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Self-Assembly of Polypeptides into Amyloid Fibrils with Structural Transitions

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We have succeeded in the design and synthesis of polypeptides that undergo a self-initiated structural transition from an α-helix to a β-sheet and self-assembly into the amyloid fibrils. A coiled-coil polypeptide structure composed of two amphiphilic α-helices with double heptad-repeats $(ALEQKLA)_2$ was designed, and hydrophobic domains, such as adamantanecarbonyl, aliphatic acyl groups, or various hydrophobic amino acids, were attached to the N-termini of the peptides to induce intermolecular peptide associations by hydrophobic interactions. These peptides initially formed the α-helix structure in a neutral aqueous solution, but gradually changed the conformation to the β-sheet structure without any environmental change. Electron microscopy showed that they self-assembled into the amyloid fibrils accompanied with the structural transition. Mutational analyses of the peptides revealed the complementary assembly into the amyloid fibrils composed of heterogeneous species. This study will divulge a system for developing peptidyl nanoscale materials as well as for controlling amyloid fibril formation of proteins.

Key words: polypeptide, amyloid fibril, α-helix, β-sheet, self-assembly

1. INTRODUCTION

Intermolecular self-assembly of a large number of polypeptide chains into macromolecular constructs occurs widely in biological systems. One of such macromolecular self-assemblages of great interest is the amyloid fibril [1-4]. The amyloid fibril is a misfolded and undesirable state for proteins as biomolecules, since it has been proposed to be a causative agent of a variety of fatal diseases known as amyloid diseases, such as Alzheimer's and prion diseases [5,6]. On the other hand, it is considered that the fibril has a highly-ordered quaternary structure, in which numerous β-stranded polypeptide chains align regularly [1], and thus this kind of fibril has the potential to be engineered into proteinaceous materials [7-10]. Amyloid fibril primarily comprises a single polypeptide species, namely it is a homogeneous self-assemblage. The pathway of protein misfolding and subsequent fibril formation involves a conformational transition, for example, from an α-helix to a β-sheet structure. This transition is especially apparent in the conversion of prion proteins from normal to abnormal isoform without any chemical modification [5]. The cellular form of the prion protein (PrP^{C}) is rich in an α-helix structure and highly water-soluble, but the scrapie isoform (PrP^{Sc}) forms amyloid fibrils with a higher β-sheet content. Similarly, the β-amyloid peptide (Aβ), which is a major component of the amyloid plaques deposited in the brains of Alzheimer's disease patients, has an α-helical propensity in some environments [4]. It is becoming

increasingly important to study the nature of the proteins in order to understand such diseases. Recent studies have suggested that relatively unstable states, which are in a partially folded/unfolded structure formed in a folding intermediate, and in some unfavorable environments or mutants, are important in determining whether proteins misfold and aggregate. In general, one cause of protein misfolding and aggregation is thought to be the exposure of the hydrophobic region of proteins in such an unstable form to an aqueous environment.

Peptides based on the de novo design have provided useful information for constructing and manipulating peptide conformation, and elucidating complex folding mechanisms [11]. The design method often utilizes the amphiphilic nature of peptide secondary structures to construct tertiary structures of artificial proteins [12]. Peptides designed for β-sheet folding have been extensively studied [13], and design of peptides to adopt a fibrillar structure like amyloid fibrils has been a current interest [14]. Moreover, designing sequences of peptides to allow conformational interconversions, e.g. helix-sheet switching, has been widely attempted by de novo sequence design [15-17]. These studies suggest that both overall and local conformations of proteins and their conversion are determined by not only local amino acid sequence (short-range interactions between nearby amino acid residues) but also by long-range interactions, including both intra- and intermolecular interactions, between secondary structures. In other words, hydrophobic clustering between amphiphilic peptides seems to determine the secondary structures and their transformation. We have found that a hydrophobic nucleation domain, *i.e.*, a hydrophobic defect, caused a structural transition of a peptide from α-helix to β-sheet [18]. Using this strategy of adding hydrophobic defects has revealed that the stability of the α-helix conformation in its initial state has a key role in determining whether or not the peptide structure will be transformed [19]. The designed peptides mimicked α-to-β transitional and amyloid forming properties of proteins such as the prion protein, a nucleation-dependent self-catalytic transformation.

This paper will focus on our recent studies that have demonstrated the importance of conformational changes in amyloidogenic proteins and protein folding, and of material design with peptide self-assembly.

2. α-TO-β TRANSITION AND SELF-ASSEMBLY TO AMYLOID FIBRILS OF ADAMANETANE-PEPTIDE

An improved understanding of protein misfolding is critical to the study of proteins related to diseases such as Alzheimer's and prion, as well as to the clarification of the folding pathway of proteins [1-4]. In general, hydrophobic clustering seems to be an important feature for peptide transformation. In this study, we applied the concept of hydrophobic defect in order to design and synthesize a peptide capable of performing an α-to-β structural transition and forming amyloid fibrils [18]. The peptide was composed of two amphiphilic α-helix segments, each modified with a 1-adamantanecarbonyl group at the N-terminal as a hydrophobic defect (Ad-2α) (Fig. 1). The two-α-helix part was constructed from amino acid sequences of coiled-coil proteins [20], which have heptad repeats $(abcdefg)_n$ with hydrophobic residues at the a and d positions. The $<P_\alpha>$ value of the core 14-peptide as calculated with Chou-Fasman parameters [21] was 1.34, and the $<P_\beta>$ value was 0.94. Therefore, judging only from the amino acid sequence, the peptide is expected to form an α-helix structure. However, when the peptide sequence is drawn as a β-sheet model, the peptide can take a kind of amphiphilic β-sheet structure [12], in which hydrophobic Leu residues and hydrophilic Glu and Lys residues are separated on the different faces (Fig. 1).

The peptide was synthesized by the solid-phase method using Fmoc chemistry [22]. The dimeric peptide (2α) was synthesized via the disulfide linkage between Cys residues at the 17th position. The peptides were purified by HPLC, and identified by MALDI-TOFMS and amino acid analysis. The CD studies revealed that the peptide folded in a 2α-helix structure at first, but the conformation changed gradually to a β-structure in a neutral aqueous solution after 4 h at

25 °C (Fig. 2). The time course of the structural transition was quite sigmoidal, resembling the autocatalytic transition process in β-amyloid and prion peptides. FTIR measurements confirmed the α-to-β structural transition of Ad-2α. The gel-filtration analysis indicated that the peptide was in a monomeric 2α-helix structure at first, and formed aggregates coinciding with the α-to-β transition. The acetylated-2α(C2-2α) lacking the hydrophobic defect formed an α-helical structure, but failed in the transition. One segmental peptide (Ad-1α) was almost in a random structure and did not show the transformation. These results suggested that both the hydrophobic defect and the initial α-helix structure were essential for the transformation. Transmission electron micrographs showed that the β-structural peptide formed amyloid fibrils (~10 nm width) (Fig. 3), and the fibrils assembled into larger deposits (10-100 μm) observable by a photomicroscope. The amyloid formation of the peptides was also examined employing an amyloid-specific dye-binding analysis [23] (Fig. 4). It is known that a fluorescent dye, thioflavin T (ThT), associates with amyloid fibrils and the binding gives rise to a significant enhancement in fluorescence according to the amount of amyloid. All peptides in α-helix did not affect ThT fluorescence. In the presence of Ad-2α which completed the α-to-β transition, ThT showed an enormously enhanced fluorescence (~35-fold). The hydrophobic association process was supported by the temperature-dependent transition and by the inhibition experiments with β-cyclodextrin, trifluoroethanol and ionic detergents. For example, β-cyclodextrin, which has a high affinity to capture the adamantane group in the cavity, inhibited the transition of Ad-2α in a concentration-dependent manner. The addition of 10 equiv. of β-cyclodextrin completely inhibited the transition. Thus, the peptide undergoing the α-to-β transition and the amyloid fibrillogenesis was successfully designed.

3. OPTIMIZATION OF HYDROPHOBIC DEFECTS USING ACYL-PEPTIDES

To clarify the effects of hydrophobic groups at N-termini on the transition, aliphatic acyl groups with a range of chain lengths (C2 - C16) were attached to the 2α- and 1α-peptides (Fig. 1) [19]. It became clear that there was the optimum hydrophobicity for the α-to-β transitions, and the transitional properties of the peptides seemed to be dependent on the initial α-helix states (Table I). The octanoyl-group was most effective for the transition of the 2α-peptide. The α-to-β transition of the 2α-peptide with octanoyl groups (C8-2α) occurred at the highest transitional rate among all of the peptides with acyl chains, the rate being almost identical to that of the adamantanecarbonyl peptide (Ad-2α). Transmission electron micrograph showed that C8-2α which was in the β-structure formed amyloid fibrils. The CD spectra of C4- and C6-2α changed over 14 and 7 h, respectively, but their α-to-β transitions were incomplete. As the chain length increased, the α-to-β transition appeared to take place more rapidly and completely. The peptides with C8 and C10 showed an almost complete α-to-β transition, though the rate of transition for C10-2α was lower than that of C8-2α. In contrast, the peptides with longer acyl chains (C12-, C14-, and C16-2α) had a more stable α-helix structure due to the formation of oligomers, and did not transform. These results indicated that the hydrophobic defect was important for the 2α-helix peptide to transform to β-structure, but there was an optimum in the hydrophobicity of the defect. The 1α-peptides capable of forming α-helix (C12-, C14-, and C16-1α) also transformed to a β-structure (data not shown). The 1α-peptides with shorter chains were in a random coil and did not transform. These results confirmed that the appropriate hydrophobic defects in the peptides and formation of α-helix are important for the transition. It should be noted that appropriate hydrophobic amino acid such as Ile and Phe can be the hydrophobic domain for the structural transition and the amyloid formation instead of the non-proteinaceous moiety such as adamantane [24].

4. MUTATIONAL STUDY (Q->A) OF THE AMYLOIDAL PEPTIDE

The peptide sequence that we designed was composed of two amphiphilic α-helices, and the two-α-helix part was constructed from amino acid sequences of coiled-coil proteins, which had heptad repeats $(abcdefg)_n$ with hydrophobic residues at the a and d positions (QQ peptide as a parent compound, Fig. 5). However, when the peptide sequence is drawn as a β-sheet model, the peptide takes a kind of amphiphilic β-sheet structure, in which hydrophobic Leu residues and hydrophilic Glu and Lys residues are separated on the different faces (Fig. 1). In this model, hydrophilic Gln residues of QQ peptide, which have a hydrogen-bonding ability, are located in the hydrophobic face of the β-strand composed of Leu residues. In order to elucidate importance of the Gln residues in the autocatalytic α-to-β structural transition and amyloid fibrillogenesis, the mutant peptides of Ad-2α with single or double Gln->Ala substitutions (QA, AQ and AA peptides) were designed (Fig. 5) [25].

The α-to-β transition of the original Ad-QQ and the double-mutant Ad-AA occurred at the highest transitional rate among the peptides with adamantanecarbonyl group (Table II). On the contrary, the transitional rates of single mutant peptides, Ad-QA and Ad-AQ, were significantly retarded, with changing the conformation from an α-helix to a β-sheet for 3 days. The amyloid-specific dye-binding analysis using thioflavin T revealed that Ad-QQ and Ad-AA formed amyloid fibrils after transition to β-structure (Table II). On the contrary, Ad-QA or Ad-AQ after the α-to-β transition lacked the ability of forming the amyloid. Transmission electron microscopic measurements also confirmed that Ad-QQ and Ad-AA in β-sheet formed fibrils (~10 nm width), but Ad-QA and Ad-AQ did not (Table II). It was shown that Ad-QA and Ad-AQ could not assemble into fibrils, although only a little difference in the secondary structure between amyloidogenic and non-amyloidogenic peptides was observed.

However, when Ad-QA and Ad-AQ were mixed at an equimolar ratio, the α-to-β transition was accelerated (completed within 9 h), and they acquired the ability to form the amyloid fibril (Table II). This finding implies that the peptides, which originally do not have the ability, are able to self-assemble into the fibrils if the complementary peptide coexists. There should be a complementary assembly mechanism to form the amyloid fibrils of homogeneous and heterogeneous assembling peptides.

It has been shown that single or double Gln->Ala substitution significantly affects the transitional properties and aggregate formation of the peptides. These results suggest that a specific interaction of hydrophobic face of β-strands (β-sheet packing) is crucial for a well-organized assembly into an amyloid fibril, although a detailed alignment of these strands is not clear. The repeated sequences, QQ and AA, appeared to prefer such an assembly, whereas non-repeated sequences, QA and AQ, disfavored.

5. SELF-ASSEMBLY OF PEPTIDES TO AMYLOID FIBRILS WITH A COMPLEMENTARY MANNER

Next, we carried out mutational analyses for the charged residues of the parent sequence (Fig. 6) [26]. According to the previous studies, a peptide composed of two amphiphilic α-helices was designed and the N-termini were acylated with the 1-adamantanecarbonyl (Ad-) group. Here, we synthesized and investigated a series of 16 mutant peptides which had substitutions in the charged residues (Lys (K) and Glu (E)). The CD, TEM and ThT-binding studies revealed that only 4 peptides (6.EEKK, 7.EKEK (parent sequence), 10.KEKE and 11.KKEE) among 16 peptides showed structural transitions to β-sheet and amyloid fibril formation. This result suggests that the charge neutralization is not sufficient for the fibril formation which stabilizes a β-sheet structure and that the positions of positive and negative charges are critical for the well-organized assembly of the β-strands. None of the negatively (1.EEEE to 5.KEEE) or positively-charged peptides (12.EKKK to 16.KKKK) was able to form the amyloid fibrils, due to that they disfavored intermolecular association.

All combinations of the equimolar mixtures of two of sixteen peptides (120 combinations) were examined employing

the thioflavin T binding analysis to search complementary pairings that enabled heterogeneous assembly of two peptide species into amyloid fibrils. It has become clear that there are complementary parings of the peptides for the fibril formation. The combinations are only four patterns of negatively- and positively-charged peptides, 2.EEEK/12.EKKK, 3.EEKE/13.KEKK, 4.EKEE/14.KKEK and 5.KEEE/15.KKKE, among 120 combinations. This result implies that two kinds of the β-strands can be arrayed in an antiparallel manner to form ion pairs in the fibrils (Fig. 7).

It has been shown that even a single amino acid substitution significantly alters the property of α-to-β transition and the ability to form amyloid fibrils of the peptides. Furthermore, the peptides which originally do not have the ability to form the fibrils are able to self-assemble into the fibrils if the complementary peptide coexists. It is considered that the fibril has a highly-ordered quaternary structure, in which numerous β-stranded polypeptide chains align regularly [1], and thus this kind of fibril has the potential to be engineered into proteinaceous materials [7-10]. Amyloid fibril primarily comprises a single polypeptide species, namely it is a homogeneous self-assemblage. Here we have demonstrated the heterogeneous assembly of designed peptides into amyloid fibrils accompanied by a drastic secondary structural transition from an α-helix to a β-sheet. The heterogeneous assembly into fibrils is accomplished by complementary electrostatic interactions between peptide pairs; each of which is not able to self-assemble. These results provide insights into studies on a number of amyloidosis-related mutant proteins, as well as a structural basis of well-organized self-assembling peptides and proteins.

6. CONCLUSION

The polypeptides that undergo amyloid fibril formation with the α-to-β structural transition have been successfully design and synthesized. The hydrophobic groups at the N-termini of the peptides and the initial α-helix formation are important for the transition and amyloid formation. The mutational studies have revealed that there are complementary recognition mechanisms for forming the amyloid fibrils with homogeneous or heterogeneous β-sheet packing. Using the mechanism of forming amyloids, we can design heterogeneous assembly of β-strand polypeptides composed of chiral polymer units of two-, three- or four-different species. These studies will lead to a novel approach which enables to develop peptidyl nanoscale materials with a regulated self-assembling ability.

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8. REFERENCES

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