Book Chapter

Implications of Metal Binding and Asparagine Deamidation for Amyloid Formation

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Published September 24, 2020

This Book Chapter is a republication of an article published by Masahiro Kawahara, et al. at International Journal of Molecular Sciences in August 2018. (Sadakane, Y.; Kawahara, M. Implications of Metal Binding and Asparagine Deamidation for Amyloid Formation. Int. J. Mol. Sci. 2018, 19, 2449.)

How to cite this book chapter: Yutaka Sadakane, Masahiro Kawahara. Implications of Metal Binding and Asparagine Deamidation for Amyloid Formation. In: Sławomir Lach, editor. Prime Archives in Molecular Sciences. Hyderabad, India: Vide Leaf. 2020.

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Acknowledgments: This work was supported by JSPS KAKENHI Grant Number JP16K08207 for Y.S. and Number JP18K06669 for M.K. We thank James Murray, PhD, from Edanz Editing (www.edanzediting.com/ac) for editing a draft of this manuscript.

Abstract

Increasing evidence supports the idea that amyloid formation, i.e. protein oligomerization and the conformational changes, is based on the pathogenesis of various neurodegenerative disorders such as Alzheimer’s disease, prion diseases, and Lewy body diseases. Among factors which accelerate or inhibit the oligomerization, we focus here two non-genetic and common characteristics of many amyloidogenic proteins: metal binding and asparagine deamidation. Both reflect the aging process and occur in most amyloidogenic proteins. All of the amyloidogenic proteins such as Alzheimer’s β-amyloid protein, prion protein, α-synuclein are metal-binding proteins and are involved in the regulation of metal homeostasis. It is widely accepted that these proteins are susceptible to non-enzymatic posttranslational modifications, and many asparagine residues of these proteins are deamidated. Moreover, these two factors can combine because asparagine residues can bind metals. We review the current understanding of these two common properties and their implications in the pathogenesis of these neurodegenerative diseases.

Keywords

Alzheimer’s disease; Oligomerization; Conformation; Prion Disease, Iron

Abbreviations

AβP- β-Amyloid Protein; AD- Alzheimer’s disease; AFM- Atomic Force Microscopy; ALS- Amyotrophic Lateral Sclerosis; AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; APP- Amyloid Precursor Protein; β2M- β2-Microglobulin; BSE-
Introduction

Amyloids are fibril-like deposits observed in various tissues including kidney, spleen, liver, and brain. In 1853, Virchow found abnormal accumulates in tissues and named them “amyloid”, since they exhibited similar characteristics to amylum, such as being stained by iodine [1]. In 1968, amyloid was determined to be oligomers of proteins. The accumulation of amyloid in various organs causes disorders termed “amyloidosis”, including familial amyloid polyneuropathy (FAP), amyloid-light chain amyloidosis, and dialysis amyloidosis [2]. All of these diseases share common properties regarding the deposition of amyloids, which are protease-resistant, insoluble fibril-like structures (amyloid fibrils), and stained by congo-red (a β-sheet specific dye). Although the composition of amyloid is identical in each disease, some of the amyloidosis are fatal, and there are no effective treatments for amyloidosis.

Recent neurochemical studies have suggested a link between amyloid formation and the pathogenesis of various neurodegenerative diseases such as Alzheimer’s disease (AD), prion diseases, Lewy body diseases (dementia with Lewy bodies (DLB) etc.). The disease-related proteins, such as β-amyloid protein (AβP) in AD, prion protein in prion diseases,
synuclein in Lewy body diseases, are identical in each disease, as shown in Table 1. Furthermore, all these proteins, termed ‘amyloidogenic proteins’, share common characteristics, namely that they form amyloids with β-pleated sheet structures, and exhibit cytotoxicity. Thus, a new concept termed “conformational disease” was proposed, suggesting that protein conformation and its misfolding is an important determinant of its toxicity, and consequently, the development of the related disease [3]. Considering that these amyloidogenic proteins are commonly found in our brain, factors that inhibit or accelerate the oligomerization process may play crucial roles in their neurotoxicity and pathogenesis [4]. As such, we focus here on two non-genetic factors: metal binding and deamidation of amino acid residues.

Considerable amounts of trace elements, such as iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) exist in the brain, and the concentration and distribution of each metal differs in each brain region [5]. These essential trace elements play crucial roles in brain functions such as energy production, synthesis of neurotransmitters, and myelination. Because an excess or deficiency of these essential trace elements disrupts normal brain functions, their concentrations are strictly regulated.

It is widely accepted that metal ions are essential factors for the regulation of protein conformations. These metals can firmly bind to metal-binding residues of proteins, such as arginine (Arg), tyrosine (Tyr), histidine (His), and phosphorylated amino acids residues, which can cause cross-linking of the proteins and influence their conformations (Figure 1A). Indeed, all of these amyloidogenic proteins possess the ability to bind metals as shown in Table 1. Furthermore, most of these proteins, or the precursor proteins, are implicated in the maintenance of metal homeostasis [6]. We have observed metal-induced oligomerization of amyloidogenic proteins using SDS-PAGE, thioflavin T (ThT) fluorescence assay, far-UV circular dichroism (CD) spectroscopy, and atomic force microscopy (AFM) imaging techniques [7-10].
Meanwhile, amyloidogenic proteins are susceptible to non-enzymatic posttranslational modifications caused by various stressors including oxidants, reducing sugars and reactive aldehydes owing to their long-life span [11,12]. Asparagine (Asn) and aspartyl (Asp) residues are hot spots for such non-enzymatic posttranslational modifications, and the structural alterations of both residues are reported in many amyloidogenic proteins (Table 1). These alterations result in a change of local charge as well as the addition of extra carbon atoms to the polypeptide backbone, which are implicated in various biological phenomena including amyloid fibril formation (Figure 1B). For determining such structural alterations, we have developed a simple method using high performance liquid chromatography (HPLC), which allowed us to analyze the amount of structurally-altered Asp residues in various proteins, including amyloidogenic peptide fragments [13,14].

In particular, the deamidation of Asn residues is a remarkable and prevalent phenomenon that occurs during protein aging, and has also been predicted by computer simulation. Over 170,000 Asn residues in 13,300 proteins for which 3D structure is known, were analyzed by an automated computational method. The calculated results revealed that at least one Asn residue in ~4 % or ~17 % proteins was estimated to undergo at least 10% deamidation within 1 day or 5 days under the physiological conditions, respectively [15]. More importantly, these predicted values accord quantitatively to the experimental results when the rates of over 1370 Asn deamidation are analyzed [16]. Taken together, considering the metal-binding ability of Asn residues, the interaction between metals and the deamidation may also occur in some diseases.

In this article, we review two common characteristics of amyloidogenic proteins: metal binding and asparagine deamidation and discuss their implications in the pathogenesis of conformational diseases.
## Table 1: Characteristics of amyloidogenic proteins and related peptides.

| Disease                        | Binding Metals | Structural Alteration of Asn or Asp | Functions of Amyloidogenic Proteins or Their Precursors |
|-------------------------------|----------------|------------------------------------|--------------------------------------------------------|
| **Alzheimer’s Disease**       |                |                                    | ✓ Neuronal proliferation and development                |
| Name                          |                |                                    | ✓ Neurite outgrowth                                      |
| **Sequence**                  |                |                                    | ✓ Fe homeostasis                                         |
| **AβP**                       | Al, Zn, Cu, Fe | Isomerization and racemization of Asp<sup>1</sup> and Asp<sup>2</sup> |                                                         |
| **Disease**                   |                |                                    | ✓ Neuronal proliferation and development                |
| Name                          |                |                                    | ✓ Neurite outgrowth                                      |
| **Sequence**                  |                |                                    | ✓ Fe homeostasis                                         |
| **Prion Diseases**            |                |                                    |                                                         |
| **Prion protein; (PrP**<sup>106–126</sup>) | Zn, Cu, Fe, Mn | Deamidation of Asn<sup>108</sup> | ✓ SOD activity                                           |
| **Lewy Body Diseases**        |                |                                    | ✓ Cu homeostasis                                        |
| α-synuclein; (NAC, a fragment of α-synuclein) | Cu, Fe, Al | Deamidation of Asn<sup>103</sup> and Asn<sup>122</sup> | ✓ dopamine release                                      |
| **Type 2 Diabetes**           |                |                                    | ✓ Fe homeostasis and ferrireductase activity            |
| Islet amyloid peptide (IAPP, amylin) | Cu, Zn | Deamidation of Asn<sup>21</sup> | ✓ a partner hormone to insulin to control blood glucose concentration |
| **Dialysis Amyloidosis**      |                |                                    | ✓ antigen presentation in the immune responses         |
| β<sub>2</sub>-microglobulin   |                |                                    |                                                         |
| **Amyotrophic Lateral Disorder (ALS)** | Cu, Zn-SOD1 | Deamidation of Asn<sup>26</sup> | ✓ SOD activity                                          |
| **Sequence**                  |                |                                    | ✓ cellular defense                                      |
| **Sequence**                  |                |                                    |                                                         |

The sequence of fragment peptide of each amyloidogenic protein (PrP<sup>106–126</sup>; NAC) is indicated by an underline. Deamidated Asn or Asp residues are shown as italic bold. In “Functions” of Alzheimer’s disease, possible functions of APP are noted. The numbers in the upper right indicate residues’ range. Same as below.
Figure 1: Metal binding and Asn deamidation in proteins. (A) Trace elements act as cross-linkers of amyloidogenic proteins. M stands for metal. (B) Deamidation of Asn residue affects the fibril formation by structural alteration of the neighboring Asn residue.

Asparagine Deamidation and its Biological Significance

There are few reviews summarizing the relationship between amyloidogenesis and Asn deamidation. Therefore, we first introduce the mechanism that generates Asn deamidation and illustrate its biological significance with some examples. The deamidation of Asn residues occurs by intramolecular rearrangement, for example via a succinimide intermediate. The side chain carbonyl groups of the Asn residue is attacked by the peptide-bond nitrogen atom of the following residue, forming a five-membered succinimide ring intermediate [17] (Figure 2). The intermediate has a half-life of hours under physiological conditions.
conditions before it is hydrolyzed, and a mixture of L-Asp and L-isoAsp is generated. The ratio of L-Asp to L-isoAsp is experimentally found to be approximately 3:1. Some L-succinimide intermediates also undergo reversible stereoinversion, which results in the formation of a D-succinimide intermediate. This intermediate is also quickly hydrolyzed, and a corresponding mixture of D-Asp and D-isoAsp is generated. Analyses using various model peptides reveal that the rate of succinimide formation is affected by both the primary amino acid sequence and the secondary structure of the protein. The amino acid residues on the carboxyl side of the asparagine/aspartyl (Asx) residue affects the succinimide formation rate. A fast rate is obtained when the carboxyl side residues are Gly, Ser and Ala [18,19]. The succinimide formation is inhibited by higher order structures of protein such as α-helixes and β-sheets [13,20]. The half-lives of degradation of Asx residue vary between about 1 and 1000 days, depending on the circumstances of the Asx residue [21]. The succinimide formation rates of Asn residues is 10 ~ 30 times faster than those of Asp residue [18].

Since the deamidation of Asn is a dominant event for protein aging, the repair enzyme for isoAsp residue, which is the main primary reaction product of deamidation, is found in various organisms from bacteria to human. Protein L-isoaspartyl O-methyltransferase (PIMT) catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to the internal α-carboxyl group of a L-isoAsp residue (Figure 2). The methylation strongly accelerates the L-succinimide intermediate formation. The repetition of this repair cycle finally results in the replacement of an isoAsp with an Asp residue because the Asp residue is not a substrate for PIMT [22]. In knockout mice lacking PIMT, the amount of damaged protein containing the L-isoAsp residue is significantly increased in the brain, heart, liver, and erythrocyte in comparison to wild-type mice. The knockout mice show significant growth retardation and underwent several tonic-clonic seizures, they then die at an average of 42 days after birth [23]. Proteomic approaches show that the substrates of PIMT are collapsin response mediator protein 2, dynamin 1, synapsin I, and synapsin II, which are characterized by having unique roles.
in neuronal function [24,25]. These shows that deamidation of Asn residues is a critical event that causes protein functional disorder associated with various physiological systems, including neuronal dysfunction. The biological significance of deamidation will be explained in the following section with some examples.

The aggregation of eye lens crystallins, which may result in the formation of cataracts in aged lenses, is a specific example of a protein functional disorder caused by the deamidation of Asn residues. In human γS-crystallin, one of the major structural protein components of the eye lens, the deamidation of Asn\textsuperscript{76} causes a decrease in stability of the protein and promotes dimer formation [26]. The deamidation of Asn\textsuperscript{76} and Asn\textsuperscript{143} enhances the protein-protein interaction, which leads to the promotion of protein aggregates [27]. αA-crystallin, obtained from elderly donors, contains D-Asp and D-isoAsp residues, and interestingly, the D/L ratios of specific Asp residues are reported to be higher than 1.0 [28,29]. The dissociation of αA-crystallin is also significantly affected by the structural alteration of Asp residues [30]. The results obtained from two types of crystallins clearly show that structural alteration of Asx residues affects the function of the proteins. [31]. Functional disorder caused by Asn deamidation is also reported in the case of calcium modulator protein, calmodulin: specifically, the deamidation of two specific Asn residues caused a 90 % reduction in activity of calmodulin [32]. The repair enzyme PIMT can partially recover the calmodulin activity (up to 40 %) because PIMT promotes the conversion of L-isoAsp residues to L-Asp, but not to L-Asn. An examination using the Xenopus oocyte assay system also showed that the aged calmodulin became unstable, suggesting the alteration of calmodulin’s 3D structure by the deamidation of L-Asn residues [33]. On the contrary, it is reported that the deamidation of specific Asn residue causes protein function to be gained. Fibronectin is an adhesive protein that mediates various cellular interactions with the extracellular matrix. Although the Asn\textsuperscript{263}-Gly-Arg (NGR) sequence of fibronectin is known to be crucial for the binding to the RGD-binding site of integrin, it is reported that the isoAsp-Gly-Arg (isoDGR) sequence, generated by Asn deamidation, is actually the sequence that binds to
integrin [34]. In ceruloplasmin, a copper-binding protein with ferroxidase activity present in the cerebrospinal fluid (CSF), the ability to bind integrin also arises by the Asn deamidation of two internal NGR sites [35]. CSF obtained from AD patients promotes the deamidation of these two Asn residues because of its pathological pro-oxidative environment, suggesting that the environments of senile dementia promotes the integrin signaling pathway and cellular adhesion activity via deamidation of Asn residues.

Figure 2: Pathways for spontaneous deaminadion, isomerization and racemization of L-Asn and L-Asp residues in the proteins. The PIMT repair system for L-isoAsp residue is also shown.
Alzheimer’s disease
Amyloid Cascade Hypothesis

AD is a severe type of senile dementia affecting a large proportion of elderly people worldwide. It is characterized by profound memory loss and an inability to form new memories. The pathological hallmarks of AD are the presence of numerous extracellular deposits (senile plaques) and intraneuronal neurofibrillary tangles (NFTs) [36]. The degeneration of synapses and neurons in the hippocampus or cerebral cortex is also observed. The major components of NFTs are phosphorylated tau proteins, and that of senile plaques are AβPs. Although the precise cause of AD remains controversial, numerous biochemical, cell biological, and genetic studies have supported the idea, termed the “amyloid cascade hypothesis”, that the AβP accumulation and consequent neurodegeneration play a central role in AD [37]. Moreover, recent studies on the identified AβP species have indicated that the oligomerization of AβP and the conformational changes are critical in the neurodegeneration process [38].

AβP is a small peptide of 39–43 amino acid long. It is derived from the proteolytic cleavage of a large precursor protein (amyloid precursor protein, APP). AβP is afforded by the cleavage of the N-terminus of APP by β-secretase (BACE), followed by the intra-membrane cleavage of its C-terminal by γ-secretase (Figure3A). Genetic studies of early-onset cases of familial AD indicated that APP mutations and AβP metabolism are associated with AD [39,40].

Yankner et al. reported that the first 40 amino acid residues of AβP (AβP1-40) caused the death of cultured rat hippocampal neurons and neurodegeneration in the brains of experimental animals [41]. Thereafter, it was agreed upon that the aggregation and the subsequent conformational change of AβP contributes to its neurotoxicity. AβP is a hydrophobic peptide with an intrinsic tendency to self-assemble into insoluble oligomers with β-pleated sheet structures. Pike et al. revealed that aged AβP1-40 (aggregated under incubation at 37°C for several days) were considerably more toxic to cultured neurons than freshly prepared AβP1-40 [42]. Simmons et al. revealed the β-sheet
content of AβP, observed by CD spectroscopy, correlates with its neurotoxicity [43].

Figure 3: Alzheimer’s disease and factors affecting AβP aggregation. (A) Structure of APP and AβP. AβP is secreted by cleavage from its precursor protein, APP by transmembrane cleavage. The mRNA of APP possesses IRE domain, and Fe regulates its expression. (B) AβP aggregation. AβP self-aggregates and forms several types of oligomers (including SDS-soluble oligomers, ADDLs, globulomers, or protofibrils) and finally forms insoluble aggregates termed amyloid fibrils. Oligomeric soluble AβPs are toxic, although...
the monomeric and fibril AβPs are rather nontoxic. The aggregation process is influenced by the acceleratory factors or the inhibitory factors. (C) Summary of Asp isomerization in AβP. The Asp isomerization positions found in an AD brain are indicated by open circles. The relationship between chemical substitution and fibril formation is also shown. (↑) acceleration or increase of fibril formation, (↓) suppression or decrease, (1) suppression of acceleration effect by Asp$^{23}$ substitution [41], (2) unchanged in vitro assay, (3) triggered the dense-core congophilic amyloid plaque formation in APP transgenic mice. The comparison between the sequence of primate (human or monkey) AβP$^{1-42}$ and rodent (rat or mouse) AβP$^{1-42}$ is also depicted and the different amino acids are indicated by underline.

Recent approaches using size-exclusion chromatography, gel electrophoresis, and atomic force microscopy have demonstrated that there are several stable types of soluble oligomers: naturally occurring soluble oligomers (dimers or trimers), AβP-derived diffusible ligands (ADDLs), AβP globulomers, or protofibrils [44]. These studies further strengthened and modified the amyloid cascade hypothesis, which suggest that AβP oligomers are neurotoxic and crucial for the pathogenesis of AD (Figure 3B) [45].

**Metals and AβP**

AβP is reportedly secreted from APP into the brain of young people or of normal subjects [46]. Therefore, factors which accelerate or inhibit the oligomerization process may become important determinants of the pathogenesis of AD. Various factors, such as the mutations, oxidations of AβP, as well as environmental factors, such as pH, composition of solvents, concentrations of peptides, and temperature, all reportedly influence the oligomerization processes (Figure 3B). Several small molecules, such as rifampicin, curcumin, carnosine, β-sheet breaker peptide, and aspirin have been reported to inhibit AβP oligomerization *in vitro* [47-51]. Some of these substances are considered to be protective agents against AD.

Among the factors that influence AβP oligomerization, trace elements are of particular interest. The accumulation of AβP is rarely observed in the brains of rodents (rats or mice) compared with those of humans or monkeys. As shown in Figure 3C, the amino acid sequence of human and rodent AβP are similar, yet
they differ by three amino acids. However, rodent AβP is less prone to oligomerization compared with that of human AβP [52]. Interestingly, these three amino acids (Arg<sup>5</sup>, Tyr<sup>10</sup>, and His<sup>13</sup>) have the ability to bind metals as shown in Figure 1A, and such trace metals have cross-linking ability. Therefore, it is highly possible that these metals might play important roles in the accumulation of AβP in the human brain. Indeed, the accumulation of these trace elements was observed in the senile plaques of AD patients [53,54].

Numerous studies reported the metal-induced oligomerization of AβP. Exley et al. first demonstrated that Al<sup>3+</sup> induces a conformational change in AβP<sub>1-40</sub> by CD spectroscopy [55]. Furthermore, exposure to Al<sup>3+</sup> causes the accumulation of AβP in cultured neurons or in the brains of experimental animals or humans. Pratico et al. found that Al-fed mice transfected with the human APP gene (Tg 2576) exhibited pathological changes similar to those of the AD brain, including a marked increase in the amount of AβP both in the secreted and accumulated form; an increased deposition of senile plaques was also observed [56]. We have shown that Al enhances the polymerization of AβP<sub>1-40</sub> and forms SDS-stable oligomers in vitro by immunoblotting and precipitation [7-9] (Figure 4A). The oligomerized AβP<sub>1-40</sub> is heat- or SDS-stable but re-dissolves on adding deferoxamine, a chelator of Al. The oligomerization induced by Al is more pronounced than that induced by other metals, including Zn, Fe, Cu, and Cd. Furthermore, while Zn-aggregated AβPs are rarely observed on the surface of cultured neurons even several days after exposure, Al-aggregated AβPs bind tightly to the surface of cultured neurons and form fibrillar deposits (Figure 4B). Bush et al. found that Zn induced the oligomerization of AβP, even at low concentrations (300 nM) [57]. They also reported that Cu markedly enhanced the AβP aggregation [58]. Zn binds to three histidine residues (His<sup>6</sup>, His<sup>13</sup>, and His<sup>14</sup>) and/or to the carboxyl group of Asp<sup>1</sup> of AβP [59]. However, the metal-induced oligomerization of AβP is complex and controversial. The morphology of AβP oligomers treated with Al, Cu, Fe, and Zn were reported to be quite different [60]. Zatta and his colleagues demonstrated that metals including Al, Cu, Fe, Zn alter the oligomerization of AβP and its toxicity in a different manner.
[61]. Cu-oligomerized AβP is reportedly more toxic than Zn-oligomerized AβP [62].

**Figure 4**: Metal-induced oligomerization of AβP\textsubscript{1-40}. A) Oligomerization of AβP\textsubscript{1-40} by various metals. The solutions of AβP\textsubscript{1-40} were incubated at 37 °C for 24 h with or without various metal ions (each 1 mM), and were separated by SDS-PAGE using the tris-tricine method. (from Ref. No. 8 with permission). B) Deposition of AβP\textsubscript{1-40} oligomers on neuronal membranes. The solutions of AβP\textsubscript{1-40} were incubated at 37 °C for 24 h with Al\textsuperscript{3+} or Zn\textsuperscript{2+}, and were applied onto cultured cortical neurons. After 2 days of exposure, cells were washed out and double-immunostained with a polyclonal antibody to AβP (green) and a monoclonal antibody to MAP2 (red). The cells were observed under a confocal laser scanning microscopy. (a): control, (b): Al-aggregated AβP, (c): Al-aggregated AβP. Bar represents 50 µm. (From Ref. No. 9 with permission).

Furthermore, APP is a metal-binding protein that has two Cu and/or Zn binding domains at its N-terminus (Figure 3A). APP reportedly possesses the ability to reduce Cu\textsuperscript{2+} to Cu\textsuperscript{+} [63]. Cu and Zn influence the expression and processing of APP and enhance AβP production [64]. Cu induces the dimerization and trafficking of the APP from the ER to neurites [65]. APP binds
to ferroportin, which controls $\text{Fe}^{2+}$ efflux, and regulates Fe homeostasis [66]. Meanwhile, the expression of APP is regulated by Fe, as well as ferritin (iron storage protein) since APP mRNA possesses an iron responsive element (IRE) [67]. These findings suggest that APP plays crucial roles in the regulation of metal homeostasis [6].

**Isomerization and Racemization of Asp Residues in AβP**

Delicate chemical analyses with AβP isolated from the deposits reveal that the predominant component of aggregation is the 42 amino acid form of AβP (AβP$^{1-42}$), and considerable structural rearrangements occur at Asp residues at the 1 and 7 positions of AβP, e.g. the Asp$^7$ residue is changed to isoAsp (~70 %) or Asp in the D-configuration (~10 %) [68-71] (Figure 3C). The amount of isoAsp residue is considerably lower in the peptide isolated from cerebrovascular amyloid in comparison to that from parenchymal amyloid plaques [70]. Several familial AD (FAD) mutations are reported to occur within the AβP region of APP. The Tottori FAD is characterized by the intra-AβP missense mutation causing the substitution of Asp$^7$ for Asn (D7N), and the two affected sisters in the Tottori kindred show the early-onset dementia, i.e. the age of onset are 60 and 65 years [72]. Both fibril formation and secondary structure transformation of the synthetic AβP (D7N) are accelerated in comparison to that of intact AβP [73,74]. The seeding effect on fibril formation also increases in AβP (D7N). Ion mobility mass spectrometry (IM-MS) reveals that the early aggregation state of AβP (D7N) is different from that of the intact peptide while the monomer structures of both peptides have no difference [75]. Another FAD mutation causing the substitution of Asp$^{23}$ for Asn (D23N), designated as Iowa, is also found, and characterized by showing symptoms of a progressive aphasic dementia, leukoencephalopathy, and occipital calcification [76]. Fibril formation of synthetic AβP (D23N) is remarkably accelerated and the protofibrils, which are observed as small globular oligomeric structures in early stage of fibril formation, also appear earlier [77,78]. Both two FAD mutations result in
increased fibril formation, suggesting that the increment is one of factors for the early onset of AD.

The presence of the isoAsp residue is confirmed by PIMT assay in the tryptic AβP\(^{17-28}\) prepared from the brain of Iowa kindred [77], which is to be expected because the rate of succinimide formation is 10 ~ 30 times faster with an Asn residue than it is with an Asp residue. Analysis using a synthetic AβP (D7isoD and D23isoD) reveals that the isoAsp\(^{23}\) residue accelerates the fibril formation, but the isoAsp\(^{7}\) residue has little effect on the formation [79]. Immunohistochemical studies using anti-isoAsp antibodies showed that the senile plaques and vascular amyloid of an AD brain are stained by anti-isoAsp\(^{23}\) antibody, but those of a control brain are not. On the contrary, anti-isoAsp\(^{7}\) antibody stains the senile plaques and vascular amyloid of both AD and control brains [80]. In addition, AβP\(^{1-42}\) (D7isoD) acts as a trigger for the formation of dense-core amyloid plaques in the APP transgenic mouse brain [81]. The phosphorylation of proteins such as tau, tubulins and matrin 3 is also accelerated by AβP\(^{1-42}\) (D7isoD) when the peptide is introduced to cultured cells [82]. The effect of Asp racemization on the fibril formation has also been examined using synthetic peptides. Stereoinversion of the Asp\(^{23}\) residue accelerates fibril formation, but that of Asp\(^{7}\) residue had little effect [83], and the stereoinversion of Asp\(^{1}\) residue strongly suppressed the acceleration of fibril formation by the D-Asp\(^{23}\) residue [84]. The drastically different rate change of fibril formation observed in AβP modified with isoAsp or D-Asp residue, as described above, suggests that the structural alteration of specific Asp residue may act as a potential trigger for AD amyloidosis. The fibril formation of AβP\(^{1-40}\) (N27D) is inhibited, indicating that the deamination also affects the formation rate [85].

**Prion Diseases**

**Pathogenesis of Prion Diseases and Prion Protein**

Prion diseases are fatal neurodegenerative diseases, such as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt-Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS) and Kuru in humans [86].
The common pathological hallmarks of prion diseases are the spongiform degeneration of glial cells and neurons. The accumulation of amyloidogenic prion protein (PrP) as the abnormal scrapie type isoform (PrP\textsuperscript{Sc}) is also observed in the brain of patients. Prion diseases are also called transmissible spongiform encephalopathies because their infection characteristics caused by the invasion of PrP\textsuperscript{Sc} in the pathogenetic tissues. Although the molecular pathogenesis and transmission pathway of prion diseases are still controversial, it is widely accepted that the conformational conversion of normal cellular prion protein (PrP\textsuperscript{C}) into an abnormal PrP\textsuperscript{Sc} is the transmissible characteristic of prion diseases.

Normal PrP\textsuperscript{C} is a 30-35 kDa cell surface glycoprotein anchored to the plasma membrane with a glycosylphosphatidylinositol (GPI) domain (Figure 5A). PrP\textsuperscript{C} is ubiquitously expressed in the body, and most notably in the brain. Both PrP\textsuperscript{C} and PrP\textsuperscript{Sc} have the same characteristic chemical modification of the same primary sequence. However, PrP\textsuperscript{C} differs from PrP\textsuperscript{Sc} in terms of resistance to protease digestion, a high content of β-sheet secondary structure, and the propensity to form insoluble amyloid fibrils. When the misfolded PrP\textsuperscript{Sc} enters into the body via the ingestion of contaminated food, for example, the protease-resistant PrP\textsuperscript{Sc} can aggregate, resulting in fibril formation that in turn promotes other PrP\textsuperscript{C} molecules in the brain to misfold and aggregate. These lines of evidence suggest that the conformational change of PrP is crucial for the pathogenesis of prion diseases.

**Prion Protein and Metals**

There are three possible neurodegenerative pathways in prion diseases: 1) supporting the “loss of the normal, protective functions of PrP\textsuperscript{C}”; 2) supporting a “gain of toxic functions of PrP\textsuperscript{Sc}”; 3) “a combination of both”. Although the physiological roles of PrP\textsuperscript{C} are not yet fully understood despite its wide distribution, increasing evidence suggests that PrP\textsuperscript{C} is a metal-binding protein and regulates metal homeostasis [87].
Regarding the first pathway, the link between Cu and prion diseases were first reported by Brown et al. in 1997 [88]. They demonstrated that the levels of Cu in the brains of PrP-knockout mice were significantly decreased compared with that of normal mice. The activity of Cu-dependent enzymes was also reduced in PrP-null mice. As shown in Figure 5, PrP<sup>C</sup> contains 208 amino acid residues and possesses a highly conserved octarepeat domain composed of multiple tandem copies of the eight-residue sequence PHGGGWGQ at its N-terminus (Figure 5A). Thus, PrP<sup>C</sup> binds to 4 Cu atoms in its octarepeat domain and binds to 2 Cu atoms in other His residues, namely His<sup>96</sup> and His<sup>111</sup> [89]. Other metals including Zn<sup>2+</sup>, Mn<sup>2+</sup>, and Ni<sup>2+</sup> bind to these binding sites with lower affinities compared with Cu<sup>2+</sup>. PrP<sup>C</sup> reportedly transports Cu<sup>2+</sup> from the extracellular space to the intracellular space via endocytosis and regulates the intracellular concentrations of Cu<sup>2+</sup>. Furthermore, PrP possesses or modulates Cu/Zn superoxide dismutase (SOD) activity in the brain and plays roles in the cellular resistance to oxidative stress [90]. Therefore, the depletion of PrP<sup>C</sup> and the resulting metal dyshomeostasis may trigger neurodegenerative processes. Indeed, PrP-deficient neurons exhibit lower glutathione activity and susceptibility to hydrogen peroxide [91]. PrP<sup>C</sup> regulates the excitability of N-methyl-D-aspartate (NMDA)-type glutamate receptor in a Cu-dependent manner [92]. Meanwhile, Cu<sup>2+</sup> influences the gene expression and cellular trafficking of PrP [93].

PrP<sup>C</sup> is also implicated in the homeostasis of other metals such as Zn and Fe. Bioinformatics analysis has revealed evolutionary similarities between prion genes and genes encoding Zrt-, Irt-like protein (ZIP)-type Zn transporters [94]. Watt et al. reported that PrP<sup>C</sup> enhanced cellular uptake of Zn<sup>2+</sup> via binding to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) -type glutamate receptor, and that PrP<sup>C</sup> acts as a Zn<sup>2+</sup> sensor in the synapse [95]. PrP facilitates Zn<sup>2+</sup> influx into the brain, regulates Zn homeostasis, and attenuates Zn-induced neurotoxicity. Furthermore, PrP<sup>C</sup> reportedly has the ferrireductase activity that converts Fe<sup>3+</sup> to Fe<sup>2+</sup>, and then modulates the cellular uptake of Fe<sup>2+</sup> [96]. Fe<sup>2+</sup> ions are oxidized to Fe<sup>3+</sup> by ferroxidases (such as ferritin or ceruloplasmin) in the blood stream, then Fe<sup>3+</sup> is transported with transferrin (an iron-binding protein that binds
two Fe$^{3+}$ ions) across the blood–brain barrier via transferrin receptors and enters neurons or glial cells. Then, the Fe$^{3+}$ is reduced to the bioactive Fe$^{2+}$ by ferrireductase and transferred to neuronal enzymes, which require Fe$^{2+}$ as a cofactor. Indeed, PrP-knockout mice exhibit altered Fe metabolism and Fe deficiency [97].

Metals are also implicated in the conformational changes and neurotoxicity of PrP$^{Sc}$, which are central for the transmission and the pathogenesis of prion disease. To investigate PrP$^{Sc}$ neurotoxicity, we and other researchers have employed synthetic fragment peptides of PrP (PrP$^{106-126}$) as a model peptide of PrP$^{Sc}$, considering the methodological difficulties of using a whole prion protein owing to its strong infectious characteristics [98]. The structure of PrP$^{106-126}$ coincides with the proposed β-sheet structures of PrP$^{Sc}$. PrP$^{106-126}$ forms aggregates with β-sheet structures, similar to amyloid fibrils, that share several characteristics of PrP$^{Sc}$ in that it causes the apoptotic death of cultured neurons or glial cells.

PrP$^{106-126}$ possesses the ability to bind to metals including Cu$^{2+}$ and Zn$^{2+}$. We found that PrP$^{106-126}$ forms β-sheet structures during the “aging” process (incubation at 37 °C for several days) as determined using the ThT fluorescence assay, CD spectroscopy, and AFM imaging [10]. Moreover, aged PrP$^{106-126}$ exhibits enhanced neurotoxicity on primary cultured rat hippocampal neurons. Thus, we added various trace elements or metal chelators to solutions of PrP$^{106-126}$ during the aging process and evaluated its conformational changes and neurotoxicity. We demonstrated that the co-existence of Zn$^{2+}$ or Cu$^{2+}$ during the aging process significantly attenuated the neurotoxicity of PrP$^{106-126}$ and oligomerization ability of PrP$^{106-126}$. Furthermore, aged PrP$^{106-126}$ forms amyloid fibrils with distinct straight and long morphology on mica plates, as observed using AFM, although we did not observe fiber-like structures in freshly prepared PrP$^{106-126}$. Moreover, aged PrP$^{106-126}$ in the presence of Cu$^{2+}$ or Zn$^{2+}$ exhibited different morphological features compared with that of aged PrP$^{106-126}$ alone. Therefore, it is possible that Cu$^{2+}$ and Zn$^{2+}$ influenced the β-sheet formation of PrP$^{106-126}$, and thereafter attenuated its neurotoxicity.
Figure 5: Prion protein structures and analysis of Asn deamidation.

A) The structure of PrPC and metal-binding sites. Octarepeat domain and neurotoxic fragments PrP\(^{106-126}\) are depicted. PrPC possesses six metal-binding sites. The HPLC profiles after incubation for 28 days at 37 °C in 50 mM phosphate buffer (pH 7.4) are shown. The PrP\(^{106-126}\) was analyzed with a mobile phase containing 20% acetonitrile, a 15 mM sodium phosphate solution (pH 5.0), and 100 mM NaCl. The amount of each peak is summarized in left graph.

B) Asn deamidation and Asp isomerization in rodent prion protein are shown.

Asn Deamidation in Prion Protein

Human or murine PrP possesses 11 or 13 Asn residues, respectively (Table 1). PIMT assay using the recombinant murine prion protein revealed that isoAsp residues increased the half-life of PrP to 33 days, and 0.8 mol isoAsp is accumulated per mol of protein after incubation of 135 days [99]. The Asn\(^{107}\) residue is the main position for isoAsp accumulation, and Asp\(^{226}\), which exists only in murine PrP is also isomerized. Asn\(^{107}\) residue is deamidated even in the PrP sample stored at -20 °C for several months; moreover, the deamidated Asn\(^{107}\) residue...
changes the sensitivity for metal ions [100]. Interestingly, the aged PrP, in which Asn\textsuperscript{107} is deamidated forms aggregates and gains proteinase-K resistance in the presence of Cu\textsuperscript{2+}. To quantify the structural alteration of Asp residues, we have established a simple method using reversed phase HPLC, with a standard octadecylsilane column, and applied it to analyze the deamidation of Asn\textsuperscript{108} in human PrP\textsuperscript{106-126} peptide (or Asn\textsuperscript{107} in murine prion) [14] (Figure 5A). Under physiological conditions, the Asn\textsuperscript{108} is deamidated with a half-life of 10 days. The different half-lives between in human and murine prions is due to the difference of the amino acid residues on the carboxyl side of the Asn residue and the highly ordered structure. D-Asp or D-isoAsp residues are also observed in PrP\textsuperscript{106-126} peptide after 28-days incubation at 37°C. Although the deamidated amino acids are accumulated with a comparatively high rate in Asn\textsuperscript{108} in human PrP, there is little information about the biological significance of deamidated PrP.

**Figure 6:** Structure of α-synuclein. The metal-binding sites and Asn deamidation sites are depicted. The sites of Asn deamidation are indicated by the closed circles.

**Lewy Body Diseases**

Lewy body diseases include Parkinson’s disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy, \textit{etc} [101]. These diseases commonly exhibit abnormal cellular inclusions called Lewy bodies, which are the accumulation of α-synuclein, and are therefore known as synucleinopathies. DLB
includes approximately 25% of all senile dementia cases and shares some pathological changes with AD, such as the deposition of senile plaques and tau protein. Moreover, the α-synuclein fragment peptide - non-amyloid component (NAC) - co-accumulates with AβP in the senile plaques of patients with AD. The oligomerization and fibrillation of α-synuclein have been implicated in the formation of Lewy bodies and the etiology of Lewy body diseases, similar to AβP and PrP. α-Synuclein is abundant in the brain, primarily in presynaptic terminals, and is thought to play roles in maintaining the supply of synaptic vesicles to the presynaptic terminals and regulating the release of the dopamine, as well as in synaptic functions and plasticity [102]. A comprehensive analysis with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) reveals that two Asn residues at the 103 and 122 positions are deamidated dominantly with a half-life of 60 days in α-synuclein [103] (Figure 6). Furthermore, α-synuclein can bind metals including Cu, Mn, and Fe. α-Synuclein has ferrireductase activity that converts Fe^{3+} to Fe^{2+}, and transfers bioavailable Fe^{2+} to many enzymes, such as thyrosine hydroxylase, and regulates the biosynthesis of neurotransmitters [104]. However, oligomerized α-Synuclein reportedly has no ferrireductase activity [105]. As shown in section 4.2, PrP<sup>C</sup> has similar ferrireductase activity. Considering that α-synuclein is localized in the presynaptic domain and PrP<sup>C</sup> is in the postsynaptic domain, these two amyloidogenic proteins may regulate neurotransmitter synthesis by controlling Fe^{2+}/Fe^{3+} ratio in the synapse [6]. In contrast, Fe regulates the expression of α-synuclein because its mRNA has an IRE domain, similar to APP and ferritin [106].
Figure 7: Summary of deamidation and isomerization in amyloidogenic proteins and peptide. Asn deamidation and Asp isomerization are indicated by the closed circles and open circles, respectively. The relationship between Asn substitution and fibril formation is also shown. (↑) acceleration or increase of fibril formation, (↓) suppression or decrease, (-) little or no effect.

Other Amyloidosis

The accumulation of islet amyloid polypeptide (IAPP or amylin) in the islets of Langerhans is a hallmark of type 2 diabetes mellitus. IAPP is a 37-residue polypeptide with a disulfide bridge between the Cys² and Cys⁷ residues, and acts as a partner hormone to insulin to control blood glucose concentration [107]. IAPP also has affinity for metals, such as Zn²⁺ or Cu²⁺[108]. Cu²⁺ inhibits the formation of amyloid by IAPP and attenuates the cell toxicity of amylin [109]. IAPP incubated for several
months under physiological conditions exhibits deamidation of at least four Asn residues at rates of 70 ~ 90 %, and isotope-labeled IR spectroscopy shows that the fibril formation of deamidated IAPP was faster than that of intact IAPP [110] (Figure6). Pramlintide is a synthetic analogue peptide of human IAPP in which residues 25, 28, 29 are replaced with proline. To examine the stability, Asn deamidation was analyzed by HPLC-MS [111]. After incubation for 45 days at 40 °C, deamidation was found in four Asn residues, at positions 14, 21, 22, and 35 of pramlintide. The seeding effect was examined by using short peptide fragment, IAPP20-29 [112]. While IAPP20-29 alone cannot form fibrils, a sample of peptide with as little as 5% deamidated peptide leads to the formation of amyloid deposits. The fibril formation of IAPP was examined by chemical substitution of Asn residues. The IAPP peptides were prepared in which three Asn residues at 14, 21, and 35 positions, had been substituted with either L-Asp or L-isoAsp and then assayed by using ThT [113]. Substitution N14D accelerates the fibril formation, the shape of which was long and thick; however, N14isoD shows little effect on fibril formation. Substitutions N21D and N21isoD drastically inhibit the fibril formation; however, N35D and N35isoD shows little effect.

β2-Microglobulin (β2M), which is a single-chain polypeptide composed of 99 amino acids is a serum protein that serves as a component of major histocompatibility complex class I. Thus, β2M is required for antigen presentation in the immune responses. However, in some pathological conditions, the protein forms amyloid fibrils and is found as the major component of deposits associated with dialysis-related amyloidosis [114]. A heterogeneity of β2M is reported in patients treated with long-term hemodialysis. Several metals including Cu^{2+}, Zn^{2+}, Ni^{2+}, and Al^{3+} can bind to β2M and influence the amyloid formation [115,116]. The sequencing analysis of the minor form of β2M reveals that it has identical amino acid sequence except for the substitution of Asn^{17} residue for Asp, suggesting that the Asn residue has been deamidated during long-term hemodialysis [117] (Figure7). Asn deamidation of β2M was analyzed by a comprehensive top-down approach with FT-ICR MS [118]. Three Asn deamidation residues at 17, 42, and 83 positions were
found in aged β2M. The recombinant β2M (N17D) is able to form amyloid fibrils faster than the intact β2M under acidic conditions [119].

SOD1 is widely used in our bodies for protection against free radicals. It was the first identified protein for familial amyotrophic lateral sclerosis (ALS), which is a lethal neurodegenerative disease caused by loss of motor neurons [120]. Over 180 different mutations in the SOD1 gene have all been linked to the cause of ALS. The missense mutations such as N86D and N139D in SOD1, which cause familial forms of ALS, change the protein’s thermodynamic stability and folding behavior [121]. The deamidation rate of all seven Asn residues of SOD1 has been calculated by a computational method developed by Robinson et al [122], which indicated that Asn residues at positions 26, 131, and 139 will be deamidated with 99, 55, and 21 % conversion after 450 days under physiological conditions, respectively. The lifetime of SOD1 is expected to be long enough to allow the accumulation of the deamidated residues. The recombinant protein (N139D) forms amyloid fibrils two times faster than that of intact SOD1, and the triple mutated protein (N26D, N131D and N139D) is also accelerated with an identical rate to that of N139D [123] (Figure 7). These results suggest that deamidation of Asn residues in intact SOD1 has the same effect on fibril formation as the familial SOD1 mutant (N139D).

Curli, observed in various bacteria including Escherichia coli, is a functional extracellular amyloid fiber and is associated with surface colonization and interaction with the host immune system [124]. The major curli subunit is CsgA protein, which has a self-polymerization ability, which allows it to form amyloid fibers. CsgA is composed of 130 amino acids and has 16 Asn residues. LC-MS/MS analysis reveals that deamidation is found in 14 Asn residues, and especially those at position 57, 87, and 102 are rapidly deamidated with a half-life of 2.5 ~ 5.3 days [125] (Figure 7). An aged CsgA incubated for 19 days shows little or no fibril formation, thus deamidation disables the fibril formation in bacterial curli.
Conclusion

Based on our findings and other numerous studies, amyloids share common characteristics such as metal binding and Asn deamidation. These two non-genetic factors can reflect the aging process, and influence the oligomerization, conformation and ultimately the neurotoxicity of amyloidogenic proteins. Moreover, as we have shown here, metal-binding Asn residues can be deamidated, and the combination of these two factors profoundly affects the amyloidogenesis, such as the effect of Cu$^{2+}$ on the aged murine PrP [100].

Considering these common characteristics, we hypothesized that the pathogenesis of conformational diseases is linked with metals and deamidation. This hypothesis may help in the development of drugs for these diseases, then substances which influence these two factors may become candidates for the treatment of these diseases. We have already focused carnosine as one of such substances because it possesses the various beneficial attributes such as, metal-chelating, antioxidant, anti-glycation, and anti-crosslinking [126,127]. In particular carnosine is used for the treatment of cataracts owing to its anti-crosslinking ability [128]. The protective activity of carnosine against the accumulation of AβP has also reported [49]. Concerning substances that can suppress Asn deamidation, further research is urgently required because there are few reports in this under-researched field. Further research about the detailed characteristics, including the conformation and the toxicity, of deamidated amyloids is necessary.

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