Heat Shock Proteins 70 and 90 Inhibit Early Stages of Amyloid β-(1–42) Aggregation in Vitro*

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Alzheimer disease is a neurological disorder that is characterized by the presence of fibrils and oligomers composed of the amyloid β (Aβ) peptide. In models of Alzheimer disease, overexpression of molecular chaperones, specifically heat shock protein 70 (Hsp70), suppresses phenotypes related to Aβ aggregation. These observations led to the hypothesis that chaperones might interact with Aβ and block self-association. However, although biochemical evidence to support this model has been collected in other neurodegenerative systems, the interaction between chaperones and Aβ has not been similarly explored. Here, we examine the effects of Hsp70/40 and Hsp90 on Aβ aggregation in vitro. We found that recombinant Hsp70/40 and Hsp90 block Aβ self-assembly and that these chaperones are effective at substoichiometric concentrations (~1:50). The anti-aggregation activity of Hsp70 can be inhibited by a nonhydrolyzable nucleotide analog and encouraged by pharmacological stimulation of its ATPase activity. Finally, we were interested in discerning what type of amyloid structures can be acted upon by these chaperones. To address this question, we added Hsp70/40 and Hsp90 to pre-formed oligomers and fibrils. Based on thioflavin T reactivity, the combination of Hsp70/40 and Hsp90 caused structural changes in oligomers but had little effect on fibrils. These results suggest that if these chaperones are present in the same cellular compartment in which Aβ is produced, Hsp70/40 and Hsp90 may suppress the early stages of self-assembly. Thus, these results are consistent with a model in which pharmacological activation of chaperones might have a favorable therapeutic effect on Alzheimer disease.

The amyloid diseases are a collection of protein misfolding disorders associated with the formation of distinctive fibrils (reviewed in Refs. 1–5). Alzheimer disease is one of the most common amyloid diseases, and it is characterized by fibrils composed of Aβ, a 39–43-amino acid proteolytic fragment of the amyloid precursor protein (reviewed in Ref. 6). Upon release from amyloid precursor protein, Aβ becomes enriched in β-sheet structure and acquires the propensity to self-assemble (7). Initial theories to explain the pathology of AD focused on the involvement of the visually striking fibrils, but more recent evidence has strongly supported a role for soluble oligomers (reviewed in Refs. 3 and 8–10). Aβ oligomers can be prepared in vitro (11, 12), biosynthesized by cultured cells (13, 14), or collected from AD tissues (15), and in all cases, these structures are highly neurotoxic. Despite these important observations, numerous questions about the basis of disease are unanswered. For example, although there is active research in this area (8, 11, 16), the number of Aβ monomers present in an oligomer has not been fully described. Moreover, the subcellular site(s) of oligomer production and the conditions that lead to their assembly are still debated; fibrils are found in extracellular space, but there is growing evidence that oligomers may be produced in intracellular compartments (8, 13, 17–19). Additional insights into the molecular mechanisms that contribute to AD are needed.

One of the important consequences of the oligomer hypothesis is that it implies that therapeutic benefits could arise from preventing early (i.e. pre-oligomeric) stages of amyloid formation. Accordingly, small molecules that directly block Aβ aggregation (20–22), reduce its production from amyloid precursor protein (23–25), decrease soluble amyloid load (26, 27), and enhance progression to fibrils (28, 29) are being actively explored (for a review see Ref. 30). The goal of most of these approaches is to intervene at a stage prior to oligomerization and prevent accumulation. Consistent with this idea, it has been suggested that stimulating natural cellular mechanisms that combat protein aggregation, including proteases that digest Aβ and chaperones that inhibit misfolding, might have therapeutic benefit (31–33).

Molecular chaperones are involved in many important aspects of protein homeostasis, including folding, degradation, and subcellular trafficking (for recent reviews see Refs. 34–36). These tasks typically involve the heat shock proteins, such as Hsp70 and Hsp90, which are chaperones that recognize misfolded polypeptides and use energy-driven cycles of substrate binding and release to favor productive folding (reviewed in Refs. 37 and 38)). One of the important consequences of chaperone binding is that deleterious protein aggregation is prevented. Consistent with this activity, numerous studies have suggested that heat shock proteins are important for the prevention of amyloid formation (31, 39–43). For example, genetic overexpression of Hsp70 has been shown to reduce amyloid-related phenotypes in mouse models of spinocerebellar ataxia type 1 (44) and Parkinson disease (45). Similar results

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2 The abbreviations used are: Aβ, amyloid β-(1–42); AD, Alzheimer disease; ATPγS, adenosine (5’-thio) triphosphate; BSA, bovine serum albumin; DMEM-F12, Dulbecco’s modified Eagle’s medium with F12 nutrients; MeSO, dimethyl sulfoxide; HSF-1, heat shock factor-1; Hsp, heat shock protein; PBS, phosphate-buffered saline; TEM, transmission electron microscopy.

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are seen in Drosophila melanogaster (46), Saccharomyces cerevisiae (47, 48), and Caenorhabditis elegans (49) models of neurodegeneration. These experiments suggest that chaperones, particularly Hsp70, may bind amyloidogenic peptides and help prevent disease by restoring the balance between aggregation and folding. This hypothesis is supported by biochemical evidence in some systems. For example, purified Hsp70 blocks aggregation of α-synuclein, a protein implicated in Parkinson disease, in vitro (50). Similarly, addition of Hsp70 and its co-chaperone partner, Hsp40, to Huntingtin, an aggregation-prone protein that causes Huntington disease, blocks oligomer formation (43, 51). In addition to these findings, other chaperones, such as Hsp20 (52) and Hsp104 (53), have been shown to limit amyloid formation in vitro. Together, the combination of genetic and biochemical evidence provides compelling support for a direct role of chaperones in these diseases.

In Alzheimer disease, genetic evidence to suggest a role for chaperones has been reported (54), but corresponding biochemical analysis is lacking. Similar to what occurs in other disease models, Hsp70 overexpression improves the viability of cells that express excess Aβ (54). Additionally, abundant chaperone levels block formation of Aβ aggregates in C. elegans (49). Biochemical evidence would help elucidate the mechanism(s) responsible for these favorable effects. For example, the type of Aβ structure (e.g. monomers, oligomers, and/or fibrils) bound by chaperone is not known. Moreover, it is not clear how much chaperone is required or if this process is ATP-driven. Here we define a role for specific molecular chaperones, Hsp70, Hsp40, and Hsp90, in the prevention of Aβ fibril formation in vitro. The results of these experiments and a comparison with what has been observed in other amyloid diseases are discussed.

EXPERIMENTAL PROCEDURES

Preparation of Amyloid β—Synthetic amyloid β-(1–42) (AnaSpec, San Jose, CA) was prepared for aggregation according to methods developed previously (55, 56). Briefly, lyophilized Aβ was resuspended in hexafluoroisopropanol, dried under a nitrogen stream, and stored as a film at −20 °C. Immediately prior to use, Aβ was resuspended in Me2SO to 10 mM and sonicated for 10 min. For experiments in which early stages of aggregation were studied, these aliquots were rapidly brought to 25 μM in phosphate-buffered saline (PBS), pH 7.2, and used immediately. Oligomers were prepared by diluting the Aβ to 25 μM with phenol red-free DMEM-F12 and incubating for 24 h at 4 °C without shaking. Fibrils were similarly prepared by incubating 25 μM Aβ in PBS at 37 °C for 24 h with vigorous shaking.

Turbidity Measurements—Human Hsp70, Hsp40, and Hsp90 were provided by Assay Designs (Ann Arbor, MI). Hsp70 (catalog number ESP-555) is endotoxin-free, recombinant human protein expressed and purified from bacteria; Hsp40 (SPP-400) is also recombinant human protein and from bacteria; Hsp90 (SPP-770) is human and purified from HeLa cells. Concentrated stocks (×100) of these proteins or buffer control were dispensed into the wells of 96-well, half-volume, clear bottom plates (Corning Glass, Corning, NY). To these solutions, Aβ in either PBS or DMEM-F12 was added. In the final volume (75 μl), Aβ was present at ~25 μM and ATP or ATPγS at 9 mM, and the heat shock proteins were at the indicated concentrations. Plates were immediately placed in a prewarmed SpectraMax M5 multimode plate reader, and the turbidity measurements were initiated. The turbidity program began with a 20-s mixing shake and was followed by absorbance readings at 330 or 350 nm every 60 s. A 20-s shaking step immediately followed each reading, followed by 40 s of settling time. The temperature was set at 30 or 37 °C (see figure legends). For Hsp70/40-treated samples, the listed concentration is the concentration of Hsp70, and the Hsp40 was held at 100-fold lower value. For example, treatment with 474 nm Hsp70/40 involved final concentrations of 474 nm Hsp70 and 4.7 nm Hsp40.

Electron Microscopy—At the conclusion of the turbidity measurements, 25-μl aliquots were removed from each well and immediately frozen at −80 °C. Thawed samples were placed on glow-discharged Formvar-coated 300-mesh copper grids (Electron Microscopy Sciences) for 1 min, washed twice with distilled water, and treated with 3% uranyl acetate for 1 min. Images were taken at 80 kV at magnifications between 46,000 and 130,000. Image quantitation was performed with NIH Image using at least 10 random fields.

Thioflavin T Experiments—Immediately following removal of samples for electron microscopy, the remaining volume from the turbidity experiments (50 μl) was treated with 75 μl of freshly prepared 50 mM glycine, pH 8.0, containing 25 μM thioflavin T. After 10 min at room temperature, the fluorescence was measured on a SpectraMax M5 multimode plate reader using an excitation of 440 nm and emission of 490 nm (475 nm cut-off). The reported values have been corrected by subtracting the background fluorescence of thioflavin T in the absence of amyloid.

Synthesis—The synthesis of SW02, SW08, and related compounds is described in more detail elsewhere. Briefly, SW02 is prepared by coupling the urea of γ-aminobutyric acid to Wang resin in dimethylformamide using standard conditions. After washing to remove excess coupling agents, an acid-catalyzed three-component Biginelli reaction was performed in the presence of four equivalents of p-bromobenzylaldehyde and ethyl acetooacetate (58). The yields and kinetics of this reaction were greatly enhanced by the use of microwave-accelerated conditions (110 °C, 40 min; Biotage Initiator EXP). The yellow product was cleaved from resin with 50% trifluoroacetic acid and purified by high pressure liquid chromatography. SW02 was characterized by mass spectrometry (calculated m/z = 438.06; observed = 439.0 [M + H]) and 1H NMR. SW08 was prepared in similar fashion (expected m/z = 737.4; observed = 738.4 [M + H]). Note that in experiments outlined in Fig. 4, SW02 was added to chaperone concentrations (119 nm) selected to be suboptimal for preventing aggregation. This was done to favor visualization of the effects of the small molecule. However, this chaperone concentration occasionally inhibited aggregation in the absence of drug, and in these cases, SW02 was unable to boost the potency further.

3 S. Wisén and J. E. Gestwicki, submitted for publication.


**RESULTS**

Recombinant Hsp70/40 Blocks Aggregation of Freshly Prepared Amyloid β-(1–42)—Results from overexpression experiments have suggested a role for Hsp70 in the aggregation of amyloid β (54), but evidence for this interaction in vitro is lacking. To explore this question, freshly prepared amyloid β-(1–42) was treated with recombinant Hsp70 and its co-chaperone Hsp40 in the presence of excess ATP. Hsp40 stimulates the ATPase activity of Hsp70 (59, 60) at a low molar ratio (100:1). Thus, the goal of these experiments was to establish if an activated Hsp70/40 combination could inhibit amyloid formation. In our initial experiments, we monitored turbidity measurements reveal that substoichiometric chaperone levels can block aggregation. The concentrations of amyloid, Hsp70/40 (molar ratio 100:1), and ATP are indicated. The blank control is buffer alone (i.e. no amyloid β). Results are the average of triplicate wells and are representative of other experiments. Error (mean ± S.D.) is represented by the error bars on the penultimate time points. B, thioflavin T fluorescence assays were performed on samples at the conclusion of the turbidity assay (90 min). Results are the average of 2–4 experiments each in triplicate. In each case, the fluorescence was normalized by subtracting the blank sample (20–50 fluorescence units). C, analysis of samples by transmission electron microscopy at the conclusion of the turbidity experiments (90 min). The inset (right panel) shows occasional (~1%) short fibrils observed in the Hsp70/40-treated samples. The dark arrows point to fibrils and the light arrows to small, roughly spherical structures.

**Thioflavin T assay.** Thioflavin T is an indicator dye that becomes strongly fluorescent in the presence of amyloid (61). Confirming the turbidity results, there was a strong dose-dependent decrease in fluorescence after 80 min of treatment with chaperones (Fig. 1B). Finally, we used transmission electron microscopy (TEM) to examine the ultrastructure of the chaperone-treated samples. These experiments revealed that, rather than forming fibrils, Aβ was redirected into roughly circular structures by Hsp70/40 (Fig. 1C). Although these spheres were prevalent, short fibrils were occasionally observed, which suggests that some Aβ escapes the block. Furthermore, the barrier to aggregation was not complete; over the time course studied, the initial rate of assembly was slowed but not entirely stopped.

The Combination of Hsp70/40 Is More Effective than Hsp70 Alone—Hsp40 is a known co-chaperone and stimulator of the ATPase activity of Hsp70 (62). Thus, we were interested in understanding if the combination of Hsp70/40 is more effective than Hsp70 alone. This result would suggest that ATPase activity is important for the anti-aggregation function of Hsp70. To test this idea, we treated Aβ with Hsp70 or the combination (100:1 Hsp70:40) and monitored aggregation by the thioflavin T assay. Consistent with reports in other systems (50), Hsp70 alone blocks aggregation (Fig. 2). However, when we added low levels of Hsp40 to Hsp70, we found an enhancement over the chaperone alone. As a control, we tested whether Hsp40 could inhibit aggregation in the absence of its partner. We found that Hsp40 failed to block Aβ self-assembly, even at 10-fold higher concentrations (50 nM) than those used in the previous experiments. Therefore, we conclude that, although both Hsp70 and Hsp70/40 prevent amyloid formation in vitro, the combination is most effective.

Recombinant Hsp90 Blocks Aggregation of Freshly Prepared Amyloid β-(1–42)—Although Hsp70 and Hsp40 have both been implicated in counteracting amyloid formation in certain neurodegenerative systems, Hsp90 has attracted less attention. In one in vitro model, Hsp90 has been shown to be insufficient to prevent amyloid formation (43). However, Hsp90 is an important and highly abundant component of the chaperone machinery that shares some pro-folding tasks with other heat shock proteins (for a recent review see Refs. 36 and 37). To explore potential roles of this chaperone in Aβ aggregation, we performed similar experiments to those described in the pre-
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As part of these studies, we also were interested in determining if any protein might substitute for the chaperones. To explore this possibility, we attempted to block amyloid formation with bovine serum albumin (BSA). At the highest concentrations used (474 nM, equivalent to the top chaperone levels employed), no effect on Aβ aggregation was observed (Fig. 4). These results are consistent with previous reports using non-chaperone proteins (43), and demonstrate that the chaperone interaction cannot be replaced by contacts with the BSA interface.

An Hsp70 Agonist Promotes Anti-aggregation Activity—Because our results indicate that ATPase activity is required for chaperones to fully block aggregation, we hypothesized that agonists (particularly those that promote turnover by Hsp70) might stimulate this function. The natural product analog, 15-deoxyspergualin, is an agonist of Hsp70 that binds outside the nucleotide-binding site (63–65). Recent work by Brodsky and co-workers (66, 67) has shown that certain synthetically accessible dihydropyrimidines, which structurally mimic 15-deoxyspergualin, can likewise enhance ATPase activity and regulate protein translocation. Based on these studies, we have recently developed a route to the modular synthesis of additional dihydropyrimidines and shown that some of these molecules modify the chaperone-mediated refolding of denatured luciferase.3 For example, we found that one of these compounds, SW02, enhanced folding ~20% in vitro. Conversely, a related compound, SW08, blocked folding by ~50%. Based on these activities, we hypothesized that SW02 (Fig. 5A) might stimulate the ability of the Hsp70 to block Aβ aggregation, and SW08 might be inhibitory. To test this theory, we started with an Hsp70 concentration, 119 nM, that was insufficient to block aggregation. Under these conditions, we propose that aggregation could only be inhibited if the chaperone activity were artificially enhanced. When SW02 was added to this suboptimal chaperone pool, significant anti-aggregation activity was gained (Fig. 5B). In control experiments, SW02 had no effect in the presence of Hsp40 or Hsp90. Moreover, in the absence of any chaperone, SW02 did not alter Aβ aggregation. Thus, these results suggest that SW02 is relatively specific for Hsp70 and that it indirectly promotes anti-aggregation activity. To gain further evidence in support of this idea, we used a higher concentration of Hsp70 (474 nM) and added the antagonist SW08. This treatment partially blocked the function of Hsp70 and restored aggregation (Fig. 5B). Thus, agonists and antagonists could be used to adjust or “tune” chaperone activity.

The Potency of SW02 Is Modest—To determine efficacy of SW02, we varied the concentration of the agonist at a constant level of Hsp70/40 (119 nM) and measured Aβ aggregation by turbidity and thioflavin T assays (Fig. 6). In both platforms, we found that high concentrations of SW02 (between 100 and 500 μM) were required to activate the chaperone. By TEM, treatment with Hsp70/40 and 500 μM compound yielded similar Aβ structures to those produced by treatment with higher concentrations of unstimulated chaperone (Fig. 6C). Together, these results suggest that, although the potency of SW02 is modest, promoting the anti-aggregation activity of Hsp70/40 can enhance inhibition in vitro.
Chaperones Recognize Amyloid Oligomers but Have Mild Effects on Mature Fibrils—Despite the characteristic presence of Aβ fibrils in the brains of AD patients, recent evidence suggests that oligomers are critical in the development of disease. Therefore, we sought to determine whether chaperones would have any effects on pre-formed oligomers or mature fibrils. The impetus for these experiments was to understand (in an in vitro system) the types of amyloid forms that can be recognized by chaperones. The answer to this question might provide insight into how chaperones counteract Aβ toxicity in vivo. Moreover, we (and others) have an interest in pharmacological stimulators of chaperone activity. These efforts would benefit from additional mechanistic knowledge about the types of amyloid structures that can be modified by chaperones. For example, if mature fibrils were dissolved by chaperones and processed into toxic oligomers, this could have a strongly negative effect on the organism. Moreover, if chaperones only act on freshly prepared Aβ samples (as used in Figs. 1–3 above), this suggests that pre-existing oligomers might be safe from chaperone intervention. Because of these questions, we decided to supplement our studies with experiments that explore the effects of chaperones on pre-formed oligomers and fibrils in vitro.

By varying the temperature and buffer conditions used to prepare Aβ, we were able to produce solutions of soluble oligomers or fibrils, consistent with previous reports (55, 56). After formation of these structures, chaperones and ATP were added and any effects measured by thioflavin T fluorescence and TEM. Treatment of preformed fibrils with Hsp70/40 or Hsp90 had little effect on the thioflavin T fluorescence (Fig. 7). Because the native cellular environment might possess both of these chaperone systems, we also combined them. In samples treated with the Hsp70/40/90 mixture, there was a small but not sta-
the fibril length tended to be shorter in the Hsp70/40/90-treated samples. To further investigate this observation, we used image analysis to quantify average fibril length. Although this result is not striking, there was a slight tendency for chaperone-treated samples to contain fewer long fibrils. However, because this effect is subtle, we conclude that these chaperones have very little impact on fibrils.

Preformed oligomers were treated with chaperones and studied by thioflavin T fluorescence and TEM. Under these conditions, Hsp70/40 did not measurably change the thioflavin T reactivity or structure of oligomers (Fig. 7). The combination of Hsp70/40/90, however, caused an initial increase in fluorescence followed by steady drop. Hsp90 alone could produce similar effects, but these changes were more pronounced in the triple chaperone-treated samples. To study the ultrastructure of the treated oligomers, we studied the resulting solutions by TEM. The oligomers in the samples treated for 240 min with Hsp70/40/90 become noticeably less defined and more diffuse. This effect was most obvious during attempts to focus the images; it was not possible to capture images in which the oligomer boundaries were well defined. Additional experiments will be required to further characterize this effect, but these results suggest that Hsp70/40/90 can alter the structure of pre-formed oligomers.

**DISCUSSION**

Self-assembly of $\alpha$-helical $\beta$-sheet (Aβ) produces a number of distinctive structures, such as dimers, oligomers, unstructured aggregates, and characteristic amyloid fibrils. Of these structures, oligomers are believed to be the most neurotoxic and important in the development of disease (3, 8, 9). Thus, any strategies to reduce amyloid-related phenotypes must avoid producing toxic oligomers from relatively benign structures, such as fibrils. In genetic models of AD, Hsp70/40 overexpression reduces cell death, which is a result that is consistent with decreased oligomer load. Moreover, in a D. melanogaster model...
of Huntington disease, Hsp70 overexpression inhibits neurodegeneration without preventing the formation of large inclusions (46). These results provide indirect evidence that chaperones specifically inhibit early stages of aggregation. However, we reasoned that biochemical support for this conclusion would enhance our understanding of the types of amyloid structures recognized by chaperones. These considerations led us to explore the interaction of chaperones with various types of Aβ structures in vitro. Specifically, we have studied the effects of Hsp70/40 and Hsp90 on three types of amyloid structures as follows: freshly prepared Aβ structures, oligomers, and fibrils. Although the exact temporal and causal relationships between these structures are unclear, we use the freshly prepared samples and oligomers as representative of earlier stages in self-assembly. This distinction is partially based on the observation that aged Aβ preparations and the brains of terminal AD patients often contain elongated fibrils (68). Using this paradigm, we conclude that Hsp70/40 and Hsp90 specifically influence aggregation at "early" stages in the process; freshly prepared Aβ, oligomers, and fibrils. Although the exact temporal and causal relationships between these structures are unclear, we use the freshly prepared samples and oligomers as representative of earlier stages in self-assembly. This distinction is partially based on the observation that aged Aβ preparations and the brains of terminal AD patients often contain elongated fibrils (68). Using this paradigm, we conclude that Hsp70/40 and Hsp90 specifically influence aggregation at "early" stages in the process; freshly prepared Aβ (Figs. 1–3) and oligomers (Fig. 7) were most susceptible, whereas fibrils were less affected (Fig. 7). This result is consistent with findings in other systems that suggest an early role for chaperones in blocking protein aggregation (43, 50, 51). One interesting question related to this observation is whether samples treated with chaperones in vitro are neurotoxic. We have not characterized toxicity in this system, but chaperones are protective in other systems (3, 8–10), and we predict that treated Aβ will also be less toxic.

What is the molecular mechanism used by chaperones to inhibit Aβ self-assembly? Although the details of this process remain unclear, we will discuss our results in the context of two specific models (Fig. 8). In the "holding" model, the chaperone binds misfolded amyloid (likely via its substrate-binding domain). We propose that this interaction decreases free monomer concentration and thereby slows the rate of self-assembly, which is known to be dependent on monomer availability (68–70). This model predicts that inhibition would be independent of ADP-ATP exchange because cycles of substrate release do not contribute to the inhibitory mechanism. Some of our results are consistent with this prediction. Specifically, we report that Hsp90 is only partially inhibited by ATP. Thus, for this chaperone, we speculate that a holding or partitioning model describes some of the observations. However, Hsp90 was effective at substoichiometric concentrations (Fig. 3). This result seems at odds with the holding model because one might presume that a 1:1 (or greater) stoichiometry should be optimal. The dependence of Aβ aggregation on monomer concentration might partly resolve this apparent conflict; decrease in available monomer is expected to directly influence rate. Thus, removal of monomer might be effective even if only a portion of
the total pool is deactivated. Similar arguments have been made for other homo-polymerization reactions, such as actin filament assembly, that are dependent on the level of competent monomer (71). Further analysis of this model awaits, but we cannot dismiss the possibility that Hsp70/40 partly accesses a holding mode, and we consider it likely that the behavior of Hsp90 is consistent with this mechanism.

A more active role for chaperones is proposed in the “refolding” model (Fig. 8B). In this model, chaperones not only bind Aβ but they also change its structure; release from chaperone is coupled to conversion of Aβ to an altered state. The altered structure is defined as being less competent for progression through the aggregation pathway (or, alternatively, better able to travel a nonproductive route (51)). How would this structural modification occur? Some clues might be gleaned by studying the function of Hsp70 during normal de novo protein biosynthesis. Current models suggest that Hsp70 acts by “entropic pulling” (72); chaperone binding to extended polypeptides precludes certain entropically accessible conformations. Thus, the presence of Hsp70 forces the peptide to sample other conformations, which likely have fewer exposed hydrophobic regions. Therefore, under this model, chaperone would bind Aβ in an aggregation-prone state (i.e. exposed hydrophobic regions and primed for self-association) and release it in a modified conformation. We propose that this model is consistent with our data on Hsp70. For example, we found that the ATPase activity of Hsp70 was required to inhibit aggregation (Fig. 4) and that its potency could be stimulated by co-chaperone (Fig. 2) or an agonist (Fig. 5). These results suggest that ATP-driven cycles of substrate binding-and-release are important. Furthermore, the refolding model predicts that a low molar ratio of chaperone could be effective if a single enzyme acts on multiple substrates. We found this to be true for both Hsp70/40 and Hsp90 (Figs. 1 and 3). Thus, although more data are certainly required, we speculate that Hsp70/40 might actively process Aβ in a refolding model. Whether this conclusion describes the behavior of other combinations of chaperones and misfolded peptides is unclear.

An important aspect of both models (Fig. 8) is that neither Hsp70/40 nor Hsp90 are effective at dealing with fibrils. Based on our results (Fig. 7) and observations from other systems (50, 51), we suggest that chaperones may have difficulty recognizing hydrophobic regions embedded in fibrils. Alternatively, the interaction energy supplied by extensive monomer-monomer contacts may be an insurmountable barrier to chaperone-mediated reorganization (at least by the chaperones tested). Regardless, our results suggest that fibrils are not readily reversible by the action of Hsp70/40 or Hsp90. Oligomers, on the other hand, seem susceptible to manipulation by the tri-chaperone system (Fig. 7). This suggests that these structures contain sufficiently exposed hydrophobic “flags” to trigger chaperone recognition and subsequent reorganization.

Recently, an interesting hypothesis was developed by Morimoto and co-workers (73, 74) to explain the favorable effects of chaperone overexpression. This “sink hypothesis” states that
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cellular toxicity might develop because chaperones and other proteins are being sequestered onto amyloid fibrils and re-directed from their normal tasks. Under this model, depleted cellular levels of these factors may trigger apoptosis by decreasing the levels below a threshold needed to maintain necessary processes (75). Thus, chaperone overexpression might circumvent the problem by supplying a less exhaustible supply. Moreover, higher chaperone levels might block other proteins from becoming trapped by the “sink.” This intriguing hypothesis has been developed around work with Huntington disease, but it might be more generally applicable to other amyloid disorders. For example, we have shown that Hsp70/40 and Hsp90 can influence early stages of Aβ aggregation but that fibrils are not significantly changed. Although direct evidence is still needed, these results suggest that chaperones might accumulate on fibrils because they are unable to process these structures.

An interesting component of these discussions is whether oligomers and pre-oligomeric Aβ are normally present in the same subcellular space as chaperones. Chaperones are expressed in all intracellular regions, including the nucleus, secretory pathway, and cytoplasm. Aβ fibrils are found in extracellular regions, but the exact location of oligomer production is unclear. Recent reports have examined extracellular (76–79) and intracellular (18, 49, 54, 80–85) models. Thus, the exact mechanism by which the protective effect of chaperones is manifested awaits definition. For example, it is not clear if chaperones simply provide a general cytoprotection or if direct physical interaction is required. Regardless, our results strongly suggest that if early Aβ structures are formed in the presence of Hsp70/40 or Hsp90, these proteins prevent further self-assembly.

Our results support a hypothesis in which stimulation of chaperone activity may be a viable means of therapy for neurodegenerative diseases. Previous studies to test this idea were conducted in mouse (86, 87) and tissue culture (88, 89) models of neurodegenerative disease. For example, an analog of the natural product geldanamycin was shown to promote the clearance of Huntingtin in a mouse model of Huntington disease. This drug is proposed to be anti-neurodegenerative by virtue of its ability to activate the transcription factor, heat shock factor-1 (HSF-1) (86, 87). HSF-1 controls transcription of proteins involved in the stress response, including Hsp70. Thus, geldanamycin may alleviate amyloid-related phenotypes via mimicking the effects of Hsp70 overexpression. In another approach, Morimoto and co-workers (57) screened a chemical library for compounds that could modify amyloid formation in a yeast model of Huntington disease. They reported the discovery of celestrols that, like geldanamycin, stimulate HSF-1 and inhibit amyloid formation. Both these strategies take advantage of cellular mechanisms for coping with stress. Here we suggest a complementary approach that involves direct stimulation of Hsp70. Based on pioneering work (66, 67), we have synthesized an agonist of Hsp70 and tested its activity in vitro. This molecule, SW02, was able to compensate for insufficient chaperone levels and promote anti-aggregation activity. Compounds that have this effect in vitro might activate endogenous Hsp70 and combat neurodegenerative diseases without concomitant involvement of a stress response. One compelling aspect of this approach is that it relies on boosting a physiological mechanism that normally protects asymptomatic individuals.

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