# Towards unfolding the prion misfolding mystery: Protein free radical chemistry in transmissible spongiform encephalopathies

YANG Chi-Ming

*( Neurochemistry Group, College of Chemistry, Nankai University, Tianjin 300071;*

*Institute for Life Sciences & Health, La Jolla (San Diego), CA 92039-2035, USA)*

**Abstract** Owing to the high oxygen-respiration in the brain of mammals, oxidative damage to prion protein has been suggested to be an additional factor. A large body of intriguing features of scrapie and prion diseases have provided multiple lines of indirect chemistry evidence, suggesting that the infectious agents may be putative forms of sequence-specific prion radicals (SSPR) and /or their immediate precursors in the transmissible spongiform encephalopathies (TSE). Here a molecular mechanism corresponding to the self-replication of scrapie protein mediated by prion free-radical processes, consonant with "protein-only" hypotheses is proposed. This new theory may not only aid our understanding of the occurrence of prions, but also provides new insight into the possible chemistry principles underlying the neurodegenerative disorders. It is anticipated that future studies based on this suggestion and chemistry principles of genetic diseases may allow us to determine an effective approach to stop mad cow disease and its human version, new variant of Creutzfeldt-Jakob disease (v CJD).

**Keywords** Sequence-specific prion radicals (SSPR), Transmissible spongiform encephalopathy (TSE), Genetic diseases, Creutzfeldt-Jakob disease (CJD), Reductive zipper motif, Protein-radical process

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## 1 Introduction

Recently, a multi-disciplinary suggestion of "prion radicals" as a putative and opportunistic pathogen[1,2] promoted research on the chemistry of prion and spur attempts to emulate it in several laboratories, meanwhile, more recent studies demonstrated that the octarepeats may be oxidized by both copper ion (II) and reactive oxygen species.[2-7]

But how to draw an equation between prion pathogen in biology and a protein free radical process in chemistry, here is a multi-disciplinary discussion.

## 2 The old question: prion diseases and scrapie

Prion diseases are mammalian neurodegenerative disorders caused by prions, a notable subviral pathogen that is believed to be devoid of nucleic acids.[8,9] The diseases occur as three types of incidences, i.e., sporadic, inherited and infectious, and includes scrapie in sheep, transmissible bovine spongiform encephalopathies (BSE or mad cow disease) in cattle and Creutzfeldt-Jakob Disease (CJD) in humans.[9,10] Over the past decades, there has been a renewal of interest in this subject, since both epidemiological study and laboratory experiments suggest a strong linkage between BSE and a new variant of human Creutzfeldt-Jakob disease (v CJD),[11] which is regarded as a human form of BSE. More recently, a dramatic increase in both British and French BSE has been observed, with up to over 90 cases of v CJD found in UK and meanwhile, up to 5 people in France succumbed to v CJD. It is therefore of particular importance to elucidate the molecular mechanism in

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prion pathogenesis and define the causative factor before preventative measures and effective therapeutic intervention could be taken.

Scrapie in sheep has been observed for more than 250 years. A mathematician[12] initially postulated "protein-only" as a highly speculative hypothesis to explain scrapie replication, and it has since been a prevailing view. Since prion research has emerged as a multi-disciplinary subject, there has been little dispute over the central role of prion protein in the disease. Three major research camps, i.e., biologists, molecular geneticists, and protein misfolding scientists, have focussed their research efforts on the unknown molecular mechanism how a normal prion protein ($PrP^{C}$) is structurally converted to the diseased form ($PrP^{Sc}$ ).[8,9,13] But until now, progress in delineating the unknown pathogenic mechanism has been hampered in several aspects. First, protein misfolding theory, supported by presumably equivocal evidence in prions, is contradictory to the long-established Anfinsen's protein folding hypothesis, in which it states "one protein, one conformation" under a certain physiological condition.[14] Second, misfolding theory is inconsistent with the phenomenon as more than 20 types of prion strains, together with a large discrepancy in the correlation between the amount of diseased protein ($PrP^{Sc}$) and infectivity titer, have been observed.[10,15] Third, a "virino" or unconventional virus hypothesis,[10,16] as an alternative, is not supported by any data for an involvement of virus-associated nucleic acids.[9]

## 3 Biochemistry of prions – it is not just the folding

Whereas prion has been a subject of intense scrutiny, a clearer understanding of its molecular pathogenic mechanism has been hindered mainly by the unknown structures of infectious prions, which is in a non-homogeneous, insoluble and multimeric form of protein aggregates.[8] The findings of more than 20 types of infectious prion strains (structures), as mentioned previously, together with coexistence of three types of prion disease incidences and many intriguing features of prions prompt us to have examined prion puzzle from a different angle.[1] Facing the prion protein sequence which contains multiple reductive amino-acid residues, a physical organic chemist will be immediately interested in whether the prion protein structural transformation is a biochemical or biophysical process, as it is known that a biochemical process involves covalent bond-breaking and/or formation while a biophysical process does not involve any covalent bond modification.[1]

Recall that earlier work indicated that prion infectivity would not be lost even after the prion agent in dried brain had been allowed to stay in air for up to two years.[17] Besides, it is well known that the prion agent is unusually resistant to gamma and UV (250 nm) irradiation, heat (80~100 °C) and harsh chemical treatment which normally kill viruses.[16] Recent investigation revealed that solubilization of prion solid is always accompanied by the loss of infectivity.[9] To free-radical mechanistic chemists, these unusual features of the transmissible agent may imply that infectious portion in prions may be protein free radicals or their precursors such as some oxidized forms of a protein. Protein free radicals or oxidized forms of a protein can be very stable when occluded in scrapie solid and may be capable of function only in the brains of mammals, where some vital and unique factors shall facilitate their infectious process. One of the most likely factors may be the high oxygen-respiration in brains.

Using modern molecular biology technique, scrapie protein has now been excellently identified and sequenced.[9,18] It is interesting, to mechanistic chemists, whether a protein molecule comprised of more than 10 Tyr, more than 10 His, and multiple Trp residues are chemically stable towards reactive oxidative species (ROS). In fact, it has been widely demonstrated that side-chains of all these three amino-acid residues are predisposed to oxidative damage by ROS.[1,19-21] In brain tissue there is a remarkably high oxygen-respiration, for instance, the human brain constitutes only about 2% of the adult body mass, but about 20% of its resting oxygen consumption of the whole body, independent of the state of mental activity. Accordingly, a protein free-radical reaction mechanism was recently suggested in prions, indicating that sequence-specific prion radicals (SSPR) together with protein cross-link may play a pivotal role in the infectious diseases.[1,22,23] Consequently, "protein-only"

notion without virus-associated nucleic acids in the infectious process may have been theoretically validated. However, classical biologists may have had difficulties in understanding the essential free-radical chemistry principles, I here describe a more detailed picture, for the importance of neurochemistry and chemistry basis of genetic diseases in the post-genome era.

## 4 A new approach to the old question: free radical oxidative damage to proteins

A free radical species is usually a short-lived chemical intermediate, defined as an identity (a molecule or an atom) which carries an unpaired electron.[24] It normally attacks other labile molecules via hydrogen-atom abstraction or free-radical addition to unsaturated (double or triple) bonds.[25-27] Free radicals such as hydroxyl radical (HO•) can therefore be very damaging towards labile biomacromolecules such as proteins.[19,20] The resulted protein radicals, as primary intermediates, may subsequently attack others both inter- and intra-molecularly in an autocatalytic manner.[28-30] Small free radicals such as hydroxyl radicals will exhibit no sequence-specificity in attacking other molecules, but it is conceivable that macromolecular free radicals, for example, a protein radical, is capable of displaying partial sequence-specificity in damaging other biomacromolecules, for a pre-requirement of sequence-specific protein-protein interaction prior to the free-radical attack.[1,23]

Free radical oxidative damage to proteins has long been implicated in the pathogenesis of many neurodegenerative disorders including Alzheimer's disease.[19,20,31-36] The pathogenic oxidative-damage is presumably mediated via rapid attack on proteins by ROS such as hydroxyl radicals, which are generated by Fenton-type reactions. Recently, a consensus "i-4, i, i+4" (chemical) reductive zipper motif has been identified from both prion protein sequence, beta-amyloid peptide sequence and free radical enzyme proteins, suggesting a possibly common molecular mechanism underlying the initiation stages of sporadic Alzheimer's disease and both sporadic and genetic prion diseases.[37] Moreover, biophysical study revealed a tight and co-operative binding of the normal mammalian prion protein with copper (II) ion,[38] implying its feasibility in generating ROS, via a number of metal ion-mediated redox chemical processes under certain physiological conditions.

## 5 Prion radicals – a vital and opportunistic subviral pathogen

### 5.1 Initiation of scrapie prion radicals

A prion protein free radical is formed via hydrogen-atom abstraction from a prion protein molecule by a hydroxyl radical, presumably at the tyrosine residues. Due to the reactive properties of free-radical intermediates, these protein radicals may subsequently damage the oxidatively labile prion protein both intramolecularly and intermolecularly, presumably accompanied by cross-link and production of secondary (new) protein radicals. Based on the unusual stability of the prion infectious properties, it may reasonably be suggested that putative forms of sequence-specific prion radicals may be responsible for the infectivity of prion particles.[1,23] In other words, the immediately causative factor may be in a free-radical form, or the precursor of the free-radical form of scrapie prion protein, which would be physically impossible to be separated from the neutral form of scrapie prion. This prion radical may be expected to be sequence-specific in both binding and damaging other normal prion proteins.[1] The difference between $PrP^{Sc}$ and $PrP^{Sc}$ radical, i.e., $PrP^{Sc}$•, is one hydrogen-atom or one electron. Shown below is the formation of simple forms of prion radicals without discussion of their detailed three-dimensional structures:

$$[PrP\text{-}H] + [HO\bullet] \rightarrow [PrP\bullet] + H_2O$$

(hydrogen-atom abstraction by ROS)

$$[PrP] - e \rightarrow [PrP\bullet^+]$$

(one-electron oxidation reaction by ROS)

### 5.2 Prion radical-mediated replication and one-electron difference puzzle

The requirements for establishing a free-radical reaction mechanism mainly include supportive evidence for a chain propagation, and inhibition of the reactions by free radical inhibitors or scavengers.[24-27] Interestingly, it seems that these major requirements

are available from prion research, including the renowned autocatalytic propagation of the diseases and inhibition of prion by electron-rich molecules including dye Congo red, polyanions and poly aromatics.[1,9,10] In addition, prion replication and a free radical chain reaction display kinetic similarity.[1]

Hence, an infectious mechanism underlying prion structural transformation via the proposed transmissible agent, prion radicals ($PrP^{Sc}$•), is as follows, where *A* represents a human species, and *B* represents cow, respectively (also see Fig.1).

$$[PrP^{Sc(B)}]+[HO\bullet]\xrightarrow{\text{activation}}[PrP^{Sc(B)}\bullet]+H_2O$$

$$[PrP^{C(A)}]+[PrP^{Sc(B)}\bullet]\xrightarrow[\text{association}]{\text{binding}}[PrP^{C(A)}]/[PrP^{Sc(B)}\bullet]$$

$$\xrightarrow{\text{free-radical transfer}}[PrP^{C(A)}\bullet]/[PrP^{Sc(B)}]\xrightarrow{\text{structural change}}$$

$$[PrP^{Sc(A)}\bullet]/[PrP^{Sc(B)}]\xrightarrow{\text{dissociation}}[PrP^{Sc(A)}\bullet]+[PrP^{Sc(B)}]$$

In this prion radical chemistry mechanistic model, $r = k\,[PrP^{C}][PrP^{Sc}][\bullet OH]$, where *r* is disease propagation rate (i.e. rate of infectious prion propagation); *k*, propagation constant; $[PrP^{C}]$, concentration of normal (cellular) prion protein; $[PrP^{Sc}]$, concentration of diseased prion protein; [•OH], a level of endogenous reactive oxidative species (ROS). A corresponding schematic free energy diagram is shown in Fig.2.

The diseased form of a prion protein, $PrP^{Sc\,(B)}$, is initially activated by ROS in vivo, to its free radical form, $PrP^{Sc\,(B)}$•. This prion radical then binds with a normal prion, $PrP^{C\,(A)}$, of the infected host, to form a prion heterodimer $[PrP^{C(A)}]$ / $[PrP^{Sc(B)}\bullet]$. Subsequently, a free radical transfer reaction may take place between $PrP^{C(A)}$ and $PrP^{Sc(B)}$• within the dimers to form new dimers $[PrP^{C(A)}\bullet]/[PrP^{Sc(B)}]$, depending on the sequence-compatibility between the two protein molecules. The feasibility of this free-radical-transfer reaction is reflected by a species barrier and crossing the barrier in mad cow disease. This newly formed dimers, $[PrP^{C(A)}\bullet]/[PrP^{Sc(B)}]$, may then undergo an irreversible free radical-induced structural transformation to form structurally damaged dimers, $[PrP^{Sc(A)}\bullet]/[PrP^{Sc(B)}]$, which are thermodynamically more stable. In the structurally transformed dimers, $[PrP^{Sc(A)}\bullet]/[PrP^{Sc(B)}]$, both of the protein molecules are in their scrapie forms: one is a neutral scrapie prion, the other is scrapie prion radical. The newly formed scrapie prion radical, $PrP^{Sc(A)}\bullet$, may further induce other normal prion proteins to scrapie prion proteins.

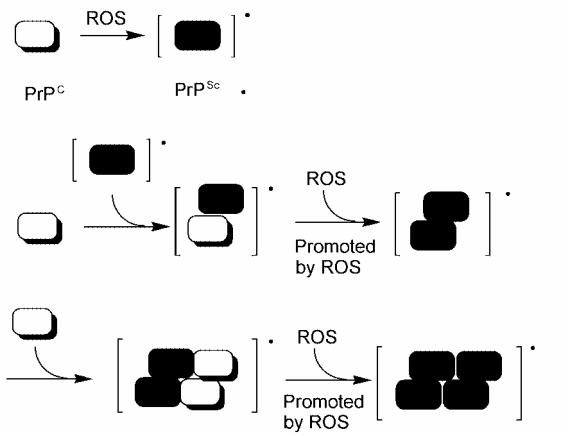


**Fig.1** A putative molecular mechanism for a structural transformation from normal prion protein ($PrP^{C}$) to diseased prion protein aggregates ($PrP^{Sc}$), mediated by both ROS (reactive oxygen species) and prion protein radicals.

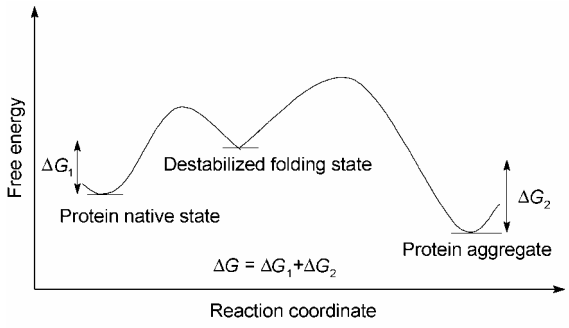


**Fig.2** A schematic free energy diagram for going from normal prion protein to diseased prion protein aggregates.

Thus, it can be understood that prion disease occurrence may be initiated by both ROS and acquired scrapie prion radicals, $PrP^{Sc(B)}$•. And the disease is promoted by several variables, which include amounts of acquired infectivity, i.e. scrapie prion radicals $PrP^{Sc}$•; appropriate sequence compatibility between $PrP^{C(A)}$ and $PrP^{Sc(B)}$•; and levels of ROS in host.

### 5.3 Why it is difficult to accept a free-radical mechanism

Systematic understanding the pivotal role of a free radical process in biological system is often con-

strained by limited visualization ability in laboratories. Moreover, strictly speaking, a molecular mechanism can never be proven and it can only be disproved. Nevertheless, a free radical reaction mechanism involved in a molecular transformation process can be supported by collective evidence. Fortunately, EPR (electron paramagnetic resonance) technique has been widely employed to visualize free-radical species. But an observable EPR signal would not necessarily mean that the observed free radical species is unquestionably involved in the right pathway leading to the interested molecular transformation process; conversely, absence of EPR signal can not always be used to argue against the likely participation of free radical species. Returning to the molecular transformation mechanism in prions, therefore, a prion radical concept is developed by invoking of a free radical mechanism through a multi-disciplinary consideration of the disease features, and this mechanistic consideration has since been supported by distinct evidence including self-catalytic chain-like polymerization and inhibition of prion chain propagation with electron-rich molecules.

## 6 Why bio-macromolecular free-radical processes are important – new tricks from an old dog: oxygen

The major focus of intensive research on prion diseases aims to promote understanding of the underlying mechanism in order to develop successful treatment and precautionary measures.[1,9,10] In fact, sequence-specific protein radical processes are important in ribonucleotide reductases and have also been proposed as reactive intermediates in site-specific DNA cleaving agents.[21] The presumed production of prion protein radicals requires participation of oxygen and oxidative damage to proteins, which may have theoretically but ironically unraveled a possible connection between the fatal prion pathogen and oxygen, which is necessary for life. According to a multi-disciplinary study, it appears that many lines of observation to date may have supported a contention that oxidative damage to prion proteins in brain is responsible for the infectious process of the elusive subviral pathogen, i.e., scrapie and prions.[23]

## 7 Crossing a concept barrier: from a sequence-specific protein-radical process to the elusive subviral pathogen

A general relationship between oxidative prion chemistry and “protein-only” notion has been explored. The idea of prion protein-radical chemistry may have received support from the following arguments, in which I present evidence from clinical, biochemical and physiological aspects of the disease.

1) Ageing has been believed to be associated with oxidative stress,[39,40] and the average age of classical CJD patients is normally above 60,[9,10] implying that ageing and hence high levels of oxidative damage in host may facilitate prion disease occurrence;

2) Scrapie prions multiply mostly in the brains,[9,10] and it is known that brain cells consume very high amount of oxygen, implying that reactive oxidative species in brain may be crucial to prion diseases;

3) Experiments revealed that elimination of the PrP gene made experimental mice totally resistant to scrapie infection,[41] and cerebellar cells lacking $PrP^C$ were more sensitive to oxidative stress and underwent cell death more readily than wild-type cells,[42] indicating that oxidative damage to PrP has the great potential to play an important role in the disease;

4) It can be suggested that PrP protein molecule with multiple oxidizable amino-acid residues is susceptible to oxidative attack;[1,23]

5) It is known that some protein radicals such as protein tyrosyl radicals are efficient in facilitating protein cross-link reactions, and oxidized prion proteins can be very stable in the solid aggregates. Moreover, free radical initiators can be transmissible, since free radical reactions can often be initiated by appropriate initiators;

6) It may be suggested that a sequence-compatible protein radical may be very damaging, sequence-specifically, to redox labile molecules such as PrP;

7) Prion radicals are unable to be chromatographically separated from the neutral form of prions;[9]

8) The kinetically autocatalytic pattern of prion disease is consistent with a protein radical-mediated

chain reaction;[1]

9) A prion protein radical process may be inhibited or quenched by antioxidants such as phenol and other electron-rich molecules, as is demonstrated by experiments.[9,10]

Therefore, the assumption that prion functions as viruses via prion protein radical reactions has led to a remarkable scenario, where most of the unusual features of prion diseases may be satisfactorily interpreted by invoking the prion free-radical chemistry. These features include prion strain diversity, i.e, multiple structures of the (damaged) diseased prion proteins; species barrier and the possibility of crossing the barriers.

More than a dozen of natural amyloidogenic proteins have been identified to be critically associated with human disorders including prion disease and Alzheimer's disease. A general mechanism is suggested here in Fig.3 to help understand their molecular structural transformation of the relevant amyloid proteins. Evidently, the riddle of the prion disease origin is becoming one of the multi-disciplinary challenges. While a detailed mechanism for ROS and prion radicals-mediated prion structural damage remain to be determined, nevertheless, it may be conceivable this one-electron difference between scrapie prion protein and its free-radical form has generated debate over the past many decades. However, whether the suggestion of prion radicals and chemistry principles of genetic diseases allow us to develop potential therapies and preventative strategies to stop mad cow disease and v CJD remains to be explored.

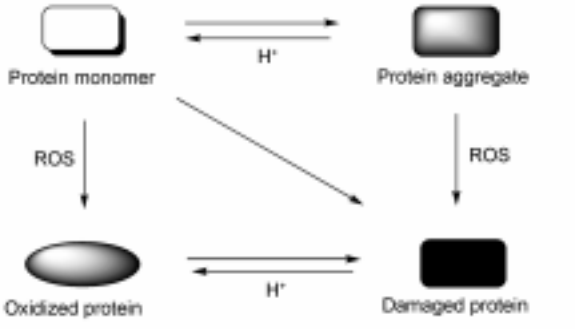


**Fig.3** A postulated general mechanism underlying the molecular structural transformation from protein monomer (white rectangle) to damaged protein aggregates (black rectangle) of amyloid proteins associated with amyloid diseases. In the scheme, two intermediates are protein aggregate (grey rectangle) formed via a mis-folding pathway as a physical process, and oxidized protein (grey ellipse) resulted from oxidative chemical damage to the protein molecule.

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