Parkinson’s disease (PD) is a neurodegenerative disorder affecting an estimated 4 million people worldwide. Intracellular proteinaceous inclusions called Lewy bodies are the histological hallmarks of PD and are primarily composed of aggregated α-synuclein (αSyn). Although the detailed mechanisms remain unclear, mounting evidence suggests that the misfolding of αSyn into prefibrillar and fibrillar species is the driving force responsible for cellular toxicity. We show here that the molecular chaperone heat shock protein (Hsp) 70 strongly inhibits αSyn fibril formation via preferential binding to prefibrillar species. Moreover, our studies reveal that Hsp70 alters the characteristics of toxic αSyn aggregates and indicate that cellular toxicity arises from the prefibrillar forms of αSyn. This work therefore elucidates a specific role of Hsp70 in the pathogenesis of PD and supports the general concept that chaperone action is a crucial aspect in protecting against the otherwise damaging consequences of protein misfolding.

A variety of interconverting conformational states is accessible to a protein under biological conditions, with the distribution among these states governed by both thermodynamic and kinetic factors (1). Just as enzymes facilitate changes in chemical states within living systems, molecular chaperones and other cellular factors regulate transitions between protein conformations (2). Molecular chaperones are likely to mediate the flux between different protein conformations, for example, by binding unfolded or partially folded regions of proteins enriched with hydrophobic amino acids, an ability common to many chaperones including heat shock protein (Hsp)\textsuperscript{\textregistered} 70 (3). The formation of amyloid fibrils, the species associated with a large family of human disorders including Alzheimer’s disease and type II diabetes (4, 5), is likely to occur via monomeric or oligomeric intermediates exhibiting exposed hydrophobic surfaces (6, 7). Thus, molecular chaperones might naturally be expected to target such intermediate species, and indeed a range of molecular chaperones has been observed to be sequestered within amylloids plaques generated in several forms of neurodegeneration (8, 9).

In addition to their role in assisting protein folding, molecular chaperones appear to have been strongly selected by evolution to protect cells against the harmful effects of protein aggregates (2, 3, 10). Amorphous aggregates are usually relatively harmless to cells, at least in part because they may eventually be degraded by the proteolytic machinery (11). Highly ordered amyloid fibrils can, however, be resistant to degradation (12), and in addition they, or perhaps more generally their precursors, can be exceedingly toxic (13). Consistent with their role in the prevention of protein misfolding, molecular chaperones have been shown specifically to inhibit fibril formation (11, 14, 15) and toxicity (15, 16) in several disease models, both in vitro and in vivo. Evidence also suggests that molecular chaperone networks are degraded in old age, which may explain the late onset of most protein misfolding diseases (17, 18). Recent work has shown, for example, that increasing the expression of small heat shock proteins in Caenorhabditis elegans results in improved longevity and resistance to protein aggregation (19). These studies therefore suggest that molecular chaperones are likely to play a critical role in the prevention of protein misfolding disorders.

Like enzymes, individual molecular chaperones are often components in coupled systems, working in tandem with co-chaperones and co-factors to select distinct protein conformations (20). Although a combination of Hsp70, Hsp40, ATP, and other factors is likely to be required for full chaperone activity (20), recent studies have shown that chaperone binding alone can have a powerful impact on protein refolding and aggregation. For example, the isolated GroEL substrate binding domain has been shown to facilitate the refolding of several proteins (21–23), and Hsp104 alone has been found to inhibit fibrillation of Sup35 prion conformers (24). Of particular significance for the present study is the finding that the substrate binding domain of the bacterial Hsp70 homologue DnaK has the same high affinity for peptide substrates as the ADP-bound full-length DnaK (25, 26), and that chaperone binding assists in both aggregation suppression and protein refolding (27). Taken together, these studies indicate that substrate binding alone reveals the most fundamental steps in chaperone function. Our objective, exemplified in the present work, is to understand the specific role of individual chaperones in distinct steps of the aggregation process in order to define factors such as substrate binding that regulate the conformational interconversion of specific proteins (2). As part of this strategy, we have investigated the effect of Hsp70 on αSyn aggregation in vitro and show that fibril formation can be strongly inhibited via chaperone binding to cytotoxic prefibrillar species, even in the absence of co-factors.
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**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Proteins**—Wild-type human αSyn (vector pT7-7) was expressed and purified as described previously (28). Recombinant, His-tagged, human Hsp70 was expressed and purified as described previously (29). Protein purity exceeded 95% as determined by SDS-PAGE.

Thioflavin T Fluorescence—Solutions contained 70 μM αSyn alone or with 7 μM Hsp70 in 50 mM Tris, pH 7.4, and 150 mM KCl. For kinetic aggregation studies, 15 μM thioflavin T (thioT) (Sigma) was included prior to aggregation. Following filtration using 0.22-μm sterile filters, aggregation was induced by heating the sample to 37 °C with magnetic stirring, and readings were taken every 30 min in a Cary Eclipse spectrophotometer. Emission at 480 nm was measured at an averaging time of 10 s. ThioT fluorescence measurements were made for 10-μl reaction aliquots by diluting them 150-fold in 50 mM glycine-OH (pH 8.5) containing 5 μM thioT using the experimental parameters described above. Hsp70 alone did not contribute to the thioT fluorescence under these conditions.

**Electron Microscopy**—Samples were prepared from 10-μl aliquots of the relevant aggregation reaction by negative staining by 2% (w/v) uranyl acetate on Formvar-coated nickel grids. Images were obtained at ×25,000 magnification using a Phillips CEM100 transmission electron microscope.

**Circular Dichroism**—Reaction aliquots of 150 μl were diluted 2-fold with buffer and analyzed on a Jasco J-810 spectropolarimeter at 20 °C. Each scan was run in triplicate and averaged. The appropriate buffer spectrum was subtracted from the spectrum of each sample, and the signal from Hsp70 in the presence of αSyn was subtracted from samples containing both αSyn and Hsp70.

**Gel Filtration**—Pre fibrillar αSyn was prepared as described previously (30). Samples consisted of pre fibrillar species (18 μM monomer concentration), Hsp70 (54 μM), or a combination of the two at these final concentrations and incubated at room temperature for 2 h. The samples (250 μl) were centrifuged and filtered to remove insoluble material and injected onto a Superdex 200 10/300 GL column equilibrated in phosphate-buffered saline at 10 °C. A flow rate of 0.4 ml/min was used, and 0.4-ml fractions were collected. Injections were performed in triplicate from independently prepared samples. The molecular mass standards used were as follows: bovine thyroglobulin, 66 kDa; yeast alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa (all from Sigma).

**NMR Spectroscopy**—Isotopically enriched α-synuclein was expressed by substituting M9 minimal medium with 1 g/liter 13C-glucose as the sole nitrogen and carbon sources, respectively. Two-dimensional gradient-enhanced 15N-1H HSQC spectra of solutions containing αSyn in the absence or presence of Hsp70 were collected on Bruker DRX500 and Avance 700 MHz spectrometers at 10 °C. The samples were made with 100 μM uniformly15N-labeled αSyn and either 0 (R2), 0.3 (R1), 1.0, or 3.0 μM Hsp70, 70 μM thioT, pH 7.4, 100 mM NaCl, and 10 μM D. HSQC spectra were collected using 16 scans per increment, with 1024 complex points for the direct dimension and 128 complex points for the indirect dimension. Chemical shift values were obtained from further analysis in cases of severe peak overlap. Resonance assignment was performed using modern triple resonance experiments (31). NMR data were processed with NMRPipe (32) and analyzed with NMRView (33).

**Cytotoxicity Assays**—Samples for cytotoxicity assays were prepared according to the conditions described for fluorescence experiments, except for the omission of thioT. Aliquots were removed at varying intervals and, if desired, centrifuged at 16,000 × g to separate aggregates into insoluble and soluble fractions. The insoluble fraction was washed three times with aggregation buffer (50 mM Tris, pH 7.4, and 150 mM KCl) to remove any residual, soluble proteoliposome complexes. Samples were then resuspended in aggregation buffer and lyophilized for storage. The retention of aggregate morphology after lyophilization and resuspension was confirmed by electron microscopy (EM). U937 (human myelomonocytic) cells were obtained from the American Tissue Type Culture Collection (ATCC; Manassas, VA) and cultured at 37 °C and 5% (v/v) CO2. To minimize the effects of endogenous extracellular chaperones such as clusterin (34), Aim V serum-free medium was used for cultures. Untreated U937 cells cultured in Aim V medium under these conditions maintained 85–90% viability. Aliquots of U937 cells suspended in Aim V medium (100 μl containing 20,000 cells) were seeded into the wells of sterile 96-well microplates (Greiner, Germany). Proteins were added from stock solutions to wells at a 5-fold dilution and incubated with the cells for 48 h. In control experiments, only aggregation buffer or a control protein (e.g. ovalbumin) was added to the same final concentration. Following a 48-h incubation, cells were removed from each well (suspended in 100 μl of Aim V), diluted by the addition of 0.2 ml of phosphate buffered saline, and stained with 1 μg/ml propidium iodide (PI) immediately before flow cytometric analysis. Flow cytometry was performed using a FACSCalibur (BD Biosciences); cells were excited with a 488-nm argon laser, and red fluorescence was collected using a 650-nm long pass filter. Flow cytometry data were analyzed using CellQuest software (version 3.1; BD Biosciences). The statistical functions of the CellQuest software were used to quantify the proportions of cells falling into each of three arbitrarily defined regions defined on the basis of forward scatter and PI fluorescence. The regions corresponded to healthy cells (with low PI staining; R1), “leaky” cells and cell ghosts (both stained to an intermediate level with PI and with either “normal” forward scatter or reduced forward scatter, respectively; R2), and intact dead cells (highly stained with PI; R3). The percentage of dead/damaged cells was defined as R2 + R3.

**RESULTS**

αSyn forms amyloid fibrils in vitro that closely resemble those found in Lewy bodies (35, 36). The assembly and propagation of such structures was quantified by the binding of thioT, a widely used amyloid-specific dye that fluoresces strongly upon binding to fibrillar species. Under our experimental conditions, αSyn forms fibrils on a time scale of 1–2 days (Fig. 1a). The kinetic curve is characteristic of a nucleation-dependent aggregation reaction in which an initial lag phase is followed by the rapid formation of thioT-binding amyloid fibrils, ending in a plateau phase for extended periods of aggregation. The mechanism for such a reaction has been studied in detail (37, 38) and is believed to be initiated by prefibrillar species capable of driving the assembly of mature fibrils such that the rate-limiting step in fibril formation is the growth of prefibrillar species. Indeed, it has been observed that the removal of such species prior to the initiation of aggregation results in a greatly extended lag phase (35). ThioT binding experiments were performed both by including initially the dye molecule in the reaction and continuously monitoring increases in fluorescence (Fig. 1a) and by aliquot analysis with the thioT addition occurring immediately prior to the fluorescence measurement (Fig. 1b); similar fluorescence intensity patterns were observed for both methods in the present work.

In preliminary studies we observed that Hsp70 could suppress fibril formation; we therefore systematically varied the Hsp70 concentration and the solution conditions to examine their effects on fibril formation. We discovered in particular that a critical but highly sub-stoichiometric concentration of Hsp70 is required for the strong inhibition of fibril formation. Thus, the addition of a 1:10 molar equivalent of Hsp70 (relative to the starting concentration of monomeric αSyn) virtually abolished the increase in thioT fluorescence that is observed in its absence (Fig. 1a, a and b). Below the critical concentration, Hsp70 does not prevent the transition from lag phase to elongation and rapid fibril formation, whereas increasing the amount of Hsp70 above the critical concentration had no further detectable effect on the thioT fluorescence. Although the addition of Hsp70 at intermediate time points during the course of an aggregation reaction resulted in a premature leveling of the fluorescence intensity, additional experiments showed that Hsp70 alone does not alter the thioT fluorescence in a reaction that has already reached its steady-state maximum. This latter result indicates that Hsp70 does not modify the structural properties of mature αSyn fibrils or cause them to dissociate, in contrast to the disaggregation behavior observed for other chaperones such as Hsp104 (24).

EM was used to monitor the morphology of aggregates generated in both the presence and absence of Hsp70. EM of αSyn aggregates in the absence of Hsp70 revealed the presence of long, highly ordered fibrils as well as large web-like networks composed of many fibrils (Fig. 1a, upper inset). In aggregation
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αSyn toxicity, but proteinaceous aggregates were still observed. Furthermore, Hsp70 has been shown to co-localize with Lewy bodies in postmortem PD brain tissue (8), implying an intimate relationship between Hsp70 and the aggregates formed in diseases of protein misfolding.

Amyloid fibril formation is typically associated with a concomitant increase in a β-sheet or “cross-β” structure (40). αSyn, which is an intrinsically disordered protein in dilute solution (41), undergoes a β-sheet transition during fibril formation (42), as observed here by circular dichroism (CD) studies (Fig. 2a, upper section). Prior to incubation, αSyn exhibits a CD signal characteristic of a random coil with a minimum at ~200 nm, but as aggregation progresses a minimum at ~220 nm is observed, indicative of the formation of β-sheet structure. In the presence of Hsp70, however, the CD spectrum of αSyn remained largely that of a random coil throughout the course of the aggregation reaction, demonstrating the inhibition of β-sheet formation (Fig. 2a, lower section). These data suggest that Hsp70 inhibits the formation of amyloid fibrils by maintaining the majority of αSyn in a soluble state. This conclusion was confirmed by SDS-PAGE analysis of the soluble fraction of αSyn reactions in both the presence and absence of Hsp70 (Fig. 2b). It is clear that in the absence of Hsp70, but not in its presence, bands corresponding to soluble αSyn grow progressively fainter with time, consistent with the formation of insoluble forms of the protein (Fig. 2b) that are removed by centrifugation and filtration before gel loading.

We conclude, therefore, that the presence of Hsp70 in the aggregation reaction shifts the distribution of αSyn from insoluble to soluble species and thereby inhibits the formation of mature amyloid fibrils.

Because the addition of Hsp70 after the completion of aggregation has no effect on thioT fluorescence, it would appear that Hsp70 inhibits fibril formation via interactions with monomeric and/or prefibrillar forms of αSyn. To probe potential interactions between Hsp70 and monomeric αSyn, we used heteronuclear NMR methods capable of detecting binding between the two proteins. A ²H,¹⁵N correlation (HSQC) spectrum of αSyn in the absence of Hsp70 yielded ~120 discrete cross-peaks, each corresponding to one of the 135 (non-proline) amino acids of αSyn (Fig. 3); severe overlap prevented the observation of the small number of remaining peaks. The resolved cross-peaks have been shown to be excellent reporters of environmental variation through the observation that substantial line-broadening and chemical shift changes result from interactions between αSyn and SDS (43, 44) and polyamines (45). To analyze potential interactions on a residue-by-residue basis, the backbone assignments of αSyn were obtained by standard triple resonance methods and supplemented with published data (43). The HSQC spectra of αSyn, in both the absence and presence of a 3-fold molar excess of Hsp70 relative to monomeric αSyn (an amount of chaperone 30 times greater than that observed to inhibit fibril formation), are virtually identical (Fig. 3), with no ²H or ¹⁵N chemical shift changes greater than ±0.01 and ±0.1 ppm respectively (Fig. 4). Only two peaks, Val-3 and His-50, approach these thresholds, and their chemical shifts were shown to be highly sensitive to slight deviations in sample conditions such as pH. A binding event, particularly at this saturating concentration of Hsp70, would trigger much larger and highly correlated chemical shift changes (43–45), whereas the changes observed here are characteristic of minor variations in solution conditions. In addition to the absence of chemical shift changes, no line broadening was observed for αSyn in the presence of Hsp70, conclusively indicating that no significant interactions between Hsp70 and monomeric αSyn occur under these conditions.

The absence of binding between Hsp70 and monomeric αSyn...
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as observed by NMR spectroscopy (Figs. 3 and 4) suggests that Hsp70 inhibits αSyn fibril formation via an interaction with prefibrillar species. Although only a 1:10 molar equivalent of Hsp70 with respect to monomeric αSyn is required to inhibit strongly the formation of amyloid fibrils during the early stages of aggregation, because the populations of prefibrillar species have been observed to be very low during the course of aggregation (30). To probe potential interactions between such an excess of Hsp70 and prefibrillar αSyn, a concentrated solution of stable, soluble species was generated by dissolving αSyn at high concentration (30).

Any mature fibrils or insoluble aggregates were removed via filtration and centrifugation, and the separation of the prefibrillar species from the remaining monomeric fraction was performed using gel filtration as described previously (30). Re-injection of the isolated prefibrillar species results in only a minor amount of monomer dissociation, indicating that these species have sufficient stability to be analyzed in gel filtration binding assays using Hsp70.

Gel filtration elution times depend not only on molecular mass but also molecular shape (46), and as such the intrinsically disordered αSyn molecule (14 kDa) elutes with an apparent molecular mass of 67 kDa (30). The prefibrillar fraction of αSyn elutes in the void volume in these studies and is thus well resolved with respect to both monomeric αSyn and Hsp70 (70 kDa) (Fig. 5a). Binding assays were independently performed on solutions of Hsp70, purified prefibrillar αSyn, and a combi-
nation of the two after incubation. Fig. 5a shows that the prefibrillar species elute in the void volume as a heterogeneous distribution, consistent with previous studies describing the many structural variations of prefibrillar αSyn and the extreme difficulty associated with the isolation of specific populations (30). The prefibrillar species appear to range in size from ~500 kDa to 1 MDa or larger; however, because monomeric αSyn elutes at an anomalously high molecular mass, it is possible that this technique overestimates the true size of these species. A small amount of dissociated monomeric αSyn is also observed at its characteristic elution time. Hsp70 alone elutes as a very large peak (because of its sizable extinction coefficient) corresponding to its known molecular mass of ~70 kDa, along with a very small quantity of dimeric species (Fig. 5c). Although Hsp70 has a tendency to self-associate into high molecular mass species under the relatively harsh conditions used for aggregation reactions, it remains almost entirely monomeric under the conditions used for the binding assays. Moreover, any large aggregates potentially present in the Hsp70 stock solution were removed prior to incubation using both centrifugation and filtration. Thus, Hsp70 was not observed to elute in the void volume or as high molecular mass species under these conditions, and analysis of the void fraction of an injection of Hsp70 alone revealed the absence of any protein, consistent with an absorbance of 0 mAU corresponding to its void fraction.

When Hsp70 was incubated with the prefibrillar species, however, an increase of nearly 25% (~1.5 mAU/ml) in void peak area was observed, along with a 5% decrease (~1 mAU/ml) in the area of the larger peak corresponding to monomeric Hsp70, signifying the co-elution of the two proteins (Fig. 5c). SDS-PAGE analysis of the void volume fractions confirmed that Hsp70 had indeed co-eluted with the prefibrillar species, because a band corresponding to Hsp70 was observed only following their co-incubation (Fig. 5b). Taken together, these data suggest that the interaction between Hsp70 and αSyn involves the prefibrillar structures formed early in the aggregation process.

To relate the structural properties of the prefibrillar species to earlier fluorescence studies (Fig. 1, a and b), the thioT binding properties of these species were assessed. Fig. 5c shows a wavelength scan of both fibrillar and prefibrillar αSyn. Whereas an intense peak at ~485 nm is observed for the mature fibrils, almost no intensity is observed for the prefibrillar species, indicating that they do not possess the structural characteristics of amyloid fibrils. These data demonstrate that the prefibrillar species to which Hsp70 binds appear to be undetectable in conventional thioT binding studies.

Recent evidence in animal and cell culture models of PD (15, 16, 47) has suggested that αSyn aggregates can be highly toxic to cells when intracellularly overexpressed. Studies of αSyn-derived peptides (42) and oligomeric species (48) have also confirmed that αSyn can induce toxicity when added exogenously to cells. To investigate this effect further, we used flow cytometry methods to study the cytotoxicity of αSyn aggregates generated either in the absence or presence of Hsp70 and then added exogenously to U937 (human myelomonocytic) cells. As demonstrated by the representative raw data (Fig. 6) as well as by data averaged over three independent experiments (Fig. 7a), aggregate samples containing αSyn alone became increasingly toxic as the total aggregation time progressed, and the addition of Hsp70 had essentially no effect on the magnitude of
cellular toxicity. Therefore, although Hsp70 strongly inhibits α-Syn fibril formation, such a suppression is not reflected in the toxicity assays.

To gain further insight into the mechanism of Hsp70 action on α-Syn aggregation, we carried out toxicity studies after separating each sample into insoluble and soluble fractions using low speed centrifugation. The toxicity induced by the insoluble and soluble samples was summed to give the total sample toxicity as shown in Fig. 7b; the net toxicity is not significantly altered by the addition of Hsp70 as described above (Figs. 6 and 7a). The toxicity profile for the insoluble fractions shows a substantial reduction (p < 0.05) in toxicity with the addition of Hsp70 (Fig. 7c), whereas that of the soluble fractions shows a concomitant increase in toxicity (p < 0.05) as a result of an Hsp70 addition (Fig. 7d). These results are consistent with an Hsp70-induced shift in the distribution of toxic α-Syn species from insoluble to soluble forms and are supported by the observation that Hsp70 inhibits the progression from soluble α-Syn species to amyloid fibrils (Fig. 2b). The samples used for the toxicity experiments shown in Fig. 7, b–d were also analyzed using thioT fluorescence (Fig. 1b). As it is clear that Hsp70 markedly reduces the thioT fluorescence normally observed during α-Syn aggregation. The lack of any correlation between thioT fluorescence and cytotoxicity suggests strongly that the large majority of toxic species do not bind thioT and, as such, are not mature amyloid fibrils. Prefibrillar species, in particular because they do not appear to bind thioT (Fig. 5c), are therefore likely to represent the most toxic form of α-Syn.

**DISCUSSION**

The conversion of α-Syn into prefibrillar species, amyloid fibrils, and Lewy bodies is of fundamental importance in the onset and pathogenesis of PD (7, 49). We have shown here that the presence of the molecular chaperone Hsp70 is sufficient to inhibit amyloid fibril formation by α-Syn, even in the absence of co-factors, as observed in thioT fluorescence studies (Fig. 1, a and b). Such chaperone action was shown to be mediated by the binding of Hsp70 to prefibrillar species (Fig. 5, a and b), resulting in a distribution shift from insoluble to soluble forms of α-Syn (Figs. 2b and 7, c and d). A similar partitioning mechanism was recently observed in a study of the effect of Hsp70 and Hsp40 on the formation of oligomeric huntingtin species (50). Although the addition of Hsp70 inhibited strongly the formation of amyloid fibrils, we observed no effect on the net cytotoxicity of the aggregation samples (Figs. 6 and 7, a and b), indicating that prefibrillar rather than fibrillar forms of α-Syn possess the highest toxicity and that such toxic species are neither degraded nor dismantled by Hsp70. Because of the intrinsic cellular toxicity of prefibrillar species, they are likely to be either degraded or converted into less toxic species in vivo, thereby preventing the onset of protein misfolding diseases.

Recent evidence suggests that protein inclusions, which were once thought to be pathogenic agents in PD and Huntington’s disease, are relatively non-toxic and may even play a protective role in neurodegeneration (8, 51, 52). For example, Hsp70 overexpression in a Drosophila model of PD, either by directed expression (8) or enhancement of the stress response (52), effectively alleviates neuronal toxicity without altering significantly the presence of inclusion bodies. Molecular chaperone networks (11, 50, 52) and proteasomal degradation pathways (53) may in fact be involved in the production of such inclusions. However, inclusion formation does not seem to be prerequisite for chaperone rescue. In a cell culture model of PD (15), overexpression of Hsp70 abrogates the cytotoxicity of a tagged form of α-Syn that forms macroscopic aggregates, as well as that of wild-type α-Syn which was not observed to form large

**FIG. 6. Raw flow cytometry data for α-Syn aggregation reactions in the presence or absence of Hsp70.** a, when stained with PI, untreated cells are found predominantly in R1, with much smaller numbers of cells found in R2 and R3. b, a similar fluorescence pattern is observed after the addition of ovalbumin, with healthy cells indicated by a dashed ellipse. c, the addition of 20% (v/v; final concentration) ethanol as a positive control results in total cell death, with all events detected in regions R2 and R3. d–f, representative fluorescence plots show that cell death increases with aggregation time for the addition of samples from an α-Syn alone reaction; aggregation times were 8 h (d), 23 h (e), and 47 h (f), and the addition of Hsp70 to an α-Syn reaction does not significantly alter the net toxicity (R2 + R3) of the aggregates; aggregation times were 8 h (g), 23 h (h), and 47 h (i). Mean values for toxicity assays performed in triplicate are presented in Fig. 7a. Au, arbitrary units.
inclusions. Additionally, Hsp70 overexpression in a transgenic mouse model of PD (15) was shown specifically to decrease high molecular mass and SDS-insoluble forms of αSyn. The observation that macroscopic aggregates do not enhance cellular toxicity highlights the importance of prefibrillar species of αSyn in the pathogenesis of PD. In the present study, the addition of Hsp70 alone to αSyn aggregation reactions inhibited fibril formation, although some aggregates were still observed (Fig. 1a, lower inset). Similar results were obtained by the addition of Hsp70 to aggregating huntingtin fragments (50), which did not result in the generation of additional aggregates. However, when co-chaperones and co-factors were included in the huntingtin aggregation reaction, an increase in the generation of aggregated species was observed (11, 50). Thus, Hsp70 action in vivo is likely to be coupled to other chaperones and co-factors, such as Hsp40 for assistance in aggregate binding, conversion, or inclusion formation (11, 50), or to another chaperone to facilitate disassembly and degradation of prefibrillar species (24, 53). However, our results indicate that Hsp70 alone preferentially binds to prefibrillar αSyn, an interaction that is likely to represent a key recognition step in the prevention of protein misfolding.

Our NMR binding studies reveal that Hsp70 does not interact with monomeric, “native” αSyn (Figs. 3 and 4), which is at first sight surprising because αSyn is believed to be an intrinsically disordered protein having no significant secondary or tertiary structure, and such an unfolded conformation would be thought to be a structural target for Hsp70. Instead, this result suggests that αSyn may possess a non-random structure that provides some measure of protection for its central and more hydrophobic region, which is thought to form the core of amyloid fibrils (54). We have recently obtained NMR data to support this hypothesis, finding that although αSyn appears to be substantially disordered, the highly charged C terminus makes long range contacts with the hydrophobic center region of the protein (55). Such contacts in αSyn have also been observed by fluorescent energy transfer techniques (56). Perturbation of these interactions is likely to expose hydrophobic stretches of αSyn, facilitating the formation of prefibrillar and fibrillar aggregates. Thus, the binding of Hsp70 to prefibrillar species is most likely mediated by the presence of increased amounts of exposed hydrophobic surfaces as compared with monomeric, native αSyn.

The cytotoxicity experiments we performed suggest the presence of an ensemble of prefibrillar species, including some that are capable of being pelleted by low speed centrifugation as a consequence of either their size or intrinsic insolubility (Fig. 7, c and d). Because of the heterogeneity of the prefibrillar fraction as observed by ourselves (Fig. 5a) and others (30), as well as the generic mechanism of Hsp70 for binding to hydrophobic sequences (39), it is probable that Hsp70 binds to a range of prefibrillar species, including very early aggregates or even non-native monomeric species. By effectively “capping” these hydrophobic species, Hsp70 appears to inhibit their maturation into amyloid fibrils and to shift their distribution to favor smaller, more soluble species (Fig. 7d). It is probable that the differential partitioning of such species will facilitate their degradation or conversion, a process that is likely to be of paramount importance in the light of our observation that αSyn toxicity arises from prefibrillar conformers.

The result that prefibrillar rather than fibrillar forms of αSyn possess the highest toxicity is supported strongly by experiments in Drosophila that suggest that proteinaceous inclusions are not harmful to dopaminergic neurons (8, 52). Similar results have also been obtained for Drosophila (16) and neuronal (51) models of Huntington’s disease. Additionally, oligomeric forms of αSyn have been shown to be toxic to neuronal cells when added exogenously (48). Prefibrillar species of αSyn and other proteins (47) have been observed to interact with and permeabilize membranes, leading to the formulation of the “amyloid pore” hypothesis (7, 47). Provided that conditions conducive to the formation of prefibrillar species are present, such a mechanism of toxicity would be likely to affect many cell types, regardless of whether or not the protein aggregates disrupt the cell membrane from the extra- or intra-cellular spaces.

Many proteins associated with debilitating human disorders such as type II diabetes, Alzheimer’s, Huntington’s, and Parkinson’s disease have been found to form prefibrillar species (47). Moreover, it has been found that even such species originating from traditionally benign proteins can be highly cytotoxic (13). This finding implies a common structure among prefibrillar species, as well as a common mechanism of pathogenesis. Recent evidence has strongly supported this concept by showing that sequence-independent conformational properties are shared among prefibrillar species derived from different proteins (48). The results presented here show that Hsp70 preferentially interacts with prefibrillar species to inhibit the formation of αSyn fibrils. We suggest, therefore, that Hsp70 may interact generally with structural features shared by prefibrillar species, regardless of their sequences, and that the recognition of such species is likely to represent a critical com-
ponent of a common cellular defense mechanism against dis-
eases of protein misfolding.

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