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The polybasic N-terminal region of the prion protein controls the physical properties of both the cellular and fibrillar forms of PrP

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SUMMARY

Individual variations in structure and morphology of amyloid fibrils produced from a single polypeptide are likely to underlie the molecular origin of prion strains and control the efficiency of the species barrier in transmission of prions. Previously, we observed that the shape of amyloid fibrils produced from full-length prion protein (PrP 23–231) varied substantially for different batches of purified recombinant PrP. Variations in fibril morphology were also observed for different fractions that corresponded to the highly pure PrP peak collected at the last step of purification. A series of biochemical experiments revealed that the variation in fibril morphology was attributable to the presence of miniscule amounts of N-terminally truncated PrPs, where a PrP encompassing residue 31–231 was the most abundant of the truncated polypeptides. Subsequent experiments showed that the presence of small amounts of recombinant PrP 31–231 (0.1–1%) in mixtures with full-length PrP 23–231 had a dramatic impact on fibril morphology and conformation. Furthermore, the deletion of the short polybasic N-terminal region 23–30 was found to reduce the folding efficiency to the native α -helical forms and the conformational stability of α -PrP. These findings are very surprising considering that residues 23–30 are very distant from the C-terminal globular folded domain in α -PrP and from the prion folding domain in the fibrillar form. However, our studies suggest that the N-terminal polybasic region 23–30 is essential for effective folding of PrP to its native cellular conformation. This work also suggests that this region could regulate diversity of prion strains or subtypes despite its remote location from the prion folding domain.

Keywords

amyloid fibrils; fibril morphology; fibril polymorphism; prion protein; electron microscopy

Introduction

Misfolding and aggregation of the prion protein underlie the pathogenic mechanisms in several fatal neurodegenerative diseases that can be infections, inherited, or sporadic ¹. The cellular isoform of the prion protein (PrP^C) is expressed predominantly on surface of neuronal and glial cells of the brain and spinal cord ^{2, 3} and consists of two domains. The N-terminal domain (residues 23–126) is highly flexible and, in the absence of Cu²⁺, lacks any identifiable

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secondary structure^{4, 5}, whereas the C-terminal domain (127–231) adopts a globular α -helical fold^{4, 6, 7}. Upon conversion into the pathological, disease-related form (PrP^{Sc}), the region ~90–231 that includes the α -helical domain acquires a protease-resistant, predominantly β -sheet rich conformation^{8, 9}. While the N-terminal region 23–89 was shown to be not essential for prion replication^{10,11, 12}, numerous studies have showed that the N-terminal domain is involved in fibrillation and is likely to determine the physical properties of disease-related forms of PrP^{13,14,15–17}. In addition, the octarepeat region (residues 57–91) within the N-terminal domain has also been thoroughly investigated because of its ability to bind Cu²⁺¹⁸.

The unstructured N-terminal region 23–56 that precedes the octarepeat domain has been largely ignored as this region appeared to be not important for PrP physiological or pathological functions. In the recent years, however, a growing number of studies have drawn attention to this region and, specifically, to the polybasic N-terminal motif $_{23}\text{KKRP}_{26}$. The N-terminal motif $_{23}\text{KKRP}_{26}$ was shown to be involved in the interaction of PrP^C with a transmembrane adaptor protein that engages the clathrin endocytic machinery¹⁹. PrP^C mutants that lack $_{23}\text{KKRP}_{26}$ failed to undergo endocytosis and accumulated on the cell surface. In addition to regulating the PrP^C life-time on the plasma membrane, the $_{23}\text{KKRP}_{26}$ motif was shown to be involved in mediating the β -secretase cleavage of amyloid precursor protein and production of neurotoxic A β ^{20, 21}. Only full-length PrP^C, but not PrP^C lacking $_{23}\text{KKRP}_{26}$ was found to inhibit the cleavage of amyloid precursor protein on the cell surface²⁰.

In the current study, we showed that the N-terminal polybasic region encompassing residues 23–30 controls the physical properties of both the monomeric α -PrP and the disease-related, fibrillar form. In the absence of residues 23–30, α -PrP was found to display reduced conformational stability, a high tendency for aggregation, and impaired folding behavior. Even more surprising, the presence of small amounts of N-terminally truncated PrP in the mixtures with full-length PrP had a dramatic impact on fibril morphology and conformation. Our studies suggest that the N-terminal polybasic region is important for effective folding of PrP to its native cellular form. This work also suggests that a region considerably distant to the prion folding domain could be a potential source of diversity of prion strains or subtypes.

Results

In the last several years, we observed that the morphology of fibrils formed *in vitro* from full-length hamster PrP varied substantially for different batches of purified recombinant protein. The variations in morphology occurred despite a very robust protocol for purification employed in our studies^{22,23}. The purification according to this protocol yields highly pure, properly folded α -PrP that lacks misfolded species or chemical adducts. In addition to purification on a Ni²⁺-column, a one-step protocol used by other laboratories^{24,25,26}, our protocol includes reverse-phase HPLC chromatography. While sufficient purity of PrP could be achieved using one-step purification (up to 95–99%), we found that PrP pools eluted from Ni²⁺-columns contained a variety of PrP species including misfolded and aggregated forms, PrP that lacks a disulfide bond, PrP with oxidized methionines, and chemical adducts of PrP. Reverse-phase HPLC chromatography separates α -PrP from misfolded species or PrP adducts (Fig 1a).

In preliminary studies, we noticed that variations in fibril morphology were largely attributed to the way the major peak corresponding to the monomeric α -PrP was collected and fractionated during elution from the C4 HPLC column (Fig. 1a). Unless specified, all fibrillation reactions in the current study were performed using the shaking protocol as described in Materials and Methods. The PrP pool collected as a complete peak including the peak's tail (this pool is referred to as *ii* in Figure 1 a) formed straight, rigid, twisted fibrils (Fig. 1b). If the peak's tail was omitted (this pool is referred to as *i*), the fibrils showed untwisted, curvy morphology (Fig. 1c). The differences in morphology of fibrils produced from pool *i* and *ii* were puzzling

considering the lack of any visible amounts of impurities in either pool, as judged by SDS-PAGE (Fig. 1a). Both PrP pools were stored and treated in the same way, and identical solvent conditions and shaking mode were used for fibrillation reactions from both pools.

In an alternative experimental set up, fibrils were formed from individual fractions eluted within the major peak (Fig. 2a). Fractions that corresponded to the beginning and the center of the peak produced curvy fibrils, whereas the fractions that corresponded to the peak's tail formed rigid fibrils (Fig. 2b). No impurities could be seen in any of the fractions as judged by SDS-PAGE stained with Commassie Blue (Fig. 2a). The fractions corresponding to the peak's tail (combined fractions 6+7), however, displayed a slightly thicker and more diffused band on a gel than other fractions, a possible indication of a minor amounts of truncated PrP polypeptide. Overstaining of SDS-PAGE with silver revealed very weak bands in the tail fractions visible as a smear. These minor bands ran just slightly faster than PrP 23–231 and, presumably, corresponded to the truncated PrP fragments. Over the last two years we conducted numerous experiments on fibril formation starting from different pools or fractions from different purifications and obtained consistent results. The fractions which smear on a gel always gave rise to rigid fibrils, whereas the fractions that did not smear produced curvy fibrils.

Once a correlation between the presence of weak bands and fibril morphology was established, we wanted to identify the minor fragments eluted in the peak's tail. The minor fragments were estimated to contribute less than 1% of the total amount of α -PrP eluted with the major peak. Because the fragments were initially found in the tail fractions, we assumed that their elution time on the C4 column was slightly longer than that of full-length PrP. To enrich the PrP pool with the minor fragments, the tail fractions from five purifications were combined together, loaded onto the C4 column and eluted using the same gradient as used for PrP purification. The HPLC profile of the tail fractions showed two overlapping peaks (Fig. 3a). As judged from SDS-PAGE, PrP 23–231 was present in all fractions (1 to 9), whereas shorter fragments were seen only in fractions corresponding to the second peak (Fig. 3b, fractions 4–9). Two bands corresponding to the shorter fragments were observed in the fractions of the second peak: the upper band migrated slightly lower than the PrP 23–231 band, while the lower band was clearly resolved. ESI-MS analysis of the fractions eluted with the subpeak identified several N-terminally truncated PrP fragments (Fig. 3c). PrP 208–231 and PrP 31–231, that presumably composed the upper band, and PrP 47–231 and PrP 51–231, that seemed to compose the lower band, were the most abundant fragments. All fragments lacked the N-terminal pentapeptide ($_{23}$ KKRPK $_{27}$) that has four positively charged amino acid residues. Lack of this polybasic N-terminal region in the truncated fragments was consistent with their slightly longer elution time on the reverse-phase column.

To confirm the cleavage sites identified by ESI-MS, we employed N-terminal Edman's sequencing. For separating the truncated PrP polypeptides from the full-length PrP, we used cation-exchange chromatography on SP-sepharose. The fractions with truncated PrPs (fractions # 4 to 9) were loaded onto the SP-sepharose column, and the polypeptides were eluted using a gradient of NaCl. The chromatography profiles showed two overlapping peaks at the beginning of the gradient and a third peak at the end of the gradient (Fig. 4a). As judged from SDS-PAGE, all truncated polypeptides eluted in the two overlapping peaks at low salt concentrations, whereas the full-length PrP eluted in a third peak at high salt concentrations (Fig. 4b). Such distant separation of the truncated polypeptides from the full-length PrP on ion-exchange chromatography was consistent with the loss of the polybasic N-terminal region in all PrP fragments. To confirm the site of fragmentation in truncated polypeptides, the lower band from fraction #2 and the upper band from fraction #5 (Fig. 4b) were subjected to N-terminal Edman's sequencing. Consistent with the previous results, the N-terminal sequencing revealed that the two most abundant PrP fragments start at the residues 31 and 47.

To test whether the N-terminal residues control fibril morphology, we produced a recombinant PrP variant that lacks the residues 23–30 (referred to as PrP 31–231). The region 23–30 is sufficiently remote from the octarepeat domain and, therefore, its deletion should not affect the Cu²⁺-binding properties of PrP.

To produce recombinant PrP 31–231, the deletion 23–30 was introduced into Syrian Hamster PrP coding DNA sequence in plasmid pET101/D-TOPO. PrP 31–231 was expressed in *E. coli* and purified using the same procedure that was used for expression and purification of full-length PrP^{22,23}. While the yield of PrP 31–231 expression was found to be similar to that of PrP 23–231, only 0.5–1.0 mg of PrP 31–231 was purified from 1 L of culture comparing to 6–10 mg/L of PrP 23–231. We also noticed that a large fraction of PrP 31–231 aggregated during the oxidative refolding that was carried out under solvent conditions, where PrP 23–231 remained soluble. To remove aggregated protein, PrP 31–231 was centrifuged at 12,000 g for 30 min and the pellet was discarded. Despite removal of aggregated PrP 31–231, we noticed an unusually high increase of pressure upon loading PrP 31–231 onto the C4 HPLC column, yet another sign of continuing aggregation. The HPLC peak corresponding to the monomeric α -helical form of PrP 31–231 was collected; the molecular weight and purity was confirmed by ESI-MS and SDS-PAGE (Suppl. Fig. 1a,b, Fig. 5a). Low purification yield and high tendency for aggregation during purification were indicative of the impaired folding behavior of PrP 31–231.

To examine whether the deletion of residues 23–30 affected the secondary structure or conformational stability of α -PrP, we recorded CD spectra and performed thermal denaturation for both PrP 23–231 and PrP 31–231 (Fig. 5 and Suppl. Fig. 1). CD spectra revealed that both proteins have identical, predominantly α -helical secondary structure (Fig. 5b and Suppl. Fig. 1c). As judged from the temperature denaturation experiment, both PrP 23–231 and PrP 31–231 displayed cooperative unfolding and showed very similar conformational stability when the temperature scans were carried out in low ionic strength buffer (Fig. 5c). In the presence of 0.3 M NaCl, however, PrP 23–231 and PrP 31–231 showed substantially different denaturation profiles. Consistent with the previous studies²⁷, 0.3 M NaCl reduced the stability of PrP 23–231. Remarkably, in the presence of salt the stability of PrP 31–231 reduced to a much greater extent than that of PrP 23–231. This experiment revealed that the global stability of the α -helical folded domain encompassed by the residues 127–231 is affected by the polybasic N-terminal region that belongs to the unstructured part of PrP. This experiment also provided some clues regarding the propensity of PrP 31–231 to aggregate.

Using purified α -PrP 31–231 we set up a series of fibrillation experiments in which increasing amounts of PrP 31–231 (0.1%, 1%, and 10% of total protein) were added to the conversion assays containing PrP 23–231, while keeping the total amount of PrP constant. As a source of PrP 23–231, we used pool *i* (Fig. 1) that gave rise to curvy fibrils. As expected, in the absence of PrP 31–231, only curved fibrils were observed (Fig. 6a). In the presence of 0.1% of PrP 31–231, we found a mixture of curvy and rigid fibrils, where the percentage of each fibrillar type varied from experiment to experiment (Fig. 6b). In the presence of 1% PrP 31–231, predominantly rigid fibrils were observed with minor amounts of curvy fibrils (Fig. 6c). A few fibrils seem to show mixed features where different fibrillar segments displayed either rigid or curvy shape. Unexpectedly, in the presence of 10% PrP 31–231, only one experiment showed rigid fibrils, whereas curvy fibrils were found in four experiments (data not shown). 100% PrP 31–231 formed ThT-positive aggregates that lacked typical fibrillar shape (Fig. 6d). Detail examination of EM images revealed that PrP 31–231 aggregates consisted of tiny protofibrils. Taken together, these data illustrate that PrP 31–231 alters fibril morphology only when present as a small percentage in mixtures with PrP 23–231. At 10% level, the effect of PrP 31–231 on morphology was not as robust as at lower concentrations. Such discrepancy could be due to

the tendency of PrP 31–231 to self-aggregate at elevated concentrations, a process that presumably depletes monomeric PrP 31–231 and competes with nucleation.

To test whether morphologically different fibrils were different with respect to their secondary structure, we recorded FTIR spectra for fibrils produced from PrP 23–231 (curvy fibrils) and from 99:1 mixture of PrP 23–231 and PrP 31–231 (rigid fibrils) (Fig. 7). The FTIR spectra revealed notable differences in the position of the peaks that correspond to the cross β -sheet structure. As we reported previously, the fibrils made from PrP 23–231 displayed a strong peak at 1626 cm^{-1} with a minor shoulder at 1613 cm^{-1} ²⁸, whereas the fibrils produced in 99:1 mixture showed a single peak at 1618 cm^{-1} (Fig. 7). As judged by PAGE under non-denaturing conditions, complete depletion of the monomeric PrPs was observed at the end of fibrillation reactions in all conversion assays regardless of the ratios of PrP 31–231 to PrP 23–231 (Suppl. Fig 2). Therefore, the differences seen by FTIR spectroscopy cannot be assigned to the presence of unconverted monomeric PrPs in the samples. We cannot completely exclude, however, the possible contribution of partially aggregated intermediate forms in the preparation of fibrils from the mixtures of PrP 23–231 and PrP 31–231.

Because the rigid fibrils from mixture of PrP 23–231 and PrP 31–231 were morphologically similar to the R-fibrils described in our previous studies²⁸, we were interested in testing whether these two fibrillar types have a similar conformation. Previously, we found that the R-fibrils could be produced from pool *i* or pool *ii* of PrP 23–231 under rotation, an alternative agitation mode alternative to shaking²⁸. Despite similarities in morphology, the R-fibrils and the rigid fibrils showed substantial differences in secondary structure (Fig. 7). Instead of strong single peak at 1618 cm^{-1} typical for rigid fibrils, the R-fibrils showed double peak at 1628 cm^{-1} and 1613 cm^{-1} and substantial peak at 1662 cm^{-1} suggesting that these two forms are different.

As an alternative method for probing fibrillar conformation, we employed a double immunostaining fluorescence microscopy assay^{28,29}. The assay consisted of immunostaining with a pair of PrP-specific antibodies (Ab), R1 (specific to the C-terminal epitope 225–231) and 3F4 (recognizes the epitope 109–112). 3F4 was used as a reference Ab, because the epitope 109–112 was found to be solvent-exposed in all types of fibrils produced *in vitro* from recombinant PrP^{29,30}. The secondary Ab to Ab 3F4 was labeled with ALEXA-546 (red) and the secondary Ab to R1 with ALEXA-488 (green); therefore, the color of fibrils in double staining reflected the extent to which the epitope 225–231 was solvent-accessible or buried (Fig. 8). Consistent with our previous observations²⁸, the R1 epitope was found to be well exposed in curvy fibrils formed by pool *i* of PrP 23–231 (Fig. 8b). As judged by fluorescence imaging, the same epitope become partially buried in the rigid fibrils formed from the 99:1 mixture of PrP 23–231 and PrP 31–231 (Fig. 8a). Analysis of the 2D-intensity scattering plots indicated a higher level of heterogeneity in rigid versus curvy fibrils with respect to the solvent-accessibility of the epitope 225–231 (Fig. 8 *insets*). Consistent with the previous observations, the R1 epitope was found to be completely buried in the R-fibrils (Fig. 8c).

DISCUSSION

The N-terminal polybasic region influences physical properties of α -PrP

In the present studies, we show that the small N-terminal region encompassing residues 23–30 controlled the physical properties of both the monomeric α -PrP and fibrillar forms. These findings are very surprising considering that the residues 23–30 are very distant from the globular folded domain in α -PrP (residues 128–231) and from the prion folding domain in amyloid fibrils (residues ~90–231). While the deletion of residues 23–30 did not seem to perturb the secondary structure of α -PrP, it dramatically reduced the conformational stability of the protein measured in 0.3 M NaCl. The ionic strength of 0.3 M NaCl solution is close to

that of the cellular environment. The very strong destabilizing effect of salt explains in part the high tendency of PrP 31–231 to aggregate and its impaired folding properties. No variations have been reported within the region ²³KKRPPK₂₈ in mammalian species ³¹ suggesting the conservation of these residues in evolution and implicating their structural importance in preventing PrP aggregation.

The mechanism of how the distant N-terminal region controls the properties of the globular α -helical domain is currently unclear. In the previous studies, the destabilizing effect of salt on full-length α -PrP was attributed to the abnormally high number of glycine residues located in the unstructured N-terminal region 23–127 and, specifically, to the favorable interactions of glycine with anions ²⁷. This mechanism, however, does not explain the more profound effect of salt on stability of PrP 31–231 than that of PrP 23–231 observed in the current work. Furthermore, because the N-terminal region 23–127 is solvent exposed in both the native and denatured states, the interactions of glycine residues with solvent anions should affect the free energies of both states to a similar extent bringing the net difference in ΔG s measured in the presence and absence of salt close to zero. It remains to be established whether the effects of the polybasic region ²³KKRPPK₂₈ on the stability of the globular domain is mediated through long-range electrostatic interactions with the rest of the charged groups in PrP and/or regulated by the conformation of two peptidyl-prolyl bonds in this motif.

Biological and pathological roles of the polybasic region

The N-terminal polybasic region has been shown to be involved in several biological functions including interaction of PrP^C with diverse cellular components. This region was found to be essential for dynein-mediated retrograde axonal transport of PrP^C ³². The same region was also shown to mediate PrP^C interaction with a transmembrane adaptor protein that controls PrP^C internalization and endocytosis ^{33,19}. The motif 23–28 is a part of the larger N-terminal region (residues 23–52) that was found to bind cellular glycosaminoglycans and proteoglycans ^{34,35}. Previous studies revealed that the deletion of the N-terminal residues significantly prolonged the presence of PrP^C on the plasma membrane ³⁶ supporting the key role of the polybasic region in endocytosis and in regulating protein turnover ^{19, 33}. A variety of N-terminally truncated forms of PrP^C were identified in normal mammalian brains ^{15, 37–40}. Substantial variations with respect to length and amounts of N-terminally truncated fragments were observed not only between different species ³⁹, but also between different parts of human brains ³⁸. The N-terminally truncated PrP molecules can be formed as a result of proteolytic cleavage or PrP self-cleavage ^{40–42}. On a cell surface, PrP^C is subject to endoproteolysis by zinc metalloproteases including ADAM10 and ADAM17 ^{40, 43, 44}. The self-cleavage was shown to predominantly target the octarepeat region and depend on reactive oxygen species and Cu²⁺ ⁴².

If the deletion of the polybasic N-terminal residues indeed reduces conformational stability and facilitates PrP misfolding and aggregation, one can speculate that N-terminally truncated PrP^C molecules might initiate the conversion into disease-related oligomeric forms in sporadic prion diseases. Consistent with this hypothesis, uninfected human brains were found to contain aggregated, proteinase K resistant PrP species that are predominantly composed of N-terminally truncated PrPs ⁴⁵. Whether these species represent an intermediate step toward PrP^{Sc} remains to be determined. Just like normal brains, the brains of patients with sporadic Creutzfeldt-Jakob disease were found to contain a variety of N-terminally truncated PrP^{Sc} forms ^{15, 40, 46, 47}. It is unclear, however, whether endogenous N-terminal cleavage precedes conversion to PrP^{Sc} or occurs after conversion.

The N-terminal polybasic region influences fibrillar morphology and structure

The current studies revealed profound effects of the polybasic N-terminal residues on fibril morphology and, possibly, substructure. These findings are unexpected, considering the distant position of the region 23–31 from the prion folding domain. In our previous studies, highly pure full-length PrP (pool *i*) was found to form two amyloid strains (referred to as S- and R-fibrils) under two different agitation modes²⁸. Slow rotation favored straight and rigid fibrils (R-strain), whereas fast shaking produced curvy fibrils (S-strain) (Fig. 9). The current work revealed that the presence of minor amounts of N-terminally truncated PrP was sufficient to abolish the pathway toward curvy S-fibrils. Instead, under shaking, pool *ii* formed rigid, R-like fibrils that appeared to be morphologically similar to the R-fibrils described before²⁸. FTIR spectroscopy and the immunoconformational assay, however, revealed substantial differences in sub-structures of the previously described R-fibrils and the rigid fibrils characterized in the current studies. The epitope 225–231 was found to be exposed to much greater extent in the rigid fibrils than in the R-fibrils (Fig. 8).

Do differences in fibril morphology reflect distinct molecular structures?

There is considerable debate as to whether diverse fibril morphology reflects differences in the structures of fibrillar cross β -sheet cores or arise due to multiple alternative modes of lateral association of structurally identical protofilaments into mature fibrils^{48,49,50}. Previous studies on several amyloidogenic polypeptides including A β and α -synuclein showed that distinct fibrillar morphologies reflect altered molecular structures of cross β -cores as probed by solid-state NMR^{51, 52}. In our recent studies, the R- and S-fibrils produced from PrP not only displayed different morphologies as judged by EM and AFM, but also exhibited substantially different cross β -core structures as probed by FTIR²⁸ and hydrogen-deuterium exchange UV Raman spectroscopy (I. Lednev, I.V. Baskakov, unpublished observation). Noteworthy, while the S-fibrils were morphologically homogeneous, the R-fibrils always displayed a high level of polymorphism^{28,49,50}. Despite such polymorphism, the cross β -cores was found to be uniform within R-fibrils, as judged by Raman spectroscopy. Therefore, within R-type fibrils, polymorphism can be attributed to the different modes of lateral association of structurally uniform protofilaments into mature fibrils⁵⁰. Scrapie fibrils derived from animals infected with prions were also found to display high levels of polymorphism within individual strains^{53,54}. Surprisingly, comparison of several scrapie strains revealed that fibrils belonging to different strains display partially overlapping morphologies⁵³. Similarly, substantial overlaps in morphological shapes were described for fibrils of the yeast prion protein Sup35 produced *in vitro* in reactions seeded with three different strains⁵⁵. Therefore, the simplified equation that each strain gives rise to a unique cross β -core structure and displays unique fibril morphology might not be accurate in describing strain-specific molecular differences.

How do the miniscule amounts of the truncated PrPs change fibril morphology?

While incorporation of PrP 31–231 into fibrils formed by the full-length PrP 23–231 can not be excluded, it is unlikely that this mechanism alone accounts for the dramatic impact on fibril morphology. The observation of two types of fibrils that were occasionally observed in mixtures of PrP 23–231 and PrP 31–231 suggests that they were formed by competing nucleation mechanisms. Competing fibrillation pathways were described previously for other amyloidogenic proteins including β 2-microglobulin⁵⁶. We suggest that PrP 31–231 is involved in forming nuclei, which under shaking conditions give rise to fibrils that are morphologically different from those formed from PrP 23–231 nuclei. Because of its reduced conformational stability and tendency for misfolding, PrP 31–231 may be selectively engaged in nucleation that occurs prior to the nucleation of PrP 23–231. This mechanism suggests that PrP 31–231 initiates or triggers the fibrillation reaction and explains why distinct types of fibril

morphology were formed even in the presence of a large excess of PrP 23–231. Yet PrP 31–231 was not effective in altering fibril morphology, when used at the relatively high proportions (10% and above). Off-pathway aggregation of PrP 31–231 that competes with the nucleation reaction could account for this effect. The mechanistic details for the two competing nucleation pathways remain to be elucidated in future studies. Nevertheless, the current studies illustrate that PrP fibrillation reactions are highly sensitive to the presence of very minor amounts of PrP truncated fragments.

The N-terminal polybasic region is involved in prion replication

Our study suggests that the polybasic N-terminal region could be one potential source of diversity for prion strains or PrP^{Sc} subtypes in sporadic forms of prion disease. While the precise effect of N-terminal cleavage on generating distinct prion strains has yet to be explored, this hypothesis is supported by the fact that the relative level of the N-terminally truncated PrP species varies in different prion strains⁵⁷. Several previous studies documented the involvement of the polybasic N-terminal region in prion replication. Using a panel of motif-grafted antibodies, Solforosi and coauthors showed that the PrP^C region 23–31 is one of three high affinity PrP^{Sc}-binding motifs⁵⁸. The reactivity of this segment was largely dependent upon the presence of multiple positively charged amino acid residues. Another study revealed that fusion of the N-terminal region 23KKRPKP₂₈ to PrP(90–231, Q218K) substantially increased the dominant-negative effect of the PrP Q218K variant in inhibiting PrP^{Sc} replication⁵⁹. Both results are consistent with the hypothesis that 23KKRPKP₂₈ forms a PrP^C-PrP^{Sc} interactive interface.

The current studies suggest that the N-terminal motif 23KKRPKP₂₈ controls the physical properties of α -PrP and is important for efficient folding of PrP to its native α -helical conformation. The same region was also found to influence conformation and morphology of PrP fibrils highlighting the potential involvement of this motif in prion replication and strain diversity.

Materials and Methods

Expression and purification of PrP 23–231 and PrP 31–231

Syrian Hamster PrP 23–231 cDNA was amplified with the following primers: 5' CACCATGAAAAAGCGGCCAAAGCCTGG 3' (forward), and 5' TTAGGATCTTCTCCCGTCGTAATAG 3' (reverse). Cloning of PCR product into pET 101/D-TOPO (Invitrogen, USA) was performed according to Invitrogen protocol, and the plasmid was verified by sequencing.

Syrian Hamster full-length recombinant PrP encompassing residues 23–231 was expressed and purified as described earlier^{22, 23} with the following modifications. After oxidative refolding, the protein solution was filtered using disposable filter units with polyethersulfone membrane (0.22 μ m, Nalge, Nunc International). For the last step of purification conducted on a 22 mm $\times$ 25 cm C4 HPLC column at flow rate 5 mL/min, the percentage of HPLC buffer B (0.1% trifluoroacetic acid in acetonitrile) was increased from 0 % to 25 % during the first 15 min. Then, a gentle gradient was applied (from 25 % to 35% of buffer B in the next 65 min) to ensure efficient separation of PrP adducts. From there, the percentage of buffer B was increased from 35 % to 100 % in 15 min; the column was washed with 100 % of buffer B for 15 min, and the gradient dropped down to 0 % within 10 min. The correctly folded, α -helical form of PrP was eluted as a major peak at 53 min (Fig. 1). The shoulder to the major peak containing PrP with oxidized methionines eluting at < 52.5 min was discarded. Fractions eluted at 52.5 – 58 min were lyophilized and stored at –20 °C for not longer than 2 weeks prior to use. As described below, the purity of the final full-length PrP was estimated to be ~99 % with

the minor fragments contributing less than 1% of the total PrP. From optical absorbance at 280, the amount of total PrP eluted in the tail fractions (fraction 6&7, Fig 2) was approximately 10% of the total PrP eluted with the whole peak. As judged from SDS-PAGE gels (Fig. 2a, Suppl. Fig. 1), the truncated fragments contributed less than 10% of the PrP eluted with the tail fractions (fraction 6&7). SDS-PAGE carried out with the mixtures of full-length and truncated PrPs was used to determine the approximate percentage of truncated PrP in the tail fractions.

Plasmid DNA encoding PrP 31–231 was obtained by introducing a deletion into the pET101/D-TOPO plasmid containing hamster PrP 23–231 DNA. Codons for residues 23–30 were excised using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) with primers 5'-GGAATTCAGGAGCCCTTCACCATGTGGAACACTGGCGGAAGCCG -3' (forward) and 5'-CGGCTTCCGCCAGTGTTCACATGGTGAAGGGCTCCTGAATTCC -3' (reverse). Amplified plasmid was transformed into supercompetent XL1 Blue cells (Stratagene). The DNAs from positive clones were checked by DNA sequencing and retransformed into BL21 (DE3) Star cells (Invitrogen, USA). Further expression and purification procedures were the same as for PrP 23–231.

Because the reverse primers for PrP 23–231 and PrP 31–231 have STOP codons, the resulting proteins do not contain 6xHis tag. PrP 23–231 and PrP 31–231 have additional methionine residue prior to the residues 23 and 31, respectively.

Formation of Amyloid Fibrils

To form amyloid fibrils, stock solutions of PrP were prepared immediately before use by resuspending lyophilized PrP powder in 5 mM MES, pH 6.0. The stock solution of PrP was diluted with MES (pH 6.0) and GdnHCl to final concentrations of 50 mM and 2 M, respectively, and to a final protein concentration of 0.5 mg/ml. The protein concentration was determined by measuring the absorbance at 280 nm. The fibrillation reactions were carried out in 1.5 mL conical plastic tubes (Fisher) at a total reaction volume of 0.4 mL at 37 °C with continuous shaking at 600 rpm using a Delfia plate shaker (Wallac). The fibril formation was monitored using a ThT binding assay¹⁷. All fibrillation reactions were completed in less than 20 hours of incubation at 37 °C.

Ion-exchange chromatography

Ion-exchange chromatography of PrP was carried out on ÄKTAprime system (Amersham Biosciences, Piscataway, NJ) using 1 mL HiTrap SP HP column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Lyophilized HPLC fractions were dissolved in 25 mM HEPES buffer, pH 7.0 and filtered through a low protein binding 0.22-µm PVDF membrane (Millex-GV, Millipore). After applying the sample on the column and washing the column with 5 volumes of sodium acetate buffer, all proteins were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. Elution fractions were analyzed with SDS-PAGE.

In order to sequence N-termini of the eluted proteins after the SDS-PAGE gel was blotted onto PVDF membrane, protein bands were stained with Coomassie Brilliant Blue R-250, cut out, and analyzed by ABI 494 Protein Sequencing System (Applied Biosciences, Foster City, CA) at Tufts University Core Facility (Boston, MA).

Mass Spectrometry

Lyophilized samples were first dissolved in water, then diluted to 0.1 µg/µL in 1:1 water: methanol containing 1% acetic acid. The solution was directly injected at 10 µL/min into a Waters ZMD single quadrupole mass spectrometer operated in positive ion mode. The instrument acquired full scan mass spectra (m/z 500 – 2000) every second for 5 min.

CD spectra and thermal denaturation scans

CD studies were performed using a J-810 CD spectrometer (Jasco, Easton, MD) equipped with a temperature controlled water-circulated quartz cell (1 mm path length). CD spectra of PrP 23–231 and PrP 31–231 (0.125 mg/mL) were collected in 1 mM Na-acetate, pH 5.0. Each spectrum represents the average of 5 individual scans. Thermal denaturation was performed using PrPs (0.25 mg/mL) prepared freshly in 25 mM MES buffer, pH 6.0, in the presence or absence of 0.3 M NaCl by monitoring the ellipticity at 222 nm with a scan rate of 1.0 °C/min.

Electron Microscopy, Atomic Force Microscopy, FTIR

Negative staining was performed on formvar-coated 200-mesh copper grids. The samples were adsorbed for 1 min, washed with 0.1 M and 0.01 M ammonium acetate for 5 s each, stained with freshly filtered 2% (w/v) uranyl acetate for 2 min and viewed in a Zeiss EM 10 CA electron microscope.

AFM imaging was performed as described earlier⁴⁹. Amyloid fibrils were imaged with a PicoSPM LE AFM (Molecular Imaging, Phoenix, AZ) operating in the AAC (acoustic alternative current) AFM mode and using a silicon cantilever PPP-NCH (Nanoscience, Phoenix, AZ) with a tip radius < 7 nm and a spring constant of ~ 42 N/m. Amyloid fibrils (10 µL) were deposited at a concentration of 5 µg/mL onto a freshly cleaved piece of mica and left to adhere for 10 min. Samples were washed with distilled H₂O and dried with nitrogen. The images (512×512 pixel scans) were collected at a scan rate of 1 line/s.

FTIR spectra were measured with a Bruker Tensor 27 FTIR instrument (Bruker Optics, Billerica, MA) equipped with a MCT detector cooled with liquid nitrogen. Fibrils were dialyzed for two hours against 10 mM sodium acetate buffer (pH 5.0), then the buffer was changed and the dialysis continued overnight. 10 µL of each sample were loaded into a BioATR II cell. A total of 1024 scans at 2 cm⁻¹ resolution were collected for each sample under constant purging with nitrogen. Spectra were corrected for water vapor, and background spectra of the same buffer were subtracted. The bands were resolved by Fourier self-deconvolution in the Opus 4.2 software package using a Lorentzian line shape and parameters equivalent to 20 cm⁻¹ bandwidth at half height and a noise suppression factor of 0.3.

SDS-PAGE

Samples were treated with denaturing sample buffer under reducing conditions (the final 60 mM Tris, 2% SDS, and 1.25% β-mercaptoethanol, 2.25 M urea, heating for 15 min at 90°C) and analyzed using 12% SDS-PAGE (precast NuPAGE gels, Invitrogen).

Immunostaining and fluorescence microscopy

PrP fibrils (2 µg/ml) were deposited onto Permanox 8-well Lab-Teks chamber slides and stained with antibody as described previously²⁹ with minor modifications. Formaldehyde fixation was omitted, and the staining was performed in the following order: (1) anti-PrP human Ab R1 (1:500, recognizes epitope 225 – 231); (2) mouse Ab 3F4 (1:1000, recognizes epitope 109–112); and (3) the mixture of secondary Abs: goat anti-human and goat anti-mouse labeled with Alexa-488 and Alexa-546, respectively (Invitrogen/Molecular Probes, 1:1000 for both Abs). Fluorescence microscopy was carried out on an inverted microscope (Nikon Eclipse TE2000-U) using 1.3 aperture Plan Fluor x100 numerical aperture objective. Collected images were processed with WCIF ImageJ software (National Institute of Health) as previously described²⁹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

The abbreviations used are

PrP^C, cellular isoform of the prion protein; PrP^{Sc}, disease associated isoform of the prion protein; PrP 23–231, full-length recombinant prion protein; PrP 31–231, recombinant prion protein encompassing residues 31–231; α -PrP, alpha-helical form of the prion protein; GdnHCl, guanidine hydrochloride; EM, electron microscopy; AFM, atomic force microscopy; ThT, Thioflavin T..

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FIGURE 1.

Purification of PrP on C4 column. (a) Typical HPLC elution profile of recombinant PrP. The major peak contains pure α -PrP (peak 2); it is separated from the peak containing PrP with oxidized methionines (peak 1) and from the peaks containing PrP-glutathione adducts (peaks 3, 4, 5). Peak 2 was collected in two different ways: avoiding the peak's tail (referred to as pool i), and including peak's tail (referred to as pool ii). Both PrP pools were analyzed by SDS-PAGE followed by Coomassie Blue staining (inset). The amounts of PrP loaded into each gel (from the first to the last lane): 5.0, 2.5, 1.25, 0.62, 0.31 μ g. Both gels showed no detectable impurities. (b) EM (left panel) and AFM (right panel) images of amyloid fibrils formed from

the pool *ii*. (c) EM (left panel) and AFM (right panel) images of amyloid fibrils formed from the pool *i*. Scale bars = 0.2 μm .

FIGURE 2.

Fractions eluted within the major HPLC peak gave rise to two types of fibrils. (a) Enlarged image of the major HPLC peak (left panel). α -PrP was collected in several fractions (# 1 to 7) and analyzed by SDS-PAGE followed by Coomassie Blue staining (inset). An arrow points at a barely visible sub-band observed in the combined fractions 6&7. SDS-PAGE of fractions 4 and 6&7 followed by prolonged silver staining is shown in the right panel. A bracket shows a weak smear observed in fractions 6&7. (b) Electron microscopy images of amyloid fibrils formed from fractions #2 (top left), #4 (top right), #6 (bottom left) and #7 (bottom right). Scale bars = 0.2 μ m.

FIGURE 3.

Identification of minor fragments eluted with tail fractions. (a) Rechromatography of the tail fractions on C4 HPLC column. (b) α -PrP collected in several fractions (# 1 to 9) and analyzed by SDS-PAGE followed by Coomassie Blue staining. The fractions 1 to 3 showed a single band corresponding to PrP 23–231, whereas the fractions 4 to 9 showed two additional bands: the upper band overlaps with the PrP 23–231 band, while the lower band was well resolved. (c) The table summarizes the PrP fragments present in HPLC fractions identified by ESI-MS.

FIGURE 4.

Ion-exchange chromatography separates truncated PrP polypeptides from PrP 23–231. (a) Chromatography profile shows elution of truncated and full-length PrP by a NaCl gradient from 0 to 0.5 M. Dashed line represents real-time change in conductivity. (b) PrP polypeptides were collected in several fractions (# 1 to 11) as shown in panel *a* and analyzed by SDS-PAGE followed by Coomassie Blue staining.

FIGURE 5.

(a) SDS-PAGE of α -PrP 31-231 (lane 2) and a 1:1 mixture of α -PrP 23-231 and α -PrP 31-231 (lane 1) stained with silver. (b) Far-UV CD spectra of α -PrP 23-231 (○) and α -PrP 31-231 (●) (0.125 mg/mL) collected in 1 mM Na-acetate, pH 5.0. (c) The thermal denaturation curves for α -PrP 23-231 (○, Δ) and α -PrP 31-231 (●, ▼) collected in the absence (circles) or presence of 0.3 M NaCl (triangles) in 25 mM MES, pH 6.0.

FIGURE 6.

EM imaging of fibrils formed in mixtures of PrP 23-231 and PrP 31-231. Fibrils were formed under standard conditions from 100% PrP 23-231 (a), from the following molar mixtures of PrP 23-231 and PrP 31-231: 99.9 to 0.1 % (b) and 99 to 1 % (c), and from 100% PrP 31-231 (d). The total protein concentration: 0.5 mg/mL. Arrows in panel (d) point to barely visible protofibrils. Scale bars = 0.2 μm in panels a, b, and c, and =2 μm in panel d.

FIGURE 7.

FTIR second derivatives of curvy fibrils produced from PrP 23–231 (pool *i*) (solid thin line), rigid fibrils produced from the mixture of PrP 23–231 (pool *i*) and PrP 31–231 with 99:1 molar ratio (dashed lines), and the R-fibrils (solid thick line). The R-fibrils were produced from PrP 23–231 (pool *i*) under rotation as previously described²⁸.

FIGURE 8.

Double staining immunofluorescence microscopy images of amyloid fibrils. (a) Fibrils produced from the mixture of PrP 23–231 (pool *i*) and PrP 31–231 with 99:1 molar ratio. Two representative fields of view are shown. (b) Fibrils produced from PrP 23–231 (pool *i*, curvy fibrils). (c) The R-fibrils produced from PrP 23–231 (pool *i*) under rotation as previously described²⁸. The microscopy images were transformed into 2D-fluorescence intensity scattering plots (*insets*)²⁹. Red fluorescence intensities (staining with 3F4) are plotted on the horizontal axis, and the green intensities (staining with R1) are plotted on the vertical axis. Scale bars = 3 μm.

FIGURE 9.

Schematic diagram illustrating that highly pure full-length hamster PrP (pool *i*) produces two strains of amyloid fibrils under identical solvent conditions but different shaking modes. At slow rotation, PrP formed fibrils with a straight, rigid shape (R-strain), whereas under fast shaking the fibrils showed a curvy S-like shape (S-strain)²⁸. The pool *ii* that contains minor amounts of the N-terminally truncated PrPs did not form S-fibrils under shaking. Instead, it produced R-like fibrils with morphologies very similar to R-fibrils.