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Review

Amyloid fibrils from the viewpoint of protein folding

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Abstract. In amyloid related diseases, proteins form fibrillar aggregates with highly ordered β-sheet structure regardless of their native conformations. Formation of such amyloid fibrils can be reproducible in vitro using isolated proteins/peptides, suggesting that amyloid fibril formation takes place as a result of protein conformational change. In vitro studies revealed that perturbation of the native structure is important for the fibril formation, and it is suggested that the mechanisms of amyloid fibril formation share the mechanisms of protein folding. In particular, amyloid fibril formation is similar to one of the common features of proteins, i.e. amorphous aggregation upon partial unfolding, which is likely driven by hydrophobic interactions through exposed protein interior. However, these molecular associations are distinct phenomena, and identifying factors that lead to amyloid fibril formation would precede our understanding of the mechanisms of amyloid fibrillization. The necessity of understanding the nature of protein denatured states is also suggested.

Key words. Amyloid fibrils; protein folding; aggregation; denatured states.

Introduction

Protein is a chain molecule that consists of 20 kinds of amino acids, which spontaneously folds into a stable structure to be functional. Protein folding is a process in which a protein acquires its native structure. Since the era of Anfinsen, numerous studies to understand protein folding mechanisms have been carried out, but we are still far from a complete understanding of protein-folding. For example, it is still very difficult to predict the structure of a protein from its sequence, and in this era of postgenomics, further understanding of protein-folding mechanisms is much in demand. In the concept of the energy landscape [1, 2], one of the most popular recent concepts describing protein folding, folding properties of proteins are depicted by a funnel-like energy profile with a rough surface defined by thermodynamic and kinetic properties. The native state usually locates at the lowest minimum of the funnel, implying that it is the most stable configuration of the polypeptide chain. However, there are some known cases that are inconsistent with the native state being the most stable. Formation of amyloid fibrils is one such case, and the mechanisms underlying the formation of amyloid fibrils have drawn intense interest in the protein science community in recent years.

Amyloid fibrils are found as deposits of insoluble aggregation in patients with a range of diseases such as systematic amyloidoses, spongiform encephalopathies and Alzheimer's disease. These fibrils are formed with a single kind of protein or peptide, not a mixture of multiple kinds in general, and about 20 proteins are known to be associated with human disease [3]. Particularly interest-

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ing is the case of prion diseases, a large group of neurodegenerative disorders that affect both animals and humans. Prion protein, a predominantly α-helical protein constituent of mammalian cells under normal conditions converts its structure into a β-sheet form responsible for amyloid fibril formation by unknown mechanisms [4]. This conformational conversion occurs without apparent additional factors such as nucleic acids or polysaccharides, and it is transmittable by the modified isoform, leading to the proposal of the 'protein only' hypothesis [5-7]. In vitro studies using isolated proteins such as Bence Jones proteins [8], β_2-microglobulin [9], insulin [10], transthyretin and its fragments [11], peptide homologues to Alzheimer β protein [12, 13] and a peptide homologue to a prion protein fragment [14] have demonstrated amyloid fibril formation with isolated proteins/peptides, supporting this hypothesis. Thus, understanding the mechanisms of conformational conversion is essential for understanding the mechanisms of amyloid fibrillization.

In early 1990s, a study based on the principles of protein folding linked the formation of amyloid fibrils and protein folding more directly: Colon and Kelly have demonstrated amyloid fibril formation of transthyretin (TTR) under acidic denaturing conditions where the tetrameric protein dissociates to a partially denatured form [15]. Based on this observation, they hypothesized that a folding intermediate converts into a 'misfolded' conformer responsible for amyloid fibril formation. This hypothesis is well supported by a series of experiments by Dobson and co-workers, in which proteins that are not relevant to amyloid disease are shown to be capable of forming amyloid fibrils under destabilized conditions [16–20]. These findings led them to propose a hypothesis: 'the potential for amyloid deposition may be a common property of proteins, and not only of a few proteins associated with disease' [16]. Thus, it is likely that the key mechanisms of amyloid fibril formation share the key mechanisms of protein folding, and therefore it is quite reasonable to study this mysterious phenomenon from the viewpoint of protein folding. In this review, we focus on structural studies toward elucidation of molecular mechanisms of amyloid fibril formation approached from this viewpoint. After describing criteria of amyloid fibrils, we introduce case studies of several well-characterized proteins. Then we highlight the similarity between general protein aggregation and amyloid fibril formation, both being linked to protein folding. Finally, we discuss the relevance between amyloid fibril formation and protein denatured states.

General properties of amyloid fibrils

Historically, Divry and Florkin demonstrated in the 1920s that Congo-red stained amyloid deposits in biologic tissues exhibit apple green birefringence under polarized light [21], which became the first criterion of amyloid fibrils. This birefringence is not always simple to demonstrate and sometimes encounters practical difficulties, such as sample requirements for a certain thickness. Due to such difficulties, a simpler dye binding assay using Congo-red [22] or another dye binding assay using thioflavin T have been widely used as indicators in amyloid studies in vitro [23, 24]. However, Khurana et al. demonstrated that the simpler Congo-red binding assay shows false positives in some cases [25], emphasizing the importance of the birefringence property.

This microscopic property of showing birefringence implies the nonamorphous structural feature of the fibrils, which was verified by the first observation of the fibrous morphology in amyloid deposits through electron micrography [26]. Later studies using electron micrography with higher resolution revealed a consensus structural feature: a twisted nonbranched fiber with 7–12 nm diameter [27].

In late 1960s, two X-ray diffraction studies of dried fibrils reported a strong meridional reflection at 0.47 nm and a weaker equatorial reflections at 0.98 nm in amyloid plaques [28, 29]. The meridional reflection indicates a regular structural repeat of 0.47 nm along the fibril axis, and the equatorial reflection indicates a structural spacing of 0.98 nm perpendicular to the fibril axis. These reflections are characteristic of a 'cross β' structure, where ordered β-pleated sheets propagate with the direction of the each strand perpendicular to the long axis of the fiber [30]. With high-resolution data obtained through intense use of synchrotron sources, Sunde et al. have demonstrated that six different ex vivo amyloid fibrils have a common protofilament substructure based on the cross β motif [31].

Because of this β-rich nature of amyloid fibrils, spectroscopic procedures such as infrared [32] or circular dichroism (CD) [33] have been widely used for low-resolution characterization. These spectroscopies are sensitive to protein secondary structure and are applicable to insoluble protein samples, in particular infrared. Therefore, they serve as a powerful tool to monitor conformational conversion of proteins/peptides upon fibril formation.

The formation of amyloid fibrils is a two-step reaction that consists of a slow nucleation step followed by a molecular assembly step [34–36]. Interestingly, seeding of preformed fibrils diminishes the lag phase of the nucleation step [37–39], suggesting that the nucleation step plays an important role in the kinetics of fibril formation.

In summary, common hallmarks of amyloid fibrils are enumerated as follows: Congo-red binding ability with positive birefringence, nonbranched fibrous morphology with dimensions of 7–12 nm, X-ray fiber diffraction pattern with unique reflections at 0.47 nm and 0.98 nm, β-

sheet rich secondary structure and a two-step reaction with a slow nucleation step followed by a faster molecular assembly step. In addition, high resistance to proteases may be included [14, 40]. Thus, study of in vitro amyloid fibril formation should be carefully examined with multiple criteria.

Amyloid fibril formation hinted by protein folding

Here, we highlight case studies of several amyloidogenic proteins, where formation of amyloid fibrils was linked with their folding behavior.

TTR

TTR is a homotetrameric protein consist of 127 residue subunits. The crystal structure of TTR exhibits a β-sandwich structure with eight strands [41]. Wild-type TTR is very stable at neutral pH [42], although it is converted into amyloid fibrils in patients with senile systematic amyloidosis [43]. In addition, about 70 single mutants have been associated with familial amyloid polyneuropathies [44]. Kelly and co-workers focused on the connection between protein stability and amyloidogenicity in the early 1990s. First, they demonstrated that partial denaturation of wild-type TTR by acidic pH drives amyloid fibril formation [15] and that destabilization upon mutagenesis also increases its amyloidogenicity [45]. Further thermodynamic characterization of the wild-type TTR revealed marked hysteresis upon guanidium chloride denaturation, which is not observable upon urea denaturation [46]. The following study showed that the charge pair of Lys15 and Lys15′ at the subunit interface in the tetramer plays an important role in protein stability and that anion shielding of the electrostatic repulsions stabilizes the tetramer, thus inhibiting amyloid fibril formation [47]. These results highlight the critical dissociation step of the tetramer into monomers. Based on these observations, Kelly and co-workers have demonstrated a few strategies to inhibit amyloid fibrillization by stabilizing the tetrameric state against dissociation, i.e. incorporation of one or two subunits with a stabilizing mutation into an amyloidogenic tetramer [48], and binding of small compounds that increases the kinetic stability of the native state [49].

A hydrogen-deuterium (H/D) exchange nuclear magnetic resonance (NMR) study of wild-type TTR revealed significant perturbation on one β sheet of the β-sandwich protein, where pathogenic single mutations are identified with high frequency [50]. The same approach with the TTR variants revealed that single mutations in the amyloid-causing variants resulted in significant destabilization and increased flexibility of the core region, while that in an amyloid-suppressing mutant led to increased stability and flexibility of the core region [51]. Despite good correlation between destabilization of the core strands and the tendency for amyloid fibril formation, there are other pathogenic variants that have mutations outside of the core region. There may be other regions important for the fibril formation of TTR.

Human lysozyme

Mutant human lysozymes, I56T, D67H, W64H and F57I have been identified in hereditary nonneuropathic systemic amyloidosis that leads to death by the 5th decade [52–55]. Human lysozyme is an $\alpha+\beta$ protein that consists of two domains (α and β domains). The residues 64 and 67 locate in the β domain, while residue 56 locates between the two domains. A comparison of crystal structures between the I56T mutant and the wild type reported almost no change in the structure except in the vicinity of the mutation site, while thermodynamic and kinetic data revealed a significant decrease in protein stability [56]. These results suggest that partially denatured structures induced by destabilization upon mutation are responsible for amyloid fibril formation. Later studies using other amyloidogenic variants reached the same conclusion [57–61]. The conclusion is further supported by direct evidence of partial unfolding in an amyloidogenic variant; H/D exchange study on the D67H variant demonstrated that this destabilized variant exhibited a significantly increased level of fluctuation in the β domain but not in the α domain [62]. The key role of structural perturbation in the β domain in fibril formation is further supported by an artificial modification of this region; insertion of the EAEA sequence in the β domain significantly enhanced the amyloidogenic propensity [63].

A recent study of the naturally occurring T70N variant that is non-amyloidogenic reports a comparative result [64]. This mutant is less active, less stable and less protected in the H/D exchange than the wild type. However, it shows a higher level of H/D protection than the wild type in the helix 3–10, suggesting this region prevents further conformational fluctuation beyond a certain level to allow competitive formation of amyloid fibrils, as seen in cases of amyloidogenic variants. These results suggest that destabilizing mutations do not always lead to amyloid fibril formation and a particular intermediate structure is required for fibril formation. Only a small modification inhibiting the formation of such a critical intermediary structure may prevent amyloid fibril formation.

β_2-Microglobulin (β_2m)

β_2-Microglobulin (β_2m) is a 12-kDa protein subunit that is necessary for cell surface expression of the class I major histocompatibility complex (MHC). Its crystal structure reveals a seven-stranded β-sandwich structure with a

disulfide bond between strands B and F, typical of proteins that belong to immunoglobulin superfamily [65]. The turnover of MHC results in release of soluble β_2m followed by degradation and excretion in the kidney. In patients with dialysis-related amyloidosis, β_2m forms amyloid fibrils mainly in the joints, resulting in a variety of arthritis. So far, full-length wild-type β_2m [66], modified and truncated versions of the protein [67], and fragmented peptides [68, 69] have been found to form amyloid fibrils, while no naturally occurring mutations have been associated with the disease. In the meantime, an in vitro study with an induced single deamidated mutation, asparagine to aspartate, on a loop region has shown that this mutation significantly destabilizes the protein and increases the amyloidogenic propensity [70]. As for the wild-type protein, it has been reported that incubation of high concentrations of β_2m at neutral pH did not lead to amyloid fibrils even in the presence of ex vivo β_2m amyloid seeds, while fibril formation was greatly enhanced at acidic pH with a high salt concentration, under which conditions this protein is partially denatured [71]. Thus, protein stability and the amyloidogenic propensity appear correlated for this protein as well as TTR and lysozyme described above. However, acid-induced protein destabilization and the resulting amyloid fibril formation are difficult to link with the pathological case of dialysis-related amyloidosis. Miranker and co-workers demonstrated that the addition of transition metals, in particular Cu^{2+}, significantly destabilizes β_2m and enhances the fibril formation of β_2m at neutral pH [72]. This finding provides a plausible scenario for the pathological case, since there is a high chance of uptake of free Cu^{2+} in the clinical dialysis procedure: a low but significant copper content in dialysate and in the dialysis membrane [72]. Later studies identified key histidine residues that are responsible for protein destabilization upon the addition of Cu^{2+}, which leads to amyloid fibril formation [73, 74].

It is noteworthy that several NMR studies provide a significant glimpse of the molecular mechanisms of amyloid fibril formation. Goto and co-workers mapped the β_2m amyloid core region through a quenched H/D exchange technique, where hydrogen-exchanged amyloid fibrils were dissolved in a dimethylsulfoxide (DMSO) solution to directly map the extent of H/D exchange on the monomeric protein [75]. Their results showed that the amyloid core region consists of four strands, including flexible loop regions. Radford and co-workers reported complementary results on a partially denatured form of β_2m, in which a region similar to the core region identified in the H/D study remained folded in an amyloidogenic intermediate form [76]. These results provide structural perspectives for protein conformation in amyloid fibrils, for which atomic-resolution structure is difficult to obtain due to the noncrystalline or insoluble nature of amyloid fibrils.

Prions

Prion diseases include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) and scrapie in animals, where cellular prion protein, referred to as PrP^C, is converted into a disease-specific oligomeric isoform, referred to as PrP^{SC}. PrP^C is a monomeric extracellular protein covalently linked to glycosylphosphatidylinositol, and it is likely involved in signal transduction [77] and/or controlling copper ion concentration [78].

A similar transmittable conformational conversion of proteins is found in yeast, leading to protease-resistant aggregation [79-81]. These proteins are called yeast prions. Unlike mammalian prions, yeast prions do not cause cell death. Two well-studied yeast prions, Sup35 and Ure2, have glutamine- and asparagine-rich regions, and a few studies have pointed out the important role of this polar region at the amino termini in the formation of amyloid fibrils [82–84].

Fourier transform infrared (FTIR) and CD studies have revealed that hamster PrP^C is highly helical (42%), with little β-sheet content (3%), while PrP^{SC} contains less α-helical structure (30%) with an elevated level of β-sheet content (43%) [85]. The PrP^C form can be converted to the PrP^{SC} form upon contact, and thus it is proposed that PrP^{SC} acts as a template that promotes the conversion of PrP^C to PrP^{SC} and that this conversion involves only conformational change [86]. The high-resolution structure of the autonomously folding PrP^C domain comprising residues 121–231 was first published by Wüthrich and co-workers through NMR method on a recombinant mouse protein [87]. Subsequently, other groups published NMR structures of recombinant hamster PrP^C (comprising residues 90–231) [88] and human PrP^C (comprising residues 90–231) [89]. These proteins essentially share the same structural features: the flexible N-terminal tail ~100 residues long and the predominantly α-helical C-terminal domain with three α helices and a short β hairpin.

The C-terminal domain unfolds in the presence of chaotropic denaturants, and the reaction is reversible. Kinetic studies of mouse PrP^C showed unusually large folding and unfolding rates with no detectable folding intermediates at physiological pH [90]. In addition, an H/D exchange study on human PrP^C supports the absence of partially unfolded intermediates in its conformational equilibrium [89]. Moreover, the data identified a region with residual structure in the unfolded state, suggesting that this small region in the unfolded state plays an important role in the conformational conversion. The authors concluded that PrP^{SC} is unlikely to be formed from a partially unfolded state but rather from a largely unfolded state, a pathway distinct from the suggested pathways of amyloid fibril formation for other proteins. The difference in mechanisms for fibril formation is also suggested by a set of mutagenesis studies, where change in

protein stability upon mutation did not correlate with the tendency for amyloid fibril formation [91].

Although PrP unfolds via a two-state mechanism at physiological pH, an alternative folding pathway is observed at acidic pH. Studies on recombinant human PrP [92] and mouse PrP [93] detected an equilibrium folding intermediate around pH 4.0 with a secondary structure rich in β sheet, and the authors proposed that this may be an on-pathway intermediate responsible for PrP^{SC} formation. Subsequent studies on human PrP^{C} have established a condition at acidic pH under which the protein folds into a soluble monomer with high β-sheet content and partial resistance to proteinase K digestion, hallmarks of amyloid fibrils [94]. Reduction of the native disulphide bond is necessary for β-sheet formation and, thus, the importance of an alternating folding pathway dependent on pH and redox potential is suggested.

Other peptides/proteins

There are a few relatively short polypeptides known to form amyloid fibrils in human diseases, such as Alzheimer β peptides (Aβ peptide, a 42-residue peptide and its variations) in Alzheimer disease and a 37-residue peptide of islet amyloid polypeptide (IAPP, sometimes called as amylin) in type 2 diabetes. Extensive studies have been carried out for these cases and many interesting aspects have been revealed. As for structural aspects, core regions in fibrils of these peptides were identified [95–97], and solid-state NMR study supported an extended parallel β-sheet conformation in the fibrils of 1–40 Aβ peptide [98–100]. Here, however, we do not present these cases in detail because of the main focus of protein folding.

There are other amyloidogenic proteins that are involved in a range of amyloid diseases such as the V_L domain of immunglobulin (I_g) light chain in monoclonal protein systemic amyloidosis [101] and gelsolin in Finnish hereditary amyloidosis [102]. However, particularly interesting in the context of amyloid fibril formation linked to protein folding is a series of disease-unrelated cases reported by Dobson and co-workers. They first demonstrated that the SH3 domain, a well-characterized small protein module that has no relevance to amyloid disease forms amyloid fibrils under acidic conditions where this protein is partially denatured [16]. Later, they demonstrated amyloid fibril formation of non-disease-related proteins such as fragments from cold-shock protein B [17], hen lysozyme [18], cytochrome c [20] and myoglobin [19] under destabilizing conditions. The SH3 domain and cold shock protein all have β structure in the native form, while hen lysozyme has $\alpha+\beta$ structure and, remarkably, cytochrome c and myoglobin have all α structure. These findings strongly support the hypothesis that the potential for amyloid deposition may be a common property of all proteins and invoke an argument that the protein native state may not necessarily be the energy minimum in the folding landscape.

Kinetic mechanisms of amyloid fibril formation in protein folding

A slow nucleation step is one of the unique features of amyloid fibril formation. Kinetic studies have reported that seeding with preformed fibrils accelerates the fibrillization reaction [37–39], highlighting the important role of the nucleation step in amyloid fibril formation. As reviewed by Kelly [103], there are models proposed based on kinetic studies such as the nucleated conformational conversion model [104], the monomer-derived conversion model [7], the template-assisted model [105], the nucleated polymerization model [35] and the off-pathway folding model [106, 107]. Recently, the dimer-driven association model has been proposed [108]. However, the presence of all of the models argues against a unique mechanism for all types of amyloid. In this context, a recent report by Kayed et al. is notable, where they found that a dozen soluble oligomers of amyloidogenic proteins/peptides tested all displayed a common structure regardless of sequence [109].

Molecular mechanisms for amyloid fibril formation

As many studies have shown, it is highly likely that partial unfolding is important for amyloid fibril formation. However, it is still not clear how such partially unfolded molecules associate into amyloid fibrils. In this section, we review proposed molecular mechanisms of amyloid fibril formation.

Polar zipper model

Four inherited neurodegenerative diseases are linked to abnormally expanded glutamine repeats in the affected protein. Based on experimental data for the polyglutamine amyloid system, Perutz and co-workers proposed the 'polar zipper' model where β sheets are stabilized with a network of hydrogen bonds involving polar residues such as glutamine and asparagine [110, 111]. Such glutamine- and asparagine-rich regions are commonly found in the N termini of both mammalian and yeast prion proteins [84]. In the literature, glutamine and asparagine are frequently found in the sequence of polypeptides known to form amyloid fibrils. Moreover, Orpiszewski and Benson found a significant increase in amyloidogenic propensity upon neutralization of aspartates, including mutation to asparagine [112], consistent with the importance of enrichment of asparagine or glut-

amine in amyloid fibril formation. Furthermore, it has been reported that a highly polar peptide formed amyloid-like fibrils by the addition of trifluoroethanol (TFE), which enhances inter- and intramolecular hydrogen bonds [38]. In these cases, the 'polar zipper' network of hydrogen bonds may be at work in stabilization of β-sheet structure. However, this polar zipper model may not be a general mechanism, since there are other cases that certainly involve few or no polar residues. For example, Blondelle et al. reported an extreme case in which alanine-rich sequences such as $KYA_{13}K$, AGAAAAGA, AGAAAAGAVVGGLGG, which formed β-sheet fibrils [113].

Domain-swapping model

Domain swapping is a mechanism wherin two or more protein molecules form a dimer or oligomer by exchanging one or multiple structural domain(s) [114]. In the past several years, the number of structures of domain-swapped proteins has increased dramatically, and it has been proposed that domain swapping may be a possible mechanism for amyloid fibril formation [115]. Recent crystallographic studies identified domain swapping in a dimer form of PrP^C [116] and an amyloidogenic protein, human cystatin C [117, 118], supporting this hypothesis. In addition, there is other experimental evidence supporting this hypothesis. Rousseau and co-workers found a good correlation between domain swapping and the tendency for aggregation for a cell cycle regulatory protein, p13suc1 [119]. Domain swapping in bovine pancreatic ribonuclease (RNaseA) was identified in a dimer form produced at acidic pH, under which conditions the protein forms higher-order oligomers through a partially unfolded state [120]. In this case, the interface is formed by an interstrand β sheet with hydrogen bonds involving asparagines, reminiscent of the polar zipper model. The authors proposed a hybrid mechanism of polar zipper and domain swapping for the dimerization of RNaseA. Although the mechanistic and thermodynamic details of domain swapping are unclear, these results suggest a significant similarity in the mechanism between dimer/oligomer formation with domain swapping and amyloid formation.

To the best of our knowledge, the sizes of polypeptides that form amyloid fibrils vary from several to a few hundred residues in length. It is obvious that very short peptides cannot undergo domain swapping. Probably such a short peptide itself may serve as an interface. Peptide studies identified very short fragments in amyloidogenic proteins, such as C10-A19 in TTR [121] and H14-D23 in Alzheimer β peptide [122]. Do these regions serve as a domain-swapped interface in the fibrils of the intact protein? As for human IAPP, for instance, very short fragments such as F15-S19, N14-H18 [97], N22-L27 [123] and T30-Y37 [124] have been revealed to be capable of forming amyloid fibrils in isolation. An extension of the domain-swapping model could predict that these regions form β strands in the fibrils of the full-length (37-residue-long) IAPP, although there is no experimental evidence at this point.

Design of interface β strand

β-Sheets are abundant in nature, while amyloid fibril formation is infrequently observed in vivo. Natural β-sheet proteins can be monomeric and soluble with special edge strands designed to avoid aggregation. For example, proteins in the PDB database typically show combinations of the following edge strand protection mechanisms: (i) continuous β-sheet formation in a cylinder or barrel, (ii) covering the edge strand with loops, (iii) shortening of the edge strand to minimize the interface surface, (iv) having charge(s) in the middle of hydrophobic strand, (v) making a β-bulge or including proline to distort the interface of the edge strand, (vi) making a tight twist, bend or reverse twist in the edge strand to distort the interface [125]. In contrast, edge strands that natively form dimers or rings (barrels) with β-sheet interface have long stretches without such protection. Thus, it is reasonable to assume that the lack of such protection mechanisms could result in amyloid fibril formation. In fact, mutagenesis studies introducing Pro in the middle of an amyloidogenic short peptide [126] or a single charge in the nonpolar edge strand interface [127] drastically inhibited amyloid fibril formation, supporting this hypothesis. However, such protection mechanisms do not appear powerful enough without a combination of multiple factors. For instance, a recent solid-state NMR study has reported that an amyloidogenic peptide fragment TTR105-115 (YTIAALLSPYS) forms an extended conformation in fibrils with the proline residue in the strand [128], suggesting that amyloid fibrils can tolerate minor incompatibilities in the edge strand interface.

Water-filled tube structure model

While introducing an unpaired charge in the strand interface converted a fibril-forming polypeptide into a monomeric β-sheet protein [127], some fibril-forming short peptides have a net charge in the context of fibrils. For example, a transthyretin fragment (TTR10-19, CPLMVKVLDA) and human and rat IAPP fragments 8–20 (ATQRLANFLVHSS and ATQRLANFLVRSS, respectively) form amyloid fibrils in acidic solution [11, 129], where they have a net charge. Fibrils of these peptides have a width of 7–12 nm, and thus these fibrils should consist of multiple layers or high-order assembly of β sheets with embedded unpaired charges. A possible mechanism to accommodate such unpaired charges be-

tween layers is provided by a water-filled tube structure model [130]. Such architecture possibly reduces the energetic penalty of burying unpaired charges, and thus amyloid fibrils could be capable of incorporating unpaired charges in the cross β structure.

An alternative model has been presented based on X-ray diffraction study of a microcrystal of a Sup35 fragment that shows amyloidogenic properties, which include high stability, cross β X-ray diffraction patterns and binding of Congo red [131]. The unusually high density of this crystal suggests a highly intermolecularly bonded, dehydrated array of densely packed β sheets. Such a densely packed β-sheet model has been proposed for crystals of poly(AG) polypeptides [132], suggesting that this dehydrated packing may be attributed to the nature of crystals. There is a significant analogy between crystal formation and amyloid fibril formation: both are formed by a single kind of protein unit; slow two-step kinetics controlled by a slow nucleation step and repeated molecular alignment. Therefore, it is possible that molecular alignment in the microcrystal is essentially the same as that in amyloid fibrils, and the difference between crystal and fibril features may result from the difference in the packing of β sheets with and without hydration.

Inverse side-chain effect in protein folding

The series of experiments by Dobson and co-workers where nonpathogenic proteins form amyloid fibrils under destabilizing conditions [16–20] support a hypothesis that the potential to form amyloid fibrils may be a common feature of all proteins [16]. Recently, Fändrich and Dobson demonstrated that polyamino acids such as polythreonine, polyglutamate and polylysine are capable of forming amyloid fibrils. These results led them to propose that in contrast to protein folding driven by tertiary interactions between side chains or side chain and backbone, amyloid fibril formation is driven by interactions between backbones, and the side-chain interactions inversely contribute (i.e. side-chain interactions in the native state contribute for prevention of fibril formation) [133]. This is consistent with the fact that amyloid fibril formation in vitro is frequently observed under destabilizing conditions where side-chain interactions are weakened and that amyloid fibrils have a similar cross β structure regardless of amino acid sequence. This view also is consistent with a β-sheet conformation of polyglycine in crystal [134] and that of glycine-rich polypeptide involved in spider-silk fiber [132]. On the other hand, if only backbone interactions are dominant and interactions involving side chains are trivial in the formation of fibrils, fibrils would be coformed with anonymous polypeptides, not necessarily formed with a single kind of polypeptide. Although it is unlikely that amyloid fibrils in vivo randomly recruit other proteins in the vicinity to form hybrid fibrils, there are several reports that are consistent with this idea. For example, an Aβ peptide or its fibrils has been reported to interact with acetylcholine esterase [135], apolipoprotein E [136], creatine phosphokinase and glutamine synthetase [137]. In addition, nonspecific interactions between an Aβ peptide and soluble proteins have been reported [138]. Thus, side-chain interactions appear to be less specific in amyloid fibril formation than they are in protein folding. Nevertheless, as supported by the fact that most proteins do not form amyloid fibrils easily and that mutations sometimes effectively induce or diminish the amyloidogenic propensity of some proteins, interactions involving side chains cannot be ruled out as a mechanism of amyloid fibril formation.

Aggregation and amyloid formation

Aggregation is a general feature of proteins and can be classified into two types: in vivo aggregation such as inclusion body formation and in vitro aggregation such as denaturation-induced aggregation. Amyloid fibril formation is an in vivo phenomenon, but it also occurs under artificial conditions in vitro. As for the formation of inclusion body, detailed mechanisms are not clear. One possible mechanism for this phenomenon has been proposed through an FTIR spectroscopy. Fink and co-workers revealed that proteins in inclusion bodies have considerable native-like secondary structure with an increased level of β-sheet content (~20–25%), suggesting that a partially unfolded intermediate conformer aggregates with others at an interface formed by a β-sheet-type interaction [139, 140]. The increase in β-sheet content was observed even in the case of all β-sheet proteins. Although it is not clear whether the partially unfolded form is an on-pathway folding intermediate, it is highly likely that the high local concentration of proteins due to overexpression is responsible for inclusion body formation. Thus, the mechanisms of inclusion body formation would be distinct from those of amyloid fibril formation, despite the common observation that a partially unfolded intermediate likely plays an important role in the molecular aggregation.

In vitro aggregations of water soluble proteins often can be observed under various situations: (i) mild denaturing conditions where proteins are partially unfolded, (ii) incubation at high temperature with relatively high protein concentration, (iii) salt at high concentration (salting out), (iv) changing the pH close to the isoelectric point, (v) presence of polar organic solvent, (vi) contacting the interface between water and air or water and lipid, (vii) presence of heavy metals or (viii) presence of abundant nonionic polymer. Although detailed mechanisms for these protein aggregation/precipitation phenomena are elusive, an exposed hydrophobic surface upon denatura-

tion or contact with a nonpolar phase is thought to be responsible for cases (i), (ii) and (vi). Aggregation/precipitation takes place in the case of (iii) because high concentrations of salt remove water molecules from proteins. In the case of (iv), proteins aggregate and precipitate due to loss of net charge on the surface, leading to loss of charge repulsion and increased intermolecular association. In the case of (v), polar solvent environment reduces hydration of the protein surface and enhances intermolecular hydrogen bonding, leading to protein aggregation. In the case of (vii), proteins make an insoluble metal complex whose structural details are not known. In the case of (viii), the amount of water available to hydrate proteins is significantly reduced. As described previously in the section on case studies, the conditions reported for in vitro amyloid formation typically match cases (i), (ii) and (vi). Does this agreement imply that amyloid fibrils and protein aggregations induced under such denaturing conditions are identical? For clarity, we refer to the general phenomenon of protein aggregation induced by partial denaturation as 'destabilized protein aggregation'. An FTIR study showed that a destabilized protein aggregation has a native-like structure with an elevated β-sheet content, which was similarly observed in inclusion bodies [139]. This similarity suggests that a partially unfolded intermediate upon mild denaturation has a structure similar to the partially unfolded intermediate responsible for inclusion bodies in vivo, and it leads to amorphous aggregation, distinct from amyloid fibrils [139, 140]. This view has been supported by the finding of two distinct folding intermediates of immunoglobulin light chain (V_L domain) [141]. In the process of acid denaturation, this protein formed an intermediate conformation at pH below 3 that is capable of forming amyloid fibrils, while at pH around 4–6 it formed another intermediate conformation that forms amorphous aggregates. This suggests that amyloid fibril formation is driven by different mechanisms from those for destabilized protein aggregation. In contrast, thermal denatured proteins at high concentration often precipitate or form a gel with considerable β-sheet content [142–144]. Although the number of studies that report characterization of protein aggregation induced in the process of thermal denaturation is small, a few studies have reported that protein aggregation induced upon thermal denaturation exhibits some of the hallmarks of amyloid fibrils [145, 146]. It is intriguing how similar these cases are.

From the observations described above, a schematic diagram is proposed as shown in figure 1. Amyloid fibril formation is a type of destabilized protein aggregation that is driven by hydrophobic interactions through exposed hydrophobic surface with a β-sheet interface, but it requires some extra factor(s) necessary for ordered β-sheet alignment into the cross β structure, otherwise the protein forms amorphous aggregates with an elevated β-sheet content. Such factors are likely different from the mechanisms that lead to protein aggregation, because electron micrography, the most direct method to observe fibrous morphology, sometimes reports coexistence of amyloid fibrils and amorphous aggregates [147–149]. In addition, a conversion from amorphous aggregates initially formed to amyloid fibrils has been observed in the process of amyloid fibril formation [150], suggesting that rearrangement of strands may be possible in an associated form. These observations are consistent with the model outlined in figure 1.

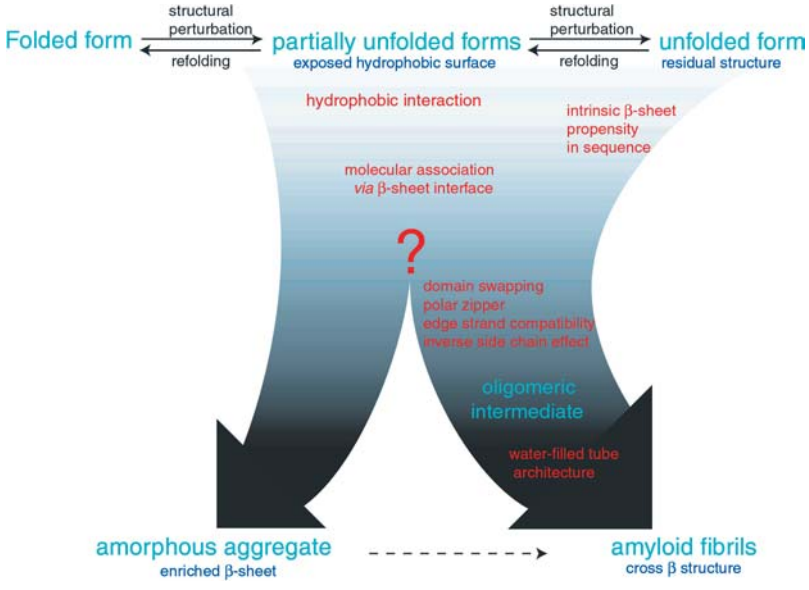

Figure 1. A schematic diagram of amyloid fibril formation and amorphous aggregation in a protein folding-refolding process, drawn based on published results. For details, see text.

In this context, three examples merit attention. The first example is found in a series of peptide studies designed from a single-layer β-sheet motif of *Borrelia* OspA, whose surfaces both exposed to solvent without contact to other domains of the protein [151]. A β-hairpin peptide designed from this single-layer β sheet has been found to form fibrils in the presence of TFE [38], while a circular permutation of the peptide that retains identical strand-strand interactions but destabilizes the turn structure resulted in the formation of amorphous aggregates rich in β sheets [152]. This result highlights an important role for turn formation in fibril formation of this peptide system. In the second example, amyloid fibril formation of α-lactalbumin under acidic pH was significantly enhanced by the reduction of disulfide bonds, while the protein with bound Zn^{2+} formed amorphous aggregation [153]. This suggests that a large conformational change is required for fibril formation by this protein, and flexibility of the polypeptide chain is critical to formation of cross β structure. The third example is seen in molecular engineering of a loop region of the SH3 domain family using the SH3 domain of phosphatidylinositol 3-kinase (PI3-SH3 domain), which is capable of forming amyloid fibrils under acidic conditions, and the Spectrin SH3 domain (SPC-SH3 domain), which does not form fibrils under the same conditions [154]. Replacing the loop region with that of SPC-SH3 domain did not inhibit amyloid fibril formation of PI3-SH3, while SPC-SH3 with the PI3-SH3 loop formed amorphous aggregates rich in β sheets. Based on these results, the authors pointed out the importance of the balance between solubilities of nonnative states to allow efficient nucleation and formation of amorphous aggregates in the denatured state. Although the factors described above may not be general, accumulation of such information is necessary for further understanding of the mechanisms of amyloid fibril formation.

Denatured states and amyloid fibril formation

In vitro studies suggest that nonnative forms of protein are responsible for amyloid fibril formation. For some proteins, such as PrP and α-lactalbumin, amyloid fibril formation requires a large degree of unfolding [89, 153], while for other proteins, such as TTR, amyloid fibril formation takes place through partially unfolded states, but not through largely/fully unfolded states [47]. This discrepancy may arise from differences in the structural features of proteins. For fibril formation of TTR, only partial unfolding may be sufficient due to its high β-sheet content in the native state. In contrast, in the cases of fibril formation of PrP and α-lactalbumin, both predominantly α-helical proteins, a large degree of unfolding would be required for the large conformational reconfiguration into the cross β structure. How do such largely unfolded molecules accomplish structural reconfiguration into the cross β structure under strong denaturing conditions? The mechanisms may be determined by the structural properties of the denatured states. In many proteins, the presence of residual structure even under strong denaturing conditions has been reported [155–159], and for PrP, a robust residual structure has been identified under strongly denaturing conditions [89]. Although there is no clear evidence that links the existence of residual structure under denaturing conditions with the ability to form fibrils, it is possible that such residual structure serves as a key nucleation site for fibril formation under strong denaturing conditions (fig. 1). In addition, the secondary structure propensity of amino acids may be involved; a sequence analysis on PrP found that the whole protein sequence except for the first helix region has relatively high β propensity, especially in the second helix [160].

There is increasing evidence that fully unfolded polypeptide chains are not random coils [161–166]. Recent studies by Shortle and co-workers proposed that a polypeptide can be viewed as a relatively stiff chain with structure encoded by local interactions between side chains and backbone [163, 164, 166]. It follows that crude structural topology is determined by the structural propensity of each amino acid and combination of amino acids, i.e. sequence. In the largely unfolded states with such a crude topology, residual structure is likely induced by local clustering of hydrophobic residues and charged pairs [167, 168]. Although transient, residual structures thus formed and local structural encoding by the sequence must be key determinants in amyloid fibril formation as well as in protein folding.

Concluding remarks

In this review we have highlighted the relevance of amyloid fibril formation to protein folding. In particular, fibril formation exhibits significant similarity to one common feature of proteins, i.e. destabilized protein aggregation. Thus, it is suggested that partially unfolded intermediates or largely unfolded denatured forms are responsible for the formation of amyloid fibril in vitro. However, there is no clear evidence that partially or largely unfolded forms are populated in vivo under physiological conditions, and it is unclear how amyloidgenic proteins undergo conformational conversion from the native form to the cross β fibrils in vivo. As seen in a critical role for Cu^{2+} ion in dialysis-related amyloidosis [72], extra factors causing a structural perturbation in vivo may be involved. Many mechanisms may cause protein structure perturbation in vivo, such as protein degradation upon turnover, interaction with membranes or posttranscriptional modifications, including phosphorylation or glycation. For example, involvement of advanced glyca-

tion end products in the β_2m amyloid fibrils has been reported [169]. From the clinical point of view, it is very important to discover the factors that link the mechanisms of in vitro amyloid fibril formation with pathogenic cases.

Recent advances in solid-state NMR have provided more detailed structural information of the polypeptide in the context of the fibrils [100, 128, 170, 171]. Together with improved structural details, results from studies approached from the viewpoint of protein folding could advance our understanding of amyloid fibrillization mechanisms. Needless to say, such knowledge would advance the treatment and/or prevention of amyloid disease. In addition, utilizing this unique potential of forming cross β assembly could also provide new perspectives in nanotechnology in materials science.

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