Amyloid Fibril Formation and Chaperone-like Activity of Peptides from αA-Crystallin[†]

Naoki Tanaka,*[,‡] Ryoji Tanaka,[‡] Mutsumi Tokuhara,[‡] Shigeru Kunugi,[‡] Yin-Fai Lee,[§] and Daizo Hamada[||]

Department of Bio-molecular Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo, Kyoto 606-8585, Japan, Department of Radiation Oncology and Biology, University of Oxford, Churchill Hospital, Headington, Oxford OX3 7LJ, United Kingdom, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan, and Department of Developmental Infectious Diseases, Research Institute, Osaka Medical Center for Maternal and Child Health, 840 Murodo, Izumi, Osaka 594-1011, Japan

Received September 6, 2007; Revised Manuscript Received December 19, 2007

ABSTRACT: αA-Crystallin (αAC), a major component of eye lens, exhibits chaperone-like activity and is responsible for maintaining eye lens transparency. Synthetic peptides which corresponded to the putative substrate-binding site of αAC have been reported to prevent aggregation of proteins [Sharma, K. K., et al. (2000) *J. Biol. Chem. 275*, 3767–3771]. In this study, we found that these peptides, αAC(70–88), the peptide corresponding to amino acids 70−88 of αAC (KFVIFLDVKHFSPEDLTVK), and αAC(71–88), suppressed the amyloid fibril formation of amyloid β protein (Aβ). On the other hand, while αAC(71–88) exhibited chaperone-like activity toward insulin, αAC(70–88) and αAC(70–88)K70D promoted rapid growth of aggregates consisting of insulin and these peptides in their solution mixtures. Interestingly, we found that αAC(71–88) itself can also form amyloid fibrils. It is possible that the chaperone-like activity of the αAC peptides is potentially related to their propensity for amyloid fibril formation. Analysis of variants of the αAC peptides suggested that F71 is important for amyloid formation, and interestingly, this same residue has previously been found to be essential for chaperone-like activity. Amyloid fibril formation was also observed with the shorter peptide, αAC(70–76)K70D, showing that the ability to form amyloid fibrils is maintained even with significant deletion of the C-terminal sequence. The formation of amyloid fibril was suppressed in αAC(70–88), suggesting that the K70 in the substrate binding site may play a role in suppressing the amyloid fibril formation of αAC, which agreed with recent proposals about the presence of an aggregation suppressor in the region flanking aggregation-prone hydrophobic sequences.

α-Crystallin (αC)[1] is the most abundant eye lens protein and is involved in maintaining eye lens transparency through its ability to prevent protein aggregation (*1, 2*). αC is a member of the family of small heat shock proteins (sHSP) which prevent protein aggregation and have received much recent interest as they have been implicated in many protein folding-related diseases such as Alzheimer's disease and Parkinson's disease (*3–6*). In eye lens, αC consists of two closely related 20 kDa subunits, αA-crystallin (αAC) and αB-crystallin (αBC) (*7, 8*). They form highly heterogeneous multimers with a molecular mass of ca. 800 kDa (*9*). The results from CD and IR spectroscopic measurements indicated that 60−70% of the αC polypeptide is arranged in β-strands and there is very little or no α-helix (*10*).

αC chaperone-like activity correlates with the extent of hydrophobicity of its molecular surface, and hydrophobic interaction has been proposed to be involved in substrate binding of αC (*11, 12*). On the basis of this idea, Sharma et al. suggested that hydrophobic sites of αC, residues 79–88 (HFSPEDLTVK) of αAC, residues 73–82 (DRFSNVLNVK), and residues 93–103 (VLGDVIEVHGK) of αBC, are involved in substrate binding (*13*). Substitution of F71 of αAC, which is highly conserved among the sHSP family, with glycine suppressed its chaperone-like activity (*14*), suggesting that this residue is also involved in substrate binding of αAC. Interestingly, synthetic peptides which have amino acid residues 70–88 of αAC [αAC(70–88)] or residues 73–92 of αBC have been reported to prevent aggregation of various proteins (*15, 16*). Chaperone-like activity was also observed for αAC(71–88), but it was significantly decreased by the removal of F71 from αAC(71–88), i.e., αAC(72–88), indicating that F71 is essential for the chaperone-like activity of the αAC(71–88) peptide (*15*). The K70D substitution of αAC(70–88) [αAC(70–88)K70D (DFVIFLDVKHFSPEDLTVK)] enhanced its chaperone-like activity (*15*) and suppressed the amyloid fibril formation of amyloid β protein

[†] This work was supported by the Association for the Progress of New Chemistry and the Sumitomo Foundation for NT and by Grant-in-Aid for Priority Area 40153770 from the Japanese Ministry of Education, Culture, Sports, Science and Technology to D.H.

* To whom correspondence should be addressed. Telephone and fax: +81-75-724-7861. E-mail: tanaka@kit.ac.jp.

[‡] Kyoto Institute of Technology.

[§] University of Oxford.

[||] Osaka University and Osaka Medical Center for Maternal and Child Health.

[1] Abbreviations: αAC, αA-crystallin; Aβ, amyloid β protein; αC, α-crystallin; AFM, atomic force microscopy; sHSP, small heat shock protein; ThT, thioflavin T.

10.1021/bi701823g CCC: $40.75
Published on Web 01/31/2008

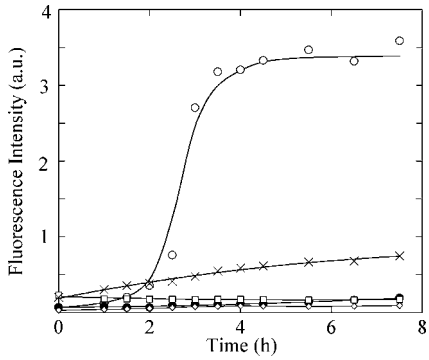

FIGURE 1: ThT fluorescence assay for the effect of αAC peptides on the amyloid fibril formation of Aβ(1–40). The time trace of the ThT fluorescence intensity of the peptide solutions incubated at 37 °C with 200 rpm shaking was monitored: (○) 0.1 mg/mL Aβ(1–40) alone, (●) 0.1 mg/mL Aβ(1–40) in the presence of 0.5 mg/mL αAC(70–88), (□) 0.1 mg/mL Aβ(1–40) in the presence of 0.5 mg/mL αAC(71–88), (◇) 0.5 mg/mL αAC(70–88) alone, and (×) 0.5 mg/mL αAC(71–88) alone.

(Aβ) (*17*). To reveal the mechanism of chaperone-like activity of the peptides from αAC (αAC peptides), we studied their effects on the kinetics and morphology of protein aggregation. We chose Aβ(1–40) and insulin as substrates because both of them form amyloid fibril at 37 °C and neutral pH within the time frame for in situ spectroscopic measurements (*18–22*). The effects on the kinetics of protein aggregation were analyzed by spectroscopic measurements, and the effects on morphology were examined by AFM measurements. We further investigated the self-aggregation potential and secondary structures using variants of the αAC peptides.

EXPERIMENTAL PROCEDURES

Peptides and Proteins. All peptides used in this study were synthesized and purified by Genescript. The purities of the peptides are >95% according to HPLC and mass spectroscopy. Bovine insulin was purchased from Sigma, and Aβ(1−40) (TFA type) was obtained from Peptide Institute (Osaka, Japan); 50 mM sodium phosphate buffer (pH 7.5) with 100 mM NaCl was used as the solvent for all experiments in this study.

Atomic Force Microscopy (AFM). The polypeptide solution in 50 mM sodium phosphate buffer (pH 7.5) with 100 mM NaCl was incubated, and an aliquot of 20 μL was diluted 20-fold in the same buffer. Twenty microliters of the diluted sample was deposited on freshly cleaved mica and dried immediately with nitrogen gas. The samples were imaged with a Nanoscope IIIa (Digital Instrument) in tapping mode, and the cantilever was set vibrating in the z direction at a resonance frequency of 290 kHz. The images were taken in air under ambient conditions using silicon tips.

ThT Fluorescence Assays. The ThT fluorescence assay was performed for Aβ(1–40), insulin, and αAC peptides in 50 mM sodium phosphate buffer (pH 7.5) with 100 mM NaCl. ThT (20 μM) was added to each of the 200 μL polypeptide solutions, which were incubated in 96-microwell plates at the indicated temperature with shaking with a ThermoStat Plus apparatus (Eppendorf). A Genios plate reader (TECAN) was used for fluorescence intensity measurement with excitation at 450 nm and emission at 485 nm as described previously (*20*). A Confort thermomixer (Eppendorf) was used for the incubation of the solution in the plate at 37 °C with a rotation rate of 200 rpm shaking for Aβ(1–40), and the incubation with a rotation rate of 900 rpm shaking for insulin.

Gel Electrophoresis. SDS−PAGE was carried out on 16.5% Tricine gels as described previously (*23*). Twenty microliters of peptide solutions was mixed with 6× SDS sample buffer and boiled for 5 min. Gels were silver-stained using the EzStain silver kit from ATTO (Tokyo, Japan).

CD Spectroscopy. The peptide secondary structure was monitored by CD spectroscopic measurement using a Jasco (Tokyo, Japan) J-720 spectrometer. An optical cell with a path length of 1 mm was used. Far-UV spectra at 25 °C were measured with a scan speed of 20 nm/min. The time-dependent CD spectral change at 60 °C was measured using a single scan with a scan speed of 100 nm/min.

RESULTS

Effect of αAC Peptides on the Aggregation of Aβ(1–40). We investigated the effects of αAC(70–88) and αAC(71–88) on the amyloid formation of Aβ(1–40). Aggregation of Aβ(1–40) was monitored using the ThT fluorescence intensity at 485 nm. When Aβ(1–40) was incubated at a concentration of 0.1 mg/mL, 37 °C, and pH 7.5 with shaking at a rotation rate of 200 rpm, the time trace of ThT fluorescence showed a characteristic sigmoidal curve (*18, 19*) with a lag phase of ∼2 h followed by a rapid fibril growth phase (○ plot in Figure 1). Our result matches a previous observation of the formation of amyloid fibrils of Aβ(1–40), which can be approximated by a nucleation-dependent process. Atomic force microscopy (AFM) also showed the presence of fibrillar aggregates in this solution (data not shown). When 0.1 mg/mL Aβ(1–40) was incubated in the presence of 0.5 mg/mL αAC(70–88) (molar ratio of ca. 1:10), ThT fluorescence did not increase throughout the incubation period (● plot in Figure 1). A similar result was obtained for 0.1 mg/mL Aβ(1–40) in the presence of 0.5 mg/mL αAC(71–88) (□ plot in Figure 1). We could not find any large fibrils in the solution mixture of Aβ(1–40) and these αAC peptides by AFM, indicating that amyloid formation of Aβ(1–40) had been suppressed by these peptides from αAC because of their chaperone-like activities. These results are consistent with previously published results which showed that αAC(70–88)K70D suppressed the fibril formation of Aβ(1–40) (*17*). While the ThT fluorescence of the αAC(70–88) solution remained at the same intensity under this condition (◇ plot in Figure 1), the ThT fluorescence intensity of the αAC(71–88) solution on its own showed a gradual increase when incubated at 37 °C with shaking (× plot in Figure 1). We confirmed that fibrillar aggregates as shown in Figure 2a were formed in this solution by AFM measurement. Therefore, αAC(71–88) itself forms amyloid fibril, but it is suppressed in the solution mixture of αAC(71–88) and Aβ(1–40).

Effect of αAC Peptides on the Aggregation of Insulin. We further investigated the chaperone-like activity of αAC peptides to see if this peptide could prevent the aggregation of other aggregation-prone proteins, e.g., insulin. To observe an increase in the intensity of ThT fluorescence within the time frame of this experiment, the insulin solution was shaken with a much faster rotation rate (900 rpm) than in

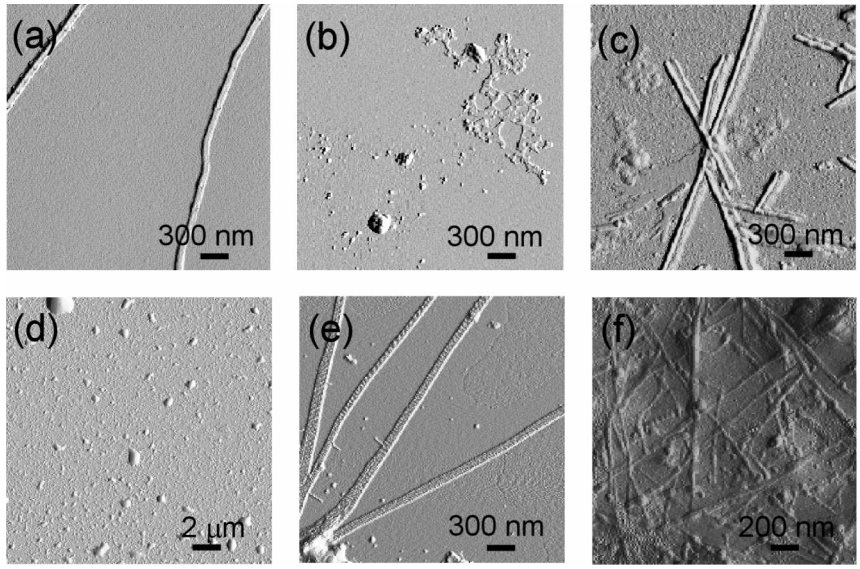

FIGURE 2: AFM images of the aggregates. (a) Amyloid fibrils obtained by incubation of 0.5 mg/mL αAC(71–88) at 37 °C with 900 rpm shaking for 12 h. Similar amyloid fibrils were obtained also by incubation with 200 rpm. (b) Aggregates obtained by incubation of the 2 mg/mL insulin solution at 37 °C with 900 rpm shaking for 12 h. (c) Amyloid fibril obtained by incubation of the solution mixture of 2 mg/mL insulin and 0.5 mg/mL αAC(71–88) at 37 °C with 900 rpm shaking for 12 h. (d) Aggregate obtained by incubation of the solution mixture of 2 mg/mL insulin and 0.5 mg/mL αAC(70–88)K70D at 37 °C with 900 rpm shaking for 6 h. (e) Amyloid fibril obtained by incubation of 0.5 mg/mL αAC(70–76)K70D at 60 °C without shaking for 24 h. (f) Amyloid fibril obtained by incubation of 0.5 mg/mL αAC(70–88)K70D at 60 °C without shaking for 24 h.

the case of Aβ(1–40) (200 rpm). The time trace of ThT fluorescence intensity of the 2 mg/mL insulin solution at 37 °C with 900 rpm shaking showed a rapid increase after an initial lag phase of 8 h (○ plots in Figure 3). AFM measurement of the solution indicated the presence of spherical aggregates rather than fibrils in this solution (Figure 2b). Therefore, the ThT fluorescence intensity shown in ○ plots in Figure 3 is monitoring the growth of spherical aggregates of insulin. The distinct morphology of insulin aggregates from that reported in previous studies (*20–22*) would be due to differences in experimental conditions such as the solvent and sample rotation system for shaking. The × plot of Figure 3a shows that the ThT fluorescence intensity of the αAC(71–88) solution gradually increased during the incubation with 900 rpm shaking. AFM measurement of this solution indicated that fibrillar aggregates were formed as shown in Figure 2a. On the other hand, the ThT fluorescence intensity of the solution mixture of 2 mg/mL insulin and 0.5 mg/mL αAC(71–88) (molar ratio of ca. 1.5:1) showed only a subtle increase after 4 h of lag time (● plot in Figure 3a), suggesting that the level of aggregation of both αAC(71–88) and insulin was reduced as in the case of solution mixtures of αAC peptides and Aβ(1–40). AFM measurement revealed a small amount of amyloid fibrils as shown in Figure 2c in the solution mixture. It is possible that this amyloid fibril is composed of αAC(71–88) alone since its morphology is similar to that obtained from the αAC(71–88) solution shown in Figure 2a.

The ThT fluorescence intensity of the αAC(70–88)K70D solution on its own showed a rapid and small increase in the initial stage as shown in the × plot in Figure 3c. AFM measurement indicated that amorphous aggregates were formed in this solution (image not shown). While the ThT fluorescence of the αAC(70–88) solution remained at the same low intensity during the incubation at 37 °C and 900 rpm (× plot in Figure 3b), AFM measurement indicated that a small amount of amorphous aggregate was also formed in this solution (image not shown). When the solution mixture of 0.5 mg/mL αAC(70–88) and 2 mg/mL insulin was incubated under the same condition, a rapid increase in the ThT fluorescence was observed after the 4 h lag time (● plot in Figure 3b). A similar result was obtained when the solution mixture of 0.5 mg/mL αAC(70–88)K70D and 2 mg/mL insulin was incubated under this condition (● plot in Figure 3c). AFM measurement indicated that large spherical aggregates were formed in these solutions as shown in Figure 2d, which have a morphology distinct from the one obtained from the solution mixture of αAC(71–88) and insulin as shown in Figure 2c. These results suggested that the self-association of αAC(70–88) and αAC(70–88)K70D is distinct from that of αAC(71–88).

To study the interaction between insulin and the αAC peptide, we performed SDS−PAGE analysis of the solution mixture after incubation for various length of time, as it has been reported previously that SDS-stable aggregates may be formed in the process of aggregation for amyloid β-protein (*23, 24*). The solution mixture was incubated at 37 °C with 900 rpm shaking for 0−6 h and then analyzed by SDS−PAGE using a slab gel with a concentration of 16.5% and a Tricine buffer system. After incubation of the solution mixture for 4 h, a high-molecular mass band appeared accompanying the reduction in the band intensities corresponding to αAC(70–88)K70D and insulin (A and B chains) as shown Figure 4. These results indicate that the SDS-stable aggregates comprising αAC(70–88)K70D and insulin were formed in the solution mixture, and a similar result was also obtained for the solution mixture of αAC(70–88) and insulin. Therefore, rapid increases in the ThT fluorescence intensities shown in ● plots in panels b and c of Figure 3 are monitoring the growth of an aggregate comprising insulin and αAC peptide. We performed Tricine SDS−PAGE analysis for the solution mixture of insulin and αAC(71–88) incubated with

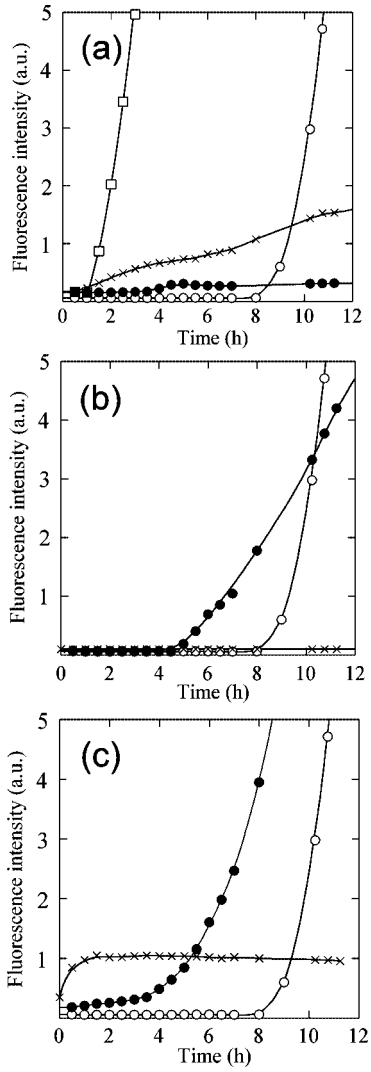

FIGURE 3: ThT fluorescence assay for the effect of αAC peptides on the aggregation of insulin. The time trace of the ThT fluorescence intensity of the peptide solutions incubated at 37 °C with 900 rpm shaking was monitored. (a) Samples of (○) 2 mg/mL insulin, (●) 2 mg/mL insulin in the presence of 0.5 mg/mL αAC(71–88), (×) 0.5 mg/mL αAC(71–88) alone, and (□) 2 mg/mL insulin in the presence of the sonicated preformed fibril of αAC(71–88). The amyloid fibril obtained by incubation of 0.5 mg/mL αAC(71–88) at 37 °C with 900 rpm shaking for 24 h was sonicated and then diluted 10-fold into the insulin solution. (b) Samples of (○) 2 mg/mL insulin, (●) 2 mg/mL insulin in the presence of 0.5 mg/mL αAC(70–88), and (×) 0.5 mg/mL αAC(70–88) alone. (c) Samples of (○) 2 mg/mL insulin, (●) 2 mg/mL insulin in the presence of 0.5 mg/mL αAC(70–88)K70D, and (×) 0.5 mg/mL αAC(70–88)K70D alone.

900 rpm shaking for 0−12 h. In contrast to the results described above, the intensities of the bands corresponding to αAC(71–88) and insulin did not change, and the band corresponding to the SDS−PAGE-stable aggregate was not observed during this incubation period (data not shown). Therefore, the majority of αAC(71–88) and insulin did not form SDS-stable aggregates in the solution mixture, which is consistent with the result from the ThT fluorescence assay that the aggregation of insulin and αAC(71–88) was suppressed in their solution mixture.

It is interesting that αAC(70–88) and αAC(70–88)K70D promoted rapid growth of aggregates comprising insulin and αAC peptide in their solution mixture, while αAC(71–88)

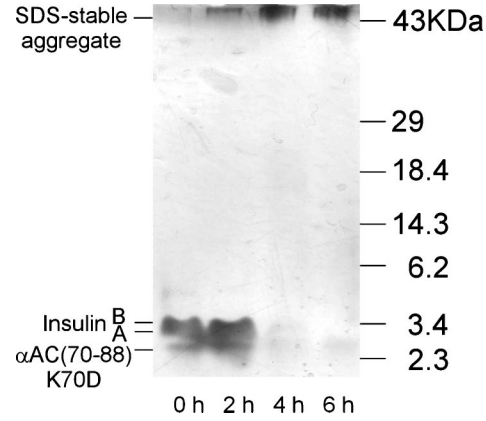

FIGURE 4: SDS−PAGE analysis of the solution mixture of insulin and αAC(70–88)K70D. A slab gel with a concentration of 16.5%, a Tricine buffer system, and silver staining were used. The solution mixture of 2 mg/mL insulin and 2 mg/mL αAC(70–88)K70D in 50 mM sodium phosphate buffer (pH 7.5) with 100 mM NaCl was incubated at 37 °C with 900 rpm shaking for the indicated period and then analyzed. Insulin is separated into the A and B chains under the reducing condition of SDS−PAGE sample buffer. The molecular mass of the SDS-stable aggregates was estimated to be higher than 100 kDa by SDS−PAGE of a slab gel with a concentration of 12% (data not shown).

suppressed the aggregation of insulin. These findings may be similar to what happened with a cross-seeding effect, where the sonicated preformed amyloid fibrils act as the "nuclei" or "seeds" to promote the amyloid formation of other protein (*22, 25*). To see whether the αAC peptide can act as the nuclei of aggregation for other polypeptides, the cross-seeding effect of amyloid fibrils of αAC(71–88) was examined. We performed a ThT fluorescence assay for insulin aggregation in the presence of sonicated fibrils of αAC(71–88), which were prepared as previously reported (*22*). As shown in the □ plot in Figure 3a, the ThT fluorescence intensity increased rapidly after the ca. 1 h lag phase in the presence of sonicated fibrils of αAC(71–88), and AFM measurement of this solution showed the presence of a mixture of large aggregates and amyloid fibrils (data not shown). Therefore, the sonicated fibril of αAC(71–88) can promote the aggregation of insulin by a cross-seeding effect. We performed a ThT fluorescence assay for amyloid fibril formation of Aβ(1–40) in the presence of sonicated fibrils of αAC(71–88) to examine its cross-seeding effect on Aβ(1–40). The ThT fluorescence intensity of this solution mixture showed a sigmoidal curve with a lag time of ca. 9 h, followed by a rapid fibril growth phase (data not shown). This lag phase was much longer than the 2 h lag time for fibril formation of Aβ(1–40) alone, indicating that the sonicated fibril of αAC(71–88) decelerated Aβ(1–40) amyloid formation. Therefore, αAC(71–88) exhibits chaperone-like activity against Aβ(1–40) even in the sonicated fibril form.

Residues Required for or Suppressing Amyloid Fibril Formation of αAC Peptides. We further investigated the αAC peptides to find the region that is important for amyloid fibril formation. We prepared a range of variants of αAC(71–88) and αAC(70–88) (Table 1) and examined their aggregation propensities. In these assays, we used the same solvent condition as before but at a higher temperature (60 °C) since the aggregation of these peptides became faster at 60 °C and this would enable us to study and compare the

Table 1: Sequence of αAC Peptide Variants and Morphology of Aggregates Obtained by Incubation at 60 °C without Shaking for 24 h

	sequence	the morphology monitored by AFM
αAC(70-88)	KFVIFLDVKHFSPEDLTVK	amorphous
αAC(70-88)K70D	DFVIFLDVKHFSPEDLTVK	amyloid[a]
αAC(71-88)	FVIFLDVKHFSPEDLTVK	amyloid
αAC(72-88)	VIFLDVKHFSPEDLTVK	not detected
αAC(70-83)	KFVIFLDVKHFSPE	insoluble
αAC(70-83)K70D	DFVIFLDVKHFSPE	amyloid
αAC(71-83)	FVIFLDVKHFSPE	insoluble
αAC(70-76)	KFVIFLD	insoluble
αAC(70-76) K70D	DFVIFLD	amyloid
αAC(71-76)	FVIFLD	insoluble

[a] Amorphous aggregates were formed when the solution was incubated at 37 °C with 900 rpm shaking. Insoluble means the peptide is insoluble in 50 mM sodium phosphate buffer (pH 7.5) and 100 mM NaCl.

aggregation propensities of these peptides in a reasonable experimental time frame. For αAC(71–88), an increase in ThT fluorescence can be observed within 2 h, and AFM measurement indicated that the morphology of αAC(71–88) fibrils formed at 60 °C is similar to that formed at 37 °C. The increase in ThT fluorescence for αAC(70–88) is significantly smaller compared to that of αAC(71–88) (ca. 10%). Interestingly, the removal of F71 from αAC(71–88), i.e., αAC(72–88), significantly decreased the propensity to form amyloid fibrils according to both ThT fluorescence and AFM. This result suggested that F71 is crucial for amyloid fibril formation of the αAC peptides. The same amino acid (F71) has also been shown to be essential for the chaperone-like activity of αAC (*14*) and αAC peptide (*15*).

To further characterize the aggregation potential of the αAC peptides, we have also made αAC(70–83), but it has very low solubility, preventing us from further characterizing its aggregation potential. Other shorter peptides prepared, including αAC(70–83), αAC(71–83), αAC(70–76), and αAC(71–76), have all been found to be insoluble, preventing further detailed characterization. On the other hand, we were able to recover the solubility of these peptides by introducing the substitution K70D [αAC(70–83)K70D and αAC(70–76)K70D], allowing us to study the aggregation propensities of the shorter peptides (Table 1). AFM revealed that both of these shorter peptides formed amyloid fibrils [αAC(70–83)K70D (data not shown) and αAC(70–76)K70D (Figure 2e)]. The FVIFLD sequence is commonly found in the peptides with the ability to form amyloid fibrils as shown in Table 1. Therefore, it is likely that the amino acids important for the amyloid fibril formation of αAC(71–88) would be located in the FVIFLD sequence.

To further study the effect of having the additional N-terminal amino acid on the self-aggregation of the αAC peptides, we investigated the effect of heat treatment on

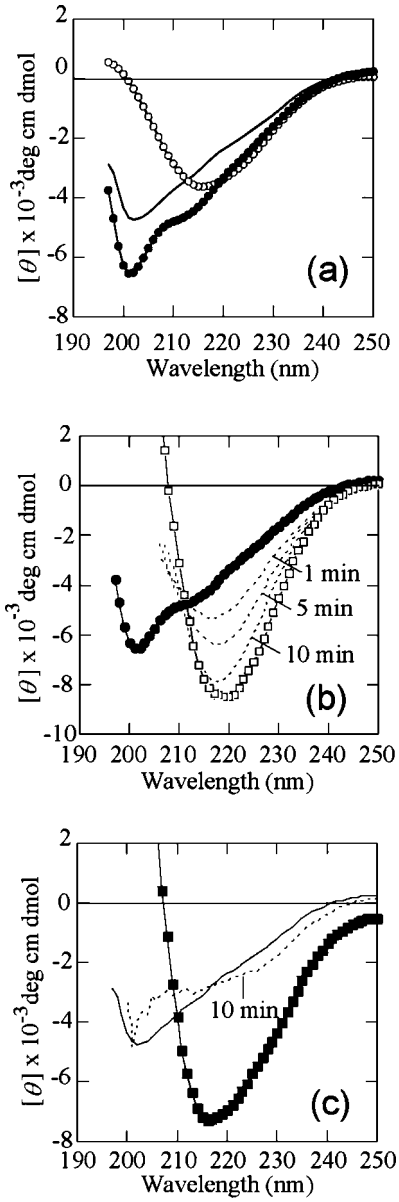

FIGURE 5: CD spectra of the αAC peptides at 0.5 mg/mL. (a) αAC(70–88) (○), αAC(70–88)K70D (—), and αAC(71–88) (●) at 25 °C. (b) Effect of temperature on the secondary structure of αAC(71–88): at 25 °C (●), measured at 60 °C after incubation for the indicated period (•••); and measured at 25 °C after incubation for 24 h at 60 °C (□). (c) Effect of temperature on the secondary structure of αAC(70–88)K70D: at 25 °C (–), measured at 60 °C after incubation for the indicated period (•••), and measured at 25 °C after incubation for 24 h at 60 °C (■).

αAC(71–88), αAC(70–88), and αAC(70–88)K70D. AFM indicated the heat treatment of αAC(70–88) at 60 °C for 24 h induced the formation of the amorphous aggregates (data not shown). On the other hand, heat treatment of αAC(70–88)K70D at 60 °C for 24 h induced amyloid fibril formation as shown in the AFM image in Figure 2f. To study the effect of K70 and K70D on the conformation of αAC(71–88), we monitored their secondary structure by measuring the far-UV CD spectra. The ○ plot in Figure 5a indicates that αAC(70–88) is in a β-sheet rich conformation at 25 °C, while αAC(71–88) (● plot in Figure 5a) and αAC(70–88)K70D (solid line in Figure 5a) are very close to a random conformation. When the temperature was increased to 60 °C, the CD spectrum of αAC(71–88)

gradually changed to a β-sheet rich profile (dotted lines and □ plot in Figure 5b), which is consistent with our experimental result in which αAC(71–88) formed amyloid fibril readily with heat treatment at 60 °C. On the other hand, as shown in Figure 5c, the random conformation of αAC(70–88)K70D did not change much shortly after the temperature was increased to 60 °C (dotted line in Figure 5c) but was later transformed to the β-sheet rich conformation on heat treatment at 60 °C for 24 h (■ plot in Figure 5c). This result together with the AFM result indicated that the K70D residue at the N-terminus of the αAC peptide decelerated amyloid fibril formation of the αAC(71–88) peptide at 60 °C. For αAC(70–88), the β-sheet rich conformation remained unchanged when the temperature was elevated to 60 °C. These results suggested that K70 may suppress amyloid fibril formation by having the β-sheet rich conformation at 25 °C. These results from CD spectroscopic measurements indicate that an additional N-terminally charged amino acid reduces the potential to form amyloid fibrils in αAC peptides, which may account for the reduced chaperone-like activity for αAC(70–88) and αAC(70–88)K70D.

DISCUSSION

We found that αAC(71–88) exhibits chaperone-like activity against amyloid fibril formation of Aβ(1–40) and the aggregation of insulin. αAC(71–88) suppressed amyloid fibril formation of Aβ(1–40) even in the sonicated fibril form, while insulin aggregation was accelerated under the same condition. A small amount of amyloid fibril was also formed in the solution mixture of insulin and αAC(71–88). A previous study of peptide mapping and alanine substitution on Aβ(1–40) revealed that the KLVFF amino acid sequence, the residues from position 16 to 20, is essential for the self-association of Aβ(1–40) monomer to form amyloid fibrils (*26*). On the basis of this result, Sharma et al. further suggested that the DFVIF sequence of αAC(70–88)K70D, which is homologous to the KLVFF sequence of Aβ(1–40), may be competing with the binding site in Aβ(1–40) to prevent it from forming fibrils (*17*). This suggestion is consistent with our finding that the FVIFLD sequence of the αAC peptide is important for its own amyloid fibril formation, which is also prevented in the solution mixture of αAC(71–88) and Aβ(1–40). Therefore, the stronger binding of the αAC peptides to Aβ(1–40) through the homologous sequence could potentially be responsible for their chaperone-like activities. Insulin does not possess a sequence homologous to that of αAC(71–88), and the interaction between αAC(71–88) and insulin may not be sufficiently strong to completely suppress amyloid formation.

On the other hand, the aggregation of insulin was accelerated in the presence of αAC(70–88) and αAC(70–88)-K70D. CD spectroscopic measurements indicated that the propensity for amyloid fibril formation of these peptides was reduced compared with that of αAC(71–88). AFM measurements also showed that αAC(70–88) and αAC(70–88)K70D have a tendency to form amorphous aggregate but not amyloid fibrils. It is possible that the chaperone-like activity of the αAC peptides could be correlated with their propensity to form amyloid fibrils. αAC(70–88) and αAC(70–88)K70D may promote the growth of aggregates composed of αAC peptide and insulin by a mechanism similar to that of the sonicated fibrils of αAC(71–88).

We also found that αAC(71–88) itself can form amyloid fibrils on its own under our incubation conditions at pH 7.5 with 900 rpm shaking. The analysis for the variants of αAC(71–88) suggested that the amino acids important for amyloid fibril formation of αAC(71–88) would be located in the FVIFLD sequence. These findings could potentially be explained by the possible mechanism of how molecular chaperones prevent the aggregation of other proteins. Aggregation-prone substrates for molecular chaperones such as partially folded or misfolded proteins expose hydrophobic clusters to water or solvents. Molecular chaperones preferentially bind to such exposed hydrophobic clusters and prevent self-association of the substrates by forming intermolecular interactions with the solvent-exposed hydrophobic regions. Therefore, the substrate binding sites of molecular chaperons tend to possess a significant amount of hydrophobic residues. However, such a high hydrophobic property required for molecular chaperones could also increase the potential to form aggregates or amyloid fibrils by the molecular chaperones themselves.

The removal of F71, which is essential for the chaperone-like activity of αAC peptide (*15*), from αAC(71–88) significantly decreased the propensity to form amyloid fibrils, suggesting that F71 is essential also for amyloid fibril formation of the αAC peptides. Recently, αAC protein has been shown to form amyloid fibril under mildly denaturing conditions (*27*). Our results suggest that F71 not only is essential for the chaperone-like activity of αAC as previously suggested (*14*) but also may play an important role also in the amyloid fibril formation of αAC. We have also found that charged residues present in the region flanking the hydrophobic sequence [αAC(70–88) and αAC(70–88)K70D] reduced the β-aggregation propensity of the αAC peptides. The presence of such an aggregation suppressor in the protein amino acid sequence has recently been proposed since the region flanking the aggregating hydrophobic sequence has been observed to be enriched in proline or charged residues such as lysine, arginine, aspartic acid, and glutamic acid (*28, 29*).

In conclusion, we showed that αAC(71–88) and αAC(70–88) suppressed the amyloid fibril formation of Aβ(1−40). αAC(71–88) also exhibited chaperone-like activity against insulin, but the formation of fibrils was not completely suppressed in their solution mixture. On the other hand, αAC(70–88) and αAC(70–88)K70D promoted the growth of aggregate comprising the αAC peptides and insulin, suggesting that the chaperone-like activities of these peptides were significantly reduced. Interestingly, we found that αAC(71–88) also formed amyloid fibrils on its own, and the potential for fibril formation was reduced in αAC(70–88) and αAC(70–88)K70D. F71, which is essential for the chaperone-like activity of αAC(71–88), is found to be important for its self-aggregation. The charged residues K70 and K70D in the region flanking the aggregating hydrophobic sequence reduced the β-aggregation propensity of αAC peptide, supporting the recent proposal about the presence of an aggregation suppressor in the region flanking pro-aggregating hydrophobic sequences in proteins. Our results provide important insight into amyloid fibril formation and chaperone-like activity of peptides from αAC.

ACKNOWLEDGMENT

Yozo Suzuki, Takashi Ohshima, Yohei Kubo, and Saori Go (Kyoto Institute of Technology) are acknowledged for technical assistance.

REFERENCES

1. Horwitz, J. (1992) α-Crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. U.S.A. 89*, 10449–10453.
2. Horwitz, J. (2003) α-Crystallin. *Exp. Eye Res. 76*, 145–153.
3. Clark, J. I., and Muchowski, P. J. (2000) Small heat-shock proteins and their potential role in human disease. *Curr. Opin. Struct. Biol. 10*, 52–59.
4. Macario, A. J., and Conway de Macario, E. (2005) Sick chaperones, cellular stress, and disease. *N. Engl. J. Med. 353*, 1489–1501.
5. Sun, Y., and MacRae, T. H. (2005) The small heat shock proteins and their role in human disease. *FEBS J. 272*, 2613–2627.
6. Shinohara, H., Inaguma, Y., Goto, S., Inagaki, T., and Kato, K. (1993) αB Crystallin and HSP28 are enhanced in the cerebral cortex of patients with Alzheimer's disease. *J. Neurol. Sci. 119*, 203–208.
7. Ingolia, T. D., and Craig, E. A. (1982) Four small *Drosophila* heat shock proteins are related to each other and to mammalian α-crystallin. *Proc. Natl. Acad. Sci. U.S.A. 79*, 2360–2364.
8. Augusteyn, R. C. (2004) Dissociation is not required for α-crystallin's chaperone function. *Clin. Exp. Optom. 87*, 356–366.
9. Groenen, P. J., Merck, K. B., de Jong, W. W., and Bloemendal, H. (1994) Structure and modifications of the junior chaperone α-crystallin. From lens transparency to molecular pathology. *Eur. J. Biochem. 225*, 1–19.
10. Thomson, J. A., and Augusteyn, R. C. (1989) On the structure of α-crystallin: Construction of hybrid molecules and homopolymers. *Biochim. Biophys. Acta 994*, 246–252.
11. Raman, B., and Rao, C. M. (1994) Chaperone-like activity and quaternary structure of α-crystallin. *J. Biol. Chem. 269*, 27264–27268.
12. Raman, B., and Rao, C. M. (1997) Chaperone-like activity and temperature-induced structural changes of α-crystallin. *J. Biol. Chem. 272*, 23559–23564.
13. Sharma, K. K., Kumar, G. S., Murphy, A. S., and Kester, K. (1998) Identification of 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid binding sequences in α-crystallin. *J. Biol. Chem. 273*, 15474–15478.
14. Santhoshkumar, P., and Sharma, K. K. (2001) Phe71 is essential for chaperone-like function in αA-crystallin. *J. Biol. Chem. 276*, 47094–47099.
15. Sharma, K. K., Kumar, R. S., Kumar, G. S., and Quinn, P. T. (2000) Synthesis and characterization of a peptide identified as a functional element in αA-crystallin. *J. Biol. Chem. 275*, 3767–3771.
16. Bhattacharyya, J., Padmanabha Udupa, E. G., Wang, J., and Sharma, K. K. (2006) Mini-B-Crystallin: A functional element of B-crystallin with chaperone-like activity. *Biochemistry 45*, 3069–3076.
17. Santhoshkumar, P., and Sharma, K. K. (2004) Inhibition of amyloid fibrillogenesis and toxicity by a peptide chaperone. *Mol. Cell. Biochem. 267*, 147–155.
18. Nichols, M. R., Moss, M. A., Reed, D. K., Lin, W.-L., Mukhopadhyay, R., Hoh, J. H., and Rosenberry, T. L. (2002) Growth of β-amyloid(1–40) protofibrils by monomer elongation and lateral association. Characterization of distinct products by light scattering and atomic force microscopy. *Biochemistry 41*, 6115–6127.
19. Naiki, H., and Gejyo, F. (1999) Kinetic analysis of amyloid fibril formation. *Methods Enzymol. 309*, 305–318.
20. Nielsen, L., Khurana, R., Coats, A., Frokjaer, S., Brange, J., Vyas, S., Uversky, V. N., and Fink, A. L. (2001) Effect of environmental factors on the kinetics of insulin fibril formation: Elucidation of the molecular mechanism. *Biochemistry 40*, 6036–6046.
21. Ahmad, A., Millet, I. S., Doniach, S., Uversky, V. N., and Fink, A. L. (2003) Partially folded intermediates in insulin fibrillation. *Biochemistry 42*, 11404–11416.
22. Hong, D.-P., and Fink, A. L. (2005) Independent heterologous fibrillation of insulin and its B-chain peptide. *Biochemistry 44*, 16701–16709.
23. Walsh, D. M., Lomakin, A., Benedek, G. B., Condron, M. M., and Teplow, D. B. (1997) Amyloid-protein fibrillogenesis. Detection of a protofibrillar intermediate. *J. Biol. Chem. 272*, 22364–22372.
24. Stine, W. B., Jr., Dahlgren, K. N., Krafft, G. A., and LaDu, M. J. (2003) In vitro characterization of conditions for amyloid peptide oligomerization and fibrillogenesis. *J. Biol. Chem. 278*, 11612–11622.
25. Yagi, H., Kusaka, E., Hongo, K., Mizobata, T., and Kawata, Y. (2005) Amyloid fibril formation of α-synuclein is accelerated by preformed amyloid seeds of other proteins: Implications for the mechanism of transmissible conformational diseases. *J. Biol. Chem. 280*, 38609–38616.
26. Tjernberg, L. O., Näslund, J., Lindqvist, F., Johansson, J., Karlström, A. R., Thyberg, J., Terenius, L., and Nordstedt, C. (1996) Arrest of β-amyloid fibril formation by a pentapeptide ligand. *J. Biol. Chem. 271*, 8545–8548.
27. Carver, J. A., Meehan, S., Knowles, T. P. J., Baldwin, A. J., Smith, J. F., Squires, A. M., Clements, P., Treweek, T. M., Ecroyd, H., Tartaglia, G. G., Vendruscolo, M., MacPhee, C. E., and Dobson, C. M. (2007) Characterisation of amyloid fibril formation by small heat-shock chaperone proteins human αA-, αB- and R120G αB-crystallins. *J. Mol. Biol. 372*, 470–484.
28. Pawar, A. P., Dubay, K. F., Zurdo, J., Chiti, F., Vendruscolo, M., and Dobson, C. M. (2005) Prediction of "aggregation-prone" and "aggregation-susceptible" regions in proteins associated with neurodegenerative diseases. *J. Mol. Biol. 350*, 379–392.
29. Rousseau, F., Serrano, L., and Schymkowitz, J. W. (2006) How evolutionary pressure against protein aggregation shaped chaperone specificity. *J. Mol. Biol. 355*, 1037–1047.

BI701823G