Contribution of a Metal-Peroxide Adduct to Neurodegeneration Is Due to Its Oxidative Protease Activity

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Many hypotheses have been developed to explain aging and age-related neurodegenerative disorders; one of the most compelling is the role of oxidative stress to induce changes in protease activity in brains of patients of Alzheimer's disease and prion disease. At the moment however, there is no clear answer how protein degradation may be achieved in the brain. We have observed that several metal compounds can degrade proteins in the presence of hydrogen peroxide, and elucidated the reaction scheme based on the new theoretical point for the reactivity of a metal-peroxide adduct with η^1-coordination mode. In this article we would like to point out the importance of a copper(II)-peroxide adduct to promote neurodegenerative diseases such as prion disease and amyotrophic lateral sclerosis through its oxidative protease function.

Progressive degeneration of a subset of neurons is the pathologic hallmark of adult-onset neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson' diseases(PD), and amyotrophic lateral sclerosis (ALS). Growing evidence points to the involvement of oxidative stress in mediating neuronal death in these diseases (Simonian and Coyle, 1996; Mills and Reiner, 1999; Olanow, 1999).

Alzheimer's disease is characterized by the deposition of amyloid plaques in the brain of patients (Mills and Reiner, 1999; Smith *et al.*, 1998). The amyloid plaques are composed mainly of an insoluble 39–43 residue amyloid β-proteins (Aβ) derived from a set of larger transmenbrane β-amyloid precursor proteins (βAPPs). In the normal state, amyloid precursor protein (APP) is cleaved at α-position (Fig. 1), but in the abnormal processing, this protein is cleaved at both β- and γ-positions, and Aβ-protein is secreted into the cell. At present, detailed information on the secretases at α- and γ-positions are scarce (Hardy, 1997; Mentlein *et al.*, 1998; Kosik, 1999).

Prions have become notorious in the past few years as potentially infectious particles implicated in a variety of spongiform encephalopathies, including Creutzfeld-Jacob syndrome, scrapie and "mad-cow disease", and certain serious psychiatric disorders. (Prusiner, 1996, 1997) Remarkably, these prion diseases are due to the prion pro-

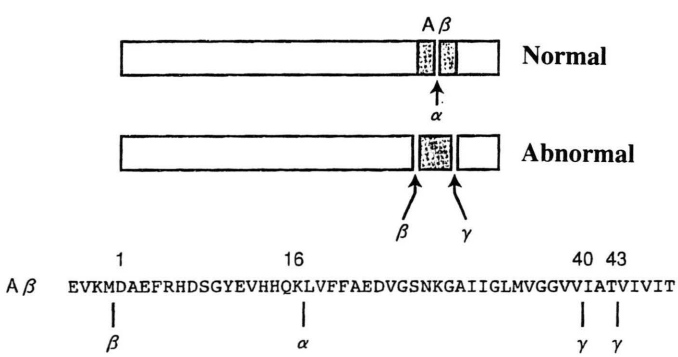

Fig. 1. Secretase cleavage sites of amyloid precursor protein APP. APP is cleaved inside the Aβ sequence by α-secretase. β- and γ- secretase cleave APP on both side of the Aβ sequence.

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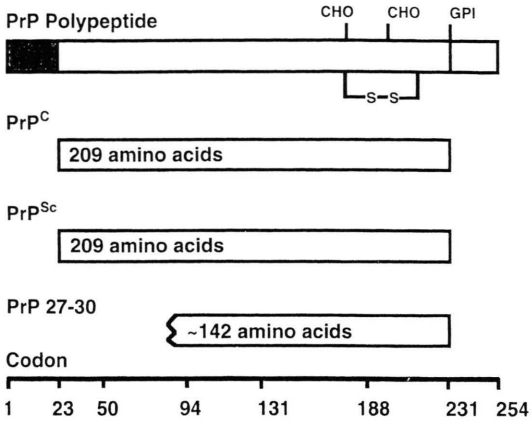


Fig. 2. Bar diagram of SHaPrP, which consists of 254 amino acids. (PrP^C, PrP^{Sc}, and SHa denote prion protein, scrapie prion protein, and Syrian hamster, respectively) (Prusiner, 1998). Attached carbohydrate and a glycosyl-phosphatidyl-inositol anchor are indicated. After limited proteolysis, the NH_2 terminus of PrP^{Sc} is truncated to form PrP 27–30.

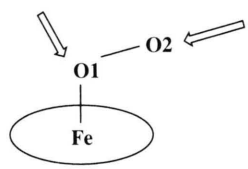
tein(PrP^c) achieving an altered prion protein(PrP^{Sc}) and also PrP 27–39 (Prusiner, 1998) (Fig. 2). The gene encoding the prion protein has been sequenced from many mammalian sources; it codes for a protein ~250 amino acid residues as illustrated in Figure 2. The structure of the prion protein has been determined by multidimensional NMR methods; (Glockshuber *et al.*, 1997) the region (51–91) of the protein contains a group of octarepeats, which have the consensus sequence, Pro-His-Gly_3-Try-Gly-Gln (Prince and Gunson, 1998). Recently it was suggested that copper ions bind to these octarepeats, and that PrP^c might act as a 'shuttle' for copper ions destined to bind to enzymes that prevent oxidative damage (Brown *et al.*, 1997, 1998). Truncated PrP^{Sc} proteins lacking the copper-binding octarepeats retain their infectivity, suggesting that abnormal cleavage of the prion proteins occurs at this region (see formation of PrP 27–30 in Fig. 2). However, it is unclear how the prion protein is degraded.

Nature has developed many hydrolytic metalloenzymes, to hydrolyze some of the important molecules of life including proteins, phospholipids, and DNA. (Chin, 1991) For proteins, zinc-containing enzymes are well-known, such as carboxypeptidase-A and -B. These enzymes cleave proteins by a hydrolytic mechanism, and are quite different from those described above for cleavage of amyloid and prion proteins. In the latter cases cleavage is due to oxidative stress including an oxygen molecule in a reaction intermediate (Behl, 1999). In this article, we will focus on the oxidative cleavage of proteins by metal compounds.

I. Protein cleavage by metal/oxygen systems

There are several reports on the protein degradation by a metal/oxygen system *in vivo* and *in vitro*. Rana and Meares (1991) found that in the presence of ascorbate and hydrogen peroxide, an iron chelate attached to Cys-212 of the enzyme human carbonic anhydrase I quickly cleaves the protein between residues Leu-189 and Asp-190 to produce two discrete fragments (Rana and Meares, 1991; Platis *et al.*, 1993). In this step, it was confirmed that the transfer of an ^{18}O atom from $[^{18}O]H_2O_2$ to the carbonyl group of Leu-189 by mass spectroscopy. They have concluded that generation of a highly nucleophilic oxygen species, such as peroxide coordinated to the iron chelate attacks a carbonyl carbon atom nearby (Fig. 3). However, their conclusion may be wrong, and the experimental result should be considered as follows.

According to our recent investigations on the reactivity of the metal-peroxide adducts, both oxygen atoms of the metal-peroxide adduct with η^1-coordination mode exhibit electrophilicity as shown below (Ito *et al.*, 1997; Nishida *et al.*, 1997, Ito *et al.*, 1998).



Thus, when the carbon atom of the carboxylic acid approaches to the terminal O atom of O_2 of the peroxide adduct in the above figure, heterolytic O-O cleavage ($O_2^{2-} \rightarrow O^{2-} + O$(neutral)) is promoted through the electronic interaction between them, and covalent bonding between the carbon atom and the terminal oxygen atom forms associated with the cleavage of C-N bond, giving the products (see the figure below). This mechanism explains the incorporation of ^{18}O of the peroxide ion in the product. The importance of electrophilicity of the peroxide adduct has been confirmed

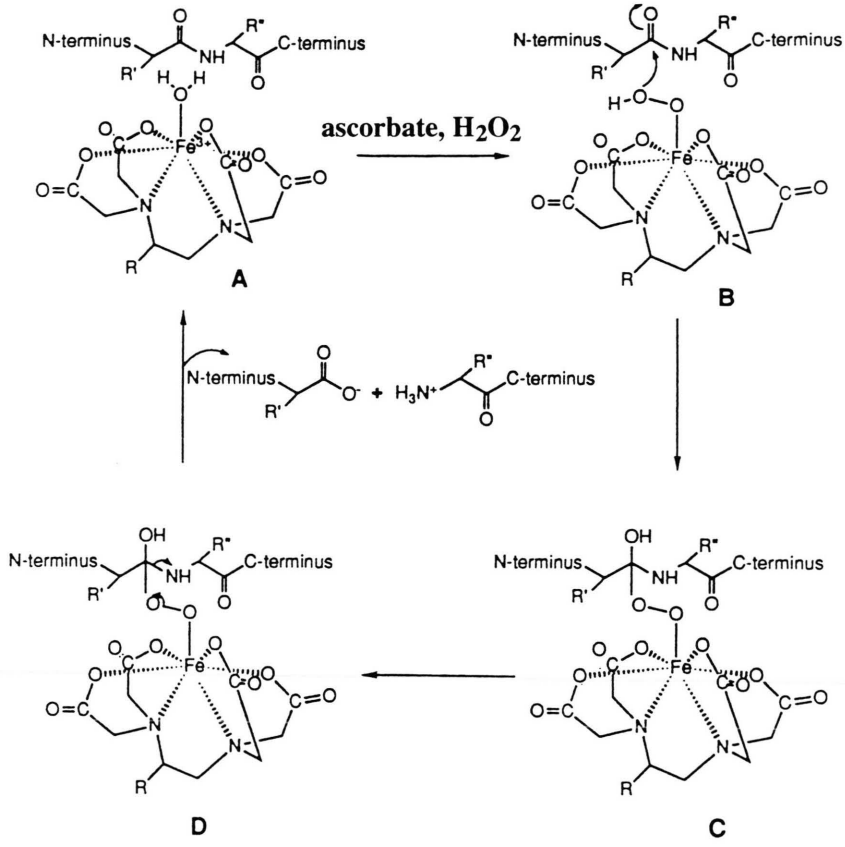
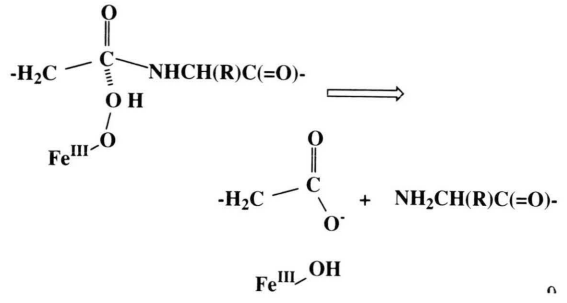
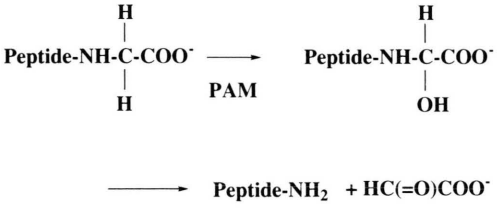


Fig. 3. Mechanism of cleavage of human carbonic anhydrase by iron(III) complex (Rana and Meares, 1991).

by experimental findings (Ito *et al.*, 1998; Montellano, 1998).



Many neuropeptides and peptide hormones require amidation at the carbonyl terminus for activity, and peptidylglycine α-amidating monooxygenase (PAM) catalyzes the amidation of these diverse physiological regulators (see below) (Prigge *et al.*, 1997).



The amino-terminal domain of the difunctional PAM protein is a peptidylglycine α-hydroxylating monooxygenase (PAM) with two coppers that cycle through cupric and cuprous oxidation states. In contrast to other dicopper proteins, the coppers in PAM are 11Å apart and do not forma binuclear center; in this respect this enzyme is very similar to dopamine β-hydroxylase (DβH) (Klinman, 1996) The reaction mechanism proposed for the PAM enzymes, is thus similar to that for the dopamine β-hydroxylase, but the mechanism for the latter

enzyme is not established yet. It should be noted that in both cases the formation of a copper(II)-peroxide adduct, Cu(II)-OOH is assumed to be an important reaction intermediate.

As illustrated above, we have been studying the reactivity of the metal-peroxide adducts, and it seems quite likely that the same discussion described for the iron(III)-OOH adduct may elucidate the reaction mechanism in these copper-containing enzymes. We have prepared several copper(II) compounds with the ligands as shown in Fig. 4, and observed that Cu(bdpg)Cl^+ complex

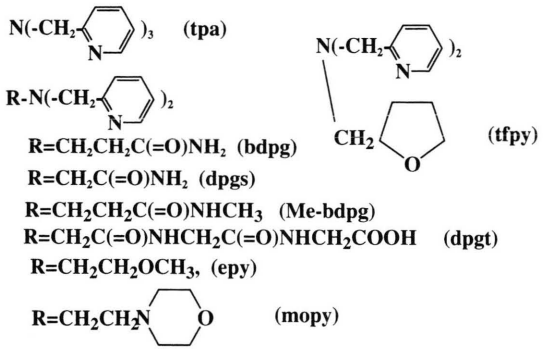
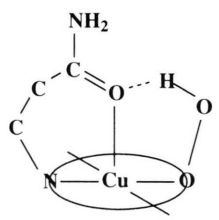


Fig. 4. The chemical structures of ligands used in our study.

exhibits abnormal high activity for hydroxylation of cyclohexane in the presence of hydrogen peroxide, (Okuno *et al.*, 1997) which has been attributed to

1) facile peroxide adduct formation through hydrogen bonding with the ligand system(see below),

2) electronic interaction between the peroxide ion and the amide group which enhances the activation of the peroxide ion through electron donating from the amide group to the peroxide ion.



This reasoning may explain the reaction mechanism for the hydroxylation reaction of cyclohexane by the Cu(bdpg)Cl^+/H_2O_2 system, and PAM and DβH enzymes as well (Okuno *et al.*, 1997).

II. Importance of Cu(II)-OOH and neurodegenerative diseases

In the above section, we showed that Cu(bdpg)Cl^+ complex is one of the best models for PAM and DβH enzymes. In addition we have observed that this complex exhibits high activity for cleaving the proteins, such as bovine serum albumin (BSA) or human carbonic anhydrase I(HCAI), as shown in Fig. 5, where the electrophoresis containing a copper(II) complex, protein, and hydrogen peroxide are illustrated (Ishikawa *et al.*, 1998). The high activity of the Cu(bdpg)Cl^+ complex relative to Cu(tpa)Cl^+ complex is noteworthy, similar to that observed with the hydroxylation of cyclohexane as reported by Okuno *et al.* (1997). This suggests that the presence of an amide group near the peroxide ion plays an important role in activating the peroxide ion, which is supported by the fact that Cu(dpgt)Cl^+ complex (Kobayashi *et al.*, 1998; see Fig. 6) also shows a high activity for degrading the protein in the presence of hydrogen peroxide.

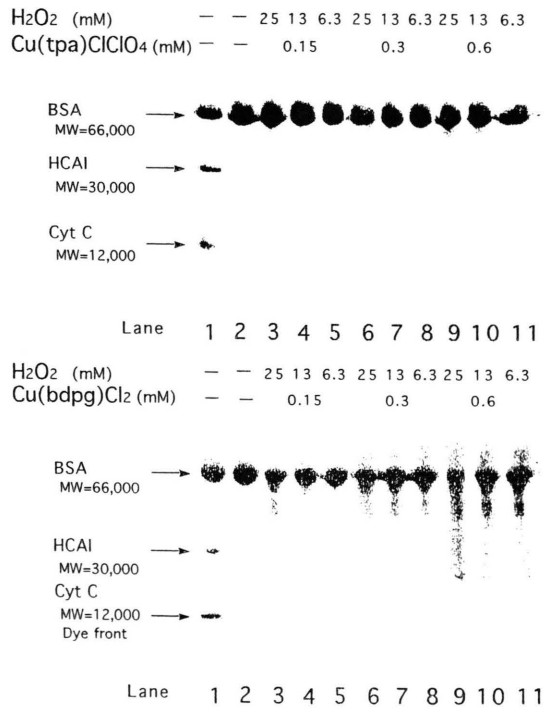


Fig. 5. Electrophoresis of the solutions containing copper(II) complex, hydrogen peroxide, and bovine serum albumin (BSA) (Ishikawa *et al.*, 1998). HCAI denotes human carbonic anhydrase I.

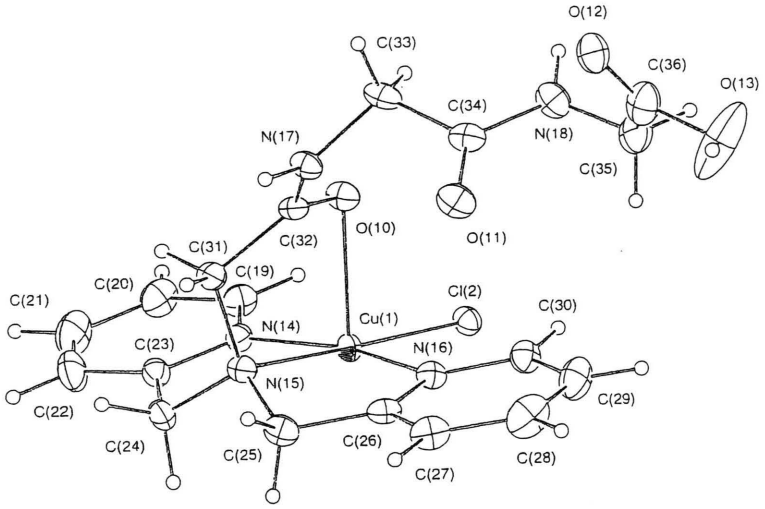
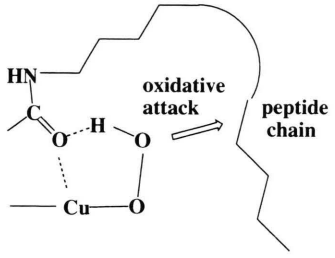


Fig. 6. Crystal structure of Cu(dpgt)Cl$^+$ (Kobayashi *et al.*, 1998) C and N denote carbon and nitrogen atoms, respectively. Small circle attached to these atoms represents hydrogen atom.

Based on above discussions, it seems quite likely that formation of PrP 27–30, which originates from the cleavage of PrP^{Sc} near the octarepeat region, may be attributed to oxidative protease activity by a copper(II)-OOH species, which is activated through interaction with the amide groups coordinated to the copper(II) ion, to cleave the peptide chain nearby (see the figure below).



A similar discussion may be applied to ALS. ALS is a neurodegenerative disease with an incidence of 1 to 10000. Approximately 1% of the cases of ALS are familial amyotrophic lateral sclerosis (FALS), and in a subset of these cases, mutation in superoxide dismutase (SOD1), which encodes copper/zinc superoxide dismutase (CuZnSOD), have been demonstrated (Deng *et al.*, 1993; Brown, 1995; W.-Pazos *et al.*, 1996). It has been hypothesized that the mutations in SOD1 lead to FALS by decreasing the enzymatic activity of CuZnSOD. However, several findings have raised question about this interpretation. (i) the determinations of low CuZnSOD activity were carried out *in vitro*, in some cases following isolation of the protein in non physiological conditions, without correlation of *in vivo* activity, (ii) the FALS-associated SOD1 mutation affect amino acid residues involved in enzyme dimerization or β-barrel turn, rather than those corresponding to the active sites. Thus, we would like to propose a new consideration on the mutation of SOD1 in FALS.

SOD catalyzes the disproportionation of superoxide ion into peroxide and oxygen molecule,

$$2O_2^- \xrightarrow[H^+]{} O_2 + H_2O_2$$

The enzymes contains copper(II) and zinc(II) ion, but only the copper(II) ion participates in the reaction,

$$Cu(II) + O_2^- \longrightarrow Cu(I) + O_2$$

$$Cu(I) + O_2^- \xrightarrow[H^+]{} Cu(II) + H_2O_2$$

The peroxide ion formed during the reaction is decomposed by glutathione peroxidase, however, it should be noted here that formation of a copper(II)-OOH , a very dangerous compound, is produced. Under normal conditions, the hydrogen peroxide formed may be decomposed rapidly, but with the mutated SOD, this process is prevented

(Fullerton *et al.*, 1998) and the copper(II)-OOH may give damage to the peptide group nearby. This will give more damaged SOD, which is consistent with the observations in patients of ALS (Rando *et al.*, 1998; Shaw *et al.*, 1998). This process also leads to release of a copper(II) ion from SOD, and the released copper(II) ion may become a dangerous ion in cells.

III. Free iron ion and neurodegenerative diseases

Iron is an essential participant in many human metabolic processes, but recent studies on neurodegenerative diseases, have revealed that a free ion, i.e., excess iron ion in the cell is potentially dangerous, and abnormalities in brain iron metabolism have been described for several disorders such as Alzheimer's and Parkinson's disease (McCord, 1996; Gerlach *et al.*, 1994; Double *et al.*, 1998; Loeffer *et al.*, 1995). We have prepared several model compounds for the "free iron(II) ion" in the cell, and discussed the free iron ion formation, the oxygen activation, cell damage, and the cancer stimulation process by the free iron ion (Nishida and Ito, 1995a; 1995b). As proposed by us, a free iron(III) ion or copper(II) ion may be captured by the chelate containing an amide group, and it is frequently observed that a free iron ion in the cell is of a dimeric structure (for example see Fig. 7; where a dimeric structure of a binuclear iron(III) compound with a ligand containing an amide-group is illustrated) (Ito *et al.*, 1996). We have found that several binuclear iron(III) compounds with an μ-alkoxo bridge, such as $Fe_2(HPTP)Cl_4ClO_4$, (chemical structure of the ligand H(HPTP) is illustrated below) can degrade proteins in the presence of hydrogen peroxide, as illustrated in Fig. 8.

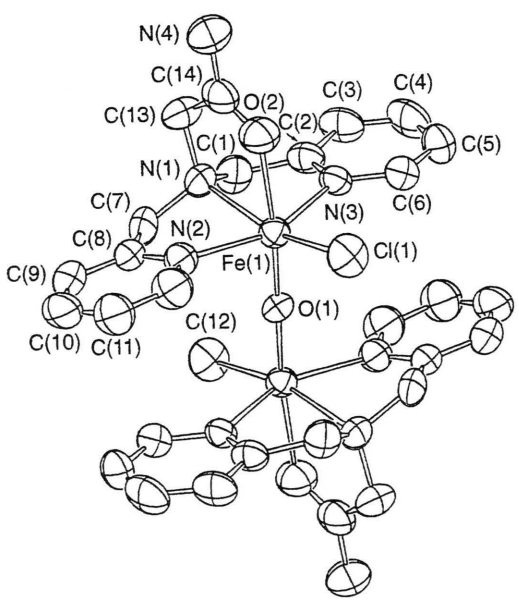


Fig. 7. Crystal structure of $Fe_2(dpgs)_2OCl_2^{2+}$ (Ito *et al.*, 1996).

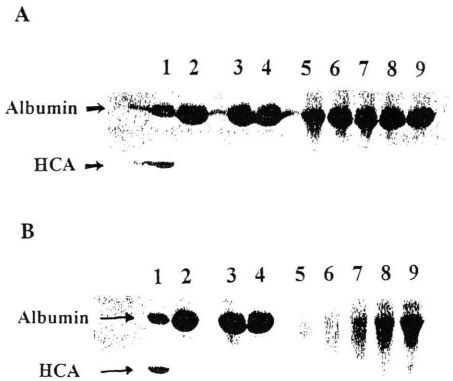
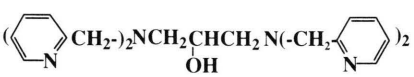


Fig. 8. Electrophoresis of a solution containing Fe(III) complex (0.3 mM) hydrogen peroxide, and bovine serum albumin (SBA) (for experimental details, see Ishikawa *et al.*, 1998) HCA is human carbonic anhydrase.
A: mononuclear $Fe(tpa)ClClO_4$;
B: dinuclear $Fe_2(HPTP)Cl_4ClO_4$.
Lane 1, albumin and I(HCA); lane 2, albumin only; lane 3, albumin + Fe(III) complex; lane 4, albumin + H_2O_2(100 mM); lane 5, albumin + Fe(III) complex + H_2O_2(100 mM); lane 6, albumin + Fe(III) complex + H_2O_2(50 mM); lane 7, albumin + Fe(III) complex + H_2O_2(25 mM); lane 8, albumin + Fe(III) complex + H_2O_2(12.5 mM); lane 9, albumin + Fe(III) complex + H_2O_2(6.25 mM).



As is well known, iron(III) and aluminum(III) ions are present in the brain of Alzheimer's disease patients (Gerlach *et al.*, 1994). A large amount of intake of Al(III) ion in brain induces lacking in neurotransmitters and increase in free iron ion (Nishida and Ito, 1995b). This suggests that cleavage at β-position in APP may be attributed to a free iron ion and hydrogen peroxide. It has been reported that the amyloid protein itself gives damage to the neural cell by decomposing it (Mills and Reiner, 1999). This may be attributed

to the formation of an iron(III) or copper(II) complex with amyloid protein, and to its oxidative protease activity in the presence of hydrogen peroxide. We have observed that amyloid β protein (1–40, from Sigma) and bovine carbonic anhydrase rapidly interact with several copper(II) complexes such as $Cu(bdpg)Cl_2$, etc. changing its conformational structure (Nishino *et al.*, 1999). These findings may give a reasonable explanation of the mechanism to form PrP^{Sc} from PrP^{C} (Fig. 2).

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