Structural attributes of mammalian prion infectivity: Insights from studies with synthetic prions

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Prion diseases are neurodegenerative disorders that affect many mammalian species. Mammalian prion proteins (PrPs) can misfold into many different aggregates. However, only a small subpopulation of these structures is infectious. One of the major unresolved questions in prion research is identifying which specific structural features of these misfolded protein aggregates are important for prion infectivity in vivo. Previously, two types of proteinase K–resistant, self-propagating aggregates were generated from the recombinant mouse prion protein in the presence of identical cofactors. Although these two aggregates appear biochemically very similar, they have dramatically different biological properties, with one of them being highly infectious and the other one lacking any infectivity. Here, we used several MS-based structural methods, including hydrogen–deuterium exchange and hydroxyl radical footprinting, to gain insight into the nature of structural differences between these two PrP aggregate types. Our experiments revealed a number of specific differences in the structure of infectious and noninfectious aggregates, both at the level of the polypeptide backbone and quaternary packing arrangement. In particular, we observed that a high degree of order and stability of β-sheet structure within the entire region between residues 89 and 227 is a primary attribute of infectious PrP aggregates examined in this study. By contrast, noninfectious PrP aggregates are characterized by markedly less ordered structure up to residue 167. The structural constraints reported here should facilitate development of experimentally based high-resolution structural models of infectious mammalian prions.

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§ The abbreviations used are: PrP(C), cellular prion protein; PrP(Sc), scrapie prion protein; PrP, prion protein; PK, proteinase K; rPrP, recombinant prion protein; PMCA, protein misfolding cyclic amplification; HXMS, hydrogen–deuterium exchange coupled with MS; UPLC, ultra-HPLC; HRF, hydroxyl radical-mediated footprinting; GdnHCl, guanidinium hydrochloride; TRF, time-resolved fluorescence.
seed PMCA conversion of rPrP in the presence of phosphatidylethanolamine as a solitary cofactor, resulting in a different prion strain with high infectivity (25).

Recently, it was shown that, in addition to these highly infectious rPrP-resRNA prions, using the same PMCA protocol and identical cofactors one can generate de novo another type of self-propagating rPrP-res aggregate, denoted rPrP-resRNA-low, that biochemically appears very similar to rPrP-resRNA but lacks any infectivity (26, 27). The availability of these two self-propagating aggregate types with dramatically different biological properties opened up new avenues for exploring the structural basis of prion infectivity.

Initial characterization of rPrP-resRNA and rPrP-resRNA-low aggregates revealed that the PK-resistant core of rPrP-resRNA is approximately 1 kDa longer compared with that of rPrP-resRNA-low (16 and 15 kDa, respectively) (27). However, using an arsenal of biochemical and immunochemical tools, we could not detect any other differences in the physicochemical properties of the two aggregate types (27). This raises the question whether the profoundly different infectivity properties of these aggregates are solely due to the small difference in the size of the PK-resistant core or whether there are more fundamental structural differences that were undetectable by the methods used. To address this fundamentally important issue, here we used an arsenal of MS-based structural tools, including hydrogen–deuterium exchange and hydroxyl radical footprinting. Our data reveal major structural differences between rPrP-resRNA and rPrP-resRNA-low, both at the level of the polypeptide backbone and quaternary packing arrangement, providing new insight into the structural basis of prion infectivity.

Results

Sequencing of PK-resistant cores

Previous Western blotting–based studies indicate that the PK-resistant core of infectious rPrP-resRNA is ~1 kDa larger compared with that of noninfectious rPrP-resRNA-low (27). To gain more detailed insight into the identity of these core regions, we used MS to sequence the longest PK-resistant fragments in both aggregate types. This analysis demonstrated the single C terminus at Ser-230, i.e. the last residue in mouse rPrP substrate. By contrast, N-terminal sequencing analysis revealed ragged N termini, with the most abundant (~45%) N-terminal fragment in rPrP-resRNA starting at residue 89, and several minor fragments starting at residues 81 and 97 (Table 1). In rPrP-resRNA-low, the most abundant (~90%) N terminus was at residue 99, with minor fragments starting at residues 100 and 101.

Backbone amide hydrogen–deuterium exchange

To gain information about the backbone conformation of rPrP-resRNA and rPrP-resRNA-low, we used the method of backbone amide hydrogen–deuterium exchange coupled with MS (HXMS). Although the exchange of backbone amide hydrogens proceeds rapidly within unstructured regions of a protein, it is much slower for the amide groups involved in systematically hydrogen-bonded structures, such as β-sheets or α-helices. The method proved especially useful for studying ordered protein aggregates, such as amyloids, as the exchange rates within β-amyloid cores are exceptionally slow (28, 29).

The prerequisite for HXMS analysis is identification of peptide fragments that can be separated under a rapid reverse-phase chromatography gradient required for hydrogen exchange experiments. Using ultra-HPLC (UPLC), we were able to separate under these conditions 31 reproducible peptic fragments that could be identified by MS with a signal-to-noise ratio sufficient for reliable analysis of deuterium incorporation. These fragments cover almost the entire PK-resistant regions of both types of rPrP aggregates studied with multiple overlaps in some regions. The only exception is the lack of hydrogen–deuterium exchange data for the N-terminal end of rPrP-resRNA-low aggregates as the peptic fragment for this region showed very weak MS signal.

The deuterium incorporation level for peptic fragments generated from rPrP-resRNA and rPrP-resRNA-low after 10-min and 24-h incubation in D2O is shown in Table S1, and color-coded hydrogen–deuterium exchange protection maps summarizing 24-h exchange data for all identified fragments are depicted in Fig. 1. Furthermore, to facilitate visual comparison of data for the two types of aggregates studied, the extent of deuterium incorporation for representative nonoverlapping (or nearly nonoverlapping) segments is shown in Fig. 2.

The striking feature of the exchange profile for the infectious rPrP-resRNA aggregates is that the vast majority of peptide fragments derived from the PK-resistant core show very low-level (less than 25%) deuterium incorporation even after 24-h incubation in D2O buffer. The notable exceptions are the C-terminal fragment encompassing residues 224–230 and the 188–196 segment, both of which show ~65% deuterium incorporation after 24 h. Remarkably, in the case of the very C-terminal 224–230 fragment, a similar high level of hydrogen exchange already after 10-min incubation in D2O (Fig. 2 and Table S1) was observed. One possible explanation of such a rapid fractional labeling could be structural heterogeneity of the sample with a large fraction of molecules (~65%) characterized by the essentially unordered 224–230 region and a smaller fraction with very high order in this region. However, this would result in a bimodal mass distribution in mass spectra, which was not observed experimentally for this or any other fragments. MS data thus favor another scenario in which three of five amide protons in the 224–230 fragment that can be followed by hydrogen–deuterium exchange analysis are completely unpro-

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**Table 1**

| Aggregate type | N-terminal tryptic fragments | Relative amount (%)a |
|---------------|-----------------------------|----------------------|
| rPrP-resRNA   | 81–105                      | 23                   |
|               | 89–105                      | 43                   |
|               | 97–105                      | 10                   |
| rPrP-resRNA-low | 99–105                     | 93                   |
|               | 100–105                     | 4                    |
|               | 101–105                     | 2                    |

a Estimate based on relative areas in chromatographic peaks.
tected, whereas the other two are highly protected (note that the first two amides in each fragment back-exchange too rapidly to retain deuterium atoms during the experiment (30, 31)). Given that the undetectable/unprotected residue in the 224–230 fragment is likely an extension of the highly protected 213–223 region, this would map the C terminus of the ordered/H9252-sheet core of infectious rPrP-resRNA aggregates to residue 227.

Within the above defined ordered core of rPrP-resRNA (residues 89–227), the only regions showing a relatively large degree of deuterium incorporation map to residues within the 188–196 and, to a lesser extent, 133–143 segments. In those cases, however, only small fractions of those segments (~30% or two of seven detectable amides in 188–196 segment and ~20% or two of 10 detectable amides in the 133–144 segment) are fully unprotected as indicated by 10-min exchange data. In the PrPC structure, the 188–196 segment largely maps to the loop between α-helices 2 and 3 (Fig. 2C). Apparently, part of this loop remains preserved upon PrPC conversion to rPrP-resRNA aggregates. Overall, backbone amide hydrogen–deuterium exchange data indicate a highly ordered structure in the 89–227 region (i.e. almost the entire PK-resistant core) of infectious synthetic prions analyzed in the present study.

Figure 1. hydrogen–deuterium exchange protection map summarizing 24-h backbone amide hydrogen–deuterium exchange data for rPrP-resRNA and rPrP-resRNA-low. The peptic fragments covered almost the entire PK-resistant core of both aggregates except the very N-terminal region of rPrP-resRNA-low. Color bars below the sequence represent the level of deuterium incorporation for individual peptic fragments.

Figure 2. A and B, deuterium incorporation by backbone amide groups for nearly nonoverlapping segments derived from rPrP-resRNA and rPrP-resRNA-low after 10-min (A) and 24-h (B) incubation in D2O. The deuterium incorporation levels shown represent direct measurements for individual peptic fragments, except data for the 89–120 and 188–196 segments that were derived from analysis of data for overlapping peptic fragments within these two regions (see “Experimental procedures” for details). Error bars indicate S.E. (three independent experiments). **, p < 0.01; ***, p < 0.001. C, protection maps for nearly nonoverlapping segments summarizing 24-h exchange data shown together with schematic representation of the secondary structure of PrPC.
from the 133–159 region in which case a high level (−45−65%) of deuterium incorporation in rPrP-resRNA-low was observed already after 10-min exchange.

Altogether, HXMS data indicate highly ordered structure in almost the entire PK-resistant region of the infectious rPrP-resRNA prions and suggest that the β-sheet core of these prions extends from residue 89 to residue 227. The noninfectious rPrP-resRNA-low aggregates prepared using the same PMCA protocol appear to have a similar highly ordered structure in the region C-terminal to residue ~167 but are markedly less ordered in the region between residues ~127 and 167. In the native PrPc structure, the latter region encompasses β-strand 1, the first α-helix, β-strand 2, and the connecting loops (Fig. 2C). Thus, conversion of this entire motif to β-sheet structure appears to be essential for prion infectivity.

**His-HXMS**

Structural properties of rPrP-resRNA and rPrP-resRNA-low aggregates were further examined using the His-HXMS method. In these experiments, the rate of hydrogen–deuterium exchange for C2 protons in the imidazole rings of histidine side chains is measured (32, 33). Even for unprotected (i.e. fully water-exposed) His residues, this exchange is relatively slow with a half-time of ~2–3 days. When the His side chain is buried in a water-protected environment, the exchange half-time is substantially increased, and these rates can be conveniently monitored by MS (32, 33). The methods of backbone amide HXMS and His-HXMS provide complementary structural information: amide HXMS probes protein structure at the level of the polypeptide backbone, whereas His-HXMS reports on the microenvironment (water accessibility) of individual histidine side chains. As shown in our previous studies, the latter method is a sensitive tool for probing the packing arrangement within ordered protein aggregates, such as amyloid fibrils (34, 35).

There are five His residues in the PK-resistant core region of infectious rPrP-resRNA aggregates (His-95, His-110, His-139, His-176, and His-186), four of which (except His-95) are also present in rPrP-resRNA-low. In the PrP monomer, all of these His side chains are exposed to water, and, as expected, their exchange half-times are ~2–3 days (Fig. 3). Upon conversion to the infectious rPrP-resRNA form, these side chains become at least partially protected from water with a particularly strong protection observed for His-176. Importantly, the accessibility to water of two side chains, His-139 and His-176, is markedly higher in rPrP-resRNA compared with that in rPrP-resRNA-low, indicating distinct packing arrangements and/or interfaces between β-sheets around residues 139 and 176 in the infectious and noninfectious rPrP aggregates.

**Hydroxyl radical footprinting**

Additional information about structural differences between rPrP-resRNA and rPrP-resRNA-low was sought using the hydroxyl radical-mediated footprinting (HRF) method. Here, aqueous protein samples are exposed to synchrotron X-rays that generate hydroxyl radicals through water radiolysis, and these radicals can oxidatively modify susceptible amino acid side chains (36–38). For a given type of side chain, the rate of this modification is proportional to solvent accessibility. Thus, akin to His-HXMS, HRF reports on the microenvironment of specific side chains in proteins. However, the latter approach is potentially more powerful as it is not limited to probing His residues. The usefulness of the HFR method has been demonstrated in structural studies of highly complex systems (39–42), including a recent study with β-amylloid peptide aggregates (39).

Different forms of mouse prion protein, including rPrP-resRNA, rPrP-resRNA-low and rPrP(89–230) monomer were exposed to a broadband X-ray beam for 0–500 ms to generate hydroxyl radicals. After sample denaturation and digestion with pepsin, fragments with and without modified residues were separated by UPLC, identified by tandem MS, and quantified by measuring areas of corresponding chromatographic peaks. The modification rates were determined by plotting the extent of modification of specific side chains versus the exposure time as illustrated for several side chains in Fig. 4.

Using this approach, for both rPrP-resRNA and rPrP-resRNA-low, we were able to determine the modification rates of 11 residues (Table S2). A larger number of residues could be followed for the rPrP(89–230) monomer. However, peptic fragments containing some of them could not be identified in mass spectra of the aggregates. Among those that could be identified for all protein forms, the modification rates of four side chains (Val-160, Tyr-161, Tyr-168, and Val-202) in rPrP-resRNA and rPrP-resRNA-low were too slow to be reliably determined by MS, indicating that these side chains are buried in an environment that is particularly strongly protected from water. To compare the microenvironment of “HRF-measurable” side chains in rPrP-resRNA and rPrP-resRNA-low, we calculated the “protection ratios” by dividing modification rates for the monomer (in which these side chains are largely exposed) by the rates observed for the same side chains in the aggregates. These protection ratios (shown in Fig. 5) normalize the data, providing a measure of the degree to which the side chain solvent accessi-
bility of a particular residue is altered upon conversion of the monomer to rPrP-resRNA or rPrP-resRNA-low aggregates. Inspection of data for the infectious rPrP-resRNA prions reveals great variability in water accessibility for individual side chains with protection ratios ranging from 1 (Tyr-156 and Met-205) to 0.5 (Glu-145) and apparently higher (Val-160, Tyr-161, Tyr-168, and Val-202). Although the present HRF data provide information about the fraction of PrP residues only, such large variability in the degree of exposure to water of individual side chains is consistent with the ordered cross-β structure of rPrP-resRNA prions as residues in the dry interface between β-sheets (steric zippers) are expected to be inaccessible to the solvent, whereas those facing in an opposite direction should be largely solvent-exposed (43, 44). HRF data also reveal major differences between rPrP-resRNA and rPrP-resRNA-low with regard to water exposure of a number of side chains. These differences are seen both in the region where, based on HXMS data, the overall structure of infectious rPrP-resRNA prions appears to be much more ordered compared with noninfectious rPrP-resRNA-low aggregates (Trp-144 and Glu-145), and in the C-terminal region characterized by a high degree of order on both aggregate types (Met-204 and Met-212). The latter data point to differences in packing of β-sheets in the C-terminal regions of rPrP-resRNA and rPrP-resRNA-low aggregates.

Conformational stability

Next we assessed how the structural differences between rPrP-resRNA and rPrP-resRNA-low revealed by MS-based methods affect the susceptibility of these aggregates to denaturation by a chaotropic agent, guanidinium hydrochloride (GdnHCl). To this end, we performed (on samples without PK treatment) a “sandwich” conformational stability assay (22, 45) using mAb 8H4 (epitope 175–185) as a capture antibody and europium-conjugated mAb 12B2 (epitope 89–92) as a detection antibody (see “Experimental procedures” for details). Detection of the unfolding event in this assay requires that both epitopes become exposed, allowing antibody binding. Unfolding curves derived from this method show large differences in conformational stability between the two types of rPrP aggregates studied with GdnHCl concentration corresponding to midpoint denaturation of 2.3 and 3.7 M for rPrP-resRNA and rPrP-resRNA-low, respectively (Fig. 6A). To further validate these data, we repeated the conformational stability assay in a different format, immobilizing the aggregates not via the capture antibody
but through nonspecific binding to the surface of plastic wells. As expected, no unfolding event could be detected in this assay for rPrP-resRNA-low (Fig. 6B) as the epitope for the detection antibody (residues 89–92) is outside the core region of these aggregates (see sequencing data above) and thus exposed even in the absence of the denaturant. By contrast, the unfolding curve for rPrP-resRNA (in which the epitope for the detection antibody is within the structured core region) was very similar to that obtained using the sandwich assay.

Discussion

It was previously shown that highly infectious mammalian prions, rPrP-resRNA, can be generated de novo from the recombinant mouse prion protein in the presence of two cofactors, mouse liver RNA and a synthetic phospholipid, phosphatidylglycerol (24). Following this finding, more recently it was demonstrated that, using the same experimental protocol and identical cofactors, one can also generate from mouser PrP another PK-resistant aggregate, rPrP-resRNA-low, that can be propagated in vitro by PMCA. However, in contrast to rPrP-resRNA that has high infectivity and causes transmissible prion disease in mice, rPrP-resRNA-low aggregates show no infectivity when tested in mice or using a cell culture–based prion infection assay (27). The availability of these two PK-resistant rPrP aggregates that can indefinitely propagate in vitro but have dramatically different infectivity properties in vivo offers unique opportunities for exploring the molecular and structural bases of prion infectivity. This model system is especially important given that the high-resolution structure of infectious prions remains unknown, and studies in this regard present enormous technical challenges.

Our previous study revealed that infectious rPrP-resRNA is characterized by a slightly larger PK-resistant core (~16 kDa) compared with that of noninfectious rPrP-resRNA-low (~15 kDa). However, besides this relatively minor difference in the core size, we were not able to detect any other significant differences between the two aggregate types, finding them essentially indistinguishable with regard to the overall morphology (as assessed by atomic force microscopy), core stability (as assessed by the resistance to digestion with increasing amounts of PK), recognition by a panel of conformational antibodies (as assessed by immunoprecipitation assay), and solubilization in the presence of increasing concentrations of GdnHCl (27).

Given the dramatic difference in the infectivity of rPrP-resRNA and rPrP-resRNA-low, this was a surprising finding and raised the question whether the size of the PK-resistant core could be the main determinant of infectivity properties of these aggregates. Another possibility, however, is that rPrP-resRNA and rPrP-resRNA-low differ in a more fundamental way with regard to their structural organization, but the biochemical tools used in the previous study were not sensitive enough to detect these differences. To address this issue, here, we examined these two aggregate types using a set of complementary MS-based structural methods that provide information at different levels of structural organization of ordered protein aggregates. Although some of these approaches have been successfully used previously for probing the structure of prions and related PrP aggregates (29, 34, 35, 46–48), hydroxyl radical footprinting is a novel tool in prion research.

Based on relatively low-resolution electron cryomicroscopic data for brain-derived glycosylphosphatidylinositol-anchorless PrPSc fibrils, it was recently proposed that the structure of mammalian prions is based on a four-rung β-solenoid motif (21). A β-solenoid structure, consisting of two rungs separated by a long (~15-residue) unstructured loop, is best documented for HET-s prions from the filamentous fungus Podospora anserina (49, 50). The presence of at least the large part of this central loop appears to be essential for HET-s prion infectivity (49). One of the findings of the present study is that the entire region encompassing residues 89–227 in the infectious rPrP-resRNA aggregates is characterized by a highly ordered and stable β-sheet structure as indicated by the very low level of deuterium incorporation by the backbone amide groups even after 24-h incubation in D2O buffer. Thus, should a four-rung β-solenoid motif also be applicable to synthetic mammalian prions under study, our data would argue that the entire ~89–227 region would have to be part of the β-solenoid structure with no longer unstructured/loop regions present. Remarkably, the hydrogen–deuterium exchange profiles identified only two loops that are at least two residues long, one within the 133–144 segment and the second one within the 188–196 region. This suggests that turns between most of the β-strands in the structure of rPrP-resRNA are likely very tight.
Structural attributes of mammalian prion infectivity

The crucial constraint in any structural model for mammalian prions is the presence of a disulfide bridge between cysteines 178 and 213 (numbering according to mouse PrP sequence). As noted previously (21), in the four-rung β-solenoid model these two cysteines would have to be located within the loop/tturn regions in the corners of two consecutive rungs. Furthermore, the backbones of these two Cys residues would have to rotate so that the side chains can form a disulfide bond parallel to the protofilament axis. Thus, one would expect the backbone amides of the two cysteines (and likely any nearby residue) to be unprotected by hydrogen bonds and accessible to hydrogen–deuterium exchange. Our present data, however, are inconsistent with this expectation as the peptic fragments containing both Cys-178 and Cys-213 show very little deuterium incorporation (5 and 2% after 10-min incubation in D₂O for the 168–180 and 213–223 fragments, respectively). Thus, the HXMS data for these peptic fragments appear to present a dilemma for the four-rung β-solenoid as a universal model for the structure of mammalian prions. In contrast, no rotation of Cys residues would be required in the context of a parallel register model of PrPSc structure (16–18), and none of the structural constraints obtained in this study are inconsistent with the latter model. However, the present data alone are insufficient to conclusively determine the specific folding motif of infectious rPrP-resRNA prions.

It should be noted that the hydrogen–deuterium exchange profile for rPrP-resRNA is generally similar to that previously reported for infectious mouse PrP aggregates propagated in the presence of phosphatidylethanolamine as a sole cofactor (51). The only significant differences are observed within the 133–143 region (that appears to be less ordered in rPrP-resRNA) and the 148–153 segment (that appears to be somewhat more ordered in rPrP-resRNA). This overall structural similarity is especially noteworthy given that the strain properties (i.e., incubation times and neuropathological and biochemical characteristics) of these two types of synthetic mouse prions are quite different (24, 52). Notably, an overall high degree of protection from hydrogen exchange within the entire ~89–227 region was also found for brain-derived mouse prion strains (46). Thus, it appears that a high degree of order within this region is a general feature of infectious prions.

The notion that highly ordered β-sheet structure within the entire ~89–227 region is important for prion infectivity is further supported by comparison of HXMS data for the infectious and noninfectious self-propagating rPrP aggregates generated in the presence of identical cofactors. This comparison revealed a similarly high degree of protection from hydrogen exchange for the C-terminal regions starting at residue ~168. However, the N-terminal part of the PK-resistant core of noninfectious rPrP-resRNA-low aggregates (especially within the 127–167 region) was found to be much less protected from hydrogen exchange compared with this region in the infectious rPrP-resRNA counterpart. Given the extent of hydrogen–deuterium exchange, the present data strongly suggest that, unlike in rPrP-resRNA, the N-terminal part of the PK-resistant core of rPrP-resRNA-low does not form a stable, systematically H-bonded β-sheet structure. However, this region is also not completely disordered as indicated by partial protection from exchange of backbone amide groups. Furthermore, His-HXMS and HRF data indicate that many of the side chains within this region are involved in relatively strong (likely intermolecular) interactions, resulting in low accessibility to water. The latter stabilizing interactions might be an important factor contributing to the resistance of this region in rPrP-resRNA-low to PK digestion.

Apart from greatly differing with regard to the protection of the backbone amide groups within the N-terminal part of PK-resistant cores against hydrogen-deuterium exchange, rPrP-resRNA and rPrP-resRNA-low show substantial differences with respect to water accessibility of many side chains as indicated by His-HXMS and hydroxyl radical footprinting data. Importantly, the latter differences are detectable throughout the entire amino acid sequence of the PK-resistant cores, indicating major differences in quaternary packing arrangements of rPrP-resRNA and rPrP-resRNA-low aggregates.

Consistent with these findings, our experiments revealed large differences in the conformational stability of both aggregate types as measured by susceptibility to GdnHCl denaturation. Interestingly, no differences in GdnHCl "denaturation" curves could be detected in the previous study using the assay based on solubility of rPrP aggregates. The assay used in the present study provides a more reliable measure of conformational stability as it directly monitors the exposure of epitopes that are hidden in the structures of rPrP-resRNA or rPrP-resRNA-low aggregates but become exposed upon unfolding of these structures by the denaturant.

In conclusion, our present study revealed large differences in the structure of two types of PK-resistant rPrP aggregates generated in the presence of identical cofactors that, despite very similar biochemical characteristics, have dramatically different biological properties. These data provide novel insight into the structural basis of prion infectivity, suggesting that a highly ordered, stable β-sheet structure within the entire region encompassing residues ~89–227 is one of the key attributes of highly infectious prions. By contrast, PrP aggregates showing no infectivity are characterized by a markedly less ordered structure up to residue ~167, especially within the 126–167 region. The structural constraints obtained in this study should facilitate future efforts to construct experimentally based high-resolution structural models for infectious mammalian prions.

Experimental procedures

Generation of rPrP-res aggregates

rPrP-resRNA and rPrP-resRNA-low aggregates were generated as described previously (24, 27). For all MS-based experiments, samples were pretreated with Benzonase (Novagen; 100 units/ml for 1 h at 37 °C) to remove any residual RNA followed by digestion with proteinase K (Roche; 25 μg/ml for 30 min at 37 °C). For the conformational stability assay, samples were treated with Benzonase but not with proteinase K. Following enzymatic treatment, samples were pelleted and stored at −80 °C.

Sequencing of PK-resistant cores

Samples of rPrP-resRNA or rPrP-resRNA-low (~2 μg) were solubilized in 55 μl of 8 M GdnHCl solution, and the disulfide bridge was reduced by incubation for 1 h at 37 °C in the pres-
ence of 1,4-dithiothreitol (DTT) (17 mM). Free Cys residues were blocked by incubation for 1 h in the dark with iodoacetamide (50 mM) followed by addition of DTT (50 mM) and further incubation for 1 h at 37 °C. The protein was then precipitated by adding 90% methanol and incubating overnight at −20 °C. Following resolubilization in 6 M urea and 10-fold dilution with 100 mM ammonium bicarbonate, the protein was digested overnight at 37 °C with trypsin (MS grade, Thermo Pierce; 200 ng). Tryptic fragments were separated on a C18 analytical column (Acclaim PepMap, Thermo Fisher Scientific) using a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific) and analyzed by MS using an LTQ Orbitrap XL instrument (Thermo Fisher Scientific) interfaced to the UPLC system. Peptide fragments were identified by tandem MS/MS using the MassMatrix search engine (MassMatrix Xtreme 3.0.9.7 Alpha; http://magneto.case.edu/mm-cgi/home.py)4 (53) against the sequence of mouse prion protein. The search was performed in “nonspecific enzyme” mode. Because the most N-terminal trypsin digestion site within the PK-resistant cores of rPrP-resRNA and rPrP-resRNA-low is between Lys-105 and Thr-106, N termini of tryptic fragments ending at residue 105 correspond to N termini of the respective PK-resistant core regions.

**HXMS**

To initiate hydrogen–deuterium exchange, rPrP-resRNA or rPrP-resRNA-low (~4 μg) was suspended in 100 μl of 10 mM phosphate buffer in D2O, pH 7.3. After 5-min or 24-h incubation at room temperature, protein aggregates were collected by centrifugation (21,000 × g for 5 min), and pellets were immediately dissociated by incubating for 5 min in 20 μl of an ice-cold solution of 7 M GdnHCl in exchange quench buffer (0.1 M potassium phosphate, 0.1 M tris(2-carboxyethyl)phosphine, pH 2.5). The samples were then immediately diluted 10 times with ice-cold 0.05% TFA in H2O and digested for 5 min on ice with agaro-agaromethylated pepsin slurry (100 μl; Thermo Fisher Scientific). The peptic fragments were collected in a C18 trap column (Symmetry C18 NanoEase, Waters), washed for 5 min to remove salts, and separated on an UPLC Ethylene Bridged Hybrid (BEH) C18 column (Waters) using a 2–45% acetonitrile gradient with a total elution time of 13 min. Separated peptic fragments were analyzed by an LTQ Orbitrap XL mass spectrometer coupled to the UPLC system. To minimize back-exchange, both the trap column and the analytical column were placed in a cooled chamber (~0 °C) integrated with a LEAP TriValve system (LEAP Technologies). The extent of deuterium incorporation for each peptic fragment was determined as described previously (46, 54). To improve resolution, deuterium incorporation for two regions covered by overlapping peptides was further sublocalized using an approach described previously (31). Specifically, deuterium incorporation for the 89–120 segment was calculated from data for the 119–126 and 89–126 peptic fragments. Deuterium incorporation for the 188–196 segment was obtained as an average of results calculated from data for the following pairs of peptic fragments: 181–189 and 181–196, 182–189 and 182–196, and 183–189 and 183–196.

**His-HXMS**

In His-HXMS experiments, rPrP-resRNA or rPrP-resRNA-low (~2 μg) was resuspended in 100 μl of 10 mM phosphate buffer in D2O, pH 9. After incubation for 5 days at 37 °C, samples were collected by centrifugation (21,000 × g for 10 min). To obtain peptide fragments containing single histidine residues, protein samples were dissociated and digested with immunoconjugated pepsin as described above for HXMS experiments followed by digestion with silica-immobilized trypsin (2 μl; Princeton Separations, Inc., Adelphia, NJ). The peptide fragments were separated on an UPLC system and analyzed by MS as described above for the sequencing experiments. The half-time of His-hydrogen–deuterium exchange was calculated as described previously (32, 33).

**Hydroxyl radical footprinting**

X-ray exposures were performed at the 17-BM X-ray footprinting (XFP) beamline of the National Synchrotron Light Source II using a high-throughput apparatus at room temperature (55). After irradiation, samples were immediately frozen in liquid nitrogen and stored at −80 °C. Before analysis by MS, samples were denatured by incubating for 30 min in 95% formic acid, which was subsequently removed by rotary evaporation. Samples were digested with pepsin (Promega) at 37 °C for 5 h at a pepsin-to-PrP weight ratio of 1:10. The peptic fragments were then separated by UPLC and analyzed by MS as described above for the sequencing experiments. Chromatographic peaks of unmodified and modified peptides were identified using the MassMatrix search engine (MassMatrix Xtreme 3.0.9.7 Alpha; http://magneto.case.edu/mm-cgi/home.py)4 (53) and manually verified. The integrated areas of chromatographic peaks of the unmodified peptide (A_u) and of the same peptide in which the residue is modified (A_m) were used to calculate the fraction unmodified: F_u = A_u/(A_u + A_m). The first-order modification rate constant k was calculated by the equation: F_u(t) = F_u(0)e^{-kt}

where t is the exposure time and F_u(0) and F_u(t) are the fractions of unmodified peptide at time 0 and time t (36, 38). Protection ratios were determined by dividing the modification rates for monomeric rPrP (89–230) by the modification rates for rPrP-res aggregates (36, 38, 39).

**Conformational stability assay**

rPrP-resRNA and rPrP-resRNA-low (without PK treatment) were separated from soluble monomers by phosphotungstate anion precipitation, and a conformational stability assay in the “sandwich format” was performed as described previously (45) using mAb 8H4 (epitope 175–185) for PrP capture and europium-labeled mAb 12B2 (epitope 89–92) for detection. The assay in a “direct format” was performed as described (22) with several modifications. In brief, phosphotungstate anion–precipitated aggregates were sonicated (3 × 5 s at 60% power) using a Sonicator 4000 (Qsonica, Newtown, CT), and the concentration of rPrP was adjusted to ~50 ng/ml. 15-μl aliquots were treated with increasing concentrations of GdnHCl using 0.25 or 0.5 M increments. After 30-min incubation at room tem-

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temperature, samples were rapidly diluted with H$_2$O containing diminishing concentrations of GdnHCl so that the final GdnHCl concentration in all samples was 0.2 M. Each aliquot was incubated at 4 °C and blocking with casein, the plates were developed with europium-labeled mAb 12B2 (epitope 89–92) and TRF values for native and unfolded conformations of prion proteins are reported. The data were fitted using a least-square method according to a sigmoidal transition model.

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