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In Vitro Formation of Amyloid from α-Synuclein Is Dominated by Reactions at Hydrophobic Interfaces

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Abstract: Most in vitro investigations of α-Synuclein (αSyn) aggregation and amyloidogenesis use agitation in the presence of air and/or Teflon to accelerate kinetics. The effect of the agitation is implicitly or explicitly attributed to mass transfer or fibril fragmentation. This paper evaluates these hypotheses by agitating αSyn under typical amyloidogenic conditions with controlled numbers of balls made of polytetrafluoroethylene (PTFE), polymethylmethacrylate (PMMA), and borosilicate glass with no headspace. Amyloid was assayed using thioflavin T fluorescence and atomic force microscopy. The observed kinetics were proportional to the PTFE surface area; the effects of PMMA and glass balls were negligible by comparison. No amyloid was observed to form in the absence of mixing balls. Agitation with only air also showed accelerated kinetics but different aggregate morphology. The results indicate that the mechanism active in agitation experiments is dominated by reactions at the hydrophobic−water interface. Of the mass transfer, fragmentation, and hydrophobic interface hypotheses, only the last is capable of explaining the data. Condition and sequence determinants of amyloidogenic propensity that have thus far been reported must be reinterpreted as being reflective of partitioning to hydrophobic−water interfaces. Comparable hydrophobic interfaces are not found in vivo.

Introduction

Parkinson's disease (PD) is a neurodegenerative disease affecting 1% of the population over 65 and 4−5% over 85.[1] α-Synuclein is present in intracellular inclusions that stain positive for amyloid; these Lewy Bodies are hallmarks of PD.[2] Mutations and triplication of α-Synuclein lead to early onset forms of PD, though sporadic PD accounts for 95% of cases.[2]

α-Synuclein (αSyn) is a 140 residue, 14.5 kDa protein found in neuronal cells especially in presynaptic termini.[2−4] It is an intrinsically disordered protein with instability in its secondary and tertiary structure.[5−9] Residues 61−95 of αSyn were first identified as a non-Aβ component (NAC) of amyloid plaques in Alzheimer's disease.[2] The NAC is mostly hydrophobic, but has one positive and one negative residue in close proximity to each other. In experiments where samples are shaken (large air−water interface), αSyn forms amyloid within a few days where αSyn without the NAC does not within 6 weeks.[10] Furthermore, the NAC alone will form seeding-competent amyloid.[10] αSyn is overall acidic with pI = 4.6;[11] at pH 7.55, the N-terminal region should have 8 negative and 11 positive charges while the C-terminal region has 3 positive and 15 negative charges. In this way, αSyn resembles a triblock copolymer with the hydrophobic NAC lying between charged terminal regions. This structure makes αSyn an excellent surfactant molecule.

The link between αSyn and PD has led to intensive in vitro studies of αSyn that aim to determine the aggregation mechanism.[2−5,10−21] αSyn amyloidogenesis shows sigmoidal kinetics that can be seeded, consistent with a nucleated process.[19,22,23] However, the lag time in the sigmoidal kinetic profile varies by 2 orders of magnitude depending on details of the incubation conditions.[3,4,16,21,24] The method used to remove pre-existing aggregates and the use of agitation both have a large impact on the kinetics.[15,25,22] Preformed aggregates, such as those created during lyophilization, can reduce the lag time from >30 days to <3 days. In samples without preformed aggregates, shaking with air headspace reduces the lag time from >30 days to several hours.[25] Agitating aqueous solutions of αSyn with air or polytetrafluoroethylene (PTFE) leads to faster formation of globular aggregates and amyloid fibrils than without.[3,4,16,21,24,25] Most αSyn in vitro experiments use shaking and/or PTFE stirrers to accelerate αSyn amyloid kinetics.[3,4,14−16,18−24,26,27]

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The effect of agitation has been attributed to mixing[23,26] and fibril fragmentation.[28,29] The mixing hypothesis suggests that aggregation reactions between fibrils and additive species are limited by mass transfer. The mass transfer rates and therefore reaction rates are proposed to be related to the rate of mixing. The fragmentation hypothesis suggests that fibrillization is limited by the number of active nucleation sites. The effect of agitation is to increase sheer forces, which break fibrils, creating more active sites. Both models assume agitation affects reaction steps that occur in homogeneous solution. Both models predict that the kinetics should be independent of the material or interfacial properties of the mixers.

An alternative hypothesis is that the dielectric interface created by bubbles and stir bars provides a driving force for the preferential partitioning of protein to the inhomogeneous interface. At the hydrophobic−hydrophilic interface, αSyn is expected to behave like a surfactant, thereby accelerating aggregation reactions by concentrating and conformationally orienting reactive protein species. This hypothesis predicts kinetics to be proportional to interfacial surface area.

Investigations into other proteins have demonstrated the sensitivity of aggregation reactions to hydrophobic−water interfaces. Insulin aggregation kinetics have been shown to be proportional to the amount of air−water or Teflon−water interface.[30] Hydrophobic, but not hydrophilic, bacteriophages are inactivated with air−water−Teflon interfaces.[31] PTFE balls were shown to accelerate the formation of amyloid for the small hormone protein, glucagon.[32] Hydrophobic nanoparticles were recently shown to nucleate cross-β aggregation of β_2-microglobulin in vitro[33] and in a model peptide in silico.[34] Grazing incidence X-ray scattering showed that Aβ is random coil in bulk solution but adopts a β-sheet when aggregated at the air−water interface.[35,36] A model peptide, LSFDNSGAITIG-NH$_2$, spontaneously forms β-sheet aggregates at an air−water interface.[37]

This hypothesis implies that the important reaction steps occur at the *heterogeneous* interface. It further implies that mutations and solution conditions that modulate kinetics report on changes in the affinity of the protein for the interface. Hydrophobic−hydrophilic interfaces like those of air−water and PTFE−water in vitro experiments have not been found in the mammalian brain and do not appear to be biologically relevant.

This paper aims to determine the mechanism by which agitation accelerates the kinetics of amyloidogenesis from αSyn. To evaluate the mixing, fragmentation, and interfacial hypotheses, αSyn was incubated under gentle agitation with mixing balls of varying number, density, and hydrophobicity. These conditions systematically changed the efficiency of convection, the local shear forces experienced during agitation, and the driving force for amphiphilic partitioning of αSyn to the interface, respectively. Monodisperse balls provide a fixed-area hydrophobic interface allowing quantitative assessment of its influence. Materials for mixing balls in this study are polytetrafluoroethylene (PTFE), polymethylmethacrylate (PMMA), and borosilicate glass. PTFE is a chemically inert, highly hydrophobic fluorinated polymer with a large water contact angle; PMMA is modestly hydrophilic; glass is hydrophilic.

Experimental Section

Mixing Balls. Grade 1, 1/16 in PTFE; 1 mm PMMA balls (Engineering Laboratories, Inc.; Oakland, NJ); and 1 mm glass balls (B. Braun Melsungen; Melsungen, Germany) were shaken in several changes of buffer to remove dust before use. Balls were produced by milling and lack a releasing agent (oil) coating. AFM shows the balls to be rough with ∼200 nm features (see Supporting Information: Figure S1). The radius of PTFE balls is 0.0625 ± 0.001 in., establishing a minimum 3% error on surface area.

Expression of αSyn. *Escherichia coli* BL21 DE3 strain (Invitrogen, Inc.) was transfected by a plasmid (pT7-7) encoding human wild-type αSyn (gifts from Prof. Jean Baum; Rutgers University, Piscataway, NJ). Expression, purification, and lyophilization of αSyn followed published protocols.[21]

Thioflavin T (ThT). ThT is a positively charged histological stain which gains characteristic absorbance and fluorescence bands at 450 and 482 nm, respectively, upon specific binding. Amyloid protofibrils and mature fibrils give strong fluorescence at 482 nm, which has led researchers to use ThT to monitor fibril production.[23,32,33] A fluorescent impurity was removed from ultrapure grade ThT (>98% by HPLC, >95% by TLC; AnaSpec, Inc., San Jose, CA) by 10× exchange of ThT/water with hexanes. A 10 mM NaPO$_4$ solution, pH = 7.55, was made from monobasic and dibasic sodium phosphate (>99% Sigma-Aldrich) in ultrapure online-filtered water (Millipore Synergy 185) and filtered (0.22 μm, Stericup).

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Incubation Sample Preparation. Preformed aggregates associated with reconstituting lyophilized protein were removed with an Amicon Ultra 100 kDa ultracentrifuge membrane filter at 3300 rpm. Strong base does not completely remove these aggregates and results in substantially reduced lag times.[15,22,25] Protein solutions were diluted to 1 mg/mL (~70 μM). A total of 30−90 μL of 2.5 mM stock purified ThT was added to make a total concentration of 8 μM. Protein and ThT concentrations were verified by absorbance measurements ($\varepsilon_{\alpha Syn}$ (276 nm) = 5800 M^{-1} cm^{-1},[24] ε_{ThT} (412 nm) = 36 000 M^{-1} cm^{-1}[38]). PTFE and PMMA balls were counted and added to reduced volume PMMA fluorescence cuvettes (Fisher). Cuvettes were overfilled and capped with polyethylene (Fisher) to remove air before being sealed with a thermoplastic (hot glue). Air samples were prepared and sealed as above before caps were pierced with a syringe needle. To create a volume of air equivalent to 40 PTFE balls, 80 μL of the solution was removed and the cap was resealed. Solutions were degassed with helium and an aerating frit for 3 min immediately before use.

The efficacy of convective mixing under the conditions of gentle headspace-free agitation was evaluated using a sample with a small amount of dye at the bottom and a single PTFE ball. Within 1 min, the sample was mixed, but still visually inhomogeneous. After 2 min, the sample appeared completely homogeneous. This time scale is orders of magnitude faster than the time scale observed for aggregation suggesting that convective mixing did not limit the reaction rates in any of the agitated samples.

Incubation. Samples were incubated at 37 °C and vertically rotated at 76 rpm on a Roto-Torque heavy duty rotator. Samples were incubated and transported in a home-built, 1.2 kg aluminum cuvette holder to minimize temperature fluctuations. All spectroscopy was performed in thermostatted cuvette holders at 37 °C. UV−vis absorbance was measured on a Cary 50 Bio spectrophotometer, fluorescence on a JY/Horiba photon counting fluorimeter. Acquisition times were 0.1 s/nm and source and observation slits were 4 nm.

Atomic Force Microscopy (AFM). AFM tapping mode measurements were performed on a Veeco MultiMode Scanning Probe Microscope with a Nanoscope IIIa controller and etched silicon probe. Scan speed was 1 Hz with image resolution of 512 × 512 pixels. Samples were deposited on clean and APTES-passivated mica as previously described.[39]

Contact Angles. Contact angles were measured on a 1.5 in. flat sample of PTFE, glass cuvette, and sections of PMMA cuvette and polyethylene cap with a model 250 Standard Contact Angle Goniometer/Tensiometer and analyzed with DROPimage Advanced v1.5.04 software (Ramé Hart; Netcong, NJ). The PTFE surface was cleaned between experiments with hexanes to remove residues and rinsed with ultrapure water to reduce triboelectric charging. Other samples were cleaned with buffer only. All samples were allowed to dry before measurement. Drops (2−5 μL) of incubation buffer were pipetted to the surface and measured within 30 s to minimize systematic evaporation errors. A total of 4−13 drops per experiment were measured to account for variations in the surface roughness.

Data Analysis. Nonlinear least-squares fitting was performed with a Levenberg−Marquardt optimization implemented in IGOR Pro v6.12. Error bars shown in figures are the sum of fit errors and the sample variability of the triplicate 20 ball samples, scaled by the number of balls. Initial slopes were determined from a fit to first linear points of fluorescence versus incubation time which terminate with the rise of the growth phase. The lag parameter was defined as the point of inflection in the data and was determined from a sigmoid-plus-line fit. The asymptotic limit was determined by averaging the last 4 data points (see Supporting Information Figur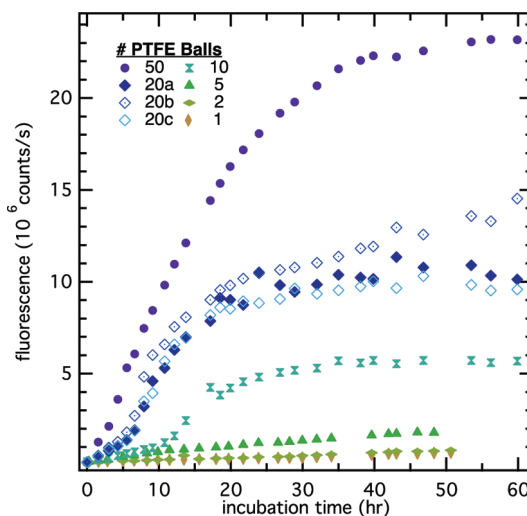e S2). Data in Figure 2 was fit with zero to fourth order polynomials. Fits more complicated than linear failed to provide $p < 0.1$ using the F-test on the residuals.

Figure 1. ThT steady-state fluorescence monitored during amyloidogenic incubation of αSyn in the presence of varying numbers of PTFE balls as labeled in the figure: the initial slope and asymptotic limit of fluorescence are simply proportional to the available PTFE surface area; lag time until growth phase is inversely proportional to PTFE surface area.

Results

Agitation with PTFE Balls. Figure 1 shows the steady-state thioflavin T (ThT) fluorescence of 70 μM αSyn incubating at 37 °C with varying numbers (1, 2, 5, 10, 20, 50) of PTFE balls. Three features of the sigmoidal kinetics appear to vary systematically with the number of PTFE balls in the data: the initial slope, lag time, and asymptotic limit of fluorescence (Figure 2). Each parameter showed a linear dependence on number of PTFE balls. AFM of all PTFE samples showed fibrils and small aggregates at the end of the incubation. (see Supporting Information Figure S3).

Agitation with Air. Samples agitated with controlled volumes of air showed sigmoidal increases in ThT fluorescence (Figure 3) but only small aggregates (*d* ≈ 20−150 nm) by AFM (see Supporting Information Figure S4) consistent with previous reports.[13] The *A* sample held its air mostly in one large bubble (*d* ≈ 3 mm) with a handful of very small bubbles (*d* ≈ 500 μm) though the large bubble occasionally broke into several smaller bubbles. The *B* sample consistently had several smaller bubbles (*d* ≈ 1−2 mm) and a handful of the very small bubbles until ~33 h in which the seal was compromised (data after 33 h was excluded). The *C* sample had the same air volume, but consisted entirely of smaller bubbles from the start that did not noticeably coalesce.

The air samples exhibited characteristic sigmoidal kinetics often reported in the literature.[20,21] *A* and *C* shared a common lag time; however, the asymptotic limit of *A* was roughly double that of *C*. All samples displayed a sharp rise to minor plateaus before the principal lag at 30 h. The times to reach this point varied by 12 h and had no clear correlation to the other kinetic parameters.

Agitation with PMMA or Glass. PMMA ($\theta_{buf} \approx 73−74°$) and glass ($\theta_{buf} \approx 40−47°$) balls provided controls for the mechanical aspects of the agitation process. Mixing and shear forces in solution depend on the hydrodynamic properties of the balls. The PMMA balls moved more slowly and more turbulently through solution than did the PTFE balls, while glass

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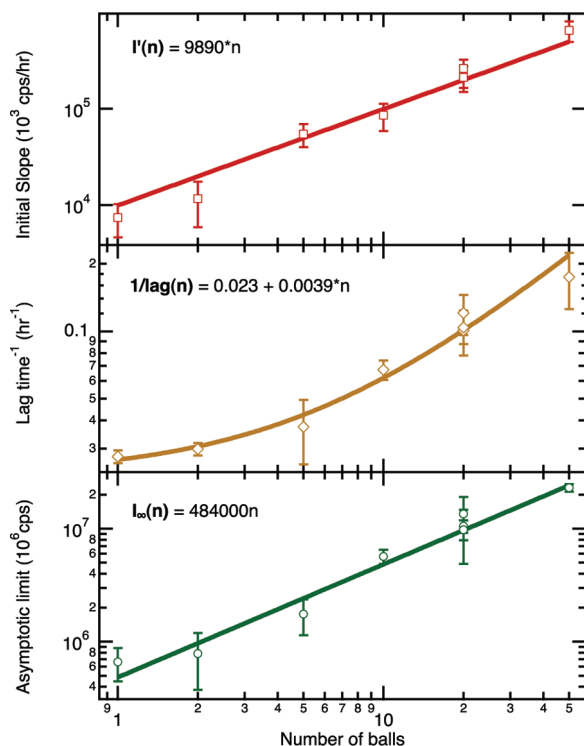(39) He, X.; Giurleo, J. T.; Talaga, D. S. *J. Mol. Biol.* **2010**, *395*, 134−154.

Figure 2. Linear fits of (top panel) initial slopes, (middle panel) lag times^{-1}, and (bottom panel) asymptotic limits as a function of the number of PTFE balls present illustrate the dependence of αSyn amyloid kinetics on hydrophobic surface area.
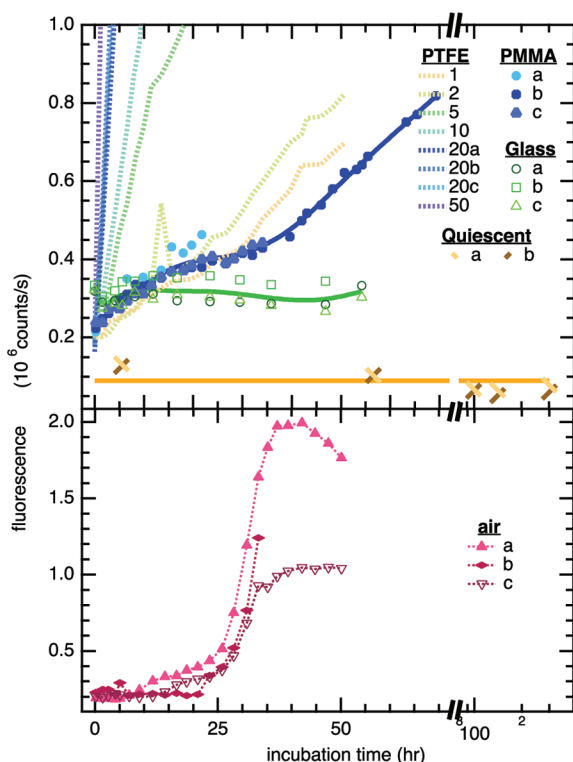

Figure 3. ThT fluorescence of PMMA, air, and quiescent samples with PTFE samples for reference: (Top panel) 50 PMMA balls (filled blue symbols) with equivalent surface area to 20 PTFE balls show ThT fluorescence slightly below that of 1 PTFE ball. A total of 50 glass balls (open green symbols) exhibit no increase in fluorescence. Quiescent data show no increase in ThT fluorescence for 300 h and is fit with a constant for reference. (Bottom panel) Air samples (pink) exhibit typical sigmoidal kinetics reported in literature.

Table 1. Measured Contact Angles of Buffer Drops on Flat PTFE Surface[a]

PTFE treatment	Contact Angle θ_{buf}
clean surface	108–121°
17 h buffer	109–124°
3 h ThT/buffer	96–120°
20 h ThT/buffer	73–108°
water rinsed	100–114°
hexanes wash	113–121°
1 h αSyn/buffer	10–13°
water rinsed	100–109°

[a] Soaking PTFE in buffer has no affect on contact angle. ThT/buffer soaking causes moderate loss of hydrophobicity over 20 h and αSyn adsorption transforms the surface to a strongly hydrophilic one within 1 h.

balls moved nearly identically to the PTFE. Fifty PMMA ($d =$ 1 mm) or 50 glass balls ($d =$ 1 mm) gave the equivalent surface area of 20 PTFE balls ($d =$ 1/16 in.). These samples showed smaller increases in fluorescence than those with a single PTFE ball (Figure 3). Fibrils were not observed by AFM for samples agitated with glass balls. (see Supporting Information: Figure S4).

Quiescent Samples. Samples containing no mixing balls or headspace showed no increase in ThT fluorescence after 300 h without agitation (Figure 3).

Effect of Sample History on Interfacial Hydrophobicity. Contact angle measurements were used to determine the degree to which the PTFE remains hydrophobic during the incubation. Ranges of measured contact angles are reported in Table 1 and likely reflect heterogeneity in surface roughness.

Soaking in buffer for 17 h did not influence the PTFE contact angle. Three and 20 h soaks of PTFE in buffer with 8 μM ThT showed a modest decrease in hydrophobicity as reflected in the decrease in contact angle. A mild water rinse returned most of the surface to its original condition indicating weak binding of ThT. A mild rinse with hexanes returned the ThT-soaked samples to their original states. A 1 h soak of the surface in 35 μM αSyn (half of the experimental concentration) resulted in a dramatic decrease in surface hydrophobicity. The surface was completely wetted and after drying the contact angle was reduced to 10−13°. The 39 charges carried by each protein transform the surface from strongly hydrophobic to strongly hydrophilic. Mild rinsing again showed 90−95% recovery of surface hydrophobicity, indicating mostly reversible binding.

Evaluation of Interfacial Viability. The steady state in amyloid sigmoidal kinetics is typically interpreted as being due to the consumption of available monomer to below the threshold required for nucleation. When clean PTFE balls were added to samples that had been incubating for 55 h, ThT fluorescence was observed to increase sharply over the time that followed. This indicates that viable protein remained in solution and that it could be nucleated into amyloid by fresh PTFE surfaces (Figure 4). Three samples that appeared to have plateaued were given fresh balls: 9, 18, and 15 fresh balls were added to the 1, 2, and 5 ball samples, respectively. In all cases, the plateau in the kinetics was eliminated and growth continued.

Discussion

The Mixing Hypothesis. This implies that aggregation kinetics are primarily limited by mass transfer rates in solution.[23,26] The effect of mixing is to eliminate local concentration gradients that may arise due to rapid fibril elongation reactions. A single PTFE ball effectively mixed the sample on a time scale much
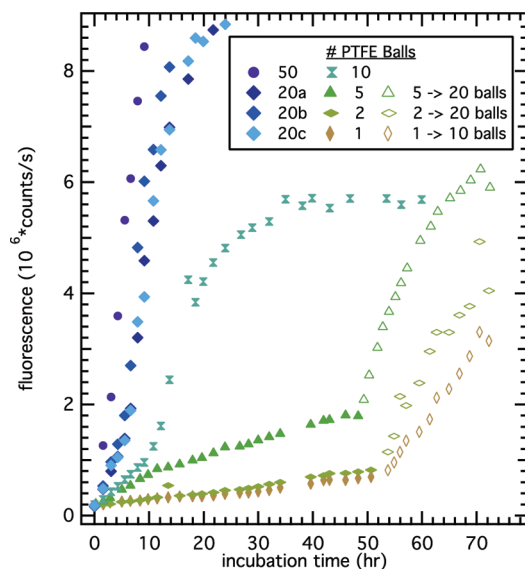
Figure 4. Extra ball data: 9 fresh PTFE balls are added to the 1 ball sample; 18 to 2; and 15 to 5 resulting in a sharp increase of ThT fluorescence.

faster than the rate of reaction ($t_{mix} \approx 2$ min $\ll t_{lag} \approx 3$ h); the number of balls should have essentially no effect under the mixing hypothesis. The linear dependence of the kinetics on the number of PTFE balls is inconsistent with the mixing hypothesis. The kinetic enhancement of mixing should be independent of the material of the balls; 50 PMMA or glass balls yield less ThT fluorescence than one PTFE ball in otherwise identical experiments. Mixing alone cannot account for the acceleration of αSyn amyloid kinetics in agitated experiments.

The Fragmentation Hypothesis. This attributes the acceleration to the production of more active fibril ends due to breakage during agitation. Fragmentation may result from sheer, torsion, and bending forces during agitation[20,40] presumably from the balls passing through solution. The fragmentation hypothesis predicts that faster amyloid production would occur in samples with more balls, consistent with the PTFE results. Samples with faster kinetics would be expected to have shorter fibrils. However, AFM fibril length appeared independent of number of balls (see Supporting Information: Figure S3). Moreover, the fluorescence data would imply that shorter fibrils produce more fluorescence per unit length than longer ones. This contradicts fluorescence lifetime results that show that longer ThT lifetimes accompany larger fibrils.[25,41] ThT luminescence is also known to occur along the entire fibril, which would imply similar brightness per unit length for long and short fibrils.[42]

The fragmentation hypothesis predicts that PMMA or glass balls should give comparable levels of acceleration to PTFE. This is inconsistent with this study, which shows less ThT fluorescence with 50 PMMA or glass balls than with 1 PTFE ball. Therefore, fragmentation of fibrils alone cannot account for the acceleration of αSyn amyloid kinetics in these agitated experiments. Under the relatively gentle agitations conditions of these experiments, significant fibril fragmentation does not

appear to be active. The plateau observed in the kinetics could be eliminated by the addition of fresh PTFE surface suggesting that the number of active ends for fibril growth was the limit reached at the kinetic plateau. Under agitation conditions (without headspace) where significant fragmentation occurred and the fibril length distribution was altered, interfacial effects would still be predicted to be the dominant effect for the initiation of the amyloidogenic aggregation reactions for αSyn.

The Hydrophobic Interface Hypothesis. This attributes the acceleration to the presence of interfaces that lower the barrier to rate-determining steps in amyloidogenesis. Both the amount and nature of the interfaces should influence the observed acceleration of kinetics, consistent with the results. PTFE interfaces accelerated production of amyloid in proportion to the exposed surface area, while other interfaces showed only limited effects. The hydrophobic interface hypothesis explains the simple dependence of the initial slope and lag on the surface area of PTFE found in the data. The contact angle experiments showed that PTFE surfaces become coated under experimental conditions, effectively removing the catalytic interface. When fresh surface was introduced to the experiment, the catalytic activity resumed, indicating the presence of protein in solution that is viable for hydrophobically activated heterogeneous nucleation.

Total ThT/Amyloid Signal. The steady-state ThT fluorescence of each PTFE sample reaches an apparent plateau by the end of the experiment (Figure 1). Figure 2 shows the linear relationship between the asymptotic limit of fluorescence and PTFE surface area. The hydrophobic interface hypothesis cannot explain this observation without modification; a catalytic surface increases the reaction rate but not the extent of reaction.

Four explanations for the interfacial-area dependence of the total amyloid signal include: there is a nonproductive parallel reaction that consumes viable monomer; there is a maximum size of amyloid fibril that can be formed; there is a fibril termination reaction; and there is a catalyst surface fouling reaction. The first two explanations both predict that new catalytic surface should have no effect on the apparent kinetic plateau. Growth resumed when fresh PTFE balls were added to samples that had reached a kinetic plateau, eliminating the first two explanations. If the hydrophobic interface becomes passivated, then the extent of the reaction would be limited by the total amount of hydrophobic interface. The contact angle experiments illustrate that the PTFE surface becomes less hydrophobic as a result of ThT association to the surface and/or due to protein association to the surface. The dependence of the total amyloid signal on the amount of interface is consistent with first-order kinetic deactivation of the catalytic hydrophobic interface and termination of the active fibril ends.

Mechanistic Implications. The present study suggests that the acceleration of amyloid formation depends strongly on the hydrophobicity of the interface. PTFE and air are both highly hydrophobic and strongly promote aggregation. Proteins with surfactant-like properties will partition to hydrophobic−hydrophilic interfaces. The hydrophobic NAC region of αSyn is expected to partition to the interface, while the charged N- and C-termini remain solvated in buffer. Association with the interface must lower the barrier of rate-determining steps in amyloidogenesis. Two possible effects of association of protein to hydrophobic/water interfaces include increases in local concentration and conformational changes. A net driving force

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for macromolecular association to the surface typically requires a conformational change to allow preferential interaction of hydrophobic side chains with the surface. The association of the NAC with the interfacial region would increase NAC−NAC contacts and is consistent with the observed acceleration of the formation of amyloid.

The orienting effect of the interface can be thought of in terms of protein folding conformational landscapes. Folding and collapse of polypeptides into structured soluble proteins typically requires a specific number of hydrophobic residues. The hydrophobic core needs to be of a critical size to offset the loss of polypeptide chain entropy. By associating with the surface, αSyn gives up some of its chain entropy. β-strand structures can expose side chains to the surface. Surface-associated side chains on different αSyn molecules can interact allowing more favorable hydrophobic interactions. Thus, aggregated αSyn may then have a pool of hydrophobic interactions large enough for a stable collapsed oligomeric structure based on NAC−NAC contacts.

Kinetics of Mutated Proteins. Kinetics of mutated proteins reported in the literature are consistent with the hydrophobic interface hypothesis. The structure of αSyn is reminiscent of a charged-hydrophobic-charged triblock copolymer and exhibits surfactant behavior.[25] Mutations that alter hydrophobicity of the NAC have been reported to have a direct effect on the observed amyloid kinetics in experiments agitated with hydrophobic−hydrophilic interfaces. Hydrophobic to charged amino acid mutations in the NAC decreased amyloid kinetics consistent with its reduced affinity for the hydrophobic−hydrophilic interface. Addition of three charges (A69K-V70T-V71K-T72E) severely reduced amyloid production while balancing the negative charge of E83 with V82K slightly increased it.[18] Neither mutation significantly affected the native conformation as measured by circular dichroism. Adding a charge to residue 76 (A76R or A76E) in the NAC slowed amyloid kinetics by a factor of 2−4.[10] Removing the hydrophobic center of the protein (residues 71−82) eliminated fibril production in a 6 week agitated experiment that produced wild-type fibers in a few days.[10] Removing two of the charged repeats from the N-terminal region increased the fibrillization rate, while adding two decreased it.[14]

Heterogeneous Nucleation. The hydrophobic interface hypothesis, unlike mixing and fragmentation, suggests the important reaction steps occur at *heterogeneous* interfaces. To evaluate the sequence and condition determinants of amyloidogenic aggregation requires either elimination of the heterogeneous interfaces present in the in vitro studies or use of an accelerating interface that mimics the cellular nucleation sites. It is not known if homogeneous or heterogeneous nucleation dominates in vivo; the cellular structures responsible for nucleation of αSyn are not known.

In vitro studies of sequence and condition determinants of αSyn amyloid kinetics that use air and/or PTFE agitation are confounded by the heterogeneous component of the kinetic mechanism. These studies measure two effects in addition to those desired; the relative affinities of the proteins for the interface and the degree to which those interfacially associated states lower the kinetic barriers to amyloidogenesis. Since amyloid formation from αSyn appears to take 1−2 orders of magnitude longer without agitation, it is reasonable to conclude that the interfacial effects dominate the sequence and condition determinants of amyloidogenesis as measured in vitro.

Air−Water Variability. Agitation with headspace creates variable amounts of hydrophobic−hydrophilic (−hydrophobic) interfaces both at the onset and, if the agitation is vigorous, throughout the experiment. Since the reaction kinetics appear dominated by interfacially promoted reactions, the stochastic and mutable nature of the air−water interface introduces another source of sample-to-sample and investigator-to-investigator variability. One approach to overcoming this problem is to increase the vigor of agitation with the added effect of introducing substantial fibril fragmentation, which will significantly complicate kinetic analysis. The PTFE−water hydrophobic interface experiment offers a measure of control over its air−water counterpart.

Conclusions

Amyloid formation kinetics in vitro depend on the amount of hydrophobic surface area available to αSyn. Previous assertions that sample agitation increases αSyn amyloidogenesis primarily by fragmentation or mixing are inconsistent with the PMMA and glass ball control experiments. The initial slope dependence and contact angle experiments show that the hydrophobic interface affects αSyn aggregation from the first hours of the experiment. PTFE/water and air/water interfaces gave different products suggesting the presence of multiple interfacially active mechanisms. Quiescent samples with no headspace showed no amyloid-related signals over the course of the experiment. PMMA did not show signs of amyloid, suggesting that a minimum interfacial tension is required for nucleation.

Some of the inconsistency often observed in αSyn kinetics is likely due to the inconsistency of the air−water interface during shaking. The use of mixing balls in the absence of air−water interfaces gives less run-to-run variability than air-shaken samples. The interfaces present during shaking are stochastic creating a variable amount of nucleation-promoting surface during the reaction. Differences in amount of air−water interface persisted in spite of rotation at 76 rpm.

Analysis of αSyn aggregation kinetics based on homogeneous nucleation theory is inappropriate when the dominant mechanism is interfacially promoted aggregation. The sequence and condition determinants of αSyn amyloidogenesis as currently understood in the literature are likely a reflection of the degree to which they influence the partitioning of αSyn to the hydrophobic interfaces present during the in vitro assays. Interfacially promoted aggregation is a different mechanism with different kinetic barriers and therefore potentially different relevant driving forces and potentially different sequence and condition determinants than the homogeneous nucleation mechanism. The degree to which interfacially promoted kinetic measurements will reflect in vivo aggregation propensities cannot be ascertained. However, PTFE/water and air/water interfaces are not physiologically relevant to PD; membranes are amphiphilic, not hydrophobic, and induce α-helical structure in αSyn. The dramatic difference between homogeneous and heterogeneous aggregation rates suggests that a biologically relevant cellular nucleation mechanism should be identified.

Supporting Information Available: Complete ref 6; AFM images of aggregation products from the PTFE, PMMA, and glass agitated incubation experiments; AFM images of PTFE and PMMA ball surfaces; fits of initial slope, lag, and asymptotic limit. This material is available free of charge via the Internet at http://pubs.acs.org.

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