Pathogenic prion protein is degraded by a manganese oxide mineral found in soils

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Prions, the aetiological agents of transmissible spongiform encephalopathies, exhibit extreme resistance to degradation. Soil can retain prion infectivity in the environment for years. Reactive soil components may, however, contribute to the inactivation of prions in soil. Members of the birnessite family of manganese oxides (MnO₂) rank among the strongest natural oxidants in soils. Here, we report the abiotic degradation of pathogenic prion protein (PrPTSE) by a synthetic analogue of naturally occurring birnessite minerals. Aqueous MnO₂ suspensions degraded the PrPTSE as evidenced by decreased immunoreactivity and diminished ability to seed protein misfolding cyclic amplification reactions. Birnessite-mediated PrPTSE degradation increased as a solution’s pH decreased, consistent with the pH-dependence of the redox potential of MnO₂.

Exposure to 5.6 mg MnO₂ ml⁻¹ (PrPTSE : MnO₂ = 1 : 110) decreased PrPTSE levels by >4 orders of magnitude. Manganese oxides may contribute to prion degradation in soil environments rich in these minerals.

Bovine spongiform encephalopathy, Creutzfeldt–Jakob disease, sheep scrapie and chronic wasting disease (CWD) of deer, elk and moose are members of a class of fatal, neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs) or prion diseases. The infectious agent appears to be primarily, if not solely, comprised of a misfolded isoform of the prion protein, designated PrPTSE. TSE agents are remarkably stable; most treatments that inactivate other infectious agents fail to eliminate prion infectivity (Taylor, 2000). Scrapie and CWD differ from other TSEs in that epizootics can be eliminated (Greig, 1940; Miller & Williams, 2003). Bovine spongiform encephalopathy, Creutzfeldt–Jakob disease, sheep scrapie and chronic wasting disease (CWD) of deer, elk and moose are members of a class of fatal, neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs) or prion diseases. The infectious agent appears to be primarily, if not solely, comprised of a misfolded isoform of the prion protein, designated PrPTSE. TSE agents are remarkably stable; most treatments that inactivate other infectious agents fail to eliminate prion infectivity (Taylor, 2000). Scrapie and CWD differ from other TSEs in that epizootics can be eliminated (Greig, 1940; Miller & Williams, 2003) and experiments with mule deer suggest a similar mode of transmission is possible with CWD (Miller et al., 2004).

The lack of clear evidence for vector-mediated TSE transmission prompted investigation of soil as a reservoir of prion infectivity (Schramm et al., 2006). Prions can persist in soils for years (Seidel et al., 2007; Brown & Gajdusek, 1991) and binding of PrPTSE by soil particles may maintain prions near the soil surface, thereby increasing animal exposure (Johnson et al., 2006; Cooke et al., 2007; Ma et al., 2007). Soil particle-associated agents are infectious orally (Seidel et al., 2007; Johnson et al., 2007). Prion sorption to some types of soil particles enhances oral TSE transmission (Johnson et al., 2007), providing an explanation for disease transmission despite presumably low levels of prions in soil environments.

Soils comprise complex mixtures of inorganic and organic constituents, and soil properties vary considerably across multiple spatial scales. The influence of soils on prion fate and environmental TSE transmission is expected to be complex, and abiotic soil components may affect the stability of prions present in soil. For example, birnessite group manganese oxide (MnO₂) minerals rank among the strongest oxidants in soils (E₄₋₀=1.29 V) (Bricker, 1965). Soils subjected to alternating reducing and oxidizing conditions, such as those occurring in seasonally waterlogged or poorly drained areas, typically contain the highest accumulations of manganese oxide minerals and experiments with mule deer suggest a similar mode of transmission is possible with CWD (Miller et al., 2004).
(Post, 1999; Tebo et al., 2004). Well-drained soils may also contain these minerals due to previous wet conditions (McKenzie, 1989). Manganese III and IV minerals can mediate the transformation of numerous organic compounds, including herbicides (Barrett & McBride, 2005) and antibiotics (Zhang & Huang, 2005; Rubert & Pedersen, 2006).

As an initial step towards understanding potential abiotic transformations of prions in soil, we investigated PrPTSE degradation by aqueous suspensions of δ-MnO₂, a synthetic manganese oxide mineral equivalent to the birmesite-family mineral vernadite (Villalobos et al., 2003). Using the method described by Murray (1974), we synthesized a poorly crystalline manganese oxide resembling δ-MnO₂ with an average Mn oxidation state of +3.94 (Gao, 2007). Brains from clinically affected Syrian hamsters experimentally infected with hamster-adapted transmissible mink encephalopathy agent (HY strain) were used as a source of TSE agent. Brain homogenate (BH), 10% w/v, was prepared in ddH₂O and PrPTSE was purified as described previously (Johnson et al., 2006). For experiments using protein misfolding cyclic amplification (PMCA), PrP-TSE was sodium phosphotungstate (PTA)-precipitated from infected BH (Safar et al., 1998). Protein concentrations were determined using the DC protein assay (Bio-Rad).

PrP TSE was incubated with suspended δ-MnO₂ under ambient O₂ conditions at room temperature for the time periods indicated in the figure legends. Reactions were conducted in 20 mM sodium acetate (pH 4.0), except for experiments examining the effect of pH, in which solutions were buffered with 20 mM sodium acetate (pH 4.0 and 5.0), MES (pH 6.0) or HEPES (pH 7.0 and 8.0). Final sample volumes were 40 μl. Reactions were terminated by dissolving MnO₂ with 25 μl 500 mM EDTA (pH 8.0) at 80 °C. Dissolving of the δ-MnO₂ (using ascorbic acid, citric acid or EDTA) liberated proteins, facilitating measurement of remaining protein without affecting PrPTSE levels or detection (data not shown); subsequent experiments used EDTA. The possibility of dissolved δ-MnO₂ interfering with PrPTSE detection was examined by dissolving the manganese oxide prior to addition of PrPTSE. After quenching reactions, the pH was stabilized by the addition of 5 μl 1 M Tris/HCl (pH 8.0) to samples. For SDS-PAGE/immunoblot analysis, 20 μl 10× sample buffer (Johnson et al., 2006) was added, and samples were heated for 10 min at 100 °C.

A modification of the method described by Saá et al. (2006) was used for PMCA detection of PrPTSE. Uninfected hamsters were perfused with PBS containing 1 mM EDTA; harvested brains were homogenized to 10% (w/v) in PBS containing 150 mM NaCl, 1% Triton X-100, 0.5% digitonin and complete protease inhibitor cocktail (Roche), then clarified by centrifugation for 5 min at 850 g. Prior to PMCA, PTA-purified PrPTSE (50 μg ml⁻¹) was incubated with 5.6 μg δ-MnO₂ ml⁻¹ (PrPTSE : δ-MnO₂ = 1 : 110) or EDTA-dissolved δ-MnO₂. Dilutions of all samples, in uninfected BH, were aliquoted into 96-well PCR plates then placed at 37 °C in a Misonix 3000 sonicator equipped with a microplate horn. Each of the 120 cycles consisted of 10 s sonication at 80% power followed by 30 min incubation. Following PMCA, 50 μl sample was mixed with 50 μl 4% N-lauroyl-sarcosine in PBS and incubated with 40 μg proteinase K ml⁻¹ for 1 h at 37 °C. Proteinase K digestion was terminated by addition of 1 μl phenylmethylsulfonyl fluoride-saturated ethanol. Samples were prepared for immunoblotting by PTA-precipitation and resuspending pellets in 5× sample buffer with 1% N-lauroyl-sarcosine.

Proteins were fractionated by SDS-PAGE (4–20% gradient) or 10% Bistris gel (Invitrogen) for PMCA experiments, transferred to polyvinyl difluoride membranes and immunoblotted with PrP-specific antibodies: mAb 3F4 (1 : 3 F4; 1 : 40 000 dilution) or full-length polyclonal antibody Rab 9, pool 2 (1 : 10 000). Detection was achieved with horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit immunoglobulins.

Following 16 h incubation with δ-MnO₂, PrPTSE immunoreactivity declined in proportion to the amount of δ-MnO₂ in suspension (Fig. 1a). At the highest δ-MnO₂ concentration tested (5 mg ml⁻¹; PrPTSE : δ-MnO₂ = 1 : 200), PrPTSE levels decreased below the limit of immunoblotting detection. The unstructured N-terminal portion of PrPTSE is susceptible to degradation; N-terminal cleavage leaves a 27–30 kDa infectious core (Bolton et al., 1982). When lower δ-MnO₂ concentrations were used, the PrP appeared similar in size to the untreated controls, suggesting that δ-MnO₂ did not selectively cleave the N terminus (Fig. 1a). We increased the amount of PrPTSE in the reactions by approximately 10-fold (from 33 to 333 μg ml⁻¹) to estimate the extent to which δ-MnO₂ degrades larger amounts of protein (Fig. 1b) and found that 3 mg δ-MnO₂ ml⁻¹ was capable of degrading a considerable fraction of the protein (PrPTSE : δ-MnO₂ = 1 : 90 to 1 : 9). As a control, δ-MnO₂ was dissolved prior to incubation with PrPTSE. The amount of PrPTSE present in the dissolved manganese control was substantially larger than in the δ-MnO₂ samples, approximately equal to the starting material (Fig. 1b). These data indicate that δ-MnO₂, not Mn²⁺ and EDTA, was responsible for the decrease in immunoreactivity.

The duration of exposure to δ-MnO₂ influenced the extent of prion protein loss (Fig. 1c). Exposure for 1 h to 1.5 or 2.6 mg δ-MnO₂ ml⁻¹ (PrPTSE : δ-MnO₂ = 1 : 60 and 1 : 100) had a limited effect on PrPTSE, whereas 24 and 168 h exposures produced substantial declines. Comparable amounts of protein remained after 24 and 168 h exposure to 1.5 mg δ-MnO₂ ml⁻¹, suggesting no further reaction (Fig. 1c). Decreases in MnO₂ reactivity toward organic molecules over the course of reactions have been noted previously (e.g. Rubert & Pedersen, 2006) and have been attributed to adsorption of reaction products to the oxide surface, shifts in surface site distribution towards less reactive sites or both (Klausen et al., 1997).
The most probable explanation for the \( \delta \)-MnO\(_2\)-dependent loss of PrP\(^{\text{TSE}}\) immunoreactivity is degradation of the protein. Birnessite dissolving following the incubation with PrP\(^{\text{TSE}}\) excludes the possibility that the decreases in immunoblotting signal are due to PrP\(^{\text{TSE}}\) sorption to mineral surfaces. In the analyses described above, we used the monoclonal antibody (mAb) 3F4, directed against a single epitope on the hydrophobic core of the PrP molecule (residues 109–112). To ensure that the observed declines in PrP\(^{\text{TSE}}\) were not due to \( \delta \)-MnO\(_2\), degradation experiments were repeated with 3F4 and a polyclonal antibody directed against full-length PrP (Rab 9, pool 2). After treatment of PrP\(^{\text{TSE}}\) with \( \delta \)-MnO\(_2\), no immunoreactivity with Rab 9, pool 2 remained and lower molecular mass breakdown products were absent (Supplementary Fig. S1, available in JGV Online). These data are consistent with the hypothesis that \( \delta \)-MnO\(_2\) degrades PrP\(^{\text{TSE}}\) by breaking the polypeptide backbone of the protein, but do not exclude the possibility that \( \delta \)-MnO\(_2\) also alters amino acid side chains.

Organic molecule degradation by \( \delta \)-MnO\(_2\) typically exhibits pronounced pH dependence (Stone & Morgan, 1984). We examined the \( \delta \)-MnO\(_2\)-mediated degradation of PrP\(^{\text{TSE}}\) as a function of pH over the range relevant for most natural soils (pH 4–8) (Supplementary Fig. S2, available in JGV Online). Under the experimental conditions employed (25 µg PrP\(^{\text{TSE}}\) ml\(^{-1}\) exposed to 3.8 mg \( \delta \)-MnO\(_2\) ml\(^{-1}\) for 16 h, PrP\(^{\text{TSE}}\) : \( \delta \)-MnO\(_2\) = 1:150), PrP\(^{\text{TSE}}\) levels dropped below the limit of detection when reactions were performed at pH 4 or 5, whereas substantial PrP\(^{\text{TSE}}\) remained following reactions at pH \( \geq 6 \).

Fig. 1. \( \delta \)-MnO\(_2\) mediates PrP\(^{\text{TSE}}\) degradation. (a) PrP\(^{\text{TSE}}\) was reacted overnight with the indicated amount of \( \delta \)-MnO\(_2\), demonstrating that \( \delta \)-MnO\(_2\) causes a dose-dependent decline in PrP\(^{\text{TSE}}\) immunoreactivity. (b) Varying doses of PrP\(^{\text{TSE}}\) were exposed to 3 mg \( \delta \)-MnO\(_2\) ml\(^{-1}\) or dissolved manganese oxide, indicating that \( \delta \)-MnO\(_2\), but not dissolved manganese, degrades PrP\(^{\text{TSE}}\). (c) Time-course of \( \delta \)-MnO\(_2\)-mediated PrP\(^{\text{TSE}}\) degradation (initial concentration 25 µg ml\(^{-1}\)). All blots used mAb 3F4. Extra lanes from the gels in (a) and (c) were excised for clarity of presentation.

Pathogenic prion proteins may be released into the environment in saliva (Mathiason et al., 2006), excreta (Safar et al., 2008) or from decomposing animal tissue in a complex mixture of biomolecules (Miller et al., 2004). To evaluate the \( \delta \)-MnO\(_2\)-mediated degradation of PrP\(^{\text{TSE}}\) in the presence of biological macromolecules, we assessed degradation of infected BH by \( \delta \)-MnO\(_2\) by assaying both total residual protein and PrP\(^{\text{TSE}}\) (Fig. 2). Following 16 h

Fig. 2. \( \delta \)-MnO\(_2\) deg granizes most proteins present in infected BH, including PrP\(^{\text{TSE}}\). Infected 10% (w/v) BH (4 µl and 1.5 µl for a and b, respectively) was incubated with \( \delta \)-MnO\(_2\) overnight, fractionated by SDS-PAGE and visualized by Coomassie blue staining (a) or immunoblotting with mAb 3F4 (b). Extra lanes from the same gels were excised for clarity of presentation.
incubation with 0.4 mg \(\delta\text{-MnO}_2\) ml\(^{-1}\), little protein was observed on Coomassie brilliant blue-stained gels, and incubation with 3.7 mg \(\delta\text{-MnO}_2\) ml\(^{-1}\) decreased total protein to undetectable levels (detection limit ~100 ng protein) (Fig. 2a). Dissolving of \(\delta\text{-MnO}_2\) prior to incubation with the BH had little effect on protein levels. The PrP\(^{\text{TSE}}\) present in infected BH was also diminished by exposure to \(\delta\text{-MnO}_2\) (Fig. 2b). Compared with experiments using preparations enriched in PrP\(^{\text{TSE}}\), more \(\delta\text{-MnO}_2\) was needed to degrade the PrP\(^{\text{TSE}}\) in BH (compare Figs 1a and 2b). This may be due to the reductive dissolution of \(\delta\text{-MnO}_2\) as it reacts with other biomolecules in BH and/or fouling the oxide surface by adsorbed biomolecules.

To semi-quantitatively assess the extent of \(\delta\text{-MnO}_2\)-mediated PrP\(^{\text{TSE}}\) degradation, we diluted PrP\(^{\text{TSE}}\) starting material to the limit of immunoblotting detection (Johnson et al., 2006; Hinkley et al., 2008). A 200-fold dilution of starting material was still detectable on immunoblots (data not shown), thus samples exhibiting no detectable immunoreactivity, such as PrP\(^{\text{TSE}}\) treated with 5 mg \(\delta\text{-MnO}_2\) ml\(^{-1}\) (Fig. 1a), contain at least 200-fold less PrP\(^{\text{TSE}}\) than the starting material. To further assess PrP\(^{\text{TSE}}\) loss, PMCA was used to determine the amount of PrP converting activity remaining after \(\delta\text{-MnO}_2\) treatment. PMCA sensitively detects prions by measuring the ability of PrP\(^{\text{TSE}}\) in a sample to convert PrP\(^{\text{C}}\) to a proteinase K-resistant form (Saá et al., 2006). Converting activity of the PTA-purified PrP\(^{\text{TSE}}\) starting material was detectable over four 10-fold dilutions (Fig. 3). Conversion activity of PTA-purified PrP\(^{\text{TSE}}\) is diminished relative to infected BH (Fig. 3 and Supplementary Fig. S3, available in JGV Online), possibly due to increased aggregation of the PTA-purified agent. When samples were incubated with 5.6 mg \(\delta\text{-MnO}_2\) ml\(^{-1}\) (PrP\(^{\text{TSE}}\): \(\delta\text{-MnO}_2\) = 1:110) and assayed by PMCA, no converting activity was observed (Fig. 3). The limit of detection for PMCA is defined by the dilutions of the PTA-purified starting material (see above), indicating that 5.6 mg \(\delta\text{-MnO}_2\) ml\(^{-1}\) decreased converting activity by at least four orders of magnitude.

Abiotic processes influence the environmental fate of many contaminants. The data presented here suggest that MnO\(_2\) can degrade PrP\(^{\text{TSE}}\) in soil environments. Previous studies reported loss of protein from solution when incubated with MnO\(_2\) and ascribed the losses to sorption to MnO\(_2\) surfaces (Naidja et al., 2002; Rao et al., 2007). Treatment of BH with \(\delta\text{-MnO}_2\) (Fig. 3) indicates that protein degradation is not specific to PrP\(^{\text{TSE}}\); \(\delta\text{-MnO}_2\) degrades most, if not all, brain proteins.

Birnessite-mediated PrP\(^{\text{TSE}}\) degradation exhibited pronounced pH-dependence (Supplementary Fig. S2). Two non-mutually exclusive factors may have contributed to this behaviour. First, MnO\(_2\) redox potential increases and surface charge becomes less negative as pH declines (Bricker, 1965). Second, solution pH may influence the degree of protein sorption to MnO\(_2\) surfaces. The point of zero charge for \(\delta\text{-MnO}_2\) occurs near pH 2.3 (Murray, 1974); \(\delta\text{-MnO}_2\) particles carried a net negative charge at all pH values examined. Protein attachment to negatively charged surfaces is often maximal at the isoelectric point (pI) of the protein and declines when pH>pI due to repellent electrostatic interactions (Quiquampoix et al., 2002). The apparent average pI of PrP\(^{\text{TSE}}\) aggregates is ~4.6 (Ma et al., 2007); prion protein aggregates carried no net charge around this pH value. In most models of degradation of organic molecules by MnO\(_2\), sorption to the oxide surface represents a critical initial step (Stone, 1987). The increase in degradation near the apparent pI of PrP\(^{\text{TSE}}\) aggregates is consistent with the importance of protein sorption to \(\delta\text{-MnO}_2\) in the overall protein degradation process.

Our data suggest that manganese oxides in soils may promote PrP\(^{\text{TSE}}\) inactivation, thereby reducing the probability of environmental TSE transmission. Previous investigation of soil manganese contributing to TSE development focused on dietary manganese and copper imbalance (Chihota et al., 2004; Gudmundsdottir et al., 2006; Ragnarsdottir & Hawkins, 2006; McBride, 2007). Attempts to correlate soil or forage manganese and copper concentrations with TSE incidence have met with limited success. Future attempts to link TSE incidence with soil manganese levels should examine the mineral form of the element in addition to total (or bioavailable) manganese concentration.

Prion fate in terrestrial environments probably depends on soil composition. Abiotic prion degradation could be expected in soil environments rich in MnO\(_2\), including young, and currently or formerly poorly drained soils (Post, 1999; Tebo et al., 2004). Our results indicate acidic soil conditions may also promote MnO\(_2\)-mediated PrP\(^{\text{TSE}}\) degradation. Under the experimental conditions employed, \(\delta\text{-MnO}_2\)-mediated PrP\(^{\text{TSE}}\) degradation occurs over

Fig. 3. Semi-quantitative assessment of PrP\(^{\text{TSE}}\) degradation by \(\delta\text{-MnO}_2\) using PMCA. Serial dilutions of PrP\(^{\text{TSE}}\) (50 \(\mu\text{g ml}^{-1}\) starting material) and PrP\(^{\text{TSE}}\) reacted with \(\delta\text{-MnO}_2\) were subjected to PMCA amplification and converted material was detected by immunoblotting with mAb 3F4. Immunoreactivity was observed in all dilutions, but no PrP was detected in \(\delta\text{-MnO}_2\)-treated samples, indicating prion converting levels were reduced by more than a factor of 10\(^{-4}\).
relatively short periods (Fig. 1c) and reduces PrP^TSE-converting activity by at least a factor of 10^4 (Fig. 3). Our findings also suggest that MnO_2 may be effective as a reactive burial material in the disposal of prion-infected materials. Use of MnO_2 for the decontamination of prion-contaminated soils also warrants investigation.

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