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Interaction of recombinant and disease-associated prion proteins with organic and mineral soil colloids

Ph.D. Dissertation
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Questa tesi è senza dubbio dedicata a Mamma e a Papà che sempre mi hanno supportato e mi supportano nella vita e negli studi.

È il tempo che hai perduto per la tua rosa che l'ha resa così importante

Antoine de Saint-Exupéry

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Chapter 1

Introduction

Prion diseases, also referred as transmissible spongiform encephalopathies (TSEs), affect a variety of mammals. These neurodegenerative diseases are fatal, with pathogenesis in the central nervous system leading slowly but inexorably to death. The most known TSE in humans is the Creutzfeldt-Jakob disease (CJD), while in animals is the bovine spongiform encephalopathy in cattle (BSE), commonly known as the "mad cow disease". One of the first known prion diseases is the sheep scrapie. It was known since at least 200 years and was described as a transmissible disease over 100 years ago. Since then, several groups of TSE diseases have been identified in humans including familial, infectious and sporadic types.

In 1982 Stanley B. Prusiner attributed the disease to a novel infections entity: "the novel properties of the scrapie agent distinguish it from viruses, plasmids, and viroids, a new term "prion" is proposed to denote a small proteinaceous infectious particle which is resistant to inactivation by most procedures that modify nucleic acids" (Prusiner, 1982). The discovery of the prion protein in the 1980s greatly accelerated knowledge of the biology and pathogenesis of TSE diseases.

A remarkable feature of this class of diseases is that the incubation time can be really long, but usually the time required for the manifestation of symptomatology varies in animals species. For instance, in humans this period may last decades, whereas for the typically laboratory rodent models this period is less than one year. According to most researchers' ideas, these diseases are

not anymore attributed to any hypothetical and unconventional virus, and show some similarity to other neurodegenerative diseases (although non transmissible) such as Parkinson disease and Alzheimer disease.

The prion hypothesis formulated by Prusiner states that a disease-associated and improperly folded form of a protein, generally named PrP^{Sc} (Sc from the sheep Scrapie), is derived from a benign membrane displayed precursor protein, PrP^C (C from Cellular) encoded on Chromosome 2 and Chromosome 20 in mice and humans, respectively (Prusiner, 1982).

The prion protein diseases cause an environmental issue because environment and, in particular soil compartment, can be contaminated and become a potential reservoir and diffuser of TSEs infectivity as a consequence of (i) accidental dispersion from storage plants of meat and bone meal, (ii) incorporation of meat and bone meal in fertilizers, (iii) possible natural contamination of pasture soils by grazing herds and (v) burial of carcasses of contaminated animals. The problem can be even more relevant in soil because of prion protein capability to preserve the infectivity over years (Brown and Gajdusek, 1991; Georgsson et al., 2006).

1.1. Prion proteins and TSEs

1.1.1. History of TSEs

Scrapie disease has been known in sheep in Europe for over 200 years. There was no knowledge about the possibility that this disease could be transmissible among sheep. The problem was discovered after appearance in healthy animals of the symptoms once they came in contact with infected flocks, which suggested that the disease might be transmissible. Experimental transmission was reported as early as 1899 (Besnoit, 1899), and later by Cuille et al. (1936). The exact mechanism of the natural transmission of scrapie is not yet completely clear. Also an indirect contact with infected sheep was reported to be able to develop the disease in healthy animals, for instance pastures contaminated by placenta and other tissues showed this capability (Race et al., 1998). This mechanism of infection due to infectious pasture can explain the findings that scrapie-free sheep became infected when introduced three years after than housed infected flocks were removed (Palsson et al., 1979). Also TSEs affecting humans like Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker syndrome (GSS), were originally thought to be not transmissible (Creutzfeldt, 1920, Gerstmann et al. 1936). The observed similarities between Kuru pathology in humans and scrapie in sheep led later to the experimentally successful transmission of the disease by inoculation from humans to chimpanzees proved by Gajdusek (1966, 1971).

The social concern about the transmissible spongiform encephalopathies related to the prions reached the maximal visibility during the past decade with bovine spongiform encephalopathy (BSE) or 'mad cow disease' in the UK and in all European regions. BSE has strongly influenced medical, agricultural, economic

and political issues in Europe. BSE was found for the first time in 1986 and then it diffused as a big epidemic disease that clinically involved more than 180000 bovines. The most reliable hypothesis of the spread of BSE is believed to be linked to the feeding of cows with meat and bone meal from infected cows or sheep. Also the hypothesis of the link of BSE with new variant of Creutzfeldt-Jakob disease (nvCJD), due to human consumption of cattle contaminated meat, contributed to the growing concern about public health. Other countries like North America have been spared the ravages of the epidemic BSE. Nowadays there is, however, alarm over the high incidence of the cervid TSE, chronic wasting disease (CWD), in wild and captive populations of deer and elk specially in the Mid-western areas of the US and Canada and the risk of possible transmission to humans (Chesebro, 2003).

1.1.2. TSEs in animals

Several are the TSEs affecting the animals and the causes can be attributed to the contact with contaminated material and infect animals or be genetically based.

The most known and socially or environmentally relevant TSEs affecting animals are Scrapie in sheep, Bovine Spongiform Encephalopathy in cattle (BSE) and the Chronic Wasting Disease in deer and elk (CWD). There are also other TSEs reported like Transmissible Mink Encephalopathy in mink (TME) and Feline Spongiform Encephalopathy in cats and other felines (FSE) (Table 1.1) (Aguzzi, 2000).

Table 1.1. Classification of TSEs in animals

Disease	Host	Mechanism of pathogenesis
Scrapie	Sheep	Infection in genetically susceptible sheep
BSE	Cattle	Infection with prion-contamined MBM[*]
CWD	Mule, deer, elk	Unknown
TME	Mink	Infection with prion from sheep or cattle
FSE	Cats	Infection with prion-contamined bovine MBM

[*]MBM meat and bone meal

1.1.2.1. Scrapie in sheep

The term "scrapie" is derived by the scraping in consequence of the intense pruritus in affected animals. This disease was first recognized in 1755 in England and it was then related to Kuru disease that affects humans. Now the scrapie is considered as the prototype of all prion diseases.

Scrapie can affect ovine and goats and can be transmitted to other mammals like mouse and guinea pigs. The typical symptoms in sheep scrapie include hyper-excitability, pruritus and myoclonus. Scrapie in sheep is characterized by a rapid progression leading to tetraparesis and ultimately to the death of animal. The symptomatology is usually visible in animals older than 2.5 years even though the incubation period can last 10 years.

1.1.2.2. Bovine spongiform encephalopathy (BSE)

BSE is probably the most known disease for animals among the transmissible encephalopathies. It was observed between 1970 and 1980 but the epidemic spread in the 1980s occurred. The cause of the diffusion of BSE is attributed to

the feeding of protein supplements contaminated with the tissues of BSE positive cattle. The problem was probably enhanced by the use of new procedures to extract proteins from meat and bone of animals lacking the use of solvents and thus the need of high temperatures, to evaporate the solvents. However, it remains unclear whether BSE was originated from an unrecognised bovine TSE case or by adaptation from an unusual strain of sheep scrapie. In 1988 containment procedures from the UK government denied the use of Specified Bovine Offal (SBO), like the brain and bone marrow from 6 months older bovine, for production of human and food, that led a successful reduction of the occurrence of the disease (http://wwwold.unict.it/fpc/monografia06.html). BSE similarly to scrapie shows in infected animal aggressiveness, loss of equilibrium, tremors, weigh loss, but the pruritus and even the typically scraping observed in sheep scrapie are not frequent. By feeding of contaminated meat and bone meal BSE has also been transmitted to other species, to ungulates and large felines in zoos and probably also to domestic cats (FSE). Transmission to humans has also been strongly suggested by the appearance of vCJD in over 130 humans in the UK (Chesebro, 2003).

1.1.2.3. Chronic wasting disease (CWD)

Chronic wasting disease (CWD) is a TSE affecting mule deer (*Odocoileus hemionus*), white-tailed deer (*O. virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*). CWD in deer and Rocky Mountain elk is another example of a TSE disease of unknown origin. It differs from scrapie and BSE by its occurrence in non domestic and free-ranging species. CWD has many features in common with scrapie, including early widespread distribution of infectious isoforms of prion protein in lymphoid tissues, with later involvement of central

nervous system and peripheral tissues. Clinical features and lesions of CWD are similar to the other animals. TSEs-like marked spongiform lesions occur in the brains after a prolonged incubation period and variable course of clinical disease (Williams, 2005). Also this disease is progressive and always fatal; animals show behavioural changes, hyperexcitability and principally weight loss. The wildlife of deer and elk could contribute to the uncontrolled spread of this disease in the environment. Miller in 2003 suggested that horizontal transmission is likely important in sustaining CWD epidemics (Miller, 2003); later he demonstrated that the presence of decomposed infected carcasses or residual excreta from infected animals on the landscape were sufficient to transmit CWD to healthy mule deer (Miller, 2004).

1.1.3. TSEs in humans

Human prion diseases have been traditionally classified into Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and Kuru, and they can be further divided into three etiological categories: sporadic, acquired, and inherited (Table 1.2).

1.1.3.1. Sporadic TSEs

Sporadic Creutzfeldt-Jakob disease (CJD) accounts for the majority of TSEs disease cases in humans (at least 85%) occurring with an incidence of 1 in 2 × 106 people world-wide.

Current hypotheses for the occurrence of the disease include stochastic initiation of spontaneous PrP^{res} (res, resistant form) formation without PrP mutation even though there is no evidence for spontaneous PrP^{res} formation in any animal or

human TSE disease. Moreover in humans the peak age incidence of sporadic CJD is 55-60 years, and the subsequence decrease of the incidence is not expected if the occurrence of PrP^{Sc} would be only stochastic. CJD primary clinical symptoms are usually dementia, cognitive disturbances and memory loss. This usually progresses to a severe dementia which can also be associated with myoclonus, cerebral symptoms such as ataxia and finally to the death (Chesebro, 2003).

Table 1.2. Classification of TSEs in humans (from Mallucci and Collinge, 2005)

Aetiology	Phenotype	Frequency
Sporadic		
Apparently random distribution with an annual incidence of 1-2 per million worldwide	Sporadic CJD: multiple distinct prion strain type associated with distinct clinopathological phenotypes; rarely associated with sporadic fatal insomnia	85%
Inherited		
Autosomal dominant with high penetrance; all are associated with *PRNP*-coding mutations	Highly variable: more than 30 mutations includes GSS disease, familial *PRNP*-coding mutations CJD and fatal familial insomnia phenotypes	~10-15%
Acquired		
Iatrogenic exposure to human prions from medical contact with human cadaveric-derived pituitary hormones, tissue grafts or contaminated instruments	Iatrogenic CJD: typical CJD following direct CNS exposure; ataxic onset following peripheral infection	<5% (most patients from USA, UK, France and Japan)
Dietary exposure to human prions through endocannibalism	Kuru	Only in a small area of Papua New Guinea; epidemic in the 1950s, with a gradual decline after the cessation of cannibalism
Environmental exposure (presumed to be dietary) to the BSE prion strain	Variant CJD	Mainly in the UK (total so far ~150), 6 in France, individual patients in several other countries

BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; GSS, Gerstmann-Sträussler-Scheinker; PRNP, the gene that encodes the prion protein.

1.1.3.2. Inherited TSEs

The inherited TSEs include familial CJD, Gerstmann-Sträussler-Scheinker (GSS) and Fatal Familial Insomnia (FFI). Clinical and pathological findings are variable as the age of onset. The primary clinical finding can be ataxia, dementia or sleep abnormality. Clinical variability occurs even within individual patients of the same family, suggesting that genes other than PrP or non-genetic factors also influence these diseases (Chapman, 1993).

1.1.3.3. Acquired TSEs

The acquired/iatrogenic group of TSEs consists of Kuru, iatrogenic Creutzfeldt-Jakob disease (CJD) and variant CJD (vCJD). In these cases the disease arises with the exposure to TSEs agents by contact with brain or other tissues contaminated. In Kuru, this occurred through ritualistic cannibalism. Iatrogenic CJD was induced by transplantation of corneal or dural tissue from patients with TSE, or by neurosurgery using instruments incompletely sterilised. Humans affected by Kuru showed loss of coordination (ataxia), and dementia before death.
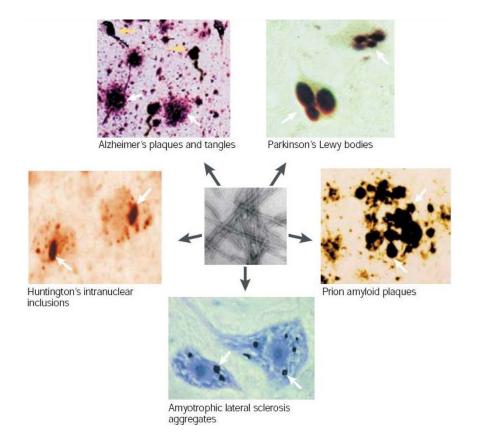
Predominant clinical symptoms are behavioural changes, anxiety, depression, ataxia, and subsequently myoclonus. Later memory disturbances arise, and finally akinetic mutism.
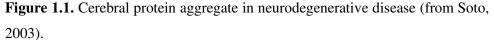
In vCJD young age range of these patients, as compared to the inherited TSEs, and distinctive pathology suggested a new clinical TSE disease in humans. Because of some similarities with BSE, the variant of Creutzfeldt-Jakob disease

is believed to be the human form of BSE. Subsequent laboratory experiments indicated a strong similarities in BSE and vCJD, based on patterns of infectable mouse strains, lesion distribution in mouse brain, PrP^{Sc} gel banding patterns, and disease pathology (Lasmezas, 2001). Based on these data, most observers agree that vCJD represents spread of BSE from cattle to humans.

1.1.4. Protein misfolding role in neurodegenerative diseases

Recently, it has been proposed a central role of misfolding of proteins and the subsequent aggregation and accumulation not only in TSEs but in many neurodegenerative diseases (Soto, 2003). The function of a protein depends on its three-dimensional structure, which is mainly determined by its amino-acid sequence. Chaperone proteins supervise protein folding so that, in most cases, mistakes are avoided and malfunctioning proteins are removed. However, there is accumulating evidence that protein misfolding and aggregation are the most likely cause of various neurological and systemic diseases. It is possible to identify diseases related to protein conformational disorders (Dobson, 1999). The hallmark feature of conformational disorders is that a particular protein can fold into a stable alternative conformation, which in most cases results in aggregates and accumulation in tissues as fibrillar deposits (Dobson, 1999; Soto, 2003). The term "amyloid" was originally used to refer to the extracellular protein deposits found in Alzheimer disease and systemic amyloid disorders, but its use has recently been extended to include some intracellular protein aggregates. There is accumulating evidence that the aggregates formed by the different misfolded proteins have the same molecular form (Figure 1.1).

Figure 1.1. Cerebral protein aggregate in neurodegenerative disease (from Soto, 2003).
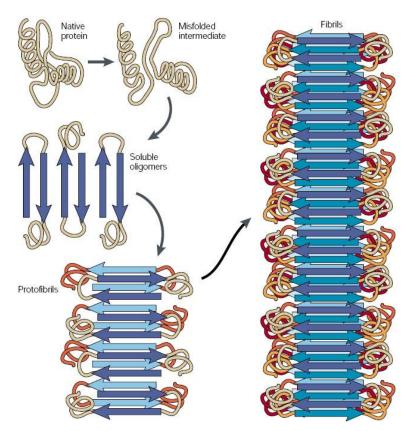
These diseases that affect thinking, skilled movements, emotional feelings, cognition, memory and other abilities include Alzheimer disease, Parkinson disease, Huntington disease (and related polyglutamine disorders such as several forms of spinocerebellar ataxia), transmissible spongiform encephalopathies and amyotrophic lateral sclerosis (Soto, 2003).

Native conformation of these proteins is composed mainly of α-helical and unstructured domains whereas the misfolded protein conformations are rich in β-sheets. The β-sheets rich conformers of these proteins are characterized by low solubility and non crystalline nature, so that high resolution studies of the

aggregates have been difficult. Recent studies with solid state nuclear magnetic resonance and X-ray fibre diffraction confirmed the β-sheets nature of protein aggregates founded in brain affected by neurodegenerative disease.

The formation of big aggregates is likely mediated by the formation of a misfolded intermediate, unstable in an aqueous environment because of the exposure of hydrophobic segments to the solvent, then stabilized by intermolecular interactions with other molecules, forming small β-sheet oligomers which, with further growth, produce amyloid-like fibrils (Figure 1.2) (Soto, 2003).

Figure 1.2. Pathway of fibrils formation through protein misfolding, oligomerization and polymerization (from Soto, 2003).

The formation of unstable intermediates in this process is likely generated by destabilization of the normal protein conformation. Environmental factors that may catalyse protein misfolding include changes in metal ions, pathological chaperone proteins, pH or oxidative stress, macromolecular crowding and increases in the concentration of the misfolding protein (Soto, 2001).

1.1.5. The cellular PrP^C, the misfolded PrP^{Sc} and mechanism of misfolding

The function of the normal PrP^C protein is not yet fully understood. Significantly, mice that have had the PrP gene knocked out exhibit no characteristic phenotype, apart from a slight disruption of diurnal cycles (Brown, 1997). They show diminished superoxide dismutase (SOD) activity in brain tissue, suggesting that PrP^C may have a Zn/Cu SOD activity (Brown, 1997). The flexible and histidine-rich octapeptide repeat segment present in the PrP molecule is theoretically able to bind Cu^{2+} ions, and the increased stability of this region to proteases is suggestive of such binding (Brown, 1997). Because of its location at the outer surface of cells, anchored by phosphatidylinositol glycolipid (GPI), PrP is a candidate for signalling, cell adhesion or perhaps even for some transport functions.

Accordingly, western blot analysis of PrP^C reveals three major bands, reflecting that PrP^C has two, one, or no glycosylation signals. PrP^C consists of an N-terminal region of about 100 amino acids, which is unstructured in the isolated molecule in solution, and a structured C-terminal segment, also approximately 100 amino acids in length. The C-terminal domain is folded into a largely α-helical conformation. There are three α-helices and a short antiparallel β-sheet, in which helices 2 and 3 are stabilized by a single disulphide bond between Cys

179 and Cys 214, while the glycosylation is located at two asparagine sites (Collinge, 2001) (Figure 1.3).
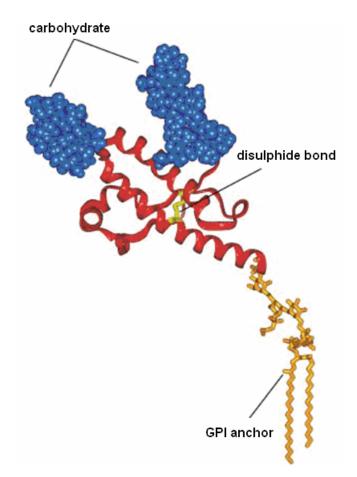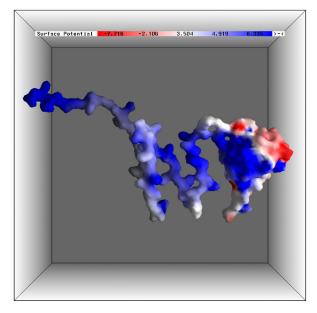
Figure 1.3. Conformational representation of PrPC (from Collinge, 2001).

Prion protein is synthesized in the rough endoplasmatic reticulum where it is cleaved of an N-terminal peptide (residues 1-22) and of a C-terminal hydrophobic segment (residues 231-253) followed by the attachment of a glycophosphatidylinositol (GPI) anchor. After the transition through the Golgi it is transported to the cell surface.

The N-terminal unstructured arm has numerous positive charges and it is thought to be utilised in the interaction with other molecules through columbic forces.

Measurements of the surface potential of a recombinant PrP showed how the charge distribution on prion molecules are different in C-terminal part and in the N-terminal part. The C-terminal globular part of protein has both negatively and positively charged areas. (Figure 1.4).
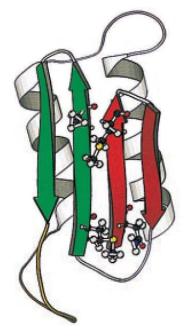
Figure 1.4. Electrostatic potential surface of a recombinant ovine PrP^C (from Rao et al., 2007)

The conformational isoform PrP^{Sc}, responsible of TSEs disease, is the seed of the protein misfolding and aggregation related to the disease. PrP^{Sc} requires the contemporary presence of PrP^C to propagate the PrP^{Sc} replication. Mice lacking the PrP as a result of gene knockout (PrnP$^{0/0}$) were completely resistant as underlined by inoculation tests with the infective protein.

PrP^C and PrP^{Sc} have an identical amino acid sequence and share the same posttranslational modifications as assessed by currently available methodology, but differ in their secondary and (presumably) tertiary structure. PrP^{Sc}, which is represented in a model in Figure 1.5, is extracted from brains of TSE affected animals as highly aggregated, detergent-insoluble material. Fourier transform infrared spectroscopic methods show that PrP^{Sc}, in sharp contrast to PrP^C, has a high β-sheet content and that is covalently indistinguishable from PrP^C. FTIR spectroscopy demonstrated also that PrP^C has a high α-helix content (42%) and no β-sheet (3%), findings that were confirmed by circular dichroism measurements. Conversely, the β-sheet content of PrP^{Sc} was 43% and the α-helix 30% (Pan, 1993).

Figure 1.5. Plausible model for the tertiary structure of PrP^{Sc} (from Prusiner, 1998)

One of the most relevant characteristic of PrP^{Sc} is resistance to the attach of proteases, due to a resident core close to the C-terminal part that is designed as PrP$^{27-30}$ according to the molecular weight evaluated on SDS-PAGE. The presence of PrP$^{27-30}$ after protease treatment provides a specific and reliable marker for the presence of the infectious agent (Figure 1.6).
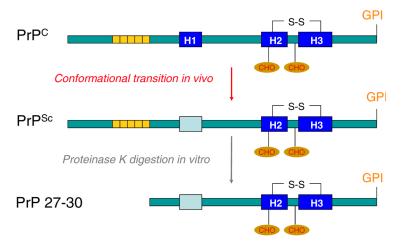
Figure 1.6. Cellular, Scrapie and PK digested prion proteins (from Watts, 2006).

The mechanism of PrP^{Sc} formation and PrP^{Sc} aggregation is still unclear. However, many investigations devoted to individuate intermediate states of the protein that should lead to the PrP^{Sc} formation, and based principally on the folding and unfolding of the protein in presence of chaotropic denaturants, demonstrated that there are no populated intermediates in the folding reaction and that the protein displays unusually rapid rates of folding and unfolding.

These findings have been reinforced by hydrogen/deuterium exchange measurements on the human protein, which shows that no partially unfolded forms or intermediates have a population greater than the unfolded state. It is thus unlikely that PrP^{Sc} is formed from a kinetic folded intermediate. In fact, on the basis of population results, it would be more likely that PrP^{Sc} is formed from

the unfolded state of the molecule. Figure 1.7 shows how the normal cellular isoform of prion protein, PrP[C], rich in α-helix (blue cylinders) can be reversibly interconverted (A, B) to a β-sheet-rich (arrows) conformation, β-PrP. The β rich isoform has an increased ability to aggregate, and the formation of a crucial seed size leads to essentially irreversible propagation of the disease-related isoform of the prion protein, PrP[Sc], through the recruitment of further β-PrP monomers (C) or unfolded PrP (D).
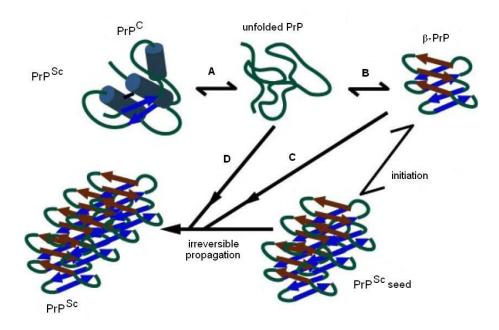
Figure 1.7. Propagation of prion protein aggregates (from Collinge, 2001).

Subsequent mechanical breakage or cleavage of elongating fibrils would lead to an exponential rise in prion titre. Such a model can accommodate the different aetiologies of human prion diseases: prion propagation might be initiated by the introduction of a seed (acquired prion disease); by spontaneous seed production as a rare stochastically mediated event that involves wild-type PrP (sporadic

prion disease); or as an essentially inevitable event with PrP that contains a pathogenic mutation (inherited prion disease). Following such initiating events, the process of propagation is driven thermodynamically by intermolecular association (Collinge, 2001) (Figure 1.7).

1.1.6. Disease transmissibility and species barrier

Prion protein disease can easily transmit from host to other animals of the same species. The transmission to other species is well documented but it is less likely to happen. Also in early works it was reported that transmission of prion diseases between different species is restricted by a "species barrier" (Pattison and Jebbett, 1971).

Usually inoculation of uninfected animal of a specie B with infectious material from a species A determines the transmission of the disease in not all the inoculated animals from species B. Relevantly also the incubation period in this first passage (from specie A to specie B) is much longer and variable than those observed after inoculations in the same species. Typically, after the second passage (from specie B to specie B) most, if not all, animals show symptoms of the disease and dye with shorter and more consistent incubation periods. Species barriers can therefore be also evaluated by measuring the fall in mean incubation period between the primary and second passage (Collinge, 2001).

Prion disease transmission is thought to be related to the prion strain. For instance transmission of CJD prions to conventional mice is difficult, with few, if any, inoculated mice succumbing after prolonged incubation periods, consistent with a substantial species barrier (Hill et al., 1997). In sharp contrast, transgenic mice expressing only human PrP are highly susceptible to CJD prions with 100% of infection attack rate and short incubation periods that resulted also

unaltered by second passage, consistently with a complete lack of species barrier (Collinge et al., 1995).

Both PrP amino acid sequence and strain type affect the three-dimensional structure of glycosylated PrP, which will presumably, in turn, affect the efficiency of the protein-protein interactions thought to determine prion propagation (Collinge, 2001).
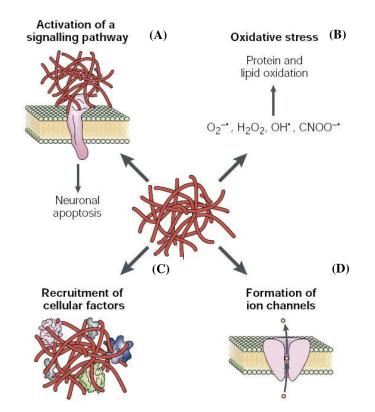
1.1.7. Prion toxicity

The most widely accepted theory of brain degeneration in neurodegenerative diseases proposes that misfolding and aggregation result in the acquisition of a neurotoxic function by misfolded protein. Experimentally neuronal apoptosis by aggregates of several misfolded proteins reported by Forloni (1993) or El Agnaf (1998) furnished a basis for this hypothesis.

Several proposed mechanisms leading to neurotoxic activity of misfolded aggregates of PrP^{Sc} in the brain were reported by Soto (2003) (Figure 1.8).

Extracellular aggregates might activate a signal transduction pathway that leads to apoptosis by interacting with specific cellular receptors (Figure 1.8A). Protein aggregates might induce oxidative stress by producing free radical species, resulting in protein and lipid oxidation, elevation of intracellular calcium and mitochondrial dysfunction (Behl et al., 1994; Hsu et al., 2000) (Figure 1.8B). Intracellular aggregation might recruit essential factors like chaperone proteins, cytoskeletal proteins and transcription factors for cell viability (Cummings et al., 1998). (Figure 1.8C).

Neurotoxicity could arise also by membrane disruption and depolarization of ion-channel formation, resulting in alteration of ion homeostasis leading to cell death (Lin et al., 1997) (Figure 1.8D).
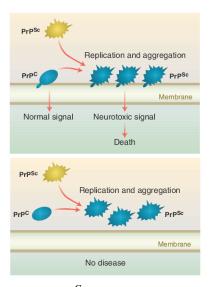
Figure 1.8. Possible mechanism of neurotoxicity related to PrPSc (from Soto, 2003)

Recent histopathological, biochemical and cell biology studies have disproved these hypotheses (Bondareff et al., 1989; Tompkins et al., 1997). In some animals affected by several neurodegenerative diseases cerebral damage and clinical symptoms have been detected before protein aggregates (Klement et al., 1998; Moechars et al., 1999). In most studies inhibitors of protein aggregation have also prevented neuronal damage. Some *in vitro* and *in vivo* studies found that, under certain conditions, prevention of aggregation did not inhibit (or even increased) cell death suggesting a possible important role of reduced dimension aggregates (Klement et al., 1998; Saudou et al., 1998).

Most recent studies suggested instead a role of glycosylphosphatidylinositol (GPI) membrane anchoring of PrP on its toxicity (Aguzzi, 2005, Chesebro et al., 2005). During its early biogenesis, PrP^C is directed to the lumen of the endoplasmic reticulum, thus entering the cellular secretory pathway. A glycosylphosphatidylinositol lipid anchor is then added to its C terminus, confining the protein to the outer side of the cell membrane. Chesebro et al. (2005) redacted a Prpn (Prpn is the gene that provides instructions to product prion protein) transgenic mice to remove the signal peptide responsible for GPI anchoring. As a consequence, the resulting GPI-negative transgenic mice expressed a monomeric, soluble secreted form of PrP^C. Chesebro et al. (2005) reported that GPI-negative transgenic mice never developed clinical prion disease after inoculation with infectious material. Quite surprisingly, though, their brains were packed with PrP^{Sc} plaques (Chesebro et al., 2005). Evidently, removal of the GPI anchor abolished susceptibility to clinical disease while preserving the competence of the soluble PrP molecule to support prion replication (Aguzzi, 2005). The supposed signalling function of PrP^C seems to be crucial in this view for prion disease manifestation. Altered PrP^C signalling due to PrP^{Sc} conversion on the membrane may be unhealthy. The lack of GPI anchor can act denying the wrong signalling into the cell and avoid the disease occurrence (Figure 1.9). If this hypothesis is true, it seems crucial to investigate the exact function of PrP^C signalling in the brain.
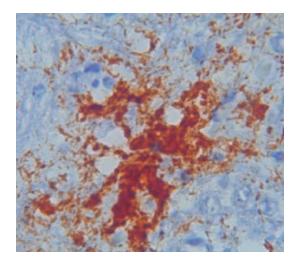
Figure 1.9. GPI anchoring of PrP^C as a factor influencing disease occurrence (from Aguzzi, 2005)

1.1.8. Detection of prions

One of the problems working with the TSEs is the detection of PrP^{Sc} putative of the disease itself. Brains of infected individuals usually exhibit pronounced vast areas affected by spongiform formation, neuronal degeneration and death, astrogliosis, and accumulation of amyloid plaques containing PrP^{Sc}. Spongiform change can be observed using standard histological procedures, and PrP^{Sc} deposits can be viewed following pre-treatment with formic acid and hydrated or hydrolytic autoclaving (Haritani et al., 1994) to reduce the immunoreactivity of PrP^C prior to staining with a PrP-specific antibody (Figure 1.10).

Figure 1.10. PrP amyloid deposit stained by immunohistochemistry (brown) (from Watts, 2006)
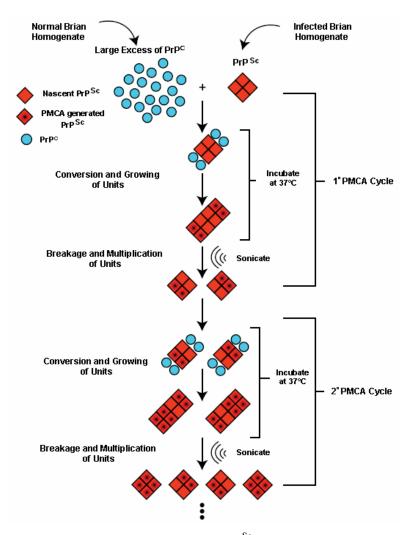
Following proteinase K treatment, PrP^{res} (PrP^{Sc}) can be detected using either a Western blot or an ELISA; these strategies are the basis for two of the most widely used commercial tests for BSE. Other methods take advantage of the differential availability of sequestered antibody epitopes between PrP^{C} and PrP^{Sc}. An antibody is used which recognizes a central epitope with differential accessibility between PrP^{C} (available) and PrP^{Sc} (not accessible until thermal or chemical denaturation).

In contrast to the direct detection of PrP^{Sc}, active diagnostics amplifies PrP^{Sc} and then infectivity both *in vivo* and *in vitro* before a detection step. The prion bioassay in mice is the most commonly used method for assaying infectivity *in vivo*. Following intracerebral inoculation of the test sample, mice typically succumb to prion disease following an incubation period of approximately 150 days (depending on the strain of mouse and prion utilized) (Watts, 2006). This bioassay is considered as an optimal standard but it is affected by at least two kinds of problems. Firstly, the procedure requires long time to obtain complete

results and thus results in a lot of labour and high costs; moreover bioassays may be subject to the "species-barrier" that can cause the prolongation of the incubation time or even inefficient transmission of disease. The individuation of species with a high susceptibility to the prion transmission like wild type bank voles can help to solve problems related to inefficient transmission.

Another active technique, much faster and cheaper than conventional bioassays, is the scrapie cell assay (Klöhn et al., 2003). This cell culture-based method utilizes sub-lines of mouse N2a neuroblastoma cells that have been selected for enriched susceptibility to prions and measures the ability of a test sample to generate PrP^{Sc}-positive cells. Unfortunately the attempt to use different prions as reported by Klöhn et al. (2003) resulted in unsuccessful prion propagation.

A third technique that is been using a lot recently is the protein misfolding cyclic amplification (PMCA). This technique allows to amplifying the titre of PrP^{Sc} in a solution in presence of such brain template and PrP^{C} that is converted *in vitro* to PrP^{Sc}. PrP^{Sc} is then detected by commonly western blot techniques. Small amounts of infected material are diluted into normal brain homogenate and *in vitro* conversion is allowed to proceed at 37 °C (Saborio et al., 2001; Saa et al., 2005) A key ingredient is a subsequent sonication step, formally analogous to thermal denaturation of complementary DNA strands in a PCR reaction. Here, mechanical energy is used to break up newly formed PrP aggregates into smaller structures, with the latter providing new seeds for PrP^{Sc} formation in reiterations of the two-step procedure (Figure 1.11).

Figure 1.11. Schematic representation of PrP^{Sc} amplification by PMCA (from Watts, 2006)

Repeated cycles of sonication are performed in order to amplify any PrP^{Sc} present in the starting sample, instead omitted sonication steps reduce PrP^{Sc} amplification.

1.2. Prion protein in soil

Soil is a natural sink or reservoir of several contaminants including prion proteins. Dispersion of prion proteins causative of BSE might be occurred in soil from meat and bone meal storage plants, use of fertilizers augmented with meat and bone, decomposition of TSE-contaminated animal carcasses buried in soil, TSE infected tissues, liquid and solid waste fragments from abattoirs, central sterilization units, operating theatres and wastes from TSE-infected animals. Differently, Scrapie and CWD, environmental sources of infectious prion protein are linked to the dispersion of infectivity by free ranging animals that can act sustaining the disease. Environmental sources of infection by prions represent strong obstacles to control the spread of the disease (Miller et al., 2004). An early study by Brown and Gajdusek (1991) demonstrated that soil retains infectivity after 3 years of infectious material internment; recently Georgsson et al. (2006) reported that may have persisted in the old sheep-house for at least 16 years, strongly highlighting prion capability to persist in soil in an infectious state. Therefore, understanding the mechanism of retention, persistence and dissemination of prion proteins in soils, is very important for dealing with environmental problems related to TSEs.

Once introduced into the soil a protein may remain free in solution, and as such to be biologically or chemically degraded, or participate in the formation of organic and organo-mineral soil complexes. Both are complex phenomena that could depend on properties of both the protein and soil. For instance, physicochemical properties of protein such the isoelectric point and flexibility of polypeptide chain may affect the adsorption phenomenon on soil colloids such as clays (Quiquampoix et al., 1995) and humic substances because of the presence of polar and non polar adsorption sites (Stevenson, 1994; Senesi and

Loffredo, 1999). It seems important to investigate the possible role of colloidal particles both mineral and organic that constitute the most reactive part of soil in the processes of immobilization and degradation of chemicals and bio macromolecules like proteins.

1.3. Soil environment

Soils exhibit unique physical and chemical sorptive qualities and dynamics, both reflective of their inorganic and organic composition. Soils are dynamic, living systems involved in cycling of carbon, nitrogen and other nutrients in nature.
 Soils are porous and open bodies containing mineral particles of many shapes and sizes and organic material which often are in a colloidal form. The solid particles are in contact to each other, and packed closely together. Soils exhibit great ranges of properties. Using measurable and observable properties, such as the kind and arrangement of soil horizons, soils can be characterized and classified. "Series" in soil taxonomy is the lowest category and all soils within a series will have developed on the same kind of parent material, with comparable drainage characteristics, and will have similar age. The effects of climate and biological activity will have been very similar. Higher levels of classification are family, subgroup, great group, suborder and order.

In soil, primary mineral particles are classified on a size distribution basis, indicating the soil texture. As the lower particle's dimension (higher specific area) is usually associated to higher reactivity, this classification is really important to understand processes acting in soil. For instance, particles larger than 2 mm in diameter are considered inert; smaller than 2 mm are classified according to USDA definition into three broad categories based on size. Particles of 2 to 0.05 mm diameter are called sand, those of 0.05 to 0.002 mm

diameter are silt, and the <0.002 mm particles are clay. These primary particles that usually undergo a natural arrangement into secondary particles involve organic matter in the formation of aggregates that determine the soil structure. Often a sandy soil may be structureless because each sand grain behaves independently of all others, while clay particles cohere to each other and adhere to larger particles under the conditions that prevail in most soils. Processes of wetting and drying, freezing and thawing, root and animal activity, and mechanical agitation are all involved in the rearranging of particles in soils.

The organic fraction of a soil, although usually representing much less than 10% of the soil mass by weight, has a great influence on soil chemical properties. Soil organic matter is composed mainly of carbon, hydrogen, oxygen, nitrogen and lower amounts of sulphur and other elements. The organic fraction serves as a reservoir for the plant essential nutrients like nitrogen, phosphorus, and sulphur, increases soil water holding capacity and cation exchange capacities; moreover soil organic matter has an important role in the formation of aggregates (Chen, 1996; Piccolo, 1996).

The most chemically active fraction of soils consists of mineral and organic colloids. Colloids are particles that remain suspended in water and their dimensions are as small as < 2 μm. Colloids are important in many soil processes, having a very large specific surface area, and usually showing a net negative charge that results in a high adsorptive capacity. They have strong influence on the physical and chemical properties of a soil. Inorganic colloids (clay minerals, hydrous oxides) are usually the main part of soil colloids. Clay minerals usually have crystalline structure (although some has low crystalline order) and have a defined chemical and physical configuration. The organic colloids include highly decomposed organic matter and reorganized in substances generally called humus. The organic colloids are thought to be the more chemically reactive and generally to have a greater influence on soil

properties than the inorganic colloids. Humic substances are amorphous and their chemical and physical characterisation is not yet well defined, as they are considered by some researcher as polymer or as supramolecular association of relatively small molecular weight molecules (Sutton and Sposito, 2005). Both inorganic and organic colloids are intimately mixed together forming organo-mineral colloids.

Silicate clays and organic matter typically possess net negative charge because of isomorphous substitution of cations with lower valence in clays (Foth and Ellis, 1997), and dissociation acid functional groups of organic matter. Positively-charged cations are attracted to these negatively-charged particles. Soil clays and organic matter adsorb and exchange cations with those present in the water of soil porosity, determining the soil cation exchange capacity (Foth and Ellis, 1997). Cation adsorption is reversible if other cations in soil solution are sufficiently concentrated to displace those attracted to the negative charge on clay and organic matter surfaces. Cation adsorption allows plants to have readily available cations, limiting their uncontrolled leaching or downward movements in soils with water (Chen, 1996). Moreover, pollutants like pesticides and other organic xenobiotics with positively charged functional groups are also attracted to cation exchange sites and may be removed from the soil solution, making them less subject to loss and potential pollution.

One of the most informative soil chemical properties and probably the most commonly measured is the soil pH. A soil with a pH in the range of 6.6 to 7.3 is considered neutral while up or below these values soils are classified as acid or alkaline (Foth and Ellis, 1997). Soil pH typically ranges from 4.0 to 8.5, but extreme situation can be found like as low as pH = 2 in materials associated with pyrite oxidation (Haraguchi et al., 2005) and acid mine drainage. Soil pH affects the quantity, activity, and types of microorganisms which in turn influence decomposition of crop residues, manures, sludge and other organics. It also

affects other nutrient transformations and the solubility, or plant availability, of many plant essential nutrients. Phosphorus, for example, is most available in slightly acid to slightly alkaline soils, while all essential micronutrients, except molybdenum, become more available with decreasing pH. Aluminium, manganese, and even iron can become sufficiently soluble at pH < 5 and high quantity could result toxic to plants. Bacteria which are important mediators of numerous nutrient transformation mechanisms in soils generally tend to be most active in slightly acid to alkaline conditions.

1.3.1. Soil mineral constituents

In soils phyllosilicates, secondary clay minerals play a profound role affecting numerous soil chemical reactions and processes.

Clay minerals are assemblages of tetrahedral and octahedral sheets. When one tetrahedral sheet is bonded to one octahedral sheet a 1:1 clay mineral results (e.g. kaolinite, halloysite). When two tetrahedral sheets are coordinated to one octahedral sheet, a 2:1 clay mineral results (e.g. montmorillonite, vermiculite, illite) (Yariv and Michaelian, 2002).

Isomorphous substitution is the substitution of one atom by another of similar size in the crystal structure without disrupting the crystal structure itself. The size of the cationic radius determines that cations can substitute in the silica and octahedral sheets. In the tetrahedral sheet Al^{3+} usually can substitute for Si^{4+} (Foth and Ellis, 1997). In the octahedral sheet Fe^{2+}, Fe^{3+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, or Cu^{2+} can substitute for Al^{3+}. As a result of this isomorphous substitution, a net negative charge develops which is associated with the 6 oxygen or hydroxyls of the octahedrons and with the 4 oxygen of the tetrahedrons and represents the permanent charge that is unaffected by external factors, such as pH. Conversely,

along the edge of some clay minerals, some of the OH$^-$ are bonded to only one cation and these hydroxyl play the major role in the development of variable charge (dependent on pH) (Foth and Ellis, 1997). A small amount of variable charge develops along the edges of phyllosilicates. Variable charge is a relatively small sources of charge in 2:1 layer minerals (e.g. montmorillonite, vermiculite, illite), but it is the major or sole sources of charge in 1:1 layer phyllosilicates, including kaolinite and halloysite

Metal oxides like aluminium, iron, and manganese oxides play extremely important roles in the chemistry of soils. Although they may not be found in large quantities, they have significant effects on many soil chemical processes because of their high specific surface areas and reactivity (Post, 1999).

The general term oxides refer to metal hydroxides, oxyhydroxides, and hydrous oxides (where non steichiometric water is in the structure). Oxides are ubiquitous in soil. They may exist as discrete crystals, as coating on phyllosilicates and humic substances, and as mixed gels. Such of most reactive oxides in soil are manganese oxides that are quite common in soil and occur as coatings on soil particles, in cracks and veins, and as nodules even as large as 2 cm in diameter. Most manganese oxides are amorphous. The most stable form of manganese oxides is pyrolusite (β-MnO$_2$) but it is uncommon in soils. Birnessite (δ-MnO$_2$) is the most prevalent Mn oxide in soils.

Surface charge of Fe, Al and Mn oxides in contact with aqueous solutions is pH dependent. At the hydroxylated or hydrated surface, positive or negative charges develop by adsorption or desorption of protons and/or hydroxyl ions or dissociation of surface species. The pH at which the net variable charge on the surface is zero is called the point of zero charge (pzc). Because of its dependency on pH the surface charge of the metal oxides is called "variable charge". The pzc of most Fe-oxides usually ranges from 7.0 to 9.5, in Al-oxides is usually higher than 8.0-8.5 whereas in Mn-oxides, like birnessite, is around

3.0. The pzc has important consequences on colloids mobility in soils increasing or decreasing the possibility of contact between metal oxides and inorganic and organic ions or biopolymers.

Organic and inorganic ligands with a strong affinity for Fe, Al or Mn are strongly adsorbed on the surfaces of metal oxides through a ligand exchange mechanism, forming inner-sphere complexes. Monodentate ligands (acetate, formate) are weakly adsorbed on oxide surfaces.

Surface area is another important physical property of soil minerals that determines the sorption capability and thus affects the mobility of contaminants in soils. The total surface area of a secondary mineral depends on both internal and external surface areas. External surface area is usually determined by measuring the adsorption of nitrogen gas at 77 K. Ethylene glycol monoethyl ether is used to measure the total surface area of a mineral. Mobilization of organic ions can also be related to other events like change in pH or presence of other ions that in turn can exchange with adsorbed ones.

1.3.2. Soil organic constituents

Organic matter (OM) in agricultural soils consists primarily of intact or partially decomposed biopolymer residues deriving from plants and animal residues, microbial biomass and humic substances (Stevenson, 1994, Zech et al., 1997). In natural soils, where soil mixing processes are reduced, as compared to agricultural soils, usually a layer deposition of OM occurs at the surface as the primary input of organic carbon represented by plant residues. A progressive transformation of this "fresh" OM occurs with depth due to the decomposition by soil micro- and macro-organisms, which utilize it as nutrient source for building up their own biomass. In this process new molecules are formed and

these can be, in turn, utilized by the microbial community providing that effective biochemical pathways are active. In forest soils "litter" is the upper layer stratification, where leaf deposition is active. Underneath, an "organic horizon" is formed, and sub-horizons can seldom be recognized (with cellular structure and plant debris no longer evident), on top of a mineral-organic layer (A horizon) where humified OM and mineral particles are intimately complexed.

Early effort to characterize humic substances (HS) conduced to a fractionation scheme based on solubility in alkaline or acid condition (Stevenson, 1994). Humin is the insoluble fraction of HS, humic acids (HA) are the fraction soluble in alkaline conditions but not acid (generally below pH 2) and fulvic acids (FA) the fraction soluble in all pH conditions (Figure 1.12). Although the different solubility at different pH underlies chemical and physical differences, the classification is only operational and does not indicate the existence of different types of organic molecules (Sutton and Sposito, 2005).
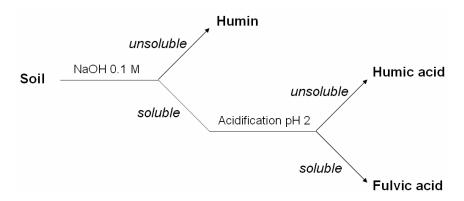
Figure 1.12. Fractionation scheme of soil humic substances.

According to the "polymer model", biopolymers resilient to degradation or suitable simple organics can react together and form complex macromolecules

with moderate to high molecular weight that are relatively stable to microbial degradation, commonly referred as humic substances (HS). In this view HS comprised of randomly coiled macromolecules that have elongated shapes in basic condition or low ionic strength solution, but become coils in acid or high ionic strength solution (Stevenson, 1994).

According to a new concept these substances are organized in a "supramolecular structure", in which many relatively small and chemically diverse organic molecules form clusters linked by hydrogen bonds (H-bonds) and hydrophobic interactions (Piccolo, 2001; Simpson et al., 2002; Sutton and Sposito, 2005). In this view these associations are capable to form a micellar structure: organic molecules in aqueous media are arranged with hydrophilic region turned exteriorly while hydrophobic regions are close to each other, oriented interiorly and separated by the aqueous solution (Von Wandruska, 1998).

Humic substances are found to contain biomolecules fragments that normally were excluded from their definition because of the conflict with acknowledged refractory nature of these materials. However, binding to humic fractions can result in the protection of biomolecules from microbial degradation (Zang et al., 2000).

A substantial fraction of the mass of humic acids has carboxylic functional groups, which have chelating properties toward positively charged multivalent ions like Mg^{2+}, Ca^{2+}, Fe^{2+}, most other "trace elements" of value to plants, as well as other ions that have no positive biological role, such as Cd^{2+} and Pb^{2+}. This chelation of ions is probably one of the most important functions of humic acids with respect to living systems. By chelating ions, HS facilitate the uptake of these ions by several mechanisms, one of which is preventing their precipitation, with positive influence on their bioavailability.

HS show hydrophobic character that allows interacting with hydrophobic molecules and favouring the adsorption and the retention of these molecules.

1.3.3. Mineral and organic associations in soil

Most of the humic substances in soil exist in close association with mineral colloids. This association tends to stabilize soluble organic matter (Stevenson, 1994). HS are retained in soil as: i) insoluble complexes of humic and fulvic acids, ii) associations of HA and FA bound by bi- and trivalent cations, such as Ca^{2+}, Al^{3+} and Fe^{3+} that also contribute to bridge soil particles together in the formation of stable aggregates, iii) substances adsorbed on mineral surfaces, iv) substances held within the interlayer of expanded clay minerals.

Several forces are involved in the adsorption of organic compounds by clay minerals: i) physical adsorption due to van der Waals' forces ii) electrostatic attraction or chemical adsorption, iii) hydrogen bonding, and iv) coordination complexes (Cornejo and Hermosin, 1996). These mechanisms may operate together depending on the characteristic of the organic molecules, the type of clay (expanding, i.e., smectite or no expanding, e.g. kaolinite), the nature of the saturating cation on the clay, the moisture of the system.

As clays are negatively charged, electrostatic repulsion has to be expected with negatively charged humic substances at pHs above the isoelectric point of HS. Laboratory studies have shown that fulvic acids can be adsorbed in the interlamellar spaces of smectites only at pH <5.5 (Schnitzer and Kodama, 1967; Theng, 1976) or in the presence of polyvalent cations (Vreysen and Maes, 2006). With this respect the adsorption in the presence of divalent cations is reversible, while for trivalent cations tightly bound complexes are formed. The SOM bound in the interlayer space of swelling minerals is biologically protected. A revealed by radiocarbon ages it can result as old as 6700 years (Theng et al., 1992).

1.4. Actual knowledge on prion protein in soil environment

Studies on interaction of prion protein with soil or soil constituents are recent and quite few. Due to soil and protein properties the problem of infectious particles in soil arises for several reasons:

- there are many sources of possible soil infection in soil;

- soil mineral constituent can increase infectivity of prion protein;

- soil humic substances could act protecting protein from degradation processes;

- soil can retain infectivity over years;

- prion protein, because of its properties, is likely to be retained in the superficial layer of soil and so remain highly available to wild animals;

- prion protein diseases are capable to spread the disease inter-species, not excluding humans.

Actual literature is focused on several aspects i) soil persistence of PrP^{Sc}, ii) dissemination and diffusion by wild animals, iii) adsorption on soil mineral constituents and whole soil, iv) role of organic matter, v) desorption and mobility of prions in soil, vi) and microbial activity and prion proteolysis

1.4.1. Persistence in soil

Since the historical article of Brown and Gadjusek that in 1991 reported that consistent amounts of residual infectivity persisted in soil buried perforated Petri dishes after 3 years, there was a growing concern about the issue of prion protein in soil. Further studies about soil persistence of prion protein in soil confirmed these data and were even more convincing of the capability of prion protein to preserve its infectious potential. PrP^{Sc} spiked soil resulted infectious

even after 29 months and also the aqueous extract was able to induce disease (Seidel et al., 2007). Georgsson et al. (2006) reported that in Iceland sheep contracted scrapie by contact with a sheep house where infected sheep were housed 16 years before.

1.4.2. Dissemination by wild animals

Scrapie and CWD are contagious diseases which show horizontal transmissibility under natural conditions (Miller et al., 2003). The regular occurrence of scrapie in affected areas and the spread of CWD in North America and Korea among mule deer, white-tailed deer and elk indicates that a contagion in the environment is responsible for the occurrence of these TSEs (Miller et al., 2004) and even raises the possibility of a cross-species transmission under natural conditions. Recent findings demonstrated that saliva from deer with CWD harbours infectivity and can transmit these TSEs upon peroral uptake (Mathiason et al., 2006). Other studies pointed to the transmission of scrapie among sheep by vectors like mites, fly larvae or other ectoparasites (Post et al., 1999).

1.4.3. Adsorption on soil mineral components and whole soil

The majority of studies on the interaction between prion protein and soil have been performed with soil mineral constituents and whole soil. A recombinant ovine form of protein, recPrP, and the PrP^{Sc} has been utilized. Highly stable adsorption of a recPrP on muscovite mica, a phyllosilicate representative of soil clays, was reported by Vasina et al. (2005). Adsorption was performed with a recPrP labelled with ^{125}I in a pH range from 4 to 9. Desorption was not observed

in the pH range 4-9, while the addition of detergent or 0.1 NaOH determined almost complete removal. The authors concluded that the driving force in adsorption has to be attributed to electrostatic forces establishing between the highly negative surface charge of the mineral and the positive charge region like the N-terminal part of recPrP utilized (Vasina et al., 2005). Therefore the authors supported the idea that prions, and likely their infectivity, could spread in the environment through animal contamination by grazing herds rather than vertical transportation.

Adsorption on mineral clays was reported to affect recPrP^C by modifying its conformation (Revault et al., 2005). The authors investigated the conformational states of the full-length ovine prion protein adsorbed on montmorillonite (that has an electronegative charged surface) as compared to its solvated state in deuterated buffer in the pD range 3.5-9.0, using FTIR spectroscopy. The trapped conformers obtained by spontaneous adsorption on the electronegative clay surface adopted a β-like structure significantly different from the β-oligomers formed in solution at acidic pH, thus it appeared unlikely that the interaction of normal prions (PrP^C) with soil clay surfaces could induce a change of conformation leading to the pathogenic form of prions (PrP^{Sc}) (Revault et al., 2005).

Adsorption experiments performed with this model recombinant protein should be confirmed by using real glycosilated protein. Ma et al. (2007) found that PrP^{Sc} aggregates showed a pI of 4.6, markedly different from pI of 9 of recombinant prion protein (Rezaei et al., 2000). Ma et al. (2007) found that the maximal PrP^{Sc} aggregates adsorption on quartz sand was at a pH close to the pI aggregates while adsorption decreased at lower or higher pHs. They suggested that disposal strategies made by burial in sites with addition of lime or fly ash to degrade prion are likely to increase PrP^{Sc} mobility.

We also focused our interest on soil minerals and in particular we investigated the oxidant capability of manganese oxides in soil toward the disease-associated prion protein as a factor potentially limiting environmental prion disease transmission. This topic is diffusely discussed in Chapter 5 (Russo et al., submitted to publication).

Johnson et al. (2006) demonstrated highly affinity of two soil clay minerals (montmorillonite and kaolinite), and four whole soils in binding the infectious PrP^{Sc}. They found high amounts of sorption of PrP^{Sc}, desorption was possible only in strongly denaturing conditions. The desorbed protein resulted cleaved of its unstructured N-terminal end even though preserved its infectivity (Johonson et al, 2006). In a subsequent work Johnson et al. (2007) found that soil particles, in particular montomorillonite loaded by PrP^{Sc} was able to increase transmissibility of the disease as evaluated by oral exposure. Authors observed also that soil-bound prions can enhance transmissibility, suggesting that latter an explanation of environmental spread of some TSEs despite the relatively low levels in the environment (Johnson et al, 2007). Leita et al., (2006) investigated the interaction of PrP^{Sc} and using directly brain homogenate of infected animals concluded that prions can be retained and accumulated in soil especially if amended with prion-containing organic fertilizer (Leita et al., 2006). Recently, Genovesi et al. (2007) reported a useful technique to detect prion infected soil using a cell-based assay of infectivity. This cell approach for detecting potential infectivity of contaminated soil is fast and cheap, resulting really useful to test infected environmental sites. Limitations of this technique, like the susceptibility of only few mouse prion strains, have to take in account (Genovesi et al., 2007).

1.4.4. Role of organic matter in soil and organo-mineral complexes

Scarce information is available on the role of soil organic matter on the fate of prion protein in soil. We examined the role of organic matter in soil, and specifically processes of formation of new humic substances (Rao et al., 2007) or natural substances in the interaction with prion protein. This part is diffusely treated in the Chapters 3 and 4.

The effect of soil organic matter has been investigated also by Pucci, 2007. Studies focused on the effect of removal layer by layer of superficial soil organic matter in undisturbed soil aggregates by Low Temperature Ashing (LTA) on adsorption capacity of recPrP. Comparison experiments were carried out in a batch system. Soil aggregates adsorption isotherms evidenced high affinity for recPrP. Experiments evidenced as the soil organic matter has a large specific adsorption capacity for recPrP, even larger than the sole mineral matrix, and that removing OM resulted in reduced adsorption of recPrP. In addition coupling PAS-FTIR measurements allowed them to demonstrate that recPrP adsorption is limited only to the external part of the aggregates.

1.4.5. Factors affecting desorption and mobility of prion protein in soil

Some studies focused their interest on possible mobilization of prions in soil (Rigou et al., 2006, Cooke et al., 2007, Cooke and Shaw, 2007). A technique to extract PrP from soil reported by Rigou et al. (2006) was based on an electroelution of the bound protein on soil and clays. Cooke et al. (2007) reported a technique to desorb prions from soil using a solution of an anionic surfactant. The addition of a proteinase K treatment (in which the N-terminal was cleaved) was needed to remove PrP^{Sc} from a clay-soil, highlighting that PrP strongly binds to one or more soil components, but especially to clay

components, as with sandy soil the PK treatment was not necessary (Cooke et al., 2007). The successful extraction of prions by electroelution, the use of an anionic surfactant suggested that binding of protein occurred mainly via the positively charged N-terminal of PrP (Rigou et al., 2006; Cooke et al., 2007). Subsequently Cooke and Shaw (2007) reported that PrP^{Sc} and even the recPrP^{C} are likely to survive buried in soil for months. Interestingly the soil mobility of prion was detected but reduced to the first centimetre. Thus, soil is likely to act as a significant barrier to the dispersion of contaminated material at storage or burial sites (Cooke and Shaw, 2007).

1.4.6. Microbial activity and proteolysis

The effect of burial of carcasses in soil on microbial activity was studied by Rapp et al. (2006). In a 1-year field experiment, the potential proteolytic activity and the substrate induced respiration (SIR) as a response of microbial community to lamb carcasses burial were investigated. Soil above carcasses and control soil exhibited low proteolytic capacity, whatever the depth of burial. Contrastingly, in soil beneath the carcasses, proteolysis was stimulated. Decomposing carcasses also stimulated SIR, i.e., microbial biomass, suggesting that proteolytic populations specifically developed on lixiviates from animal tissues. The ability of soil proteases to degrade the β form of prion protein *in vitro* was also demonstrated. Results reported by Rapp et al. (2006) suggest that proteases active toward prion protein are very likely produced in soil followed stimulation by animal tissues. In the case of animals buried in spring, prion protein could be degraded rapidly in spring or summer with a reduced risk of potential dissemination. Degradation of β form of prion protein by proteases has to be verified by using infectious PrP^{Sc} aggregates.

Other studies on biodegradability of recPrP were made through earthworms gut passage. Nechitaylo (2007) found a change in earthworms microbial fauna gut associated. Many bacterial isolates (depending on taxonomic affiliation, up to 33%) from earthworms did digest recPrP in pure cultures. Gamma proteobacteria, Bacilli, Actinobacteria, and fungi were the most active potential degraders of recPrP *in vitro*. Non-specific proteolytic activity of soil strongly increased during the transit through the earthworm gut. The major contribution to that were the earthworm-produced enzymes. However, this augmentation along with modification of microbial population in the earthworm gut environment did not enhance the recPrP digestion. Most likely, the active enzymatic fraction for recPrP proteolysis in the soil enzyme pool was constituted by trypsin- and chymotrypsin-like proteases. The contribution to this pool of the earthworm itself and earthworm gut microflora seems to be minimal. Thus, studied microbial-earthworm gut systems did not produce proteases notably affecting the prion proteolysis.

These studies of biodegradability of recombinant prion protein needs to be confirmed by using a disease associated prion protein that notably has a core highly protease resistant that maintains infectious capacity (Watts, 2006).

1.4.7. Prion relevance in the environment

According to most of the recent findings, recently reviewed by Schramm et al. (2006), soil can act as reservoir and a diffuser of the prion disease. Furthermore, due to the transmissibility character of the disease, interspecies passages in the environment are likely to happen.

The role of soil appears essentially relevant in the case of CWD and Scrapie that might be transmittable through the environment and in which lateral transmission is the primary mode of disease spread.

Infectious prion protein is persistent in soils also really hard to be degraded in the environment and its infectivity may last for decades. Even many inactivation procedures active towards different contaminated environmental materials, such as ultraviolet, ionizing radiation, treatment with proteases and contact with strong chemical disinfectants, high dry temperature, are often inefficient with prions, highlighting again the extreme persistence character of prions.

Interaction of the prion protein with soil, minerals and organic colloids is strong, thereby self vertical migration in soil through percolating water is unlikely to happen. Therefore, infectivity could result close to the soil surface and thus be available to animal contact through intentional and/or non intentional soil ingestion by herbivores (Schramm et al., 2006). Furthermore, as attachment to clay minerals enhances prion infectivity via oral route of exposure (Johnson et al., 2007) it can be concluded that soil may play an important role in sustaining environmental diffusion of these diseases.

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Chapter 2

Aims

Studies on prion and soil problematic have mainly addressed the interactions of prion proteins with whole soils and soil mineral components. Soil and soil mineral components revealed a high retention capability of prion proteins that are likely confined to the more superficial layers of soils and thus resulting more accessible to other animals. Furthermore, it was demonstrated that in such environmental conditions, prion proteins infectivity can persist for years in natural soils.

The persistence of the prion protein in soil environment is due to its intrinsic properties and to the several processes that it can undergo in soil. In fact, its pathogenic form PrP^{Sc} results only partially cleaved by proteases, abundantly present in soils, and the cleavage does not affect its own infectious capability. The high resistance of PrP^{Sc} is also highlighted by the inefficiency of the most common and even hard disinfecting procedures toward this protein.

The interaction of proteins with soil is a complex matter and in some cases proteins can be involved into protections processes such as the immobilization on both mineral clays and organic matter that in turn can reduce degradation processes.

While several experimental findings have demonstrated that clays were able to bind on their surface high amounts of PrP^{Sc}, whose infectivity resulted unaltered or even enhanced, no specific indications on the interaction of prion proteins in any of its possible forms with soil organic matter are available. However, humic substances can involve in their structure soil proteins (e.g., enzymes, since the majority of studies dealt

with enzymatic proteins) that can result protected by humic networks, being their pores large enough to permit the passage of substrates and reaction products but not that of larger molecules such as proteases. Processes leading to humus formation can even more easily involve other molecules like chemical exogenous proteins and xenobiotics in generals. Some protection processes observed with soil enzymes can results also with exogenous proteins arriving to soil.

In addition, clay minerals and humic material are usually present in soil in association, forming stable complexes with properties sensibly different from the pure mineral or organic components. For instance, properties fundamental to processes of protein interaction with clays like surface charges and surface specific areas can strongly change in soil minerals after organic matter coverage.

Therefore, many could be the arising questions to answer concerning the fate of prion proteins in soil.

The experimental work reported in the present thesis, as part of a large European Research Project entitled "Biotic and Abiotic mechanisms of TSE infectivity Retention and Dissemination in Soil", has addressed only a limited aspect of this complex topic. In particular, it has been devoted to study the role played by organic matter in the fate of the prion protein in soil.

The research has been essentially based on the use of simplified model systems. This has allowed all the parameters and conditions, which influence their transformation to be controlled. The justification for using model systems is to create the conditions to observe directly the cause-effect relationships between the fate of prion protein and its interaction with soil organic matter. The studies have been performed by using a benign recombinant purified ovine protein, recPrP, expressed in

Escherichia coli, which mimics the native prion protein and by simulating the formation of soil organic matter by both abiotic and biotic catalysts in the presence and absence of the protein according to different experimental conditions.

As organic matter is also an important constituent of soil with properties markedly different from mineral constituents, investigations were also dedicated to elucidate, the differences in the interaction of the protein with soil mineral, organo-mineral complexes and organic matter alone as well as to compare the immobilization efficiency by organic matter entrapment.

Furthermore, the degradation capability of one of the most strong oxidant soil mineral as birnessite towards the disease-associated PrP^{Sc} has been investigated. This aspect is an important goal in the research of environmental processes acting in the degradation of the pathogenic form of the prion protein that can contribute to reduce the environmental risk of infection

Chapter 3

Interaction of a recPrP with organo-mineral complexes[1]

Prion proteins are regarded as the main agents of transmissible spongiform encephalopathies. Understanding their fate in soil may be crucial to elucidate the dissemination of the prion in the environment, associated with a possible transmission of infectivity.

Little experimental evidence is available on the adsorption to and entrapment in organic/mineral soil components of prion proteins with regard to the persistence, movement and infectivity of TSEs in the environment.

In this Chapter studies performed with simplified model systems, derived from the birnessite-mediated oxidative polymerization of catechol, resembling humic-mineral complexes formation in soil will be presented. As a model, a benign full-length recombinant purified ovine protein (residues 23-234), as well as a truncated form ovPrP (103-234) were utilized.

The disappearance of protein molecules from the solution, the decrease of UV-Visible absorbance of supernatants, and the FT-IR spectra and the

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elemental analyses of solid phase residues indicated that both the full-length as well as the N-truncated form of PrP were involved in catechol polymerization by birnessite.

Clearly flocculation of soluble catechol-protein polymeric products in solid aggregates was observed when prion proteins were added to the supernatants.

Different kinds of extracting agents were not able to desorb/extract the prion proteins from the formed solid aggregates, thereby highlighting the high stability of protein-organic and protein-organo-mineral complexes.

Further FTIR investigations addressed to the immobilized prion protein allowed to study its interaction with organic coated MnO_2 surfaces, and obtain useful information on conformational changes correlated to the organic surface coatings.

3.1. Introduction

As previously reported and widely discussed a growing concern is dedicated to study prion protein interaction with soil or its components (Revault et al., 2005; Vasina et al., 2005; Johnson et al., 2006; Georgsson et al., 2006; Leita et al., 2006; Rigou et al., 2006, Schramm et al., 2006; Cooke et al., 2007; Cooke and Shaw, 2007; Johnson et al, 2007; Genovesi et al. 2007; Ma et al., 2007; Seidel et al., 2007).

Indeed, soil considered as a natural sink of several contaminants, including prion proteins may play an important role in the retention as well as the dissemination of such infectious agents into the environment. Therefore, understanding the retention and/or the dissemination of prion proteins in soils may be important for dealing with the dissemination of TSE infectivity. Brown and Gadjusek (1991) demonstrated that infectious particles conserved its infectivity after 3 years internment in soil. More recent studies have also experimentally proved that scrapie prions can persist in an active form in soil for years (Seidel et al., 2007), and that infected soil is able to transmit the disease to healthy animals even after 16 years from the contamination event (Georgsson et al. 2006).

Once introduced into the soil a protein may remain free in solution, and as such to be biologically or chemically degraded, be associated with preformed organic and inorganic soil components by adsorption, or participate in the formation of organic and organo-mineral soil complexes. Relatively stable protein-soil colloid complexes may be formed. A strong retention and accumulation of proteins on the formed complexes as well as their release can occur under some specific environmental conditions.

As demonstrated by several studies (Ladd and Butler, 1975; Burns, 1986; Boyd and Mortland, 1990; Quiquampoix, 2000; Gianfreda et al., 2002; Revault et al., 2005) profound structural alterations and conformational changes of protein molecules associated to soil colloids may take place with variations of the protein functionality. Structural molecular alterations may also result upon protein release from the soil aggregates under favourable environmental conditions.

Electrostatic and hydrophobic interactions of proteins with solid surfaces induce structural changes in the adsorbed proteins (Norde, 2000). Adsorption on clay is mainly driven by electrostatic interactions (Quiquampoix et al., 2002). Proteins having a high flexibility can be strongly retained on electronegative surfaces and undergo a large conformational rearrangement giving rise to newly formed intermolecular β-sheet and protein association and aggregation (Baron et al., 1999; Servagent-Noinville et al., 2000; Lecomte et al. 2001).

As a consequence, the interaction of prion proteins, either the PrP^{Sc} or PrP^{C}, with soil organic and organo-mineral complexes or their release from these latter may become an important step in the risk of prion infectivity dissemination in soil.

Little attention has been paid to the role of soil organo-mineral complexes in the interaction with prion protein. In soil, organo-mineral matrices are expected to be more common than isolated humic or clay components. Organic matter association with mineral constituents can contribute to the adsorption/entrapment of molecules and macromolecules entering the soil.

Several researches have determined that inorganic components, such as manganese and iron oxides and idroxides, react with organic substances and, similarly to enzymes, are responsible of humic substances formation (Shindo and Huang, 1982, 1984; McBride, 1987, 1989; Huang, 1991).

Polymerization of simple organic monomeric precursors occurring in the presence of oxidative enzymes or manganese and iron oxides is considered one of the most important processes contributing to the formation of humic substances in soil (Bollag et al., 1995). The process is very fast and produces a population of polymeric products of different molecular structures, sizes, shapes and complexity (Huang, 1995). Further polymerization, self-assemblage of different polymeric products, and/or association with mineral constituents quickly lead to the formation of more complex organic and organo-mineral soil colloids (Huang, 1995).

Several are the manganese oxides presents in soils, most of them are poorly crystalline and have high surface area. Oxidation of organic substances takes place on the solid surface of these minerals. In the case of phenols the first step is the interaction of hydroxylic or carboxylic groups of these molecules with the mineral surface, then the phenol transfers electrons to the metal oxide surface and then there is a subsequent release of a quinone or semiquinone and soluble reduced manganese (Mn^{2+}). Quinone and semiquinone can further react and proceed toward the complete mineralization or be involved in the oxidative coupling processes leading to the polymer formation. The O_2 in solution is utilized in the process (McBride, 1989).

Among manganese oxides birnessite (δ-MnO_2) is a naturally occurring, poorly crystalline manganese oxide, abundantly present in soil, and promotes the oxidative transformation of phenols (Bollag, et al., 1995; Huang, 1995; Rao et al., 1999).

The concentration of birnessite in soil controls the transformation rate of hydroxylated aromatics transformation and the nature of reaction products. In soil low birnessite concentrations can determine minimal phenol-phenol coupling while the coupling with soil organic matter would be significant.

At increasing birnessite concentrations, the formation of polymers becomes predominant and the coupling with soluble organic matter decreases, finally high birnessite concentration leads to larger polymers that become strongly associated with birnessite surface or other natural occurring sorbents (Weber et al., 2000). In this view, if active proteins are involved in the process of polymerization of phenol or phenol like compounds, protein-organic and protein-organo-mineral complexes with different structural and functional properties may form.

Studies on soil enzymes have proved that enzymes and proteins in soil are associated with clay and humic matrix being adsorbed or entrapped. Actually, only humic-enzyme complexes have been extracted from soil and characterized (Nannipieri, 2006).

Usually, the interaction of proteins with the prevalently hydrophobic surfaces of organic matter might cause structural conformational changes in the immobilized protein. In the case of the prion protein, such conformational changes may result in the transformation from the non-pathogenic to the pathogenic form of the protein. To our knowledge, no information is available on the interaction of prion proteins with organic and organo-mineral soil components.

In this study we focused our attention on the polymerization of catechol promoted by birnessite in the presence or absence of a recombinant ovine protein, structurally well characterized (Rezaei et al., 2000; Eghiaian et al., 2004), and leading to organo-mineral complexes that resemble soil organo-mineral colloids. The recombinant protein is used as a model of the natural cellular prion protein PrP^C (Leclerc et al., 2001; Somerville, 2002; Legname et al., 2004). Catechol, a very common humus precursor, is frequently present in soil, and may be involved in polymerization processes

by biotic and abiotic catalysis (Huang, 1995; Naidja et al., 1998, Matocha et al., 2001).

The properties of the deriving complexes, the allocation and the stability of the protein in/on the complexes as well as its potential release from the formed complexes and its possible structural alteration were investigated. In order to evaluate the role of the N-terminal part of the protein in the interaction with catechol and birnessite, comparative studies were also performed with the C-terminal part of the protein, i.e., missing the N-terminal fragment.

In this study the entity of recombinant prion protein conformational changes toward β sheet-like structures found in the pathogenic (PrP^{Sc}), as a result of an interaction with a birnessite and birnessite surfaces catechol-coated, was also investigated.

3.2. Material and Methods
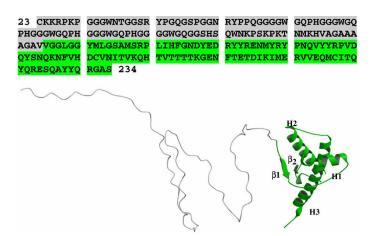
3.2.1. Chemicals

Reagent grade catechol (Cat) (>99.0% purity) and High Performance Liquid Chromatography (HPLC)-grade solvents were purchased from Sigma Aldrich (Germany).

Sarkosyl (Sodium Lauroyl Sarcosinate) was from Fluka (Germany). All other chemicals, reagent grade, were supplied by Analar, BDH Ltd, (Germany), unless otherwise stated.

Birnessite (δ-MnO$_2$) (Bir) was synthesized with KMnO$_4$ and HCl according to McKenzie (1989).

A purified full-length recombinant ovine ARQ genetic variant (mM 23 kDa, residues 23-234) prion protein, for simplicity named PrP in this, and its C-terminal fragment (15 kDa, residues 103-234**)**, indicated as tPrP, were prepared according to Rezaei et al. (2000) and Eghiaian et al. (2004), respectively (Figure 3.1). Both were kindly furnished by Dr. Jeanne Grosclaude, from the Virologie et Immunologie Moléculaires, INRA (Jouy-en-Josas, France). The full-length protein is formed by a well-folded C-terminal domain (residues 125-234) and a rather flexible N-terminal arm (residues 23-124) (Eghiaian et al., 2004). It has an isoelectric point of 9.2, a molar extinction coefficient of 57930 M^{-1} cm^{-1} at 280 nm, and its structural stability is preserved in the pH range from 4.6 to 7.2 (Rezaei et al., 2000). Above and below these pH values the protein may partly loose its α helix conformation to achieve a β sheet character (Rezaei et al., 2000). The C-terminal fragment used in this study has a theoretical pI of 8.84 and a molar extinction coefficient of 18005 M^{-1} cm^{-1} at 280 nm (Eghiaian et al., 2004).

Figure 3.1. Ribbon representation of ovine PrP structure. PrP C-terminal domain (green) contains three α-helices (H1, H2, H3) and two β strands (β₁ β₂). The flexible PrP N-terminal arm (gray) was modelled as containing

mainly polyproline II (Blanch et al., 2004). The inset shows the sequence of the PrP form under investigation.

3.2.2. Circular dicroism (CD) spectroscopy measurements

The behavior of PrP in all buffers and components was checked by CD measurements and only those which preserved PrP stability and solubility were successively used.

CD measurements were carried out in the far-UV (250-190 nm) using a Jasco J-810 spectropolarimeter equipped with a Peltier type temperature control system (Model PTC-423-S). CD spectra were recorded with a 0.1 cm optical path length of the cell, a time constant of 4 s, a 2 nm bandwidth, and a scan rate of 5 nm min^{-1}. All spectra were signal-averaged over at least three scans and the baseline corrected by subtracting the buffer spectrum. Concentration of PrP used for CD experiments was in the range 0.2-0.5 mg mL^{-1}.

CD spectra were recorded on PrP-MnCl$_2$ mixtures using 0.25 mg mL^{-1} PrP in 10 mM acetate buffer at pH 5.5 and containing MnCl$_2$ amounts corresponding to Mn^{2+}: PrP molar ratios equal to 10, 40, 100 and 1000, very far exceeding those possibly occurring in the polymerization conditions. The solutions were incubated for 18 h at 20 °C prior CD measurements.

Thermal unfolding curves were measured in the temperature mode at 222 nm in the range 20-80 °C using a scanning rate of 1 °C min^{-1}. To control the reversibility of the unfolding process, PrP refolding was followed using the same scanning rate, and the conservativeness of the melting temperature was checked.

In order to identify experimental conditions suitable for extraction experiments, the behaviour of PrP in solutions of Cu^{2+} was studied by recording CD spectra at various incubation times (10 min, 30 min, 18 h, 30 h) in 10 mM phosphate buffer at pH 7.5. This pH was chosen to keep His residues of the protein N-terminal arm, devoted to Cu^{2+} binding, in their uncharged state. Solutions were prepared using a PrP:CuCl$_2$ ratio of 1:4, 1:10, 1:30, and 1:100. Best conditions, which did not produce protein precipitation, were used for the extraction experiments (see above). Similarly, the stability of PrP in pyrophosphate solutions was measured by recording CD spectra after incubation times of 10 min, 30 min, 2 h, and 5 h.

3.2.3. PrP conformational studies by FT-IR

Catechol and birnessite, used for FT-IR conformational studies of adsorbed recombinant prion protein, were treated in D_2O to obtain H/D exchange. One gram of birnessite was suspended in 5 mL of D_2O and then freeze dried, this procedure was repeated 2 more times. The same procedure used for birnessite was also used for catechol, where the amounts used for catechol and D_2O were 140 mg and 2 mL, respectively. The buffer used for H/D exchange of protein, and in all FT-IR conformational studies was MOPS (3-[N-morpholino] propanesulfonic acid) 20 mM in D_2O, adjusted at 5.8 pD by addition of DCl or NaOD.

In the case of Bir-PrP sample PrP was 4 mg mL^{-1}, Bir was 160 mg mL^{-1}, while in Cat-Bir-PrP and Cat-Bir+PrP samples PrP was 4 mg mL^{-1}, Bir was 40 mg mL^{-1} and Cat was 40 mM. In any cases Cat/Bir ratio was selected to obtain a complete catechol removal; in addition the ratio of PrP and Bir or

Bir loaded by catechol and catechol products, was selected in order to obtain a complete protein adsorption.

FTIR-spectra were recorded on a Bruker Equinox spectrometer equipped with a MCT detector. Resolution was set at 4 cm^{-1} using a boxcar apodization. The spectrometer was continuously purged with dry air, the sample was maintained at 25 °C.

The deuterated PrP solution was placed in a CaF$_2$ cell with a 50 µm spacer. The first FTIR absorbance spectrum was obtained at 8 min of incubation in the deuterated medium.

Absorbance spectra of PrP sample with Bir and Cat suspensions were obtained using a CaF$_2$ cell with a 25 µm spacer.

Difference spectra were obtained by subtracting the reference spectra of D$_2$O or D$_2$O-clay from spectra of PrP in solution or of PrP-clay-suspension recorded at similar pD and time before spectral analysis of Amide I bands. The broad Amide I band in the spectral range 1600-1700 cm^{-1} was attributed to the peptide carbonyl stretching vibrational modes and revealed the extent of secondary structure of the protein.

Suitable spectra decomposition for the analysis of the Amide I band was used for both PrP in standard buffer condition and in PrP adsorption/entrapment experiments. This decomposition reported by Revault et al. (2005) is also reported in Table 3.1. Each identified Amide I' component band is correlated to the amount of peptide units involved in specific ordered structures (α-helix or β-sheet) of the protein.

Conformational studies as reported in this Paragraph were carried out CNRS-Université PARIS VI, Laboratoire Dynamique Interaction et Réactivités, Groupe "Systèmes d'intérêt biologique" Thiais, France under the supervision of Dr. Silvie Noinville.

Table 3.1. Assignments of absorption infrared component-bands in the
Amide I' band PrP in deuterated buffer

Wavenumber (cm^{-1})	Assignments
1682	"Free" CO in hydrophobic environment
1671	"Free" CO in polar environment
1661	"Free" CO in polar environment
1650	H-bonded CO in α-helices
1641	Hydrated CO
1630	Intramolecular H-bonded CO in bent or β-sheets
1614	Intermolecular H-bonded CO in β-sheets or in protein self-association

3.2.4. Formation of catechol-birnessite-protein complexes

Catechol and birnessite mixtures were prepared by incubating in Erlenmeyer tubes 1 mL of 0.1 M sodium acetate buffer pH 5.5, increasing amounts of birnessite (1-5 mg mL^{-1}) and increasing concentrations of catechol (1-5 mM) for 1, 2 and 24 h at 25 °C. After incubation, the mixtures were centrifuged for 30 min at 10000 g and at 4 °C, and residual catechol and soluble manganese concentrations in the supernatants were determined. Final pH of the mixtures was measured as well. Appropriate controls with either acetate buffer, Cat or Bir only were also carried out.

The interaction of either PrP or tPrP with catechol and birnessite was investigated using 3 and 5 mM catechol, 0.5 mg mL^{-1} protein (by adding suitable amounts of a freshly prepared protein solution whose concentration was determined by absorbance at 280 nm) and 5 mg mL^{-1} birnessite in 1 mL of 0.1 M sodium acetate buffer pH 5.5. Catechol, birnessite and PrP or

tPrP were incubated in different trials. Two ternary systems catechol-birnessite-protein were produced: Cat-Bir-PrP or Cat-Bir-tPrP where the three components were mixed at the same time, and Cat-Bir+PrP or Cat-Bir+tPrP where Cat and Bir were incubated for 2 h before protein addition. Three binary systems were also produced: Cat-Bir, Cat-PrP or Cat-tPrP and Bir-PrP or Bir-tPrP. Samples with only Cat, PrP, tPrP or Bir served as controls.

All mixtures were incubated for a total of 4 h at 25 °C. At the end of incubation samples were centrifuged (30 min at 10000 g and 4 °C) and the supernatants were analyzed for residual catechol, soluble Mn concentrations, final pH and UV-Vis spectra. The presence of PrP or tPrP in the supernatants was revealed by SDS-PAGE electrophoresis and their amounts were measured by HPLC analysis. Indeed, the presence of catechol and its polymeric products, absorbing at 280 nm, prevented the determination of protein concentration from direct UV measurements. Occasionally, a colorimetric method, the Protein Assay ESL (Roche, Germany), was used (detection limit 20 μg of protein). No interference under all the experimental conditions was observed, thus allowing the concentration of PrP or tPrP to be determined in the presence of catechol or catechol polymers.

The precipitates were washed twice with 0.02 M NaCl and then with bi-distilled water until acetate free, lyophilized and stored at 4 °C for further analyses as reported below.

Experiments of catechol polymerization were also performed by adding catechol to birnessite that had previously reacted with PrP. In 1 mL of 0.1 M sodium acetate pH 5.5, 5 mg of birnessite and 0.5 mg of PrP were left to interact for 2 h. Two test tubes were supplemented with 0.1 mL of 0.05 M catechol and left to interact for other 2 h. Controls without catechol were

produced to determine the amount of PrP in the supernatants. At the end of incubation, the mixtures were processed as previously described.

3.2.5. Experiments with soluble catechol polymers

Experiments were performed adding 0.5 mg mL^{-1} (final concentration) PrP or tPrP to soluble catechol polymers obtained in the supernatant of the two binary systems Cat-Bir (3 and 5 mM catechol). After 2 h incubation time at 25 °C some aliquots were analyzed by UV-Vis and CD spectroscopy, the remaining parts were centrifuged for 30 min at 10000 g and at 4 °C. After removal of precipitates the amounts of PrP or tPrP were quantified by HPLC and electrophoresis analyses.

3.2.6. Desorption/extraction tests

Sequential extraction tests were carried out by suspending suitable amounts of the solid phases (usually 5 mg) obtained from centrifugation of the ternary mixtures with PrP or tPrP in 1 mL of 0.1 M phosphate buffer at pH increasing from 7.0 to 8.5. The buffered suspensions were kept under mixing at 25 °C for 1 h. Further extraction experiments were performed at 25 °C using for 1 h in a shaker either 0.14 M pyrophosphate at pH 7.0 or 0.7 mM $CuCl_2$ solution in 0.1 M phosphate buffer pH 7.5. Sequential extractions using a water solution containing 10% (v/v) propanol and incubation under magnet stirring for 24 h and further 72 h as well as treatments for 1 h with an extraction solution composed by 1% Sarkosyl in 0.1 M phosphate buffer pH 7.5 were also carried out.

After the treatment, all the samples were centrifuged for 30 min at 10000 *g* and 4 °C and the presence of PrP or tPrP released in the supernatants was determined by protein determination assays, HPLC and electrophoresis analyses.

When measurable amounts of PrP or tPrP were detected in the extracts, CD measurements were performed to evaluate, if any, the variation of the protein conformational structures.

3.2.7. High performance liquid chromatography analyses

Catechol, PrP and tPrP concentrations were determined by HPLC analysis with a Shimadzu instrument equipped with a variable-wavelength absorbance detector set at 280, 222 and 227 nm, respectively. A Spheri-5-RP18 22 cm by 4.6 mM C18-80 BrownLee (Chebios) column with a 5 μm particle size and a BrownLee Spheri-5-RP300 7 μm particle size (4.6 x 30 mM) guard column for catechol, a Biosep Sec-S2000 300 x 7.80 mM Phenomenex column and Biosep Sec-S2000 75 x 7.80 mM Phenomenex guard column for both the proteins were used. Catechol analysis was performed in isocratic elution at a flow rate of 1 mL min^{-1} with a mobile phase composed of acidified water (3 mL of 95% H_3PO_4 in 1 L of water, at pH 2.95 with 0.5 M NaOH, low in carbonate) and acetonitrile at a ratio of 70:30 (v:v). Full-length PrP and tPrP were eluted at a flow rate of 1 mL min^{-1} with 6 M guanidine hydrochloride and 20 mM phosphate buffer pH 7.5, respectively. Before injection, all samples were filtered with Acrodisck LC 13 PVDF 0.45 μm filters. Retention time for catechol, PrP and tPrP were 3.5, 6.5, and 9.8 min, respectively.

3.2.8. Atomic absorption spectroscopic and elemental analyses

Atomic absorption spectroscopic measurements of soluble Mn were performed at 279.5 nm with a Perkin-Elmer Analyst 700 by using Flam Adsorption Spectroscopy. Before each investigation standard solutions (0.5, 1.0 and 2.0 mg L^{-1}) were freshly prepared by diluting the $MnCl_2 \cdot 4 \; h_2O$ standard solution in HCl. The detection limit for Mn^{2+} determination was 0.0082 mg L^{-1}.

Carbon and nitrogen contents of lyophilized insoluble products (see above) were measured by the ash combustion procedure with a Fisons 1108 Elemental Analyzer. Calibration of the Fisons instrument with appropriate standard (acetanilide) was carried out. Accuracy (<0.05%) and recovery of C and N (for both instrument detection limit 10 mg kg^{-1}) were checked analyzing a sample of the standard material after each set of eight sample analyses.

3.2.9. UV-Visible and FT-IR analyses

The UV-Visible spectra of products remaining in the supernatants of the catechol-birnessite and catechol-birnessite-PrP or -tPrP mixtures were obtained with scanning from 900 to 200 nm using a Perkin Elmer spectrophotometer Lambda 25 instrument.

The Fourier Transform Infrared spectra of insoluble products were recorded by the Universal Attenuated Total Reflectance (UATR) method using a Perkin Elmer FT-IR spectrometer. Each spectrum represents a collection of 16 scans recorded at a 4 cm^{-1} resolution. Before FT-IR

spectrum analyses the insoluble samples were washed twice with NaCl 0.02 M to avoid acetate buffer signals.

3.2.10. Electrophoresis analysis

Denaturing SDS-PAGE electrophoresis with 15% or a gradient of 10-20% (w/v) acrylamide was performed using a Bio-Rad apparatus and the LaemMLi discontinuous system. Gels were stained with Coomassie brilliant blue R250.
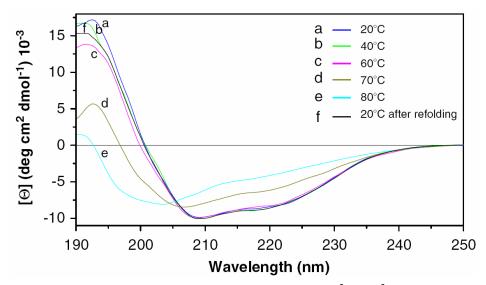
3.3. Results

3.3.1. Catechol-birnessite-protein interactions

3.3.1.1. Preliminary studies by CD spectroscopy

Preliminary experiments were carried out to establish proper experimental conditions, which did not affect protein stability and/or did not produce aggregation effects. A particular care was dedicated to the analysis of PrP behaviour in the presence of Mn^{2+}, which is produced in detectable amounts in the polymerization reaction. Indeed, PrP is known to have a strong tendency to form aggregates in the presence of metal ions such as Cu^{2+} and Mn^{2+} (Giese et al., 2004). Therefore, this preparative task was aimed at avoiding PrP aggregation under the experimental conditions used. No aggregation was induced by Mn^{2+} at any of the Mn^{2+}: PrP ratios, as described in Material and Methods. Indeed, CD spectra of Mn^{2+}: PrP

solutions after 18 h incubation were completely superposable to those of PrP obtained in the absence of Mn^{2+} (data not shown). Furthermore, the presence of Mn^{2+} did not affect PrP stability, since its melting temperature, as measured by thermal denaturation, remained unaltered (Tm=79 at pH 5.5). It is worth noting that thermal denaturation leads, under these experimental conditions, to a random coil state and not a β structure. Indeed the CD spectrum recorded at 80 °C shows a clear minimum close to 200 nm, but not at 214 nm (Rath et al., 2005). Notably, also in the presence of Mn^{2+} the denaturation process was fully reversible (Figure 3.2).

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Figure 3.2. CD spectra of PrP in the presence of Mn^{2+} (Mn^{2+}:PrP ratio = 100). Spectra recorded in the temperature range 20 - 80 °C and at 20 °C after refolding.

Further CD measurements were performed to evaluate possible effects by the chemicals used in the extraction/desorption studies reported below.

While no effects on PrP stability were observed with phosphate, a clear influence by pyrophosphate on PrP aggregation was noted.

Indeed, CD spectra performed on PrP before and after incubation for 18 and 24 h with pyrophosphate showed that it induced protein aggregation, as the minimum at 208 nm (typical of α-helix) became less deep after 18 and 24 h incubation time (data not shown).

At this time a slight opalescence was also observed and, consistently, the CD signal decreased. This roughly corresponded to a protein loss of 20%. Interference on CD analysis was observed when catechol polymers alone (supernatants of Cat-Bir mixtures) were mixed with pyrophosphate, probably because of the release of polymeric materials by pyrophosphate (data not shown). No interference occurred with pyrophosphate or Cat-Bir alone.

3.3.1.2. Formation of catechol-birnessite-protein complexes

Preliminary experiments were performed with only catechol and birnessite to select the most appropriate conditions to utilize in the formation of catechol-birnessite-protein complexes. These conditions should simulate two possible situations encountered by a protein in soil: protein molecules interacting with soluble organic matter in the first phases of its formation or with and soluble polymers which already underwent partial or strong condensation.

Investigations performed by increasing catechol concentrations from 1 to 5 mM and birnessite amounts from 0.2 to 5 mg mL^{-1} under buffered conditions (0.1 M Na acetate, pH 5.5) showed that after 2 h incubation catechol removal increased from 20% (at 1 mM catechol and 0.2 mg mL^{-1}

birnessite) up to 100% at the highest catechol and birnessite amounts. A corresponding release of soluble Mn^{2+} occurred; after 2 h incubation of 5 mM catechol and 5 mg mL^{-1} birnessite, the concentration of Mn^{2+} raised up to 0.131 mg mL^{-1}. Neither catechol removal nor Mn^{2+} release occurred in the controls lacking birnessite or catechol, respectively. Moreover, under buffered conditions at pH 5.5, the pH of the mixtures was stable (a maximum increase of 0.3 pH units was detected) and the protein was not subjected to unfolding phenomena at room temperature.

Soluble polymers were obtained at 3 mM catechol, whereas at 5 mM catechol insoluble polymers prevailed, as demonstrated by several experimental evidences. Indeed, the absorbance in the visible region 900-350 nm of the supernatant of 3 mM catechol-birnessite mixtures was much higher than supernatants of 5 mM catechol mixtures. For instance, at 400 nm the absorbance value of 3 mM catechol supernatants was 1.15 against 0.65 measured at the same wavelength for 5 mM catechol samples, where insoluble materials simultaneously precipitated at the bottom of the incubation tubes, with a corresponding, visible at naked eye clarification of the supernatants.

The formation of insoluble catechol polymers at 5 mM catechol was supported by elemental analyses. Although the oxidative coupling reaction with catechol caused the dissolution of 0.2 mg birnessite (corresponding to 0.116 mg of Mn^{2+} determined in solution by AAS), the weight of the precipitate did not change when compared to the initial MnO_2 amount, since new insoluble organic materials enriched it. Detectable amounts of carbon (C, deriving exclusively from catechol, because no C can be released by birnessite, and the solid samples were acetate-free) were measured by elemental analysis, thus confirming the formation of insoluble catechol polymers adsorbed on the surfaces of birnessite. For instance,

0.131 mg of carbon corresponding to about 36% of the initial catechol quantity was found in the solid phase of Cat5-Bir samples.

FT-IR spectra of precipitates also supported the above results (data not shown). As reported by Russo et al. (2005), the higher the catechol/birnessite ratio (reducent/oxidant ratio), the lower the intensity of the peak at 512 cm^{-1}, characteristic of birnessite, and the higher the intensities in the range 1620-1000 cm^{-1}, thus confirming the corresponding consumption of birnessite and the appearance of more complex products.

On the basis of these results, the interaction of PrP or tPrP with birnessite and catechol was studied in 0.1 M acetate buffer at pH 5.5, using 5 mg mL^{-1} Bir, 0.5 mg mL^{-1} PrP or tPrP and 3 and 5 mM catechol in four independent experiments.

Full-length PrP or tPrP molecules neither affected the extent of catechol removal (100% in all samples whether present or not the two proteins) nor the corresponding release of soluble Mn^{2+} (in larger amounts with 5mM catechol) (Table 3.2). A simultaneous, complete removal of the proteins from the mixture at the two catechol concentrations was measured both when they were incubated at the beginning with catechol and when added after catechol polymerization (Table 3.2). Neither removal of catechol and PrP or tPrP, nor release of soluble Mn^{2+} occurred in the relative controls Cat, PrP, tPrP or Bir. Whereas, when PrP or tPrP interacted with only birnessite in the absence of catechol (Bir-PrP and Bir-tPrP samples), 65 and 55% of proteins were removed from the solution and adsorbed on birnessite surfaces, respectively (Table 3.2). All of these results were supported by electrophoresis and HPLC analyses performed on the relative supernatants of all samples. Neither protein bands nor protein amounts were detected in any of the supernatants, except for those of Bir-PrP and Bir-tPrP samples.

Notably, catechol polymerization still occurred with birnessite loading PrP molecules previously adsorbed on its surfaces. When 5 mM catechol was added to birnessite coated by PrP (65% of adsorption) on its surfaces, the complete catechol removal was observed, as when protein-free birnessite was used.

Table 3.2. Protein removal and Mn^{2+} released after catechol polymerization

Samples	Protein removal (%)		Mn^{2+} (mg mL^{-1})	
	PrP	tPrP	PrP	tPrP
Cat3-Bir-protein	100±0	100±0	0.064±0.003	0.063±0.002
Cat5-Bir-protein	100±0	100±0	0.132±0.005	0,130±0.004
Cat3-Bir+protein	100±0	100±0	0.069±0.003	0,067±0.003
Cat5-Bir+protein	100±0	100±0	0.126±0.006	0,128±0.005
Cat3-Bir	-	-	0.061±0.003	0.061±0.003
Cat5-Bir	-	-	0.116±0.005	0.116±0.005
Bir-protein	65±2	55±1	0.003±0.001	0.003±0.001
Protein	0±0	0±0	nd	nd
Bir-protein+Cat5	65±2	nd	0.132±0.005	nd

Cat3 = 3 mM catechol; Cat5 = 5 mM catechol; nd = not determined.

3.3.1.3. Features of catechol-birnessite-protein complexes

The presence of PrP strongly affected the patterns and the UV-Vis absorbance values of the supernatants of both 3 and 5 mM catechol mixtures and the formation of insoluble catechol-birnessite-PrP aggregates. The absorbance of supernatants of Cat3-Bir-PrP in the visible region was still higher than that of Cat5-Bir-PrP catechol samples, thus confirming the

presence of a greater amount of soluble polymers at lower catechol concentration (Figure 3.3). When PrP was added after catechol polymerization (Cat3-Bir+PrP, and Cat5-Bir+PrP samples), the absorbance of the supernatants markedly diminished in the whole UV-Visible interval, with a decrease much more conspicuous in the visible region (Figure 3.3), and an evident and rapid formation of brown insoluble material, visible at naked eye, accumulated spontaneously at the bottom of the incubation flasks. Similar UV-Vis spectra were obtained with tPrP (data not shown).
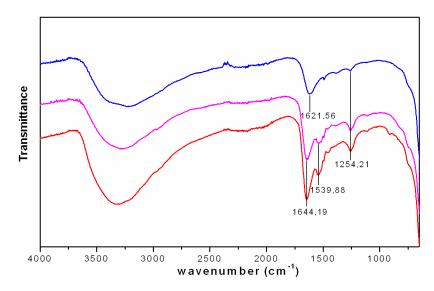
Figure 3.3. UV-Vis spectra of catechol-birnessite-PrP supernatants after 4 hours incubation. Spectra of Cat3-Bir and Cat5-Bir are also reported (see insets).

The presence of PrP in the precipitates with birnessite was also confirmed by the elemental analyses. Indeed, an increase of C content and the appearance of detectable amounts of N were measured, compared to the corresponding samples without protein. For instance, in the samples obtained with 5 mM catechol, the C content increased from 0.131 up to 0.398 mg mL^{-1} and 0.060 mg m^{-1} of N were detected. While the measured

values of C did not allow to revealing the presence of the protein in the solid phase, the detection of N (deriving exclusively from proteic material) demonstrated that PrP was present in it.

FT-IR measurements of precipitates were performed. They supported the hypothesis that the majority of the reaction products of catechol polymerization, with and without PrP, were apparently adsorbed on birnessite surfaces, and thus could not be detected in the supernatants. Figure 3.4 shows the FT-IR spectra of Cat5-Bir, Cat5-Bir-PrP and Cat5-Bir+PrP solid aggregates. The two signals at 1622 cm^{-1} (aromatic C=C, H-bonded C=O or alkenes in conjugation with C=O) and 1254 cm^{-1} (C-O stretching and aromatic C=C) in the Cat5-Bir spectrum are characteristic spectroscopic features of humic-like aromatic compounds. As respect to the Cat5-Bir sample, any modification observed in both Cat5-Bir-PrP and Cat5-Bir+PrP spectra must account for the amide I (C=O stretching at 1644 cm^{-1}) and amide II (N-H bending at 1520 cm^{-1}) bands of the protein. Moreover, the reinforcement of the band at 1254 cm^{-1} (weak in the Cat5-Bir sample) could be attributed to the presence in the same region of the bending (amide III) signal of the protein. Similar indications were provided by the spectra of the catechol-birnessite-PrP complexes obtained at 3 mM catechol (data not shown).

Figure 3.4. Fourier transformed infrared spectra of Cat5-Bir, Cat5-Bir-PrP and Cat5-Bir+PrP solid aggregates.

Conformational studies were based on the decomposition of amide I signal. Standard spectra were collected by using 4 mg mL^{-1} PrP in MOPS deuterated buffer (pD 5.8). Decomposition of this standard spectra provided the percentage contributions of component-bands of amide I. Grouped components can give indications on the total α-helix, β-structure and unstructured domains in the protein (Table 3.3).

Table 3.3. Grouped decomposed component-bands and their contribution in amide I.

cm^{-1}	Assignments	Contribution %
1614; 1617	Intermolecular β-sheet	6.5
1630	β-sheet	17.5
1650	α-helix	31.5
1641; 1661; 1671; 1682	Random domains	45.5

Using these assignments and contribution as a reference it was possible to rule out the conformational changes occurring when the protein interacted with birnessite surface coated or not by catechol.

In the sample Bir-PrP, the overall loss of α-helix character was 5.2% while the increase of β-sheet was 3.6 (Figure 3.5). No substantial differences in sample Cat-Bir-PrP, in which catechol was also present, were observed as respect to the standard.
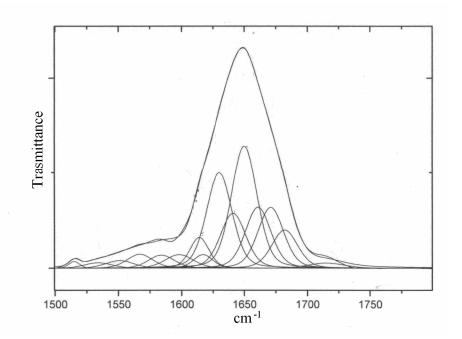
Figure 3.5. Decomposed amide I in Bir-PrP.

Different results were obtained with Cat-Bir+PrP, where PrP was added to birnessite partially coated by catechol polymers. The loss of α-helix character reached 7.7% and the increase of β-sheet reached 8.9%. Signals attributed to intermolecular β-sheet, that can be related to β-aggregation,

contributed for only 3.6% of the β-sheet character of adsorbed PrP (Figure 3.6). When incubation of Cat-Bir+PrP lasted overnight conformational changes of PrP did not substantially increased.

In order to distinguish the contribution of the soluble catechol polymers (i.e., in the absence of the mineral part loading or not organic material on its surfaces) to the interaction with either PrP or tPrP, the proteins were added to the supernatants of Cat3-Bir and Cat5-Bir mixtures when after 2 h incubation precipitates were discarded. Protein-catechol insoluble-products formed.
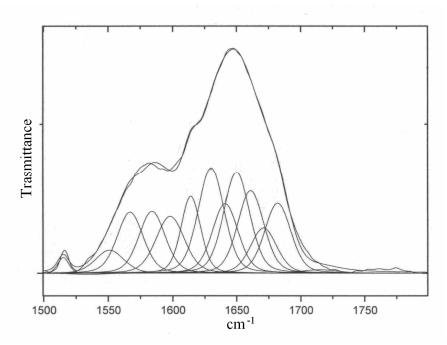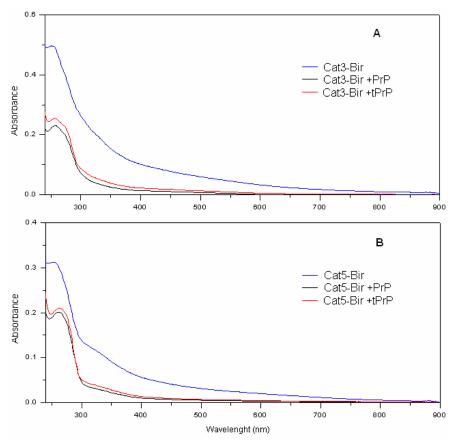
Figure 3.6. Decomposed amide I in Cat-Bir+PrP.
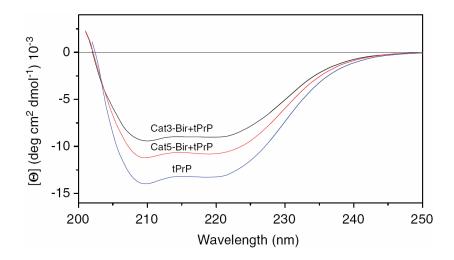
3.3.2. Interaction of PrP and tPrP with catechol polymers in the absence of birnessite

A rapid, immediate flocculation of insoluble, brown material possibly including the protein occurred with a concurrent clarification of the solution. This decolouration was confirmed by a progressive decrease of the UV-Visible signal in the wavelength range 900-200 nm (Figure 3.7A and B).

Figure 3.7. UV-Vis spectra of supernatant of Cat3-Bir (A) and Cat5-Bir (B) after 2 hours incubation with PrP and tPrP.
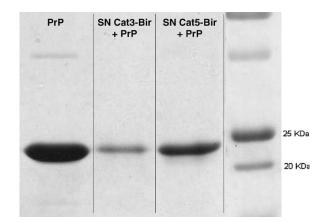
CD spectra, recorded after solution preparation and followed for a few hours, also showed an almost immediate and progressive decrease of the CD signal in the whole wavelength range (Figure 3.8).

Figure 3.8. CD spectra of supernatants of Cat3-Bir and Cat5-Bir mixture after 2 h incubation with 0.5 mg mL^{-1} tPrP. The spectrum of t PrP in 10 mM acetate buffer, pH 5.5, is also reported as a reference. For clarity, only samples containing tPrP are shown.

In the case of PrP, a removal of 92 and 74% was detected after 2 h contact time with Cat3-Bir and Cat5-Bir supernatants, respectively, by HPLC and confirmed by SDS-PAGE electrophoresis (Figure 3.9).
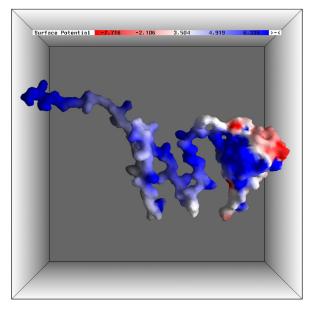
Figure 3.9. Detection of PrP stained by blue Coomassie in SDS-PAGE of supernatants of Cat3-Bir and Cat5-Bir after addition of 0.5 mg mL^{-1} PrP.

The greater protein removal in Cat3-Bir supernatants is likely due to the higher amounts of soluble polymers in these samples which can interact with protein molecules (Figure 3.3).

A significantly reduced removal of tPrP was observed for tPrP by HPLC (15% with Cat5-Bir and 33% with Cat3-Bir), consistent with CD spectra (Figure 3.8) and SDS-PAGE electrophoresis (data not shown). A less intense flocculation of insoluble material was also observed.

After 8 h incubation, electrophoresis and HPLC analyses on aliquots of the sample supernatants showed that both the proteins completely disappeared from the reaction mixtures, thereby indicating an immediate and progressive interaction of the proteins with soluble catechol polymers to form insoluble aggregates which accumulated in the precipitate.

These results indicate that (i) in the presence of soluble catechol polymers, the removal of both PrP and tPrP is significantly slower than in the corresponding heterogeneous systems including birnessite and (ii) the lack of the N-terminal fragment (residues 23-93) in tPrP significantly slows

down the process of protein-catechol polymer interaction. This is consistent with the presence in the protein N-terminus of positively charged residues, which are potential interacting moieties (Figure 3.10).

Figure 3.10. Electrostatic potential surface of PrP as calculated using GRASP (Nicholls et al., 1992).

3.3.3. Extraction/desorption tests

Different experimental conditions were tested to desorb and/or to extract PrP or tPrP from the insoluble complexes obtained.

The pellets obtained after centrifugation of each sample were subjected to sequential extraction tests with 0.1 M phosphate at pH 7.0, 8.0 and 8.5. No detectable removal of PrP or tPrP from all the samples was observed after 1 h of contact, as assessed by protein determination by HPLC and SDS-

PAGE electrophoresis. A positive response to electrophoresis analysis was observed only with the Bir-PrP sample.

With 0.14 M pyrophosphate pH 7.0, the Protein Assay ESL identified detectable amounts of extracted PrP (30, 18 and 15% for Cat3-Bir-PrP, Cat3-Bir+PrP and Cat5-Bir-PrP, respectively). However, these results were not confirmed by electrophoresis analyses (no detection of protein bands) indicating a false positive response likely due to the capability of pyrophosphate to release polymeric material from the formed catechol-protein complexes. Pyrophosphate was proven to be very effective in the extraction of proteins from soil and/or soil organic complexes (Tabatabai and Fu, 1992; Nannipieri et al., 1996).

PrP extraction tests were also performed using $CuCl_2$ or propanol solution by incubating for 1 h in a shaker or for longer time (72 h) with a magnet stirring procedure. Cu ions show a strong affinity for the prion protein (Brimacombe et al., 1999; Brown et al., 2000; Brown, 2001; Giese et al., 2004) and propanol is capable of interfering with the hydrophobic interactions possible established between the protein and the organic complexes (Wershaw, 2004). In both experimental conditions with either the copper or propanol solutions the protein was not detected in the extracts.

Finally, Sarkosyl, a strong anionic detergent, usually utilized for the cleaning of glass and instruments in laboratories, was tested. Release of PrP or tPrP was revealed neither by HPLC nor by electrophoresis analyses, even from Bir-PrP or Bir-tPrP samples.

3.4. Discussion

The results of the present work highlighted the strong and irreversible interactions of recombinant prion proteins with organic and organo-mineral compounds, thereby emphasizing the importance that organic or organo-mineral soil colloids may play in the fate of prion proteins in soil. For these studies, soil colloid models were derived by birnessite-mediated polymerization of catechol. Although the complexity of natural soil systems was not reproduced, these model systems can simulate some natural processes occurring in soil.
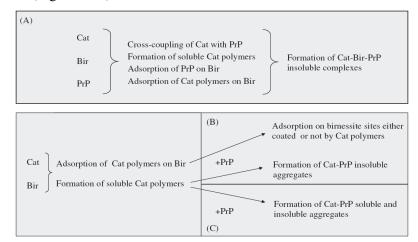
The experiments were performed under buffered conditions at pH 5.5 to favor the formation of insoluble over soluble phenol polymers (Naidja et al., 1998) and to preserve the α-helix conformation of the PrP (Rezaei et al., 2000).

A complete removal of both PrP and tPrP from the solution was observed under all experimental conditions investigated. Strong interactions of PrP occurred with the oxidative polymerization products whichever soluble polymers or insoluble aggregates (Table 3.2). These conclusions were drawn from several experimental evidences such as the disappearance of protein molecules from the solution, the decrease of UV-Visible absorbance of supernatants, the FT-IR spectra and elemental analyses of solid-phase residues, and the nil amounts of PrP or tPrP detected by HPLC and SDS-PAGE electrophoresis.

Moreover, the interaction of PrP or tPrP with birnessite and birnessite-mediated catechol polymers was so strong that both proteins could not be released from the formed solid aggregates neither by a) phosphate and

pyrophosphate buffers, b) organic solvents, c) solutions of copper, nor by a strong anionic detergent like Sarkosyl.

As reported also by Matocha et al. (2001) the oxidation of catechol by Mn(IV) oxides is a rapid multistep, redox-surface process, leading to the formation of soluble and highly insoluble organic products accumulating on birnessite surfaces. Given some constant experimental conditions (e.g., incubation temperature and time, pH, amount of birnessite), the transformation of catechol in either soluble or insoluble polymers depends on the initial catechol concentration. Our results have clearly indicated that soluble polymers prevailed over insoluble ones using a catechol concentration of 3 mM whereas an opposite situation occurred at 5 mM (Figure 3.3).

When the protein was also present in the reaction mixture along with catechol and birnessite, a rather complicated interplay of physically mediated processes and chemical reactions probably occurred, thereby leading to a very complex sequence of pathways and products being formed (Figure 3.11).

Figure 3.11. Scheme of possible physical and chemical processes occurring in the model systems studied.

The investigated systems differed substantially from each other whether the protein was mixed with catechol and birnessite at the same time (Cat-Bir-PrP) (Figure 3.11A), after the preventive incubation between catechol and birnessite (Cat-Bir+PrP) (Figure 3.11B), or with catechol polymers in the absence of birnessite (Figure 3.11C).

To attempt an explanation of the obtained results, it is necessary to separately analyze the contribution of the different phenomena to the whole process. Namely, the proteins interact with i) birnessite alone, ii) catechol polymers alone, iii) birnessite partly covered by catechol polymers and iv) birnessite and catechol.

The most complex situation holds when the proteins, catechol and birnessite were mixed at the beginning of the incubation (Figure 3.11A). In this case, along with the formation of soluble catechol polymers, the involvement of NH and OH groups of the proteins in cross-coupling reactions with phenoxy radicals produced upon birnessite catalysis is likely to occur (Figure 3.11A). Several findings have demonstrated that amino acids, humic substances and phenols may give rise to cross-coupling products during the oxidative polymerization of phenols promoted by birnessite (Bollag et al., 1995; Huang, 1995). In this process, the higher number of NH groups in PrP may have favored the entrapment of the protein in catechol-birnessite insoluble precipitates. This phenomenon is less probable when the protein was added after 2 h incubation where free catechol was completely transformed (Cat-Bir+PrP and soluble catechol polymer+PrP samples; Figure 3.11B and C).

In this reactive context, we also evidenced the adsorption of either PrP or tPrP on birnessite surfaces (Figure 3.11A), since high percentages of the two proteins were removed from the solution in the presence of the sole birnessite. This finding was consistent with the presence of amide I and

amide II bands in the FT-IR spectra of Bir-PrP insoluble complex. At the adopted experimental pH (5.5), birnessite surfaces having point of zero charge 1.81, according to Matocha et al. (2001), and PrP or tPrP are negatively and positively charged, respectively. Therefore, strong electrostatic interactions may be established, as already observed in the literature (e.g., tyrosinase was adsorbed on birnessite surfaces up to 89% of its initial amounts, Naidja et al., 2002). In addition, an interaction of PrP with birnessite sites coated with catechol polymers was also likely to occur (Figure 3.11B), in particular for Cat3-Bir+PrP and Cat5-Bir+PrP samples (where PrP is added after a preventive incubation of catechol and birnessite). In this case (Figure 3.11A and B) it is possible to hypothesize that the two phenomena, i.e., adsorption of the PrP on birnessite surfaces and its interaction with phenol polymers adsorbed on these latter, may simultaneously occur. Conformational studies carried out by decomposition of amide I indicated that in the studied conditions, the solely adsorption process of PrP on Mn oxide surface did not lead to a remarkable conformational β-conversion. Instead PrP interaction with organic coated birnessite surfaces produced 9% increase of β-sheet character that can be rendered as a partial β-conversion, suggesting a possible role of organic matter in conformational rearrangement processes of prion protein during adsorption. However these results are from a β-aggregation that is supposed to happen during the PrP[Sc] aggregation.

Besides interacting with birnessite-catechol complexes and with birnessite alone, proteins may also interact with soluble catechol polymers, a situation which we simulated by incubating either PrP or tPrP with the supernatants of Cat5-Bir and Cat3-Bir samples (Figure 3.11C). Protein interactions with soluble phenolic polymers during or after their formation involve several phenomena like i) multidentate binding of phenolic polymers to the protein,

a process which can become irreversible with time and form insoluble phases (Ladd and Butler, 1975); ii) protein-catechol polymers association through colombic and/or hydrophobic interactions; iii) protein-phenolic polymeric complexes may transform from flexible soluble structures into insoluble, reticulated structures and microporous micelles in the presence of positive charges (Gianfreda et al., 2002); iv) quinoid compounds may covalently interact with the protein (Bittner, 2006; Suderman et al., 2006). Multidentate binding is strictly influenced by the structural features of both phenolic polymers and protein and is assisted by conformational flexibility. In this respect, the long flexible N-terminal end in PrP is likely to play a strong role in PrP interaction with catechol polymers along with charge interactions favored by the polycation-like behavior of the PrP N-terminal region. This region contains as many as six positively charged residues (Figure 3.10) that are probable candidates for establishing favourable columbic interactions with catechol polymers. Also, PrP N-terminus (residues 23-93) adopts mainly polyproline II and β conformations (Blanch et al., 2004). Polyproline II is a secondary structural motif with the peculiar feature of lacking any intra-helix backbone hydrogen bonds (Berisio et al., 2006), and as such involved in the molecular recognition processes and/or in molecular interactions (Rath et al., 2005). All of its backbone hydrogen bond donors/acceptors are non-saturated and are prone to form hydrogen bonds with neighbouring molecules. Therefore, the abundance of positively charged residues in PrP as well as the capability of PrP N-terminal arm to form hydrogen bonds concurs to account for the different behaviour observed for PrP and tPrP in the interaction with soluble catechol polymers.

A role of the N-terminal arm of PrP was also suggested by Revault et al. (2005) and Rigou et al. (2006) in their studies with montmorillonite.

Electrostatic interactions between the positive charges of the Arg and Lys residues at the N-terminal of PrP and the negative surfaces of montmorillonite were hypothesized (Revault et al., 2005). A cleavage at N-terminal site probably due to a strong interaction in this region was observed by Johnson et al. (2006) when the protein was desorbed from montmorillonite with denaturing extracting agents.

The investigations performed on soils with different physico-chemical characteristics demonstrated that PrP entirely bound on soils, and desorption occurred only in conditions which may potentially denature protein molecules, and therefore very far from natural phenomena (Rigou et al., 2005; Johnson et al., 2006). Besides the well-known contribution of clay minerals, our findings indicate that in the complex soil system humic materials may likely play an important role also in the interaction with PrP molecules, thus exerting a stabilizing effect (Wershaw, 2004).

In conclusion, overall the results reported here have contributed to shedding light on the interaction of prion protein with synthetic organic- and organo-mineral complexes simulating soil organic and organo-mineral colloids. Both in the protein only theory (Prusiner, 2004) and in the more general conception of "prion", PrP is recognized as an infectious protein particle. The irreversible interaction of PrP demonstrated both with catechol polymers and furthermore in birnessite-catechol complexes, which simulate humic substances and humic-mineral complexes, suggests that prion proteins should be strongly retained in soils in which processes of organic matter reorganization are active, with very low risks of release and subsequent dissemination in soil.

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Chapter 4

Interaction of a recPrP with natural humic acids and humic-like substances[2]

The previous Chapter was dedicated to study the interaction of a recPrP with organo-mineral complexes. Adsorption and/or entrapment phenomena were observed and the interaction established with the protein appeared irreversible as no recPrP was extracted from the protein-organo-mineral complexes, at least under the investigated conditions. Some mechanisms were proposed to justify the obtained results. As a general conclusion, it was demonstrated that the mineral phase had a dominant role. Indeed, its presence during the process strongly influenced the interaction of the protein with the organo-mineral complexes and in turn affected the final features of the formed complexes.

This Chapter is dedicated to investigate and better elucidate the role of natural organic matter in the binding of a recombinant ovine prion protein to soil. Preformed humic substances and humic-like substances during their formation were investigated. In all the examined situations the interaction of recPrP occurred in the absence of mineral components. Binding of recPrP to soil organic matter as humic acids or humic-like substances, obtained by enzymatic polymerization of catechol, was followed by measuring the removal of recPrP from the solution, and UV-Vis investigations. The formed insoluble aggregates were characterized and

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[2] A version of this chapter has been prepared for publication as:
Rao Maria A., Russo Fabio, Scotti Riccardo and Gianfreda Liliana. Fate of prions in soil: interaction of a recombinant PrP with natural humic acids and synthetic humic-like substances.

studied for the presence of recPrP by FT-IR analyses. The strength of the binding between recPrP and the organic materials was investigated by different extraction procedures. Particular attention was devoted to understanding whether the involvement of the protein in the formation of the organic matter resulted in an increased stability of the interaction as compared to the adsorption of the protein on preformed humic acids.

4.1. Introduction

The recent great attention devoted to the fate of prions in the soil environment has been mainly focused on the interaction of prions with whole soils or soil mineral constituents (Revault et al., 2005; Vasina et al., 2005; Johnson et al., 2006; Georgsson et al. 2006; Leita et al., 2006; Rigou et al., 2006, Schramm et al., 2006; Cooke et al., 2007; Cooke and Shaw, 2007; Genovesi et al., 2007; Johnson et al, 2007; Ma et al., 2007; Seidel et al., 2007). As extensively addressed in the previous Chapters, this interest derives from the awareness that soil plays a role in the accumulation and dissemination of prions in the environment with consequences on the environmental and human health. Therefore, understanding the fate of prions in soil could be critical to the development of environmental defence strategies.

Recent investigations carried out with either infective or benign recombinant forms of prion proteins have clearly demonstrated that fast, active and strong interactions occur in soil between these proteins and whole soils or mineral soil constituents. High amounts of prion proteins actually and firmly bound to clays (Revault et al., 2005; Vasina et al., 2005; Johnson et al., 2006; Rigou et al., 2006, Ma et al., 2007), organo-mineral complexes (Rao et al., 2007), and whole soils (Johnson et al., 2006; Leita et al., 2006; Rigou et al., 2006) and they do not easily desorb in natural like conditions.

Binding of PrP^{Sc} on clay minerals in the absence of organic matter or on whole soils resulted in an increased infectious capacity of the disease transmission by PrP^{Sc}, while an organic matter rich soil failed increasing infectivity (Johnson et al., 2007).

The interactions of prion protein with soil organic matter alone have been not fully clarified and the role played by this important soil component in the retention, mobility and even the effect on the infectivity of prions in soil has to be elucidated.

As discussed in Chapter 3 and reported by Rao et al. (2007), we demonstrated that a full-length recombinant purified ovine protein as well as its N-truncated form strongly and irreversibly interacted with birnessite-catechol polymers, resembling humic-mineral complexes. The system reproduced the interaction of recPrP with soluble organic matter or with insoluble organic matter in association with a soil mineral during or after formation of catechol polymers. It was highlighted that i) the solid inorganic phase had a dominant role in influencing the nature and properties of the formed complexes and the retention of the protein in them; ii) no release of the protein occurred at all from the formed complexes even when the interaction of the protein occurred in the absence of the inorganic solid phase.

The systems investigated represent however only a limited number of the possible situations encountered by a protein in soil and involving organic matter. They implies that the interaction of the protein might have occurred in any moment in the presence of an ongoing polymerization phenomenon obtained by birnessite. Therefore, the irreversible retention of the prion protein observed in those systems could have been due to the involvement of protein molecules in the polymerization phenomenon with probably strong contributions due to the inorganic phase.

In this Chapter the interaction of the prion protein with preformed humic substances or its involvement in the formation of humic-like substances has been investigated, with particular attention to avoid the presence of any

inorganic mineral phase. Biotic catalysts of phenolic polymerization, like laccase and peroxidase, were used to assure complete homogenous system. These processes are more common in surface soils, where the interactions of proteins, in general, and prion proteins in particular, with humic substances very likely occur and consequently also the transmissibility of the disease from an individual to another is more expected. Indeed, surface soils are very likely the most involved in the complex interactions soil-plant-micro and macro-fauna.

Phenoloxidases and peroxidases are two groups of oxidoreductases, produced by a large number of living cells (microorganisms, plants and animals) and usually present in soil. The main producers of both groups are white-rot fungi that explicate a major role in lignin transformation (Mayer, 1987; Hammel, 1989; Messerschmidt, 1994; Gianfreda et al., 1999).

Phenoloxidases that include tyrosinases and laccases require molecular oxygen for activity, whereas peroxidases that comprise horseradish peroxidase, lignininases (i.e., lignin- and manganese-peroxidases), and chloroperoxidases utilize hydrogen peroxide. In some cases, e.g. manganese peroxidases, the reaction depends on the presence of other components such as divalent manganese and particular types of buffers.

Both groups catalyze, by different mechanisms, the oxidation of phenolic and non-phenolic, aromatic compounds through an oxidative coupling reaction that results in the formation of polymeric products of increasing complexity. Cross-coupling reactions between substrates of different nature as well as the oxidation of relatively inert substrates by the co-presence of more reactive molecules may occur (Gianfreda and Bollag, 2002). The reactions catalysed by laccases proceed by the monoelectronic oxidation of phenols, aromatic and aliphatic amines to the corresponding reactive radical. The redox process takes place with the assistance of a cluster of

four copper atoms that form the catalytic core of the laccase enzyme (Riva, 2006). The overall results of laccase catalytic cycle are the reduction of one molecule of oxygen to two molecules of water and the concomitant oxidation of four substrate molecules to produce four radicals (Claus, 2004; Solomon et al., 1996). The final step of the whole oxidative coupling process is the reaction of unstable radical intermediates producing dimers, oligomers and polymers. Polyphenoloxidase transformation of phenols is reported to produce humic-like substances (Dec and Bollag, 1990; Naidja et al., 1997, 1998). Experimental evidences have demonstrated that polymeric products obtained by oxidative coupling mediated transformation of phenols have usually properties and features very different from those of the products obtained by inorganic (birnessite)-mediated catalysis (Naidja et al., 1998).

In this view, biotic transformation of monomeric compounds toward bigger molecules can lead, in natural conditions, to the involvement of other molecules, including bio-macromolecules like proteins. Bollag (1992) proposed the addition of laccase to soil to enhance the natural process of xenobiotic binding and incorporation into the humus.

Prion proteins may also interact with preformed humic acids, already present in soil as stable entities, and give rise to organic proteic aggregates also not loading inorganic phases, at all. It is known that encapsulation in humic substances can protect soil endogenous or exogenous proteins from proteolysis (Zang et al., 2000), and that this effect is more marked when these proteins are entrapped through oxidative coupling in synthetic humic-like materials rather than when adsorbed on preformed polymers (Burns, 1986). Soil proteins, mainly enzymes, can be protected in soil by a network of humic molecules with pores large enough to permit the passage of substrates and reaction products but not that of larger molecules such as al

proteases (Nannipieri et al., 1980, 1988). Similar protection effect is reasonable acting also with exogenous proteins like prion proteins, and their interaction with preformed soil humic substances and/or their involvement in the formation of new humic matter can result in the immobilization, protection of the protein and finally affect its infectious capability.

The main purpose of this Chapter has been to study model systems formed by recPrP and humic acids or humic-like substances during their formation by using characterized soil extracted humic acids (HA) or simulated humic-like substances (HLS) obtained trough the oxidative polymerization of catechol, a phenolic humic precursor, catalyzed by laccase or peroxidases. Furthermore, a more complex model system in which catechol and laccase were added to the recPrP after its preventive interaction with humic acids was studied to simulate the possible stabilizing effect deriving from the formation of new polymeric material and resembling the continue supply of fresh humic substances.

4.2. Material and Methods

4.2.1. Chemicals

Catechol (Cat) (>99.0% purity) and all other reagents have been previously described in Chapter 3. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) or ABTS, used to assay laccase activity, was purchased from Boheringer Biochemia (Mannheim, Germania).

The purified full-length recombinant ovine prion protein indicated for simplicity PrP, was used in all the experiments. All details about this protein are reported in Paragraph 3.2.1.

4.2.2. Laccase and Peroxidase

A commercial laccase (L) (benzenediol oxygen oxidoreductase, EC 1.10.3.2) from *Trametes versicolor* was purchased from FLUKA (Switzerland). The initial laccase activity was assayed at 25 °C using 10 mM ABTS, as the substrate, in 100 mM glycine/HCl buffer pH 3.65. Oxidation of ABTS was followed by absorbance increases at 420 nm ($\varepsilon_{420} =$ 36 mM^{-1} cm^{-1}). The production of the chromogenic compound was monitored with a Shimadzu UV-Vis spectrophotometer. Enzymatic activity expressed in enzymatic units (EU) was calculated by the following relation:

$$EU = \Delta DO \cdot \Delta t^{-1} \cdot Vtot / \varepsilon \cdot v$$

where $\Delta DO \cdot \Delta t^{-1}$ is the absorbance variation rate;

Vtot is the total volume;

ε is the molar extinction coefficient of reaction product;

v is the volume of used enzyme.

One unit of enzymatic activity was defined as the amount of enzyme required to transform one micromole of ABTS per minute at 25 °C. Enzymatic activity of solutions of 2 mg mL^{-1} of laccase was measured by using 1.5 mL of ABTS 10 mM at pH 3.65 and 50 μl of laccase solution.

An essentially salt-free peroxidase (POD) (donor hydrogen peroxide oxidoreductase, EC 1.11.1.7) from horseradish was purchased from Sigma-

Aldrich, containing 1100 Units for mg of solid (one enzymatic unit oxidizes 1 μmole of ABTS, for minute at 25 °C and pH 5.0). The hydrogen peroxide solution (1%) was prepared daily diluting the commercial product (30% H_2O_2). The real H_2O_2 concentration was checked using the extinction coefficient of 39.4 M^{-1} cm^{-1} at 240 nm. Enzymatic activity of solutions of peroxidase was measured by using 0.55 mL of ABTS 100 mM in acetate buffer pH 5.0, 0.75 mL acetate buffer pH 5.0, 0.2 mL of H_2O_2 and 50 μL peroxidase solution.

4.2.3. Humic acids

Humic acid was extracted from soil with a solution of 0.5 M NaOH-0.1 M $Na_4P_2O_7$ purging with N_2. Extracts were separated and purified according to the IHSS standard procedure (Schnitzer e Khan, 1972). The main properties of the extracted humic acid are reported in Table 4.1.

Table 4.1. Humic acid properties.

Properties	Values
Total acidity, meq g^{-1}	6,00
-COOH, meq g^{-1}	4,12
-OH phenolic, meq g^{-1}	1,88
Ash, %	2,5
H_2O, %	14,1
C, %	54,1
N, %	1,3
H, %	3,9
S, %	0,4
O, %	40,3

4.2.4. Formation of peroxidase- or laccase-catechol-PrP complexes

Catechol polymerization was promoted by 0.2 UE mL^{-1} laccase or 0.01 U mL^{-1} POD by incubating in 5 mL of 0.1 M sodium acetate buffer pH 5.5 increasing concentrations of catechol (1-5 mM) in Erlenmeyer tubes for 2 and 24 h at 25 °C. After incubation, the mixtures were centrifuged for 15 min at 10000 *g* and 4 °C, and residual catechol concentrations in the supernatants were determined. Appropriate controls with either Cat, L or POD only were also carried out.

The interaction of 0.5 mg mL^{-1} PrP with catechol-laccase and catechol-POD mixtures was investigated using only 3 and 5 mM catechol in 5 mL of 0.1 M sodium acetate buffer pH 5.5. The three components, Cat, L or POD, and PrP, were mixed at the same time (L-Cat-PrP, POD-Cat-PrP) or C and the catalyst (L or POD) were incubated for 2 h before supplying PrP (L-Cat+PrP, POD-Cat+PrP). Samples with only Cat, PrP, L or POD, and mixtures Cat-L and C-POD served as controls.

All mixtures were incubated for a total of 4 h at 25 °C. At the end of incubation samples were centrifuged for 15 min at 10000 *g* and 4 °C. The residual catechol concentration, the residual laccase and peroxidase activity, pH, UV-Vis spectra of supernatants and PrP in solution were measured.

For FT-IR analyses recovered precipitates were washed twice with 0.02 M NaCl and once with bi-distilled water, lyophilized and stored at 4 °C.

Experiments were performed in triplicate.

4.2.5. Formation of humic acid-PrP complexes

In 2 mL of 0.1 M sodium acetate buffer pH 5.5 increasing amounts of humic acid (0.144, 0.430, 0.720, 1.5 mg mL^{-1}) and 0.5 mg mL^{-1} PrP were incubated for 2 h at 25 °C. After incubation, 600 µL of 0.5 M CaCl$_2$ was added to humic acid-PrP mixtures and then the precipitates were recovered by centrifugation for 15 min at 10000 *g* and 4 °C. Controls with humic acid and PrP without adding CaCl$_2$, with only humic acid or PrP were also prepared following the same procedure. Supernatants were analyzed for UV-Vis spectra and PrP concentration. For FT-IR analyses recovered precipitates were washed twice with 0.02 M NaCl and once with bi-distilled water, lyophilized and stored at 4 °C.

Experiments were performed in triplicate.

4.2.6. Formation of humic acid-PrP-laccase-catechol complexes

A solution of 2 mL of 0.1 M sodium acetate buffer pH 5.5 containing 5 mM catechol and 0.2 UE mL^{-1} laccase was added to the pellets of humic acid-PrP samples recovered as reported before. The mixtures were incubated for 4 h at 25 °C and the pellets were recovered by centrifugation for 15 min at 10000 *g* and 4 °C. Residual supernatants were analyzed by SDS-PAGE for PrP residual concentration.

Experiments were performed in triplicate.

4.2.7. Desorption/extraction tests

Extraction tests were performed on the pellets recovered from the various investigated systems. Pellets were previously resuspended in 0.1 M acetate buffer pH 5.5 and than recovered again by centrifugation for 15 min at 10000 g and 4 °C to remove any residual not adsorbed PrP. Extractions were performed at room temperature using a magnet stirring in vials with 1 mL of the following solutions: a) 0.1 M phosphate buffer at pH 7.0 and 8.0 for 1 h; b) Sarkosyl 1% in 0.1 M phosphate buffer pH 7.5 for 24 h; c) sodium hexametaphosphate 50 g l^{-1} for 1 h. After the different treatments, all the samples were centrifuged for 30 min at 10000 g and 4 °C and the extracted PrP was detected by SDS- PAGE.

Extraction was also performed using 0.2 mL of SDS-PAGE sample buffer (2.5 g of SDS, 12.5 mL glycerol, 0.15 g blue bromophenol, 0.5 mL β-mercaptoethanol) at 100 °C for 5 min; in this case after a short spin down samples were loaded in a gel for SDS-PAGE analyses.

Experiments were performed in triplicate.

4.2.8. Electrophoresis analysis

Denaturing SDS-PAGE electrophoresis with 15% acrylamide was used (for details see also Paragraph 3.2.10). Image processing software, ImageJ (Abramoff et al. 2004), was used to acquire a semiquantitative measure of the protein concentration by evaluating the band density on gels. A regression line ($R^2 > 0,97$) fitted between 5 or 6 PrP standards from 0.2 to 8 μg was used to calculate the amounts of PrP detected in samples.

4.2.9. UV-Visible, HPLC and FT-IR analyses

HPLC analyses were used to detect residual catechol concentration, as described in detail in the Paragraph 3.2.7. UV-Vis spectroscopy was used to evaluate UV-Vis absorbance of polymeric products in the supernatants and FT-IR spectra to characterize complexes as previously described in the Paragraph 3.2.9.

4.3. Results

4.3.1. Formation of laccase-catechol-protein complexes

Preliminary studies were performed to estimate the optimal conditions to form phenolic insoluble aggregates entrapping PrP molecules. According with numerous references in literature (Gianfreda et al., 2006 and references therein) laccase and peroxidase promote oxidative polymerization of catechol. Preliminary studies were carried out using 0.2 U mL^{-1} of laccase, and increasing catechol concentration (1, 2, 3 and 5 mM) incubated for 2 h at pH 5.5 and 25 °C.

Quite complete phenol transformation of catechol, ranging from 95 to 100% by laccase was always achieved also at lower catechol concentrations (i.e., 1, 2 and 3 mM), and at 3 and 5 mM catechol concentrations appreciable amounts of insoluble precipitates useful for further analyses were observed.

The oxidative reaction was also carried out with 0.01 and 0.1 U mL^{-1} peroxidase and 3 and 5 mM catechol solutions for 2 h. The lower

concentration of peroxidase promoted 97 and 92% removal of 3 and 5 mM catechol respectively; these results were close to those obtained with laccase (100 and 95% removal of 3 and 5 mM catechol, respectively). Thus, the experimental conditions suitable to favour the complete transformation of catechol and the formation of insoluble aggregates were selected as 3 and 5 mM of catechol, and 0.2 U mL^{-1} of laccase or 0.01 U mL^{-1} of peroxidase.

The immobilization of PrP was performed by adding PrP at the starting of catechol polymerization process (Cat3-POD-PrP, Cat5-POD-PrP, Cat3-L-PrP, Cat5-L-PrP) or after catechol and laccase/peroxidase interacted for 2 h (Cat3-L+PrP, Cat5-L+PrP, Cat3-POD+PrP, Cat5-POD+PrP). At 5 mM catechol concentration, the presence of PrP molecules since the beginning of the oxidative reaction of catechol catalyzed by laccase (Cat5-L-PrP), caused a hindrance on the polymerization process, in fact the reduction of catechol was only 82%, sensitively lower than in the control lacking PrP (95%) (Table 4.2). Instead, when PrP molecules were added after the polymerization process (Cat5-L+PrP), a similar catechol removal (99%) was observed (Table 4.2). In this case, despite the higher catechol removal the intensity of polymerization process was probably lower, as shown by the yellow colour of Cat5-L+PrP aggregates in comparison with the brown colour observed in Cat5-L. In any case, the presence of PrP favoured a greater amount of insoluble precipitates that resulted more stable and better separated from the solution. In fact, when the PrP added to the solution, it interacted with catechol transformation products and caused the flocculation of yellow aggregates that turned to dark yellow, whereas polymerization of catechol in the absence of PrP led to the formation of organic dark brown aggregates.

In all experimental conditions in which catechol polymerization was catalyzed by laccase, the complete immobilization of protein molecules was reached (Table 4.2). Complete immobilization was also achieved in the samples where PrP was added to the already formed peroxidase-mediated catechol polymers (Cat3-POD+PrP, Cat5-POD+PrP) (Table 4.2). While laccase favoured the complete removal of 3 mM catechol in any sequence of PrP adding (Cat3-L-PrP, Cat3-L+PrP) and high catechol removal in Cat5-L-PrP sample, peroxidase, although all suspensions browned, did not induce a total catechol removal as well as PrP immobilization. For instance, PrP entrapment was only 57 and 90% in Cat3-POD-PrP and Cat5-POD-PrP, respectively. Substantial increase in the immobilization process was observed when PrP was added after catechol polymerization (Cat3-POD+PrP and Cat5-POD+PrP samples) which determined the complete removal of PrP from the solution.

Table 4.2. PrP entrapment and catechol removal in organic complexes obtained in the presence of peroxidase and laccase.
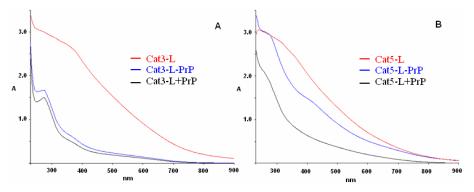
Samples	PrP entrapment %	Catechol removal %
Cat3-POD	-	97 ± 1
Cat5-POD	-	92 ± 3
Cat3-POD-PrP	57 ± 5	99 ± 1
Cat5-POD-PrP	90 ± 4	85 ± 5
Cat3-POD+PrP	100 ± 0	97 ± 1
Cat5-POD+PrP	100 ± 0	62 ± 6
Cat3-L		100 ± 0
Cat5-L	-	95 ± 1
Cat3-L-PrP	100 ± 0	100 ± 0
Cat5-L-PrP	100 ± 0	82 ± 4
Cat3-L+PrP	100 ± 0	100 ± 0
Cat5-L+PrP	100 ± 0	99 ± 1

The insoluble aggregates formed with peroxidase catalysis were less abundant than those formed under laccase action and thus not sufficient to be further analysed. Therefore, FT-IR and extraction tests were performed only with samples obtained by laccase.
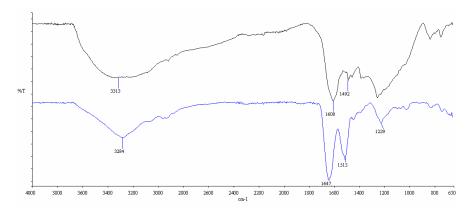
The interaction of PrP with catechol polymers was also been confirmed by UV-Vis spectra. Spectra collected on samples obtained by using laccase showed that the absorbance of Cat3-L and Cat5-L supernatants in the entire UV-Vis region was higher then that of samples obtained with PrP (Figure 4.1). In particular the UV-Vis spectra showed a stronger absorbance reduction in Cat3-L+PrP and Cat5-L+PrP in which PrP was added after catechol polymerization rather than in Cat3-L-PrP and Cat5-L-PrP in which it was added since the beginning of the reaction (Figure 4.1). Prion protein molecules interacted with soluble polymers of catechol and favoured the formation of insoluble polymers precipitating at the bottom of the test tube.

Figure 4.1. UV-Vis spectra of laccase-catechol-PrP at 3mM catechol (A) and 5mM catechol (B)

The spectra of the supernatants of samples with POD compared to samples with laccase were characterized by very higher absorbance values in the

entire range of wavelength observed (data not shown). The high values of absorbance and the darkness of samples were consistent with the low amounts of precipitates observed in POD catechol transformation.

The formed precipitates obtained by laccase oxidative polymerization of catechol in the presence of PrP were analyzed by FT-IR analyses and allowed to characterize the insoluble material (Figure 4.2).

Figure 4.2. FT-IR spectra of insoluble complexes formed with catechol and laccase in the absence of PrP (Cat5-L) (black line) and in the presence of PrP (Cat5-L+PrP) (blue line).
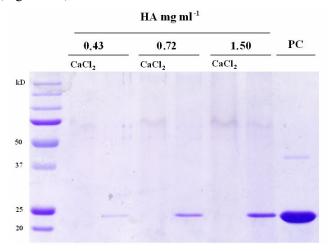
In the FT-IR spectrum of the Cat5-L sample, as well as in that of Cat3-L (not shown) characteristic peaks of OH phenolic groups present in catechol standard at 3446 and 3323 cm^{-1} disappeared, whereas C-C bound signals of aromatic rings at 1609 e 1490 cm^{-1} were visible indicating that catechol was transformed and new insoluble aromatic material lacking phenolic OH groups was present in the precipitates. The broad band at 3312 cm^{-1} was due to non phenolic -OH groups, bound through hydrogen bridges. The spectrum of Cat5-L-PrP sample markedly showed the PrP signals; in particular there were peaks at 3281 cm^{-1} corresponding to NH bound

stretching, at 1645 cm^{-1} attributed to the amide I, and at 1515 cm^{-1} corresponding to amide II. Similar results were obtained with Cat3-L-PrP samples.

4.3.2. Formation of humic acid-PrP complexes

Interaction of PrP with natural extracted humic acid, resembling the interaction of PrP with stabilized soil organic matter, was performed in order to obtain information closer to natural conditions. To obtain indications comparable to experiments performed with laccase/POD and catechol all the incubations were performed in the same conditions previously reported (i.e., 2 h-incubation at 25 °C in 0.1 M acetate buffer at pH 5.5). In particular, 0.43 and 0.72 mg mL^{-1} of humic acid (HA2 and HA3) were used. They corresponded in term of organic carbon to 3 and 5 mM of catechol used in the synthetic systems described above. Two further concentrations 0.14 and 1.50 mg ml^{-1} (HA1 and HA4) were also used. Even if incubation of PrP in the presence of natural humic acid resulted in reduced PrP amounts in solution, the complete removal of PrP did not occur. The bands of PrP shown in Figure 4.3 indicate that residual amounts of the protein were present in the incubation mixture at the end of 2 h incubation. The removal of PrP from the solution caused also an increased formation of visible precipitates after centrifugation. Subsequently to the addition of PrP to humic acid in solutions at 0.43, 0.72 and 1.5 mg mL^{-1} a flocculation was observed and was attributed to the interaction of the protein with soluble humic acid products and to a consequent formation of insoluble aggregates.
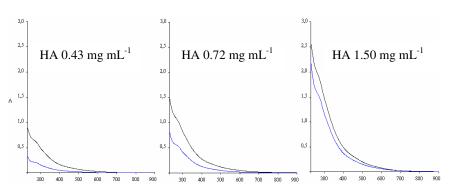
This hypothesis was also confirmed by the reduction of the UV-Vis absorbance spectra of the supernatants of all the PrP-humic acid reaction mixtures (Figure 4.4).

Figure 4.3. Immobilization of PrP with humic acid in presence or not of CaCl2
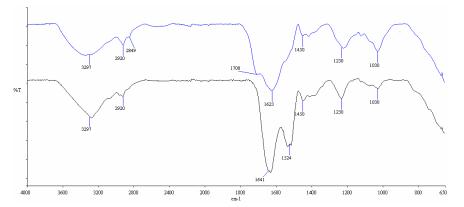
The complete PrP removal from solution was achieved by adding $CaCl_2$ that increased the flocculation of the humic acid added involved further PrP molecules (Figure 4.3).

The prion protein free in solution was determined by evaluating the intensity of the bands in the electrophoretic gel. Results showed that 0.43 mg mL^{-1} of humic acid, the lowest amount utilized in this experiment, determined the highest PrP removal with only 4% residual PrP in solution, whereas at 0.72 and 1.50 mg mL^{-1} humic acid 12 and 14% of PrP were not removed, respectively (Figure 4.3). Other experiments performed at even lower humic acid amounts (0.144 mg mL^{-1}) also determined PrP immobilization (data not shown).

Figure 4.4. UV-Vis of HA (black line) and HA+PrP (bleu line) at different concentration of HA.

The formed insoluble complexes of humic acid and PrP were characterized by FTIR analyses. Humic acid in the absence of PrP showed the presence of typical signals (Figure 4.5).

Figure 4.5. FT-IR spectra humic acid (blue line) and humic acid-PrP complex (black line).

The broad band around 3300 cm^{-1} was due to inter- and intra-molecular hydrogen bonding (Chapman et al., 2001). Absorptions at 2920-2850 and 1450 cm^{-1} were characteristic of the CH_2 stretching and bending vibrations, respectively (Chen et al., 2002). Moreover the shoulder at 1708 cm^{-1} and

the signal centred around 1250 cm^{-1}, could be assigned to the C=O and C-O bonds in protonated carboxylic, respectively. Signal centred at 1623 cm^{-1} were attributed to C=C in aromatics and alkenes. The strong band at about 1030 cm^{-1} could be attributed to C-O stretching vibrations of polysaccharide (Stevenson, 1982; Haberhauer et al., 1998).

The samples in which HA reacted with PrP highlighted strong signals of the amide I centred around 1650 cm^{-1} and the amide II at 1525 cm^{-1} attributed to the presence of the proteinaceous material relative to PrP.

4.3.3. Desorption studies

Once the PrP was immobilized in synthetic humic-like substance and natural humic acid, desorption tests of PrP were performed by using weak and stronger agents as reported in the Paragraph 4.2.7.

The effectiveness of the extraction and the percentages of desorbed PrP obtained by the different agents and experimental conditions are summarised in Table 4.3. The weakest agent, phosphate buffer at pH 7.0 and 8.0, was not able to desorb PrP from all the PrP-humic acid or PrP-humic-like systems. By contrast a solution of Sarkosyl, an anionic detergent buffered at pH 7.5, or hexametaphosphate, an agent favouring the dispersion of aggregates (Plouffe et al., 2001), were able to desorb detectable amounts of PrP immobilized onto humic acid previously treated or not with CaCl$_2$.

Sarkosyl was able to desorb from 10 to 27% of PrP immobilized on humic acid in the absence of CaCl$_2$ treatment, and from 2 to 25% of PrP immobilized on humic acid treated with CaCl$_2$. By using hexametaphosphate higher desorption values from humic acid-PrP samples

(treated with $CaCl_2$) were obtained. In particular desorption of PrP molecules was higher when PrP was immobilized in higher amount of humic acid HA4-PrP (52%) as compared with HA3-PrP (44%) and HA3-PrP (20%).

Table 4.3. Extracted PrP from humic-like and humic acid complexes

Systems	Phosphate pH 7.0 and 8.0	HMP	Sarkosyl	SDS
Cat3-L-PrP	0 ± 0	0 ± 0	0 ± 0	+
Cat3-L+PrP	0 ± 0	0 ± 0	0 ± 0	+
Cat5-L-PrP	0 ± 0	0 ± 0	0 ± 0	+
Cat5-L+PrP	0 ± 0	0 ± 0	0 ± 0	+
HA1-PrP	nd	nd	10 ± 7	nd
HA2-PrP	0 ± 0	nd	22 ± 4	+
HA3-PrP	0 ± 0	nd	27 ± 5	+
HA4-PrP	0 ± 0	nd	23 ± 4	+
HA1-PrP+CaCl$_2$	nd	nd	2 ± 1	nd
HA2-PrP+CaCl$_2$	0 ± 0	20 ± 8	25 ± 5	+
HA3-PrP+CaCl$_2$	0 ± 0	44 ± 8	23 ± 4	+
HA4-PrP+CaCl$_2$	0 ± 0	52 ± 7	24 ± 5	+
HA2-PrP+CaCl$_2$ + Cat5-	nd	0 ± 0	0 ± 0	+
HA3-PrP+CaCl$_2$ + Cat5-	nd	0 ± 0	0 ± 0	+
HA4-PrP+CaCl$_2$ + Cat5-	nd	0 ± 0	0 ± 0	+

HMP= hexametaphosphate; SDS = Sodium dodecyl sulphate. nd not detected.
HA1; HA2; HA3; HA4 = 0.14; 0.43; 0.72; 1,50 mg mL^{-1} humic acid respectively.
+ = high amounts of PrP extracted.

Extractions carried out with harsher conditions by using solution of SDS-PAGE sample buffer at high temperature (100 °C for 5 minutes) were successful to extract from all the complexes very high amounts of PrP. However all the SDS extractions resulted in positive signals on gels with poor quality and homogeneity that not allowed gel quantification by densitometry (the sign "+" indicate high positive extractions in Table 4.3).

In order to evaluate the stabilization of humic-PrP complexes by the formation of new polymeric material, extraction tests were performed also on PrP-humic acid complexes to which a fresh laccase-mediated catechol mixture, was added to favour the formation of new catechol polymers (HA2-PrP+Cat5-L, HA3-PrP+Cat5-L, HA4-PrP+Cat5-L). PrP molecules resulted strongly immobilized and no desorption was observed by using Sarkosyl and hexametaphosphate, confirming the stabilizing effect of newly formed organic polymeric products observed also in laccase catechol-PrP systems (Table 4.3). Even in this case, only the stronger extraction performed by SDS-PAGE sample buffer resulted effective, suggesting that only the complete destruction of the complexes allowed PrP release.

4.4. Discussion

Soil organic matter has a fundamental role in many processes acting in soil, influencing the fate of nutrients, ions, pollutants as well as exogenous biomacromolecules like prion proteins. As an important step to understand processes of interaction of prion proteins in soil, the interaction of prion proteins with soil organic matter and its involvement humic substance formation was investigated.

Catechol is regarded as a common humic starting material, and its polymerization by soil enzymes can be considered to resemble such processes of humic substance formation. In the experimental design, involvement of PrP in these humic matter complexes simulated the involvement of PrP molecules in soil humic substances formation, while

interaction of PrP with soil humic acid material resembled interaction with stable preformed soil humic substances.

The overall reduced removal of catechol by both laccase and peroxidase in the presence of PrP indicated that the enzymatic transformation of catechol was inhibited by the presence of this protein. In the case of laccase this effect was visible only in Cat5-L-PrP, but not in Cat5-L+PrP. Indeed, catechol removal reaction is a fast process and in the second case after the first two hours of incubation without PrP catechol was almost completely removed. The different effect of the presence of PrP on catechol removal observed in the system catalyzed by POD was probably due to a different rate of reaction.

PrP strongly interacted with humic-like substances generated by catechol transformation by laccase. Several findings proved this hypothesis: i) the PrP immobilization was reached in a time of incubation as short as 2 hours; ii) the supernatants of catechol-laccase systems were clarified by the subsequent addition of PrP to indicate the great affinity of PrP for catechol polymers; iii) FT-IR analyses markedly highlighted the presence of PrP in the formed precipitates; iv) PrP was extracted from insoluble precipitates only by strong extractants.

PrP interacted with catechol transformation products but not with catechol itself as no remarkable reduction of PrP concentration occurred in the catechol-PrP mixture (data not shown). Thus, it can be deduced that PrP interacts with catechol reactive radicals generated in the first steps of catechol oxidation or also with oligomers and polymers formed further in the reaction (Riva, 2006). From this point of view the lower reduction of the UV-Vis absorbance observed in the Cat5-L-PrP sample as respect to Cat5-L+PrP could be reasonably explained by the fact that in Cat5-L+PrP sample the prion protein interacts with the catechol polymerization

products already formed determining the formation of protein-catechol aggregates and their subsequent precipitation (Figure 4.2). In the Cat5-L-PrP, instead, PrP interacts with catechol oxidation products as soon as they are generated, leading to a quite fast disappearance of the PrP in solution meanwhile residual catechol is continuing to react and to generate new polymers (Figure 4.2). This effect is less visible in Cat3-L-PrP and Cat3-L+PrP because of the lower concentration of catechol and subsequently of the lower concentration of catechol polymers in solution.

Also in humic-like complexes obtained by POD catalysis the protein was completely immobilized and showed a strong and irreversible binding with the polymeric products. In POD systems the high UV-Vis absorbance, the darkness of supernatant of samples and the little amounts of formed precipitates suggested that with this catalyst the formation of more soluble rather than insoluble protein-catechol aggregates occurred.

In all the laccase-mediated systems investigated the PrP was strongly retained as not removed by any of the extracting conditions used. Only very harsh extractions performed by using SDS sample buffer solutions at high temperature, that very likely destroy organic aggregates, were able to release prion protein.

Natural humic acid also interacted with PrP as proved by its reduction in solution, the decrease of UV-Vis absorbance of the supernatants and its effective presence in the insoluble precipitates as revealed by FT-IR analyses.

Differently from the humic-like systems, the interaction of PrP with natural humic acids led only to a partial immobilization, even if interactions occurred under similar experimental conditions including the amount of organic carbon present in the reaction mixture. Surprisingly, immobilization of PrP with humic acid resulted higher with lower

concentrations of humic acid. This apparent unexpected behaviour is in agreement with the micellar model of humic acids that at high concentrations is expected to form a multimicellar structure (Piccolo, 2001; Sutton and Sposito, 2005) and thus probably leading to a reduced availability of the reactive sites on humic acids. Humic similar compounds were reported to adsorb more efficiently pesticides at low rather than at high concentrations (Sannino et al, 2007).

The complete immobilization of the PrP in this system was only achieved by using a flocculating agent like CaCl$_2$. However, the use of even not extreme hard extractions, like with simple surfactant solutions, led to considerable desorption of PrP regardless of the use of flocculating procedures.

It is a reasonable conclusion that the contact between the protein and humic acid, representing the already formed soil organic substances present in soil as relatively stable material, can only lead to superficial and weak adsorption processes, whereas ongoing transformation of organic material can more strongly involve PrP molecules, mainly during the polymerization processes, very likely occurring in soil.

The stabilizing effect of polymer formation was also confirmed in the further experiment where humic acid-PrP complexes were put in contact with the ongoing polymerization of catechol. Indeed the extraction conditions previously used resulted not anymore able to release the immobilized PrP.

Overall the results here reported highlight the importance of soil organic matter in the immobilization of prion proteins in soil, and particularly of those processes involving *in* and leading *to* its formation.

They strengthen that in addition to clays, vastly treated in literature, the organic soil components have an important role in the involvement of PrP in those processes that result in a strong PrP retention.

It is obviously needed to understand many other aspects of the fate of prion proteins in soil related to the organic soil components. Firstly it seems important to confirm this data with disease associate PrP[Sc]. The infectious capability of the PrP[Sc] immobilized in soil humus material should be investigated to understand if prions infectivity can be reduced by binding with organic matter or humic-like compounds and consider these latter as a potential tool for environmental remediation.

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Chapter 5

Disease-associated Prion Protein degradation by soil mineral Birnessite: an environmental component potentially acting against TSEs diffusion[3]

As widely discussed, prions, the etiological agents of transmissible spongiform encephalopathies, exhibit extreme resistance to degradation. Soil has been hypothesized to maintain prion infectivity in the environment for years. Among the several processes undergoing by prion protein in soil, direct interaction of the protein with reactive soil components might occurs and contribute to the inactivation of prions in soil. The manganese oxide, birnessite (MnO_2), ranks among the strongest natural oxidants found in soil. This Chapter reports the first evidence for abiotic degradation of prions under simulated environmental conditions. Aqueous suspensions of birnessite degraded the disease-associated prion protein (PrP^{Sc}) as evidenced by decreased immunoreactivity and diminished ability to seed protein misfolding cyclic amplification (PMCA) reactions. Birnessite-mediated degradation of PrP^{Sc} increased as solution pH decreased, consistent with the pH-dependence of the redox potential of MnO_2. Exposure to 5.6 mg mL^{-1} MnO_2 decreased prion levels by ≥ 4 log units.

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[3] A version of this chapter has been submitted for publication as:
Russo Fabio, Johnson Christopher J., Johnson Chad J., McKenzie Debbie, Aiken Judd M. and Pedersen Joel A. The soil mineral birnessite degrades the disease-associated prion protein: a factor potentially limiting environmental prion disease transmission. *Emerging Infectious Diseases.*

Manganese oxides may contribute to infectious prion degradation in the environment. As related to MnO_2 degradation prion fate in soils is expected to depend on both soil mineralogy and pH.

The work reported in this Chapter was performed at the University of Wisconsin-Madison in cooperation with Dr. Christopher. J. Johnson and under the supervision of Prof. Joel. A. Pedersen.

5.1. Introduction

The infectious agent responsible for TSEs appears to be primarily, if not solely, comprised of a misfolded isoform of the prion protein, designated PrP^{Sc} (Watts et al., 2006). TSE agents exhibit remarkable resistance to degradation; most treatments that inactivate other infectious agents fail to eliminate prion infectivity, although they do reduce TSE agent titer (Taylor, 2000). Resistance of agent to degradation has led to unexpected forms of disease transmission: residual infectivity in meat and bone meal propagated BSE epizootics (Morley et al., 2003), residual infectivity on cleaned medical devices caused iatrogenic transmission of CJD (Brown et al., 2000) and prions released into the environment resulted in scrapie (Palsson, 1979; Georgsson et al., 2006) and CWD (Miller et al., 2004) transmission.

To underline the environmental issues related to the prions in soil it is useful to remind again how prion diseases can be spread environmentally by animals and their ability to persist in soil. In particular, sheep scrapie and cervid CWD differ from other TSEs in that epizootics contribute to maintain the disease diffusion by horizontal transmission from infected to naïve animals (Hoinville, 1996; Miller and Willimas, 2003), and transmission can be mediated by an environmental reservoir of infectivity. The spread of scrapie to naïve sheep after exposure to pastures previously holding infected animals was first noted nearly 70 years ago (Greig, 1940). More recent epizoological evidence has confirmed that scrapie can be transmitted via exposure to contaminated pastures (Palsson, 1979), and that the disease agent can persist in the environments for ≥ 16 years (Georgsson

et al., 2006). Controlled field experiments demonstrated that housing mule deer in paddocks previously containing CWD-infected deer, carcasses or feces is sufficient to transmit disease (Miller et al., 2004).

The lack of clear evidence for vector-mediated transmission of scrapie or CWD (Detwiler and Bylis, 2003; Miller et al., 2006), has led to the hypothesis that soil may serve as an environmental reservoir of infectivity (Schramm et al., 2006). The survival of prions in soil has been demonstrated by the detection of infectivity in aqueous extracts of experimentally spiked soils samples after years of burial (Brown and Gajdusek 1991; Seidel et al., 2007). Furthermore, soil and soil minerals bind PrP^{Sc} potentially maintaining prions near the soil surface and increasing the potential for animal exposure (Johnson et al., 2006; Cooke et al., 2007; Ma et al., 2007; Leita et al., 2006). Clay mineral-bound prions remain infectious intracerebrally (Johnson et al., 2006) and soil-associated agent is infectious orally (Johnson et al., 2007; Seidel et al., 2007). Sorption of prions to the soil mineral montmorillonite and to natural soils can enhance the oral transmission of prion disease (Johnson et al., 2007), providing a possible explanation for environmental disease transmission despite the presumably low levels of prions shed by infected animals.

Soils are complex mixtures of inorganic and organic constituent. Their properties vary considerably across multiple spatial scales. The influence of soils on the fate of prions and the environmental transmission of TSEs is expected to be complex and some examples have been reported in the previous Chapters. Moreover, it has been demonstrated that while two soils significantly enhanced the oral transmission of prion disease, one failed to do so, potentially due to its high organic carbon content (Johnson et al., 2007). The proteolytic activity of soil microorganisms may diminish PrP^{Sc} survival in soil. Microbial proteases have been shown to degrade a β-

sheeted recombinant form of ovine prion protein (recPrP) (Rapp et al., 2006).

Abiotic components of soils could conceivably influence the stability of prions released into soil environments. For example, the manganese oxide mineral, birnessite (MnO_2), ranks among the strongest oxidants in soils (E_H° = 1.29 V) (Bricker, 1965). Soils subjected to alternating reducing and oxidizing conditions, such as those occurring in seasonally waterlogged areas or areas with reduced drainage, typically contain the largest accumulations of manganese minerals and oxides (Post, 1999; Tebo et al., 2004). Many well-drained soils, however, also contain these minerals due to previous wet conditions (Allen and Hajek, 1989). Manganese III and IV minerals can mediate the transformation of numerous organic compounds, including substituted anilines (Laha and Luthy, 1990) and phenols (Stone, 1987), herbicides (Wang et al., 1999; Barrett and McBride 2005) and antibiotics (Zhang and Huang, 2005; Rubert and Pedersen, 2006). In the Chapter 3 it has been reported how MnO_2 promoted oxidative polymerization of catechol and recPrP interactions with humic-like substances from the catechol transformation occurred (Rao et al., 2007). Naidja et al. (2002) found evidence of conformational changes in tyrosinase structure upon sorption to MnO_2 surfaces, but did not investigate the stability of the sorbed protein (Naidja et al., 2002). As an initial step toward understand potential abiotic transformations of prions in soil, we investigated the degradation of PrP^{Sc} by aqueous MnO_2 suspensions.

Protein misfolding cyclic amplification (PMCA) is a newly devised technology that involves the amplification of PrP^{Sc} *in vitro* (Saborio et al., 2001). A normal brain homogenate is used as source of PrP^{C}, while small amounts of PrP^{Sc} are used as a seed. By repeated incubation and intermittent sonication, PrP^{Sc} is able to convert PrP^{C} to the protease-

resistant form PrP^res, resembling the PrP^Sc in infected animals brains, and these PrP^res then become new nuclei, thereby facilitating the amplification of others PrP^res in the cyclic incubation process. PMCA as useful method to amplify the content of PrP^Sc was used here to high sensitively detect residual PrP^Sc after exposure with birnessite.

5.2. Materials and Methods

5.2.1. TSE agent and PrP^Sc purification

Syrian hamsters (cared for according to all institutional protocols) were experimentally infected with the HY strain of hamster-adapted TME agent (Bessen and Marsh, 1992). Brain homogenates (BH) of TSE affected animals were prepared at 10% (w/v) in distilled deionised water (ddH$_2$O). PrP^Sc was purified to a P4 pellet from brains of infected hamsters using a modification (McKenzie et al., 1998) of the procedure described by Bolton (Bolton et al., 1987). The P4 pellet prepared from four brains was resuspended in 1 mL of 10 mM Tris pH 7.4 with 130 mM NaCl.

For experiments relying on protein misfolding cyclic amplification (PMCA) before PrP^Sc measurement, PrP^Sc was previously precipitated from infected brain homogenate with sodium phosphotungstate (PTA) as described by Safar et al. (1998). Briefly, PTA was added to 5% (w/v) brain homogenate containing 2% Sarkosyl to a final PTA concentration of 0.25%. Samples were incubated for 16 h at 37 °C with agitation and centrifuged at 16000 *g* for 30 min at ambient temperature. The PrP^Sc pellet from 1 mL of brain homogenate was resuspended in 50 µL of ddH2O as a

source of agent. Concentration of PMCA-amplified samples was also performed using the PTA protocol prior to SDS-PAGE and immunoblotting.

Protein concentrations were determined using the Bio-Rad DC protein assay as directed by the manufacturer's instructions.

5.2.2. MnO$_2$ preparation

MnO$_2$(s) was prepared according to the method of Murray (1974). Briefly, 16 mL of 0.1 M NaMnO$_4$ was added to 328 mL of N$_2$-sparged ddH$_2$O, and then with 32 mL of 0.1 M NaOH. Manganese oxide was precipitated from solution by drop wise addition of 24 mL of 0.1 M MnCl$_2$ to the basic permanganate solution under constant stirring. The precipitate was recovered by 15 min centrifugation at 6500 *g* and washed with 400 mL of ddH$_2$O. The precipitate was washed a further five times using the same sedimentation and resuspension conditions. The MnO$_2$ concentration was determined by drying and weighing four 1 mL aliquots of the suspension. MnO$_2$ was aliquoted and stored at -80 °C until use. Prior to use, the MnO$_2$ suspension was thawed, vortexed and sonicated. MnO$_2$ synthesized by this method was previously determined to have specific surface area of 333.28 m^2 g^{-1} (Gao, 2007)

5.2.3. Reaction of PrP^{Sc} with MnO$_2$

PrP^{Sc} reacted with suspended MnO$_2$ under ambient O$_2$ conditions at room temperature for the indicated time periods. Except for experiments

examining the effect of pH, reactions were conducted in 20 mM sodium acetate (pH 4.0). To examine the effect of pH on PrP^{Sc} degradation, solutions were buffered with 20 mM sodium acetate (pH 4.0 and 5.0), 20 mM 4-Morpholineenthanesulfonic acid (MES; pH 6.0), or 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; pH 7.0 and 8.0). The final sample volume was 40 µL. Reactions were halted by dissolving the MnO$_2$ with 25 µL of 500 mM EDTA (pH 8.0) at 80 °C. For control experiments, MnO$_2$ was dissolved prior to addition of PrP^{Sc}. In preparation for analysis, 5 µL of 1 M Tris-HCl (pH 8.0) was added to the sample. For immunoblot analysis, 20 µL of sample buffer (100 mM Tris pH 8.0, 10% SDS, 7.5 mM EDTA, 100 mM dithiothreitol, 30% glycerol) were added, and samples were heated for 10 min at 100 °C.

5.2.4. **Protein misfolding cyclical amplification (PMCA)**

The PMCA detection of PrP^{Sc} was accomplished using a modification of the method described by Saa et al. (2006). Briefly, healthy, uninfected hamsters were perfused with phosphate buffer saline (PBS) containing 1 mM EDTA, and brains were harvested, frozen in liquid nitrogen and stored at -80 °C until homogenization. Brains were homogenized in PMCA buffer [10% w/v in PBS, 1% Triton X-100, 0.5% digitonin and Complete™ protease inhibitor cocktail (Roche, Switzerland)]. Homogenates were clarified by 5 min centrifugation at 850 *g*, and supernatants were aliquoted and frozen at -80 °C until use.

The effectiveness of the technique to amplify PrP^{Sc} has been tested by using Hyper-infected BH (10% w/v in PBS) as a source of PrP^{Sc} and uninfected hamsters BH as a sources of PrP^{C} (Figure 5.1). After a brief

sonication, ten-fold dilutions of the agent were made in freshly thawed clarified uninfected brain homogenate in PMCA buffer. Sonication was performed at 37 °C for 120 cycles. Each cycle consisted of 10 s sonication at 80% power followed by 30-min incubation. As a control, a matched dilution series was incubated at 37 °C without sonication. After completion of the PMCA cycling, 50 μL of each sample were treated with 50 μg mL^{-1} of Proteinase K (PK) and enriched using PTA precipitation. Samples were heated to 100 °C in 20 μL SDS-PAGE buffer and 20 μL 2% Sarkosyl in PBS for 15 min and sonicated for 10 s at 100% power. An 8-μL aliquot was separated on a 10% Bis-Tris gel, and immunoblotted as reported in the Paragraph 5.2.5 (Figure 5.1).
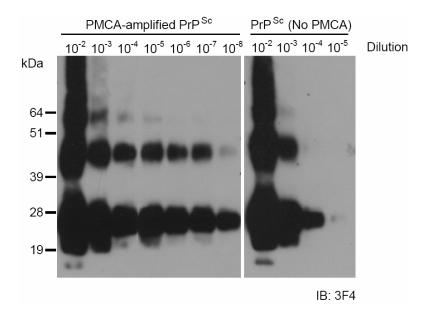

Figure 5.1. PMCA Amplification of PrP^{Sc} from BH.

For PMCA analysis, PTA-purified PrP^{Sc} (50 μg mL^{-1}) was incubated with 5.6 mg mL^{-1} MnO$_2$(s) or EDTA-dissolved MnO$_2$ using the conditions

described above. Series of 10 fold dilutions of samples and controls in freshly thawed clarified brain homogenate were made in 96 well PCR plates.

PMCA was carried out on a Misonix model 3000 sonicator (Farmingdale, NY, USA) equipped with a microplate horn for 120 cycles at 37 °C. Each cycle consisted of 10 s sonication at 80% power followed by 30 min incubation.

Following PMCA, 50 μL samples were mixed with 50 μL of 4% N-lauroyl-sarcosine in PBS and 2 μL of 2 mg mL^{-1} PK and incubated for 1 h at 37 °C. PK digestion was halted by addition of 1 μL phenylmethylsulfonyl fluoride (PMSF) saturated ethanol. Phosphotungstic acid (PTA) enrichment of PrP^{Sc} was carried out as described above. Samples were prepared for Western blot by re-suspension of the pellet in 20 μL of 2% Sarkosyland 20 μL 10× SDS-PAGE buffer, brief sonication and 15 min heating at 100 °C.

5.2.5. SDS-PAGE and Immunoblotting

Proteins from degradation experiments were separated under reducing conditions by SDS-PAGE (4-20% gradient) (BioRad, Hercules, CA, USA) or 10% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) for PMCA experiments, transferred to polyvinyl difluoride membranes, and immunoblotted with PrP-specific antibodies: monoclonal antibody (mAb) 3F4 (1:40,000 dilution) and full-length polyclonal antibody (pAb) Rab 9, pool 2 (1:10,000). Detection was achieved with HRP-conjugated goat anti-mouse and anti-rabbit immunoglobulin G, respectively.
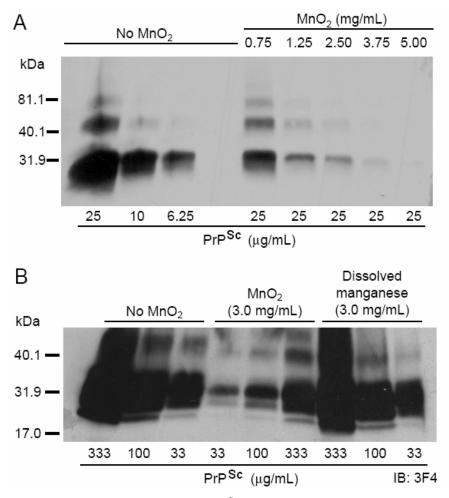
5.3. Results

The effect of MnO$_2$ on the stability of PrP^{Sc} was assessed using an *in vitro* system. Degradation of PrP^{Sc} was assessed by immunoblotting residual protein following co-incubation with and subsequent dissolution of MnO$_2$. Dissolution of the solid-phase oxidant liberated any protein sorbed to mineral surfaces and allowed measurement of all remaining protein in the sample. In preliminary experiments we found that ascorbic acid, citric acid and EDTA (used in all subsequent experiments) could each be used to dissolve MnO$_2$, and had no effect on PrP^{Sc} levels or immunodetection (data not shown).
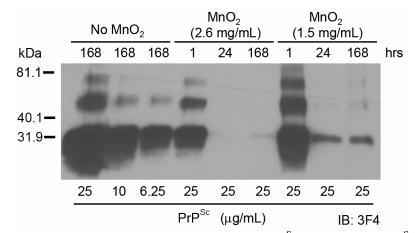
5.3.1. Birnessite-mediated degradation of PrP^{Sc}

Following 16 h incubation with MnO$_2$, PrP^{Sc} immunoreactivity declined in a manner proportional to the amount of MnO$_2$ in suspension (Figure 5.2 A). At the highest concentration of MnO$_2$ tested (5 mg mL^{-1}), PrP^{Sc} immunoreactivity decreased below the limit of immunoblotting detection. The unstructured N-terminal portion of PrP^{Sc} is susceptible to degradation by proteases and other treatments; removal of the N-terminus leaves a 27-30 kDa infectious core (Bolton et al., 1982; Riek et al., 1996). When lower concentrations of MnO$_2$ were used, the PrP detectable immunoblotting signal appeared similar in size to that in the unexposed controls, suggesting that MnO$_2$ does not selectively cleave the N-terminus (Figure 5.2 A). Analysis of protein degradation near the limit of immunoblotting detection may overestimate the extent of proteolysis. We therefore substantially increased the amount of PrP^{Sc} added to each reaction to estimate the extent

to which MnO_2 would be able to degrade larger amounts of protein (Figure 5.2 B) and found that 3 mg mL^{-1} of MnO_2 was capable of degrading a considerable fraction of the protein. As a control, we dissolved the MnO_2 prior to incubation with PrP^{Sc}. The immunoreactivity present in the dissolved manganese samples was substantially more intense than in the sample containing solid MnO_2 and approximately equal to the starting material (Figure 5.2 B). These data indicate that the presence of MnO_2, but not Mn^{2+} and EDTA, is responsible for the loss of immunoreactivity and that 3 mg mL^{-1} of MnO_2 causes substantial loss of immunoreactivity.

The duration of exposure to the MnO_2 influenced the extent of loss of immunoreactivity (Figure 5.3). A 1 h exposure to 1.5 or 2.6 mg mL^{-1} MnO_2 had a limited effect on the protein, whereas 24 and 168 h exposures caused substantial declines in PrP^{Sc} signal. Comparable amounts of protein remained after 24 and 168 h exposure to 1.5 mg mL^{-1} MnO_2, suggesting no further reaction (Figure 5.3). The most probable explanation for the MnO_2-dependent loss of PrP^{Sc} immunoreactivity is degradation of the protein. Dissolution of the birnessite at the completion of exposures excludes the possibility that losses in immunoblotting signal are attributable to sorption of PrP^{Sc} to mineral surfaces. In the analyses described above, we used the monoclonal antibody 3F4 directed against a single epitope on hydrophobic core of the PrP molecule (residues 109-112) (Kascsak et al., 1987).

Figure 5.2. MnO_2 mediates the PrP^{Sc} degradation. (A) MnO_2 causes dose-dependent decline in PrP^{Sc} immunoreactivity. PrP^{Sc} (25 µg mL^{-1}) reacted overnight with the indicated amount of MnO_2. Each concentration of MnO_2 decreased PrP levels, and 5.0 mg mL^{-1} of MnO_2 reduced immunoreactivity below the level of immunoblotting detection. (B) MnO_2, but not dissolved manganese, degrades PrP^{Sc}. The indicated doses of PrP^{Sc} were exposed overnight to 3.0 mg mL^{-1} MnO_2 or an equivalent amount of dissolved manganese oxide. Samples lacking manganese served as controls. Dissolved manganese had no detectable effect on PrP^{Sc} immunoreactivity whereas MnO_2 degraded the protein. All immunoblots used mAb 3F4.
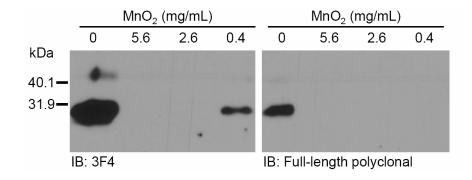
Figure 5.3. Time course of MnO$_2$-mediated PrP^{Sc} degradation. PrP^{Sc} (25 μg mL^{-1}) was reacted with two concentrations of MnO$_2$ (1.5 and 2.6 mg mL^{-1}) for 1, 24 or 168 h (7 days). A MnO$_2$-free control was processed in parallel and sampled at 168 h. Both concentrations of MnO$_2$ caused substantial degradation of the protein following 24 or 168 h of incubation, but had less effect at the 1 h time point. A 24 h incubation period appears sufficient to achieve maximal degradation. Immunoblots used mAb 3F4.
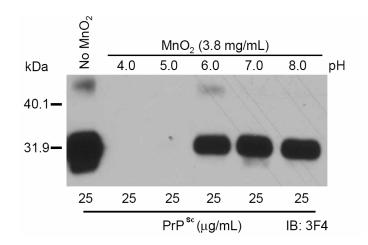
To exclude the possibility that the observed declines in immunoreactivity were not merely an effect of MnO$_2$ on the 3F4 epitope, rather than more complete degradation of PrP^{Sc}, we repeated the above experiments then compared the immunoreactivity remaining using two antibodies, 3F4 and a polyclonal antibody directed against full-length PrP (Rab 9, pool 2). The polyclonal antibody is composed of multiple immunoglobins specific to epitopes along the PrP molecule. After treatment of PrP^{Sc} with 0.4 to 5.6 mg mL^{-1} MnO$_2$, no immunoreactivity against Rab 9 remained and evidence of lower molecular mass breakdown products was lacking (Figure 5.4). The sensitivity of mAb 3F4 was superior to that of the polyclonal antibody as some residual PrP immunoreactivity was detected in the sample incubated with 0.4 mg mL^{-1} MnO$_2$. These data are consistent with the idea that MnO$_2$

degrades PrP^{Sc} by breaking the polypeptide backbone of the protein, but do not exclude the possibility that MnO$_2$ also alters amino acid side chains.

Figure 5.4. Degradation of PrP^{Sc} by MnO$_2$ affects the entire protein. PrP^{Sc} (25 μg mL^{-1}) was incubated overnight with the indicated amounts of MnO$_2$, and the remaining protein was analyzed by immunoblotting using either mAb 3F4 (epitope consisting of residues 109-112) or a PrP-specific pAb generated against the full-length protein. Both immunoblots indicated degradation of PrP^{Sc} by MnO$_2$.

Degradation of organic molecules by MnO$_2$ typically shows a pronounced pH dependence (Stone and Morgan, 1984). We examined the MnO$_2$-mediated degradation of PrP^{Sc} as a function of pH over the range relevant for most natural soils (pH 4 to 8) (Figure 5.5). Under the experimental conditions employed (25 μg mL^{-1} exposed to 3.8 mg mL^{-1} MnO$_2$ for 16 h), PrP^{Sc} levels dropped below the limit of immunoblotting detection when reactions were performed at pH 4.0 or 5.0. Substantial PrP^{Sc} immunoreactivity remained following reactions at pH $\geq$ 6.0.
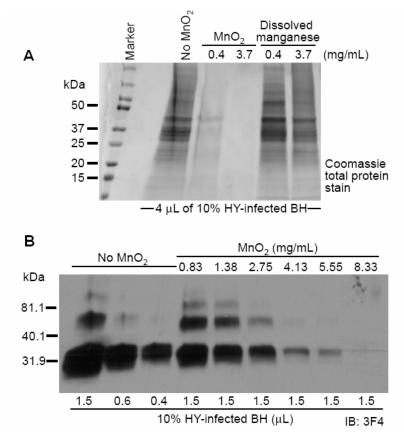
Figure 5.5. Low pH favours MnO_2-mediated degradation of PrP^{Sc}. PrP^{Sc} (25 μg mL^{-1}) was reacted with 3.8 mg mL^{-1} of MnO_2 for 16 h at the indicated pH values (4.0-8.0). An equal amount of PrP^{Sc} was incubated in the absence of the mineral at pH 4.0 as a control. Solutions at pH 4.0 or 5.0 promoted extensive MnO_2-mediated degradation, whereas in higher pH solutions PrP^{Sc} immunoreactivity was only slightly diminished. Immunoblot used mAb 3F4.

5.3.2. Degradation of PrP^{Sc} in brain homogenates (BH)

Pathogenic prion proteins may be released into the environment in secretions (e.g. saliva), excreta (e.g., urine) or from decomposing animal tissue (e.g., carcasses, placenta) (Schramm et al., 2006). In the case of decomposing animal tissue, PrP^{Sc} enters the environment with a complex mixture of biomolecules. In Figure 5.6, we assessed the degradation of infected BH by MnO_2 by measuring (A) total residual protein or (B) PrP^{Sc}. Following incubation with 0.4 mg mL^{-1} MnO_2, little remaining protein was detected by staining with Coomassie brilliant blue, and 3.7 mg mL^{-1} of MnO_2 decreased protein to undetectable levels (Figure 5.6 A). Dissolution

of MnO_2 prior to incubation with the BH failed to substantially decrease protein levels.
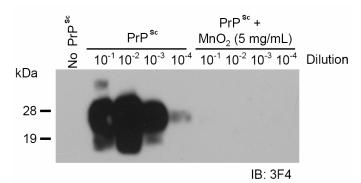
Figure 5.6. MnO_2 degrades most proteins present in infected BH (A), including PrP^{Sc} (B). (A) Infected BH (4 µL, 10% w/v) was reacted overnight with the indicated concentrations of MnO_2. Samples lacking MnO_2 or containing dissolved manganese served as controls. All samples were subjected to SDS-PAGE, and proteins were stained with Coomassie brilliant blue. MnO_2 at 0.4 mg mL^{-1} degraded most brain proteins, and increasing the dose to 3.7 mg mL^{-1} reduced protein levels below the limit of detection. (B) Infected BH (1.5 µL, 10% w/v) was incubated with the indicated concentrations of MnO_2, and PrP^{Sc} degradation was assessed by immunoblotting with mAb 3F4. The highest tested MnO_2 concentration (8.33 mg mL^{-1}) decreased PrP^{Sc} levels below the limit of immunoblotting detection.

The amount of PrP^{Sc} present in infected BH was also diminished by exposure to MnO$_2$ (Figure 5.6 B). Compared to experiments using purified pathogenic prion protein, more MnO$_2$ was needed to degrade the PrP^{Sc} in BH (cf. Figures 5.2 A and 5.6 B). This may be due to the reductive dissolution of MnO$_2$ as it reacts with other biomolecules in BH and/or to the fouling of oxide surface by adsorbed biomolecules.

5.3.3. Quantification of residual PrP^{Sc} by PMCA

The starting PrP^{Sc} material was diluted up to the limit of immunoblotting detection to allow comparison with samples lacking immunoreactivity. Immunoreactivity of PrP^{Sc} starting materials was still detectable after 200-fold dilution (data not shown). Samples lacking immunoreactivity, such as PrP^{Sc} treated with 5 mg mL^{-1} MnO$_2$ (Figure 5.2 A), therefore, contain at least 200-fold less PrP^{Sc} than the starting material.

To more quantitatively assess the loss of PrP^{Sc}, PMCA assay to measure the amount of PrP converting activity present in selected MnO$_2$-treated samples was used. PMCA sensitively detects prions by measuring the ability of PrP^{Sc} in a sample to convert PrP^{C} to a PK-resistant form (Saa et al., 2006). Converting activity of the PTA-purified PrP^{Sc} starting material used in degradation experiments was present over at least four 10-fold dilutions (Figure 5.7). To be best of our knowledge, this is the first report of PMCA amplification of PTA-purified PrP^{Sc}. The sensitivity of PMCA amplification is reduced when PTA-purified PrP^{Sc}, rather than infected BH, is used as a source of agent (Figures 5.1 and Figure 5.7), but amplification does occur. A potential explanation of this result is increased aggregation of the PTA-purified agent limiting PrP^{C} conversion.

When samples were treated with 5.6 mg mL^{-1} MnO$_2$, no converting activity remained (Figure 5.7). The limit of detection for PMCA is defined by the dilutions of the PTA-purified starting material (*vide supra*). These results therefore indicate that 5.6 mg mL^{-1} MnO$_2$ decreased converting activity by at least four orders of magnitude.

Figure 5.7. Quantification of PrP^{Sc} degradation by MnO$_2$ using PMCA. Dilution series of PrP^{Sc} (50 µg mL^{-1}) starting material and PrP^{Sc} treated with 5.6 mg mL^{-1} MnO$_2$ were subjected to PMCA amplification, and immunoreactivity was detected by SDS-PAGE and immunoblotting. Immunoreactivity was observed in all dilutions of the starting material (dilution factors of 10^{-1}-10^{-4}), but no PrP was detected in MnO$_2$-treated samples, indicating prion converting levels have reduced by more than a factor of 10^{-4}. The presence of dissolved manganese oxide in the reaction did not affect conversion efficiency (data not shown).

5.4. Discussion

Abiotic processes influence the environmental fate of many contaminants. The data presented here suggest that MnO$_2$ can degrade PrP^{Sc} in soil environments.

Investigations reported in the Chapter 3 and in Rao et al. (2007) addressed the interaction of recPrP with polymers formed by catechol polymerization by birnessite. In this study, MnO$_2$ degradation of the protein was not

investigated. The ratio of recPrP and MnO_2 used were, however, too high to allow sensitive abiotic prion degradation. Moreover, recPrP is not suitable to abiotic degradation tests because of its sensitivity to proteases (Rapp et al., 2006). Previous studies reported loss of protein from solution when incubated with MnO_2 (Naidja et al., 2002; Rao et al., 2007). The reason for the observed losses was ascribed to sorption to MnO_2 surfaces (Naidja et al., 2002; Rao et al., 2007). To our knowledge, the results here reported are the first demonstration of abiotic protein degradation by MnO_2. Treatment of BH with MnO_2 (Figure 5.6) indicates that protein degradation is not specific to PrP^{Sc}, but that MnO_2 is capable of degrading most, if not all, brain proteins.

Birnessite-mediated PrP^{Sc} degradation exhibited pronounced pH-dependence (Figure 5.5). The redox potential and surface charge of MnO_2 increase as pH declines (Bricker, 1965). Solution pH may also impact PrP^{Sc} degradation by influencing the degree of protein sorption to MnO_2 surfaces. As the point of zero charge for MnO_2 occurs at pH 2.25 (Murray, 1974), 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 decreases at pH > pI because of repulsive electrostatic interactions (Quiquampoix et al., 2002). The apparent average isoelectric point of PrP^{Sc} aggregates is ~4.6 (Ma et al., 2007); the protein aggregates possess a net neutral 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; Pizzigallo et al., 1998). The increase in degradation near the isoelectric point of PrP^{Sc} is consistent with the importance of protein sorption to MnO_2 in the overall protein degradation process.

Degradation of prions released into soil environments could be expected in soil environments rich in MnO_2, including young, currently and formerly poorly drained or sediment-rich soils (Post, 1999; Tebo et al., 2004). Our results indicate that soil pH may substantially influence the degradation of prions by MnO_2: acidic soil conditions will promote MnO_2-mediated PrP^{Sc} degradation. Under the experimental conditions used in our study, MnO_2-mediated degradation of PrP^{Sc} occurs over relatively short periods (Figure 5.3) and reduces PrP^{Sc} converting activity by at least a factor of 10^4 (Figure 5.7). These data suggest that under appropriate soil conditions, MnO_2 can rapidly reduce PrP^{Sc} levels. Our findings also suggest that MnO_2 may be employed as a reactive burial material in the disposal of prion-infected materials. Use of MnO_2 for the decontamination of prion-contaminated soils may warrant investigation.

Observations that Mn^{2+} can cause PrP^C to adopt a β-sheet-enriched conformation *in vitro* (Brown, 2001) has led researchers to hypothesize that a dietary imbalance in copper and in manganese may contribute to prion disease development. High soil manganese levels coupled with reduced copper content has been hypothesized to promote TSE development via dietary imbalance and prion protein misfolding (Purdey, 2000). Attempts to restrict bovine dietary copper while maintaining normal manganese intake or supplementing manganese levels (Legleiter et al., 2007) do not alter physicochemical characteristics of the prion protein. The relationship between soil or forage manganese and copper levels and TSE incidence is unclear. Some studies reported a correlation between the location of TSE clusters and soil or forage manganese or copper (Purdey, 2003; Gudmundsdottir et al., 2006; Ragnarsdottir et al., 2006), while other investigators failed to identify such a link (Chihotaet al., 2004; McBride, 2007). Our data suggest that soils containing manganese oxides (the most

common form of oxidized Mn in soils (Tebo et al., 2004) may reduce the probability of environmental TSE transmission. Future studies attempting to correlate TSE incidence with soil manganese levels should examine the mineral form of the element in addition to total Mn concentration.

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Chapter 6

Conclusions

The purpose of the present research was to study some of the processes undergone by prion proteins in soil. The study was mainly devoted to investigate the role of soil organic matter in the interaction and immobilization of prions in soil. Indeed, the knowledge of the interactions occurring between prion protein and soil, with particular reference to organic colloids, can provide useful information about the fate of this agent in the environment.

The capability of a soil strong oxidant like birnessite to degrade the real infectious prion protein putative of TSE (PrP^{Sc}) was also investigated.

Involvement of a recombinant prion protein in humic substances in formation was investigated by using two synthetic model systems i) organo-mineral complexes, with humic-like substances obtained by polymerization of catechol by birnessite, ii) organic complexes, in which the humic-like substances were obtained by enzymatic polymerization of catechol in the absence of any mineral component. The direct interaction with stabilized natural humic substances was also evaluated.

Either the full-length recombinant prion protein or its N-truncated form strongly and irreversibly interacted with organo-mineral complexes. In fact, complete immobilization of the protein and no extraction from the complexes was observed. The N-truncated form of the recPrP interacted in lower amounts with soluble organic polymers than the full-length protein, suggesting a specific role of the unstructured positively charged amino

terminal arm of the protein in the interactive process. The high stability of the interactions of both the recombinant prion forms in organo-mineral complexes was attributed to the presence of the solid mineral component as well as the involvement of the protein in the catechol polymerization process.

Interaction with organic complexes in the absence of solid mineral contributed to clarify the role of the sole organic matter. Biotic polymerization of organic monomers in the presence of prion protein determined its involvement and thus its immobilization, resulting also not extractable despite of the lack of the mineral component.

Comparative experiments to the biotic polymeric systems were then conducted with natural humic acids to better understand on one hand the importance of involvement of prion protein in polymeric material formation and on the other hand the direct interaction with stabilized soil organic matter. Humic acids allowed high, although not complete, adsorption of the recPrP, and considerable amounts of recPrP were more easily extractable.

Soil organic matter as humic acids has a high capability of binding prion proteins and the interactions of these proteins can be even stronger when protein is involved in organic matter formation processes. The strength of binding between prion proteins and soil organic colloids was enhanced by new organic matter arriving in soil, that in turn act stabilizing other interactions, like with humic substances themselves as supported by the achieved results. Prion proteins can result through these mechanisms bound to soil in the superficial organic rich layers and as such more available to free ranging animals.

The capability of a strong soil oxidant as birnessite to degrade the disease associated prion protein PrP^{Sc} was also studied. Degradation of PrP^{Sc} was

reached in several experimental conditions simulating those likely encountered in real environmental conditions.

Overall the results achieved in the present work have contributed to shedding light on the interaction of prion protein with organic- and organo-mineral complexes simulating soil organic and organo-mineral colloids. It is important, to confirm these results by comparative experiments with the real infectious prion protein. Furthermore, evaluating infectivity of PrP[Sc] immobilized in organo and organo-mineral complexes are needed to understand the final result of these interactions.

Moreover, findings obtained in the direct interaction between birnessite and the infectious protein putative of TSE seem to suggest that birnessite may be employed as a reactive burial material in the disposal of prion-infected materials. Birnessite, being a natural occurring mineral, could be used for the decontamination of prion-contaminated soils and reducing the disease diffusion. While the majority of studies regarding the fate of prions in soil suggest that soil can only enhance the diffusion of TSE, the presence of such components could act reducing the environmental risk related to prions.

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