrPSc molecules into an alternative conformation (9–11). Recently, it has been shown that the accumulation of even small quantities of misfolded PrP in the cytosol is strongly neurotoxic in cultured cells and transgenic mice (12, 13). However, the subcellular localization of the conformational transition of PrPc into PrPSc is controversial (14). There are indications that it takes place either at the cell surface, where the average interstitial milieu of the brain (15, 16) has a pH of 7.3, or after internalization of PrPSc into endosomes (17–19), where pH values range between 4.7 and 5.8 (20).

The in vitro conversion of human brain PrPc to a PrPSc-like form is enhanced at acidic pH (21). Biophysical studies have shown that the free energy of unfolding of hPrP-(90–231) is lower at acid pH than at neutral pH (22) and that in acidic guanidinium chloride hPrP-(90–231) forms a folding intermediate that contains a large amount of β-sheet secondary structure. A β-sheet-rich folding intermediate has also been observed for mouse PrP-(121–231) at low pH in urea but is not seen at neutral pH (23). NMR structures are available for several recombinantly expressed mammalian prion proteins (24–27) but only in acidic solution conditions between pH 4.5 and 5.5. A crystal structure of human PrP-(90–231) has recently been determined from crystals grown in pH 8 solution, where two globular domains are linked through interchain disulfide bonds (28).

In an attempt to investigate the possible effects of pH on the structure of PrPc, we have studied the recombinant human prion protein globular domain of residues 121–230 in pH 7.0 solution. We describe a high quality NMR structure of monomeric hPrP-(121–230), amide hydrogen exchange experiments monitored by NMR, and thermal unfolding experiments monitored by CD. These results are compared with the previously published structural and thermodynamic data of hPrP-(121–230) obtained at pH 4.5 (26) and with the crystal structure of hPrP-(90–231) determined at pH 8 (28).

EXPERIMENTAL PROCEDURES

Sample Preparation—13C- and 15N-labeled recombinant hPrP-(121–230) was expressed and purified as described previously (29). NMR samples were 0.5–1.2 mM in protein concentration in buffer solution containing 10 mM sodium phosphate at pH 7.0 and 0.05% sodium azide. Dynamic light scattering and size exclusion chromatography measurements show that under these conditions samples are homogeneous and monomeric. Samples were prepared either in 95% H2O, 5% D2O or in 99.9% D2O.

Circular Dichroism and Thermal Denaturation Experiments—Circular dichroism spectra were recorded with a Jasco J720 spectropolarimeter interfaced with a Peltier-type temperature control unit with 1-mm path length cuvette. Thermal denaturation experiments were performed by monitoring the circular dichroism at 222 nm while changing the temperature from 10 to 90 °C or vice versa with a constant temperature gradient of 50 °C/h. Reference spectra were collected at 10 °C before and after each experiment. Denaturation curves were analyzed

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The abbreviations used are: PrP, prion protein; hPrP, human prion protein; HSQC, heteronuclear 1H-15N single-quantum coherence; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

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assuming a two-state unfolding model (30), where the temperature dependence of the Gibbs free energy change of denaturation, $\Delta G_{U-N}$, is given by Equation 1 (31),

$$\Delta G_{U-N} = \Delta H_r (1 - T/T_m) - \Delta C_p (T - T + T \ln(T/T_m)) \quad (Eq. 1)$$

where $U$ is unfolded protein, $N$ is native protein, $\Delta H_r$, is the change in enthalpy at the midpoint of the denaturation temperature, $T_m$, $\Delta C_p$ is the difference in heat capacity between denatured and native protein ($\Delta C_p$ of hPrP-(121–230) was estimated by assuming 12 cal mol$^{-1}$ deg$^{-1}$ amino acid (32); $T$ is the absolute temperature in kelvin; $R$ is the gas constant.

**NMR Measurements and Structure Determination**—NMR spectra were recorded on Bruker DRX600 and DRX750 spectrometers. The programs Prosa (33) and Xeasy (34) were used for data processing and spectral analysis, respectively. Sequence-specific resonance assignments were derived by adapting the assignments at pH 4.5 (26) to pH 7.0 and confirmed by standard triple-resonance NMR experiments (35).

Distance constraints for the structure calculation were obtained from three NOESY spectra recorded at a proton frequency of 750 MHz with a mixing time of 40 ms: a three-dimensional $^1$H,$^1$HNOESY, a three-dimensional $^1$H,N- and $^1$H,$^1$HNOESY, and a two-dimensional $^1$H,$^1$HNOESY spectrum. The program DYANA (36) was used to convert NOE intensities into upper distance bounds according to the inverse sixth power-volume-distance relationship (37). Final structure calculations using the torsion angle dynamics protocol of DYANA with 8,000 steps were started from 100 randomized conformers. The 20 conformers with the lowest final DYANA target function value were energy-minimized in a water shell with the program Opalp (38) using the Amber force field (39). Figures of molecules were prepared with the program Molsim (40).

**Amide Proton Protection Factors**—The exchange rate of amide protons in proteins with deuterium is generally analyzed in terms of a two-state equilibrium between the protected, closed form of the protein and the unprotected, open form. The exchange of amide protons with deuterium takes place from the open form with the intrinsic exchange rate constant $k_{int}$.

$$NH(closed) \leftrightarrow NH(open) \rightarrow exchanged$$

**Scheme 1**

where $k_i$ is the first order rate constant for the opening of the folded protein, $k_r$ is the rate constant for its return to the closed state, and $k_{int}$ is the intrinsic rate constant for exchange with solvent for an unprotected amide proton that is known from model-peptide studies (41). Under folding conditions $k_i \gg k_r$; thus the observed exchange rate $k_{obs}$ becomes

$$k_{obs} = (k_i/k_r)k_{int} \quad (Eq. 2)$$

In the limiting case of $k_i \gg k_{int}$ (EX2 exchange regime), Equation 1 can be simplified to

$$k_{obs} = (k_i/k_r)k_{int} \quad (Eq. 3)$$

In the EX2 regime the exchange data can be used to measure the local equilibrium constant between “open” and “closed” backbone hydrogen bonds. This equilibrium constant is given by the ratio $k_i/k_r$, which defines the protection factor ($P$) of each amide proton.

$$P = k_{int}/k_{obs} \quad (Eq. 4)$$

The protection factors for each individual residue can be related to the free energy of the apparent opening reaction that dominates exchange under EX2 conditions according to the equation,

$$\Delta G_{EX} = RT \ln P \quad (Eq. 5)$$

The amide proton exchange rates were calculated following the decrease of two-dimensional $^{15}$N,$^1$HHSQC cross-peak intensities over time after dissolving the lyophilized protein in D2O. The resulting decaying curves were fitted to a single exponential decay equation. The intrinsic exchange rates $k_{int}$ were calculated taking into account the effect of neighboring side chains and corrected for temperature and pH effects (41).
which connects β-strand 2 and helix α2, is poorly defined, and its resonance signals show line broadening because of dynamic processes in the millisecond time scale, which in some cases prevents the detection of NMR signals (42).

Thermal Stability and Amide Proton Protection Factors—The thermal stability of hPrP(121–230) was measured following circular dichroism at 222 nm during heat denaturation and subsequent renaturation. At pH 7.0, hPrP-(121–230) undergoes a highly cooperative and reversible two-state transition with a melting temperature of 71 °C and a free energy of unfolding of $\Delta G_{U-N} = 30 \text{ kJ mol}^{-1}$. This value is similar to $\Delta G_{U-N} = 28 \text{ kJ mol}^{-1}$ of mouse PrP-(121–231) (43) as determined from urea equilibrium denaturation.

Exchange of backbone amide protons against deuterium was measured after dissolving lyophilized hPrP-(121–230) in D$_2$O and recording of two-dimensional [15N,1H]HSQC spectra over a time period of 3 days. The data were analyzed on the assumption of an EX2 exchange regime, where the rate constant of refolding is at least one order of magnitude larger than the intrinsic exchange rate constant ($k_{\text{intr}}$). An estimation of the rate constant for refolding of hPrP-(121–230) can be obtained from data measured on mouse PrP-(121–231) showing that folding is extremely fast and in the range of 4000 s$^{-1}$ (43), whereas the calculated values of $k_{\text{intr}}$ at 20 °C and pH 7.0 are in the range of 1–20 s$^{-1}$.

The calculated backbone amide proton protection factors (P) at pH 7.0 are shown in Fig. 3. All of the amide protons with protection factors P > 100 are localized within the regular secondary structure elements. Moreover, all of the secondary structure elements of hPrP-(121–230) show measurable protection factors, with the sole exception of β-strand 1 and the C-terminal part of helix α2. The lower thermodynamic stability of the C-terminal end of helix α2 is also indicated by the
Hydrogen exchange was measured at 20 °C in 99.9% D₂O containing 10 mM sodium phosphate at pH 7.0. The locations of the regular secondary structure elements are given at the top.

FIG. 3. Gibbs free energy of exchange ($\Delta G$) and protection factors (P) of hPrP-(121–230) at pH 7.0 versus the amino acid sequence. Hydrogen exchange was measured at 20 °C in 99.9% D₂O containing 10 mM sodium phosphate at pH 7.0. The locations of the regular secondary structure elements are shown at the top.

FIG. 4. Superposition of the mean NMR structures of hPrP-(121–230) determined at pH 7.0 (cyan) and pH 4.5 (magenta). The best fit shown here is the superposition of backbone heavy atoms of residues 125–228. The radius of the cylindrical rods is proportional to the mean global backbone displacement per residue of the 20 energy-minimized conformers. The side chains of the His residues from the pH 7.0 structure are shown in yellow.

FIG. 5. Plots of surface distribution of electrostatic potential. Blue indicates positive values and red negative values. Proteins in panels a and c have about the same orientation as in Figs. 1 and 4, whereas those in panels b and d have been rotated 180° around the vertical axis.

FIG. 6. Difference of the free energy of exchange ($\Delta\Delta G_{\text{ex}}$) of individual backbone amide atoms between pH 7.0 and 4.5. The $\Delta\Delta G_{\text{ex}}$ values, where $\Delta G_{\text{ex}} = G_{\text{ex,7.0}} - G_{\text{ex,4.5}}$, are displayed on a ribbon diagram of the three-dimensional structure of hPrP-(121–230) determined at pH 7.0. Individual amino acids are colored according to $\Delta G_{\text{ex}}$.

Discussion

Comparison of the Structure of hPrP-(121–230) at Neutral pH Versus Acidic pH—The three-dimensional structures of hPrP-(121–230) at pH 7.0 and 4.5 show both global similarities and local differences. The global structure at neutral pH is similar to that at acidic pH (26), with a root-mean-square deviation value of 1.3 Å between the backbone heavy atoms of residues 125–228 in the mean structures determined at the two pH values (Fig. 4). Significant differences between the two structures are localized at the C-terminal ends of helices a1 and a2.

At pH 7.0, residues 153–156 at the end of helix a1 adopt a $\beta_1$-helix conformation, whereas at acidic pH the same residues show a less regular conformation (26). Interestingly, an elongated helix a1 has also been described for the structure of hPrP-(90–231), which was determined from crystals grown in pH 8 solution (28). Helix a1 is extremely hydrophilic and has a low capacity to form hydrophobic contacts (45), indicating that its regular secondary structure must be stabilized by electrostatic interactions. The protonation of His$^{155}$ at acidic pH, therefore, might contribute to the destabilization of helix a1 by introducing an unfavorable second positive charge close to Arg$^{156}$.

Within the framework of the preserved global structure, the backbone atoms of residues 188–194 at the C-terminal end of helix a2 show a better precision for the bundle of conformers calculated at pH 7.0 than at pH 4.5 (Fig. 4). The helical content within this region is also more populated at pH 7.0, as evidenced by the comparison of $^{13}$Cα chemical shifts shown in Fig. 2. The same helical segment in hPrP-(121–230) at pH 4.5 is in equilibrium with unfolded conformations (26). In this regard it is interesting to note that in the crystal structure of dimeric hPrP-(90–231) residues 189–198 of helix a2 serve as a hinge region for the rearrangement of helix a3 into the neighboring molecule in the crystal (28). The reduced stability of this region at low pH could be due to the protonation of His$^{157}$, as positively charged histidine side chains in the middle of α-helices have been shown to have a destabilizing effect because of unfavorable interaction with the helix macrodipole (46).
The surface of the NMR structure of mouse PrP-(121–231) determined at pH 4.5 is reportedly characterized by a markedly uneven distribution of positively and negatively charged residues (24), and the same uneven distribution is seen also in human PrP-(121–230) (26). It has been proposed that this dipolar arrangement of charges might stabilize the orientation of PrPSc with its positively charged surface and also the nearby hydrophobic patches toward the cell membrane (24). The two glutamine sites at Asn$^{181}$ and Asn$^{187}$ would then be located on the opposite, negatively charged site. The electrostatic surface potentials of the human PrP globular domain at pH 4.5 and 7.0 are compared in Fig. 5. At pH 4.5, there is an uneven distribution of positive and negative charges (Fig. 5, c and d), whereas at pH 7.0 the polarity of the electrostatic surface potential is less obvious (Fig. 5, a and b). Although the negatively charged surface is similar at the two pH values (Fig. 5, b and d), the net charge at the opposite surface is less positive at the neutral pH value (Fig. 5, a and c). A major contribution to the positive net charge at acidic pH (Fig. 5c) comes from the protonated side chains of histidine residues 155 and 170 (49). These residues 90–165 convert into a β-strand 2 at low pH (47), where the protein core, consisting of helix α2 and αhelix α3, was maintained, whereas a conformational transition and instability was particularly prominent at the N-terminal part of the protein and within helix α1 and the β-sheet.

Implications of pH Dependence for PrP$^C$ to PrP$^SC$ Conversion—From the combination of structural and thermodynamic data, the decreased local stability of helix α1 and β-strand 2 at low pH thus might provide a “starting point” for the processes that ultimately leads to the conformational switch from α-helix secondary structure in PrP$^C$ into β-sheet secondary structure in PrP$^{SC}$ during prion propagation. Consistent with this structural model for PrP conversion, a recent structural model of PrP$^{SC}$ based on electron crystallography (48) suggests that helix α2 and helix α3 adopt a very similar conformation in both PrP$^C$ and PrP$^{SC}$, whereas the peptide segment comprising residues 90–165 converts into a β-sheet-rich conformation. The impact of His$^{150}$ on the structure and stability of helix α1 is intriguing, as cell-free conversion experiments with chimeric mouse/hamster PrP have shown that the PrP$^{SC}$ epitope of hamster PrP$^C$ includes Met$^{139}$, Asn$^{155}$, and Asn$^{170}$ (49). These results are consistent with the structural conversion of PrP$^C$ into PrP$^{SC}$ taking place at acidic pH, i.e. along the endosome pathway (17–19).

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