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Comparing the Extent of Methionine Oxidation in the Prion and Native Conformations of PrP

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ABSTRACT: Scrapie is a prion disease of sheep and goats. Prions (PrP^{Sc}) replicate by inducing a natively expressed protein (PrP^{C}) to refold into the prion conformation. PrP^{C} and PrP^{Sc} contain a disproportionately large number of methionines. Surface exposed methionines are more prone to chemical oxidation. Chemical oxidation is a means of measuring the surface exposure of the methionines in a prion, as these covalent changes are retained after an oxidized prion is denatured prior to analysis. Scrapie prions and recombinant sheep prion protein were oxidized in 0, 10, 20, or 50 mM solutions of hydrogen peroxide. The samples were digested with trypsin or trypsin followed by chymotrypsin to yield a set of peptides (TNMK, MLGSAMSR, ENMYR, IMER, VVEQMCIT-QYQR) containing the methionines present in sheep PrP. The

mass spectrometry based multiple reaction monitoring (MRM) method was used to analyze these peptides. Analysis of the rPrP samples showed that surface exposed methionines (132, 137, and 157) were more oxidized than those less surface exposed (209 and 216). The extent of methionine oxidation in sheep scrapie PrP^{Sc} is 216 > 137 > 132 > 157 > 209 > 112. These results demonstrate that this approach can be used to map the surface exposure of the methionines in order to distinguish among PrP conformations and effect a kind of conformational sequence.
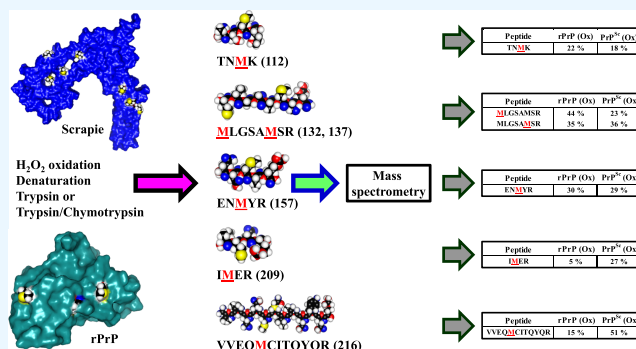
INTRODUCTION

Scrapie is a transmissible disease of sheep, first described in the late 17th or early 18th century.[1] Scrapie is caused by an infectious protein or prion (PrP^{Sc}) that induces the natively expressed prion protein (PrP^{C}) to refold into the prion conformation to propagate its pathology.[2,3] Detailed mass spectrometry-based analysis showed that PrP^{C} and PrP^{Sc} possess identical primary structures and post translational modifications and differ only in their conformations.[4] Although PrP^{C} and PrP^{Sc} possess identical covalent structures, PrP^{C} is not infectious, is monomeric, and is sensitive to proteinase K (PK) digestion, while PrP^{Sc} is infectious, multimeric, and possesses resistance to PK digestion.[2] The most infectious prion particle size has been estimated to be between 14−18 monomers.[5] The secondary structure of PrP^{C} is composed of random coil, α-helix and a small amount of β-sheet.[6−9] PrP^{Sc}'s secondary structure is composed largely of β-sheet.[10,11] The geometry of the β-sheet constrains adjacent amino side chains to project in opposite perpendicular directions from the plane of the β-sheet. This means that the side chains of adjacent amino acids may be in different chemical environments and react differently with the same reagent. Therefore, a change in the β-sheet register can dramatically change the chemical environment of an amino side chain.

Classical scrapie is transmitted among sheep by natural behaviors and from contaminated scrapie environments and susceptibility is highly dependent upon the PrP^{C} expressed by the sheep. Polymorphisms at positions 136, 154, and 171 strongly influence sheep susceptibility to scrapie[12−17] and the incubation period of the disease.[18] Among naturally infected scrapie flocks, sheep with valine (V) at position 136 are infected at higher rates than those with alanine (A) at the same position.[12,15,19,20] Polymorphisms at position 171 strongly influence scrapie susceptibility; sheep expressing glutamine (Q) are very susceptible, sheep expressing lysine (K) are more resistant, and those expressing arginine (R) have near complete resistance to classical scrapie.[12,13,21,22]

Two β-sheet geometries have been proposed for the prion's structure. Cryo-EM studies of hamster and mouse PrP^{Sc} have experimentally demonstrated a parallel in-register intramolecular β-sheet arrangement (PIRIBS) of the prion β-sheets.[23−25] A competing computational model proposed a 4-rung β-solenoid (4RβS) arrangement of the β-sheets.[26] A computational model showed that the PIRIBS geometry can act as a

template for a 4RβS structure, so the PIRIBS and 4RβS structures are not necessarily incompatible.[27]

Mammalian cells express enzymes that reduce oxidized methionines and this may be a general mechanism that cells use to minimize reactive oxygen species damage.[28,29] These mechanisms presumably maintain PrP^C's methionines in their native reduced state. When induced to adopt the prion conformation, the same would be true of PrP^{Sc}. Both PrP^C and its pathogenic conformer (PrP^{Sc}) possess a disproportionately greater number of methionines compared to other mammalian proteins.[30]

Even though all methionines are intrinsically susceptible to oxidation, those on the surface of a protein and, therefore, exposed to oxidants will be more susceptible to oxidation.[31] This suggests that the surface exposure of a prion's methionines can be measured by oxidizing them with chemical oxidants and then quantifying the extent of methionine oxidation.[32,33] These covalent modifications will be retained after a prion has been inactivated by denaturation prior to analysis. Unlike other oxidants,[34−38] hydrogen peroxide will oxidize methionine without significantly perturbing the prion conformation, as measured by a loss of infectivity that is within the estimation error of the incubation time interval assay used to measure the loss of infectivity.[35,39−41]

Hydrogen peroxide has been used to study the surface exposure of methionines of hamster recombinant PrP. Matrix assisted laser desorption ionization (MALDI) based mass spectrometry was used to detect some of the oxidized methionines.[42] The extent of methionine oxidation was consistent with the surface exposure of those methionines. An alternate mass spectrometry method, the multiple reaction monitoring (MRM) method (Figure S1), has been used to perform an analogous analysis of the tryptic, chymotryptic and tryptic/chymotryptic peptides derived from the enzymatic digestion of hamster recombinant PrP and hamster prions.[33] An improved method employed trypsin or trypsin followed by chymotrypsin digestion of PrP to yield a set of peptides that contains all of the methionines present in PrP.[32]

The conformation dependent differences in reactivity imparted by hydrogen peroxide oxidation are retained after different conformers are denatured prior to analysis. In principle, this approach can be used to define the extent of surface exposure of the methionines present in sheep scrapie and, thereby, sequence a conformation.

An MRM-based analysis of the extent of methionine oxidation after hydrogen peroxide of sheep scrapie was performed and reported herein.

■ MATERIALS AND METHODS

Chemicals. Chemicals (LC/MS grade acetonitrile and water and dithiothreitol) were purchased from Fisher Scientific (Pittsburgh, PA). Chymotrypsin (α; 3× crystallized zymogen) was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Other reagents, including trypsin (recombinant) were purchased from Sigma-Aldrich (St. Louis, MO). ^{15}N-ammonium chloride (^{15}NH$_4$Cl; 99.7% ^{15}N) was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA).

The instrument parameters were optimized using natural abundance (^{14}N) synthetic peptides that were purchased from Elim Biopharmaceuticals (Hayward, CA). The peptide sequences were verified by mass spectrometry. The peptides were of high (>95%) chemical purity as determined by LC/UV analysis. High incorporation (>99%) of the ^{15}N label into the

uniformly ^{15}N-labeled peptides was confirmed by mass spectrometry.

Samples and Sample Preparation. A sample of frozen brain tissue from a sheep naturally infected with classical scrapie was homogenized in enough sarkosyl solution (10% sarkosyl (sodium N-dodecanoyl-N-methylglycinate)) to make a 10% brain homogenate.[43] The sheep's PrP^C was homozygous for alanine (A) at position 136, arginine (R) at position 154 and glutamine (Q) at position 171 (ARQ).

The scrapie prions were enriched using a modified Bolton et al. procedure.[44] Briefly, the homogenate was clarified by centrifugation (16,000g; 18 min). A set of 12 identical aliquots from the sample was prepared: 12 mL of clarified homogenate was mixed with 24 mL of a 10% sarkosyl solution to yield a 36 mL solution. The 36 mL solution was evenly divided among 12 ultra centrifuge tubes (Beckman Coulter Quick-Seal; 16 mm × 38 mm polypropylene bell top sealable tubes). Each tube was underlaid with 1 mL of a 20% sucrose solution, sealed and placed in a Beckman 70.1 Ti ultracentrifuge rotor. The rotor was placed in a Beckman L8 80 M ultracentrifuge and ultracentrifuged (145,000g (r-average); 75 min; 20 °C). After the ultracentrifugation was completed, each tube was unsealed, the supernatant removed, and the pellet retained. Each pellet was resuspended in 90 μL of reaction buffer (0.1% n-Tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (Z 3−14) (w/v), 20 mM Tris pH 8.5) and transferred to a fresh microcentrifuge tube. These 12 samples were stored at −80 °C until ready for reaction with hydrogen peroxide.

Reacting the Scrapie Samples with Hydrogen Peroxide. The 12 sheep scrapie samples were divided into four sets of three samples, each in separate microfuge tubes. Each set of three was thawed at room temperature and each of the three tubes was sonicated (4 × 45 s bursts at maximum) using a microplate cup horn sonicator (Misonix S-4000; Qsonica LLC, Newtown, CT). A 10 μL portion of a 0.0, 0.1, 0.2, or 0.5 M hydrogen peroxide solution was added to each tube of the three-tube set and mixed to yield a final concentration of 0, 10, 20, or 50 mM hydrogen peroxide. Each three-tube set was placed in a 37 °C water bath and incubated for 15 min. The reaction was quenched by adding 50 μL of an aqueous L-methionine solution (355 mM L-methionine) to each tube and incubated for an additional 15 min in the water bath. 450 μL of 8 M guanidinium chloride solution (GdnCl) was added to each reaction mixture and allowed to stand for 24 h to inactivate the prions.[45] The inactivated prions were removed from the BSL2 laboratory to a BSL1 laboratory.

Reacting Recombinant PrP with Hydrogen Peroxide. A lyophilized tube of ARQ rPrP was solubilized in 450 μL of buffer (1 M GdnCl, 25 mM Tris pH 8.0). The tube was sonicated for 5 min in a sonicating water bath (Cole-Parmer Ultrasonic bath) at 50 °C.[42] Four 90 μL aliquots were removed and placed in separate microcentrifuge tubes. A 10 μL aliquot of a 0.0, 0.1, 0.2, or 0.5 M hydrogen peroxide solution was added to each of the four tubes to yield four reaction mixtures with 0, 10, 20, or 50 mM hydrogen peroxide. Each tube was mixed and incubated in a 37 °C incubator for 15 min. 50 μL of an aqueous L-methionine solution (355 mM L-methionine) was added and incubated for an additional 15 min in the 37 °C incubator.

Reduction and Alkylation of rPrP and Inactivated Scrapie Prions. Fresh aqueous solutions of 1 M dithiothreitol (DTT) and 500 mM iodoacetamide (IA) were prepared. rPrP

pellets were dissolved in 100 μL of 6 M μL GdnCl. Samples of inactivated prions were processed in the GdnCl from inactivation.

Enough 1 M Tris-HCl (pH 8.0) was added to yield a final concentration of 25 mM Tris-HCl (pH 8.0). Enough of the DTT solution was added to produce a final concentration of 25 mM DTT. The sample was reduced for 30 min (50 °C; 30 min) and sonicated at 0 and 15 min for 5 min. The reduced solution was cooled (25 °C). Enough IA was added to make a 75 mM solution. This solution was placed in the dark and allowed to react for 45 min at room temperature. The IA reaction was quenched by the addition of enough DTT to make a 25 mM solution. Cold methanol (−20 °C; 1.3 mL) was added to 200 μL of reduced and alkylated protein. The tube was stored in a −20 °C freezer for at least an hour. After storage for at least an hour, the solution was centrifuged (−11 °C; 20,000g; 20 min). The supernatant was removed and discarded. The pellet was resuspended in 500 μL of cold aqueous methanol (−20 °C; 85% v/v) and then centrifuged again (20,000g; −11 °C; 20 min). The supernatant was removed and discarded. After the pellet was dried, it was stored at −80 °C until ready for enzymatic digestion.

Preparation of Recombinant PrP. The gene encoding the mature (25−233) ARQ (GenBank accession #JX187517.1) sheep prion protein was cloned into the pET11a vector. The preparation of the ARQ clone was described previously.[46] The vector was cloned into BL21 (DE3) cells and stored as a glycerol stock (700 μL of an overnight Luria broth (LB) + 300 μL of sterile 50% aqueous glycerol) at −80 °C until needed.

A small sample of a glycerol stock was streaked out on a carbenicillin (100 μg/mL) LB agar plate that was incubated overnight at 37 °C. The minimal medium cultures were inoculated with a single colony from the overnight plate. A sterile solution of natural abundance (^{14}N)-M9 minimal medium (84.5 mM Na_2HPO_4, 44.4 mM KH_2PO_4, 17.1 mM NaCl, 37.4 mM $^{14}NH_4Cl$, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 33.2 μM thiamine, 22.2 mM glucose, and trace metals) and ^{15}N-M9 (same as ^{14}N-M9, except $^{14}NH_4Cl$ is replaced with $^{15}NH_4Cl$) were prepared.

To prepare a natural abundance (^{14}N-labeled) recombinant PrP (rPrP), the inoculum was added to 25 mL of ^{14}N-M9 medium supplemented with carbenicillin in a 250 mL baffled flask and cultivated overnight in a shaker incubator (37 °C; 250 rpm). Ten mL of the overnight culture was inoculated into 150 mL of ^{14}N-M9 medium (1 L baffled flask + 100 μg/mL carbenicillin) and cultivated in a shaker incubator (37 °C; 250 rpm). The OD_{600} of the culture was regularly measured until log growth was achieved (0.4 and 0.6). Once the log growth was achieved, the culture was supplemented with a sterile solution (1 M) of isopropyl β-D-thiogalactopyranoside (1 mM IPTG final concentration; 150 μL) and cultivated for 4 more hours (37 °C; 250 rpm). The culture was removed from the shaker incubator and centrifuged (10,000g; 5 min) to pellet the cells. The pellet was retained, and the supernatant discarded. The cell pellet was stored at −20 °C until it was processed.

Uniformly ^{15}N-labeled rPrP was prepared in an analogous manner. Two 250 mL baffled flasks were filled with 25 mL of ^{15}N-M9 medium and supplemented with carbenicillin (100 μg/mL) before inoculation. The first ^{15}N-M9 minimal medium was inoculated with a single colony and allowed to grow overnight (37 °C; 250 rpm). A 1 mL aliquot of the culture was placed in a microcentrifuge tube and centrifuged (3,000g; 3

min). After discarding the supernatant, 1 mL of sterile water was used to resuspend the pellet. Ten microliters of that supernatant was used to inoculate the second 250 mL baffled flask containing 25 mL of ^{15}N-M9 medium supplemented with carbenicillin (100 μg/mL) and allowed to grow overnight (37 °C; 250 rpm). Ten milliliters of the second overnight culture was used to inoculate a 1 L flask (150 mL of ^{15}N-M9 + 100 μg/mL carbenicillin) and processed in the same manner as the ^{14}N-labeled rPrP. After processing, the pellet contained uniformly ^{15}N-labeled cells.

The ^{14}N- or ^{15}N-labeled pellets were subjected to an inclusion body (IB) isolation procedure using standard molecular biology techniques. The IBs were resuspended in IB buffer (6 M GndCl 100 mM $NaPO_4$ pH 8.0, and 10 mM Tris-HCl pH 8.0) and centrifuged (10,000g, 5 min, 25 °C) to pellet insoluble cellular debris. Previously described procedures employing immobilized metal affinity chromatography (IMAC) were used to purify the supernatant. The uniformly ^{14}N- or ^{15}N-labeled rPrP-containing fraction was dialyzed overnight against ammonium acetate (100 mM, pH 4.5). After the overnight dialysis, the buffer was replaced fresh (50 mM ammonium acetate, pH 4.5) for an additional 2 h. The retentate was aliquoted (0.5 mL) and lyophilized overnight. The uniformly ^{14}N- or ^{15}N-labeled lyophilized rPrP samples were stored at −80 °C until needed.

Enzymatic Digestion. A solution of chymotrypsin (1 mg/mL chymotrypsin) in a storage buffer (1 mM HCl, 2 mM $CaCl_2$) was prepared in accordance with the manufacturer's instructions. A solution of trypsin (1 mg/mL) in storage buffer (1 mM HCl) was prepared in accordance with the manufacturer's protocols. These stock solutions were stored according to the manufacturer's recommendations at 4 °C until needed.

Each sample was digested with trypsin. The samples consisted of reduced and alkylated rPrP or inactivated, reduced, and alkylated prions. The samples were redissolved in 20 μL of buffer (0.01% β-octylglucopyranoside (BOG), 1 pmol/μL methionine, and 8% acetonitrile) and sonicated (50 °C; 5 min). Seventy-nine microliters of digestion buffer (25 mM ammonium bicarbonate (ABC), 0.01% β-octylglucopyranoside (BOG), 1 pmol/μL methionine, and 8% acetonitrile) was added. The samples were further sonicated (50 °C; 5 min). Each sample was digested with trypsin, by the addition of 1 μL of the trypsin stock solution, gently mixed and incubated overnight (37 °C). Half of the overnight trypsin digest (50 μL) was placed in a new microcentrifuge tube. Addition of 1.25 μL of a 10% aqueous formic acid solution quenched the reaction. The trypsin digest was filtered through a twice washed (0.5 mL of sterile water) 10,000 MWCO centrifugal filter (14,000g; 12 min). Until the filtrate was ready for mass spectrometry-based analysis, it was stored at −80 °C.

The unquenched 50 μL of the trypsin digest was digested with chymotrypsin. Chymotrypsin (0.5 μL stock solution, 2 mM $CaCl_2$ per reaction; 30 °C) was added and allowed to digest for 5, 10, 15, 30, 45, or 60 min, depending on the experiment. After the timed completion of the digestion, 1.25 μL of a 10% aqueous formic acid solution was added to stop digestion. The chymotrypsin digest was filtered through a twice washed (0.5 mL of sterile water) 10,000 MWCO centrifugal filter (14,000g; 12 min). Until the filtrate was ready for mass spectrometry-based analysis, it was stored at −80 °C.

Peptide Optimization. The required natural abundance peptides were obtained from a commercial vendor (Elim

Biopharmaceuticals, Hayward, CA). The optimal MRM parameters for each peptide were empirically determined using previously described methods.[47] A summary of the relevant MRM parameters may be found in Table S1 (^{14}N) and Table S2 (^{15}N).

Mass Spectrometry. A combined autosampler, column switcher, and liquid chromatography system (Applied Biosystems Tempo nanoflow LC system (AB Sciex LLC; Framingham, MA)) was used to deliver the mobile phase. Each digest (6 μL) was loaded onto a trapping cartridge (C-18; Acclaim PepMap100, 5 μm, 100 Å, 300 μm ID × 5 mm (Thermo Scientific Dionex, Sunnyvale, CA)) and washed for 3−5 min with loading buffer (acetic acid (0.5%)/acetonitrile (1%)/heptafluorobutyric acid (0.02%)/water (98.48%)) at a flow rate of 5 μL/min. After the loading/wash step was completed, the flow was reversed, and the trapped peptides eluted onto a reversed-phase column (Vydac Everest C18 column (HiChrom, Leicestershire, UK) 238EV5.07515, 75 μm ID × 150 mm). The samples were chromatographed using a binary gradient composed of eluants A (2% acetonitrile with 0.5% acetic acid in water) and B (99.5% acetonitrile with 0.5% acetic acid).

A 30 min binary linear gradient (300 nL/min) was initialized with 95% buffer A and 5% B and ended with 10% A. After 30 min, the new binary eluant mixture (10% A; 90% B) was maintained for 20 min. After 20 min, the binary eluant was returned to the starting condition over 5 min and maintained at this composition for 5 more minutes. An Applied Biosystems Nanospray II electrospray assembly was used to continuously nebulize the eluant through a spray tip (Non coated; FS360−20- 10-N-20-C12, New Objective Inc., Woburn, MA).

An Applied Biosystems (AB Sciex LLC; Framingham, MA) model 4000 Q-Trap instrument was used for quantitative mass spectrometry. The instrument was operated in positive multiple reaction monitoring (MRM) mode. The instrument's settings allowed it to alternate between detecting the sample's (^{14}N) peptides and their stable isotope (^{15}N-) labeled analogs. Previously described methods were used to determine the optimal MRM parameters for each peptide and its ^{15}N-labeled analog.[47] A tabular summary of these parameters is included in the Supporting Information Tables S1 and S2. Quantitation of the natural abundance and ^{15}N-labeled peptides was accomplished with the IntelliQuan quantification algorithm (Analyst 1.6.3 software; AB Sciex).

Preparing ^{15}N-Labeled Internal Standards. The appropriate uniformly ^{15}N-labeled sheep rPrP polymorphism (vide supra) was reduced and alkylated. The appropriate protein was digested overnight with trypsin, quenched with formic acid, and centrifugally filtered (10,000 MWCO; 14,000g; 12 min; 25 °C) to yield a set of uniformly ^{15}N-labeled tryptic peptides (Figure S2), PGGGWNTGGSR, YPGQGSPGGNR, TNMK, YYR, ENMYR, YPNQVYYR, GENFTETDIK, IMER, VVEQMCITQYQR, and ESEAYYQR. These peptides were used as uniformly ^{15}N-labeled internal standards for analysis and preparation of calibration curves.

The uniformly ^{15}N-labeled MLGSAMSR peptide was prepared by digesting an unquenched portion of the overnight trypsin digest with chymotrypsin for an additional 15 min, quenching it with formic acid, and then centrifugally filtering (10,000 MWCO; 14,000g; 12 min; 20 °C) it to yield the necessary uniformly ^{15}N-labeled MLGSAMSR peptide.

Analysis of Sample Data. The natural abundance (^{14}N) and ^{15}N-labeled peptides were quantitated using the IntelliQuan quantification algorithm (Analyst 1.6.3 software; AB Sciex). The integrated areas of the MRM signals for the optimized peptides in the method were reported by the software. The reported area of a methionine-containing peptide is the sum of the MRM signals from the unoxidized and oxidized forms of the peptide. The areas of methionine-free peptides correspond to the reported area of the MRM signal for the methionine free peptide.

Statistical and regression analysis was performed with Excel. PyMOL was used to calculate relative solvent-accessible surface area (SASA).

Safety Considerations. Scrapie prions are infectious and must be handled in a BSL2 laboratory that is certified and inspected by the Animal and Plant Health Inspection Service (APHIS) of the USDA (www.aphis.usda.gov/permits/). The sixth edition of the CDC's biosafety manual, Biosafety in Microbiological and Biomedical Laboratories describes the proper procedures for handling these agents.[48] Scrapie prions were diluted with enough 8 M guanidinium chloride (GdnCl) and thoroughly mixed to make a 6 M GdnCl solution to inactivate them. The solution was allowed to stand for at least 24 h at room temperature.[45] The inactivated prions in 6 M GdnCl were transferred to new microcentrifuge tubes and relocated to a BSL-1 laboratory for further processing. After PrP digestion with proteases, the samples were filtered by centrifugation through a 10 kDa MWCO filter.

■ RESULTS AND DISCUSSION

Optimized MRM and Chromatographic Parameters of Relevant Peptides. Four tryptic peptides, TNMK, ENMYR, IMER and VVEQMCITQYQR, and one peptide derived from a combined tryptic and chymotryptic digestion, MLGSAMSR, were used in this analysis. The MRM parameters for each peptide were determined by previously described methods. These optimized MRM parameters are summarized in Tables S1 and S2.

A reversed phase trapping cartridge was used to retain the peptides and wash off the extraneous buffer ions. There was concern that some peptides may not be retained on the trapping cartridge compared to others after a wash step. The peptides ENMYR, IMER, VVEQMCITQYQR, and MLGSAMSR were retained on the trapping cartridge after a 3, 4, or 5 min wash step (data not shown). The same was true of the oxidized analogs (data not shown). The TNMK peptide behaved differently.

The retention properties of the unoxidized TNMK peptide were explored. Unoxidized TNMK was obtained from the tryptic digestion of unoxidized ^{14}N-recombinant sheep PrP (rPrP; ARQ = 136 alanine (A), 154 arginine (R), 171 glutamine (Q)) (99% unoxidized). The unoxidized sample was loaded onto the trapping cartridge with 2, 3, 4, or 5 min of washing (Figure S3). There was a statistically significant difference ($p < 0.02$) between the MRM signal intensity ($n = 6$) observed for 2 min vs 3, 4, or 5 min. There was no statistically significant difference ($p > 0.34$) between the MRM signal intensity for 3, 4, or 5 min of washing. The loading properties of the oxidized TNMK peptide were then evaluated.

A sample of oxidized ^{14}N-rPrP (ARQ) was digested overnight with trypsin to yield the oxidized TNMK peptide (∼95% oxidized). The graph of the MRM signals from the oxidized TNMK peptide after loading for 2, 3, 4, or 5 min of
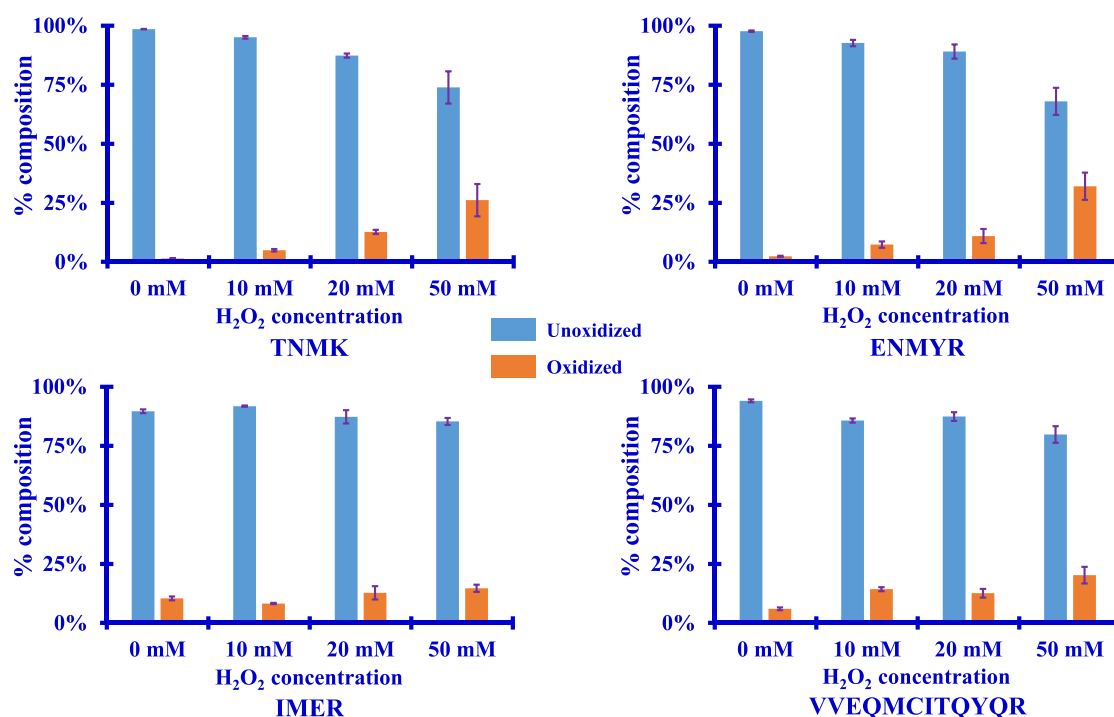
Figure 1. Proportion of the oxidized and unoxidized forms of tryptic peptides from oxidized ^{15}N-sheep rPrP (ARQ). ^{15}N-Sheep rPrP (ARQ) was oxidized with either 0, 10, 20, or 50 mM hydrogen peroxide, reduced, alkylated, and then digested overnight with trypsin. The tryptic peptides, ^{15}N-TNMK (250.1 → 397.2 transition), ^{15}N-ENMYR (361.1 → 343.2 transition), ^{15}N-IMER (278.1 → 441.2 transition), ^{15}N-VVEQMCITQYQR (524.9 → 705.3 transition), were analyzed by mass spectrometry (n = 4 or 8). The proportion of unoxidized and oxidized peptides for each condition were graphed. The location of these peptides can be found in Figure S2. Methionines 112, 157, 209, and 216 are contained in the ^{15}N-TNMK, ^{15}N-ENMYR, ^{15}N-IMER, and ^{15}N-VVEQMCITQYQR peptides, respectively.

washing are summarized in Figure S4. The MRM signal intensity drops ~98% after 4 min of washing compared to 3 min. The MRM signal intensity increases 1.6 fold after 3 min compared with 2 min ($p < 0.03$).

Another sample of ^{14}N-rPrP was oxidized to a lesser extent (~75% oxidized TNMK) and analyzed. The sample was loaded onto the trapping cartridge and washed for 2, 3, 4, or 5 min. The proportion of the oxidized and unoxidized peptides in the samples were graphed (Figure S5). There is no statistical difference in the proportion of the oxidized and unoxidized peptide with a washing time of 2 or 3 min ($p > 0.9$). Significant differences ($p < 0.01$) were observed after 4 or 5 min of washing. The optimal washing time for the TNMK peptide was determined to be 3 min.

Optimization of the Peptide to Measure the 132 and 137 Methionine Oxidations. A sample of uniformly ^{15}N-sheep rPrP (ARQ) was oxidized with hydrogen peroxide. The oxidized protein was digested overnight with trypsin. A portion was further digested with chymotrypsin for different times (5, 10, 15, or 30 min) to yield the MLGSAMSR peptide. The percentage of the unoxidized peptide, met132 oxidized, met137 oxidized, and met132 plus met137 oxidized peptides were calculated. The sum of the signal intensities for the unoxidized and oxidized peptides was recorded. These data are summarized in Figures S6 and S7.

These results indicate that overnight digestion of rPrP with trypsin yields the MLGSAMSR peptide, but the proportion of oxidation is not representative of the actual protein (Figure S6). When the tryptic digest is further digested with chymotrypsin, more of the peptide is produced (Figure S7). The distribution of oxidation from the overnight trypsin

digestion is statistically significantly different from the subsequent chymotryptic digestion at 5, 10, 15, or 30 min (Figure S6). After either 5 or 15 min of additional chymotrypsin digestion the distribution of oxidation was not statistically different ($p > 0.05$). Furthermore, the signal intensity of the MLGSAMSR peptide after 15 min of chymotrypsin digestion is twice the intensity of that observed after 5 min of chymotryptic digestion (Figure S7).

A sample of scrapie (ARQ) was oxidized with 50 mM hydrogen peroxide. The sample was processed and digested overnight with trypsin. The overnight digest was divided into five samples; one portion was quenched immediately. The other four were further digested with chymotrypsin for 5, 10, 15, or 30 min and then quenched. The unoxidized and oxidized forms of the MLGSAMSR peptide were analyzed for each sample. The percentage of the unoxidized peptide, singly oxidized (met132 or met137), and doubly oxidized (met132 + met137) peptides were calculated and graphed (Figure S8).

These results are comparable with those observed when oxidized rPrP (ARQ) is digested overnight with trypsin and subjected to a timed chymotryptic digestion. These results clearly show that the amount of the MLGSAMSR peptide produced by an overnight tryptic digestion is insufficient for analysis of a scrapie sample. Further digestion with chymotrypsin results in more MLGSAMSR peptide production ($p < 0.001$). Further digestion results in a change of the proportion of oxidized and unoxidized MLGSAMSR peptides in the samples between 5 and 30 min of digestion ($p < 0.02$). There was no statistical difference ($p > 0.07$) in the composition of the peptide mixture between a 5 and 15 min chymotryptic digestion. Furthermore, a 15 min chymotrypsin

digest yields ~13% more MLGSAMSR peptide than a 5 min chymotryptic digestion. Thus, an overnight digestion with trypsin followed by a 15 min digestion is appropriate to generate the MLGSAMSR peptide from scrapie samples.

Ensuring That There Are No Confounding Molecules in PrP^Sc Digests. Approximately 95% of the prions in a sample are isolated by the method of Bolton et al.[44] Prions are generally resistant to Proteinase K (PK) digestion, so PK is often used to digest the other PK labile proteins isolated with PrP^Sc. Up to 80% of sheep scrapie is PK-sensitive (sPrP^Sc) and can be lost by PK digestion.[46] To avoid this loss, PK was not used. This also means that the molecules produced by the tryptic or tryptic/chymotryptic digestion of coisolated, non-PrP^Sc proteins may interfere with this analysis.

To test this, a sample of scrapie (ARQ) was digested overnight with trypsin, both without and with a subsequent 15 min chymotryptic digestion. The samples were analyzed by mass spectrometry with the addition of an appropriate uniformly ^15N-labeled internal standard. The MRM chromatograms of the tryptic peptides (TNMK, ENMYR, IMER, and VVEQMCITQYQR) and the tryptic/chymotryptic peptide (MLGSAMSR) were plotted (Figure S9) and examined to determine if a confounding molecule was present in the scrapie samples. This analysis revealed that the samples contained no confounding molecules that would interfere with this analysis.

Oxidizing Recombinant PrP. The native conformation of the ARQ sheep rPrP was oxidized with hydrogen peroxide. Although rPrP lacks the GPI anchor and the asparagine linked glycosylation of PrP^C, when the NMR structures of bovine rPrP and PrP^C were compared they were determined to be "essentially identical".[49] The structure of sheep ARQ rPrP has been determined to a 2.5 Å resolution.[50] Thus, recombinant sheep PrP (ARQ) is a suitable surrogate for sheep PrP^C (ARQ) and the utility of quantifying methionine oxidation to map the surface of rPrP can be evaluated.

Hydrogen peroxide was used to map the surface of the rPrP (ARQ) prion protein, whose structure is known.[50] If a methionine is on the surface of a protein and exposed to water, then it is more susceptible to oxidation by hydrogen peroxide than a methionine in the hydrophobic region that is not exposed to water. ^15N-Sheep rPrP was oxidized with hydrogen peroxide, reduced, alkylated, digested with trypsin and then analyzed by mass spectrometry. The percentage of oxidized and unoxidized methionine-containing tryptic peptides was determined for TNMK (methionine 112), ENMYR (methionine 157), IMER (methionine 209) and VVEQMCITQYQR (methionine 216) and summarized in Figure 1. These results show that when the oxidation initially present in the sample (0 mM added H_2O_2) is subtracted from the 50 mM reaction, only 4% ± 2% of methionine 209 (IMER) is oxidized and 14% ± 4% of methionine 216 VVEQMCITQYQR is oxidized. In contrast, 32% ± 6% of methionine 157 (ENMYR) is oxidized. 25% ± 7% of methionine 112 (TNMK) is oxidized. These results indicate that methionines 209 and 216 have limited surface exposure, while methionine 157 and 112 are more surface exposed.

Methionines 132 and 137 are contained in the MLGSAMSR peptide. This peptide is derived from an overnight trypsin digest followed by a 15 min chymotryptic digestion. A portion of ^15N-Sheep rPrP that was oxidized with hydrogen peroxide, reduced, alkylated, and digested with trypsin, was further digested with chymotrypsin for 15 min, and then analyzed by mass spectrometry. The percentage of oxidized and unoxidized

methionine containing tryptic/chymotryptic peptides was determined for unoxidized and oxidized forms of the MLGSAMSR (methionines 132 and 137) peptide. These results are summarized in Figure 2. Most (67% ± 2%) of the
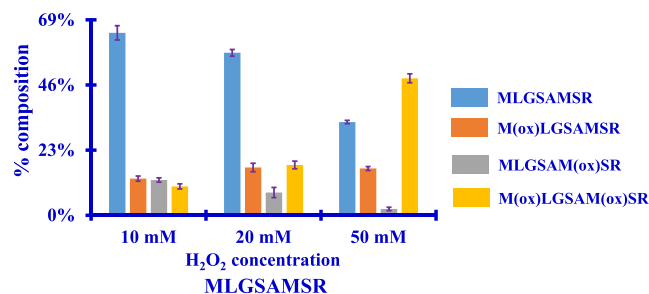

Figure 2. Proportion of the oxidized and unoxidized forms of tryptic/chymotryptic peptides from oxidized ^15N-sheep rPrP (ARQ). ^15N-Sheep rPrP (ARQ) was oxidized with either 10, 20, or 50 mM hydrogen peroxide, reduced, alkylated, digested overnight with trypsin, and then digested with chymotrypsin for an additional 15 min. The tryptic/chymotryptic peptide, ^15N-MLGSAMSR (432.2 → 617.3 transition), and the oxidized analogs, ^15N-M(ox)LGSAMSR (440.2 → 617.3 transition), ^15N-MLGSAM(ox)SR (440.2 → 633.3 transition), ^15N-M(ox)LGSAM(ox)SR (448.2 → 633.3 transition), were analyzed by mass spectrometry (n = 4). The proportion of unoxidized and oxidized peptides for each condition were graphed (background oxidation subtracted). The location of this peptide can be found in Figure S2. Methionines 132 and 137 are contained in the ^15N-MLGSAMSR peptide.

MLSAMSR peptide was oxidized with 50 mM hydrogen peroxide. 48% ± 2% was doubly oxidized, 16.5% ± 0.7% of methionine 132 was oxidized and 2.2% ± 0.6% of methionine 137 was oxidized. These results indicate that methionines 132 and 137 are surface exposed with methionine 132 being more exposed than 137.

The structure of sheep ARQ rPrP has been determined by X-ray crystallography.[50] A table of the relative solvent-accessible surface area (SASA) of rPrP's methionines are shown in Figure S10. Based on these calculations, methionines 132 and 137 are surface exposed, with methionine 132 being more exposed than 137. Methionine 157 is also surface exposed. Methionines 209 and 216 have minimal surface exposure. The crystal structure only includes amino acids 114–234, so it can offer no guidance as to the surface exposure of methionine 112. Methionine 112 is part of the random coil and so would be expected to be susceptible to oxidation, although the extent of the exposure is not known. These results show that the covalent modification of methionines by hydrogen peroxide is consistent with relative SASA calculations based on a known structure, as has been previously observed with hamster rPrP.[33,42]

The scrapie samples were isolated by a modified version of the method of Bolton et al.[44] The method of Bolton et al. isolated both proteinase K sensitive and resistant PrP^Sc.[51] Proteinase K sensitive and resistant PrP^Sc are infectious and share "a basic structure".[52] Previously, we showed that when this method is applied to uninfected sheep (ARQ/ARQ) brain tissue, we isolate 23 ± 5 fmol/50 mg of brain tissue.[53] For comparison, 50 mg of sheep brain contains approximately $7 \times 10^3 ± 1 \times 10^3$ fmol of PrP^C.[54] This means that the Bolton et al. procedure removes more than 99% of the PrP^C in a sample. Previously, we determined that $4.6 \times 10^4 ± 2 \times 10^3$ fmol per gram of PrP^Sc is present in analogous samples to the ones used
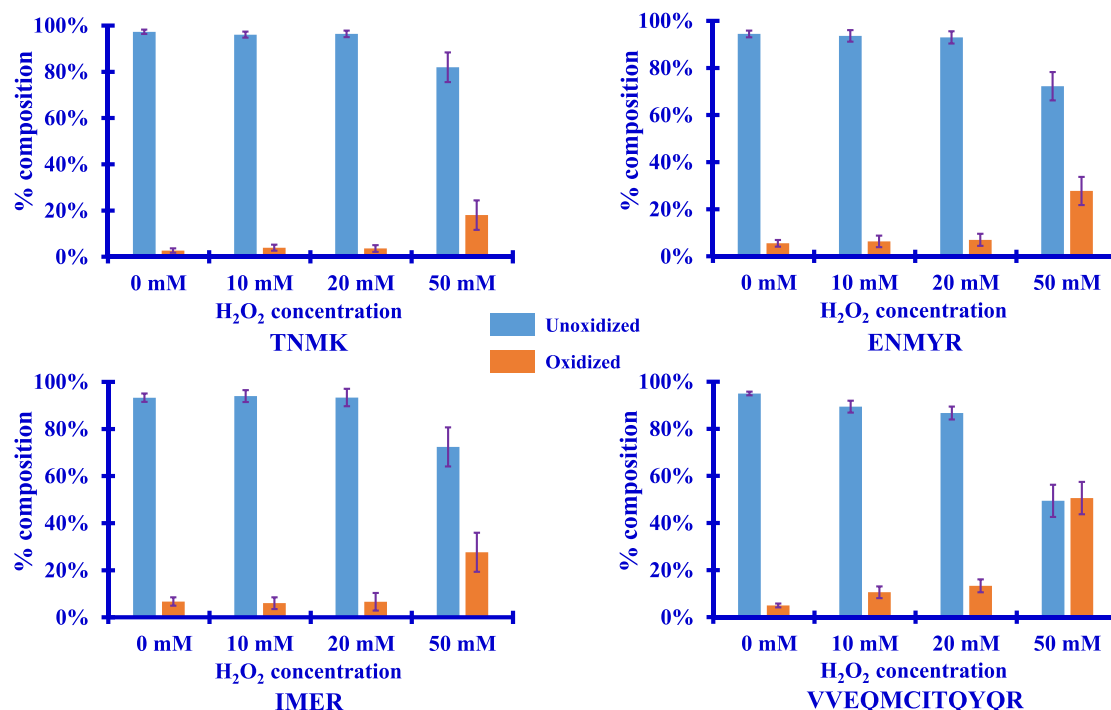
Figure 3. Proportion of the oxidized and unoxidized forms of tryptic peptides from oxidized sheep scrapie (ARQ; $n = 3$). Sheep scrapie (ARQ) was oxidized with either 0, 10, 20, or 50 mM hydrogen peroxide, reduced, alkylated, and then digested overnight with trypsin. The tryptic peptides, ^{14}N-TNMK ($247.1 \rightarrow 392.2$ transition), ^{14}N-ENMYR ($356.7 \rightarrow 338.2$ transition), ^{14}N-IMER ($274.1 \rightarrow 435.2$ transition), ^{14}N-VVEQMCITQYQR ($518.9 \rightarrow 695.3$ transition), were analyzed by mass spectrometry ($n = 4$ or 8). The proportion of unoxidized and oxidized peptides for each condition were graphed. The location of these peptides can be found in Figure S2. Methionines 112, 157, 209, and 216 are contained in the ^{14}N-TNMK, ^{14}N-ENMYR, ^{14}N-IMER, and ^{14}N-VVEQMCITQYQR peptides, respectively.

in this study.[43] Furthermore, we showed that PrP isolated by this method is proteinase K resistant.[43,46] Thus, the PrP in the samples we analyzed contains both PK sensitive and PK resistant forms of PrP^{Sc} and is predominantly (99%) of PrP^{Sc}.

The prion samples were redissolved in buffer before oxidation. Four different concentrations (0, 10, 20, or 50 mM) of hydrogen peroxide were used to oxidize three separate samples (12 total). Each prion sample was inactivated by GdnCl, reduced, alkylated, and then digested with trypsin overnight. A portion of the overnight trypsin digestion was further digested with chymotrypsin. This resulted in the production of a set of four tryptic peptides and one tryptic/chymotryptic peptide containing all of the methionines in sheep PrP.

Mass spectrometry was used to analyze the 12 samples, in triplicate, at each hydrogen peroxide concentration. The data for the tryptic peptides (TNMK, ENMYR, IMER, and VVEQMCITQYQR) were graphed and summarized in Figure 3. The data for the tryptic/chymotryptic peptide MLGSAMSR is graphically summarized in Figure 4.

The methionines in the scrapie conformation have different surface exposures compared to that observed in the native cellular prion protein conformation (rPrP conformation) after being oxidized in 50 mM hydrogen peroxide. Methionine 112 (TNMK) is less oxidized in the scrapie conformation (18% ± 6%) compared to the rPrP conformation (25% ± 7%). The extent of methionine 157 oxidation is less (22% ± 6% vs 32% ± 6%) in scrapie than in rPrP ($p < 0.04$). Methionine 209 (IMER) is much more ($p < 0.001$) exposed (21% ± 8%) in the scrapie conformation than in the native conformation (4% ± 2%). In the prion conformation, methionine 216 is the most
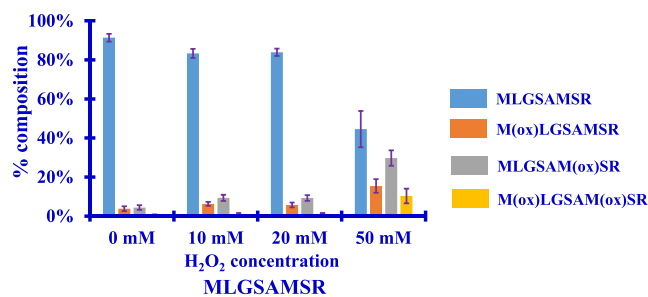

Figure 4. Proportion of the oxidized and unoxidized forms of the tryptic/chymotryptic peptide, MLGSAMSR, from oxidized sheep scrapie (ARQ; $n = 3$). Sheep scrapie (ARQ) was oxidized with either 0, 10, 20, or 50 mM hydrogen peroxide, reduced, alkylated, digested overnight with trypsin, and then digested with chymotrypsin for an additional 15 min. The tryptic/chymotryptic peptide, ^{14}N-MLGSAMSR ($426.7 \rightarrow 608.3$ transition), and the oxidized analogs, ^{14}N-M(ox)LGSAMSR ($434.7 \rightarrow 608.3$ transition), ^{14}N-MLGSAM-(ox)SR ($434.7 \rightarrow 624.3$ transition), ^{14}N-M(ox)LGSAM(ox)SR ($442.7 \rightarrow 624.3$ transition), were analyzed by mass spectrometry ($n = 8$). The proportion of unoxidized and oxidized peptides for each condition were graphed (background oxidation subtracted). The location of this peptide can be found in Figure S2. Methionines 132 and 137 are contained in the ^{14}N-MLGSAMSR peptide.

exposed ($p < 0.001$) to oxidation (46% ± 7%) compared to the native conformation (14% ± 4%). Methionines 132 and 137 (MLGSAMSR) are susceptible to oxidation in the native conformation, since the unoxidized peptide comprises much less (32.9% ± 0.6%) of the mixture than the doubly oxidized one (methionines 132 and 137; 48% ± 2%). In the native conformation, methionine 132 is more susceptible to oxidation

than methionine 137 ($p < 0.001$). In the prion conformation methionines 132 and 137 are less susceptible to oxidation, as the unoxidized peptide comprises more (45% ± 9%) of the mixture than the doubly oxidized peptide (10% ± 4%). In the prion conformation, methionine 137 (36% ± 5%, when the doubly oxidized peptide is included; Figure S11) is more susceptible ($p < 0.001$) to oxidation than is methionine 132 (23% ± 5%, when the doubly oxidized peptide is included; Figure S11). These results show that quantifying the oxidation of methionine in PrP can be used to distinguish among PrP conformers.

The chemical environment of the same methionine in different prion strains propagating in the same species expressing the same PrP^C will vary in a conformation-dependent manner. Such differences in the conformation (strain) dependent differences in the chemical environment can be seen in Cryo-EM parallel in-register intramolecular β-sheet (PIRIBS) structures of two mouse-adapted prions, RML and Me7 (Figures S12 and S13).[24,55] SASA calculations associated with the figures include the surface exposure of the methionines on the surfaces at the ends of a fibril and in the interior of the fibril. Since prions are multimeric, with the infectious multimers being greater than five monomers, the interior environment of the fibril will predominate over the surfaces at the ends of the fibril. Methionines 128, 205, and 212 (mouse numbering) have little surface exposure while methionines 153 and 204 have much greater surface exposure in the RML and Me7 strains. Methionines 133 and 137 are more surface exposed in the Me7 strain than in the RML strain. The SASA calculations predict that oxidation of the Me7 and RML prions would result in greater oxidation of methionines 133 and 137 in the Me7 strain than in the RML strain. In principle quantifying differences in the oxidation of the same methionine in different prion strains that refolded the same PrP^C in a species can be used to fingerprint prion strains.

In elk, a polymorphism at position 132 (elk numbering; methionine (M) or leucine (L)) determines strain differences.[56,57] Homozygous LL132 elk propagate a different prion strain than either MM132 or ML132 elk.[56,57] The PrP^C that is refolded into PrP^{Sc} has different amino acids at position 132 (M or L). This necessitates an adaptation of the MRM method to analyze the samples that contain peptides with either an M or L polymorphism at position 132.[32,58] The two elk prion strains are conformationally different which may place the same methionines in an environment with greater surface exposure in one conformation (strain), than in the other. The same approach can be used to analyze the prions from polymorphic sheep.[32,53,59] The MRM method can be adapted to quantify the extent of methionine oxidation in prion strains refolded from polymorphic variants of PrP^C from the same species. In principle, this can be used to fingerprint strains with polymorphic differences.

Using this approach to compare the exposure of methionines in two different species is complicated. The MRM method can be adapted to quantify the extent of methionine oxidation in many species.[32] The sequence alignments of six different species are shown in Figure S14. PrP^C from sheep, elk, and deer has the same number of amino acids (210) and differ only in their polymorphic composition. PrP^C from bank vole and hamster has one less amino acid than does PrP^C from sheep, elk, or deer. It is very near the N-terminus, the portion that is cleaved by proteinase K. The glycosylphosphatidylinositol (GPI) anchor is attached to the C-terminus. These five

sequences also align at the C-terminus. In contrast, mouse PrP^C contains three fewer amino acids in the N-terminus and an extra amino acid in the C-terminus. When mouse PrP^C refolds into the prion conformation of another species, this extra C-terminal amino acid may change the register of the mouse-adapted prion's β-sheet. If this were to occur, the change in register may dramatically alter the surface of methionines in the mouse-adapted prion, compared to the progenitor prion. This may result in the pattern of methionine oxidation of the mouse-adapted prion differing from that of the methionine oxidation pattern of the progenitor prion. Thus, the methionine oxidation patterns or fingerprints of the progenitor and new species-adapted prions may be very different.

The structures of a hamster prion strain (263K) and a chronic wasting disease (CWD) prion from a white-tailed deer have been determined by high resolution cryo-EM.[23,60] Both have a PIRIBS architecture (Figures S15 and S16). The relative SASA calculations for each methionine in these prion structures is included for each structure and a table translating the position of the methionines in sheep PrP to their position in the analogous hamster, mouse, and white-tailed deer PrP numbering systems is also included (Figures S15 and S16). The methionine at position 216 in sheep PrP^{Sc} is the most oxidized, implying that it has significant surface exposure. The analogous methionines in mouse (212), hamster (213), and deer (216) have little surface exposure, except on the surfaces of the fibril ends, suggesting that sheep PrP^{Sc} may have a different register in this region than mouse, hamster, or deer PrP^{Sc}. In the sheep PrP^{Sc}, methionines 157 and 209 are comparably ($p > 0.5$) oxidized, 22% ± 6% and 21% ± 8%, respectively, suggesting comparable surface exposures. In contrast, the analogous methionines in mouse (153 and 205), hamster (154 and 206), and deer (157 and 209) are dissimilarly surface exposed, suggesting that methionine 209 in sheep PrP^{Sc} is in a different register than that of the homologous methionine in mouse or hamster PrP^{Sc}. In sheep PrP^{Sc}, methionine 137 is more surface exposed than methionine 132, based on the relatively greater extent of its oxidation. In mouse (128 and 133) and hamster (129 and 134) PrP^{Sc}, methionines 128 and 129 are less surface exposed than methionines 133 and 134, respectively. In deer CWD both methionines (132 and 137) are similarly not surface exposed. These results indicate that the structure of sheep scrapie is different from that of hamster, mouse or deer PrP^{Sc}. It also indicates that this approach may provide useful, albeit qualitative and necessarily limited information about the structure of prions that have not been determined by Cryo-EM.

■ CONCLUSIONS

Prions are primarily composed of β-sheet secondary structure, where the side chains of adjacent amino acids project in opposite perpendicular directions from the plane of the β-sheet. Changes in the conformation of a prion may result in a different register or other subtle differences in structure that may place a methionine in a significantly different chemical environment in one conformation compared to another. This may result in the same methionine being more surface exposed and, therefore, susceptible to oxidation in one prion conformation or strain than in another. Measuring the extent of oxidation in homologous methionines from different prions in the same or different species may, in principle, provide a

means of characterizing conformations by differences in the extent of oxidation in homologous methionines. Alignment of the PrP sequences of bank vole, mouse, hamster, sheep, elk and deer show five homologous methionines, 132, 137, 157, 209, and 216 in the sheep numbering system (Figure S16). In principle conformational sequencing can be applied to prions from different species, as they share five homologous methionines. As more prions are analyzed by this method, its utility should become more apparent. Conformation dependent differences in methionine oxidation are retained after prions are denatured; quantifying the extent of methionine oxidation is a form of conformational sequencing.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c08892.

Multiple Reaction Monitoring (MRM) method scheme; MRM parameters for ^{14}N tryptic/chymotryptic peptides; MRM parameters for ^{15}N tryptic/chymotryptic peptides; Cartoon of sheep PrP^C; Signal intensity of the ^{14}N-TNMK peptide; Signal intensity of the ^{14}N-TNM(ox)K peptide; Proportion of the unoxidized and oxidized forms of the ^{14}N-TNMK peptide; Proportion of the oxidized and unoxidized forms of the ^{14}N-MLGSAMSR peptide; Graph of the sum of the MRM signals of the oxidized and unoxidized forms of the ^{14}N-MLGSAMSR peptide; Proportion of the oxidized and unoxidized forms of the ^{14}N-MLGSAMSR peptide; MRM chromatograms of tryptic peptides from scrapie; PyMol rendering of recombinant sheep PrP; Proportion of unoxidized and oxidized 132 and 137 methionines in scrapie derived MLGSAMSR peptide; Image of the 263 K prion; Image of the RML prion; Image of the Me7 prion; Alignment of 263 K, RML, Me7, and sheep PrP sequence; Alignment of the mature PrP sequences for six mammals (PDF)

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Notes

The authors declare no competing financial interest.

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