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Oxidation of Methionine 216 in Sheep and Elk Prion Protein Is Highly Dependent upon the Amino Acid at Position 218 but Is Not Important for Prion Propagation

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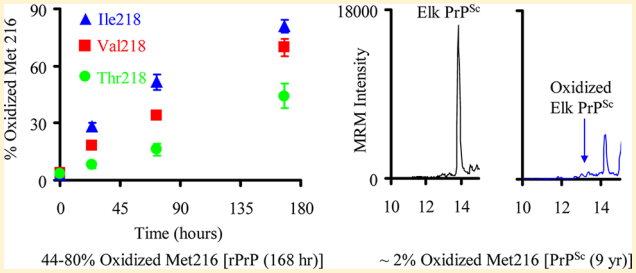
Supporting Information

ABSTRACT: We employed a sensitive mass spectrometry-based method to deconstruct, confirm, and quantitate the prions present in elk naturally infected with chronic wasting disease and sheep naturally infected with scrapie. We used this approach to study the oxidation of a methionine at position 216 (Met216), because this oxidation (MetSO216) has been implicated in prion formation. Three polymorphisms (Ile218, Val218, and Thr218) of sheep recombinant prion protein were prepared. Our analysis showed the novel result that the proportion of MetSO216 was highly dependent upon the amino acid residue at position 218 (I > V > T), indicating that Ile218 in sheep and elk prion protein (PrP) renders the Met216 intrinsically more susceptible to oxidation than the Val218 or Thr218 analogue. We were able to quantitate the prions in the attomole range. The presence of prions was verified by the detection of two confirmatory peptides: GENFTETDIK (sheep and elk) and ESQAYYQR (sheep) or ESEAYYQR (elk). This approach required much smaller amounts of tissue (600 μg) than traditional methods of detection (enzyme-linked immunosorbent assay, Western blot, and immunohistochemical analysis) (60 mg). In sheep and elk, a normal cellular prion protein containing MetSO216 is not actively recruited and converted to prions, although we observed that this Met216 is intrinsically more susceptible to oxidation.



Chronic wasting disease (CWD) in elk and scrapie in sheep are characterized by a long incubation period followed by a short disease period that ends with the death of the host.[1,2] These diseases are caused by a prion (PrP^{Sc}), which is an infectious misfolded isoform of a normal cellular prion protein (PrP^{C}).[3] Detailed structural analysis of hamster and mouse-adapted scrapie has shown that PrP^{Sc} and PrP^{C} are both glycolipoproteins with identical amino acid sequences and identical covalent structures.[4] They both possess similarly diverse asparagine-linked sugar antennae.[5,6] The sugar components of the glycosylphosphatidylinositol (GPI) anchor are similarly varied.[7]

During the mass spectrometry-based analysis of covalent modifications of hamster PrP^{Sc}, it was noted that some of the methionines present in the protein were oxidized (position 213; Met213) to the corresponding methionine sulfoxide (MetSO213). The origin of these modifications was unclear.[8] Other researchers detected the presence of MetSO213 using Western blotting and posited that this oxidized methionine is a prion-specific covalent signature.[9−11] A recent mass spectrometry-based analysis showed that the relative amount of MetSO213 present in PrP^{Sc} from hamster-adapted scrapie strains is similar to that found in hamster PrP^{C} (Met213 in hamsters is analogous to Met216 in sheep and elk).[12]

Although the hamster-adapted prion strains originated from goats, sheep, and mink, they are now adapted to hamsters and, as a result, have very different transmission properties.[13−16] Under experimental conditions, hamster-adapted prions can be transmitted from orally treated hamsters to uninfected hamsters, via the prion-contaminated feces.[17] By contrast, sheep scrapie and cervid CWD are naturally transmitted horizontally (animal to animal) and vertically (mother to offspring) and via exposure to contaminated environments.[18,19] Oxidation is a known means of inactivating prions.[20,21] It is not

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clear if the oxidation of methionine has a role in the observed spread of sheep and cervid prions.

A number of studies have been performed to assess the role of oxidized methionine in the formation of prions. Replacing methionine with a more polar amino acid homologue in PrP leads to the instability of the protein.[22] Other model studies suggest that oxidation of methionine leads to a destabilization of PrP.[23−25] In model systems, the region containing methionine (Met213) has been shown to readily form amyloid fibers without the need for oxidation.[26] Researchers studying the oxidation of methionine in hamster recombinant PrP showed that it had little effect on the structure of that protein and that Met213 was very resistant to oxidation.[27] Other studies showed that the oxidation of other methionine residues results in the inhibition of fibrillization of recombinant hamster PrP.[28] The role, if any, of oxidized methionine residues in the formation of prions is unresolved.

Oxidative damage is a feature of prion diseases. There is experimental evidence to indicate that mouse, hamster, and human PrP^{Sc} contain substantial amounts of oxidized methionine.[9,10] The proteins present in the brains of CJD-infected humans and scrapie-infected hamsters show evidence of oxidative damage.[29] Specifically, the lysine residues of hamster PrP^{Sc} are covalently modified by the products (aldehydes) of metal-catalyzed oxidation, lipoxidation, and glycoxidation. This indicates that PrP^{Sc} is exposed to products of reactive oxygen species.

The methionine sulfoxide reductase (Msr) system is responsible for repairing the oxidative damage done to proteins. The enzymes reverse the damage by reducing the oxidized methionine back to methionine. When transgenic mice lacking MsrA are inoculated with prions, there is no difference in incubation between the transgenic mice and the same strain of wild-type mice.[30] The same is true when both the MsrA and MsrB components of the Msr system are inactivated.[30] These results indicate that the inability to repair oxidative damage does not influence the incubation period of the prion disease.

Methionine may be oxidized by a variety of common oxidants to yield the corresponding sulfoxide. A methionine residue is oxidized by the electrospray ionization necessary to perform mass spectrometry.[31,32] It is oxidized during the sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) process necessary for Western blotting.[33,34] Methionine residues can even be oxidized during the general handling of biological samples.[35−37] Methionine residues can be oxidized as a consequence of normal metabolism, which is why a set of methionine reductases is present in cells to reduce the oxidized methionines that exist during normal cellular metabolism.[38,39] It is therefore important to ascertain whether the observed oxidation in prions is an artifact of sample preparation.

We have developed a mass spectrometry-based approach to quantitating elk and sheep PrP. We have used this method to study the chemistry of methionine oxidation and to quantitate the prions and the MetSO216:Met216 ratio present in the brains of prion-infected sheep and elk. We report the results here.

EXPERIMENTAL PROCEDURES

Chemicals. The iodoacetamide derivatives of the VVEQM-CITQYQR peptides (sheep and elk), the corresponding methionine sulfoxide, and the isotopically enriched peptide analogues (internal standard) were synthesized by Anaspec (Fremont, CA). These peptides and their corresponding isotopically enriched analogues differ only in terms of the isotopic content of the N-terminal valine, either natural isotopic abundance or [$^{13}C_5$,^{15}N]valine. The peptide and the sulfoxide-containing peptide have different chromatographic mobilities in our chromatographic system. The chromatographic mobilities of the isotopically labeled internal standards and their corresponding peptides or oxidized peptides are identical.

The chemical composition of all of these synthetic peptides was confirmed by mass spectrometry. The chemical purity was >90% as shown by LC/UV. The isotopic purity was >99.8% as verified by MRM analysis of the isotopically labeled internal standard. The structure of each peptide was verified by amino acid analysis and mass spectrometry (Anaspec).

High-performance liquid chromatography (HPLC) grade water was purchased from Burdick and Jackson (Muskegon, MI). Acetonitrile, HPLC grade, was purchased from Fisher Scientific (Fairlawn, NJ). Trypsin (porcine, sequencing grade, modified) was purchased from Promega (Madison, WI). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Animal Handling and Sample Preparation. The brains from the sheep and elk were harvested from naturally infected animals that were humanely euthanized, some of them in the terminal stages of the disease. All of the animals tested positive for the presence of disease by Western blotting (WB) (except elk 1 to 4), an enzyme-linked immunosorbent assay (ELISA) (TeSeE, Bio-Rad, Hercules, CA), and immunohistochemical (IHC) analysis. The scrapie strain infecting the sheep was consistent with the WB and IHC profiles of classical scrapie and not "atypical" or Nor98 strain.

PrP^{Sc} was isolated according to the methods of Bolton et al. with some minor modifications.[40] Briefly, a 10% brain homogenate (w/v) was made using an Omni GLH general laboratory homogenizer and disposable Omni Tip plastic generator probes, in homogenization buffer [10% (w/v) *N*-lauroylsarcosine, 9.5 mM sodium phosphate (pH 8.5), and 1% (v/v) Sigma protease inhibitor cocktail], and allowed to stand for 30 min at room temperature. The homogenate was then centrifuged for 18 min (16000*g* and 20 °C) in an Eppendorf 5810R refrigerated centrifuge, to remove large particles. The supernatant was retained. A portion (100 μL) of this supernatant was diluted to 3 mL with homogenization buffer and transferred to an ultracentrifuge tube (4.2 mL, 16 mm × 38 mm). The contents of the tube were underlaid with 1 mL of 20% (w/v) sucrose, and the tube was sealed. The sample in the sealed tube was centrifuged for 75 min at 150000*g* (46000 rpm and 20 °C) with a floating Noryl spacer in a Beckman 70.1 Ti rotor to obtain an insoluble pellet. This pellet was resuspended in 250 μL of buffer [0.1% (w/v) 3-(*N*,*N*-dimethylmyristylammonio)propanesulfonate and 20 mM Tris (pH 8.5)] by a brief sonication using closed tubes in a microplate horn (four 45 s bursts; Misonix 3000 sonicator).

The resuspended PrP^{Sc} pellet was either retained for analysis, reprecipitated with phosphotungstic acid (PTA), or partially digested to yield PrP^{Sc}, PTA-precipitated PrP^{Sc}, or PrP 27−30, respectively. PTA-precipitated PrP^{Sc} was obtained by adding 100 μL of a 20% (w/v) sarkosyl solution and 80 μL of a 4% PTA/170 mM $MgCl_2$ (pH 7.4) solution to the sonicated buffer. The solution was diluted with PBS (pH 7.4) to yield a final volume of 1 mL [2% (w/v) sarkosyl, 0.32% (w/v) PTA (pH 7.4), and 13.6 mM $MgCl_2$ (final concentration)] and incubated with rotation for 1 h at 37 °C.[41] After this incubation, the

solution was centrifuged at room temperature for 30 min at 15800*g*. The supernatant was removed and discarded. The pellet was washed with 500 μL of a solution of 0.1% sarkosyl, 150 mM EDTA, and PBS (pH 7.4) to remove excess magnesium salts and sarkosyl and then centrifuged for 15 min at 15800*g*. The supernatant was again discarded. PrP 27–30 was obtained by treating the pellet with 5 μg/mL PK (Invitrogen; ≥20 units/mg) for 1 h at 37 °C. The PK reaction was stopped by the addition of a protease inhibitor cocktail [2% (v/v) Sigma protease inhibitor cocktail].

The PrP^{Sc} pellet solution was denatured by addition of a sufficient amount of 8 M guanidine hydrochloride to achieve a final concentration of 6 M.[42] The solution was allowed to stand for 24 h at room temperature. The denatured protein was precipitated with ice-cold methanol (85% methanol to 15% protein solution) and centrifuged for 20 min at 20000*g* for 20 min in a cold rotor (−9 °C) with an Eppendorf (Hamburg, Germany) model 5417R centrifuge.

The PTA-precipitated pellets were brought up in 200 μL of a solution of 6 M guanidine hydrochloride and sonicated to solubilize the pellet. After denaturation for 24 h, the resulting PrP was precipitated with ice-cold methanol (85% methanol to 15% protein solution) and centrifuged for 20 min at 20000*g* and 0 °C to obtain an insoluble, denatured pellet.

The pellet resulting from the methanol precipitation was subjected to reduction, alkylation, and tryptic cleavage as described previously.[12,43] After tryptic cleavage, the resulting peptides were filtered through a 10000 molecular weight cutoff filter (VWR International, San Francisco, CA).

Preparation of the Sheep Recombinant PrP Polymorphisms (Val218 and Thr218). The sheep prion protein gene corresponding to amino acids 25–233 and other polymorphisms were generated using the standard megaprimer method of site-directed mutagenesis and cloned into the pET11a vector (EMD Millipore, Billerica, MA) using standard methods.[44,45] The resulting rPrP was purified by previously described methods.[46] The molecular weight of the purified proteins was verified by mass spectrometry.

Shaking Samples. The samples were placed in 1.5 mL screw cap microfuge tubes (Eppendorf North America, Hauppauge, NY) and put on an IKA Vibrax VXR basic S1 shaker with a type VX 2E platform (IKA Works, Wilmington, NC) and allowed to shake at 1700 rpm for the duration of the experiment. Samples were removed after being shaken or standing at room temperature for 0, 24, 72, or 168 h and analyzed by mass spectrometry.

Quantitative Mass Spectrometry: Nanospray LC–MSMS. An Applied Biosystems (ABI/MDS Sciex, Toronto, ON) model 4000 Q-Trap instrument equipped with a nanoelectrospray source was used to perform nanospray LC–MS/MS. The chromatography conditions and the instrument response optimization procedures have been described previously.[12,46,47]

The mass spectrometer was operated in multiple-reaction monitoring (MRM) mode, alternating between detection of three peptides and the appropriate internal standards. The mass settings for the tryptic peptides and the MetSO216 analogues common to sheep and elk have been described previously.[46] The mass settings for the elk ESEAYYQR peptide were empirically determined [precursor ion at m/z 523.2, product ion at m/z 466.2 (y_3 ion, CE 24.5) or 629.3 (y_4 ion, CE 26)]. The mass settings for the mono-oxidized peptides [^{15}N]VVEQMCITQYQR [precursor ion at m/z 794.9, product ion at m/z 980.6 (y_7 ion, CE 45), 996.5 (y_7 ion + O, CE 45), or 1128.6 (y_8 ion + O, CE 45)], VVEQMCVTQYQR [precursor ion at m/z 778.8, product ion at m/z 954.5 (y_7 ion, CE 45), 970.5 (y_7 ion + O, CE 45), or 1101.5 (y_8 ion + O, CE 45)], and [^{15}N]VVEQMCTTQYQR [precursor ion at m/z 788.8, product ion at m/z 968.4 (y_7 ion, CE 50), 984.4 (y_7 ion + O, CE 45), or 1116.5 (y_8 ion + O, CE 45)] were experimentally determined. Both quadrupoles were operated at unit resolution (full width at half-maximum of 0.7 Da). The fragmentation of the ESEAYYQR peptide to yield the characteristic y_3 or y_4 ion was optimized by adjusting the Q2 offset voltage (collision energy) to a characteristic value for the y_3 ion (CE 24.5) or y_4 ion (CE 26). Quantitation was done with the Intelliquan quantitation algorithm using Analyst version 1.4.1.

HAZARDOUS PROCEDURES

Acetonitrile is hazardous and was manipulated in a dedicated chemical safety hood. Scrapie and CWD prions can infect sheep and cervids, so all prion-containing samples were manipulated in a dedicated biosafety level 2 (BSL-2) laboratory [certified and inspected by the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (http://www.aphis.usda.gov/permits/)] using procedures outlined in the 5th edition of the CDC's biosafety manual, Biosafety in Microbiological and Biomedical Laboratories (http://www.cdc.gov/biosafety/publications/bmbl5). The infectious material was inactivated before removal from the dedicated BSL-2 laboratory by the addition of a sufficient volume of 8 M guanidine hydrochloride to make a 6 M solution that was thoroughly mixed and allowed to stand for at least 24 h at room temperature. The solution of inactivated prions was transferred to clean fresh tubes and removed from the BSL-2 laboratory.

RESULTS

Three clones with methionine at position 216 (Met216) and isoleucine (Ile), valine (Val), or threonine (Thr) at position 218, i.e., Ile218, Val218, and Thr218, were prepared. The Ile218 protein is analogous to the sheep, elk, and human protein. The Val218 and Thr218 proteins are analogous to the mouse and hamster proteins, respectively. The clones were grown in minimal medium supplemented with either NH_4Cl or $^{15}NH_4Cl$ to yield a protein in which each nitrogen contains either the natural isotopic abundance (0.4% ^{15}N) or ^{15}N (99.7%) isotope, respectively. The molecular weight of the purified proteins was confirmed by mass spectrometry. The isotopic abundance of the starting $^{15}NH_4Cl$ was 99.7%, and this level of incorporation was observed in the purified proteins.

These purified recombinant proteins were used to determine the relationship between Met216 oxidation and the amino acid at position 218. Samples of ^{15}N-labeled Ile218, ^{14}N-labeled Val218, and ^{15}N-labeled Thr218 were prepared, aliquoted, reduced, alkylated, and then digested with trypsin. One set (n = 3) was allowed to stand at room temperature, and the other set (n = 3) was placed in an orbital shaker and vortexed (1700 rpm) at room temperature. Periodically, samples were removed from each of the sets and quantitated by mass spectrometry. These results are summarized in Figure 1. The sum of the oxidized and unoxidized peptides differed by <20% from the start to the end of the experiment for all of the peptides, either standing or vortexed. If the samples were allowed to stand at room temperature, then the MetSO216:Met216 ratio increased

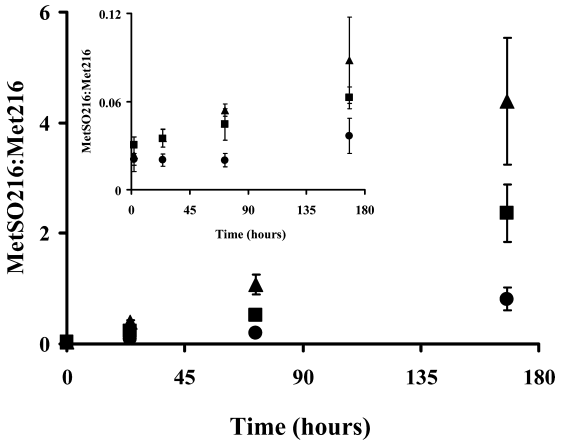

Figure 1. Graphical representation of the ratio of MetSO218 to Met218 in a solution containing peptides VVEQMCITQYQR (▲), VVEQMCVTQYQR (■), and VVEQMCTTQYQR (●). Each mixture was either vortexed (n = 3) or allowed to stand (inset) at room temperature (n = 3). Aliquots of the mixtures were periodically removed and analyzed by mass spectrometry (t = 0, 24, 72, or 168 h).

over time for all three peptides but remained below 0.12 (Figure 1, inset). The MetSO216:Met216 ratio for the Ile218 and Val218 peptides was higher than that of the Thr218 peptide in the standing samples (Figure 1, inset). There was a statistically significant difference between ratios of the Ile218 and Thr218 peptides in these samples at 24, 72, and 168 h (P < 0.01). If the samples were constantly exposed to oxygen by vortexing, then a more pronounced time-dependent increase in this ratio was observed (Figure 1), and there was a significant difference (P < 0.01; t = 72 and 168 h) in the susceptibility to oxidation of the three peptides: Ile218 > Val218 > Thr218.

Purified sheep rPrP (Ile218) was reduced, alkylated, digested with trypsin, and then analyzed directly. The MetSO216:Met216 ratio was observed to be 0.04 ± 0.01. The reduction and alkylation procedure necessary for trypsin digestion was previously determined to result in a measurable but negligible (~0.1%) reduction of sulfoxide.[12] Electrospray ionization was observed to oxidize Met216, but this was not significant and did not contribute to the measured oxidation. When samples were precipitated with methanol prior to the reduction step and then subjected to analysis, the observed ratio was 0.06 ± 0.01. Analogous experiments using the purified Val218 and Thr218 PrP polymorphisms showed similar results (data not shown). This indicates that the general handling necessary to prepare the PrP^{Sc} samples introduced some minor artifactual oxidation of Met216.

There are two sulfur-containing amino acids in the analyte peptide, Met216 and the alkylated cysteine (Cys217), and we wished to determine which sulfur was oxidized. The a_2 ion (containing amino acids VV) was used to quantitate the peptide, but it could not be used to reveal the location of the oxidized amino acid.[47] To determine the exact location of the oxidation, we included the transitions for the oxidized y_8 ion (containing the MCXTQYQR amino acids, where X is I, V, or T) and the unoxidized and oxidized y_7 ions (containing the CXTQYQR amino acids, where X is I, V, or T). Over the course of the experiment, the signal for the oxidized y_8 ion was observed to increase (Figures S1–S3 of the Supporting Information). There was no observable signal for the corresponding oxidized y_7 ion (Figures S1–S3 of the Supporting Information). The signal for the unoxidized y_7 ion was present in all samples (Figures S1–S3 of the Supporting Information). This indicates that neither the alkylated cysteine (Cys217) nor the other amino acids in the y_7 peptide (including the I, V, and T analogues) were oxidized. In addition, no signals were observed for any of the doubly oxidized peptides. These results indicate that only Met216 was oxidized.

Three peptides were selected for analyzing the PrP^{Sc} found in scrapie-infected sheep and CWD-infected elk. The instrument response was optimized for each of the peptides. The instrument responses for the confirmatory peptides GENFTETDIK (sheep and elk) and ESQAYYQR (sheep) have been reported previously.[46] The response for the confirmatory peptide ESEAYYQR (elk) was determined empirically (vide infra). Sheep and elk share a common analyte peptide VVEQMCITQYQR (containing Met216) and its oxidized homologue VVEQM(SO)CITQYQR (containing oxidized Met216). The settings for the analyte peptide and its oxidized homologue were used to generate calibration curves relating the area ratio of a variable amount of the synthetic analyte peptide (1–80 fmol) to a fixed amount of the isotopically labeled internal standard (10 fmol).[12,46,47] The correlation coefficient for these quadratic curves was excellent (R^2 = 0.99), and the coefficient of variation (CV) for these area ratios was small (0.9–3.8%). The CV for the analyte retention time was low

Table 1. Summary of the Quantitation of PrP^{Sc} and Oxidized PrP in 10 Elk Samples[a]

	age (years)	sex	IHC grade	ELISA OD	PrP^{Sc} (MS) (fmol/g)	MetSO216:Met216 ratio	
						without PTA	with PTA
1	2	male	2	3.044	$6 \times 10^4 \pm 5 \times 10^3$	0.03 ± 0.01	0.03 ± 0.02
2	6	female	3	>3.5	$7 \times 10^4 \pm 2 \times 10^4$	0.03 ± 0.01	0.09 ± 0.01
3	9	female	3	>3.5	$5 \times 10^4 \pm 9 \times 10^3$	0.02 ± 0.01	0.10 ± 0.01
4	4	female	3	>3.5	$8 \times 10^4 \pm 8 \times 10^3$	0.05 ± 0.02	0.10 ± 0.01
5	3	male	3	>3.5	$5 \times 10^4 \pm 2 \times 10^4$	0.04 ± 0.01	0.09 ± 0.02
6	N/A[b]	male	3	>3.5	$1 \times 10^4 \pm 1 \times 10^4$	0.04 ± 0.04	0.11 ± 0.04
7	3	female	2	3.381	$2 \times 10^4 \pm 2 \times 10^3$	0.06 ± 0.01	0.12 ± 0.02
8	4	female	3	>3.5	$3 \times 10^4 \pm 1 \times 10^4$	0.07 ± 0.02	0.13 ± 0.02
9	4	male	3	>3.5	$4 \times 10^4 \pm 7 \times 10^3$	0.05 ± 0.01	0.19 ± 0.04
10	8	male	2	3.384	$4 \times 10^3 \pm 3 \times 10^3$	0.06 ± 0.01	0.15 ± 0.03

[a]All animals were positive by Western blotting (animals 1–4 were not tested by WB), ELISA, and immunohistochemistry (IHC) (vide infra) analysis of obex tissue. The mass spectrometry-based analysis was conducted with non-obex tissue. All animals are homozygous for methionine (M/M) at position 132. [b]Not available.

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Table 2. Summary of the Results of the Quantitation of PrP^{Sc} and Oxidized PrP in 10 Sheep Samples[a]

	age (years)	sex	IHC grade	ELISA OD	PrP^{Sc} (MS) (fmol/g)	MetSO216:Met216 ratio without PTA	MetSO216:Met216 ratio with PTA
1	3	female	2	2.686	$1 \times 10^4 \pm 3 \times 10^3$	0.06 ± 0.01	0.06 ± 0.01
2	3	female	2	2.574	$2 \times 10^3 \pm 6 \times 10^2$	0.09 ± 0.04	0.09 ± 0.01
3	3	male	3	2.170	$5 \times 10^3 \pm 2 \times 10^3$	0.11 ± 0.03	0.08 ± 0.02
4	2	female	1	2.223	$5 \times 10^2 \pm 5 \times 10$	0.2 ± 0.1	0.2 ± 0.2
5	3	female	3	2.650	$4 \times 10^3 \pm 2 \times 10^3$	0.09 ± 0.03	0.11 ± 0.04
6	3	female	3	3.036	$2 \times 10^4 \pm 3 \times 10^3$	0.04 ± 0.01	0.05 ± 0.01
7	3	female	2	3.244	$3 \times 10^3 \pm 2 \times 10^2$	0.07 ± 0.02	0.07 ± 0.02
8	3	female	3	3.150	$5 \times 10^2 \pm 1 \times 10^2$	0.09 ± 0.06	0.4 ± 0.1
9	3	female	1	2.578	$2 \times 10^3 \pm 2 \times 10^2$	0.09 ± 0.06	0.3 ± 0.2
10	2	female	1	2.705	$2 \times 10^4 \pm 4 \times 10^3$	0.07 ± 0.01	0.05 ± 0.01

[a]All animals were positive by Western blot, ELISA, and immunohistochemistry (IHC) (vide infra) analysis of obex tissue. The mass spectrometry-based analysis was conducted with non-obex tissue. All animals have the genotype ARQ/ARQ (positions 136, 154, and 171) and are cross-bred (CB).

(~2.0%). The results indicate that this method provides an accurate and reproducible way of quantitating the analyte peptide and its oxidized homologue.

The 20 scrapie-infected sheep and CWD-infected elk originated from two Canadian farms that were depopulated by the Canadian Food Inspection Agency. Some of the animals displayed clinical signs, and all were naturally infected with scrapie or CWD (Tables 1 and 2). Each animal was humanely euthanized, and the brain of each was surgically removed. The age of the elk varied between 2 and 9 years. They were evenly divided between males and females. The age of the sheep was 2 or 3 years. Nine of the sheep were ewes, and the other was a ram. The scrapie or CWD diagnosis was based on immunohistochemical (IHC) analysis of brain tissue.

The obex of the medulla oblongata was used for the ELISA, Western blot, and IHC analysis. The IHC results were reported on a scale of 0−4. A result of 0 showed no evidence of PrP^{Sc} staining, and a result of 1−4 was evidence of mild to severe pathology and a corresponding increase in the intensity and extent of staining.[48] The ELISA test (sha31 antibody) used approximately 60 mg of brain tissue per sample. The OD values for the ELISA and the pathology scores are included in Tables 1 and 2. All of the animals used in this study tested positive for all three of these tests, except for four of the elk (animals 1 to 4; Table 1), which were positive by IHC and ELISA but were not tested by WB. The samples for the mass spectrometry-based analysis were taken from non-obex portions of the brain stem near the obex of the medulla oblongata.

We developed a method of isolating PrP^{Sc} from these samples that did not require the use of proteinase K (PK) digestion to remove the endogenous PrP^{C}. When PrP^{Sc} was isolated from combined CWD-infected elk brain tissues, we obtained $1 \times 10^4 \pm 1 \times 10^3$ fmol/g of PrP^{Sc}. If a PK digestion step was included in the isolation procedure, then the yield was reduced to $1.3 \times 10^3 \pm 1 \times 10^2$ fmol/g of PrP^{Sc} (~8%). When we performed an analogous experiment using scrapie-infected sheep brain tissue, we determined that only ~20% of the sheep PrP^{Sc} was PK resistant. Using PK to remove the endogenous PrP^{C} in scrapie-infected sheep or CWD-infected elk tissues resulted in unacceptable losses of PrP^{Sc}.

We used phosphotungstic acid (PTA) to isolate PrP^{Sc} from the brains of sheep and elk. The elk and sheep PrP^{Sc} was first concentrated using a modified version of the ultracentrifugation procedure developed by Bolton et al.[40] While the yield of PrP^{Sc} was high, the resulting pellet contained a small amount of PrP^{C}. We determined that $2.1 \times 10^3 \pm 6 \times 10^2$ fmol of PrP^{C} was present in the pellet from 1 g of healthy sheep brain tissue. To remove this small amount of PrP^{C}, a second step, employing low-speed centrifugation and PTA, was performed.[41] The yield of PrP^{Sc} was dependent upon the concentration of magnesium and the choice of polyoxometalates,[49] so this step was optimized for the sheep and elk samples. The optimal concentrations of PTA and $MgCl_2$ for the isolation of PrP^{Sc} from elk or sheep samples were determined to be 0.31% (w/v) and 13.6 mM, respectively.

These samples were prepared without the use of proteinase K, so there is a possibility that trace amounts of PrP^{C} or some other molecule might produce signals that would interfere with this analysis. The internal standards were of sufficiently high isotopic purity that no signal was observed for the analyte peptide when only the isotopically labeled internal standard was analyzed. To test for interference from other molecules present in the homogenized tissue, brains from uninfected sheep and elk were removed and subjected to the prion isolation procedure (vide supra). The resulting pellets were reduced, alkylated, digested with trypsin, and then subjected to mass spectrometric analysis using the internal standards. A careful examination of the resulting chromatograms revealed that there was no signal greater than noise for the analyte peptide or its oxidized homologue (Figures 2 and 3) in samples from uninfected sheep or elk. These results were similar to those previously reported for the analyte peptide and the oxidized form of the analyte peptide in hamsters.[12,43,46] We concluded, therefore, that there were no interfering molecules present in a healthy brain and that the signals we observed from infected brains result from inactivated PrP^{Sc}.

Our mass spectrometry-based approach required approximately 600 μg of tissue for each mass spectrometric analysis. The representative signals from the analyte peptide (VVEQMCITQYQR) and the confirmatory GENFTETDIK (sheep and elk), ESQAYYQR (sheep), and ESEAYYQR (elk) peptides from an elk and sheep sample are shown in Figures 4 and 5. The results of our mass spectrometry-based quantitation are listed in Tables 1 and 2 and are reported as femtomoles per gram of tissue per sample.

We applied the mass spectrometry-based approach to determine the MetSO216:Met216 ratio in the PrP^{Sc} present in CWD-infected elk. This ratio was somewhat higher in samples isolated with PTA than without. In the elk samples, this ratio varied from 0.03 to 0.07 in samples isolated without

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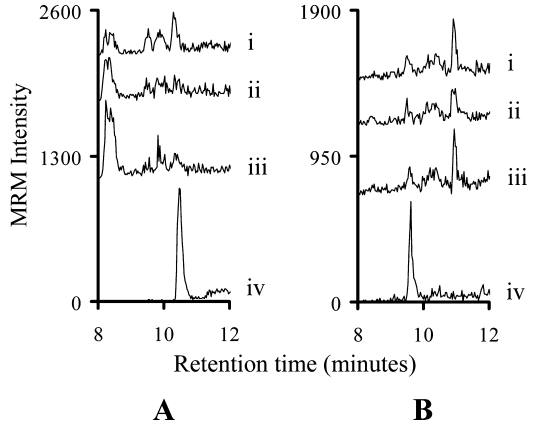

Figure 2. Graphs comparing the signal intensities and chromatographic properties of the isotopically labeled unoxidized VVEQMCITQYQR peptide (A, iv) and the oxidized analogue VVEQM(SO)CITQYQR (B, iv) with those from the PTA precipitation of brain homogenates from uninfected elk (i–iii in each panel). The graphs are offset for the sake of clarity. There is no endogenous molecule that interferes with the analysis. Retention times are relative to the internal standard.

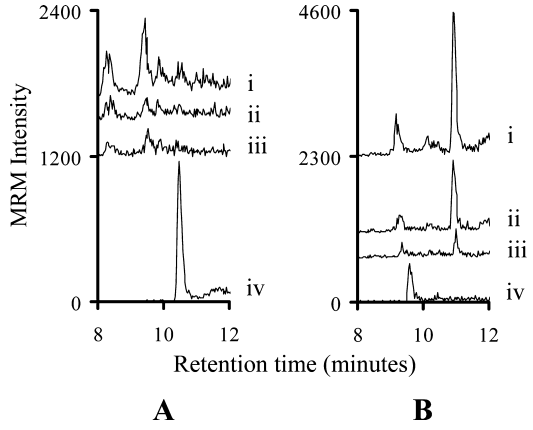

Figure 3. Graphs comparing the signal intensities and chromatographic properties of the isotopically labeled unoxidized VVEQMCITQYQR peptide (A, iv) and the oxidized analogue VVEQM(SO)CITQYQR (B, iv) with those from the PTA precipitation of brain homogenates from uninfected sheep (i–iii in each panel). The graphs are offset for the sake of clarity. There is no endogenous molecule that interferes with the analysis. Retention times are relative to the internal standard.

PTA and from 0.03 to 0.19 in samples isolated with PTA (Table 1). This difference was most likely due to artifactual oxidation caused by the extra handling inherent in the PTA isolation procedure (vide supra). Because the amount of PrP^{C} estimated to be present in each sample represented a small portion of the total PrP content, simple removal of this small amount of PrP^{C} could not account for the observed increases in the ratio. Older animals had a similarly low level of oxidation when compared to younger animals [compare animals 7 and 10 and animals 1 and 3 (Table 1)]. Elk with the largest and smallest amount of PrP^{Sc} [animals 4 and 10, respectively (Table 1)] had similar levels of Met216 oxidation. The MetSO216:Met216 ratio of 0.03–0.07 in the samples isolated without the use of PTA was comparable to that observed from general handling of sheep recombinant PrP (rPrP) samples used in this study (0.04 ± 0.01).

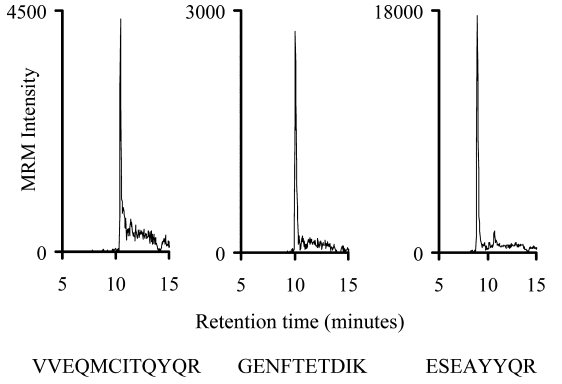

Figure 4. Comparison of the signal intensity of three characteristic tryptic peptides isolated from the brain of a CWD-infected elk. The graphs correspond to peptides from elk sample 7 (3.6 fmol/injection). Retention times are relative to the internal standard.

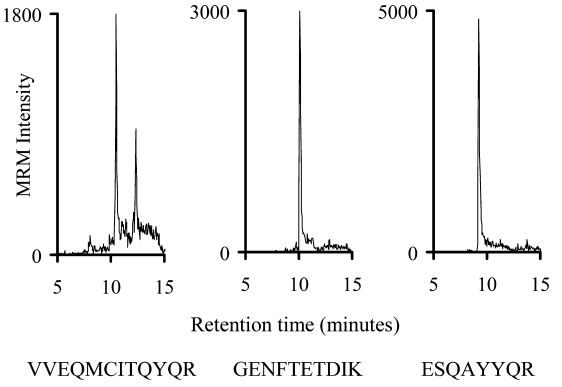

Figure 5. Comparison of the signal intensity of three characteristic tryptic peptides isolated from the brain of a scrapie-infected sheep. The graphs correspond to peptides from sheep sample 7 (2.4 fmol/injection). Retention times are relative to the appropriate internal standard. The signal with a retention time of 10.47 min corresponds to the VVEQMCITQYQR peptide.

The MetSO216:Met216 ratios observed in the sheep samples were comparable to those observed for the elk samples. The samples isolated with PTA had a generally higher MetSO216:Met216 ratio (0.05–0.4) than the same samples isolated without it (0.04–0.2). Again, this difference was most likely due to the PTA isolation procedure. The MetSO216:Met216 ratio present in the sheep samples was greater than that in the elk samples. This was undoubtedly due to the smaller amounts of PrP^{Sc} present in the sheep samples (Table 2). Small amounts exaggerated the contribution of noise to the observed signal and the small amount of oxidation that occurs during general handling. Even with this variation, the MetSO216:Met216 ratio in the sheep samples was similar to that observed in hamsters.[12]

DISCUSSION

Our mass spectrometry-based approach was able to detect the presence of PrP^{Sc} in all of the samples (Tables 1 and 2). We developed an isolation procedure to obtain PrP^{Sc} that was not dependent upon the use of PK to remove PrP^{C} from the sample. This isolation procedure removed any molecule that had the same chromatographic and physicochemical properties as the analyte peptide as shown by the absence of interfering signals originating from the brains of healthy uninfected

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animals (Figures 2 and 3). We used the analyte peptide to quantitate the amount of PrP^{Sc} in the 20 infected sheep and elk. The presence of PrP^{Sc} was confirmed by detecting the confirmatory peptides in all of the sheep and elk samples (Figures 4 and 5).[46] The concentrations of PrP^{Sc} in the elk samples varied 20-fold (from ~4 to 80 pmol/g) (Table 1). The concentrations of PrP^{Sc} in the sheep samples varied 40-fold (from ~0.5 to 20 pmol/g) (Table 2). The immunohistochemistry (IHC) required half of the obex, and the ELISA test required approximately 60 mg of brain tissue. In contrast, MS analysis required only 0.6 mg of brain tissue. Thus, this mass spectrometry-based approach was more sensitive (~0.6 mg) than the ELISA-based approach (~60 mg), even though the detected PrP^{Sc} came from non-obex tissue. It also meant that we had sufficient material to perform other experiments beyond simple detection of prions in the samples.

The chemistry of methionine oxidation is complicated and dependent upon the oxidant. The methionine analogous to Met216 in hamsters (Met213) is resistant to oxidation by hydrogen peroxide.[27] For hydrogen peroxide to oxidize a methionine, it needs to be in a hydrophilic environment. Empirical observations indicate that a methionine near polar amino acids (E, D, and Q) is more readily oxidized with hydrogen peroxide than one near nonpolar amino acids, while a methionine adjacent to cysteine is observed to be significantly less susceptible to hydrogen peroxide oxidation.[50] The methionine present in all three of the sheep protein polymorphisms (Met216) also has E, Q, and C in its proximity. Because the oxidant used in this work was molecular oxygen and not hydrogen peroxide, it should not be surprising that the empirical results for hydrogen peroxide-mediated oxidation do not necessarily apply to this work. The order of the susceptibility of Met216 to oxidation (Ile218 > Val218 > Thr218) observed in this work was consistent with that previously observed for the hydroxyl radical-mediated abstraction of the tertiary hydrogen from the side chain of the amino acid at position 218.[51] This suggests that the nature of the oxidant in combination with proximal amino acids influences the oxidation of methionine. These results apply to proteins in general and not just to the PrP^{Sc} present in scrapie-infected sheep and CWD-infected elk.

Oxidized methionines have been experimentally observed in prions using Western blot-based detection.[9,10,23] Unfortunately, methionine and other amino acids are oxidized by the reactive oxygen species generated during the running of an SDS−PAGE gel under standard conditions,[33,35] so the presence of oxidized proteins after Western blot analysis is not surprising. These artifacts can be eliminated by removing dissolved oxygen and adding reactive oxygen species scavengers.[33] We have previously reported that the artifactual oxidation of hamster PrP 27−30, as a consequence of running an SDS−PAGE gel, occurred when no reductant (DTT or BME) was added to the loading buffer and was lessened when the reductant was added to the loading buffer.[12] Using any SDS−PAGE-based analysis (e.g., Western blotting) to detect the oxidation of proteins is problematic because of its inherent propensity to oxidize proteins.

Met216 of sheep and elk is intrinsically more susceptible to oxidation than other analogues by virtue of its proximity to Ile218. By analogy, the same is true of the human peptide and, to a lesser extent, the mouse peptide. This enhanced tendency to oxidize in the presence of reactive oxygen species may explain the observed oxidation found in human and mouse samples by researchers using Western blot-based analysis.[9,10,23] Other researchers have tested the antiserum used to detect oxidized methionines in prions[11] and determined that it does not distinguish between the oxidized and unoxidized forms of methionine.[52] Even if the antibodies could distinguish between the oxidized and unoxidized methionine, using Western blot-based analysis to detect the presence of oxidation in proteins, with its propensity to oxidize proteins, is an inherently problematic approach.

Our results show that the observed amount of oxidized Met216 in sheep and elk is no greater than would be expected from the general handling of the samples. The PrP^{Sc} in these sheep and elk was presumably exposed to oxidants, yet Met216 remained largely unoxidized, even though some animals showed clinical signs when they were euthanized. The observed oxidation was not age-related, because 2-year-old elk and 9-year-old elk both had similarly low levels of oxidation at Met216. This suggests that Met216 in sheep and elk PrP^{Sc} was relatively protected from oxidation. If oxidative destabilization were an important factor for the prion-mediated conversion of PrP^{C} to PrP^{Sc}, then the PrP^{C} containing MetSO216 would be preferentially recruited and converted to PrP^{Sc}. If this occurred to a significant extent, then the amount of MetSO216 should be more than that due to general handling. It should also be greater in samples with more PrP^{Sc} than those with less and greater in samples from older animals than younger ones. This was not observed in this work (sheep and elk), nor has it been observed in other work on hamsters.[12] These combined results indicate that oxidation of Met216 is not important for prion replication, even in the case of scrapie-infected sheep and CWD-infected elk, where the protein is intrinsically more susceptible to oxidation and the animals are fairly old.

■ ASSOCIATED CONTENT

Supporting Information

Detailed procedures for the cloning, expression, and purification of the sheep rPrP proteins and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PrP^{C}, normal cellular prion protein isoform; PrP^{Sc}, prion isoform; PrP, prion protein; PrP 27−30, characteristic proteinase K resistant fragment of PrP^{Sc}; PK, proteinase K; CWD, chronic wasting disease; PTA, phosphotungstic acid; MRM, multiple-reaction monitoring.

REFERENCES

(1) Detwiler, L. A., and Baylis, M. (2003) The epidemiology of scrapie. *Rev. Sci. Tech. 22*, 121−143.

(2) Williams, E. S. (2005) Chronic wasting disease. *Vet. Pathol. 42*, 530−549.

(3) Prusiner, S. B. (1998) Prions. *Proc. Natl. Acad. Sci. U.S.A. 95*, 13363−13383.

(4) Stahl, N., Baldwin, M., Teplow, D. B., Hood, L. E., Beavis, R., Chait, B., Gibson, B. W., Burlingame, A. L., and Prusiner, S. B. (1992) Cataloging post-translational modifications of the scrapie prion protein by mass spectrometry. In *Prion diseases of humans and animals* (Prusiner, S. B., Collinge, J., Powell, J., and Anderton, B., Eds.) pp 361−379, Ellis Horwood, New York.

(5) Rudd, P. M., Endo, T., Colominas, C., Groth, D., Wheeler, S. F., Harvey, D. J., Wormald, M. R., Serban, H., Prusiner, S. B., Kobata, A., and Dwek, R. A. (1999) Glycosylation differences between the normal and pathogenic prion protein isoforms. *Proc. Natl. Acad. Sci. U.S.A. 96*, 13044−13049.

(6) Stimson, E., Hope, J., Chong, A., and Burlingame, A. L. (1999) Site-specific characterization of the N-linked glycans of murine prion protein by high-performance liquid chromatography/electrospray mass spectrometry and exoglycosidase digestions. *Biochemistry 38*, 4885−4895.

(7) Stahl, N., Baldwin, M. A., Hecker, R., Pan, K. M., Burlingame, A. L., and Prusiner, S. B. (1992) Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. *Biochemistry 31*, 5043−5053.

(8) Stahl, N., Baldwin, M. A., Teplow, D. B., Hood, L., Gibson, B. W., Burlingame, A. L., and Prusiner, S. B. (1993) Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. *Biochemistry 32*, 1991−2002.

(9) Canello, T., Engelstein, R., Moshel, O., Xanthopoulos, K., Juanes, M. E., Langeveld, J., Sklaviadis, T., Gasset, M., and Gabizon, R. (2008) Methionine sulfoxides on PrPSc: A prion-specific covalent signature. *Biochemistry 47*, 8866−8873.

(10) Canello, T., Frid, K., Gabizon, R., Lisa, S., Friedler, A., Moskovitz, J., Gasset, M., and Gabizon, R. (2010) Oxidation of Helix-3 methionines precedes the formation of PK resistant PrP. *PLoS Pathog. 6*, e1000977.

(11) Oien, D. B., Canello, T., Gabizon, R., Gasset, M., Lundquist, B. L., Burns, J. M., and Moskovitz, J. (2009) Detection of oxidized methionine in selected proteins, cellular extracts and blood serums by novel anti-methionine sulfoxide antibodies. *Arch. Biochem. Biophys. 485*, 35−40.

(12) Silva, C. J., Onisko, B. C., Dynin, I., Erickson, M. L., Vensel, W. H., Requena, J. R., Antaki, E. M., and Carter, J. M. (2010) Assessing the role of oxidized methionine at position 213 in the formation of prions in hamsters. *Biochemistry 49*, 1854−1861.

(13) Bessen, R. A., and Marsh, R. F. (1992) Identification of two biologically distinct strains of transmissible mink encephalopathy in hamsters. *J. Gen. Virol. 73*, 329−334.

(14) Carp, R. I., Kim, Y. S., and Callahan, S. M. (1990) Pancreatic lesions and hypoglycemia-hyperinsulinemia in scrapie-injected hamsters. *J. Infect. Dis. 161*, 462−466.

(15) Kimberlin, R. H., Cole, S., and Walker, C. A. (1987) Temporary and permanent modifications to a single strain of mouse scrapie on transmission to rats and hamsters. *J. Gen. Virol. 68*, 1875−1881.

(16) Kimberlin, R. H., Walker, C. A., and Fraser, H. (1989) The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. *J. Gen. Virol. 70*, 2017−2025.

(17) Safar, J. G., Lessard, P., Tamguney, G., Freyman, Y., Deering, C., Letessier, F., Dearmond, S. J., and Prusiner, S. B. (2008) Transmission and detection of prions in feces. *J. Infect. Dis. 198*, 81−89.

(18) Miller, M. W., and Williams, E. S. (2003) Prion disease: Horizontal prion transmission in mule deer. *Nature 425*, 35−36.

(19) Hoinville, L. J. (1996) A review of the epidemiology of scrapie in sheep. *Rev. Sci. Tech. 15*, 827−852.

(20) Rutala, W. A., and Weber, D. J. (2001) Creutzfeldt-Jakob disease: Recommendations for disinfection and sterilization. *Clin. Infect. Dis. 32*, 1348−1356.

(21) Ding, N., Neumann, N. F., Price, L. M., Braithwaite, S. L., Balachandran, A., Belosevic, M., and El-Din, M. G. (2012) Inactivation of template-directed misfolding of infectious prion protein by ozone. *Appl. Environ. Microbiol. 78*, 613−620.

(22) Wolschner, C., Giese, A., Kretzschmar, H. A., Huber, R., Moroder, L., and Budisa, N. (2009) Design of anti- and pro-aggregation variants to assess the effects of methionine oxidation in human prion protein. *Proc. Natl. Acad. Sci. U.S.A. 106*, 7756−7761.

(23) Colombo, G., Meli, M., Morra, G., Gabizon, R., and Gasset, M. (2009) Methionine sulfoxides on prion protein Helix-3 switch on the α-fold destabilization required for conversion. *PLoS One 4*, e4296.

(24) Lisa, S., Meli, M., Cabello, G., Gabizon, R., Colombo, G., and Gasset, M. (2010) The structural intolerance of the PrP α-fold for polar substitution of the helix-3 methionines. *Cell. Mol. Life Sci. 67*, 2825−2838.

(25) Younan, N. D., Nadal, R. C., Davies, P., Brown, D. R., and Viles, J. H. (2012) Methionine oxidation perturbs the structural core of the prion protein and suggests a generic misfolding pathway. *J. Biol. Chem. 287*, 28263−28275.

(26) Gasset, M., Baldwin, M. A., Lloyd, D. H., Gabriel, J. M., Holtzman, D. M., Cohen, F., Fletterick, R., and Prusiner, S. B. (1992) Predicted α-helical regions of the prion protein when synthesized as peptides form amyloid. *Proc. Natl. Acad. Sci. U.S.A. 89*, 10940−10944.

(27) Requena, J. R., Dimitrova, M. N., Legname, G., Teijeira, S., Prusiner, S. B., and Levine, R. L. (2004) Oxidation of methionine residues in the prion protein by hydrogen peroxide. *Arch. Biochem. Biophys. 432*, 188−195.

(28) Breydo, L., Bocharova, O. V., Makarava, N., Salnikov, V. V., Anderson, M., and Baskakov, I. V. (2005) Methionine oxidation interferes with conversion of the prion protein into the fibrillar proteinase K-resistant conformation. *Biochemistry 44*, 15534−15543.

(29) Pamplona, R., Naudi, A., Gavin, R., Pastrana, M. A., Sajnani, G., Ilieva, E. V., Del Rio, J. A., Portero-Otin, M., Ferrer, I., and Requena, J. R. (2008) Increased oxidation, glycoxidation, and lipoxidation of brain proteins in prion disease. *Free Radical Biol. Med. 45*, 1159−1166.

(30) Tamguney, G., Giles, K., Glidden, D. V., Lessard, P., Wille, H., Tremblay, P., Groth, D. F., Yehiely, F., Korth, C., Moore, R. C., Tatzelt, J., Rubinstein, E., Boucheix, C., Yang, X., Stanley, P., Lisanti, M. P., Dwek, R. A., Rudd, P. M., Moskovitz, J., Epstein, C. J., Cruz, T. D., Kuziel, W. A., Maeda, N., Sap, J., Ashe, K. H., Carlson, G. A., Tesseur, I., Wyss-Coray, T., Mucke, L., Weisgraber, K. H., Mahley, R. W., Cohen, F. E., and Prusiner, S. B. (2008) Genes contributing to prion pathogenesis. *J. Gen. Virol. 89*, 1777−1788.

(31) Chen, M., and Cook, K. D. (2007) Oxidation artifacts in the electrospray mass spectrometry of Aβ Peptide. *Anal. Chem. 79*, 2031−2036.

(32) Morand, K., Talbo, G., and Mann, M. (1993) Oxidation of peptides during electrospray ionization. *Rapid Commun. Mass Spectrom. 7*, 738−743.

(33) Sun, G., and Anderson, V. E. (2004) Prevention of artifactual protein oxidation generated during sodium dodecyl sulfate-gel electrophoresis. *Electrophoresis 25*, 959−965.

(34) Swiderek, K. M., Davis, M. T., and Lee, T. D. (1998) The identification of peptide modifications derived from gel-separated proteins using electrospray triple quadrupole and ion trap analyses. *Electrophoresis 19*, 989−997.

(35) Chowdhury, S. K., Eshraghi, J., Wolfe, H., Forde, D., Hlavac, A. G., and Johnston, D. (1995) Mass spectrometric identification of amino acid transformations during oxidation of peptides and proteins: Modifications of methionine and tyrosine. *Anal. Chem. 67*, 390−398.

(36) Guan, Z., Yates, N. A., and Bakhtiar, R. (2003) Detection and characterization of methionine oxidation in peptides by collision-induced dissociation and electron capture dissociation. *J. Am. Soc. Mass Spectrom. 14*, 605−613.

(37) Kotiaho, T., Eberlin, M. N., Vainiotalo, P., and Kostiainen, R. (2000) Electrospray mass and tandem mass spectrometry identi-

fication of ozone oxidation products of amino acids and small peptides. *J. Am. Soc. Mass Spectrom. 11*, 526−535.
(38) Boschi-Muller, S., Gand, A., and Branlant, G. (2008) The methionine sulfoxide reductases: Catalysis and substrate specificities. *Arch. Biochem. Biophys. 474*, 266−273.
(39) Weissbach, H., Resnick, L., and Brot, N. (2005) Methionine sulfoxide reductases: History and cellular role in protecting against oxidative damage. *Biochim. Biophys. Acta 1703*, 203−212.
(40) Bolton, D. C., Rudelli, R. D., Currie, J. R., and Bendheim, P. E. (1991) Copurification of Sp33−37 and scrapie agent from hamster brain prior to detectable histopathology and clinical disease. *J. Gen. Virol. 72*, 2905−2913.
(41) Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F. E., and Prusiner, S. B. (1998) Eight prion strains have PrP(Sc) molecules with different conformations. *Nat. Med. 4*, 1157−1165.
(42) Prusiner, S. B., Groth, D., Serban, A., Stahl, N., and Gabizon, R. (1993) Attempts to restore scrapie prion infectivity after exposure to protein denaturants. *Proc. Natl. Acad. Sci. U.S.A. 90*, 2793−2797.
(43) Onisko, B. C., Silva, C. J., Dynin, I., Erickson, M., Vensel, W. H., Hnasko, R., Requena, J. R., and Carter, J. M. (2007) Sensitive, preclinical detection of prions in brain by nanospray liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom. 21*, 4023−4026.
(44) Barik, S. (1995) Site-directed mutagenesis by double polymerase chain reaction. *Mol. Biotechnol. 3*, 1−7.
(45) Xu, Z., Colosimo, A., and Gruenert, D. C. (2003) Site-directed mutagenesis using the megaprimer method. *Methods Mol. Biol. 235*, 203−207.
(46) Silva, C. J., Onisko, B. C., Dynin, I., Erickson, M. L., Requena, J. R., and Carter, J. M. (2011) Utility of Mass Spectrometry in the Diagnosis of Prion Diseases. *Anal. Chem. 83*, 1609−1615.
(47) Onisko, B., Dynin, I., Requena, J. R., Silva, C. J., Erickson, M., and Carter, J. M. (2007) Mass spectrometric detection of attomole amounts of the prion protein by nanoLC/MS/MS. *J. Am. Soc. Mass Spectrom. 18*, 1070−1079.
(48) Spraker, T. R., Balachandran, A., Zhuang, D., and O'Rourke, K. I. (2004) Variable patterns of distribution of PrP(CWD) in the obex and cranial lymphoid tissues of Rocky Mountain elk (*Cervus elaphus nelsoni*) with subclinical chronic wasting disease. *Vet. Rec. 155*, 295−302.
(49) Lee, I. S., Long, J. R., Prusiner, S. B., and Safar, J. G. (2005) Selective precipitation of prions by polyoxometalate complexes. *J. Am. Chem. Soc. 127*, 13802−13803.
(50) Ghesquiere, B., Jonckheere, V., Colaert, N., Van Durme, J., Timmerman, E., Goethals, M., Schymkowitz, J., Rousseau, F., Vandekerckhove, J., and Gevaert, K. (2011) Redox proteomics of protein-bound methionine oxidation. *Mol. Cell. Proteomics 10*, M110006866.
(51) Nukuna, B. N., Goshe, M. B., and Anderson, V. E. (2001) Sites of hydroxyl radical reaction with amino acids identified by ^{2}H NMR detection of induced ^{1}H/^{2}H exchange. *J. Am. Chem. Soc. 123*, 1208−1214.
(52) Wehr, N. B., and Levine, R. L. (2012) Wanted and wanting: Antibody against methionine sulfoxide. *Free Radical Biol. Med. 53*, 1222−1225.