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Breakdown of supersaturation barrier links protein folding to amyloid formation

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The thermodynamic hypothesis of protein folding, known as the “Anfinsen’s dogma” states that the native structure of a protein represents a free energy minimum determined by the amino acid sequence. However, inconsistent with the Anfinsen’s dogma, globular proteins can misfold to form amyloid fibrils, which are ordered aggregates associated with diseases such as Alzheimer’s and Parkinson’s diseases. Here, we present a general concept for the link between folding and misfolding. We tested the accessibility of the amyloid state for various proteins upon heating and agitation. Many of them showed Anfinsen-like reversible unfolding upon heating, but formed amyloid fibrils upon agitation at high temperatures. We show that folding and amyloid formation are separated by the supersaturation barrier of a protein. Its breakdown is required to shift the protein to the amyloid pathway. Thus, the breakdown of supersaturation links the Anfinsen’s intramolecular folding universe and the intermolecular misfolding universe.
Many globular proteins can form amyloid fibrils, misfolded ordered aggregates associated with serious amyloidosis 

3–5. Current concepts argue that folding and misfolding are alternative reactions of unfolded proteins 

6–8. However, which principles govern the relationship between folding and misfolding is unknown. Now that the atomic structures for various amyloid fibrils 

3–5,9,10 show that they are indeed ordered structures achieved by hydrophobic interactions, hydrogen bonds, and van der Waals interactions, it is important to establish a unifying mechanism explaining both folding/unfolding and amyloid polymerization/depolymerization.

Denatured proteins exist in vivo often at concentrations which are supersaturated concerning solubility 

11–14. Supersaturation is a fundamental phenomenon of nature, determining the phase transition of substances. It is required for formation of crystals and involved in the super-cooling of water prior to ice formation. The same will be true for crystal-like amyloid formation. Here, an additional trigger (e.g., ultrasonic agitation) can disrupt this metastable state leading to amyloid formation 

14–16.

To test which factors determine the accessibility of the native, unfolded, and amyloid states for a protein, we focused on the breakdown of supersaturation as a critical factor for a pathway to amyloid fibrils. We heated proteins with or without stirrer agitation and monitored amyloid formation via the amyloid specific thioflavin T (ThT) fluorescence and the total amount of aggregates via light scattering (LS) (Supplementary Fig. 1). The proteins included not only typical amyloidogenic proteins, but also several textbook proteins used previously in folding/unfolding studies 

Table 1. Many of them showed Anfinns-like reversible unfolding/refolding upon heating, but formed amyloid fibrils with or without agitation at high temperatures. This behavior is explained by the persistence of supersaturation, which depends on the flexibility of denatured states.

Table 1. Target proteins/peptides used in this study.

| Class | Name | General properties | Experimental conditions | Transition type |
|-------|------|--------------------|------------------------|----------------|
| Short peptides | K3 | 22, 2.5 | 463, 0.0 | A |
| Immunoglobulin folds | IgG | 140, 8.6 | 2940, 0.2 | B |
| α IDPs | pOVA | 23, 2.0 | 481, 0.5 | A |
| β IDPs | TDP-43 | 414, 5.8 | 8694, 0.2 | A |
| β amyloidogenic proteins | β2m | 99, 12 | 2079, 0.1 | A |
| lysozyme | HEWL | 129, 14 | 2709, 0.1 | A |
| Glucagon | 29, 3.5 | 609, 0.1 | A |
| C α,β IDPs | RNaseA | 124, 14 | 2604, 0.0 | B |
| Protein names | | | | |
| General properties | | Me (GDa) | pI | Ave. hydrophobicity |
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V L (PAT-1) exhibited an increase in ThT fluorescence at high temperatures. This behavior is explained by the persistence of supersaturation, which depends on the flexibility of denatured states.

Small globular proteins. We also examined various small globular proteins with tightly folded native structure. Hen egg white lysozyme (HEWL) is one of the most extensively studied proteins in terms of protein unfolding/folding and it is well known that HEWL forms amyloid fibrils at high temperatures under acidic conditions 

22,23. Our methodology revealed that stirrer agitation

suppression of folding and unfolding transitions. We also observed that CH-clamping induced stabilization and unfolding transition on lysozyme, which supported the previous reports 

59, 60.

Results

Proteins with an immunoglobulin fold. We first examined proteins with an immunoglobulin fold-like β2m-microglobulin (β2m), a blood protein responsible for dialysis-related amyloidosis: the variable (V L) 

3,18 and constant (C L) 

19 domains of the immunoglobulin light chain. An excess amount of specific variants of monoclonal light chains or V L fragments secreted into the blood stream forms amyloids causing AL amyloidosis, whereas the C L domain is assumed to modulate V L’s amyloidogenicity 

3,18,20. At pH 7.0, upon heating under stirring, V L (PAT-1) exhibited an increase in ThT fluorescence and the total amount of aggregates via light scattering (LS) (Supplementary Fig. 1). The proteins included not only typical amyloidogenic proteins, but also several textbook proteins used previously in folding/unfolding studies 

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was required for amyloid formation of HEWL at pH 2 (Fig. 1 and Supplementary Fig. 2). Based on differential scanning calorimetry (DSC) measurements, heating-induced amyloid fibrils of HEWL had seeding activity, as previously observed for β2m16 (Supplementary Fig. 4a, b). Seeding activity is a defining amyloid property, which reflects the crystal-like mechanism of amyloid formation11,12.

Transthyretin (TTR) is a tetrameric transporter protein in the plasma, which has been studied intensively because of its association with fatal amyloidoses such as senile systemic amyloidosis and familial amyloid polyneuropathy3,9. In addition to a large number of pathogenic familial mutants, wild-type TTR can form amyloid fibrils in patients24. However, spontaneous amyloid formation of wild-type TTR in vitro is difficult unless fragmentation is induced by proteolysis25. We found that wild-type full length TTR exhibited amyloid formation upon incubation under stirring at pH 2.0 and 50 °C, whereas TTR retained a native-like conformation without stirring (Fig. 2a–c). This reaction occurs in a narrow range of NaCl concentrations, from 50–150 mM (Fig. 2d). It has been reported that TTR readily forms ThT-negative and seeding-incompetent filamentous aggregates26 with a curvilinear morphology24. In contrast, the amyloid fibrils we prepared at pH 2.0 and at 50 °C had seeding activity under the same solvent conditions (Fig. 2e).

We also investigated the behavior of ribonuclease A (RNaseA) and α-lactalbumin (αLa), two model proteins used in many folding studies17,27. The hinge loop-expanded mutant of RNaseA was reported to generate amyloid-like fibrils via 3D domain swapping, whereas the wild-type RNaseA did not28,29. Most interestingly, we successfully induced amyloid formation of wild-type RNaseA with 1.0 M NaCl at pH 5.0, for which stirring was essential (Fig. 1 and Supplementary Fig. 2). Seeding activity was also confirmed by DSC measurements (Supplementary Fig. 4c). For αLa, it has been reported that amyloid fibrils can be formed at low pH or upon reduction of disulfide bonds30. In our experiments, intact αLa produced amyloid fibrils upon heating under agitation, consistent with previous reports, although stirring was required in our case (Supplementary Fig. 5).

Ubiquitin is a small protein which tags other proteins for degradation31. It shows reversible unfolding17,32. Recently, amyloid-like fibril formation of heat-treated poly-ubiquitin chains was reported33. We examined whether mono-ubiquitin can form amyloid fibrils by heating under agitation. An increase in ThT fluorescence was noted upon incubation at 90 °C and pH 2, for which stirring was essential (Supplementary Fig. 6). Of note, the ThT intensity was weak despite strong LS even after cooling to 25 °C. The CD spectra reflected a β-rich conformation and fibrous aggregates were observed by TEM.

Highly amyloidogenic short peptides. We then examined amyloidogenic short peptides without ordered structures. Amyloid β peptides 1–40 (Aβ40) or 1–42 (Aβ42) are two of the most well-known amyloidogenic peptides3,10. Amyloid formations of Aβ40 and Aβ42 have been suggested to play key roles in Alzheimer’s disease, in which senile plaques in the brain contain a large amount of Aβ40 and Aβ42 fibrils. In our experiments, Aβ40 formed amyloid fibrils at pH 7.0 under agitation monitored by ThT fluorescence, CD, and TEM (Fig. 3 and Supplementary Fig. 7). In contrast, without agitation, no amyloid formation was observed by ThT fluorescence or CD. However, even without agitation, the heat treatment and subsequent overnight incubation caused amyloid formation according to TEM (Supplementary Fig. 7).

We obtained similar results for the 29-residue peptide hormone glucagon34. Although no increase in ThT fluorescence was observed without agitation during the heating experiments, fibrous aggregates were observed by TEM upon overnight incubation after the heating experiments (Fig. 3 and Supplementary Fig. 7).

Islet amyloid polypeptide (IAPP), also known as amylin, is a 37-residue peptide hormone and the amyloid deposition of IAPP in pancreatic β-cells may cause type II diabetes3. Upon heating,
**Fig. 2 Amyloid formation of wild-type TTR.**

**a** ThT assays of TTR upon incubation in the presence (upper) or absence (lower) of stirring. $n = 3$. **b** TEM images of the samples after the ThT assay. Scale bar; 500 nm. **c** CD spectra of the samples before and after the ThT assays. **d** Dependence of TTR amyloid formation on NaCl concentration. Lag times and ThT maximal intensities are indicated by black and blue lines, respectively. $n = 5$. **e** ThT assays with (solid lines) or without (dashed lines) 5% seeds under the same conditions as (**a**). $n = 3$.

**Fig. 3 Effects of heating and agitation on type A proteins.**

**a** ThT assays of Aβ$_{40}$, glucagon, IAPP, and insulin upon heating in the presence (upper) or absence (lower) of stirring. $n = 3$. **b** CD spectra of each sample before and after the ThT assays.
IAPP exhibited amyloid formation both in the presence and absence of stirring at pH 7, which was confirmed by ThT fluorescence, CD, and TEM (Fig. 3 and Supplementary Fig. 7), suggesting that the supersaturation barrier is not high.

Insulin, one of the peptide hormones secreted by the pancreatic islets in addition to IAPP, is composed of a 21-residue A chain and a 30-residue B chain, which are linked by two disulfide bonds. Insulin forms amyloid fibrils upon heating and the degree of agitation was reported to affect the fibril morphology. Under agitation, ThT fluorescence increased at ~80 °C (Fig. 3a and Supplementary Fig. 7). Without stirring, no increase in fluorescence was observed upon heating. Upon cooling, LS increased markedly, suggesting the formation of amorphous aggregates (Fig. 3a and Supplementary Fig. 7). It seems that insulin entered an alternative pathway leading to amorphous aggregation upon heating and cooling under quiescence.

Relatively large IDPs. Lastly, we examined the heating-induced amyloid formation of several intrinsically disordered proteins (IDPs). IDPs are excluded from An structure, even if the ensemble of non-native structures is still determined by sequence. One of the most amyloidogenic IDPs is α-synuclein (αSyn). Amyloid formation of αSyn is highly associated with three types of synucleinopathies: Parkinson’s disease, dementia with Lewy bodies, and multiple system atrophy. According to the ThT assays under stirring at pH 7, which was confirmed by ThT fluorescence, CD, and TEM (Fig. 3 and Supplementary Fig. 7), suggesting that the supersaturation barrier is not high.

Discussion

Our study on the formation of amyloid and amorphous aggregates states of proteins revealed principles that determine the shift in pathways. According to their aggregation behavior, three types of proteins can be defined (Table 1 and Supplementary Table 1).

Type S proteins. The first type of proteins shows a strict dependence on agitation for amyloid formation at high temperatures. We call this transition the “strictly supersaturation-dependent transition” or “S transition”. Proteins exhibiting S transitions include those with a native conformation (β2m, V1, C1q, HEWL, and RNaseA) and αSyn. TTR can also be included in Type S. Although the linkage of unfolding and amyloid polymerization destabilizes the native state, this is not sufficient for amyloid formation as it is strictly dependent on agitation or seeding.

Type A proteins. The second type of proteins exhibits spontaneous amyloid formation at high temperatures even without agitation. We refer to this type of transition as “autonomous amyloid-forming transition” or “A transition”. Type A proteins include insulin, glucagon, IAPP, and Aβ1-42. In other words, the high amyloidogenicity of these relatively short amyloid peptides do not exhibit intrinsic barriers preventing amyloid formation.

Type B proteins. The third type of proteins often produces amorphous aggregates at high temperatures without a lag phase. We call this transition the “boiled egg-like transition” or “B transition”. Type B proteins (i.e., TDP-43, tau, and OVA) are relatively large and it is possible that their overall amorphous characteristics include amyloid cores (β-spines) producing a “fuzzy coat” morphology.

We found that three types (S, A, and B) of transitions with distinct responses to heating can be located on a general aggregation phase diagram dependent on the driving forces of precipitation and protein solubility (Fig. 5a). The S, A, and B transitions are indicated by green, orange, and purple arrows, respectively. This type of diagram is often used to illustrate the crystallization and amorphous precipitation of native proteins and, moreover, for solutes in general. The driving force of precipitation is influenced by various parameters such as salt concentration or temperature. Hydrophobic interactions and conformational entropies, which respectively favor and disfavor protein interactions, both increase with temperature. Therefore, amyloid formation and protein folding exhibit cold- and heat-denaturation phenomena. We previously studied with β2m at pH 7.0 the relationship between the temperature dependence of solubility and amyloid formation, showing both of lowest solubility and maximal amyloid formation occurred at ~70 °C. In this study, only heating-dependent promotion of
amyloid formation was focused on, where hydrophobic interactions increase with an increase in temperature.

The general phase diagram dependent on temperature consists of a soluble region (Region I), metastable region (Region II), labile region (Region III), and glassy region (Region IV)\(^{15,16,37,39}\). Below solubility (Region I), monomers are thermodynamically stable. In the metastable region (Region II), supersaturation is assumed to persist in the absence of seeding or agitation. In the labile region (Region III), spontaneous nucleation occurs after a certain lag time. Finally, the glassy region (Region IV) is dominated by amorphous aggregation occurring without a lag time possibly by concomitant formation of many nuclei.

Thus, the S, A, and B transitions represent those from below solubility (Region I) to metastable region (Region II), labile region (Region III), and glassy region (Region IV), respectively. These indicate that, with an increase in driving force of precipitation at high temperatures, the aggregation behavior followed exactly as expected for solutes in general\(^{39}\).

In terms of the phase diagram of conformational states (Fig. 5a), stirring or ultrasonication is a kinetic factor modifying the apparent phase diagram. It is likely that the boundary between the metastable and labile regions is shifted downward upon agitation, decreasing the barrier of supersaturation and inducing spontaneous amyloid formation\(^{37}\). At a molecular level, we proposed a theoretical model of ultrasonication-induced amyloid formation\(^{42}\), where denatured monomers are captured on the bubble surface during its growth and highly condensed by subsequent bubble collapse, so that they are transiently exposed to high temperatures. Thus, the dual effects of local condensation and local heating contribute to dramatically enhance the

**Fig. 4 Boiled egg-like aggregation of type B proteins.** a ThT assays of tau and TDP-43 upon heating in the presence (upper) or absence (lower) of stirring. \(n = 3\). b CD spectra of each sample before and after the ThT assays.

**Fig. 5 General schematic conformational phase diagram and the three transitions.** Three types of amyloidogenic proteins were plotted on a general phase diagram of aggregation (a) and diagrams of average hydrophobicity vs. number of amino acid residues (b) or \(\Delta S_{\text{conf}}\) (c). In a, an increase in the salt concentration or temperature often increases driving force of precipitation (abscissa), thus decreasing protein solubility (ordinate). In c, \(\Delta S_{\text{conf}}\) represents an increase upon denaturation of the main-chain with (points with frame) and without (points without frame) the contribution of disulfide bonds (Table 1).
nucleation reaction. We further suggested that the local condensation of the denatured proteins at the water–air interface forms seed-competent conformation43. Consistent with this, marked suppression of amyloid nucleation of Aβ was shown under agitation without water-air interface43.

To address the mechanism underlying the distinct amyloidogenic transitions, we examined the relationship between transition types (i.e., S, A and B types) and various factors, which might determine the types. Although determining the solubility of denatured proteins experimentally at high temperatures will be important, it is impractical at this stage. Thus, we examined factors related to solubility although they are relative values estimated at ambient temperatures. These include average hydrophobicity score44, CamSol score44, protein solubility score45, AGGRESCAN score46, and Tango AGG score46 as well as net charge (Supplementary Table 1). For the proteins and peptides examined, the scores of various factors were plotted against the number of amino acid residues (Fig. 5b, Supplementary Fig. 12). The plots included our results using short amyloidogenic peptides obtained from full-length proteins, i.e., K347 and OVA peptide (pOVA)21.

It is evident that the total residue number (abscissa) is the most dominant factor determining the different types. Then, hydrophobic score or AGGRESCAN score (Supplementary Fig. 12) (ordinate) showed notable correlation with distinct amyloid types. On the other hand, CamSol score, protein solubility score, Tango AGG score, and net charge did not distinguish the types. When viewed from the size and hydrophobicity (Fig. 5b), the S proteins had a moderate size and moderate hydrophobicity, the A proteins had a short length and high hydrophobicity, and the B proteins had a long length and low hydrophobicity. Once short peptides with high hydrophobicity were prepared from S or B proteins, they became the A type (e.g., K347 and pOVA)21.

Although intrinsic properties such as the total residue number and hydrophobicity are important factors to determine the types, it is also obvious that the transition type depends on the solvent conditions. When the driving forces of precipitation are increased for a particular protein, the transition type changes from S to A and B, as for example, the amyloidogenic transition exhibited by acid-denatured β2m15,16. Here, changes from S to A and B was observed upon increasing the salt concentration. Although TTR exhibited the S-type transition in this study, it also showed a B-type transition with a curvilinear morphology24,26 possibly because the driving forces were excessive. The driving forces also increase with an increase in protein concentration; therefore, the type of transition will change from S to A and B at higher protein concentrations. This change of transition type occurred for β2m at pH 7.0, where the S-type transition at 0.1 mg/ml changed to the B-type transition at 10 mg/ml (Supplementary Fig. 10). This corresponds to a move along the x-axis in the phase diagram (Fig. 5a) and therefore automatically leads to a phase border crossing.

Another important factor is the disulfide bond47,48. The reduction of disulfide bonds often reduces the amyloidogenicity, as demonstrated for β2m: the S-type transition under acidic conditions changed to B-type. These roles of disulfide bonds suggested that a more appropriate scale for evaluating the different types of amyloidogenic proteins is “conformational flexibility of the denatured state”.

Although the estimation of conformational entropy in denatured state as well as that of disulfide bonds is not straightforward, we tentatively employed the methodology often used for the analysis of conformational stability of globular proteins17,49,50. Assuming that the main chain contributes 21 KJ−1 per mole of residues49, we estimated the intrinsic conformational entropies of the denatured state “without” disulfide bonds (ΔS_conf), which were plotted against various possible factors (Table 1, Fig. 5c, Supplementary Table 1, Supplementary Fig. 13). Again, hydrophobicity and AGGRESCAN scores were factors highly correlating with transition types, suggesting the importance of hydrophobicity-related interactions.

Although the effects of disulfide bonds in reducing the conformational entropy have been addressed17,50, they are in fact small in comparison with intrinsic ΔS_conf (Table 1, Fig. 5c, Supplementary Fig. 13). More importantly, the disulfide bonds stabilize hydrophobic cores that persist in the denatured state and thus increase amyloidogenicity, as demonstrated for acid-denatured β2m17,48. Taken together, synergic effects of disulfide bonds (i.e., decreasing the intrinsic conformational entropy and stabilizing the hydrophobic cores, the latter not included in Fig. 5c) lead to the large decrease in flexibility of the denatured states.

Finally, one of the most important phenomena related to amyloid formation is liquid–liquid phase separation observed increasingly in disordered proteins51,52. There are cases that the amyloid formation is preceded by the liquid–liquid phase separation. For an example, low-complexity domain of FUS protein formed the phase-separated droplets before formation of more stable amyloid fibrils53. The results are consistent with the Ostwald’s ripening rule of crystallization, in which morphologies of crystals change with time guided by their kinetic accessibilities and thermodynamic stabilities14,23,54. We assume that the “macroscopic” phase diagram of conformational states as Fig. 5a will be also useful for understanding the liquid–liquid phase separation, where “microscopic” phase diagram might apply to each droplet system.

For β2m, a type S protein, a three-state mechanism has been proposed16 where both folding and misfolding purely depend on the Gibbs free energy change (Supplementary Fig. 11 and Supplementary Movie 1). Thus, we can apply this in general to S type proteins. In this concept, before the breakdown of supersaturation, a “protein concentration-independent” two-state mechanism applies. Upon the breakdown of supersaturation, a three-state mechanism between the native, unfolded, and “protein concentration-dependent” amyloid states determines the overall equilibrium. The transition from the two-state mechanism to the three-state mechanism shifts the overall equilibrium to the direction of amyloid fibrils, apparently destabilizing the native state by the law of mass action16.

In conclusion, our results provide another example that the effects of extreme conditions of temperature or pressure on folding/misfolding are important for clarifying the phenomena under physiological conditions7,51,55,56. Combined effects of thermodynamics (i.e., reversible unfolding) and kinetics (i.e., breaking supersaturation) have profound implications in deviations from Anfinsen’s dogma and possible onset of amyloidoses. The seemingly disparate perturbations (i.e., reversible unfolding, agitation and breakdown of supersaturation) act cumulatively each other, which seems common to various proteins. The linkage of folding/misfolding and the law of mass action enable amyloid formation even under physiological conditions where only a low amount of unfolded precursor exists. Although the validity of Anfinsen’s dogma is often questioned under high protein concentrations where intermolecular interactions are favored8, the persistence of supersaturation and the difficulty of amyloid formation under quiescent conditions have excluded to address the question experimentally. Our considerations indicates that specifically the breakdown of the kinetic barriers of supersaturation links the Anfinsen’s intramolecular folding universe with the “outer” intermolecular misfolding universe.
overnight water at 4 °C and then lyophilized. Purity was confirmed by Novo Nordisk A/S (Gentofte, Denmark) was kindly given and used.

Methods

Proteins and chemicals

αSyn Recombinant human αSyn gene was amplified from cDNA of human brain (Cap.seq cdna DT: Nippon gene) by PCR, subcloned into the Ndel and Xhol multicloning site of the expression vector pET23a+(+) (Novagen), and expressed in Escherichia coli BL21(DE3) (Novagen)22. Cells were suspended in purification buffer (50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 0.1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride), disrupted using sonication, and centrifuged (14,000 rpm, 30 min). Streptomycin sulfate (final 2.5%) was added to the supernatant to remove nucleic acids. After removal of nucleic acids by centrifugation, the supernatant was heated to 90 °C for 15 min and then centrifuged. In this step, αSyn remained in the supernatant. The supernatant was precipitated by the addition of solid ammonium sulfate to 70% saturation, centrifuged, and dialyzed overnight and then applied onto a Resource-Q column (Amersham Biosciences) with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride as running buffer. Samples were eluted with a linear gradient of 0–1 M NaCl.

Protein concentration of αSyn was determined by using a molar absorption coefficient reported.

V34 Plasmid encoding human V34 (PAT-1)(1–112) was generated by QuikChange mutagenesis PCR (Agilent, Santa Clara, CA, USA) according to the manufacturer’s protocol. The plasmid was transformed into E. coli BL21 (DE3)-star cells. Protein expression was induced at 37 °C in 1 ml induced with 1 mM IPTG at an OD600 of 0.6–0.8. Cells were harvested after overnight protein expression, and inclusion bodies were recovered. The pellet was solubilized and unfolded in 25 mM Tris-HCl (pH 8.0), 5.0 mM ethylenediaminetetraacetic acid (EDTA), 8 M urea and 2 M mercaptoethanol at room temperature for at least 2 h. The solubilized protein was injected on a Sepharose column equilibrated in 25 mM Tris-HCl (pH 8.0), 5.0 mM EDTA and 5 M urea. The proteins were all eluted in the flow-through fraction and subsequently refolded by dialysis against 250 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5.0 mM EDTA, 1.0 mM oxidized glutathione and 0.5 mM reduced glutathione at 4 °C overnight. To remove aggregates and remaining impurities, the proteins were purified using a Superdex 75 16/60 column (GE Healthcare, Uppsala, Sweden) equilibrated in PBS buffer. The recovery and purity of intact protein was verified by SDS-PAGE.

C14 Isoelectric focusing protein type C14 (1–106) domain was cloned into an E. coli expression vector, pAE14 and expressed in BL21 (DE3) pLys3 cells (Agilent, Santa Clara, CA). The recombinant protein was confirmed in inclusion bodies. Inclusion body from 1 ml culture was resuspended in 3 ml of 100 mM Tris-HCl (pH 8.5) containing 10 mM DTT and solubilized by the addition of 0.6 g of solid guanidine hydrochloride (Gdn-HCl). The crude C14 was purified over a Superdex 75 column (GE Healthcare, Uwakake, WI) pre-equilibrated with 6 M urea in 10 mM Tris-HCl (pH 8.5) and then reduced with 20 mM DTT, followed by dialysis against 10 mM Tris-HCl (pH 8.5) with 6 M urea at 4 °C for air oxidation for >48 h. The oxidized sample was dialyzed against 10 mM Na-acetate (pH 4.7) and purified using a Resource S cation-exchange column (GE Healthcare) equilibrated with 10 mM Na-acetate (pH 4.7). TTR C14 was determined using a molar extinction coefficient of 10,950 M−1 cm−1 at 280 nm, which was determined on the basis of amino acid composition.

TTR Human wild-type TTR was expressed using a pET3a vector containing the full-length cDNA for human TTR in E. coli BL21(DE3)44. Expression colonies were grown overnight on Luria-Bertani broth containing 100 µg ml−1 ampicillin at 37 °C; protein synthesis was induced with 0.8 mM isopropyl-β-D-thiogalactoside at 16 °C overnight. The following day cells were harvested by centrifugation at 2100 g for 30 min, the pellet was suspended in buffer containing 25 mM Tris-HCl, 2 mM EDTA, 0.1% Triton, pH 7.4 and sonicated for ten cycles (1 min on/1 min off). The intracellular proteins were fractionated by two cycles of ammonium sulfate precipitation. TTR, which precipitated between 30 and 60% ammonium sulfate, was dialyzed in 50 mM Tris-HCl, pH 7.5. The supernatant was heated to 65 °C for 30 min. After removal of pellet by centrifugation, the supernatant was applied onto a Resource-Q column (GE Healthcare Life Science) equilibrated and eluted with a linear gradient of 0.1–0.35 M NaCl. TTR-enriched fractions were dialyzed overnight water at 4 °C and then lyophilized. Purity was confirmed by SDS-PAGE and electrospray ionization mass spectrometry.

Glucagon. Pharmaceutical grade human glucagon expressed in E. coli and purified (> 98.9%) by Nova Nordisk A/S (Gentofte, Denmark) was kindly given and used.

TDP-43. The purified human brain TDP-43 was kindlygifted from Prof. Yun-Ru Chen (Academia Sinica, Taipei, Taiwan). The TDP-43 was expressed in E. coli BL21(DE3) (Novagen). The cells were harvested and lysed by BugBuster (Merck) including benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM mg/mL lysozyme. TDP-43 accumulated in inclusion bodies. After removal of contaminant by centrifugation, the pellet was dissolved in 50 mM Tris-HCl (pH 8.0) containing 8.0 M urea, 1 mM DTT. The sample was subjected to a HiTrap FF (GE Healthcare) equilibrated with 8.0 M urea, 50 mM Tris-HCl (pH 8.0), and then dialyzed at 4 °C. The sample was eluted with 50 mM Tris-HCl buffer at 8 °C and then dialyzed at 25 °C Tris-HCl (pH 8.0), 4.0 M urea, 1 mM DTT and then subjected to a Resource-Q column (Amersham Biosciences) equilibrated with 25 mM Tris-HCl (pH 8.0), 4.0 M urea, 1 mM DTT. The proteins were eluted with a linear gradient of NaCl (0–1.0 M). The fraction containing 0.1 mM trypsin and SDS was dialyzed against 5 mM Tris-HCl (pH 9.0). Purified His-tagged TDP-43 protein was run on SDS-PAGE and identified by Coomassie blue staining. The recombinant TDP-43 contained extra N-terminal residues MGSSHHHHHHHSSGLVPR GSHMLE. Protein concentration was quantified after background subtraction by absorption at 280 nm with the extinction coefficient of 44,380 M−1 cm−1 according to the equation described by Pace et al.

Tau40. A PET23a-hTau40 gene was constructed by ligation of a synthesized hTau40 gene optimized for E. coli expression (Thermo Fisher, MA, USA) with a DNA fragment obtained from the expression vector PET-23a(+). Human Tau40 was expressed in E. coli BL21(DE3) (Nippon gene Co., Ltd, Tokyo, Japan). Chemically synthesized human Aβ40 and human IAPP were purchased from Peptide Institute, Inc. (Osaka, Japan). All other reagents, including HEWL, were obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

ThT assays with heating. All proteins were dissolved in deionized water, except C14, Aβ40, TDP-43, and glucagon. For the exceptions, buffer was used to make a complete dissolution: C14, 0.05% (w/v) ammonia solution; TDP-43, 10 mM Tris-HCl buffer (pH 8.0) containing 0.4 M urea; glucagon, 20 mM HCl. Then, the pH was adjusted to those indicated in Table 1 with a small volume of 20 mM sodium phosphate buffer (pH 7.0) or 20 mM HCl. Protein concentrations were measured spectrophotometrically using individual molecular extinction coefficients.

The assays of heating-dependent protein aggregation distinguishing amyloid fibrils and amorphous aggregates were carried out using a Hitachi F4500 fluorescence spectrophotometer (Tokyo, Japan) in the same manner as previously reported in the equipment setup is shown in Supplementary Data Fig. 1.

CD and TEM measurements. Far-UV CD spectra (approximately 200–250 nm) were obtained using a model J-720 spectropolarimeter (Jasco Co., Ltd, Tokyo, Japan) at 25 °C using a quartz cell with a 1-mm path length. CD data were expressed as the mean residue ellipticity. To calculate the mean residue ellipticity, the total protein concentration of the sample solution was used. This obviously makes it difficult to convert to molar ellipticity when a large portion of the protein forms aggregates, as observed for glucagon and insulin with stirring (Fig. 3B). TEM images were obtained on a transmission electron microscope (H-7650, Hitachi High-Technologies Corporation) using 5 µL of 0.5% (w/v) hafnium chloride each as a staining agent.

Seeding reactions. Seeds were obtained from the preformed fibrils of heat-induced spontaneous amyloid formation under stirring and were moderately sonicated before seeding experiments. In the case of ThT assays, the conditions for sample preparation and measurements were the same as for standard experiments, except for the addition of 5% (v/v) seeds.

Calorimetric measurements were carried out using a Microcal VP-DSC, calorimeter (Malvern Panalytical, Ltd, Worcestershire, UK). The sample solution contained 0.5 mg/ml of each protein and the solution conditions were the same as those for the individual ThT assays with or without seeds. Sample and buffer solutions were carefully loaded into the DSC sample and reference cells respectively, after being properly degassed in an evacuated vessel for 3 min at 25 °C. After the buffer–buffer baseline was subtracted from the sample data, apparent heat capacity (Cp) corresponding to the whole sample solution was evaluated using ORIGIN software (Microcal Inc.).

Statistics and reproducibility. Kinetic traces of three independent experiments are shown. When plate wells were reproduced, the data are expressed as the mean ± s.e.m. for n = 5 wells. CD spectral and DSC measurements were performed twice.
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Author contributions

M.N. expressed and purified β2m and αSyn. Y.A.-O. and Y.H. expressed and purified C$_4$, T.S. E.C., and V.B. expressed and purified TTR. M.N. performed most of the experiments and analyzed the data together with Keiichi Yamaguchi, M.S. and Keisuke Yuzu. Y.K., K.I., H.M., V.B. supported the experiments and data analysis with αSyn and transthyretin. M.N., J.K., D.E.O., V.B., J.B., and Y.G. designed the study and wrote the manuscript with input from all co-authors.

Competing interests

The authors declare no competing interests.

Additional information

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