Amyloid conformation-dependent disaggregation revealed by a reconstituted yeast prion system

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Abstract

Disaggregation of amyloid fibrils is a fundamental biological process required for amyloid propagation. However, due to the lack of experimental systems, the molecular mechanism of how amyloid is disaggregated by cellular factors remains poorly understood. Here, we established a robust, *in vitro* reconstituted system of yeast prion propagation and found that Hsp104, Ssa1, and Sis1 chaperones are essential for efficient disaggregation of Sup35 amyloid. Real-time imaging of single-molecule fluorescence coupled with the reconstitution system revealed that amyloid disaggregation is achieved by ordered, timely binding of the chaperones to the amyloid. Remarkably, we uncovered two distinct, prion strain conformation-dependent modes of disaggregation, fragmentation and dissolution. We characterized distinct chaperon dynamics in each mode and found that transient, repeated binding of Hsp104 to the same site of amyloid results in fragmentation. These findings provide a physical foundation for otherwise puzzling *in vivo* observations and for therapeutic development for amyloid-associated neurodegenerative diseases.

Introduction

A number of neurodegenerative diseases, including Alzheimer's, Huntington's, and prion diseases, are elicited by misfolding of causal proteins and subsequent formation of β -sheet-rich fibrillar protein aggregates called amyloid¹. Therefore, disaggregation of amyloid into monomeric proteins potentially provides a means for intervening the intractable diseases². However, disaggregation could result in production of new amyloid fragments (seeds), which can enhance successive accumulation of amyloid and thereby exacerbate disease pathophysiology. In fact, disaggregation-mediated production of seeds may contribute to cell-to-cell transmission of amyloid, which is universal and at least partly account for disease mechanisms in many neurodegenerative diseases^{3,4}. These notions highlight the importance of our better understanding of amyloid disaggregation process.

Mechanistically, propagation of protein aggregates or amyloids is achieved by the two key processes, template-dependent extension (growth) and disaggregation of amyloid^{5,6}. Until now, many efforts have delineated molecular mechanisms of nucleation and amyloid growth processes⁷. In contrast, however, details of disaggregation mechanism have remained elusive, although the rate of amyloid disaggregation provides greater impacts on efficiency of amyloid replication and ultimately cellular phenotypes, such as prion strain phenotypes, than that of amyloid growth⁸. Notably, our poor understanding of amyloid disaggregation stems from the lack of technology and experimental systems that allow us to thoroughly investigate the process of amyloid disaggregation *in vitro*, as compared with the disaggregation mechanism of amorphous protein aggregates^{9,10}.

To solve this fundamental problem in amyloid biology, yeast prions have been employed to dissect molecular mechanisms of amyloid disaggregation process^{11–14}. Previously, the hexameric AAA⁺ ATPase Hsp104 was shown to play crucial roles in amyloid formation and disaggregation of Sup35NM

¹⁵, which consists of intrinsically disordered N-terminal and highly charged middle domains in Sup35 (a translation termination factor and protein determinant of yeast prion [*PSI*⁺] state) and is sufficient for amyloid formation *in vitro* ¹⁶. However, it has long been controversial whether Hsp104 alone is sufficient ^{15,17,18} or additional cellular factors are required to disaggregation of Sup35NM amyloid ^{9,10,19,20} though *in vivo* studies suggest that molecular chaperones such as Ssa1 (Hsp70) and Sis1 (Hsp40) play critical roles in propagation and maintenance of [*PSI*⁺] yeast ^{21–25}. More importantly, it has been unclear how Hsp104 cooperates with other cellular factors, if any, to regulate the disaggregation process of Sup35 amyloid, and whether amyloid disaggregation mechanisms are similar or different between distinct strain conformations, which are widely observed and crucial for manifestation of distinct disease phenotypes in many neurodegenerative diseases ³. Therefore, our understanding of amyloid disaggregation mechanism is highly limited.

To address these key questions, we developed a highly robust *in vitro* reconstitution system with three recombinant chaperones, Hsp104, Ssa1, and Sis1. This system recapitulated the yeast prion propagation *in vitro*, including *de novo* amyloid formation and amyloid disaggregation. Notably, we showed that all the three chaperones are essential for efficient disaggregation of Sup35NM amyloid. Next, in order to dissect disaggregation mechanisms, it is critical but has been challenging to simultaneously monitor and visualize dynamics of distinct chaperones in real time during the process of amyloid disaggregation. Therefore, we established a platform of single-molecule, multi-wavelength total internal reflectance fluorescence (TIRF) imaging analysis, coupled with the reconstitution system, and simultaneously observed dynamics of Hsp104, Ssa1 and Sis1 in real-time during disaggregation of Sup35NM amyloid. Remarkably, we identified and characterized two, distinct amyloid conformation-dependent modes of disaggregation, fragmentation and dissolution, in which we found chaperone dynamics are markedly different. These findings will provide a basis for developing novel therapeutic strategies for neurodegenerative diseases in which amyloid propagation is involved.

Results

Disaggregation of Sup35NM amyloid requires Hsp104, Ssa1 and Sis1 chaperones.

First, we established a method of accelerating formation and disaggregation of Sup35NM amyloid fibrils *in vitro* by optimizing the methods of purification and quality control of three chaperones (Hsp104, Ssa1, and Sis1) and the biochemical assays conditions (Supplementary Fig. 1). Notably, our analytical ultracentrifugation (AUC) analysis showed that Hsp104 exclusively formed a hexamer (Supplementary Fig. 1b) ²⁶ When Sup35NM monomer was incubated with the three chaperones, the intensity of thioflavin T (ThT) fluorescence showed a remarkably rapid increase, indicating acceleration of amyloid formation (Fig. 1a). However, amyloid formation was not observed when Hsp104 was replaced with Hsp104KT, an ATPase-deficient mutant ²⁷, with Ssa1 and Sis1, indicating acceleration of amyloid formation is ATPase-activity dependent. Amyloid formation of Sup35NM in the presence of only Ssa1 and Sis1 was inhibited, relative to no chaperones, consistent with the previous reports ^{28,29}.

Next, we examined how Sup35NM fibrils grow during the acceleration process by atomic-force microscopy (AFM) (Fig. 1b). The number of small fibrils increased during 30 min between the time points 15 min and 45 min. After the ThT fluorescence intensity reached the plateau point, the length of Sup35NM fibrils that formed in the presence of Hsp104/Ssa1/Sis1 was considerably shorter than that of the spontaneously formed fibrils (Fig. 1c). These results suggest that amyloid fragmentation occurred during the acceleration process (Fig. 1c). To gain further insights into the acceleration process, we investigated interactions between Alexa488-labeled Sup35NM monomer and the chaperones by AUC with a fluorescence detector (Supplementary Fig. 1d) ³⁰. Sup35NM was dominantly monomeric as evidenced by the peak at 1.5 S, as reported previously ³¹. A new peak appeared at 16 S by the addition of Hsp104 in the presence of ATPγS, an ATP analog which slowly hydrolyzes ATP (Supplementary Fig. 1d, left), but not ADP (Supplementary Fig. 1d, right). This result indicates ATP state-dependent interaction of Sup35NM monomer with Hsp104.

We next attempted to develop a robust disaggregation assay using Sup35NM amyloid by optimizing various reaction conditions such as the concentrations of chaperones (Supplementary Fig. 1e-g). We used Sc4 amyloid, which has an amyloid core region spanning residue 1–42 and induces strong *[PS⁺]* phenotypes ^{32–34}. Our ThT assay and sedimentation assay showed that Sc4 amyloid was efficiently disaggregated by Hsp104, Ssa1, and Sis1 (Fig. 1d and Supplementary Fig. 1h, i), but neither Hsp104 alone nor Ssa1/Sis1 showed disaggregation, suggesting all the three chaperones are essential for amyloid disaggregation. When Hsp104 was titrated in the ThT assay, Hsp104 showed concentration-dependent disaggregation activity (Supplementary Fig. 1e), which is consistent with the *in vivo* studies showing Hsp104 concentration-dependent propagation of *[PS⁺]* state ¹⁴. Similar to the acceleration assay, disaggregation potential was also abolished by replacing Hsp104 with Hsp104KT, indicating that Hsp104 disaggregates amyloid fibrils in an ATPase-activity dependent manner (Fig. 1d). AFM images corroborated the small size of fibrils by disaggregation in the presence of Hsp104 wild-type (WT)/Ssa1/Sis1, but not Hsp104 KT/Ssa1/Sis1 (Fig. 1e). To investigate whether our chaperone system can also disaggregate *in vivo*-derived Sup35 prions, our recombinant chaperones were incubated with lysates of *[PS⁺(Sc4)]* yeast which was generated by infection of non-prion *[psi⁻]* yeast with Sc4 amyloid ³². Importantly, semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) analysis ³⁵ revealed that Sup35p migrated to the position of monomeric Sup35 in the presence of Hsp104/Ssa1/Sis1 (Fig. 1f). Taken together, these results demonstrate that our chaperone system can efficiently disaggregate both *in vivo*- and *in vitro*-derived Sup35 amyloid.

Ssa1 and Sis1 bind to Sup35NM amyloid in an ATP-activity dependent manner.

To investigate how Hsp104, Ssa1, and Sis1 cooperate to regulate the disaggregation activity for Sc4 amyloid, we used TIRF microscopy and observed the disaggregation process of fluorescently-labeled Sc4 amyloid, which was partially tethered to a glass surface by biotinylated Sup35NM (Fig. 2a). Neither Hsp104 alone nor Hsp104/Ssa1 had effects on amyloid disaggregation, whereas Hsp104, Ssa1, and Sis1 together showed dramatic disaggregation within 300 sec in our conditions (Fig. 2b and Supplementary

Video 1). However, a combination of Hsp104KT, Ssa1, and Sis1 showed no effect, consistent with the biochemical assays. During the chaperone-mediated disaggregation, the number of nick in fibrils substantially increased with incubation time (Fig. 2b), and Sc4 amyloid mostly disappeared within 300 sec. This result revealed that Hsp104/Ssa1/Sis1 efficiently fragments Sc4 amyloid and yields a number of fiber fragments (seeds) during the disaggregation process.

We next examined how Ssa1 and Sis1 impacted the binding of Hsp104 to Sc4 amyloid. To address this question, we performed simultaneous dual-color TIRF imaging analysis to observe fluorescently-labeled SNAP-tagged Hsp104 with unlabeled Ssa1 and Sis1 during disaggregation. Hsp104-SNAP549 showed ATPase and disaggregation activities indistinguishable from untagged Hsp104 (Supplementary Fig. 2a, b). We used a low concentration of Hsp104-SNAP549 (40 nM) for this TIRF experiment to reduce the fluorescence background, which is required for single-molecule observations. Hsp104 showed repeated binding and dissociation on Sc4 amyloid (Fig. 2c and Supplementary Video 2). To evaluate the effects of Ssa1/Sis1 on Hsp104 binding to Sc4 amyloid, we counted the number of Hsp104-SNAP549 binding events to Sc4 amyloid in the presence or absence of Ssa1/Sis1. Strikingly, the binding of Hsp104 to Sc4 amyloid was approximately 50-fold higher in the presence of Ssa1/Sis1, whereas either Ssa1 or Sis1 alone did not induce the binding of Hsp104 to Sc4 amyloid (Fig. 2d).

The binding of all the three chaperones induced fragmentation of Sc4 amyloid, which prevented us from determining a binding affinity of Hsp104 to the amyloid in the presence of Ssa1/Sis1. Therefore, we used a Hsp104trap mutant³⁶, which cannot hydrolyze ATP and is therefore unable to disaggregate Sc4 amyloid (Supplementary Fig. 2c). As expected³⁶, we found that Sup35NM monomer is not released from Hsp104trap but from Hsp104 WT (Supplementary Fig. 2d, e). Remarkably, however, Hsp104trap showed repeated binding and dissociation on Sc4 amyloid (Supplementary Fig. 3a, b), which allowed us to evaluate a binding affinity of Hsp104 to Sc4 amyloid by excluding the effects caused by amyloid disaggregation. The binding affinity of Hsp104trap to Sc4 amyloid was increased by the presence of Ssa1/Sis1, yielding a K_d value of 81 nM compared with 14 μ M in the absence of Ssa1/Sis1. Therefore, Ssa1/Sis1 enhanced the binding of Hsp104 to amyloid by more than 170-fold (Supplementary Fig. 2f). We next investigated whether Ssa1-Alexa488 interacts with Sc4 amyloid in the presence or absence of Sis1. Ssa1-Alexa488 showed disaggregation activity indistinguishable from unlabeled Ssa1 (Supplementary Fig. 4a), and Ssa1-Alexa488 alone showed no interaction with the Sc4 amyloid. In contrast, Ssa1-Alexa488 with Sis1 was first associated with Sc4 amyloid as patches at distinct sites, then the patches gradually spread throughout the fibrils (Fig. 2e). Remarkably, in the presence of ADP or ATPyS, Ssa1-Alexa488 with Sis1 did not bind to Sc4 amyloid (Fig. 2f). Therefore, Ssa1 interacted with Sc4 amyloid through its ATP hydrolysis. Furthermore, we found that Sis1-Cy3 binding to Sc4 amyloid requires Ssa1 (Supplementary Fig. 4c, d). These findings demonstrate that both Ssa1 and Sis1 cooperatively bind to Sc4 amyloid in an ATP-activity dependent manner.

Ssa1 and Sis1 simultaneously bind to Sup35NM amyloid, then recruit Hsp104.

To explore the binding order of Ssa1 and Sis1 to Sc4 amyloid, we performed three-color TIRF imaging analysis to simultaneously observe the binding of Ssa1-Alexa488 with Sis1-Cy3 to STELLA650-labeled Sc4 amyloid in real time (Fig. 3a). The fluorescence intensities of both Ssa1 and Sis1 along Sc4 fibrils were plotted at each time point (Fig. 3b). Sis1 and Ssa1 were accumulated at the same sites at ~ 100 sec, and their fluorescence intensities increased until ~ 800 sec. To investigate the kinetics of Ssa1 and Sis1 binding, the fluorescence intensities of Ssa1 and Sis1 in each peak in Fig. 3b were plotted as a function of time (Fig. 3c). We determined the binding rates of Ssa1 and Sis1 at each accumulation site in Sc4 amyloid (Fig. 3d) and found a high correlation between the two rates (Slope = 0.93, $R^2 = 0.83$), indicating that Ssa1 and Sis1 almost simultaneously bind to Sc4 amyloid (Fig. 3d).

To investigate the relationships between Hsp104 and Ssa1/Sis1 for their binding to Sc4 amyloid, we next performed three-color TIRF imaging analysis in real time, using Hsp104-SNAP549, Ssa1-Alexa488, unlabeled Sis1 and STELLA650-labeled Sc4 amyloid (Fig. 3e). Binding of Hsp104-SNAP549 was detected (at ~ 200 sec) at the sites where Ssa1-Alexa488 had already been bound (at ~ 100 sec). In addition, Sc4 amyloid was fragmented (at ~ 900 sec) at the sites where Hsp104 was recruited (Fig. 3f). These results indicate that Ssa1 and Sis1 prime fragmentation sites and play important roles in recruiting Hsp104 to the specific sites. We found the difference in the arrival time between Hsp104-SNAP549 and Ssa1-Alexa488 was ~ 73 sec (Fig. 3g). This ordered chaperone binding suggests that Ssa1/Sis1 may first remodel the Sc4 amyloid conformation and the altered structure is targeted by Hsp104.

Repeated binding of Hsp104 to the same site is crucial for amyloid fragmentation.

We clearly detected appearance of nicks (*i.e.* fragmentation) in the Sc4 amyloid during the incubation with the three chaperones. However, we also found non-fragmented sites in Sc4 amyloid, even though the binding of Hsp104 and Ssa1/Sis1 to the amyloid was observed (Fig. 3e, right). What factors differentiate fragmentation from non-fragmentation within the same fibril? To determine binding parameters of the chaperones required for efficient amyloid fragmentation, we took an advantage of the single-molecule analysis by TIRF, and compared the intensity profiles of Sc4 amyloid, Ssa1 and Hsp104 between fragmented and non-fragmented sites within the same fibril (Fig. 4a, b). To observe a single molecule of Hsp104, we used 1 μ M of unlabeled Hsp104 including 30 nM of Hsp104-SNAP549. We measured the fluorescence intensities of Sc4 amyloid, Hsp104, and Ssa1 in a 3×3 pixel area to examine the chaperone dynamics at fragmented and non-fragmented sites. At the fragmented sites, the fluorescence intensity of Sc4 amyloid rapidly dropped after Ssa1's and subsequently Hsp104's binding (Fig. 4b, top). In contrast, at non-fragmented sites, the fluorescence intensity of Sc4 amyloid gradually declined (Fig. 4b, bottom). Importantly, this decline was not caused by photobleaching (Fig. 4b, black), and thus we defined this mode of disaggregation as dissolution. We found that the first arrival time of Ssa1 was not different between fragmented and non-fragmented sites, whereas the cumulative intensity of Ssa1 at fragmented sites was higher than that at non-fragmented sites (Fig. 4c, e). Therefore, the accumulation, but not the rapid binding, of Ssa1 is associated with efficient fragmentation of Sc4 amyloid (Fig. 4e). The difference in arrival time between Ssa1 and Hsp104 at fragmented sites was approximately 50 sec, which was significantly shorter than that at non-fragmented sites (Fig. 4d). The cumulative intensities of Ssa1 and

Hsp104 at fragmented sites were significantly higher and lower than those at non-fragmented sites, respectively (Fig. 4e, f). These results indicate that accumulation of Ssa1, but not Hsp104, is favorable to fragmentation of Sc4 amyloid. Consistently, when we counted the number of Hsp104 appearance on Sc4 amyloid in a 3×3 pixel area (*i.e.* the number of repeated binding of Hsp104 to the same site) from 0 to 600 sec, we found the number at fragmented sites was significantly larger than that at non-fragmented sites (Fig. 4g). Together, these results suggest that transient, repeated binding of Hsp104 to the same site of Sc4 amyloid is crucial for efficient amyloid fragmentation.

Two distinct modes of chaperone-mediated disaggregation were observed between Sc4 and Sc37 amyloids.

Amyloid conformation is a critical determinant of prion strain phenotype, which is largely regulated by amyloid disaggregation rate, rather than amyloid growth rate⁸. Therefore, to investigate the possibility of amyloid conformation-dependent disaggregation, we performed three-color TIRF imaging analysis and examined the binding of Hsp104-SNAP549 and Ssa1-Alexa488 to Sc37 amyloid, which has a longer amyloid core region (residues 1–72)^{33,34} and induces weak [*PSI*⁺] phenotypes. In contrast to the previous result of Ssa1/Sis1-independent disaggregation¹⁸, Sc37 amyloid was disaggregated by the three chaperones, but with less efficiently than Sc4 amyloid (Fig. 5a, b and Supplementary Fig. 6a). We found that the cumulative number of nick within Sc37 amyloid fibrils was significantly smaller than that of Sc4 amyloid (Fig. 5c). Importantly, however, the fluorescence intensity of Sc37 amyloid disappeared gradually, indicating uniform disaggregation (*i.e.* dissolution) throughout the fibrils (Fig. 5b), as observed in non-fragmented sites of Sc4 amyloid (Fig. 4b, bottom). Analysis of the intensity profile in a 3×3 pixel area showed that Ssa1 recruits Hsp104 to Sc37 amyloid, in a manner similar to Sc4 amyloid (Fig. 6a, b); the first arrival time of Ssa1 and the time difference of the first arrival time between Ssa1 and Hsp104 was almost indistinguishable between Sc4 and Sc37 amyloid (Fig. 6c, d). Interestingly, however, amounts of both Ssa1 and Hsp104 accumulated on Sc37 amyloid were remarkably larger than those on Sc4 amyloid (Fig. 6e, f). In addition, we observed a smaller number of appearance of Hsp104 on Sc37 amyloid than on Sc4 amyloid (Fig. 6g). This result shows that the number of repeated binding of Hsp104 to the same site was reduced for Sc37 amyloid, similar to what we found at non-fragmented sites in Sc4 amyloid (Fig. 4g). These observations suggest that the enhanced binding of both Ssa1 and Hsp104 to Sc37 amyloid may result in a “locked” conformation of Hsp104 within the amyloid, which may prevent Hsp104’s repeated binding and following extraction of polypeptides from the amyloid, leading to less efficient amyloid fragmentation.

To gain further insight, we investigated a dwell time of Hsp104-SNAP549 at fragmented or non-fragmented sites on each Sc4 and Sc37 amyloid, using Hsp104-SNAP549 (30 nM) and unlabeled Hsp104 (1 μ M) with Ssa1 and Sis1 (Supplementary Fig. 5a-c). The majority of the dwell time of Hsp104 on Sc4 amyloid was 3–30 sec at fragmented sites, whereas a fraction of a long dwell time (30–300 sec) was larger at the non-fragmented sites in Sc4 amyloid. This result implies that the shorter dwell time of Hsp104 at fragmented sites is simply due to the dissociation of Hsp104 from Sc4 amyloid by amyloid fragmentation (Supplementary Fig. 5c). In contrast, the majority of dwell time of Hsp104 on Sc37

amyloid was longer (30–300 sec), suggesting that Hsp104 stayed on Sc37 amyloid due to its lower efficiency in fragmenting the amyloid or its intrinsic high affinity to Sc37 amyloid structures. In order to address it, we used Hsp104trap, as our finding that Hsp104trap lacking a disaggregation activity repeatedly binds to the same site of amyloid allowed us to determine a dwell time by excluding the effects caused by amyloid disaggregation (Supplementary Fig. 3b). As a result, a dwell time of Hsp104trap on Sc37 amyloid was longer than that on Sc4 amyloid (Supplementary Fig. 3c), indicating an intrinsic higher affinity of Hsp104 to Sc37 amyloid conformation. Furthermore, we hypothesized that if Hsp104 repeatedly binds to the same site of Sc4 amyloid due to multiple rounds of extraction of Sup35NM monomers from the amyloid in order to complete a fragmentation process, Hsp104trap will not show such repeated binding to Sc4 amyloid due to a lack of its disaggregation activity. As predicted, we found that the number of Hsp104trap binding to Sc4 amyloid in a 3×3 pixel area in 600 sec was small and approximately similar to that of Hsp104trap on Sc37 amyloid (Supplementary Fig. 3d) and also that of Hsp104 WT on Sc4 non-fragmented sites (Fig. 4g). These results together with the longer dwell time of Hsp104trap on Sc37 amyloid (Supplementary Fig. 3c) indicate that an intrinsic higher affinity of Hsp104 to Sc37 amyloid than that to Sc4 amyloid prevents repeated binding of Hsp104 to the same site of amyloid, resulting in less efficient fragmentation.

Differences in amyloid structure determine chaperone binding and amyloid disaggregation modes.

Previous studies showed that the core of Sc37 amyloid is located around residues 2–72, whereas that of Sc4 amyloid around residues 2–42^{31,33,34}. Therefore, it was surprising that even though the exposed amino acid region is shorter in Sc37 amyloid, a substantially larger amount of both Ssa1 and Hsp104 bound to Sc37 amyloid than Sc4 amyloid (Fig. 6e, f). Since Hsp104 is recruited to Sup35NM amyloid by Ssa1 (Fig. 3f), the enhanced binding of Ssa1 to Sc37 amyloid structure (Fig. 6e) is likely to be a causal factor for the enhanced accumulation and longer dwell time of Hsp104 on Sc37 amyloid (Fig. 6f and Supplementary Fig. 3c, 5c right). To investigate it, we acquired super-resolution microscopy images, using fluorescently labeled Ssa1 in Sc4 or Sc37 amyloid. Sc4 amyloid showed wavy and helical structures, whereas Sc37 amyloid exhibited relatively straight morphology (Fig. 6h), which was consistent with independent observations by electron microscopy (Supplementary Fig. 6b). Remarkably, Ssa1 was accumulated preferentially at the specific sites where the fluorescent intensity of Sc4 amyloid is high. In contrast, Ssa1 uniformly bound to Sc37 amyloid throughout the fibrils, consistent with the enhanced accumulation of Ssa1 on Sc37 amyloid by TIRF observation (Fig. 6e). These results suggest that reduction of Ssa1 concentration may decrease the binding of Sc37 amyloid, altering the disaggregation mode from dissolution to fragmentation. When we used a low concentration of Ssa1 in the disaggregation assay with Sc37 amyloid by TIRF, amounts of Ssa1 and Hsp104 on Sc37 amyloid were significantly reduced (Supplementary Fig. 7f, g), as expected. Importantly, however, neither the frequency of fragmentation (Supplementary Fig. 7a–c) nor the number of repeated binding of Hsp104 increased (Supplementary Fig. 7h). Rather, the disaggregation (dissolution) activity was simply decreased (Supplementary Fig. 7d, e). These results indicate that the tertiary or quaternary structure of amyloid is more critical in determining a disaggregation mode than the numbers of chaperones residing on amyloid.

Together, we surmise that the difference in amyloid structure between Sc4 and Sc37 prion strain conformations is responsible for their marked difference in amyloid-chaperone interactions and underlies the remarkable difference in disaggregation mode between fragmentation and dissolution.

Discussion

We developed a robust *in vitro* reconstitution system, in which both of the *de novo* amyloid formation and disaggregation were recapitulated *in vitro*. Our data demonstrated that all the three chaperones, Hsp104, Ssa1, and Sis1 are essential for efficient disaggregation of Sup35NM amyloid. More importantly, the reconstituted yeast prion system allowed us to dissect the amyloid disaggregation process. Under our TIRF conditions, Sis1 and Ssa1 simultaneously bound to Sup35NM amyloid. After the ~ 73 sec interval, Hsp104 was recruited to the specific sites in Sc4 amyloid where Ssa1/Sis1 had already bound. This result, together with the previous report that Hsp70 can unfold protein aggregates^{37,38}, suggests that Ssa1/Sis1 first remodel amyloid structures and then the loosened polypeptide region may be easily captured and extracted by Hsp104. Once a Sup35NM monomer is extracted from amyloid, newly created breaking regions in the Sc4 amyloid will be exposed and thereby easily targeted again by Hsp104. Such multiple rounds of monomer extraction may account for the observed transient, repeated binding of Hsp104 to the same site of amyloid, followed by fragmentation of Sc4 amyloid (Fig. 7). In addition to the fragmentation, our TIRF imaging analysis revealed another mode of amyloid disaggregation – dissolution. Interestingly, the fragmentation was favorable to Sc4 amyloid while the dissolution was a predominant disaggregation process of Sc37 amyloid. Therefore, our work revealed prion strain-conformation dependent, distinct modes of amyloid disaggregation.

Our results resolved the long-standing, puzzling question whether Hsp104 alone is sufficient or additional factors are required for amyloid disaggregation^{9,10,15,17–20}. Our findings support the previous *in vitro* study¹⁹ and a number of *in vivo* studies that described the necessity of additional factors^{21–24}. Furthermore, it has been a decade-long mystery that Hsp104 is not easily identified as a Sup35 prion-associated protein *in vivo* despite its major role in disaggregation. Our yeast prion reconstitution system revealed that Hsp104 binds transiently to the Sc4 amyloid, whereas Ssa1 associates with the Sc4 amyloid in a more stable capacity (Fig. 7), thus explaining the difficulty of detecting Hsp104, but not Ssa1, in prior *in vivo* studies³⁹. Importantly, TIRF imaging analysis allowed us to directly compare physical parameters of chaperones dynamics between fragmented and non-fragmented sites within the same amyloid, and also between Sc4 amyloid and Sc37 amyloid. The binding of Ssa1/Sis1 to specific sites of Sc4 amyloid and following repeated binding of Hsp104 induces fragmentation (Fig. 1d, 2b), whereas more stable interactions of Hsp104 in non-fragmented sites of Sc4 or in Sc37 amyloid results in dissolution (Fig. 4b bottom, 5a and b). The intrinsic higher affinity of Hsp104 to Sc37 amyloid (Supplementary Fig. 3c) may induce non-productive binding of Hsp104 to the amyloid, impairing its multiple rounds of extraction of polypeptides from Sc37 amyloid that is required for fragmentation. The detailed mechanism that determines such distinct disaggregation modes and kinetics requires further investigation. Nonetheless, our data show that variation of amyloid conformations constitutes the

physical foundation to dictate the differences in chaperone binding, disaggregation mode, and prion strain phenotype.

This study provided unexpected, novel findings which were not easily understood in the previous studies, but might be specific to the disaggregation process of amyloid in general or that of Sup35NM amyloid. First, we found that Sis1 and Ssa1 almost simultaneously bound to Sup35NM amyloid, though the previous studies indicate Hsp40 first binds to a substrate, then recruits Hsp70^{40,41}. Second, an ADP-bound Hsp70 is known to be a substrate-binding form^{40,41}, but neither ADP- nor ATPγS-bound Ssa1 with Sis1 interacted with Sup35NM amyloid. Rather, only ATP-bound Ssa1 together with Sis1 was able to bind to Sup35NM amyloid, indicating that the ATPase activity (*i.e.* ATP hydrolysis) of Ssa1 is essential for binding of Ssa1 to the amyloid.

Notably, the prion conformation-dependent, distinct modes of disaggregation showed in this study provides a molecular explanation for the previous findings where the underlying mechanisms had remained elusive. First, the preference of fragmentation (to dissolution) of Sc4 amyloid leads to an increased number of Sc4 fiber fragments. Therefore, the preferred fragmentation process would be responsible for the observed larger number and shorter size of propagons in strong [*PSI*⁺(Sc4)] strains than that of weak [*PSI*⁺(Sc37)] strains^{8,32}. Second, previous studies indicated that Hsp104 is more immobile in weak [*PSI*⁺] strains than that in strong [*PSI*⁺] strains, suggesting that more Hsp104 proteins interact with weak [*PSI*⁺] prions⁴². Consistently, the immunoprecipitation assay revealed that more Hsp104 and Ssa1 proteins bind to “weak” [*PSI*⁺] prions than “strong” [*PSI*⁺] prions⁴³. These results might had been paradoxical if one assumes that interactions of more Hsp104 and Ssa1 proteins with Sup35NM amyloid are advantageous to disaggregation. However, our results indicate that enhanced binding of the chaperones with weak [*PSI*⁺] prions (Fig. 6e, f and Supplementary Fig. 5c) underlie the preference for dissolution, rather than fragmentation, which yields less propagons and elicits weak [*PSI*⁺] strain phenotypes.

Given the potential application of Hsp104-mediated disaggregation for neurodegenerative diseases², the mechanistic insights in this study provide broad implications beyond the yeast system. We suggest that selective enhancement of the dissolution process, but not fragmentation, provide a means for therapeutic intervention of pathological protein aggregates or amyloid, because enhanced fragmentation events, thereby increased fiber fragments (seeds), may accelerate amyloid formation and exacerbate disease pathophysiology. Therefore, the distinct modes of amyloid disaggregation that we uncovered in this study will provide a novel direction for the development of therapeutic intervention. Furthermore, the robust disaggregation assay for amyloid fibrils in this study will help diagnose or classify disease (strain) phenotypes at the molecular level by evaluating susceptibility of *in vivo* amyloid to the disaggregation machinery.

Methods

Plasmid construction and protein purification

For expression and purification of tagged Sup35NM proteins in *Escherichia coli*, we followed the previous protocols³¹. Briefly, Sup35NM with a 7xHis or 7xHis-Cys tag were overexpressed in *E. coli* Rosetta (DE3) cells (Nonagen) in LB media and purified by Ni-NTA affinity chromatography (GE healthcare) and anion-exchange chromatography under the denatured condition^{31,34,44}. The buffer exchange to acetonitrile/water was performed using HPLC (Hitachi). The sample was lyophilized and stored at -20°C until use.

cDNAs of Hsp104, Sis1 and their mutants were cloned into pET28a vector including N-terminally His-MBP-tag⁴⁵. A plasmid of Hsp104-SNAP was generated by addition of a SNAP-tag to the C-terminus in the expression vector. cDNA of Sis1 with a C-terminal cysteine was introduced into the pET28a plasmid. Hsp104 and Sis1 were overexpressed in *E. coli* Rosetta (DE3) in LB media and purified by Ni-NTA affinity chromatography (GE Healthcare), as previously described with modifications⁴⁶. Briefly, the eluent from Ni-NTA resin was purified by amylose-resin affinity chromatography (BioLabs). After digestion of a His-MBP-tag by the TEV enzyme, Hsp104 and Sis1 was purified by anion-exchange chromatography using HiTrap Q 6 ml column (GE Healthcare) with the gradient system. To remove any remaining His-tagged proteins, the eluent was subjected to the 2nd Ni-NTA gravity flow purification and the flow-through was obtained. Only pure fractions (>95%) were pooled, and concentration and buffer exchange were performed. Expression and purification of Ssa1 with a C-terminal 7xHis tag was followed by the previous report^{47,48}. Briefly, Ssa1 was purified by Ni-NTA affinity (GE Healthcare) and HiTrap Q (GE Healthcare) columns with the gradient system. Only pure fractions (>95%) were pooled, and concentration and buffer exchange were performed. Purity of all the proteins were rigorously checked throughout the purification procedures by Coomassie Brilliant Blue staining and the absorbance at 280 nm. Quality control of Hsp104 hexamer was performed by AUC as described later. All of the proteins were aliquoted into a small volume, flash frozen by liquid nitrogen and stored at -80°C. Typically, we used up the proteins within 1-2 months to prevent a decrease of their activities.

As a plasmid for expressing TEV, pRK793 was purchased from Addgene (RRID:Addgene_8827)⁴⁹. A TEV protease with a 7xHis tag was overexpressed in *E. coli* Rosetta (DE3) in LB media and was purified by Ni-NTA, as previously reported⁴⁵. The pure fractions of the eluent from Ni-NTA affinity chromatography was used for digestion of the tagged proteins. A plasmid for expressing ClpP was kindly provided by Dr. Axel Mogk (Universität Heidelberg). ClpP was overexpressed as a C-terminally 6xHis tagged protein in *E. coli* Rosetta (DE3) in LB media and purified by Ni-NTA affinity chromatography (GE Healthcare), as previously described⁵⁰.

Fluorescence and chemical labeling of proteins

For fluorescence labeling of Sup35NM, Sup35NM with a 7xHis-tag-Cys tag was mixed with a 10-fold excess of STELLA650-maleimide (GORYO Chemical) or Alexa-488-maleimide (ThermoFisher Scientific). An excess amount of fluorescence dye was removed by passing the protein through a Bio-Gel P-6 Gel

(Biorad #154130). Labeling efficiencies were spectrophotometrically determined (Hitachi U-3900), using the absorbances at 646 nm (extinction coefficient: $110,000 \text{ M}^{-1} \text{ cm}^{-1}$) for STELLA-650-maleimide, at 493 nm (extinction coefficient: $72,000 \text{ M}^{-1} \text{ cm}^{-1}$) for Alexa-488-maleimide, combined with the absorbance at 280 nm (extinction coefficient: $29,800 \text{ M}^{-1} \text{ cm}^{-1}$) for Sup35NM protein. For biotin labeling of Sup35NM, Sup35NM including an Avi-tag was overexpressed in *E. coli* Rosetta (DE3) that contains a pBirA plasmid in LB media. After optical density of the culture reaches ~ 0.6 , d-biotin (Nacalai, the final concentration of 50 mM) and IPTG (the final concentration of 0.6 mM) were added for biotin labeling. Biotinylated Sup35NM was purified as described above, and the incorporation of biotin and elimination of non-biotinylated Sup35NM were confirmed by anti-Biotin antibody (Jackson Immuno Research Laboratories, Inc.).

For fluorescence labeling of a SNAP tag, Hsp104 with a SNAP-tag was mixed with a 2-fold excess of SNAP-Surface549 (Biolabs) together with 1 mM DTT for 1 hour in dark. An excess dye was removed by passing of the protein through a P6 column as above. Labeling efficiencies were spectrophotometrically determined using the absorbance at 558 nm (extinction coefficient: $140,300 \text{ M}^{-1} \text{ cm}^{-1}$), combined with the absorbance at 280 nm (extinction coefficient: $49,070 \text{ M}^{-1} \text{ cm}^{-1}$) for Hsp104-SNAP protein. Ssa1 was labeled on the cysteine residue by a 5-fold excess of Alexa-488-maleimide (ThermoFisher Scientific) with 1 mM TCEP. The labeling efficiency of Ssa1 was determined by the spectrophotometer using the absorbance at 493 nm (extinction coefficient: $72,000 \text{ M}^{-1} \text{ cm}^{-1}$) for Alexa488, combined with the absorbance at 280 nm (extinction coefficient: $18,490 \text{ M}^{-1} \text{ cm}^{-1}$) for Ssa1. Sis1 was labeled on the cysteine residue with a 5-fold excess of Cy3-maleimide (GE healthcare) with 1 mM TCEP for 1 hour in dark. The labeling efficiency of Sis1 was spectrophotometrically determined using the absorbance at 550 nm (extinction coefficient: $150,000 \text{ M}^{-1} \text{ cm}^{-1}$) for Cy3, combined with the absorbance at 280 nm (extinction coefficient: $22,330 \text{ M}^{-1} \text{ cm}^{-1}$) for Sis1. The efficiency of fluorescent labeling of chaperones were typically in the range of 90–98%.

Analytical Ultracentrifugation (AUC)

The sedimentation velocity experiments of purified Hsp104, Ssa1 and Sis1 was performed using a proteome Lab XL-A analytical ultracentrifuge system with an AN60Ti rotor (Beckman Counter). All chaperone concentrations were approximately 30 mM in AUC Buffer (20 mM Tris (pH 7.5), 75 mM NaCl, 75 mM KCl, 20 mM MgCl_2 , 2 mM TCEP). All protein concentrations refer to the concentration of monomeric protein. Ultracentrifugation analysis was performed as follows; Hsp104 (Velocity: 40,000 rpm, Number of scans: 280, Scan interval: 2 min, Temperature: 20°C), Ssa1 or Sis1 (Velocity: 40,000 rpm, Number of scans: 140, Scan interval: 6 min, Temperature: 20°C). Acquired data were analyzed with the SEDFIT software (NIH) using the c(S) distribution method⁵¹. and the parameters of the density 1.0018 g cm^{-3} and viscosity 1.023 cP were determined using the SEDNTERP software (<http://www.jphilo.mailway.com/download.htm>).

ATPase activity assay

An ATPase activity of 0.25 mM Hsp104 and 1 mM Ssa1 was measured using a commercially available kit (Innova) in the ATPase buffer (40 mM Hepes-KOH pH 7.4, 150 mM KCl, 20 mM MgCl₂, 2 mM DTT) including 1 mM ATP. The assay was followed by manufacturer's protocol and the previous report ⁵².

Disaggregation assay

The Sc4 or Sc37 amyloid was freshly prepared by mixing 5μM Sup35NM with 5% (mol/mol) of corresponding Sc4 or Sc37 seeds, respectively, as previously reported ^{16,32}. The disaggregation assay of Sc4 or Sc37 amyloid was typically performed in the presence of 1 mM Hsp104, 2 μM Ssa1, 2 μM Sis1 with ATP or an ATP regeneration system (10 mM creatine phosphate and 0.1 mg/ml creatine kinase) in the disaggregation buffer (25 mM Hepes-KOH (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate, 2 mM DTT) at 30°C. Hsp104KT (K218T, K620T), an ATPase-deficient mutant of Hsp104, was used as a negative control. The extent of amyloid disaggregation was measured by thioflavin T fluorescence, sedimentation assay, atomic force microscopy (AFM). The thioflavin T (ThT) assay was followed by the previous report ³¹. ThT fluorescence was measured by SpectraMax M2 (Molecular devices) without shaking and data were collected by the SoftMaxPro software (Molecular devices). For the spin down assay, soluble fraction of Sup35NM (S) were separated by ultracentrifugation (200,000 *g*, 30 min at 4°C) from the total fraction. The supernatant (S) and total fraction (T) were subjected to the western blotting with a polyclonal anti-Sup35NM antibody ³¹. Corresponding horseradish peroxidase-conjugated secondary antibodies were used (GE Healthcare), and images were visualized by ImageQuant LAS 4000 mini (GE Healthcare). For morphological imaging of amyloid fibrils by AFM, the samples were applied to mica (Agar Scientific) for 30 s and observed by MultiMode8 Scan Asyst for NanoScope V (Bruker). The AFM images were analyzed by Nanoscope Analysis software (Bruker).

For disaggregation of cell lysates containing *in vivo* Sc4 prions, [*PSI*⁺] Sc4 (74D694, *PIN*⁺, *MAT a*) was cultured until the OD reached ~0.8. Yeast cells were collected by centrifugation (1000 *g*, 5 min) and lysed in SDD-AGE lysis buffer (30 mM Tris (pH 7.5), 150 mM NaCl, 1% TritonX, 1 mM DTT, 2 mM PMSF, inhibitor cocktail) ⁵³. Cell debris were removed by brief centrifugation (1000 *g*, 5 min, 4°C). The cell lysate (5 mg/ml) was incubated with 2 μM Hsp104, 4 μM Ssa1, 4 μM Sis1 with ATP and an ATP regeneration system in the disaggregation buffer at 30°C for 2 hours. Sup35 prions and monomers in the lysate were separated by Semi-Denaturing Detergent Agarose Gel Electrophoresis (SDD-AGE), as previously described ^{35,54}, and Sup35 was detected by a rabbit polyclonal anti-Sup35NM antibody ³¹.

Acceleration assay of amyloid formation

For acceleration of amyloid formation, 5 μM Sup35NM monomer was incubated with 5 μM Hsp104, 5 μM Ssa1 and 5 μM Sis1 with ATP and an ATP regeneration system in the fiber acceleration buffer (20 mM Tris-Cl pH 7.4, 75 mM KCl, 75 mM NaCl, 20 mM MgCl₂, 2 mM DTT) at 15°C. The extent of amyloid formation was measured by ThT fluorescence, as described above. After the formation of Sup35NM amyloid, aggregate structures were observed by AFM as described above.

Fluorescence-detected sedimentation velocity AUC

To detect only Sup35NM in the mixture with chaperones by AUC, Sup35NM with a C-terminal cysteine was labeled by Alexa488-maleimide. 20 mM Sup35NM including 20 nM Alexa488-labeled Sup35NM was incubated at 20°C with 20 mM Hsp104, 20 mM Ssa1 and 20 mM Sis in the presence of 5 mM ATPgS or ADP. AUC was performed as follows; velocity: 42000 rpm, number of scans: 600, scan interval: 90 sec, temperature: 20°C³⁰. The acquired data were analyzed using the SEDFIT software, and parameters of the density of 1.0068 g cm⁻³ and the viscosity of 1.023 cP were determined using the Sednterp software.

Microscale thermophoresis (MST)

5 mM Sc4 amyloid was sonicated (20%, 14 sec) and diluted to 200 nM by the MST buffer (25 mM Hepes-KOH pH 7.5, 150 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 0.05% tween20). The diluted amyloid solution was mixed with 20 nM (final) fluorescence dye using a Monolith His-Tag labeling Kit RED-tris-NTA 2nd Generation (Nano Temper Technologies) and incubated at room temperature for 30 min. Before the measurements, the efficiency of the His-tag labeling was confirmed by a pre-test. For the binding affinity assay, a serial dilution of Hsp104 trap, an ATPase-deficient mutant of Hsp104, in the MST buffer including 5 mM ATP and the regeneration buffer was prepared in 16 tubes and the same amount of labeled Sc4 amyloid from the above solution (100 nM protein, 10 nM dye) was added to each vial. For evaluation of cooperative binding of Hsp104 with Ssa1 and Sis1 to Sc4 amyloid, 2 mM Ssa1 and Sis1 were included in the labeled Sc4 amyloid solution, and were mixed with a serial dilution of Hsp104. Binding affinity assays were performed using Monolith NT.115 Pico Microscale Thermophoresis (Nano Temper Technologies) and data were collected by the MO. Control software (Nano Temper Technologies). The MST power was set to high and the excitation power used was 20% in each analysis. To determine *K_d*, the curve fitting of normalized intensity was performed using the MO. Affinity Analysis software (Nano Temper Technologies).

Fluorescence anisotropy

0.5 mM Sup35NM-Alexa488 monomer was incubated with 5 mM Hsp104 WT or Hsp104 trap at 30°C for 5 min in disaggregation buffer. After 5 min incubation, 0.5 mM ATPgS was added to the mixture, following addition of 20 mM ADP. Fluorescence anisotropy was measured using a black 96-well plate (ThermoFisher Scientific) on a SpectraMax iD5 (Molecular Devices, San Jose, CA, USA) and data were collected by the SoftMaxPro software (Molecular devices). The measurement volume was 120 µl. Excitation/emission wavelengths for anisotropy measurements of Sup35NM-Alexa488 was 490/530 nm. Three independent experiments were performed (*n* = 3).

Total intensity reflection fluorescence (TIRF) microscopy

All coverslips (18 mm×18 mm and 24 mm×45 mm) were cleaned by sonication in 1M KOH for 30 min, in H₂O by 3 times for 5 min each, then in methanol for 10 min as described⁵⁵. The coverslips were coated by amino silane using the amino silane buffer (2% of N-(2-Aminoethyl)-3-aminopropyltrimethoxysilane

(Tokyo Chemical Industry), 135 mM Acetic acid (Wako Pure Chemical cooperation), 4% H₂O in methanol (Wako Pure Chemical Industries, Ltd.)) at room temperature for 3 hours as described ⁵⁵. The coverslips were layered with 0.1 M NaHCO₃ (Wako Pure Chemical Industries, Ltd.) including 200 mg ml⁻¹ mPEG-succinimidyl valerate (Laysan Bio) and 2 mg /ml of Biotin-PEG-succinimidyl valeric acid ester (Laysan Bio) and incubated at room temperature for 3 hours ⁵⁵. After the PEG coating, all coverslips were washed by H₂O and dried by N₂ blow and stored in methanol. All PEG-coated coverslips were used up within 1 weeks.

Before TIRF experiments, PEG-coated coverslips were rinsed by H₂O and flow-cells (18 mm×18 mm and 24 mm×45 mm) were assembled by attaching them, using double-sided tape. The flow cell was incubated with 0.2 mg/ml neutravidin protein (ThermoFisher Scientific) for 10 min and then incubated with Sc4 amyloid (5 mM final, 10% STELLA650 labeled, 1% biotinylated) or Sc37 amyloid (5 mM final, 10% STELLA650 labeled, 1% biotinylated) for 10 min and equilibrated by the TIRF reaction buffer (25 mM Hepes-KOH pH7.5, 150 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 50 unit ml⁻¹ glucose-oxidase, 50 unit ml⁻¹ catalase, 4.5 mg ml⁻¹ glucose 5 mM ATP, 0.1 mg/ml BSA, 10 mM Creatine Phosphate and 0.05 mg ml⁻¹ Creatine kinase). Chaperone mixtures in the TIRF reaction buffer were incubated at 30°C for 5 min before flowing into the flow-cell.

TIRF imaging analysis was performed using an inverted microscope, eclipse Ti-E with TIRF module (Nikon Corp., Tokyo, Japan), equipped with Continuous Wave (CW) Solid-State Laser, Sapphire (Coherent Inc., CA, USA), a TIRF-objective lens, Apo TIRF 100x NA1.49 (Nikon Corp., Tokyo, Japan) and an EMCCD camera, Ixon3 (Andor Technology Ltd, Belfast UK). Solid-State lasers were used for excitation at wavelength of 488 nm (Sapphire, 20 mW, Coherent Inc.), 561 nm (Sapphire, 20 mW, Coherent Inc.), and 640 nm (CUBE, 40 mW, Coherent Inc.). STELLA650 fluorescence dye was excited by 640 nm. Alexa488 fluorescence dye was excited by 488 nm. Cy3 fluorescence dye was excited by 561 nm. During the measurements, optical focus was maintained by the perfect focus system (Nikon Corp., Tokyo, Japan). Images were acquired every 500 msec for Fig. 2b, 2c, 4a, 5b, 6a and Supplementary Fig. 3, 5, and 7 and every 10 sec for Fig. 2e, 3a, 3e and Supplementary Fig. 4c.

TIRF data analysis

Acquired image sequences were converted to 16-bit TIFF files using NIS Elements (Nikon Instruments Inc.) and all images was analyzed by ImageJ (NIH, Bethesda, MD). All images were automatically aligned using micropattern images by a plugin, Template Matching and Slice Alignment ⁵⁶. Background fluorescence was subtracted using the background subtraction tool (rolling ball radius: 50 pixel) in ImageJ. We calculated the fragmentation frequency of Sc4 and Sc37 amyloids in Fig. 5c and Supplementary Fig. 7c by manually counting fragmentation events. The number of fragmentation events was divided by the initial length of amyloid.

The number of Hsp104 binding on Sc4 amyloid in Fig. 2d was counted per 600 sec and the number was subtracted by the number of control experiments without Sc4 amyloid. For binding of Ssa1-Alexa488 or

Sis1-Cy3, Sc4 amyloid-STELLA650 was traced over time, and the traces were used to determine the corresponding fluorescence profiles in other channels. Mean fluorescence intensities of Ssa1 and Sis1 were normalized to the length of Sc4 amyloid (Fig. 2f and Supplementary Fig. 4d). The same method of the trace analysis was used for our preparation of spatiotemporal profiles by the Plot Profile tool. The binding rates of Ssa1 and Sis1 to Sc4 amyloid in Fig. 3d were determined by fitting the fluorescence data with a single exponential function with x offset constant using IgorPro (Wavemetrics, OR, USA).

For binding of Hsp104-SNAP549 and Ssa1-Alexa488, Sc4 amyloid-STELLA650 was traced over time using the plot profile tool. Then, the trace was used to determine the corresponding fluorescence profiles in other channels. The time of the first appearance of Hsp104-SNAP549 ($T_{\text{Hsp104 arrival}}$) and Ssa1-Alexa488 ($T_{\text{Ssa1 arrival}}$) in Fig. 3g was determined by analysis of the time when fluorescence reached 10% of the maximum intensity in a 3x3 pixel area on amyloid. Distribution of the interval times of Hsp104 and Ssa1 was calculated as $T_{\text{Hsp104 arrival}} - T_{\text{Ssa1 arrival}}$.

To determine fragmented and non-fragmented sites on amyloid, the spots in a 3x3 pixel area where amyloid was finally fragmented or not fragmented were traced, and fluorescence intensities were measured. The same pixel regions were also used to determine the corresponding fluorescence profiles in other channels. To subtract background signals, fluorescence intensities at the spots in a 3x3 pixel area that did not contain amyloid were traced. Fragmented sites were defined as the sites showing a more than 70% decrease of the initial fluorescence intensity at 600 sec, while that of non-fragmented (dissolution) sites showed over 30% of the initial fluorescence intensity at 600 sec. The first appearance time of Ssa1 was determined when fluorescence intensity reached 10% of the maximum in a 3x3 pixel area (Fig. 4c, 6c). The time of the first appearance of Hsp104-SNAP549 ($T_{\text{Hsp104 arrival}}$) and Ssa1-Alexa488 ($T_{\text{Ssa1 arrival}}$) was determined when fluorescence intensity reached 10% of the maximum intensity in a 3x3 pixel area on amyloid (Fig. 4d, 6d). Cumulative intensity of Hsp104 and Ssa1 fluorescence was integrated from 0 to 600 sec (Fig. 4e, 4f, 6e, 6f, Supplementary Fig. 7f, g). The number of Hsp104 appearance on amyloid in a 3x3 pixel area, which represents the number of Hsp104 binding to the same site, was counted from 0 to 600 sec (Fig. 4g, 6g and Supplementary Fig. 3d, 7h). Dwell time of Hsp104 was determined as the period of time when fluorescence intensity was over 100 a.u..

Negative staining with electron microscopy

Sc4 or Sc37 (5 mM Sup35NM monomer) amyloid was prepared as described above. Sc4 or Sc37 amyloid was centrifuged (10,000 *g*, 10 min, 4°C) to eliminate small aggregates or monomers. We removed 90% of the supernatant and resuspend the pellet fraction with the same volume of disaggregation buffer. A 3 ml volume of the samples was applied to the carbon-coated holey microgrid (EM Japan). Subsequently, the applied sample was negatively stained with 2% (w/v) uranyl acetate three times. Images were recorded on a Falcon II direct electron detector using a Tecnai TF20 transmission electron microscope (Thermo Fisher Scientific) at the acceleration voltage of 200kV and at the magnification of 62,000.

Confocal microscopy

All coverslips and labeled proteins (Sc4 or Sc37 amyloid-STELLA650 and Ssa1-Alexa488) were prepared as described by TIRF analysis. First, fluorescence images of Sc4 or Sc37 amyloid-STELLA650 immobilized on cover glass was acquired. Next, 2 mM Ssa1-Alexa488 and 0.5 mM Sis1 was flowed into the flow-cell and then fluorescence images at the identical place were acquired. A Leica TCS SP8 confocal microscope equipped with 638 nm diode and 488 nm OPSL laser (Leica Microsystems, Wetzlar, Germany) was used to collect z-stacks of the amyloid images, and these images were processed by deconvolution using the Leica Application Suite X (LAS X) software (Leica Microsystems, Wetzlar, Germany). Each image was compiled by Image J software

Quantification and statistical analysis

Statistical significance was tested using two-tailed, unpaired, Student's t-test in the GraphPad Prism version 8 (GraphPad Software, La Jolla, CA) and Excel (Microsoft).

Declarations

Competing Interests statement:

The authors declare no competing interests.

Author Contributions:

Y. N. and M.T conceived and designed the whole study. Y. N. prepared all the proteins and performed all the experiments except for fluorescence-detected sedimentation velocity AUC and negative staining electron microscopy experiments. Y.T., T.Y, and M.S performed negative staining experiments with Y. N. E.K. and S.U performed the experiments of fluorescence-detected sedimentation velocity AUC experiments and analyzed the data. C. H. S and S.S. prepared a part of the plasmids and proteins. K.O, M.I, T.N, Y.S, and H.T provided suggestions for TIRF imaging experiments and data analysis. Y. N. and M.T. wrote the manuscript and all authors provided comments. M.T and H.T supervised the project.

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Figures

Fig. 1

Figure 1

Disaggregation of Sup35NM amyloid requires Hsp104, Ssa1, and Sis1 chaperones. **a**, 5 μ M Sup35NM monomer was incubated with various combinations of 5 μ M Hsp104 or Hsp104 K218T/K620T (Hsp104KT), 5 μ M Ssa1, and 5 μ M Sis1 for 2,880 min at 15°C. The kinetics of acceleration of amyloid formation was monitored by thioflavin T fluorescence. Data were averaged from three independent experiments ($n = 3$). Error bars indicate SD. Boxes B and C show the time of sample collection for AFM

imaging. b-c, Morphology of Sup35NM amyloid in acceleration of amyloid formation was observed by AFM. (b) Morphology of Sup35NM protein during the acceleration process of amyloid formation (0-45 min). (c) Morphology of Sup35NM amyloid fibrils formed spontaneously or by Hsp104/Ssa1/Sis1 or Hsp104KT/Ssa1/Sis1 after plateau (~ 48 hours). d, Kinetics of disaggregation of 0.5 μ M Sc4 amyloid with various combinations of 1 μ M Hsp104 or Hsp104 K218T/K620T, 2 μ M Ssa1, and 2 μ M Sis1 was monitored at 30°C by thioflavin T fluorescence. Data were normalized by the initial fluorescence intensities. Data were averaged from three independent experiments (n = 3). Error bars indicate SD. e, Representative AFM images of 0.5 μ M Sc4 amyloid incubated with 1 μ M Hsp104 or Hsp104 KT, 2 μ M Ssa1, 2 μ M Sis1 for ~48 hours incubation at 30°C. f, Lysates of strong [PSI⁺] and [psi⁻] yeast were incubated with 2 μ M Hsp104, 4 μ M Ssa1, and 4 μ M Sis1 for 120 min at 30°C. Disaggregation of in vivo Sup35 prions was investigated by SDD-AGE.

Fig. 2

Figure 2

Real-time TIRF imaging revealed an ATPase activity-dependent binding of Ssa1 to Sc4 amyloid. **a**, Schematic representation of the single molecule imaging of Sup35NM amyloid disaggregation. STELLA650 labeled Sup35NM amyloid (Sc4 or Sc37 amyloid) was immobilized on flow-cells with PEG coating and neutravidin treatment. Fluorescently labeled chaperones were introduced into the flow-cell, and fluorescence signal intensities were detected when chaperone(s) interacted with Sup35NM amyloid.

b, Representative TIRF images from Supplementary Video 1 showing disaggregation of Sc4 amyloid-STELLA650 at various time points. Sc4 amyloid-STELLA650 was observed after 1 μ M Hsp104, 2 μ M Ssa1 and 2 μ M Sis1 were mixed and flowed. Scale bar, 10 μ m. Arrow heads indicate fragmented sites in Sc4 amyloid. c, Representative time-lapse TIRF images from Supplementary Video 2 showing interactions between Sc4 amyloid-STELLA650 and Hsp104-SNAP549. Sc4 amyloid-STELLA650 (red) was observed after various combinations of 40 nM Hsp104-SNAP549 (green), 2 μ M Ssa1 and 2 μ M Sis1 are mixed and flowed. Scale bar, 3 μ m. Images were acquired every 500 msec. d, Number of binding events of Hsp104-SNAP549 toward Sc4 amyloid (per μ m) during 600 sec. All data were averaged from three independent fibers (n = 3). Error bars indicate SD. e, Representative time-lapse TIRF images from Supplementary Video 3 showing interactions between Sc4 amyloid-STELLA650 and Ssa1-Alexa488. Sc4 amyloid-STELLA650 (red) was observed after various combinations of 2 μ M Ssa1-Alexa488 (cyan) and 0.5 μ M Sis1 with a distinct status of ATP were mixed and flowed. Images were acquired every 10 sec. Scale bar, 3 μ m. f, Mean intensity of Ssa1-Alexa488 fluorescence on Sc4 amyloid (per μ m) with or without Sis1 with a distinct state of ATP. All data were averaged from three independent fibers (n = 3). Error bars indicate SD.

Fig. 3

Figure 3

Ssa1 and Sis1 simultaneously bind to Sc4 amyloid, then recruit Hsp104 after a delay time. a, Representative time-lapse TIRF images from Supplementary Video 4 showing interactions between Sc4 amyloid-STELLA650, Alexa488-labeled Ssa1 and Cy3-labeled Sis1. Sc4 amyloid-STELLA650 was observed after 2 μ M Alexa488-labeled Ssa1 (blue) and 0.5 μ M Cy3-labeled Sis1 (green) were mixed and flowed. Images were acquired every 10 sec. Scale bar, 10 μ m. b, Representative spatiotemporal profiles of

Ssa1-Alexa488 (blue) and Sis1-Cy3 (green) on Sc4 amyloid-STELLA650 (red). Each fluorescence intensity of Ssa1 (blue) and Sis1 (green) was plotted along Sc4 amyloid. A dashed box indicates a representative binding site of Ssa1 and Sis1. c, Representative intensity profiles of Ssa1-Alexa488 (blue) and Sis1-Cy3 (green) fluorescence was shown at the peak in the dash box in Fig. 3b. d, Binding rates of Ssa1 and Sis1 toward Sc4 amyloid in Fig. 3b ($n = 27$) were calculated by fitting the data in (Fig. 3c) with a single exponential function between 0 and 1,000 sec. k_{Ssa1} and k_{Sis1} represent the rate constants of Ssa1 and Sis1 binding. e, Representative time-lapse TIRF images from Supplementary Video 5 showing interactions between Sc4 amyloid-STELLA650 and Ssa1-Alexa488, after 50 nM Hsp104-SNAP549 (green), 2 μ M Ssa1-Alexa488 (blue) and 0.5 μ M Sis1 were mixed and flowed. Images were acquired every 10 sec. Scale bar, 5 μ m. In the magnified image (bottom), white and yellow arrow heads show the sites where Sc4 amyloid was fragmented and non-fragmented, respectively. f, Representative spatiotemporal fluorescence profile of Hsp104-SNAP549 (green) and Ssa1-Alexa488 (blue) on Sc4 amyloid-STELLA650 (red). Two dashed boxes indicate representative binding sites of Hsp104 and Ssa1. White arrow heads indicate the fragmented sites of Sc4 amyloid. g, Distribution of the interval time of the first appearance between Hsp104-SNAP549 and Ssa1-Alexa488 on Sc4 amyloid ($n = 157$).

Fig. 4

Figure 4

Repeated binding of Hsp104 to the same site is more frequent in fragmented sites than non-fragmented sites of Sc4 amyloid. a, Representative time-lapse TIRF images showing interactions between Sc4 amyloid-STELLA650, Hsp104-SNAP549 and Ssa1-Alexa488, after 1 μM Hsp104 including 30 nM Hsp104-SNAP549 (green), 2 μM Ssa1-Alexa488 (blue) and 0.5 μM Sis1 were mixed and flowed. Images were acquired every 500 msec. White and yellow arrow heads show the sites where Sc4 amyloid was

fragmented and non-fragmented (dissolution), respectively. White and yellow boxes indicate the fragmented and non-fragmented sites shown in Fig. 4b. Scale bar, 3 μm . b, Representative normalized intensity profiles of Sc4 amyloid-STELLA650 (red), Hsp104-SNAP549 (green) and Ssa1-Alexa488 fluorescence (blue) in a 3x3 pixel area at the site where Sc4 amyloid was fragmented (white box) or not fragmented (yellow box) in the merged image in Fig. 4a. Notably, due to the mixture of fluorescently labeled and unlabeled Hsp104 proteins, a decrease of the fluorescence signal intensity of Sc4 amyloid was observed even though Hsp104 did not apparently bind (Fig. 4b, bottom). c-d, (c) First arrival time of Ssa1 (TSsa1 arrival) and (d) interval time of the first appearance between Hsp104 and Ssa1 (THsp104 arrival – TSsa1 arrival) for fragmented and non-fragmented sites in Sc4 amyloid. Mean $\pm$ SD values are shown in the dot plot, and significance was determined by the two-tailed Student's t-test. e-f, Normalized cumulative fluorescence intensity of (e) Ssa1 and (f) Hsp104 during 600 sec in the fragmented and non-fragmented sites of Sc4 amyloid. The cumulative intensities of Hsp104 and Ssa1 were normalized by averaged intensities of Hsp104 and Ssa1 in fragmented sites, respectively. Mean $\pm$ SD values are shown in the dot plot, and significance was determined by the two-tailed Student's t-test. g, Number of Hsp104 appearance on the fragmented and non-fragmented sites of Sc4 amyloid during 600 sec. ** $P < 0.01$ **** $P < 0.0001$. Mean $\pm$ SD values are shown in the dot plot, and significance was determined by the two-tailed Student's t-test.

Fig. 5

Figure 5

Frequency of amyloid fragmentation is lower in Sc37 amyloid than Sc4 amyloid. a, Kinetics of disaggregation of 0.5 μM Sc37 amyloid (solid line) or Sc4 amyloid (dashed line) with various combinations of 1 μM Hsp104, 2 μM Ssa1, 2 μM Sis1 was monitored at 30°C by thioflavin T fluorescence. Data were normalized by the initial fluorescence intensities. Data were averaged from three independent experiments ($n = 3$). Error bars indicate SD. b, Representative time-lapse TIRF images of

Sc37 amyloid-STELLA650 after 1 μ M Hsp104, 2 μ M Ssa1 and 2 μ M Sis1 were mixed and flowed. Images were acquired every 500 msec. Scale bar, 10 μ m. c, Analysis of fragmentation rate of Sc4 (black) and Sc37 (red) amyloid. Each data point shows the cumulative number of fragmentation events (per μ m amyloid) at various time points. Data were averaged from three independent fibers (n = 5). Error bars indicate SD. d, Kymographs of Sc4 (top) or Sc37 (bottom) amyloid-STELLA650 from Fig. 2b for Sc4 amyloid and Fig. 5b for Sc37 amyloid from 0 to 900 sec. Scale bar, 3 μ m.

Fig. 6

Figure 6

Chaperone binding is more enhanced and repeated binding of Hsp104 is less frequent in disaggregation of Sc37 amyloid. a, Representative time-lapse TIRF images showing interactions between Sc37 amyloid-STELLA650, Hsp104-SNAP549 and Ssa1-Alexa488, after 1 μ M Hsp104 including 30 nM Hsp104-SNAP549, 2 μ M Ssa1-Alexa488 and 0.5 μ M Sis1 were mixed and flowed. Images were acquired every 500 msec. Scale bar, 3 μ m. b, Representative normalized intensity profiles of Sc37 amyloid-STELLA650 (red), Hsp104-SNAP549 (green) and Ssa1-Alexa488 (blue) fluorescence in a 3x3 pixel area in Fig. 6a. c-d, (c) First arrival time of Ssa1 (TSsa1 arrival) and (d) Interval time of the first appearance between Hsp104 and Ssa1 (THsp104 arrival – TSsa1 arrival) at the fragmented and non-fragmented sites. Mean $\pm$ SD values are shown in the dot plot, and significance was determined by the two-tailed Student's t-test. e-f, Normalized cumulative fluorescence intensity of (e) Ssa1 and (f) Hsp104 during 600 sec on Sc4 or Sc37 amyloid. The cumulative intensities of Hsp104 and Ssa1 were normalized by averaged intensities of Hsp104 and Ssa1 in Sc4 amyloid, respectively. Mean $\pm$ SD values are shown in the dot plot, and significance was determined by the two-tailed Student's t-test. g, Number of Hsp104 appearance on Sc4 and Sc37 amyloids during 600 sec. ** $P < 0.01$ **** $P < 0.0001$ Mean $\pm$ SD values are shown in the dot plot, and significance was determined by the two-tailed Student's t-test. h, Z-stack images of Sc4 and Sc37 amyloid-STELLA650 by super-resolution microscopy before (left) and after (right) Ssa1-Alexa488 and Sis1 were introduced into the flow-cell.

Fig. 7

Figure 7

A model of two distinct, strain conformation-dependent modes of amyloid disaggregation. In Sc4 amyloid, Ssa1 and Sis1 bind to the amyloid, then recruit Hsp104. Following repeated binding of Hsp104 to the same site of amyloid results in “fragmentation” of Sc4 amyloid. In Sc37 amyloid, enhanced accumulation of both Ssa1 and Sis1 recruits more Hsp104 proteins and induces “dissolution”.

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