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# Evidence of Rapid Coaggregation of Globular Proteins during Amyloid Formation

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## Supporting Information

**ABSTRACT:** The question of how an aggregating protein can influence aggregation of other proteins located in its vicinity is particularly significant because many proteins coexist in cells. We demonstrate *in vitro* coaggregation and cross-seeding of lysozyme, bovine serum albumin, insulin, and cytochrome *c* during their amyloid formation. The coaggregation process seems to be more dependent on the temperature-induced intermediate species of these proteins and less dependent on their sequence identities. Because amyloid-linked inclusions and plaques are recognized as multicomponent entities originating from aggregation of the associated protein, these findings may add new insights into the mechanistic understanding of amyloid-related pathologies.

The conversion of soluble normal proteins into higher-order amyloid aggregates is one of the foundational causes for the onset of many health complications such as Alzheimer's disease, Parkinson's disease, and Huntington's disease.<sup>1–3</sup> Until now, ~30 different proteins, including huntingtin,  $\alpha$ -synuclein,  $\beta$ 2-microglobulin, and lysozyme, have been found to form  $\beta$ -amyloid aggregates that lead to severe pathologies.<sup>2–4</sup> Though the aggregation process of a specific protein is usually thought to be associated with its related amyloid disease, it is not clearly known whether such an aggregation process would affect the functionality of other proteins. Most proteins coexist in different microenvironments or subcellular compartments performing vital life processes, including protein–protein interactions and formation of protein complexes. Hence, the question of how the aggregation process of a particular protein would influence the aggregation properties of other proteins present in its vicinity is very significant. Few investigations have certainly indicated the coexistence of two different amyloid-linked diseases in individual patients.<sup>5–7</sup> In one earlier report, the presence of both Alzheimer's disease (AD) and Huntington's disease (HD) is observed in the same patient.<sup>5</sup> Recent clinicopathologic studies have also revealed the coexistence of Huntington's disease and amyotrophic lateral sclerosis (ALS) in patients.<sup>6</sup> Another recent investigation reports the coaggregation of  $\alpha$ -synuclein and Cu/Zn superoxide dismutase protein.<sup>7</sup> Moreover, investigations at the cellular level have also revealed the potential interactions between two different amyloidogenic proteins.<sup>8,9</sup> For example, Lauren et al. have shown the possible interactions between cellular prion

proteins and amyloid- $\beta$  oligomers.<sup>9</sup> Despite the evidence of coexistence of two amyloid diseases, the underlying principles of coaggregation of different globular proteins are surely missing from our current understanding of amyloid formation. Knowledge of the interactions between an aggregating protein and the proteins located in its surrounding offers new opportunities to understand the mechanism of amyloid formation and to shed light on the critical role of amyloid-linked higher-order entities such as inclusions, plaques, and Lewy bodies.

To address these unexplored fundamental issues, we have investigated the occurrence of coaggregation and cross-seeding between different globular proteins [bovine serum albumin (BSA), lysozyme, insulin, and cytochrome *c*]. Though there have been no reports that refer to the existence of any diseases linked to coaggregation of these proteins, individual amyloid formation of these proteins has been well-established. Here, we have selected these proteins as convenient model systems for examining the occurrence of coaggregation between them. Two issues have been addressed in this *in vitro* investigation. First, we have examined the occurrence of coaggregation between proteins by mixing their monomers under aggregating conditions (see the Supporting Information). Second, to address the occurrence of cross-seeding, we tested whether the amyloid fibrils of a particular protein are capable of driving the aggregation of other globular proteins. Using a combination of different biophysical techniques, including thioflavin T assays, we report here the rapid coaggregation between proteins and the surprising cross-seeding efficiencies of individual amyloid fibrils. Furthermore, we find that low-level sequence identities exist between these coaggregating proteins. Finally, on the basis of all experimental evidence, we hypothesize that regardless of their sequence identities, the population of amyloid prone intermediate species is critical for the onset of both coaggregation and cross-seeding.

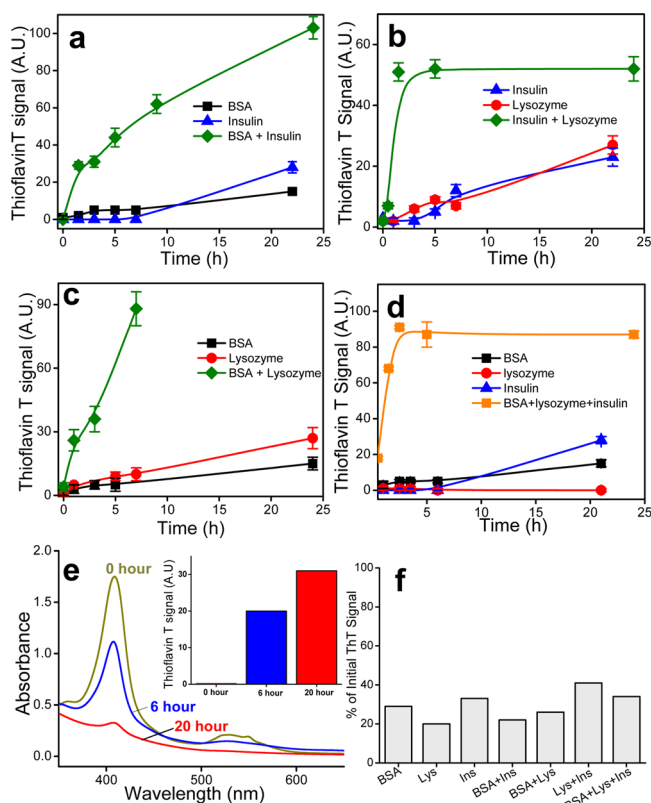
Amyloid aggregation of BSA, lysozyme, and insulin was conducted by incubating the samples at an elevated temperature, 70 °C [close to their  $T_m$  values (Table S2 of the Supporting Information)], and their kinetics were monitored by recording the thioflavin T signal at regular intervals (see materials and methods in the Supporting Information). Individual reactions of BSA [Figure 1 (■)], insulin [Figure 1

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**Figure 1.** Coaggregation studies of mixed monomers of different globular proteins in PBS at ~70 °C. (a) Coaggregation of BSA and insulin: (■) ~4.9  $\mu$ M BSA, (blue triangles) ~45.4  $\mu$ M insulin, and (green diamonds) ~2.9  $\mu$ M BSA and ~27.5  $\mu$ M insulin. (b) Coaggregation of insulin and lysozyme: (red circles) ~27.9  $\mu$ M lysozyme, (blue triangles) ~45.3  $\mu$ M insulin, and (green diamonds) ~27.5  $\mu$ M insulin and ~12.5  $\mu$ M lysozyme. (c) Coaggregation of BSA and lysozyme: (red circles) ~25.9  $\mu$ M lysozyme, (■) ~4.9  $\mu$ M BSA, and (green diamonds) ~2.9  $\mu$ M BSA and ~12.5  $\mu$ M lysozyme. (d) Coaggregation of BSA, lysozyme, and insulin: (■) ~4.9  $\mu$ M BSA, (blue triangles) ~40.2  $\mu$ M insulin, (red circles) ~18.9  $\mu$ M lysozyme, and (orange squares) ~1.9  $\mu$ M BSA, ~8.4  $\mu$ M lysozyme, and ~18.4  $\mu$ M insulin. (e) Coaggregation of mixed monomers of ~4.5  $\mu$ M BSA and ~24.5  $\mu$ M cytochrome *c*. The absorbance profile of cytochrome *c* monomers at different time points: 0 (green), 6 (blue), and 20 h (red). The inset shows the progress of amyloid aggregation of the sample by thioflavin T readings. (f) Stabilities of the amyloid fibrils against GdnHCl (1 M, 30 min at room temperature).

(blue triangles)], and lysozyme [Figure 1 (red circles)] showed the typical kinetic curves comprising a lag phase, a growth phase, and a saturation phase. Transmission electron microscopy images of the fibrils show the typical morphology as seen for normal amyloid fibrils (Figure S1 of the Supporting Information). These results confirm the conversion of soluble proteins into higher-order amyloid fibrils. To conduct coaggregation studies, we first explored four combinations of mixed monomers (lysozyme and BSA, lysozyme and insulin, BSA and insulin, and BSA, lysozyme, and insulin). The aggregation profiles of these four combinations of protein monomers showed kinetics faster than the kinetics of individual aggregation reactions [Figure 1a–c (green diamonds) and Figure 1d (orange squares)]. We did not observe any distinct lag phases for these coaggregation reactions.

To confirm the occurrence of coaggregation further, we have examined the aggregation kinetics of individual proteins at a

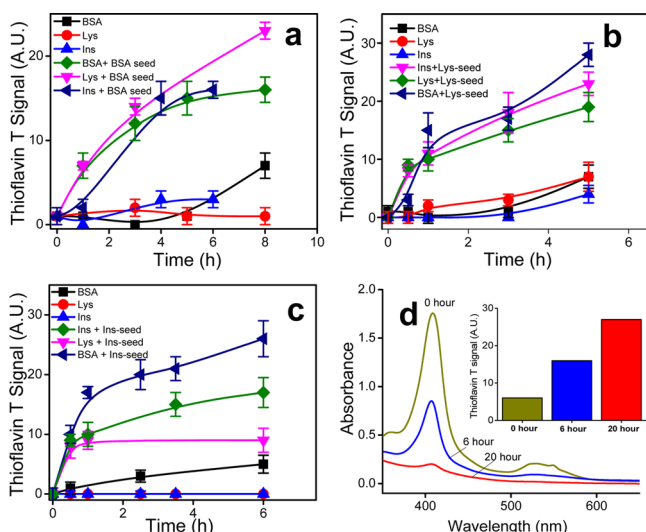
concentration relatively higher than the concentration of any of these coaggregation reactions. Results indicate that the individual aggregation kinetics are much slower (Figure S2 of the Supporting Information) than the kinetics of the reactions comprising mixed monomers. For example, in the case of the BSA/lysozyme mixed monomer sample (~2.9  $\mu$ M BSA and ~12.5  $\mu$ M lysozyme), the aggregation shows faster kinetics without a lag phase [Figure S2 of the Supporting Information (blue triangles)], whereas the aggregation profile of only lysozyme at ~48.9  $\mu$ M [Figure S2 of the Supporting Information (red circles)] or only BSA [Figure S2 of the Supporting Information (■)] at ~10.5  $\mu$ M shows much slower kinetics with a prolonged lag phase of ~6 h.

To gain more insights into the occurrence of coaggregation, we mixed BSA with a visibly detectable protein cytochrome *c* and followed the aggregation kinetics by monitoring the monomer concentration (see materials and methods in the Supporting Information). Because the absorbance profile of cytochrome *c* gives distinct peaks at ~550 and ~410 nm,<sup>10</sup> its presence can be easily detected when it is mixed with BSA monomers. The results (inset of Figure 1e) show a rapid coaggregation between BSA and cytochrome *c* into amyloid fibrils. The decrease in the absorbance value at 550 nm with an increasing thioflavin T signal magnitude during the progress of aggregation further supports the occurrence of coaggregation between BSA and cytochrome *c* (Figure 1e). Additional experiments indicate a delayed aggregation process for the sample containing only cytochrome *c* (Figure S3 of the Supporting Information).

Given that the coaggregation reactions are so rapid without any lag phases, there is a possibility that once the aggregation-promoting amyloid nuclei or oligomers are formed, they may have the potential (as seeds) to recruit the monomers of other proteins present in the sample mimicking a self-seeded aggregation process.

To understand this issue of cross-seeding, as a next step, we mixed preformed amyloid fibrils (or seeds) of individual proteins with the monomers of different proteins in separate vials and monitored the kinetics of each of these aggregation reactions by recording thioflavin T signals. Data shown in Figure 2 clearly indicate that amyloid fibrils of these proteins can efficiently initiate aggregation of the monomer samples of each other, leading to cross-seeding. Furthermore, the efficacy of cross-seeding is found to be similar to the self-seeding abilities of each of these proteins (Figure 2a–c). We have also observed the decrease in the cytochrome *c* monomer concentration during the progress of amyloid formation initiated by BSA seeds (Figure 2d and inset). These data are highly consistent with the coaggregation data. It is also possible that both coaggregation and cross-seeding can occur at the same time, leading to a faster aggregation process.

Additionally, we conducted circular dichroism (CD) experiments for structural characterization of the fibrils generated through coaggregation. For the reaction that starts with the mixed monomers of BSA, insulin, and lysozyme, a gradual change in the nature of the CD curve was observed (Figure S4 of the Supporting Information). Such conformational changes indicate the gradual formation of  $\beta$ -structures during the progress of aggregation [Figure 1d (orange squares) and Figure S4 of the Supporting Information]. Electron micrographs of the coaggregated fibrils are found to be typical amyloid type (Figure S1 of the Supporting Information). To understand the nature of the coaggregated fibrils further, we measured their



**Figure 2.** Cross-seeding of proteins during amyloid aggregation. (a) BSA at  $\sim 7.5 \mu\text{M}$  (■), lysozyme at  $\sim 27.9 \mu\text{M}$  (red circles), insulin at  $\sim 52.3 \mu\text{M}$  (blue triangles), BSA and BSA seed (green diamonds), lysozyme and BSA seed (pink triangles), and insulin and BSA seed (navy blue triangles). (b) BSA at  $\sim 6.0 \mu\text{M}$  (■), lysozyme at  $\sim 27.9 \mu\text{M}$  (red circles), insulin at  $\sim 52.3 \mu\text{M}$  (blue triangles), BSA and lysozyme seed (navy blue triangles), lysozyme and lysozyme seed (green diamonds), and insulin and lysozyme seed (pink triangles). (c) BSA at  $\sim 4.5 \mu\text{M}$  (■), lysozyme at  $\sim 18.9 \mu\text{M}$  (red circles), insulin at  $\sim 40.2 \mu\text{M}$  (blue triangles), BSA and insulin seed (navy blue triangles), lysozyme and insulin seed (pink triangles), and insulin and insulin seed (green diamonds). (d) Aggregation of cytochrome *c* monomers (at  $\sim 24.5 \mu\text{M}$ ) in the presence of BSA amyloids (as seeds). The inset shows the thioflavin T readings.

stabilities against a chemical denaturant. Comparison of the thioflavin T signal of the fibril suspensions in the absence and presence of GnHCl (1 M, incubated for 30 min) is shown in Figure 1f. This result does not show any significant differences in the stabilities of the fibril samples. Furthermore, the thioflavin T binding efficiencies of individual fibrils and coaggregated fibrils look similar (Figure S5 of the Supporting Information).

It is well-known that the population of temperature-induced partially folded species plays a critical role in the amyloid aggregation of proteins.<sup>11–13</sup> One of the reasons behind the higher propensities of intermediate states to undergo aggregation is their solvent-exposed hydrophobic moieties that promote intermolecular interactions.<sup>11</sup> In this study, we have observed both aggressive coaggregation and cross-seeding reactions between different proteins at 70 °C. Because 70 °C is close to the  $T_m$  values (Table S1 of the Supporting Information) of the studied proteins under physiological buffer conditions, it is likely that they would contain higher levels of their aggregation prone intermediate species. To confirm this hypothesis, we conducted cross-seeding experiments with lysozyme, BSA, and insulin at 37 °C where these proteins are expected to remain mostly in their native states. Results, as shown in Figure S6 of the Supporting Information, confirm the lack of aggregation, coaggregation, and cross-seeding at 37 °C. This suggests the critical role of temperature-induced partially folded protein species for the onset of coaggregation.

Recently, the coaggregation of two differently labeled polyQ sequences was reported using mixed-isotope infrared spectroscopy.<sup>14</sup> A previous study of aggregation of multidomain protein

constructs indicated that a >70% sequence identity would highly favor aggregation whereas a <30–40% sequence identity would not promote aggregation.<sup>15</sup> We have conducted bioinformatics analysis of the proteins, and it appears that there is no significant similarity between their sequences (Table S2 of the Supporting Information). Hence, the driving force for coaggregation and cross-seeding seems to be more dependent on the temperature-induced aggregation prone intermediate species and less dependent on their sequence identities. Furthermore, it is important to notice that the aggregation process of any individual protein sample (fundamentally, between species that have 100% identical sequences) shows kinetics slower than that of any mixed protein samples (Figure S2 of the Supporting Information). This observation suggests two important clues. (1) There may be some conserved regions within each protein's sequence to hinder its self-association. (2) There is a net gain of additional intermolecular interactions during coaggregation.

An increase in thioflavin T fluorescence and a decrease in monomeric protein concentration [e.g., BSA and cytochrome *c* (Figures 1e and 2d)] clearly suggest some form of coaggregation. However, these methods are not sufficiently sensitive to characterize the structural properties of coaggregated fibrils and individual fibrils. It is possible that amyloid type fibrils are formed by coaggregation in which both proteins in a sample interact via backbone H-bonds in a single amyloid cross- $\beta$  architecture. It is also possible that binding (and no coaggregation) of one species with another stabilizes fibers or intermediate species and enhances the aggregation kinetics. For example,  $\alpha\beta$ -crystalline binds to fibers of A $\beta$  peptide, without inserting into the framework of cross- $\beta$  structure.<sup>16</sup>

In conclusion, this study provides enough evidence of rapid coaggregation and cross-seeding between different globular proteins into amyloid fibrils. The onset of coaggregation is found to be more dependent on the amyloid prone intermediate species of the participating proteins. These results may be directly relevant to the mechanism of coexistence of two amyloid-linked diseases in individual patients. Moreover, because amyloid-linked hallmarks such as plaques and inclusions are poorly understood, these new findings of coaggregation may help improve our understanding of amyloid formation and its associated diseases.

## ■ ASSOCIATED CONTENT

### ⑤ Supporting Information

Figures S1–S6, Tables S1 and S2, and Methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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