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Peroxymonosulfate Rapidly Inactivates the Disease-associated Prion Protein

Alexandra R. Chesney[1], **Clarissa J. Booth**[1,†], **Christopher B. Lietz**[2], **Lingjun Li**[1,2,3], and **Joel A. Pedersen**[1,2,4,*]

[1]Molecular and Environmental Toxicology Center, University of Wisconsin, Madison, WI 53706, USA

[2]Department of Chemistry, University of Wisconsin, Madison, WI 53706, USA

[3]School of Pharmacy, University of Wisconsin, Madison, WI 53706, USA

[4]Department of Soil Science, University of Wisconsin, Madison, WI 53706, USA

Abstract

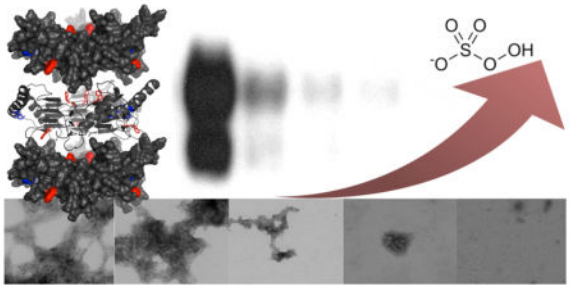
Prions, the etiological agents in transmissible spongiform encephalopathies, exhibit remarkable resistance to most methods of inactivation that are effective against conventional pathogens. Prions are composed of pathogenic conformers of the prion protein (PrP^{TSE}). Some prion diseases are transmitted, in part, through environmental routes. The recalcitrance of prions to inactivation may lead to a persistent reservoir of infectivity that contributes to the environmental maintenance of epizootics. At present, few methods exist to remediate prion-contaminated lands. Here we examined the ability of peroxymonosulfate to degrade PrP^{TSE} as an initial step toward developing an *in situ* chemical oxidation process to inactivate prions. We find that peroxymonosulfate rapidly degrades PrP^{TSE} from two species. Transition metal-catalyzed decomposition of peroxymonosulfate to produce sulfate radicals appears to enhance degradation. We further demonstrate that exposure to peroxymonosulfate significantly reduced PrP^{C}-to-PrP^{TSE} converting ability as measured by protein misfolding cyclic amplification, used as a proxy for infectivity. Liquid chromatography-tandem mass spectrometry revealed that exposure to peroxymonosulfate results in oxidative modifications to methionine and tryptophan residues. This study indicates that peroxymonosulfate may hold promise for *in situ* remediation of prion-contaminated surfaces.

Graphical abstract

*Corresponding Author: tel: (608) 263-4971; fax: (608) 265-2595; joelpedersen@wisc.edu.
†Current Address: Pace Analytical, Minneapolis MN

Supporting Information Available.

Supplementary methods and figures. This information is available free of charge via the Internet at http://pubs.acs.org.



Keywords

Chronic wasting disease; scrapie; transmissible spongiform encephalopathy; oxone; *in situ* chemical oxidation

INTRODUCTION

Prions are the etiological agents of transmissible spongiform encephalopathies (TSEs) or prion diseases.[1,2] Prion diseases are a group of fatal, neurodegenerative disorders affecting a number of mammalian species including humans (kuru, Creutzfeldt-Jakob disease), sheep and goats (scrapie), cattle (bovine spongiform encephalopathy; “mad cow” disease), and North American members of the deer family (chronic wasting disease; CWD). Prions appear to be composed primarily, if not solely of misfolded conformers (PrP^{TSE}) of the host-encoded cellular prion protein (PrP^{C}).[1,2] The central molecular event in prion diseases is the conformational conversion of PrP^{C} into PrP^{TSE},[1,3] which induces profound changes in the biophysical properties of the protein. Whereas PrP^{C} is primarily α-helical, detergent soluble and labile with respect to proteolysis, PrP^{TSE} exhibits high β-sheet content, is largely insoluble in water and most detergents, and displays remarkable resistance to a variety of chemical and physical inactivation methods.[4–7] Treatments that are effective for inactivating conventional microbial pathogens (e.g., boiling, ultraviolet irradiation, ethanol, formalin, conventional autoclaving) do not eliminate prion infectivity.[8] Sterilization procedures recommended by the World Health Organization include > 1 h exposure to 1 N sodium hydroxide or 2% sodium hypochlorite, or autoclaving in 1 N sodium hydroxide at 121 °C for 30 min.[8]

Chronic wasting disease of North American members of the deer family (cervids) and scrapie in sheep and goats are spread in part via environmental reservoirs of prion infectivity.[9] Past studies indicate that indirect transmission via environmental routes may play a important role in the long-term dynamics of CWD in North America.[10] Infected animals shed prions in feces, urine, and saliva.[11–15] Prion infectivity can persist in the environment for years.[13,16–18] The persistence of prions in the environment is attributed to the intrinsic stability of PrP^{TSE} fibrils and may be enhanced by association with soil constituents,[19–21] one explanation invoked for the preservation of intrinsically labile organic matter.[22,23]

Remediation of prion-contaminated lands poses a challenge; decontamination methods that have been demonstrated to be effective in inactivating prions are difficult to apply in the environment.[24] Several oxidants used for *in situ* chemical oxidation of recalcitrant organic contaminants[25–29] have been investigated for their ability to degrade PrP^{TSE} including hydrogen peroxide,[30] ozone,[31–33] permanganate,[34–36] and the Fenton reagent.[37–39] Table 1 provides a summary of selected studies that have demonstrated the ability of oxidants to inactivate prions. Most oxidants investigated to date are either insufficiently effective against prions (e.g., H_2O_2, permanganate) or possess features that may limit their utility for application to soils (e.g., the gaseous nature of O_3, low pH needed for optimal application of Fenton reagent). Peroxymonosulfate (HSO_5^-) has been investigated for *in situ* chemical oxidation,[40–43] but has not previously been investigated for its ability to inactivate prions. Peroxymonosulfate is a monosubstituted derivative of H_2O_2 that is less prone to spontaneous decomposition in water than hydrogen peroxide and is frequently more reactive than H_2O_2 kinetically despite having only a slightly higher standard reduction potential (E_H^0 (HSO_5^-/HSO_4^-) = +1.82 V vs. E_H^0 (H_2O_2/H_2O) = +1.776 V).[44–46] Peroxymonosulfate is reactive over a broad pH range, but its stability declines as p$K_{a,2}$ is approached (9.9 at 20 °C).[47,48] Peroxymonosulfate can oxidize organic moieties including acetals to alcohols,[49] alkenes to ketones,[50] sulfides to sulfones,[51] and phosphines to phosphine oxides.[52]

Peroxymonosulfate can be activated thermally,[45] radiolytically,[53] photolytically ($\lambda < 419$ nm),[54] and in the presence of transition metals to produce sulfate radical ($SO_4\cdot^-$).[41,55–57] Much previous work with peroxymonosulfate used Co(II) to catalytically decompose peroxymonosulfate to $SO_4\cdot^-$.[40–43,54,57,58] The estimated standard reduction potential of $SO_4\cdot^-$, E_H^0 ($SO_4\cdot^-/SO_4^{2-}$), is 2.5 to 3.1 V,[59] similar to or higher than that for hydroxyl radical (·OH; E_H^0 ($\cdot OH/H_2O$) = 2.59 or 2.7 V).[60] The reduction potential for sulfate radical does not vary with pH for proton activities typical of the environment while that for hydroxyl radical does, making the formal potential of $SO_4\cdot^-$ larger than that of ·OH at pH > 3.4 (assuming $E_H^0(SO_4\cdot^-/SO_4^{2-})$ and E_H^0 ($\cdot OH/H_2O$) are 2.5 V and 2.7 V, respectively; all activities unity other than that for protons). Cobalt-activated peroxymonosulfate has been employed to treat diesel-contaminated soil,[40] polychlorinated biphenyls,[61] atrazine,[54] and landfill leachates.[62] Activated peroxymonosulfate can degrade peptidic cyanobacterial toxins[63] and some amino acids.[64] The ability of (activated) peroxymonosulfate to inactivate prions has not been previously reported.

The objectives of this study were to determine the extent to which peroxymonosulfate degrades and inactivates pathogenic prion protein unaided, and when activated by cobalt. To achieve these objectives we used immunoblotting to investigate degradation of PrP^{TSE} from white-tailed deer (*Odocoileus virginianus*; CWD agent) and golden hamsters (*Mesocricetus auratus*; HY agent) by (Co(II)-activated) peroxymonosulfate as a function of time and peroxymonosulfate concentration. We used protein misfolding cyclic amplification (PMCA) to examine (activated) peroxymonosulfate-induced reductions in the PrP^C-to-PrP^{TSE} converting ability of prions as a proxy for infectivity. We investigated modifications to the primary structure of PrP^{TSE} due to exposure to (activated) peroxymonosulfate by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

EXPERIMENTAL

Prion protein sources

Hamster-adapted transmissible mink encephalopathy agent (HY strain) and cervid (CWD) agent were obtained from brain tissue of experimentally inoculated Syrian golden hamsters and an experimentally inoculated white-tailed deer,[65] respectively. Animals were cared for in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Wisconsin – Madison (Assurance Number 3464-01). Brain tissue was homogenized in 1× Dulbecco's phosphate buffered saline (DPBS; 137 mM NaCl, 8.1 mM $NaHPO_4^{2-}$, 1.47 mM H_2PO_4; pH 7), and the resultant 10% (w/v) brain homogenate (BH) was stored at −80 °C until use. When elimination of PrP^C was needed, BH was treated with 50 μg·mL^{-1} PK (final concentration) for 1 h at 37 °C. Proteinase K activity was halted by addition of PMSF to a final concentration of 4 mM. Some experiments were conducted with purified HY PrP^{TSE}, wherein the P4 pellet was isolated from eight hamster brains following the procedure of Bolton et al.[3] as modified by McKenzie et al.[66] The P4 pellet was resuspended in 1× DPBS, pH 7.4. Total protein concentration in the purified preparation was measured using a BCA protein assay (Pierce), and PrP^{TSE} concentrations were estimated to be > 90% of total protein.[20] Purified, full-length (23–230) recombinant murine PrP (residues 23–231, lacking a Histag) in the α-helix-rich conformation (α-mo-recPrP) was acquired from Prionatis AG (RPA0101, Alpnach Dorf, Switzerland).

Reaction of peroxymonosulfate with prions

Pathogenic prion protein, either in the form of purified preparation (HY strain) or 10% BH (HY or CWD strains) was mixed with solutions of peroxymonosulfate and/or $CoCl_2$ under the conditions indicated in the Results and Discussion. All concentrations presented for peroxymonosulfate, $CoCl_2$, and quenching agents reflect final concentrations. The molar ratio of peroxymonosulfate to $CoCl_2$ was 125:3 unless otherwise specified. All experiments were conducted at room temperature (~25 °C) in polypropylene microcentrifuge tubes in the dark. Some experiments employed α-mo-recPrP. Reactions with peroxymonosulfate and/or $CoCl_2$ were halted by addition of an equal volume of 0.5 M sodium thiosulfate (unless otherwise specified). In some cases, samples were pre-treated with sodium thiosulfate to prevent oxidation by peroxymonosulfate and radical species. All experiments were conducted in triplicate unless otherwise specified. Final solution pH of peroxymonosulfate was stable at approximately 1.5. For experiments with α-mo-recPrP pH was controlled by preparing peroxymonosulfate solutions in 100 mM phosphate and brought to the desired pH with 0.1 M NaOH or 0.1 M H_2SO_4.

Immunoblot detection of PrP^{TSE}

Following treatment, PrP^{TSE} samples were prepared for SDS-PAGE as previously described.[67] Briefly, a 20 μL aliquot was removed from the reaction vessel and mixed with 10 μL of 10× SDS sample buffer (100 mM Tris; pH 8, 10% SDS, 7.5 mM EDTA, 100 mM dithiothreitol, 30% glycerol). Samples were then heated at 100 °C for 10 min and fractionated on 12% bis-tris polyacrylamide gels (Invitrogen). Proteins were electrotransferred from the gel to a 0.45 μm polyvinyl difluoride membrane (Millipore). Membranes were blocked with 5% nonfat dry milk (prepared in 1× Tris-buffered saline

containing 0.1% Tween 20) overnight at 4 °C. Membrane-bound hamster PrP was probed with monoclonal antibody 3F4 (Covance, 1:40,000 dilution; epitope: 109–112) or SAF83 (Cayman Chemical, 1:200, epitope: 126–164). Cervid PrP and murine recPrP were probed with monoclonal antibodies 8G8 (Cayman Chemical, 1:1000, epitope: 97–102) and Bar224 (Cayman Chemical, 1:10000, epitope: 141–151). Primary antibodies were detected with horseradish peroxidase-conjugated goat-anti-mouse immunoglobulin G (BioRad, 1:10,000 dilution) and Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology). Densitometric analysis of immunoblot bands was conducted using Image J Software.

Protein misfolding cyclic amplification

Bead-assisted protein misfolding cyclic amplification (PMCA) was conducted following the methods of Johnson et al.,[68] adapted to a microplate format[69] and designated mbPMCA. Detection of CWD prions was achieved using PrP^C in normal brain homogenate from uninfected transgenic mice hemizygous for the cervid prion gene (Tg(CerPrP)1535$^{\pm}$ mice)[70] as substrate for the PMCA reaction. Mice were euthanized by CO_2 asphyxiation and immediately perfused with 1× modified DPBS without Ca^{2+} or Mg^{2+} (Thermo Scientific, amended with 5 mM EDTA). Brains were rapidly removed, flash frozen in liquid nitrogen, and stored at −80 °C until use. Brain tissue was homogenized on ice to 10% (w/v) in PMCA conversion buffer (Ca^{2+}- and Mg^{2+}-free DPBS supplemented with 150 mM NaCl, 1% Triton X-100, 0.05% saponin (Mallinckrodt), 5 mM EDTA, and 1 tablet Roche Complete EDTA-free protease inhibitors cocktail (Fisher) per 50 mL conversion buffer). Brain homogenates were clarified by centrifugation (2 min, 2,000*g*). Supernatant was transferred to pre-chilled microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at −80 °C until use.

Seeds for mbPMCA were prepared from CWD-positive white-tailed deer brain tissue by serially diluting 10% BH (made up in DPBS) five-fold in normal brain homogenate (NBH) to generate a dilution series. Sample dilutions were used to seed 36 μL NBH (4 μL seed) in 96-well PCR microplate (Axygen, Union City, CA, USA) with one 2.38 mm Teflon® bead (McMaster-Carr, #9660K12). Experimental plates were placed in a rack in a Misonix S-4000 microplate horn, and the reservoir was filled with ultrapure water. Each round of mbPMCA consisted of 96 cycles (30 s sonication at 40–60% of maximum power, 1770 s incubation at 37 °C). At the completion of 96 cycles, new NBH was reseeded with 4 μL of the reaction product for serial mbPMCA. After completing the last round of mbPMCA, 20 μL of each sample was PK digested (50 $\mu g \cdot mL^{-1}$, 1 h, 37 °C) and analyzed by SDS-PAGE with immunoblot detection (*vide supra*).

Liquid chromatography-tandem mass spectrometry

To prepare samples for LC-MS/MS analysis, we exposed ~2.4 μg of HY PrP^{TSE} (PK-treated purified preparation) with 9.6 mM peroxymonosulfate in the absence or presence of 230 μM $CoCl_2$ for 15 or 60 min. At the end of the exposure, samples were quenched with an equal volume of 0.5 M sodium thiosulfate, and the total sample volume (~26 μL) was dried down using a SpeedVac™ (Thermo Scientific). To achieve high sequence coverage in LC-MS/MS analysis, samples were digested with multiple proteases: samples were digested by a combination of endoprotease Lys-C and trypsin (which cleaves C-terminal to lysine and arginine), or by chymotrypsin (which cleaves C-terminal to tryptophan, tyrosine,

phenylalanine, and leucine) as detailed in the SI. Dry pellets were resuspended in 20 μL of 8 M urea (in 50 mM Tris-HCl, pH 8) and sonicated for 30 s. Cysteine residues were reduced and alkylated as outlined in the SI.

Peptides were extracted and desalted using 100 μL reversed-phase C18 Omix SPE pipette tips (Agilent Technologies) following manufacturer recommendations and dried down using a SpeedVac (Thermo Scientific). The sample was then resuspended in 0.1% formic acid (prepared in LCMS grade water). The peptide fragments were analyzed on a nanoAcquity UPLC (Waters Corp.) with an integrated nano-electrospray ionization (nESI) emitter. Upon elution from the column, sample was electrosprayed into the nESI source of an Orbitrap Elite mass spectrometer (Thermo Fisher). The selected ions were isolated and fragmented individually, and each fragmentation spectrum was recorded. Samples were prepared in two technical replicates and analyzed in duplicate by LC-MS/MS. Details on the method and subsequent data analysis are described in the SI.

RESULTS AND DISCUSSION

Peroxymonosulfate rapidly degrades pathogenic prion protein

Prion strains exhibit different susceptibilities to inactivation methods.[71,72] We therefore examined the ability of peroxymonosulfate to degrade PrP^{TSE} from two prion strains: laboratory model strain HY and an environmentally relevant CWD strain. We exposed 10% HY brain homogenate, purified HY preparation or 10% CWD brain homogenate to 125 mM peroxymonosulfate for 60 min. Peroxymonosulfate reactions were quenched with up to 1 M $Na_2S_2O_3$. (This concentration of $Na_2S_2O_3$ did not affect detection of PrP^{TSE} by immunoblotting; Figure S1.) Densitometry values from the resulting immunoblots are presented in Figure 1A (inset) relative to the control (PrP^{TSE} in ultrapure water). Following a 1-h treatment with peroxymonosulfate, immunoreactivity of all forms of PrP^{TSE} was reduced to ≤ 13% of control (i.e., 13 ± 1%, 8 ± 3%, and 8 ± 2% of control immunoblot intensity for HY BH, HY prep, and CWD BH, respectively). The reproducibility of PrP^{TSE} immunoblots was consistent with that of general protein immunoblotting, in which errors associated with quantitative comparisons are often ≥ 10%.[73] The results described here for HY were obtained using monoclonal antibodies (mAbs) 3F4 and SAF83 (SI), which bind to non-overlapping residues 109–112 and 126–164 respectively in hamster PrP; immunoblots for CWD were probed with mAbs 8G8 and Bar224, which are directed against residues 97–102 and 141–151, respectively, of deer PrP (epitopes are mapped onto a molecular model for PrP^{TSE} in Figure S2).[74] The results in Figure 1A (inset) indicate that HSO_5^- either induced alteration of at least two separate antibody epitopes in PrP (fragments) from each species or fragmented the protein so extensively that the fragments were not retained on the gel (i.e., fragment molecular mass ≤ 10 kDa) or both. All epitopes probed contain easily oxidizable amino acid residues (i.e., histidine (H), tryptophan (W), and tyrosine (Y), and methionine (M).[75] The cervid and hamster prion proteins both contain 42 easily oxidizable residues spaced approximately evenly throughout the protein (Figure S3), leading to the expectation of oxidation of residues throughout the protein. These results demonstrate that peroxymonosulfate is able to degrade pathogenic prion protein from two different prion strains (HY and CWD) and furthermore, that this degradation can occur even in the presence

of a large excess of other biomolecules (e.g., other proteins, nucleic acids, lipids, glycans in brain homogenate).[76–78]

We investigated the concentration-dependence of PrP^{TSE} degradation by peroxymonosulfate by incubating PrP^{TSE} (10% CWD brain homogenate) with a range of peroxymonosulfate concentrations for 30 min at a constant ratio of 125:3. Immunoblot band intensity for CWD PrP^{TSE} was significantly reduced at peroxymonosulfate concentrations ≥ 25 mM ($p < 0.0001$) (Figure 1A). These data suggest that substantial PrP^{TSE} degradation (≥ 75%) is achieved within 30 min at 25 mM peroxymonosulfate.

We then examined the kinetics of PrP^{TSE} degradation by peroxymonosulfate at two concentrations. We added 15 μL 10% BH (CWD) to ultrapure water, or 25 or 125 mM peroxymonosulfate for 1 to 60 min (Figure 1B). Immunoreactivity of CWD PrP^{TSE} was diminished after 5 min incubation ($p < 0.01$) with 125 mM peroxymonosulfate, and after 15 min incubation, CWD PrP^{TSE} was substantially reduced, and in some replicates, no longer detectable. Contact with 125 mM peroxymonosulfate for durations exceeding 1 min significantly reduced immunoreactivity compared to controls ($p < 0.01$). In contrast, contact with 25 mM peroxymonosulfate did not result in significantly reduced band intensity until 15 min exposure ($p < 0.01$). At both peroxymonosulfate concentrations, CWD PrP^{TSE} immunoreactivity was undetectable after 60 min exposure (Figure 1B, $p < 0.01$).

To gain further insight into the degradation of PrP^{TSE} achieved by exposure to HSO_5^- we imaged protein aggregates from purified prion preparations[79] by TEM (Methods in SI) after 10, 30, and 60 min and 24 h exposure to 50 mM peroxymonosulfate (Figure S4). The control (unexposed) and samples exposed for 10 min exhibited definitive rod-like structures characteristic of pathogenic prion protein fibrils. Samples exposed to HSO_5^- for longer time periods had fewer fibril-like structures; the 24 h exposure showed no discernable structures. These data indicate that peroxymonosulfate extensively altered prion protein aggregates.

Peroxymonosulfate degrades recombinant prion protein in the absence of transition metal catalysts

Peroxymonosulfate is a strong oxidant capable of direct oxidation of primary amines, alkenes, azides, and sulfides in aqueous solution without activation to form radical species.[80,81] The results presented above indicate that peroxymonosulfate can degrade PrP^{TSE} in brain homogenate and in purified preparations without addition by an exogenous transition metal (i.e., cobalt) to activate HSO_5^-. Brain homogenate and purified PrP^{TSE} preparations contain transition metals (e.g., iron, copper)[82,83] that can activate HSO_5^-.[56,84–86] For example, ~2.4 μg Cu^{2+} per mg PrP^{TSE} has been estimated to be present in brain tissue of sporadic Creutzfeldt-Jakob disease patients.[83] The benign, normal form of the prion protein, PrP^C, associates with copper with a binding stoichiometry of approximately two copper atoms per PrP molecule under physiologically relevant conditions;[82] the binding stoichiometry of PrP^{TSE} has not been worked out, however some replacement of copper by manganese and zinc has been reported.[83,87]

We sought to establish whether HSO_5^- could transform prion protein in the absence of transition metals that might activate peroxymonosulfate to produce radical species. To do

this we used α-mo-recPrP because available sources of PrP^{TSE} were expected to include transition metals. We incubated ~15 ng of α-mo-recPrP with 12.5 mM peroxymonosulfate in the absence and presence of 300 μM cupric chloride ($CuCl_2$) for 1 h, and quenched the reaction at the end of the incubation period or pre-treated samples with 50 mM $Na_2S_2O_3$. Recombinant prion proteins are typically purified using immobilized metal affinity chromatography, and His-tagged proteins purified in this manner may contain transition metal contaminants.[88] To exclude this possibility, we used α-mo-recPrP not bearing a His-tag. Under the experimental conditions employed, exposure to HSO_5^- completely eliminated α-mo-recPrP immunoreactivity (Figure S5) indicating that activation of peroxymonosulfate to form radical species was not prerequisite to degrade prion protein. Exposure to peroxymonosulfate + $CuCl_2$ also eliminated α-mo-recPrP immunoreactivity. Pre-treatment of samples with sodium thiosulfate prevented loss of α-mo-recPrP immunoreactivity in treatments of peroxymonosulfate with and without $CuCl_2$ (S5). These results indicate that peroxymonosulfate can directly oxidize prion protein without metal activation.

Cobalt accelerates degradation of pathogenic prion protein exposed to peroxymonosulfate

We next assessed whether activation of peroxymonosulfate by Co(II) accelerates degradation of PrP^{TSE}. We incubated 10% HY brain homogenate, purified HY preparation or 10% CWD brain homogenate with 125 mM peroxymonosulfate and 3 mM $CoCl_2$ for 60 min and found immunoreactivity to be diminished to 5 ± 0.3%, 3 ± 1%, and 2 ± 1% of control (identical to treatments except that HSO_5^- was excluded), respectively (Figure 2A, inset). Examination of the concentration-dependence of Co(II)-activated peroxymonosulfate transformation of PrP^{TSE} (10% CWD brain homogenate) revealed substantial decrease immunoreactivity at 25 mM HSO_5^- by 30 min exposure (Figure 2A); in contrast, substantial degradation by peroxymonosulfate without added $CoCl_2$ was not observed at concentrations below 50 mM HSO_5^- for this exposure period (Figure 1A). Exposure of PrP^{TSE} to 125 mM HSO_5^- in the absence or presence of $CoCl_2$ resulted in elimination of immunoreactivity by 15 min (Figure 1B and 2B). When PrP^{TSE} was exposed to 25 mM HSO_5^- in the presence of 600 μM $CoCl_2$, immunoblot band intensity was substantially reduced after 15 min ($p = 0.0013$) and completely eliminated at longer exposure times (Figure 2B). In the absence of cobalt, equivalent PrP^{TSE} degradation required longer exposure (1 h, Figure 1B). These data demonstrate that addition of cobalt enhances the effectiveness of PrP^{TSE} degradation by peroxymonosulfate.

The experiments described above used a 125:3 molar ratio of HSO_5^- to Co^{2+}. This ratio is within ranges reported for optimal degradation of diesel-contaminated soil and 2,4-dichlorophenol.[57,40] We investigated the effect of the HSO_5^--to-$CoCl_2$ ratio on degradation at constant HSO_5^- concentrations of 25 and 125 mM and found no difference ($p > 0.05$) in the extent of degradation after 20 min for HSO_5^--to-$CoCl_2$ ratios between 10:1 and 100:1; after 1 h, immunoreactivity was no longer detectable for these treatments (data not shown).

Formation of sulfate and hydroxyl radicals is not necessary for rapid degradation of PrP^{TSE}

Activation of peroxymonosulfate by transition metals produces sulfate and hydroxyl radicals. To assess whether the formation of either of these radical species was required for

the rapid transformation of PrP^{TSE}, we employed ethanol and *tert*-butyl alcohol as radical quenchers in exposures of PrP^{TSE} to peroxymonosulfate with or without added $CoCl_2$. Ethanol reacts rapidly with hydroxyl and sulfate radicals (·OH: $k' = 1.2$ to 2.8×10^9 $M^{-1}s^{-1}$; $SO_4{\cdot}^-$: $k' = 1.6$ to 7.7×10^7 $M^{-1}s^{-1}$),[41,56,60,89,90] quenching reactions with these radicals. (We note that ethanol also quenches chloride radical (Cl·), $k' = 1 \times 10^9$ $M^{-1}s^{-1}$).[91] In contrast, the second-order rate constant for *tert*-butyl alcohol reaction with ·OH ($k' = 3.8$ to 7.6×10^8 $M^{-1}s^{-1}$) is three orders of magnitude larger than that for $SO_4{\cdot}^-$ ($k' = 4.0$ to 9.1×10^5 $M^{-1}s^{-1}$),[60,90] allowing it to be used to discriminate between the two radical species.[43,56]

We exposed PrP^{TSE} (10% CWD brain homogenate) to 25 mM PMS ± 600 μM $CoCl_2$ for 5 min, and either quenched at the end of the incubation period with $Na_2S_2O_3$ or pre-treated samples with 17.1 M ethanol or 10.4 M *tert*-butyl alcohol (molar ratios of 684:1 and 416:1 for ethanol- and *tert*-butyl alcohol-to-HSO_5^-, respectively). Degradation of PrP^{TSE} proceeded with sufficient rapidity to abolish detectable immunoreactivity within 5 min in the presence of the alcohol quenchers (Figure S6). This result indicates that the formation of hydroxyl or sulfate (or chloride) radicals is not requisite for rapid transformation of PrP^{TSE}. The species responsible for transformation of PrP^{TSE} may be HSO_5^- itself. Peroxymonosulfate is not quenched by ethanol and has been shown to transform all common proteinogenic amino acids investigated (cysteine not tested) with the largest pseudo-first-order rate constants for methionine, tryptophan, tyrosine and histidine.[92] A second possibility is peroxymonosulfate radical ($SO_5{\cdot}^-$), a species formed from the oxidation of peroxymonosulfate by either sulfate radical or hydroxyl radical.[92] The second-order rate constant for $SO_5{\cdot}^-$ reaction with ethanol ($k'_{EtOH,SO5\cdot-} < 10^3$ $M^{-1}{\cdot}s^{-1}$) is small compared to that of sulfate radical or hydroxyl radical ($k'_{EtOH,i} > 10^7$ $M^{-1}{\cdot}s^{-1}$, $i = SO_4{\cdot}^-$ or ·OH),[59,60,89] making ethanol an inefficient quencher of reactions with this radical.

Interestingly, for samples exposed to peroxymonosulfate alone, pre-exposure of PrP^{TSE} to ethanol or *tert*-butyl alcohol appeared to enhance degradation (compare lanes 9 and 12 with lane 3 in Figure S6). This result may be attributable to partial denaturation of the protein by the alcohol, allowing HSO_5^- access to more of the structure. Ethanol can induce (partial) unfolding of proteins in a concentration-dependent manner.[93] Increased branching of the hydrocarbon portion of alcohols generally reduces their effectiveness in denaturing proteins making *tert*-butyl alcohol a less effective denaturant than ethanol.[93]

Effect of peroxymonosufate on the *in vitro* converting ability of PrP^{TSE}

The biochemical data presented thus far demonstrate that exposure to peroxymonosulfate in the absence or presence of Co^{2+} effects substantial degradation of PrP^{TSE}. The detection method employed (immunoblotting) has two limitations that are important for assessing the effectiveness of HSO_5^- for inactivating prions: (1) immunoblotting has detection limit (~4 ng PrP) that is orders of magnitude higher than the amount of PrP^{TSE} required to initiate disease (for mice, 725,000 times less sensitive than intracerebral inoculation);[94] and (2) declines in immunoreactivity do not always parallel reductions in prion infectivity.[69,95–97] Protein misfolding cyclic amplification exploits the ability of PrP^{TSE} to induce conformational conversion of PrP^C to misfolded, protease K-resistant forms of the protein and has emerged as a sensitive technique for detecting low levels of pathogenic prion protein

(detection limit of 1×10^{-12} dilution of 10% BH from hamsters infected with the 263K strain of hamster-adapted scrapie; estimated to be equivalent of 1.3 ag PrP^{TSE}, or approximately 26 molecules).[94,98] Here we use a form of PMCA adapted to a microplate format and including Teflon beads (mbPMCA) to measure the *in vitro* converting ability of prions as a proxy for infectivity and to sensitively detect PrP^{TSE}.

We exposed PrP^{TSE} (15 μL PK-treated 10% CWD BH) to 125 mM HSO_5^- with and without 3 mM $CoCl_2$ for 1 h. Samples were either quenched at the end of the exposure or pretreated with 0.5 M $Na_2S_2O_3$. A 4 μL aliquot of the reaction mixture was then added to 36 μL NBH and subjected to two rounds of 96 cycles of mbPMCA. Immunoreactivity was not detected at any dilution examined in samples that had been exposed to HSO_5^- regardless of whether Co^{2+} had been added to the sample (Figure 3). Pre-treatment with thiosulfate allowed detection of PrP^{TSE} immunoreactivity for dilutions of CWD-positive deer BH to the $10^{-7.6}$ dilution. These results indicate that 1-h treatment with 125 mM peroxymonosulfate + 3 mM $CoCl_2$ decreased the PrP^{C}-to-PrP^{TSE} converting ability of PrP^{TSE} by a factor of at least $10^{5.9}$. This factor was arrived at by considering that the lowest (10^{-2}) and highest dilutions ($10^{-7.9}$) at which untreated controls could be amplified and treated samples could not.

Modifications to PrP^{TSE} induced by exposure to peroxymonosulfate

The immunoblotting results presented above demonstrate that peroxymonosulfate in the absence or presence of added $CoCl_2$ is capable of degrading pathogenic prion protein. To gain insight into modifications to pathogenic prion protein induced by exposure to peroxymonosulfate ± $CoCl_2$, we analyzed PrP^{TSE} following such treatment by LC-MS/MS. We incubated PrP^{TSE} (PK-treated purified HY preparation) with 9.6 mM HSO_5^- in the absence and presence of 230 μM $CoCl_2$ for 15 or 60 min. Samples were either quenched at the end of each time point or pre-treated with 0.5 M sodium thiosulfate.

Exposure to peroxymonosulfate in the absence and presence of $CoCl_2$ resulted in oxidative modifications to tryptophan and methionine residues. Reproducible tryptophan hydroxylation was observed on chymotryptic peptide fragments containing W^{56} (GNDW(o)EDRY) and W^{10} (GQGGGTHNQW(o)), where (o) indicates oxidative modification. In Figure 4, we show the intensity ratios of these peptide fragments with and without modified tryptophan residues for samples exposed to HSO_5^- for 15 min. Substantially higher levels of oxidized tryptophan residues were present at both locations in samples containing unquenched peroxymonosulfate, consistent with a previous report of oxidative modification of free tryptophan.[92] The location of these residues are displayed on the molecular model of PrP^{TSE} (Figure S2).

Reproducible methionine modifications were detected near the C-terminus of the protein on tryptic (+ endoprotease Lys-C) peptides containing M^{117} (GENFTETDIKIM(sulfone)ER) and M^{124} (VVEQM(sulfone)CTTQYQK) (Figure 4C, and D; mapped onto the molecular model of PrP^{TSE}, Figure S2). The modified methionine residues were absent in samples not exposed to peroxymonosulfate or pre-treated with sodium thiosulfate. Following incubation with peroxymonosulfate in the absence of cobalt, the methionine sulfone-containing peptides were detected at similar intensities after both 15- and 60-min exposures. The same modification, but at substantially lower intensities was observed in 15- and 60-min reactions

with peroxymonosulfate + $CoCl_2$ (data not shown). This result may reflect a true reduction in intensity of the sulfone modification, or enhanced protein degradation by peroxymonosulfate + $CoCl_2$ leading to reduced production of these particular peptide fragments, consistent with individual methionine amino acid modifications seen previously.[92] Similar results were seen by Requena et al.[30] wherein hydrogen peroxide exposure led to the oxidation of methionine residues 109, 112, 129, and 134 in recombinant PrP (SHa, 29–231).

Environmental Implications

Prions are remarkably resistant to inactivation,[8] and their persistence in the environment creates a need to develop effective *in situ* remediation methods for prion-contaminated lands. We have demonstrated that peroxymonosulfate can rapidly degrade and inactivate pathogenic prion protein. We further showed that Co(II)-catalyzed decomposition of HSO_5^- to $SO_4\cdot^-$ accelerated prion degradation. The extent of prion inactivation suggested by the reduction of template-directed misfolding ability as measured by mbPMCA (≥ 5.9 $\log^{10}$ reduction for 1-h exposure to 125 mM HSO_5^- ± 3 mM $CoCl_2$) compares favorably with that for other oxidants that have been tested for their ability to inactivate prions (Table 1). Peroxymonosulfate possesses advantages over some of the other oxidants listed in Table 1 for *in situ* chemical oxidation. Peroxymonosulfate does not require low pH as does Fenton's reagent for optimal hydroxyl radical production[99] or the addition of toxic metals as does Cu^{2+}-catalyzed decomposition of H_2O_2 to produce ·OH.[100] Reductions of infectivity of >6 log have been achieved via exposure to the Fenton reagent, but at elevated temperature (50 °C for 22 h).[39] Heterogeneous photocatalytically produced ·OH have been shown to reduce prion infecitivty,[101] but the utility of such methods for in situ chemical oxidation is limited because the photic zone in soils is on the order of 0.5 mm.[102,103] While ozone has been demonstrated to inactivate prions,[31–33] its use to inactivate prions in soil is complicated by the gaseous nature of the oxidant. In some soil environments, the deposition of SO_4^{2-} may represent a concern for the application of peroxymonosulfate for *in situ* chemical oxidation.

Oxidative modifications to proteins can make them more susceptible to proteolytic degradation.[104] This suggests that if peroxymonosulfate effected only partial PrP^{TSE} degradation in the environment, the resulting oxidative modifications may render it susceptible to *in situ* degradation by native or added proteases. Our results strongly suggest that peroxymonosulfate, even without activation to radical species via transition metal activation, high temperatures or UV exposure, holds promise for *in situ* remediation of prion-contaminated land surfaces. Future research directed at the efficacy of peroxymonosulfate in degrading pathogenic prion protein and inactivating prions associated with soil constituents is warranted. We also note that peroxymonosulfate may prove useful in decontaminating medical instruments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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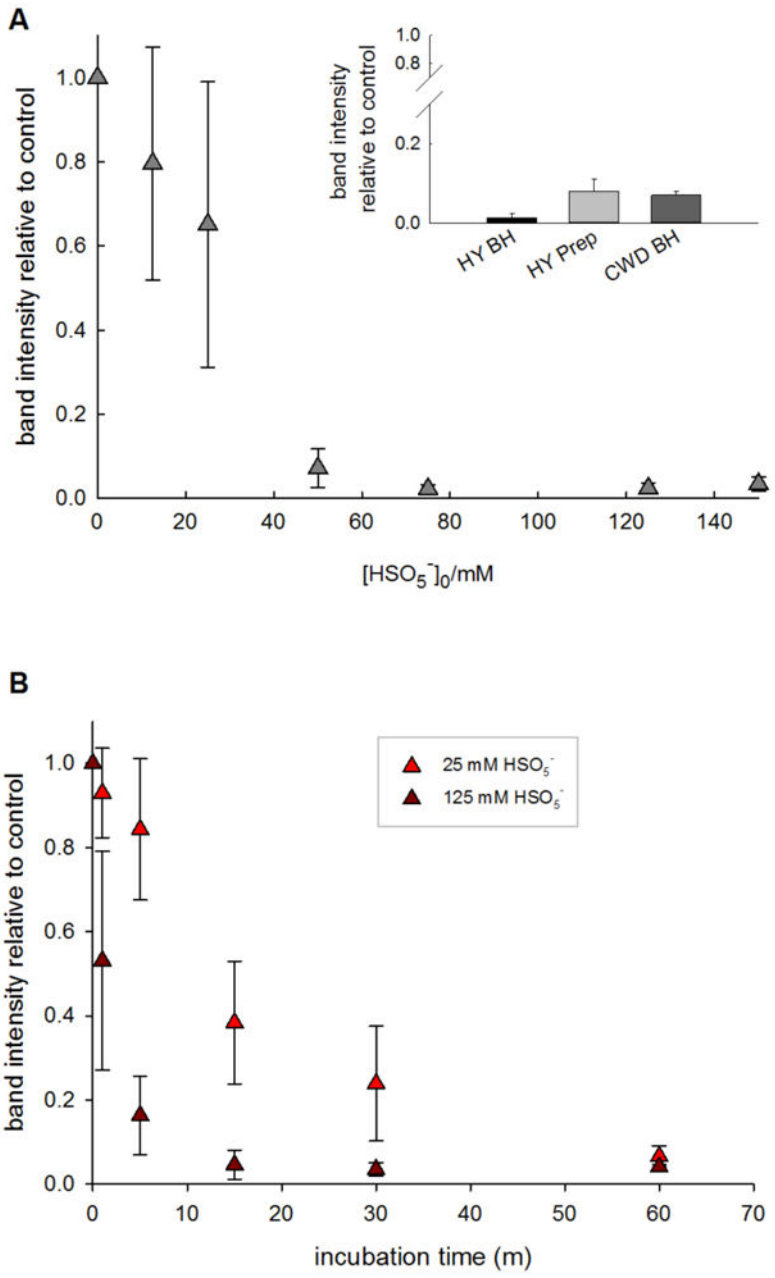

Figure 1.

Peroxymonosulfate degrades pathogenic prion protein in the absence of transition metal cations. (A) CWD BH (10% wt/vol) was incubated with between 0 and 150 mM peroxymonosulfate for 30 min. Substantial degradation of PrP^{TSE} was seen at 50 mM. (Inset) Peroxymonosulfate substantially degrades CWD and HY prions (PrP^{TSE} (10% HY BH (15 μL), purified HY preparation (0.5 μg) or 10% CWD BH (15 μL) was incubated with 125 mM peroxymonosulfate for 1 h. (B) Immunoblot detection of PrP^{TSE} was substantially reduced after 5 min when exposed to 125 mM peroxymonosulfate, and after 15–30 min when exposed to 25 mM peroxymonosulfate. All immunoblot detection was eliminated when PrP^{TSE} was exposed to both concentrations after 1-h incubation. PrP^{TSE} (15 μL 10%

CWD BH) was incubated with between 0 and up to 150 mM for 30 min. Immunoblot densitometry values normalized to PrP^{TSE} in ultrapure water ($n = 4$). Error bars represent standard deviations of experimental triplicates. Immunoblots were probed with mAb 3F4 (HY) or mAb 8G8/Bar224 (CWD). Error bars represent one standard deviation.

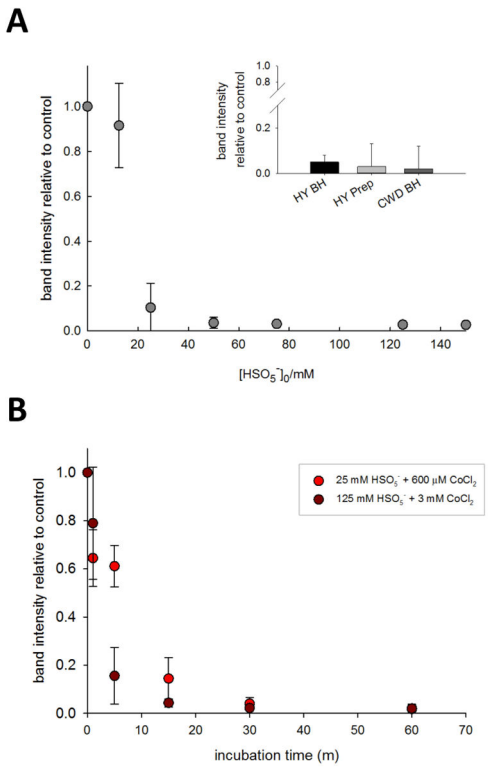

Figure 2.

Activation of HSO_5^- by $CoCl_2$ enhances degradation of PrP^{TSE}. (A) CWD BH (10% wt/vol) was incubated with between 0 and 150 mM peroxymonosulfate in the presence of $CoCl_2$ (125:3 molar ratio) for 30 min. Substantial degradation of PrP^{TSE} was seen at 25 mM. (Inset) Peroxymonosulfate activated by $CoCl_2$ substantially degrades CWD and HY prions. PrP^{TSE} (10% HY BH (15 µL), purified HY preparation (0.5 µg) or 10% CWD BH (15 µL) was incubated with 125 mM peroxymonosulfate for 1 h. (B) Immunoblot detection of PrP^{TSE} was substantially reduced after 5 min when exposed to 125 mM peroxymonosulfate with 3 mM $CoCl_2$, and after 15 min when exposed to 25 mM peroxymonosulfate with 600 mM $CoCl_2$. All immunoblot detection was eliminated when PrP^{TSE} was exposed to both concentrations after 30 min incubation. Error bars represent standard deviations of experimental triplicates. Immunoblots were probed with mAb 3F4 (HY) or mAb 8G8/Bar224 (CWD). Immunoblot densitometry values normalized to PrP^{TSE} in ultrapure water ($n = 4$). Error bars represent one standard deviation.

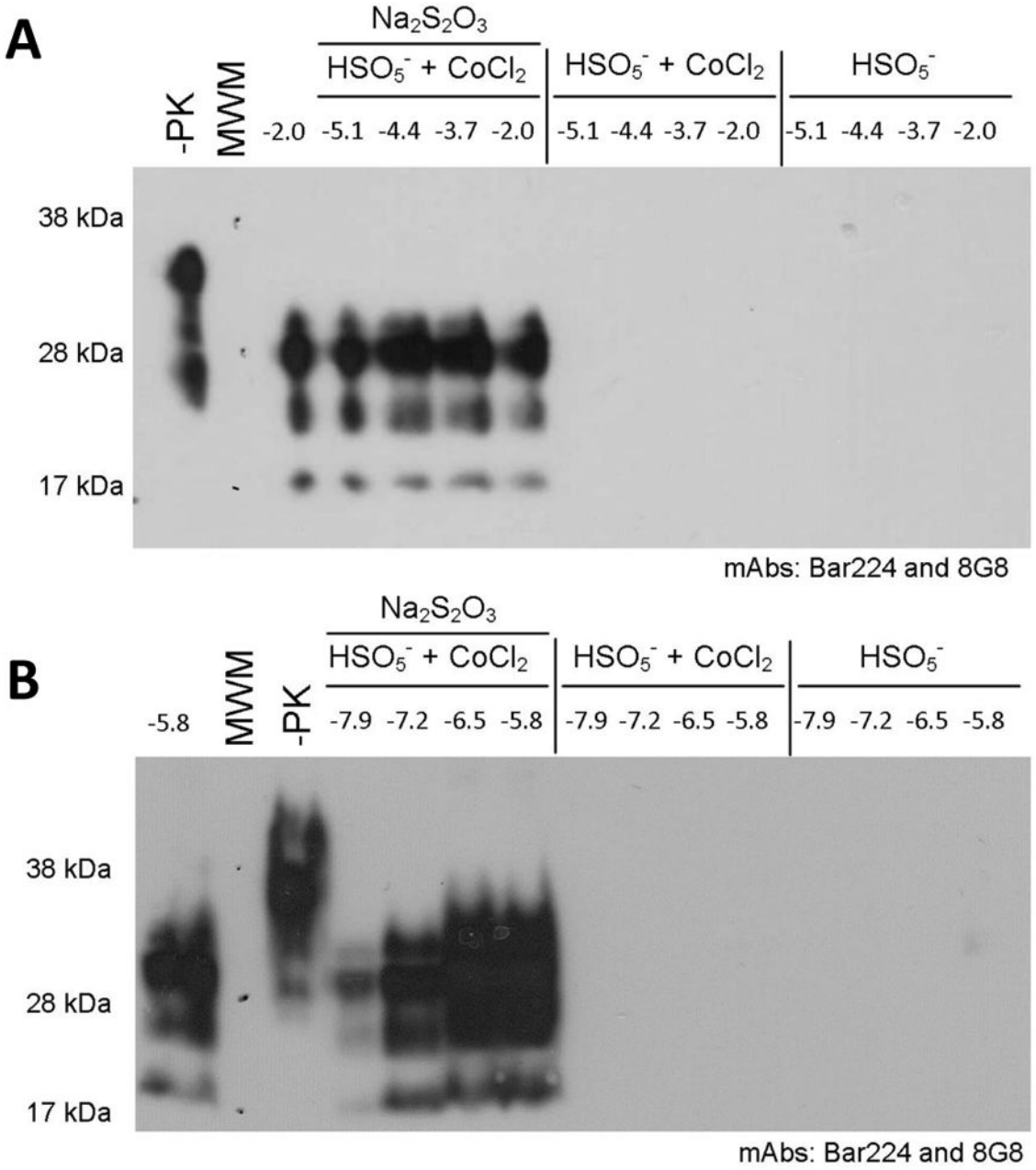

Figure 3.

Treatment with peroxymonosulfate ± $CoCl_2$ reduces the *in vitro* PrP^C-to-PrP^{TSE} converting ability of CWD agent by a factor of at least $10^{5.9}$. PrP^{TSE} (15 μL PK-treated 10% CWD BH) was incubated with 125 mM peroxymonosulfate with or without 3 mM $CoCl_2$ for 1 h. Samples were either quenched at the end of the experimental time point or pre-treated with 0.3 M $Na_2S_2O_3$. A 4 μL aliquot of resulting sample was added to 36 μL NBH and subjected to two rounds of 96 cycles of PMCAb. (A) PrP^{TSE} immunoreactivity is present in a $10^{-7.9}$ dilution from 10% brain homogenate from a white-tailed deer clinically affected by chronic wasting disease when peroxymonosulfate and $CoCl_2$ is pre-treated with thiosulfate, but not detected at any dilution (up to $10^{-2.0}$, B) examined when exposed to peroxymonosulfate +

$CoCl_2$ or peroxymonosulfate alone. Values above lanes indicate dilution from CWD brain homogenate. Immunoblots were probed with mAbs 8G8 and Bar224. Blot is representative of three replicates.

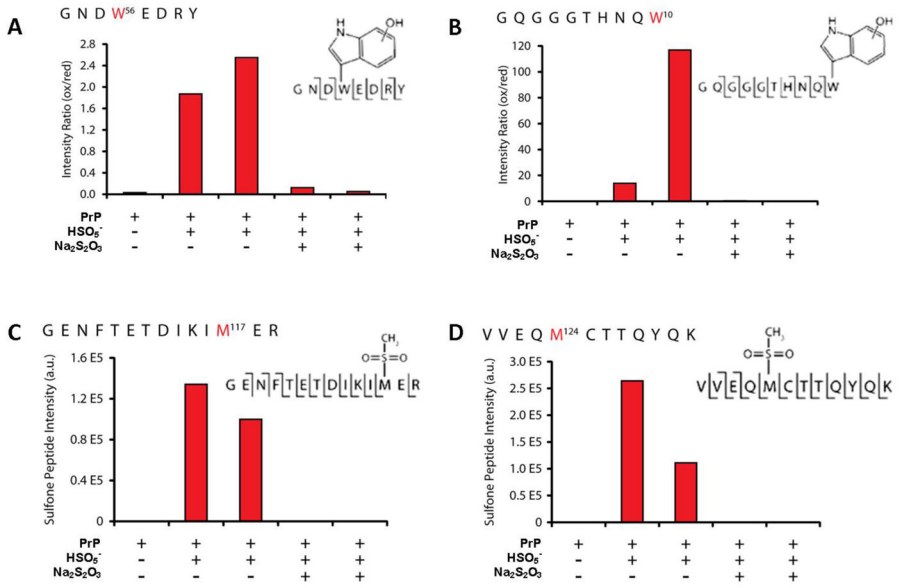

Figure 4. Exposure of pathogenic prion protein to peroxymonosulfate results in oxidative modification to tryptophan and methionine residues

PrP^{TSE} (proteinase K-treated purified HY preparation) was incubated with ultrapure water or 9.6 mM peroxymonosulfate for 15 min. After quenching with 0.5 M $Na_2S_2O_3$, samples were digested with chymotrypsin (panels a and b) or trypsin + endoprotease Lys-C (panels c and d) and analyzed by LC-MS/MS. Hydroxytryptophan (W^{56}, a; W^{10}, b) and methionine sulfone (M^{117}, c; M^{124}, d) were detected in samples treated with peroxymonosulfate that were not pre-quenched with 0.5 M $Na_2S_2O_3$. Bars indicate replicates of precursor ions.

Table 1

Inactivation of prions by chemical oxidants

Oxidant	Concentration (mM)	Contact Time (min)	Reduction in Infectivity $\log_{10}$	Ref.
HOCl	19[b]	30	4.4	36
O_3	0.3 (pH 4.4, 4 °C)	5	>4.1	33
Fenton(-like) reagent (·OH)	$[H_2O_2]$ = 100, $[Cu^{2+}]$ = 0.5	30	>5.2	100
	$[H_2O_2]$ = 2131, $[Fe^{2+}]$ = 15.8 (50 °C)	1320	>6	39
Heterogeneous photocatalysis (·OH)	$[H_2O_2]$ = 118, $[TiO_2]_s$ = 50	720	ND	101
HSO_5^-	125	60	≥5.9[c]	this study
H_2O_2	1279	1, 24	0.84	36
permanganate	253–506	15, 60	1, 2–2.3	35
	6.3	15, 60, 1440	0.51, 1.17, 0.84	36
δ-MnO_2(s)	102 (pH 4)	960	>4[c]	35, 105

[a] ND, not determined.

[b] Attributing all free residual chlorine to HOCl.

[c] Based on reduction of template-directed conversion ability as measured by PMCA.