Supplemental Information for Miller *et al*.

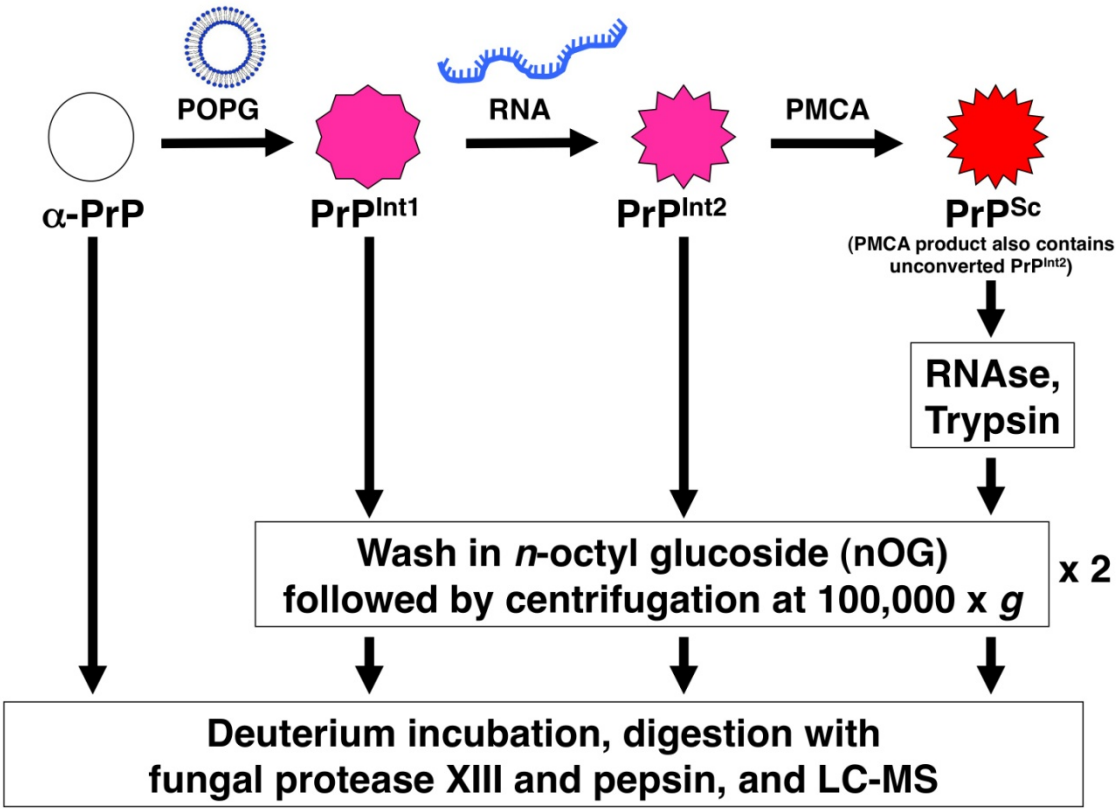
Supplemental Data

Table S1
Optimization of conditions for recovery of prion protein peptic peptides, Related to Figure 1B

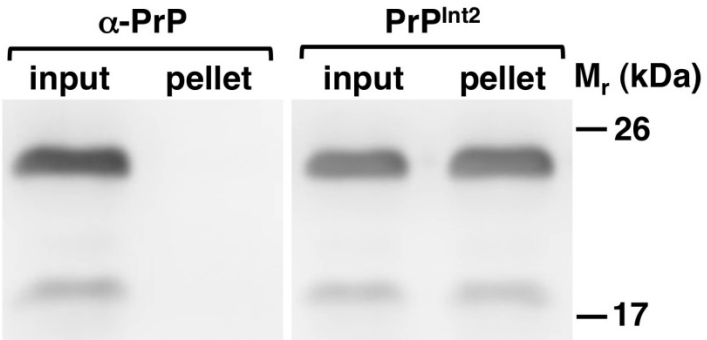
Sample preparation variable			# PrP peptides[c]
PrP quantity	**Quench[a]**	**Protease digestion[b]**	
4 μg	15 mM TCEP, ~10 sec.	~30 sec.	40
1 μg	10 mM TCEP, 10 min.	~30 sec.	31
10 μg	50 mM TCEP, 10 min.	~30 sec.	75
10 μg	50 mM TCEP, 10 min., pH 1.5	~30 sec.	61
10 μg	50 mM TCEP, 10 min., pH 1.5, room temperature	~30 sec.	60
10 μg	50 mM TCEP, 10 min., room temperature	~30 sec.	87
10 μg	50 mM TCEP, 10 min.	~2 min.	82
10 μg	50 mM TCEP, 10 min.	10 min.	85
7.5 μg	100 mM TCEP, 10 min.	10 min.	74
10 μg	100 mM TCEP, 10 min.	10 min.	96
10 μg	100 mM TCEP, 10 min.	5 min.	103
10 μg	100 mM TCEP, 2 min.	10 min.	112
10 μg	100 mM TCEP, 2 min.	5 min.	91
10 μg + detergents[d]	100 mM TCEP, 2 min.	5 min.	92
1 μg (Orbitrap[e])	100 mM TCEP, 2 min.	3 min.	160

a: All quench conditions included 4.3 M guanidine HCl, pH 2.5, and 0°C unless otherwise specified.
b: Digestion under continuous flow estimated at ~30s contact time per PrP molecule. Longer times were obtained by reducing flow or ceasing flow on column for indicated amount of time.
c: Number of PrP peptides includes highest and good quality peptide matches, as determined by DXMS program and manual scoring verification.
d: Trace amounts of Triton X-100 and n-octyl glucoside (nOG) detergents were added to this sample to test their effect on peptide recovery.
e: Initial sample preparation optimization experiments were performed with an LCQ Classic mass spectrometer. When an Orbitrap Elite instrument was available, it was used to final condition optimization and all reported deuterium-exchange experiments. This more sensitive instrument permitted detection of lower quantities of protein.

a


POPG
RNA
PMCA
α-PrP
PrPInt1
PrPInt2
PrPSc
(PMCA product also contains unconverted PrPInt2)
RNAse, Trypsin
Wash in n-octyl glucoside (nOG) followed by centrifugation at 100,000 x g
x 2
Deuterium incubation, digestion with fungal protease XIII and pepsin, and LC-MS

b


α-PrP
PrPInt2
input
pellet
input
pellet
Mr (kDa)
26
17

c

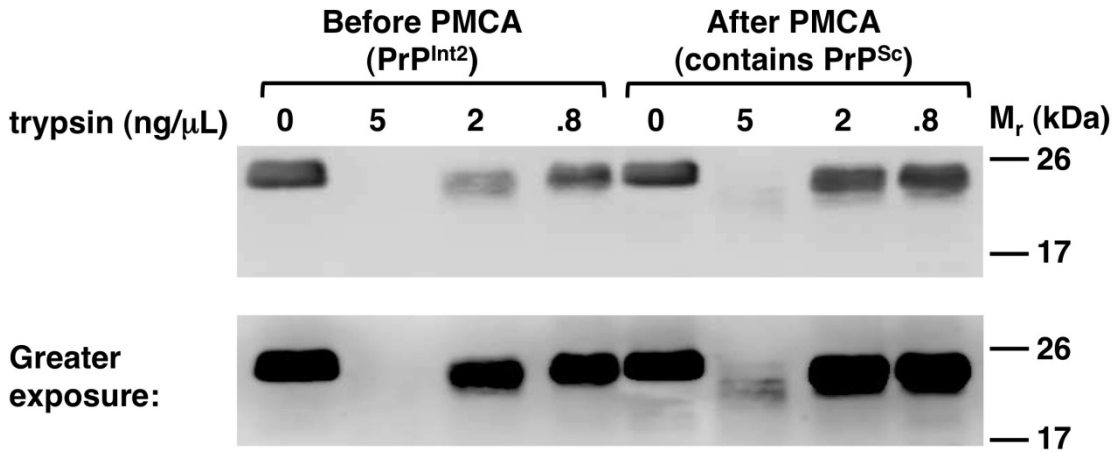


Figure S1, Related to Experimental Procedures and Figure 1A. (a) Schematic illustration of preparation methods for the four different PrP conformation analytes examined in this study. α-PrP was used directly after purification. PrP^{Int1} and PrP^{Int2} were washed with *n*-octyl-glucoside (nOG), utilizing their insolubility to collect by ultracentrifugation after each wash. The PMCA product was treated with RNAse and trypsin to yield PrP^{Sc}, which was then washed with nOG in the same manner as the other insoluble PrP forms. Following preparation, each analyte was incubated in deuterium, fragmented by fungal protease XIII and pepsin, and analyzed by liquid chromatography-mass spectrometry. **(b) Solubility of prion protein conversion intermediate 2 (PrP^{Int2}).** Purified recombinant PrP was diluted into tris-buffered saline pH 7.5 with 0.5% Triton X-100 alone (α-PrP) or incubated with the phospholipid POPG and mouse liver RNA in buffer to form PrP conversion intermediate 2 (PrP^{Int2}). Each preparation was then centrifuged at 100,000x*g* for 1 hr. at 4°C, and the pellets were resuspended to the original volume. Input and pellet samples for each preparation were analyzed for PrP by SDS-PAGE and immunoblot using anti-PrP antibody 27/33. **(c) Effect of trypsin protease treatment on PrP^{Int2} and PrP^{Sc}.** Prion protein conversion intermediate PrP^{int2} (before PMCA, formed by mixing PrP with POPG and RNA) and PMCA reaction product (after PMCA, containing PrP^{Sc}) were incubated for 12 hr. with soluble trypsin of varying concentrations, then analyzed by SDS-PAGE and immunoblot with the PrP-directed antibody 27/33. A greater exposure of the same immunoblot is shown below to better illustrate the fraction of PMCA product that is trypsin-resistant PrP^{Sc}. As described in *Materials and Methods*, PrP^{Sc} was formed by subjecting PrP^{Int2} to protein misfolding cyclic amplification (PMCA), which converts a portion of PrP^{Int2} into PrP^{Sc}.

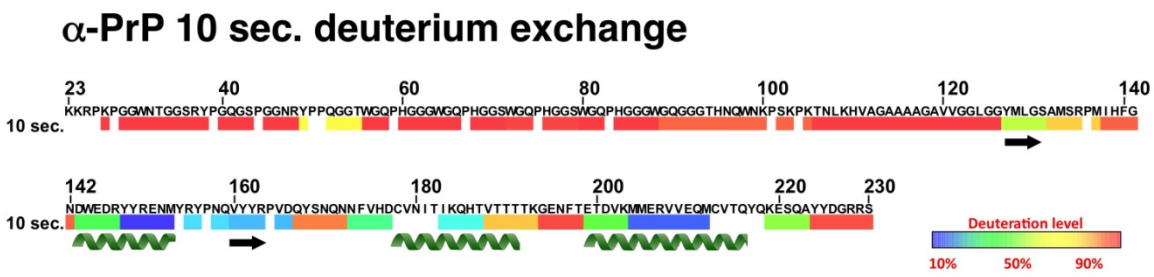


Figure S2. Regional solvent accessibility of α-PrP measured by 10 sec. deuterium incubation, Related to Figure 2A. α-PrP was incubated in D_2O for 10 sec. The reaction was quenched, and analyzed by proteolytic fragmentation, liquid chromatography and mass spectrometry. Deuterium incorporation was determined for each peptide, and information from all peptides covering each amino acid was computed. Deuterium incorporation is indicated by color, with areas of protein with low deuterium incorporation (highly protected) are indicated in blue, while areas with high deuterium exchange (highly exposed) are indicated in red. The α-helix and β-strand secondary structural motifs from α-PrP NMR (Riek et al., 1996) are indicated below the solvent accessibility map.

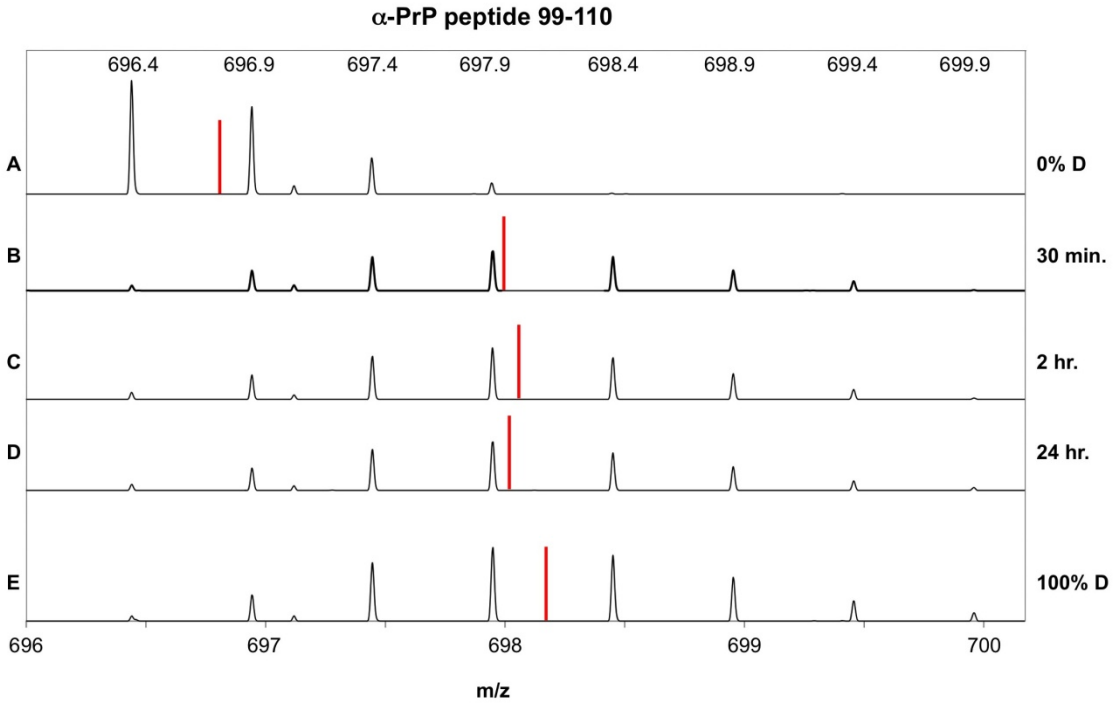


Figure S3. Mass spectra for α-PrP peptide 99-110, Related to Figure 2A. The relative signal amplitude for the isotopic envelope of α-PrP peptide 99-110 (NKPSKPKTNLKH), charge state 2, is plotted for each deuteration state against the mass-to-charge ratio (m/z). Proteolytic peptides were detected after PrP incubation without deuterium (0%, A) or in the presence of deuterium for 30 min. (B), 2 hr. (C), 24 hr. (D), or equilibrium deuterated for ≥72 hours (100%, E). The monoisotopic m/z value of 696.4 for this peptide of charge state +2 indicates that the peptide has a monoisotopic mass of 1390.8 Da. The red bar identifies the isotopic envelope centroid for each condition, which was used to calculate relative deuterium incorporation.

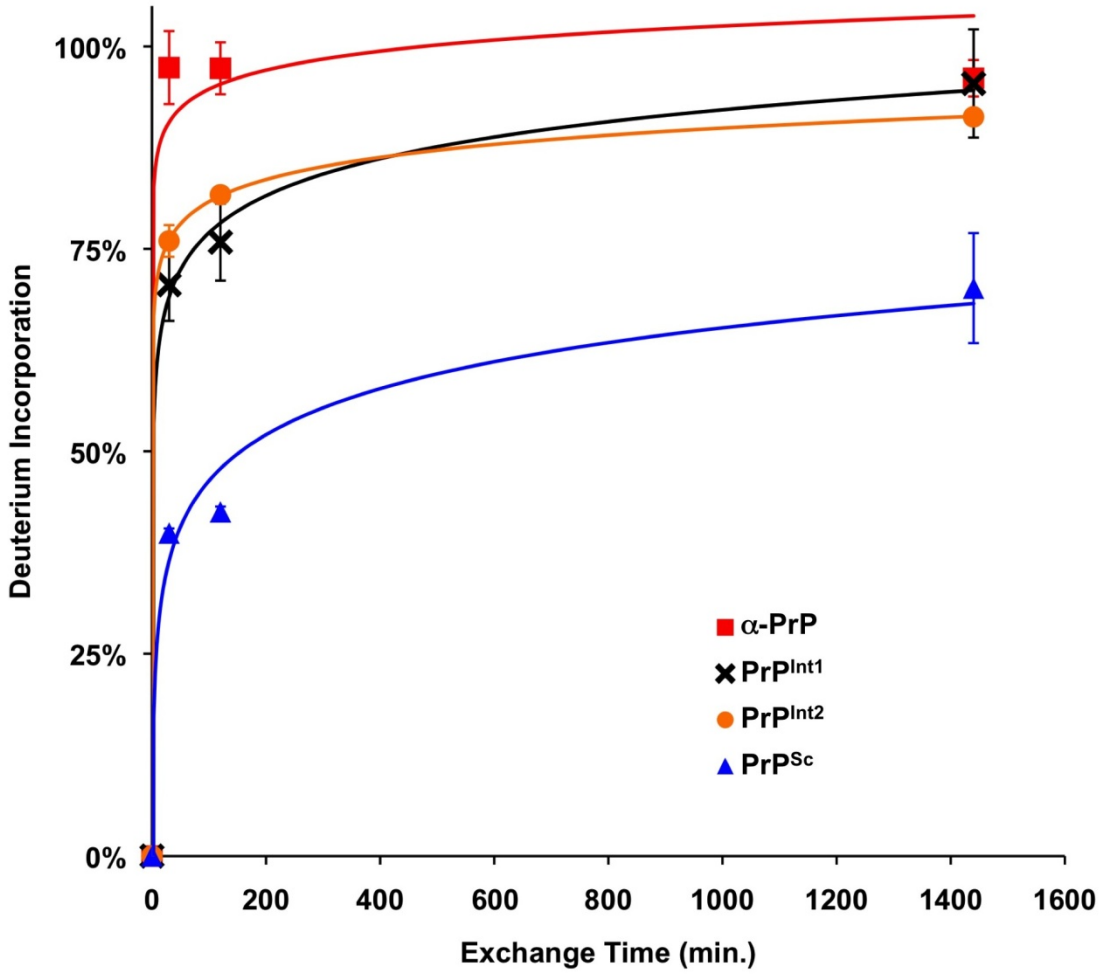


Figure S4. Kinetic curves for deuterium incorporation by peptide fragment PrP 196-201, Related to Figure 2. The following preparations of PrP were incubated at 22°C in D_2O for 30 min., 2 hr. (120 min.), or 24 hr. (1500 min.): α-PrP (red square, ■), PrP + POPG (black x, ✖), PrP + POPG + RNA (orange circle, ●), and PrP^{Sc} (blue triangle, ▲). Data points and bars, indicating standard error of the mean, are based on ≥2 independent experiments for each PrP conformation, as described in *Materials and Methods*. Logarithmic best-fit lines are shown to highlight the incorporation trend of each PrP conformation.

Supplemental Experimental Procedures

Western immunoblot detection of prion protein
Samples were mixed with SDS sample buffer, incubated at 95°C for 10 min., separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and detected with C-terminus-directed anti-PrP primary monoclonal antibody 27/33 and horseradish peroxidase (HRP)-conjugated anti-mouse sheep IgG secondary antibody (GE Healthcare, Piscataway, NJ). Signals were detected by enhanced chemiluminescence (ECL) (SuperSignal West Femto Substrate, Pierce, Rockford, IL) and visualized by a Fuji (Fujifilm) LAS-3000 chemiluminescence documentation system.

Deuterium exchange and quenching
PrP^{Int1}, PrP^{Int2}, or PrP^{Sc}, generated as described above, were mixed (1μg per sample) at 10 μg/mL with D_2O (buffered with 8.3 mM tris, 150 mM NaCl, pH* 7.2) or mock-exchange buffered H_2O, pH 7.2. pH* refers to reading of pH meter, without adjustment for hydrogen isotope effect. From this mixture, samples were aliquoted to separate tubes for different time points, and incubated at room temperature (22°C) to permit deuterium exchange for the indicated duration. For PrP conversion intermediates and PrP^{Sc} (which are insoluble) samples were centrifuged at 100,000x*g* at 4°C for the final 30 min. of the exchange period to collect the protein, discarding the supernatant. For α-PrP, 2 μL of 0.5 μg/mL α-PrP was mixed with 2 μL D_2O (buffered with 8.3 mM tris, 150 mM NaCl, pH* 7.2) and incubated for given duration as with the other analytes. Each deuterium exchange reaction was quenched by addition of ice-cold 0.8% formic acid, 6.4 M guanidine hydrochloride denaturant,150 mM tris(2-carboxyethyl)phosphine (TCEP) reducing agent, and incubated on ice for 2 min. while mixing by rapid micropipetting. Samples were then diluted with 3 volumes of 0.8% formic acid, 16.6% glycerol, transferred to autosampler microvials (product C4011-10, National Scientific, Rockwood, TN), frozen on crushed dry ice, sealed (11mm caps, product 200154, Sun Sri, Rockwood, TN) by 11mm manual crimper (National Scientific, Rockwood, TN), and stored at -70°C until analysis. The concentration of guanidine hydrochloride was calculated to be 4.3M during quenching and 1.1M after dilution. Prior to subsequent analysis, experimental samples were confirmed by Western blot to contain 1 μg of PrP.

Analysis of Deuterium Incorporation
Quenched samples were thawed at 0°C with a cryogenic autosampler, treated with in-line immobilized *Aspergillus saitoi* fungal protease XIII and pepsin digestion, then analyzed by reversed phase liquid chromatography and mass spectrometry as described previously (Burns-Hamuro et al., 2005). Specific to this study, samples were passed over an in-line immobilized *Aspergillus saitoi* fungal protease XIII column (30 mg/mL, 66 μL bed volume, Poros 20 AL medium) at 20 μL/min, with a 3 min. pause to increase digestion, followed by in-line immobilized porcine pepsin (30 mg/mL, 16 μL bed volume, Sigma-Aldrich, St. Louis, MO) on Poros 20 AL medium. Peptides were collected contemporaneously by a C18 column (Michrom MAGIC C18AQ 0.2x50), then eluted with a linear gradient of 8-48% solvent B over 30 min. (solvent A: 0.05% trifluoroacetic acid in water, solvent B: 80% (v/v) acetonitrile, 20% (v/v) water, 0.01% trifluoroacetic acid). All columns were immersed in ice. Eluted peptides were detected by an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with sheath gas flow of 8 units, a voltage of 4.5 kV, and a capillary temperature at 200ºC. Using a peptide pool generated

by tandem MS-MS and Proteome Discoverer software (Thermo Fisher Scientific, San Jose, CA), parent peptides and their isotopic mass were identified. Non-deuterated and deuterated peptides were verified by isotopic envelope comparison to calculated theoretical values with the use of DXMS Explorer software (Sierra Analytics, Modesto, CA). Of 355 total unique peptides identified with high confidence, including different charge states, an average of 160 were recovered in each experiment. Deuterium incorporation was determined by calculating the difference between the theoretical centroid of the experimental sample and a non-deuterated control that had been incubated in standard hydrogen water, relative to that of the equilibrium-deuterated PrP sample. Using the method of Zhang and Smith (Zhang and Smith, 1993), deuterium incorporation levels for each peptide were calculated as follows:

$$\text{Deuterium level (\%)} = \frac{m(S) - m(N)}{m(E) - m(N)} \times 100\%$$

m(S), m(N), and m(E) are the centroid values of the sample peptide, non-deuterated peptide, and equilibrium-deuterated peptide, respectively. Mean deuteron recovery was ~60%, measured by analysis of equilibrium-deuterated α-PrP prepared by incubation for ≥72 hours, as described previously (Hamuro et al., 2002). Deuterium incorporation values were normalized to 100% by applying a uniform multiplication factor of 0.9. Following initial identification, further peptide verification was performed by evaluation of consistency of deuterium incorporation between identical and overlapping peptides from each sample. Deuterium incorporation levels were sublocalized using overlapping peptides.

Data for each species of PrP were obtained by computing the mean deuteration levels from multiple replicate experiments (PrP^{C}: 3, PrP+POPG: 2, PrP+POPG+RNA: 2, PrP^{Sc}: 4). For the 2 hour time point of PrP+POPG+RNA, only one high-quality experiment was available for computation. For PrP^{Sc}, two experiments were performed for the 30 min. and 2 hr. time points.

Construction of ribbon maps to illustrate deuterium incorporation level was performed as described previously (Burns-Hamuro et al., 2005). To minimize artifact from back-exchange, the first time point for each peptide was treated as the minimum level of deuteration. Deuterium incorporation levels for each peptide were not applied to prolines, which lack an amide hydrogen, or to the first two amino acid residues of each peptide, which rapidly exchange due to proteolytic end effects (Bai et al., 1993). To minimize artifactual noise, ribbon diagrams were computed by averaging composite information of the surrounding 5 amino acids (Burns-Hamuro et al., 2005). Ribbon diagrams for changes between PrP conformers were prepared by subtracting the initial conformer local deuterium incorporation value from the subsequent conformer value on a 0-100% scale. The exchange-colored structure of the cellular prion protein was generated using the PyMOL Molecular Graphics System (Schrödinger) and the nuclear magnetic resonance structure of mouse PrP 121-231 (PDB 2L39) published by Damberger *et al*. (Damberger et al., 2011). This PDB structure showed only PrP 118-230 (using the mouse PrP numbering scheme consistent with this report), so the coloring for the unstructured N-terminus was added manually to the structure diagram.

Optimization of Pepsin Fragmentation of Prion Protein and Peptide Identification for Deuterium Exchange Mass Spectrometry (DXMS) Analysis

A number of quench and digestion conditions were tested to maximize recovery of identifiable PrP peptides (**Table S1**) with an LCQ Classic mass spectrometer (Thermo Scientific, Waltham, MA). These experiments were performed using α-PrP. Briefly, peptide recovery was optimized over quench time, concentration of guanidine in quench, concentration of TCEP in quench, and pepsin digestion time. A sample was also tested with trace amounts of Triton X-100 (0.0035% final) and n-octyl glucoside (nOG, 0.046% final) to determine if carryover from sample preparation would interfere with peptide analysis. This is described in greater detail in the following paragraphs:

To apply deuterium exchange mass spectrometry to investigate the conformational events that occur during PrP^{C} conversion to PrP^{Sc}, we developed a method to fragment the prion protein. This step, enabling analysis of deuterium uptake to the level of fine resolution, typically utilizes pepsin, a protease which is active at the low pH condition optimal for reducing deuterium back-exchange (Engen, 2009). We developed this method using PrP expressed recombinantly in *E. coli*, purified, and refolded *in vitro* to an α-helical structure (α-PrP) considered to have the same molecular structure as native PrP^{C} but lacking glycans and the glycolipid (glycosylphosphatidylinositol, GPI) anchor (Riek et al., 1997). Initial experiments using 1-4 μg of recombinant α-PrP permitted recovery of 30-40 identified peptic peptides from PrP on LC-MS (Supplementary Table 1), producing a map covering less than half of the mature prion protein sequence. Substantial gaps existed in the C-terminus, notably around each cysteine residue, suggesting interference from the disulfide bond in PrP (Turk et al., 1988) which might be ameliorated by greater reducing agent. The N-terminal and central regions of PrP were nearly devoid of peptide coverage.

In a series of experiments, we optimized the conditions for denaturing PrP and digesting with pepsin to substantially improve peptide coverage of the protein sequence. We found that increased protein input (to 10 μg PrP on the LCQ Classic spectrometer), increased tris(2-carboxyethyl)phosphine (TCEP) reducing agent, increased denaturation time in the quench reaction, and increased pepsin digestion time improved peptide recovery. Following optimization of these conditions, we consistently recovered 90-100 peptides (**Table S1**), spanning the entire prion protein sequence. This included robust coverage of the central and C-terminal portions of PrP, which together can form infectious prions without the N-terminus (Oesch et al., 1985). Though TCEP-mediated disulfide reduction was increased, improving recovery of peptides around the disulfide bond regions, recovery of peptides containing the actual cysteine residues was limited. We further experimented with conditions to retain peptide coverage while reducing handling and incubation time to minimize loss of incorporated deuterons from deuterated samples (back-exchange). We arrived at a set of conditions that included incubating the PrP in reaction quench solution for 2 min. at 0°C and digesting with pepsin for 5 min. We also determined that inclusion of detergents at low concentration would not compromise peptide recovery, in order for the protocol to be applicable to PrP samples treated with detergent. Finally, with the acquisition of an Orbitrap Elite mass spectrometer and the utilization of *Aspergillus saitoi* fungal protease type XIII, we were able to increase the recovery to an average of 160 high-confidence peptides per experiment (**Table S1**). A total of 355 unique peptides (including different charge states) were identified among all of the experiments reported in this study (**Fig. 2**).

Supplemental References

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