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Inhibition of Peptide Amyloid Formation by Cationic Peptides with Homologous Sequences

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Abstract—We have studied the model peptides that undergo self-initiated structural transition from α-helix to β-sheet and self-assembling into amyloid fibrils. We here constructed an inhibition system of amyloid formation utilizing homologous recognition and assembly of peptides with increased solubility. Among 20 peptides with homologous sequences examined here, cationic peptides showed the stronger inhibition ability against the amyloid formation of a model peptide.

Conformational alternation and fibril formation of proteins have a key role in a variety of amyloid diseases including Alzheimer's and prion diseases.[1–3] We have successfully developed peptides that undergo self-initiated structural transition from α-helix to β-sheet and self-assembling into amyloid fibrils.[4–9] Peptides could be manipulated to assemble into amyloid fibrils in single and multiple (two, three, four) species.[7–9] These studies afforded the idea that homologous sequences of peptides have key roles in the β-sheet assembly and amyloid formation. Generally, the amyloid peptides and proteins undergo self-assembling into fibrous structures. That is, the peptides and proteins make amyloid fibrils homogeneously, thus exhibiting the single-species recognition in amyloid transmission and amplification.[1–3] Taking these aspects into consideration, utilization of peptides with homologous sequences will lead to control of the conformational transition and subsequent amyloid fibril formation. In this study, we attempted to construct an inhibition system of amyloid formation of peptides using this concept. There have been some reports on the inhibition against aggregation of amyloid β-protein of Alzheimer's diseases by hydrophobic peptides homologous to the native sequence.[10,11] In this study, we focused on the hydrophilic regions of the model amyloid peptides, EKEK (Fig. 1). To inhibit structural transition and aggregation of the amyloidogenic peptide, the homologous peptides whose sequences were systematically substituted at charged amino acids of EKEK were employed.[8,9] We found that cationic peptides strongly prevented the structural transition and amyloid formation of EKEK. Hence, the inhibition abilities of peptides analogous to a cationic peptide KKKK were also studied. Consequently, the peptides with higher homology to EKEK showed stronger inhibition abilities.

Results and Discussion

The peptide 7.EKEK that undergoes α-to-β structural transition and amyloid fibril formation has been studied as a mother compound of amyloidogenic peptides that we used (Fig. 1a).[4] It is composed of two amphiphilic α-helices with double heptad repeats of $(ALEQKLA)_2$, and the two polypeptide chains are linked by a disulfide bond between Cys residues at the C-termini. Although an ideal amphiphilic α-helix is designed, the sequence also has the potential to form an amphiphilic β-strand. The 1-adamantanecarbonyl (Ad) group is attached to the N-termini of the peptide and thus exposed to the solvent, inducing intermolecular peptide associations through hydrophobic interactions followed by interactions between β-strand peptides in an antiparallel manner.[4,7–9] To find out an inhibition system of the α-to-β structural transition and amyloid formation of 7.EKEK,

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(a)

7.EKEK (Ad-EKEK-2α)

Ad-βAla-AL**E**Q**K**LAAL**E**Q**K**LA-βAla-C-NH_2

Ad-βAla-AL**E**Q**K**LAAL**E**Q**K**LA-βAla-C-NH_2 (Ad- = 1-adamantanecarbonyl)

Peptide	X_1	X_2	X_3	X_4	Charge
1.EEEE	E	E	E	E	-8
2.EEEK	E	E	E	K	-4
3.EEKE	E	E	K	E	-4
4.EKEE	E	K	E	E	-4
5.KEEE	K	E	E	E	-4
6.EEKK	E	E	K	K	0
8.EKKE	E	K	K	E	0
9.KEEK	K	E	E	K	0
10.KEKE	K	E	K	E	0
11.KKEE	K	K	E	E	0
12.EKKK	E	K	K	K	+4
13.KEKK	K	E	K	K	+4
14.KKEK	K	K	E	K	+4
15.KKKE	K	K	K	E	+4
16.KKKK	K	K	K	K	+8

(b)

Ad-KKKK-2α (16.KKKK)

Ad-βAla-AL**K**Q**K**LAAL**K**Q**K**LA-βAla-C-NH_2

Ad-βAla-AL**K**Q**K**LAAL**K**Q**K**LA-βAla-C-NH_2

Ac-KKKK-2α

Ac-βAla-AL**K**Q**K**LAAL**K**Q**K**LA-βAla-C-NH_2

Ac-βAla-AL**K**Q**K**LAAL**K**Q**K**LA-βAla-C-NH_2

Ad-KKKK-1α Acm

Ad-βAla-AL**K**Q**K**LAAL**K**Q**K**LA-βAla-C-NH_2

Ac-KKKK-1α Acm

Ac-βAla-AL**K**Q**K**LAAL**K**Q**K**LA-βAla-C-NH_2

Ad-KKKK-1/2α

Ad-βAla-AL**K**Q**K**LA-NH_2

Ac-KKKK-1/2α

Ac-βAla-AL**K**Q**K**LA-NH_2

Figure 1. (a) Structures of the amyloid peptide EKEK (Ad-EKEK-2α) and 15 peptides analogous to EKEK, substituted at Glu (E) and Lys (K) residues; (b) inhibitors of amyloid formation of EKEK derived from KKKK (Ad-KKKK-2α). Ac; acetyl, Acm; acetamidomethyl, Ad; 1-adamantanecarbonyl.

a series of 15 homologous peptides substituted at charged amino acids of 7.EKEK were employed (Fig. 1a).[8,9]

Structural transition of EKEK in the presence of peptides with homologous sequences was examined by circular dichroism (CD) spectroscopy. EKEK alone underwent the structural transition from α-helix to β-sheet when it was incubated in the buffer solution for 24 h at 25 °C (Fig. 2a). By contrast, the peptide maintained the α-helix structure, when EKEK was incubated with the homologous peptide KKKK. The other cationic peptides (12.EKKK–15.KKKE) also inhibited the α-to-β structural transition of EKEK while neutral peptides (6.EEKK–11.KKEE) did not (Fig. 2b). Anionic peptides (2.EEEK–5.KEEE) slightly inhibited the structural transition of EKEK, but their potentials were much weaker than those of the cationic peptides. The most anionic peptide EEEE was not used in this study, because it was not highly soluble in the assay system. As a result, cationic peptides had stronger inhibition abilities for the structural transition of the amyloidogenic EKEK.

Amyloid formation of EKEK in the presence of the homologous peptides was then examined by an amyloid-specific dye binding analysis. It is known that a fluorescent dye, thioflavin T (ThT), associates with amyloid fibrils and the binding give rise to a significant enhancement in fluorescence according to the amount of amyloid fibrils.[4,12] EKEK was incubated with each of the homologous peptides for 24 h at 25 °C, and then fluorescence spectra of ThT were measured. Fluorescence intensities of ThT at 480 nm were shown in Figure 3a. EKEK alone showed a strong intensity when the structural transition to β-sheet was accomplished. By contrast, intensities of ThT were extremely decreased,

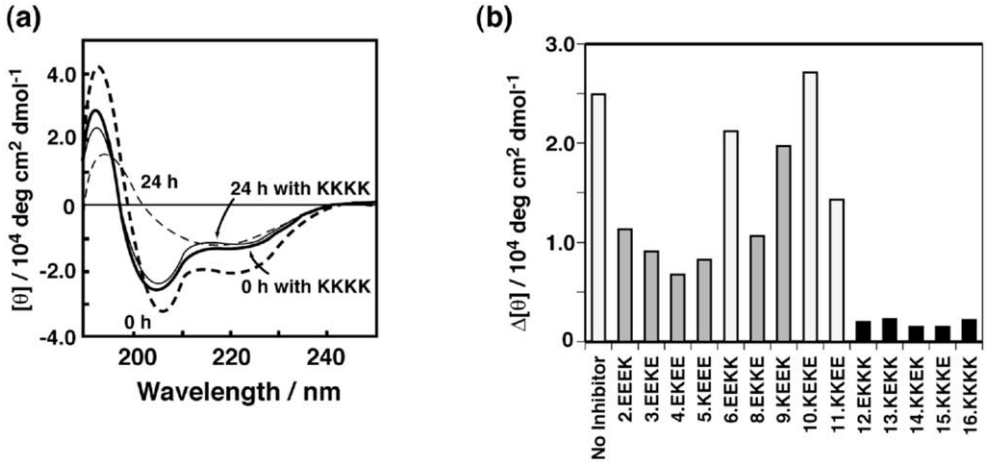

Figure 2. (a) Time-dependent CD spectral changes of EKEK with or without the inhibitor KKKK at 0 and 24 h. The solid lines denote the spectra of EKEK with KKKK at 0 and 24 h, and the dashed lines are those without KKKK. (b) CD spectral changes of EKEK with various analogous peptides. Δ[θ] represents the difference in ellipticity at 205 nm between 0 and 24 h. [EKEK] = 12 μM and [KKKK and others] = 20 μM in 20 mM Tris–HCl buffer (pH 7.4)/2.5% TFE at 25 °C.

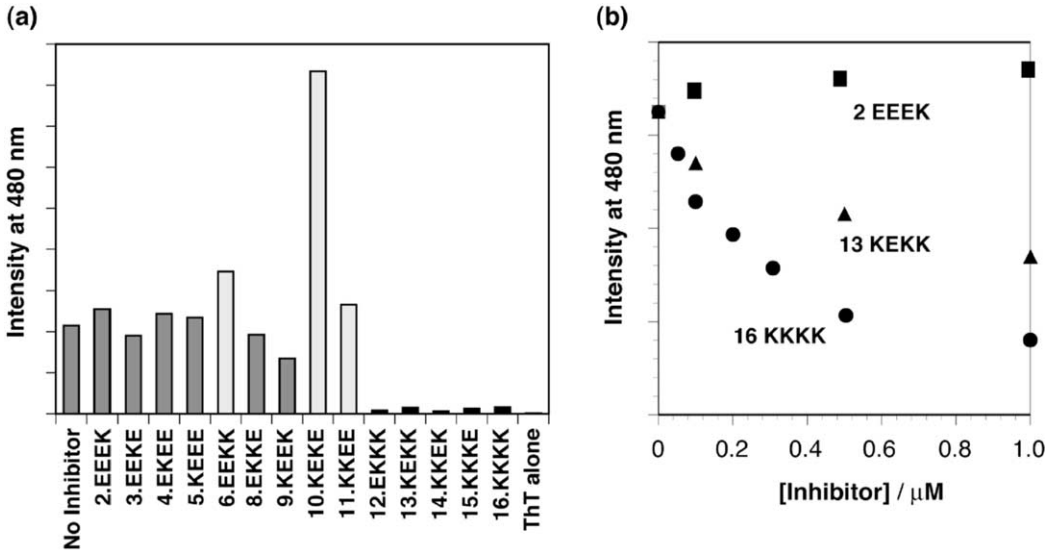

Figure 3. Thioflavin T (ThT) fluorescence analyses of EKEK with the analogous peptides. (a) Fluorescence intensities of ThT in the presence of EKEK with or without the analogous peptides after 24 h incubation; (b) titration curves of the fluorescence intensities in the presence of EKEK with the analogous peptides. EKEK (12 μM) was incubated with or without peptides (20 μM) for 24 h at 25 °C, and then ThT (6 μM) was added in 20 mM Tris–HCl buffer (pH 7.4)/2.5% TFE at 25 °C, and $\lambda_{ex} = 435$ nm; $\lambda_{em} = 480$ nm.

when EKEK was incubated with cationic peptides (12.EKKK–16.KKKK). These results imply that the cationic peptides had the strong inhibition abilities for the amyloid formation as well as the structural transition of EKEK, whereas the non-cationic peptides (2.EEEK–11.KKEE) were defective. The intensities of ThT were increased when EKEK was incubated with 6.EEKK, 10.KEKE or 11.KKEE, because these three peptides have the potential of amyloid formation in single species.[8]

We hence supposed that the inhibition abilities of the cationic peptides were attributed to their high solubility to the water and cationic repulsion between peptides. The cationic peptides may associate with EKEK with some affinity and increase the number of cationic charges and solubility of peptide complexes, thus inhibiting further assembling and the amyloid formation of peptides. The solubility of anionic peptides appeared to be lower than that of the cationic peptides. Therefore, the anionic peptides could not inhibit the amyloid formation of EKEK strongly. To examine the inhibition abilities of cationic peptides in more details, titration experiments of cationic peptides were carried out using the ThT analyses (Fig. 3b). All the cationic peptide decreased remarkably the fluorescence intensity of ThT with EKEK, when their concentrations were as low as at 1.0 μM. Especially, KKKK appeared to have the strongest inhibition abilities. CD studies of secondary structure were carried out with EKEK being mixed with KKKK (1:1, mol/mol). The α-helicity of mixture was higher than that of solution containing each single species ($\Delta[\theta]_{220} = -3000$ deg cm^2 dmol^{-1}). These results suggested that KKKK was associated with EKEK, and then the resulted assembly increased the α-helicity and solubility of peptides.

KKKK and EKEK have homologous amino acid sequences except for the substitutions of four Lys residues for Glu residues in EKEK. To examine how homologous peptide inhibits the amyloid formation, a series of peptides analogous to KKKK were designed and synthesized,[13,14] such that the Ad groups were eliminated and replaced with acetyl groups or the sizes were decreased to those of a single-chain and a half-length (Fig. 1b). Inhibition abilities of KKKK analogous peptides for EKEK were evaluated by the ThT binding analyses after 24 h incubation at 25 °C (Fig. 4a). Consequently the peptides with a higher homology to EKEK had a stronger inhibition ability. Ad-KKKK-2α (KKKK) completely inhibited the amyloid formation of EKEK as describe above, and no fibrous structure was observed by transmission electron microscopy (TEM) (Fig. 4b). The two-stranded Ac-KKKK-2α and the single-stranded Ad-KKKK-1α also showed the strong inhibition ability such that the fibrous structure was almost broken in TEM analyses. Ac-KKKK-1α showed a moderate level of inhibition, in which the ThT intensity was decreased and the fibrils density in TEM was obviously decreased compared to that without the inhibitor. However, the short peptides Ad- and Ac-KKKK-1/2α scarcely had the inhibition ability when their concentrations were more than 50 μM. CD studies also showed that KKKK analogous peptides with a higher homology to EKEK maintained the α-helical structure of EKEK after 24 h incubations at 25 °C (data not shown). As a result, the cationic residues especially in the homologous sequences were important to inhibit the assembly and amyloid formation of EKEK.

In conclusion we have succeeded in inhibition of the amyloid formation of model peptide by cationic peptides with homologous sequences, and found that the peptides with a higher homology to EKEK had a stronger inhibition ability. The finding that peptides with a homologous structure show the inhibition activity has a relevance to the fact that amyloidogenic peptides can assemble homogeneously by themselves and

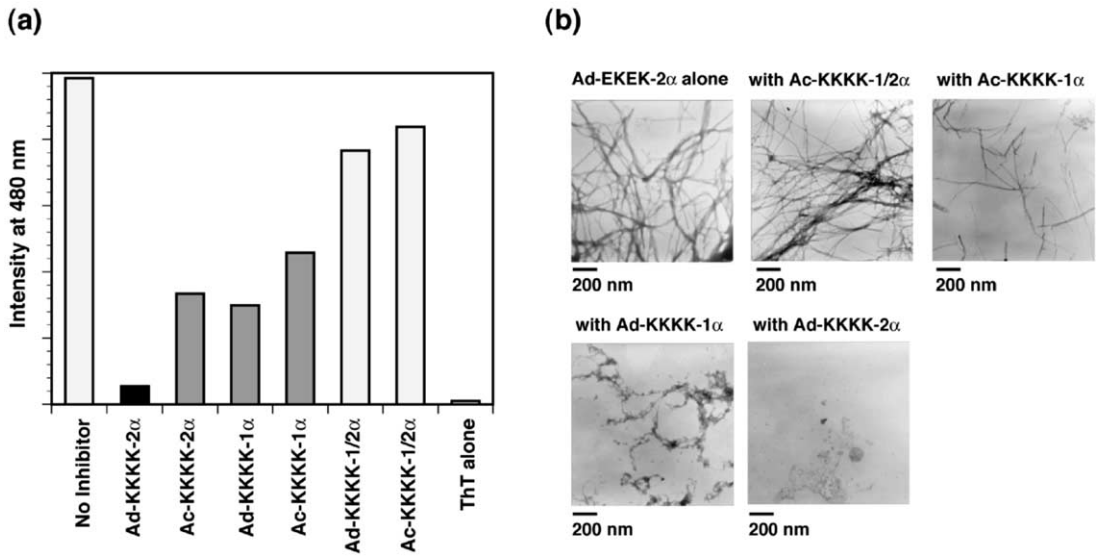

Figure 4. (a) Thioflavin T (ThT) binding analyses of Ad-EKEK-2α (EKEK) with KKKK-analogous peptides. Fluorescence intensities of ThT in the presence of Ad-EKEK-2α with or without inhibitors after 24 h incubation. Ad-EKEK-2α (11 μM) was incubated with or without inhibitors (10 μM) for 24 h at 25 °C, and then ThT (6 μM) was added in 20 mM Tris–HCl buffer (pH 7.4)/2.5% TFE at 25 °C, and $\lambda_{ex} = 435$ nm; $\lambda_{em} = 480$ nm. (b) Analyses by transmission electron microscopy (TEM) of the amyloid formation of Ad-EKEK-2α (EKEK) with or without inhibitors; from the top left, Ad-EKEK-2α alone, Ac-KKKK-1/2α (a similar result was observed inAd-KKKK-1/2α), Ac-KKKK-1α, Ad-KKKK-1α (a similar result was observed in Ac-KKKK-2α), Ad-KKKK-2α.

show species-specificity in transmission of fibrils.[1–3] Some hydrophobic peptides homologous to the Alzheimer's Aβ-peptide showed the inhibition of the amyloid formation.[10,11] This new inhibition strategy focusing on the hydrophilic regions of peptides will give a useful insight for the therapeutic and diagnostic studies of amyloid diseases such as Alzheimer's and prion diseases.

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