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Small heat-shock proteins prevent a-synuclein aggregation via transient interactions and their efficacy is affected by the rate of aggregation

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Abstract

The aggregation of a-synuclein (a-syn) into amyloid fibrils is associated with neurodegenerative diseases, collectively referred to as the a-synucleinopathies. In vivo, molecular chaperones, such as the small heat-shock proteins (sHsps), normally act to prevent protein aggregation; however, it remains to be determined how aggregation-prone a-syn evades sHsp chaperone action leading to its disease-associated deposition. This work examines the molecular mechanism by which two canonical sHsps, aB-crystallin (aB-c) and Hsp27, interact with aggregation-prone a-syn to prevent its aggregation in vitro. Both sHsps are very effective inhibitors of α -syn aggregation, but no stable complex between the sHsps and a-syn was detected, indicating that the sHsps inhibit a-syn aggregation via transient interactions. Moreover, the ability of these sHsps to prevent a-syn aggregation was dependent on the kinetics of aggregation; the faster the rate of aggregation (shorter the lag phase), the less effective the sHsps were at inhibiting fibril formation of a-syn. Thus, these findings indicate that the rate at which a-syn aggregates in cells may be a significant factor in how it evades sHsp chaperone action in the a-synucleinopathies.

Keywords

proteins, prevent, a-synuclein, aggregation, via, transient, small, interactions, heat-shock, their, efficacy, affected, rate

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Small heat-shock proteins prevent α -synuclein aggregation via transient interactions and their efficacy is affected by the rate of aggregation

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Running title: *Small heat-shock proteins interact transiently with α -synuclein*

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Key words: protein misfolding, molecular chaperone, amyloid fibril, Parkinson's disease

ABSTRACT

The aggregation of α -synuclein (α -syn) into amyloid fibrils is associated with neurodegenerative diseases, collectively referred to as the α -synucleinopathies. *In vivo*, molecular chaperones, such as the small heat-shock proteins (sHsps), normally act to prevent protein aggregation; however, it remains to be determined how aggregation-prone α -syn evades sHsp chaperone action leading to its disease-associated deposition. This work examines the molecular mechanism by which two canonical sHsps, α B-crystallin (α B-c) and Hsp27, interact with aggregation-prone α -syn to prevent its aggregation *in vitro*. Both sHsps are very effective inhibitors of α -syn aggregation, but no stable complex between the sHsps and α -syn was detected, indicating that the sHsps inhibit α -syn aggregation via transient interactions. Moreover, the ability of these sHsps to prevent α -syn aggregation was dependent on the kinetics of aggregation; the faster the rate of aggregation (shorter the lag phase), the less effective the sHsps were at inhibiting fibril formation of α -syn. Thus, these findings indicate that the rate at which α -syn aggregates in cells may be a significant factor in how it evades sHsp chaperone action in the α -synucleinopathies.

INTRODUCTION

α -Synuclein (α -syn) is a protein primarily found in neuronal tissue, where it is predominantly localised to presynaptic terminals (1). The definitive function of α -syn is yet to be established, however it has been implicated in modulating synaptic activity through membrane processes, including membrane biogenesis, vesicular trafficking and neurotransmitter release (2,3). Although it has traditionally been considered an intrinsically disordered protein (4,5), there have been recent studies indicating that it may form an alpha-helical tetramer *in vivo* (6,7). In addition, α -syn aggregates to form amyloid fibrils under physiological conditions. The deposition of fibrillar α -syn into inclusion bodies, known as Lewy bodies or Lewy neurites when they are present in the cell body or processes respectively, is a pathological hallmark of a number of neurodegenerative disorders, including Parkinson's disease, dementia with Lewy bodies and multiple system atrophy, collectively referred to as the α -synucleinopathies (8). Moreover, multiplication of *SNCA*, which encodes for α -syn, or missense mutations (A53T, A30P and E46K) in this gene, cause familial early-onset Parkinson's disease (9-11). Under conditions of physiological

pH and temperature, purified recombinant α -syn forms fibrillar aggregates *in vitro* via a nucleation-dependent mechanism (12). The lag phase in this process, which is the rate-limiting step, corresponds to the formation of oligomeric nuclei from aggregation-prone intermediates. This is followed by the rapid growth of nuclei into fibrillar species (elongation phase) until thermodynamic equilibrium is reached (plateau phase) between monomeric and aggregated α -syn forms (13).

While the main component of Lewy bodies and Lewy neurites is α -syn, auxiliary proteins, including small heat-shock proteins (sHsps), have also been identified in these inclusions (14-16). The expression of sHsps is dramatically upregulated in response to cellular stress (17), ageing (18) and in some protein aggregation disorders, including the α -synucleinopathies (15). The sHsps are an integral component of the cellular network that acts to maintain protein homeostasis (proteostasis) (19,20). This family of molecular chaperones is defined by the relatively small molecular mass of their monomeric subunits and the presence of a central, conserved α -crystallin domain, which is flanked by variable N- and C-terminal regions (21). The sHsps are often referred to as 'holdase' chaperones, although this does not fully describe their chaperone action (22). The sHsps prevent protein aggregation by recognising, interacting with and stabilising partially folded (hydrophobic) intermediate states of target proteins that have left the protein on-folding pathway and entered an off-folding pathway (23-25). As their chaperone action is ATP-independent, sHsps are specialised for preventing inappropriate associations that could otherwise lead to aggregation in the energy-depleted cell (26). A defining feature of some mammalian sHsps (there are 10 in humans, HSPB1-10), such as Hsp27 (HspB1) and α B-crystallin (α B-c; HspB5), is that they form large polydisperse oligomers in solution which undergo rapid subunit exchange (27,28). The consensus model of sHsp chaperone action is that small, dissociated species are the chaperone active species as these likely contain a higher relative degree of exposed hydrophobicity than the larger oligomers (27). The larger oligomers are therefore regarded as reservoirs of these chaperone-active dissociated forms. Based on this model, the rate of

subunit exchange governs the speed of production of chaperone-active species capable of interacting with aggregation-prone states of target proteins. Phosphorylation at three key residues, Ser15, Ser45 and Ser59 in α B-c (29) or Ser15, Ser78 and Ser82 in Hsp27 (30,31), has been suggested to be an additional regulatory mechanism with regards to chaperone function of sHsps during times of cellular stress (32-34).

The ability of sHsps such as α B-c and Hsp27 to prevent the fibrillar formation of α -syn *in vitro* is well established (35-37). However, the mechanism by which sHsps achieve this inhibition remains to be definitively established (38). Moreover, given the α -synucleinopathies (and other diseases) are associated with protein aggregation, it is clear that, under certain circumstances, aggregation-prone proteins can evade the chaperone action of the sHsps. This failure of the sHsps to prevent aggregation is often attributed to them being "overwhelmed" in the context of disease (39-41). However, specific factors that lead to sHsp chaperone activity being overwhelmed are yet to be determined. In this study, we have directly addressed this by characterising the nature of the interaction between the archetypal sHsps, α B-c and Hsp27, and monomeric α -syn. We show that these chaperones prevent α -syn aggregation through transient interactions and that the chaperone efficacy of these sHsps in preventing α -syn fibril formation is highly dependent upon the rate at which α -syn aggregates.

RESULTS

sHsps inhibition of α -syn aggregation is concentration dependent – The amyloid fibrillar aggregation of wild-type α -syn has been well characterised *in vitro* (35). As expected, there was no increase in Thioflavin-T (ThT) fluorescence when Hsp27, BSA or buffer were incubated alone. However, incubation of α -syn under these experimental conditions resulted in an increase in ThT fluorescence over time indicative of fibril formation (Figure 1A). This increase in ThT fluorescence was inhibited by the addition of Hsp27 at a 1:1 (α -syn:Hsp27) molar ratio, but was not inhibited by the presence of the non-chaperone control protein bovine serum albumin (BSA). The change in ThT associated with α -syn aggregation can be fitted by a Boltzmann sigmoidal curve.

This reveals that when α -syn was incubated alone there was a lag phase of 15 ± 3 h, and the maximum ThT fluorescence occurred after 36 ± 3 h (Figure 1B). Addition of the sHsps α B-c and Hsp27 prevented the fibrillar aggregation of α -syn in a concentration-dependent manner (Figure 1B). At a 1:10 molar ratio (sHsp: α -syn), α B-c and Hsp27 inhibited the change in ThT fluorescence associated with α -syn fibril formation by $65 \pm 4\%$ and $74 \pm 5\%$ respectively (Figure 1C).

In preventing the fibrillar aggregation of α -syn the sHsps do not form stable, high molecular mass sHsp-target protein complexes – To investigate whether the sHsps inhibit the fibril formation of α -syn via the formation of high molecular mass complexes, as has been reported for the interaction with amorphously aggregating proteins (22,42,43), the samples containing a 1:1 molar ratio (i.e. a concentration corresponding to near complete inhibition of fibril formation of α -syn, see Fig. 1B) were collected after the aggregation assay for subsequent analysis by size-exclusion chromatography (Figure 2A). Prior to incubation, in the absence of sHsp, α -syn eluted from the column in a peak centred at 17 mL. Following incubation of α -syn in the absence of sHsp, there was very little soluble α -syn present in the sample, consistent with its aggregation into fibrils. When present alone in solution, α B-c eluted as a well resolved peak at 13 mL, consistent with it being a large polydisperse oligomer of average mass ~ 650 kDa under these solution conditions (44,45). In the sample containing both α B-c and α -syn, there was no detectable shift in the elution volume or size of the individual peaks corresponding to monomeric α -syn and oligomeric α B-c, or the appearance of any additional peaks. Thus, there was no significant difference in the amount of soluble oligomeric α B-c and monomeric α -syn in this sample compared to when each of these (non-aggregated) proteins were analysed alone. The presence of only α B-c in the peak eluting at 13 mL and only α -syn in the peak eluting at 17 mL in this sample was confirmed by immunoblotting (Figure 2B) (the detectable limits of the immunoblotting procedure used in this work was ~ 30 nM for each protein). Even when a crosslinker, bis[sulfosuccinimydyl] (with a spacer arm length of 8 atoms or 11.4 Å), was added to the sample following incubation and prior to size

exclusion chromatography, there was no evidence of co-elution of α B-c and α -syn from the column (data not shown). Thus, under the conditions used in this work, we found no evidence of a stable high-molecular mass complex formed between α B-c and aggregation-prone α -syn. Similar results were obtained for samples containing Hsp27 and α -syn (Figure 2B, D).

We sought to further investigate the interaction between these sHsps and aggregation-prone α -syn by analytical ultracentrifugation (AUC). Due to the propensity of the wild-type sHsps to form large, high molecular mass, polydisperse oligomers in solution (see Fig 2A), variant forms of these sHsps that either mimic phosphorylation (Hsp27_{3D}) or consist of only the core α -crystallin domain (α B-c_{core}) were selected for use in these experiments. This is because these variants exist predominately as either monomers or dimers (46,47) and therefore their molecular masses more closely match that of α -syn (an important factor in these absorbance-based AUC experiments as it ensures that both species sediment at similar rates). We first established that both these variant forms also inhibit the fibrillar aggregation of α -syn at similar levels to the wild-type proteins (Figure 3A). Notably, the ability of the core domain (i.e. α B-c_{core}) to inhibit α -syn aggregation indicates that the ability of the sHsps to prevent α -syn fibril formation is inherent to the α -crystallin domain, and does not require the N- or C-terminal regions.

Sedimentation velocity (SV) profiles for α -syn alone, and in the presence of either Hsp27_{3D} or α B-c_{core} are shown in Figure 3. These profiles were fitted using a c(s) model which considers both sedimentation and diffusion to determine sedimentation coefficients for the species in solution (48). Single peaks for α -syn, α B-c_{core} and Hsp27_{3D} correspond to sedimentation coefficients of approximately 1.1, 1.7 and 2.7 S respectively. Fitting the SV profile resulting from co-incubation of α -syn with α B-c_{core} (Figure 3D) results in a bimodal distribution, with peak positions of approximately 1.2 and 1.9 S (Figure 3E). The small shift of the peak positions may suggest that these proteins interact transiently. Comparison of the predicted weight-average sedimentation coefficient for the two components with the weight-average sedimentation coefficient calculated for the mixture shows a small shift from

1.37 S to 1.40 S. Similarly, co-incubation of α -syn with Hsp27_{3D} (Figure 3F) results in a bimodal distribution, with peaks corresponding to sedimentation coefficients of approximately 1.1 and 2.7 S (Figure 3G). In contrast to the mixture of α -syn with α B-c_{core}, the peak positions for α -syn and Hsp27_{3D} do not change significantly. The absence of additional peaks confirms the lack of a detectable stable complex between these two proteins.

The use of fluorescently labelled protein allows specific detection of one component of a complex mixture using fluorescence detected AUC. As such, CF488A-labelled wildtype Hsp27 was used in fluorescence-based AUC experiments to obtain SV profiles with a range of α -syn concentrations (Figure 4). As above, these profiles were fitted using the c(s) model and the resulting distribution had three maxima (Figure 4B). The shoulder centred at 0.9 S results from non-sedimenting fluorescence, and is attributed to residual free CF488A dye in solution from the labelling process. The other two main peaks, centred at approximately 2.4 and 10.5 S, reflect the equilibrium characteristic of wildtype Hsp27 in which smaller species (most likely predominately dimers) dissociate from polydisperse oligomers in order to provide chaperone-active subunits (27). Importantly, with increasing unlabelled α -syn concentration, there is no concentration-dependent increase in the weight-average sedimentation coefficient that would otherwise be indicative of a stable, high molecular mass complex being formed between Hsp27 and α -syn. Rather, the weight-average sedimentation coefficient decreases as the concentration of α -syn increases (Figure 4C), indicating that the average size of Hsp27 species in solution decreases with increasing amounts of α -syn. This trend is supported by a reduction in the signal at 10.5 S and an increase in the signal at 2.4 S with increasing (unlabelled) α -syn concentration. Together, these data suggest that the presence of aggregation-prone α -syn causes the dissociation of Hsp27 polydisperse oligomers in a concentration-dependent manner.

The ability of sHsps to prevent the aggregation of α -syn is dependent on the kinetics of the aggregation process – We next sought to identify possible mechanism(s) by which aggregation-prone α -syn may ‘overwhelm’ sHsps, leading to the formation of amyloid fibrils

associated with disease. To determine whether the rate at which α -syn aggregates affects the ability of the sHsps to prevent fibril formation, we used two methods to alter the kinetics of aggregation of α -syn which avoid the confounding effect a change in solution conditions (e.g. pH, temperature) may have on the chaperone activity of the sHsps (28,49-51). First, we exploited the nucleation-dependent mechanism of amyloid fibril formation of α -syn, whereby an increase in the concentration of aggregation-prone protein increases the rate of aggregation (13). Since the dissociation of small chaperone-active species from larger polydisperse oligomers is proposed to be a key component of the chaperone activity of sHsps (33,52,53), we also determined the rate of subunit exchange over the sHsp concentration range to be used in these experiments (i.e. 3 – 90 μ M) using FRET. During subunit exchange, mixing of labelled and unlabelled sHsp subunits resulted in an exponential decrease in the emission fluorescence intensity of the acceptor, which can be used to calculate the rate of subunit exchange (Figure 5A). Over the concentration range used in this work, the rate of subunit exchange of α B-c reached a maximum at \sim 30 μ M (Figure 5B). Importantly, this rate was not significantly affected by the presence of α -syn (Figure 5B). In contrast to α B-c, the oligomeric state of Hsp27 (46) and its subunit exchange rate (Figure 5C) are both significantly affected by concentration. Therefore, Hsp27 was unsuitable for use in these experiments due to these confounding effects.

The disease-associated A53T variant of α -syn was incubated at concentrations from 150 – 750 μ M in the absence and presence of α B-c (Figure 5D). In the absence of the chaperone, increasing the concentration of α -syn led to an increase in the rate and maximum ThT fluorescence associated with α -syn fibril formation, and a decrease in the lag phase (from 9 ± 2 h to 3 ± 1 h over this concentration range). When α B-c was present, such that the molar ratio of α -syn: α B-c remained constant (i.e. 1:10 α B-c: α -syn), the ability of α B-c to inhibit α -syn fibril formation was dependent on the kinetics of the aggregation process (Figure 5E-F). Thus, when the kinetics of α -syn aggregation were relatively slow (i.e. longer lag phase and slower rate of aggregation), α B-c was a more effective inhibitor of aggregation. When the kinetics of aggregation

increased (i.e. shorter lag phase and faster rate of aggregation), α B-c was a less effective chaperone, only decreasing the amount of aggregation of α -syn by ~20%.

The second method employed to alter the aggregation kinetics of α -syn aggregation was the use of disease-related mutants which aggregate at different rates (35). Recombinant α -syn mutant proteins were incubated at 300 μ M in the absence or presence of either α B-c or Hsp27 (1:10 molar ratio, sHsp: α -syn). As expected, each of the mutant proteins displayed different aggregation kinetics, with α -synA30P having a similar lag phase, elongation rate and maximal fibril formation to that of α -synWT (Figure 6A). In contrast, α -synA53T had the shortest lag phase (3 ± 1 h), slowest elongation rate and lowest maximum increase in ThT fluorescence. The α -synE46K variant was characterised by a slow rate of elongation, high maximal ThT fluorescence, and longest lag phase (42 ± 9 h) of any of the α -syn variants tested. Whilst both α B-c and Hsp27 were able to inhibit the aggregation of the α -syn proteins (Figure 6A), their ability to do so was dependent upon the isoform of α -syn. Both chaperones were least effective at inhibiting the aggregation of α -synA53T, which aggregated the fastest (i.e. shortest lag phase), whereas they were most effective at inhibiting α -synE46K, which aggregated the slowest (Figures 6B and C).

DISCUSSION

The sHsps play a critical role in maintaining cellular proteostasis by preventing protein aggregation associated with disease. Here, we confirm that the sHsps α B-c and Hsp27 are potent inhibitors of α -syn fibril formation *in vitro* (37,54,55). The phosphomimicking variant of Hsp27 was also an effective chaperone at inhibiting α -syn fibril formation, adding to the work demonstrating that phosphorylation of sHsps acts to increase their chaperone activity (56). Notably, the α B-c core domain inhibited the aggregation of α -syn with similar efficacy to the wild type (full length) protein, demonstrating that the sites required to inhibit α -syn aggregation are present in the core domain of these sHsps. The finding that the core domain of α B-c is sufficient to prevent α -syn aggregation is consistent with previous studies showing that this region is

capable of preventing other target proteins from forming amorphous or fibrillar aggregates (47,57).

Although the ability of the sHsps to inhibit the amyloid fibrillar aggregation of a range of target proteins *in vitro* is well established, the mechanism by which they do so is not clearly defined and may be dependent on the target protein (22,58-60). We therefore sought to characterise the molecular mechanism by which α B-c and Hsp27 interact with α -syn to prevent its aggregation. Our findings that both Hsp27 and α B-c increase the lag phase of α -syn aggregation and inhibit the elongation phase suggest that they primarily act through stabilising aggregation-prone monomeric α -syn to prevent it forming fibrils. However, we were unable to detect a high molecular mass complex formed between α -syn and α B-c or Hsp27 under the experimental conditions used in this work. Thus, in the absence of any evidence of a stable interaction, we conclude that these proteins interact transiently and this acts to prevent α -syn aggregation.

Transient interactions between the sHsps and α -syn are difficult to detect. Small shifts in the c(s) distributions and calculated weight-average sedimentation coefficient for the α -syn/ α B-c_{core} mixture may suggest a transiently interacting, rapidly exchanging system. That these shifts were not observed for Hsp27_{3D} does not rule out that α -syn and Hsp27_{3D} interact transiently. Rather it indicates that this interaction was below the detection limit of the AUC experimental conditions. This may be due to differences in the binding kinetics or the amount of α -syn interacting with Hsp27_{3D} compared to α B-c_{core}. If the fraction of species in this interacting population is too small, or if the interaction occurs too fast, it may not be able to be resolved in the sedimentation experiment (61-63). Transient interactions between α B-c and α -syn (and also of α B-c with other fibril-forming target proteins) are apparent from NMR spectra acquired on mixtures of these proteins due to general broadening of spectra of the target protein with no indication of specific binding site(s) (37,64-66). We therefore conclude that these sHsps prevent α -syn aggregation via transient interactions in a similar way as has been previously described for apoC-II and reduced and carboxymethylated α -lactalbumin (22). In doing so, the manner by which these sHsps act as chaperones to prevent α -syn aggregation is not

through a 'holdase' mechanism, as has been well characterised for sHsp interaction with amorphaously aggregating target proteins under stress conditions, particularly elevated temperature (42,43). Rather, these data support a model in which sHsps can act as protein stabilisers, interacting transiently with relatively ordered protein intermediates that have entered off-folding pathways, which facilitates them re-entering the on-folding pathway (67). Considering the cellular implications of this type of interaction, a transient mechanism is favourable as it does not deplete the pool of sHsps available to interact with aggregation-prone proteins in the cells. In contrast, a holdase-type chaperone mechanism, in which stable high molecular mass complexes are formed, occurs with more disordered intermediates that expose higher degrees of hydrophobicity prior to undergoing hydrophobic collapse and aggregation, as occurs under significant stress conditions with the formation of amorphous aggregates (22).

Importantly, it is unclear at this stage whether the interaction between the sHsps and α -syn impacts the conformation of aggregation-prone monomeric α -syn. However, previous studies have demonstrated that depletion of α B-c from aggregation-inducing conditions containing amyloidogenic substrate can allow aggregation to proceed (22). This implies that transient interactions between aggregation-prone species and sHsps may not significantly alter the conformation or aggregation propensity of the target protein.

Our previous work has extensively investigated the interaction of α B-c with a variety of amorphaously aggregating target proteins under conditions of elevated temperature, reductive and chemical stress. As a result of these studies, it is apparent that there are similarities between the mechanisms of sHsp chaperone interaction with amorphous and fibrillar aggregating target proteins. As observed in the current study with fibril-forming α -syn, sHsps are more efficient chaperones when interacting with slowly (amorphaously) aggregating target proteins (68,69). They also interact with target proteins early along their aggregation pathway, i.e. monomeric forms that are in a disordered, intermediate state (68,69). Dynamic, transient interactions between the target protein and the

sHsp are also crucial factors in determining chaperone efficacy with target proteins (70).

Given that both α B-c and Hsp27 are highly effective molecular chaperones at inhibiting α -syn fibril formation *in vitro*, the question remains as to how α -syn intermediates escape the sHsps to form fibrils and plaques in the context of the α -synucleinopathies? A key factor explored in this study was whether the rate of aggregation has a significant effect on the ability of these chaperones to prevent aggregation. Similar to the reported effect of metals (71), pesticides, membrane lipids (72) and molecular crowding (73,74), increases in the initial monomer concentration of α -syn promotes nucleation during the rate-limiting step, increasing the kinetics of aggregation (73,75-77). Furthermore, the three most studied disease-related mutations in α -syn exhibit markedly different aggregation kinetics (35), which was also observed in this study. We therefore exploited both increases in α -syn monomer concentration and disease-related mutants of α -syn in order to alter the kinetics of fibrillar aggregation of α -syn. In both cases, increasing the rate of α -syn aggregation led to a decrease in the chaperone ability of α B-c and Hsp27 to prevent fibril formation. In particular, there was a marked correlation between the efficacy of the chaperone and the lag phase of aggregation, i.e. the longer the time taken to form nuclei, the more efficacious the chaperones were in preventing aggregation. These results also support our previous work in which we increased the rate of α -syn fibril formation by adding the inert crowding agent dextran, under which conditions α B-c was a much poorer chaperone (37). However, the presence of crowding agent also reduces the rate of subunit exchange of α A-c (78), which may have contributed to the decrease in chaperone efficacy of α B-c in this case.

The effect of aggregation kinetics on the ability of sHsps to prevent fibril formation is significant given the association of mutations and duplication or triplication of the *SCNA* gene with early onset Parkinson's disease (9-11,79,80). Moreover, the link between increased aggregation rate and a decrease in chaperone inhibition provides a potential mechanism for aggregation-prone α -syn overwhelming the chaperone capability of the sHsps. While fluorescence-based AUC suggested that the presence of aggregation-

prone α -syn causes the dissociation of Hsp27 oligomers, this technique provides a measure of the oligomeric distribution at equilibrium. The real-time availability of chaperone-active subunits, through their dissociation from large polydisperse oligomers, which are able to interact with aggregation-prone proteins, is governed by the subunit exchange rate (81). The rate of subunit exchange for α B-c increased with concentration up to $\sim 30 \mu\text{M}$, after which it remained constant, which supports the notion that subunit exchange is a result of dissociation from sHsp oligomers, as opposed to oligomeric collisions, similar to that demonstrated for α A-crystallin (28). Moreover, factors that increase the rate of α -syn aggregation in cells (such as mutation, gene multiplication or macromolecular crowding) overwhelm the protective capacity of the sHsps due to an insufficient supply of chaperone-active subunits capable of interacting with the aggregation-prone α -syn monomer. Such a mechanism is also consistent with the dissociated subunits of sHsps being the chaperone-active species in cells.

This study aimed to characterise the interaction of sHsps with monomeric species. This required the addition of chaperone to monomeric samples before aggregation has commenced, similar to many other studies examining sHsp chaperone action (33,35,37,82). Recently, the interaction of sHsps with preformed amyloid fibrils has also been investigated, and α B-c has been shown to bind along the length of amyloid fibrils with moderate (μM) affinity (36,83,84). Addition of monomeric units to fibril ends is essential during the elongation phase of aggregation. In addition, sites of exposed hydrophobicity along the face of the fibril have been identified as possible locations for secondary nucleation (via fragmentation), leading to rapid fibril growth and dominating the overall kinetics of aggregation (85-87). Interaction with these species may contribute to the ability of sHsps to inhibit aggregation, by competing with monomeric units for access to fibril ends or occluding sites of potential secondary nucleation (36). These mechanisms undoubtedly play a role in the sHsp inhibition of α -syn aggregation, and may complement the interaction of the sHsps with monomeric α -syn.

The current study contributes to a greater understanding of the molecular mechanisms by

which sHsps interact with disease-related target proteins to prevent their aggregation. We provide further support for the reclassification of the sHsps as protein stabilisers rather than holdase chaperones since the latter does not fully describe the manner by which they can interact with aggregation-prone proteins (22). Thus, although the ability to form complexes with destabilised, particularly amorphously aggregating, proteins is a key element of their chaperone activity (26), this work adds to a growing body of evidence highlighting that this is not the only mechanism by which sHsps inhibit protein aggregation (22,35,37,58,65). Furthermore, we demonstrate that the rate of aggregation is a significant factor that governs the relative ability of α B-c and Hsp27 to prevent α -syn fibrillar aggregation. This provides a potential rationale for how sHsp chaperone activity is 'overwhelmed' in the context of diseases associated with protein aggregation, i.e. factors that increase the rate at which aggregation occurs also compromise the ability of sHsps to prevent it.

EXPERIMENTAL PROCEDURES

Materials – The pET24d or pET24a bacterial expression vectors, containing the human *HSPB1* (Hsp27), *HSPB5* (α B-c), or *SNCA* (α -syn) genes, were used for expression of recombinant wild-type proteins. Disease-related mutants of α -syn and sHsp variants used in this work were produced via site-directed mutagenesis of the wild-type gene (GenScript, Piscataway, USA). In particular, a variant of Hsp27 designed to mimic phosphorylation was generated by mutation of serine residues to aspartic acid at sites that are modified *in vivo* (i.e. Ser15Asp, Ser78Asp and Ser82Asp) to produce Hsp27_{3D}. Plasmids for the expression of the core domain of α B-c, i.e. residues 68 – 153 (α B-c_{core}), was a kind gift from Professor A. Laganowsky (Texas A&M Health Science Center, USA). All recombinant proteins were expressed in *Escherichia coli* BL21(DE3) cells transformed with each plasmid, and purified as described previously (12,88,89). Dithiothreitol (DTT) was purchased from Amresco (Solon, USA), and protein molecular mass standards used in gel electrophoresis were Protein Precision Plus Dual Colour obtained from Bio-Rad Laboratories (Hercules, USA). Protein mass calibrants for size exclusion chromatography, containing bovine

thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B12 (1.35 kDa), was also purchased from Bio-Rad Laboratories (Hercules, USA). All other chemicals, including Thioflavin T (ThT), were obtained from Sigma-Aldrich (St Louis, USA), unless otherwise stated. Protein concentrations were determined using a NanoDrop 2000c spectrophotometer (ThermoFischer Scientific, Waltham, USA), based upon $A_{280nm}^{0.1\%}$ values of $0.44 \text{ M}^{-1}\cdot\text{cm}^{-1}$ for α -syn calculated using the ExPASy ProtParam tool (90), $0.83 \text{ M}^{-1}\cdot\text{cm}^{-1}$ for α B-c and $1.65 \text{ M}^{-1}\cdot\text{cm}^{-1}$ for Hsp27 (91). Mouse monoclonal anti- α B-c antibody (clone 1B6.1-3G4) and mouse monoclonal anti-Hsp27 antibody (clone G3.1) were purchased from Abcam (Cambridge, UK). Both the mouse monoclonal anti- α -syn antibody (clone Syn211) and the peroxidase conjugated anti-mouse IgG secondary antibody were obtained from Sigma-Aldrich (St. Louis, USA).

Aggregation assays – Fibrillar aggregation of α -syn in the absence or presence of sHsps was monitored using a ThT fluorescence assay via a previously described method (33) with adaptations. Briefly, α -syn was incubated at $300 \mu\text{M}$ in 50 mM phosphate buffer containing 100 mM NaCl (pH 7.4) and 0.01% sodium azide unless otherwise indicated. Assays were conducted in triplicate using clear 384 microwell plates (Greiner Bio-One, Frickenhausen, Germany) with each well containing $30 \mu\text{L}$ of sample. Plates were incubated in a POLARstar OPTIMA plate reader (BMG Labtechnologies, Melbourne, Australia) at 37°C , with the plate sealed to prevent evaporation. The ThT fluorescence was measured using excitation and emission filters of 440 nm and 490 nm respectively and readings were taken every 625 sec for a period of up to 60 h . Plates were subjected to linear shaking at 600 rpm for 540 sec after each reading.

At the end of each assay, data for the change in ThT over time were fitted with Boltzmann sigmoidal curves using GraphPad Prism v 4.02 (GraphPad Software Inc., San Diego, USA). Data were only used when the R^2 coefficient of determination was > 0.8 . Parameters from these fits were used to derive the length of the lag phase and rate of α -syn aggregation using equations previously derived (92), with modification (Figure

7). The equation of the Boltzmann curve is given as:

$$F = F_i + \frac{(F_f - F_i)}{1 + e^{\frac{(t_{50}-t)}{k}}}$$

The slope of the line at any point is:

$$\frac{dF}{dt} = \frac{\frac{(F_f - F_i)}{k} \times e^{\frac{(t_{50}-t)}{k}}}{\left(1 + e^{\frac{(t_{50}-t)}{k}}\right)^2}$$

When $t = t_{50}$ (i.e. the inflexion point, when the elongation rate is maximal):

$$\frac{dF}{dt} = \frac{\frac{(F_f - F_i)}{k} \times e^0}{(1 + e^0)^2}$$

Therefore the rate of α -syn aggregation at t_{50} was calculated according to the equation:

$$\text{Rate}_{t_{50}} = \frac{(F_f - F_i)}{4 \times k} \quad (1)$$

where F_i corresponds to the initial fluorescence value, F_f corresponds to the final fluorescence value, t_{50} refers to the time taken to reach half the maximal fluorescence, and k describes the gradient of the curve.

The lag phase (t_{lag}) is when

$$\left\{ \frac{(F_f - F_i)}{4 \times k} \right\} \times t_{50} + b = F_i \quad (2)$$

To solve for b , at t_{50} (i.e. the mid-point between F_f and F_i):

$$F_{t_{50}} = \frac{(F_f - F_i)}{2}$$

$$b = \left\{ \frac{(F_f - F_i)}{2} \right\} - \left\{ \frac{(F_f - F_i)}{4 \times k} \right\} \times t_{50}$$

So substituting b into equation (2):

$$F_i = \left\{ \frac{(F_f - F_i)}{4 \times k} \right\} \times t_{lag} + \left\{ \frac{(F_f - F_i)}{2} \right\} - \left\{ \frac{(F_f - F_i)}{4 \times k} \right\} \times t_{50}$$

Solving for t_{lag} :

$$\begin{aligned} 4 \times k \times F_i &= \{(F_f - F_i) \times t_{lag}\} \\ &+ \{2 \times k (F_f - F_i)\} \\ &- \{(F_f - F_i) \times t_{50}\} \\ (F_f - F_i) \times t_{lag} &= 4 \times k \times F_i + \{(F_f - F_i) \times \\ &t_{50}\} - \{2 \times k (F_f - F_i)\} \end{aligned}$$

Therefore, t_{lag} is determined using the equation:

$$t_{lag} = \frac{4 \times k \times F_i}{(F_f - F_i)} + t_{50} - \{2 \times k\}$$

The relative efficacy of the sHsps to inhibit α -syn fibril formation was determined by calculating the protection provided by each sHsp at the conclusion of the assay, according to the difference in maximum ThT fluorescence in the absence and presence of the chaperone using the equation:

$$\% \text{ Protection} = \frac{\Delta I - \Delta I_{\text{chaperone}}}{\Delta I \times 100}$$

where ΔI and $\Delta I_{\text{chaperone}}$ correspond to the change in ThT fluorescence of α -syn in the absence and presence of the sHsp respectively. In each case, samples were assayed in triplicate and the percent protection is reported as a mean \pm S.E.M of at least 3 independent (biological) replicates.

Analytical size exclusion chromatography

– The nature of the interaction between the sHsps and α -syn was analysed via size exclusion chromatography of the samples at the end of the aggregation assays. Samples containing 300 μ M α -syn in the absence or presence of 300 μ M α B-c or Hsp27, were collected immediately following incubation and centrifuged at 14 000 \times g for 10 min at 4°C to remove any insoluble protein. Supernatants were collected and loaded onto a Superose-6 size exclusion column (GE Healthcare, Uppsala, Sweden), pre-equilibrated with 50 mM phosphate buffer containing 100 mM NaCl (pH 7.4) and 0.01% sodium azide, at a flow rate of 0.5

mL/min. Protein elution was monitored using an in-line UV detector, and concentrations were determined using the peak integration function of PrimeView v5.0 (GE Healthcare, Uppsala, Sweden), to monitor any loss of protein following centrifugation. Eluate fractions (1 mL) were collected and analysed via SDS-PAGE and immunoblotting. Representative results are presented from two independent experiments from two separate aggregation assays.

Immunoblotting – Following SDS-PAGE, in which proteins were resolved on 15% (v/v) gels, proteins were transferred to nitrocellulose membrane at 100 V for 1 h. Membranes were blocked with 5% (w/v) skim milk powder in Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h at room temperature. Blots were then incubated with primary antibodies directed against α B-c, Hsp27 or α -syn, diluted 1:5000 in 5% (w/v) skim milk powder in TBS containing 0.05% Tween-20 (TBS-T) for 1 h at room temperature. Blots were washed four times (10 min) in TBS-T, and incubated with the peroxidase-conjugated anti-mouse secondary antibody (1:5000 dilution in 5% (w/v) skim milk powder in TBS-T) for 1 h at room temperature. Blots were washed as above and labelled proteins were detected with SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, USA).

Fluorescent labelling of sHsps – Recombinant proteins of interest were fluorescently labelled with either succinimidyl ester or maleimide variants of CF488A or CF647 dyes (Biotium, Hayward, USA) according to the manufacturer's instructions. Briefly, those samples intended for maleimide labelling were pre-incubated with a 10 fold molar excess of Tris(2-carboxyethyl)phosphine (TCEP) for 30 min at room temperature. Recombinant protein was then loaded onto a PD10 column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with degassed phosphate buffered saline (PBS; 2.7 mM KCl, 1.75 mM K_2HPO_4 , 135 mM NaCl, 10 mM NaH_2PO_4 , pH 7.4) and 500 μ L fractions were collected. Protein elution was monitored by determining the absorbance of each fraction at 280 nm. Fractions containing protein were pooled and dye conjugates were added in a 1.5 molar (succinimidyl ester) or 10 molar (maleimide)

excess relative to the protein concentration for labelling. Labelling reactions were incubated overnight at 4°C with agitation, and then labelled protein was separated from unreacted dye using a pre-equilibrated PD10 column as described above. The concentration and degree of labelling of each protein were calculated as per the manufacturer's instructions and the latter was found to be greater than 60% in all cases. Labelled proteins were stored at -20°C. Protein labelling with the fluorophore was also confirmed via electrospray ionisation mass spectrometry.

Analytical ultracentrifugation (AUC) – UV absorbance-detected AUC was performed as previously described (83). Briefly, the maximum concentration was calculated for each protein such that the absorbance at 230 or 280 nm was within the optimal detection range of the instrument. Samples (380 μ L) were prepared at room temperature and incubated for 1 h before being loaded into a 12 mm double-sector epon-filled centrepiece, alongside the relevant reference solution (400 μ L). Samples were centrifuged in an XL-I analytical ultracentrifuge (Beckman Coulter, CA, USA) at 50 000 rpm using either a Ti60 or Ti50 rotor at 20°C. Radial absorbance scans were collected at 230 nm or 280 nm at 6 min intervals with a radial step size of 0.003 cm for a total of 10 h. Sedimentation velocity (SV) profiles were analysed with SEDFIT software using a continuous $c(s)$ distribution model and regularization by maximum entropy. Using a regularisation parameter of $p=0.95$ and 200 sedimentation coefficient increments, the data were fitted to produce $c(s)$ distributions. Weight-average sedimentation coefficients of each sample were then calculated by integration of the sedimentation distributions over the given range. The buffer density (ρ), buffer viscosity (η) and partial specific volume (\bar{v}) used for analysis were estimated using SEDNTERP software (<http://sednterp.unh.edu>).

Fluorescence-detected AUC was performed as previously described (83), using Hsp27-WT labelled with CF488A and unlabelled, monomeric α -syn. Briefly, samples (350 μ L) were prepared at room temperature and incubated for 1 h before being loaded into a 12 mm double-sector epon-filled centrepiece, and covered with 50 μ L of FC-43 perfluorotributylamine (Scientific Instrument Services Inc., NJ, USA). Samples were

centrifuged in an XL-A analytical ultracentrifuge fitted with a fluorescence detection system (FDS; Aviv Biomedical, NJ, USA) at 50 000 rpm using either a Ti60 or Ti50 rotor at 20°C. Excitation was at 488 nm and fluorescence above 505 nm was measured. Radial fluorescence scans were collected at 3 min intervals with a radial step size of 0.002 cm for a total of 5 h. Sedimentation velocity profiles were analysed as described above, fitting time-independent noise and according to a fixed sample meniscus position.

Bulk Förster Resonance Energy Transfer (FRET) analysis of sHsp subunit exchange rate – Subunit exchange rates of the sHsps were determined by monitoring changes in Förster Resonance Energy Transfer (FRET) between fluorescently labelled sHsp oligomers. Aliquots of fluorescently labelled sHsp, composed of an equimolar mixture of CF488A- and CF647-maleimide labelled protein, were prepared in PBS (pH 7.4) at concentrations ranging from 3 – 90 μ M. Samples were incubated at 37°C with shaking at 60 rpm using a VorTemp incubator (Labnet, Edison, USA) for 1 h, to allow complete mixing of the two labelled populations (confirmed by monitoring the quenching of donor fluorescence at 495 nm). The labelled sHsps were then diluted 10 fold into a sample of unlabelled sHsp at an equivalent concentration so as to maintain the overall concentration of the sHsp. The loss of FRET due to subunit exchange of labelled sHsps with unlabelled sHsps was determined by the decrease in acceptor fluorescence at 670 nm and monitored using a POLARstar Omega platereader (BMG Labtechnologies, Melbourne, Australia). Experiments were performed in the absence or presence of a 10-fold molar excess of α -synWT, in black polystyrene clear-bottom 384-well plates (Greiner Bio-One, Frickenhausen, Germany). Readings were taken every 120 s over a period of 4 h, with linear shaking at 600 rpm for 90 s after each cycle. The rate constant was determined by fitting the data to a one-phase exponential decay curve using GraphPad Prism v 4.02 (GraphPad Software Inc., San Diego, USA). The rate of subunit exchange was derived from the equation:

$$Y = (Y_0 - Y_t) \times e^{-kt} + Y_t$$

where k is defined as the rate constant of the reaction, and Y_0 and Y_t correspond to the

fluorescence intensity at time = 0 and t respectively.

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Author contributions: HE conceived and coordinated the experiments. HE, DC, JC and MG designed the experiments. DC, ES and MG performed the experiments. All authors analysed and reviewed the data. DC and HE wrote the paper. All authors read and approved the final manuscript.

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FOOTNOTES:

The abbreviations used are: α -syn, α -synuclein; α B-c, α B-crystallin; AUC, analytical ultracentrifugation; sHsp, small heat-shock protein; SV, sedimentation velocity; ThT, Thioflavin-T; TCEP, Tris(2-carboxyethyl)phosphine

FIGURE LEGENDS

Figure 1: sHsp inhibit α -syn aggregation in a concentration-dependent manner. Recombinant α -syn was incubated at 300 μ M in 50 mM sodium phosphate buffer with 100 mM NaCl and 0.01% NaN₃ (pH 7.4), in the absence or presence of sHsps or the non-chaperone control protein BSA. Fibril formation was monitored by the change in ThT fluorescence at 490 nm over time. (A) A representative trace of 2 independent experiments is shown for α -syn in the absence or presence of Hsp27 or BSA at a 1:1 molar ratio (Hsp27/BSA: α -syn). Hsp27, BSA and buffer alone samples are also included for comparison, and the data for these overlay one another along the x-axis due to them showing no change in fluorescence over the course of the assay. (B) A representative trace of 4 independent experiments is shown for α -syn in the presence of various ratios of wildtype α B-c. Data were fitted with a Boltzmann sigmoidal curve using GraphPad Prism v 4.02. (C) Values obtained for the maximum change in ThT fluorescence from these fits was used to determine the percent protection afforded by wildtype α B-c or Hsp27 at a range of molar ratios. Results are presented as mean \pm S.E.M (n \geq 4).

Figure 2: sHsps prevent α -syn aggregation but, in doing so, do not form a stable high molecular mass sHsp-target protein complex. (A, B) Size-exclusion chromatograms of soluble α -syn (300 μ M), α B-c (300 μ M), Hsp27 (300 μ M) or post-aggregation samples of soluble α -syn (300 μ M) in the absence and presence of 1:1 molar ratio of α B-c or Hsp27. (C, D) Immunoblot analysis of the eluate fractions collected from the size-exclusion column after loading with the sample from A or C containing α -syn and α B-c or Hsp27. Aliquots from every fraction (1 mL) collected between 8–20 mL were loaded on to a SDS/PAGE gel, transferred to nitrocellulose membrane and blotted with an anti- α B-c, anti-Hsp27 or anti- α -syn antibody. Results shown are representative of two independent experiments.

Figure 3: Analysis of the interaction between sHsps and aggregation-prone α -syn by absorbance-based AUC. Recombinant α -syn was incubated at 300 μ M in 50 mM sodium phosphate buffer with 100 mM NaCl and 0.01% NaN₃ (pH 7.4), in the absence or presence of sHsps variants (30 μ M). Fibril formation was monitored by the change in ThT fluorescence at 490 nm over time, and data were fitted with a Boltzmann sigmoidal curve using GraphPad Prism v 4.02. Sedimentation velocity analysis of 5 μ M (B) α -syn, (C) α B-c_{core} or (D) Hsp27_{3D}. Data collected at 60 min intervals is presented, overlaid with theoretical fits to the c(s) model generated by SEDFIT. Sedimentation velocity profiles of α -syn co-incubated with (E) α B-c_{core} or (G) Hsp27_{3D} are also shown, along with c(s) distributions for these profiles (F and H respectively).

Figure 4: Aggregation-prone α -syn induces the dissociation of oligomeric Hsp27. Sedimentation velocity analysis of 0.5 μ M CF488-labelled Hsp27 in the absence or presence of increasing concentrations of α -syn (0 – 50 μ M). (A) Radial scans are displayed for the highest α -syn concentration overlaid with theoretical fits to the c(s) model generated by SEDFIT. Data are collected at 60 min intervals is presented. (B) c(s) distributions for each profile are shown, and (C) the weight-average sedimentation coefficient was calculated via integration from 1 - 25 S for each concentration of α -syn. Data were then fitted with a linear regression model.

Figure 5: The ability of α B-c to inhibit the fibrillar aggregation of α -syn is dependent on the kinetics of aggregation. (A) Fluorescently labelled α B-c was incubated for 1 h at 37°C in PBS (pH 7.4) at concentrations ranging from 3 – 90 μ M, consisting of an equimolar mixture of fluorescently labelled protein capable of FRET. Samples were diluted 10-fold into unlabelled α B-c, in the absence or presence of α -syn at a 1:10 (α B-c: α -syn) molar ratio, and the loss of fluorescence in the acceptor fluorescence channel was used to calculate (B) the rate of subunit exchange in the absence and presence of α -syn. (C) The rate of subunit exchange was similarly calculated for Hsp27, in the absence and presence of α -syn. (D) Recombinant A53T α -syn was incubated at concentrations ranging from 150 – 750 μ M in 50 mM phosphate buffer containing 100 mM NaCl and 0.01% NaN₃ (pH 7.4), in the presence or absence of a

1:10 molar ratio of α B-c. Samples were incubated at 37°C for 60 h and aggregation was monitored via the change in ThT fluorescence at 490nm. A representative plot is shown for α -syn in the absence of α B-c with Boltzmann-sigmoidal curves fitted to the data. Values obtained from D were used to calculate the lag phase, elongation rate and plateau phase for each α -syn concentration. The percent protection afforded by α B-c when present in the sample was calculated and correlated with the (E) duration of the lag phase and (F) rate of elongation. Symbols represent the calculated parameters from each of three independent repeats, with each point corresponding to values calculated from a fit of triplicate samples and shaded according to the concentration of α -syn as indicated in panel D. Data in B, E and F were fitted with a non-linear regression model and data in C were fitted with a linear regression model. The R^2 coefficients of determination are shown.

Figure 6: The ability of the sHsps α B-c and Hsp27 to prevent the aggregation of disease-associated mutant forms of α -syn. Recombinant WT or disease-related mutant forms of α -syn (A30P, A53T or E46K) were incubated at 300 μ M in 50 mM phosphate buffer containing 100 mM NaCl and 0.01% NaN₃ (pH 7.4), in the presence or absence of 30 μ M α B-c or Hsp27. Samples were incubated at 37°C for 60 h and aggregation was monitored via the change in ThT fluorescence at 490 nm. (A) Representative plots of the four independent repeats, each consisting of triplicate samples, are shown for each of the α -syn proteins, including Boltzmann-sigmoidal curves fitted to the data. Values obtained from these fits were used to calculate the lag phase for each protein, which was then correlated with the percent protection when (B) α B-c or (C) Hsp27 was present in the sample. Data shown are the results of four independent repeats, with each point corresponding to values calculated from a fit of triplicate samples. Non-linear regression analysis of the correlation between the lag phase and the percent protection was generated using GraphPad Prism v 4.02.

Figure 7: Schematic illustration of the Boltzmann sigmoidal curve used to describe the increase in ThT fluorescence upon α -syn fibril formation. F_i corresponds to the initial fluorescence value, F_f corresponds to the final fluorescence value, t_{50} refers to the time taken to reach half the maximal fluorescence (F_{i50}), t_{lag} is the lag time and k denotes the steepness of the curve.

FIGURES

Figure 1

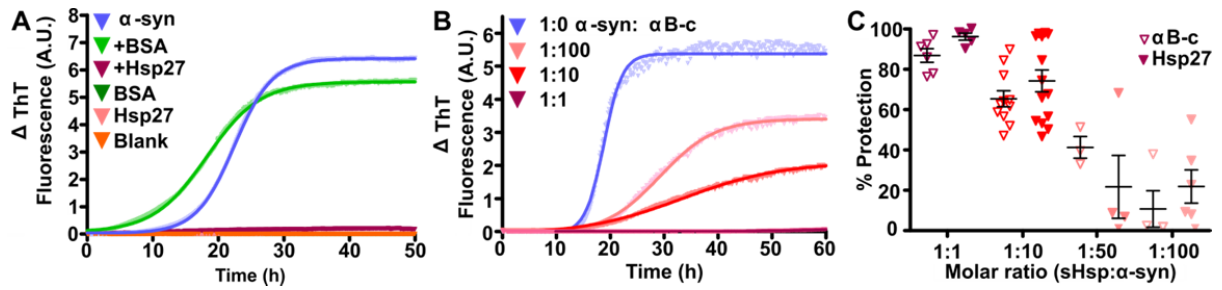


Figure 2

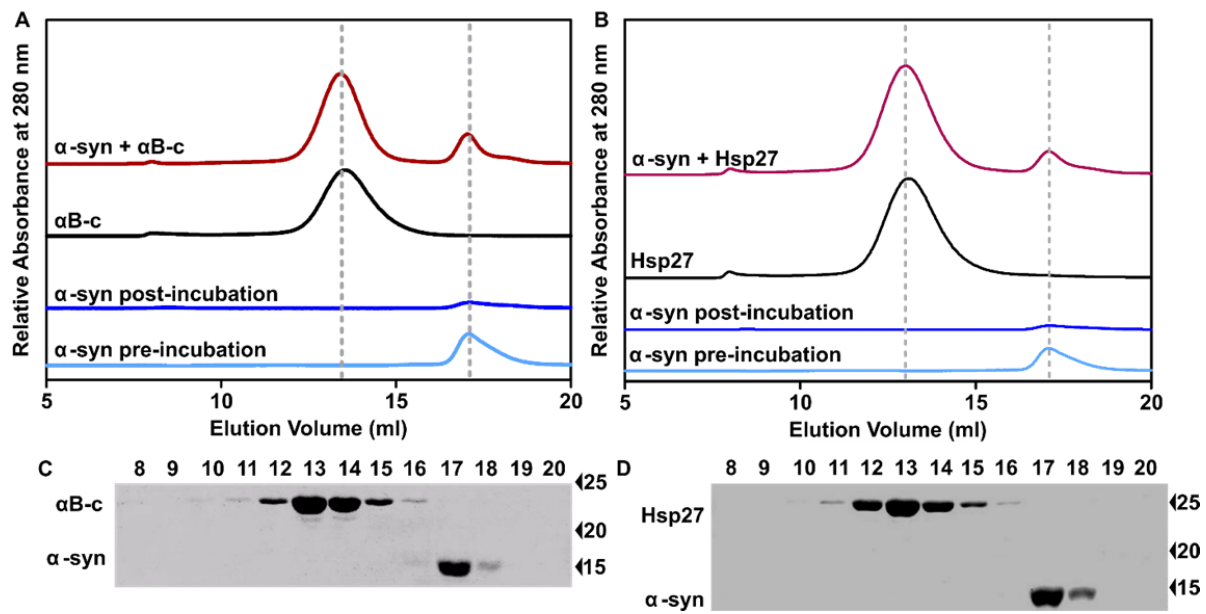


Figure 3

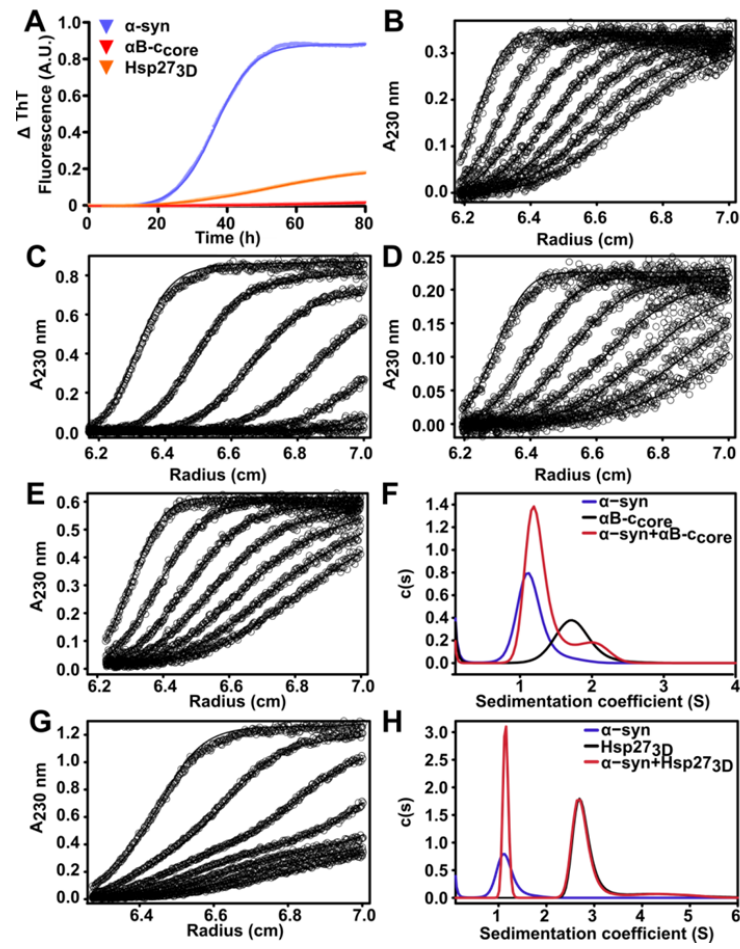


Figure 4

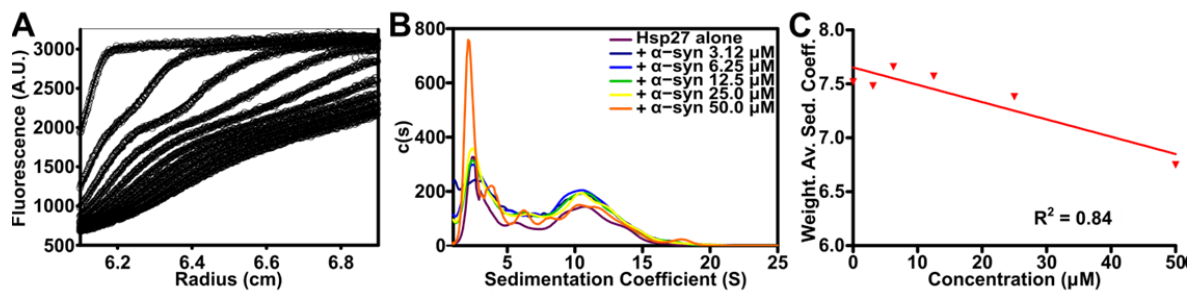


Figure 5

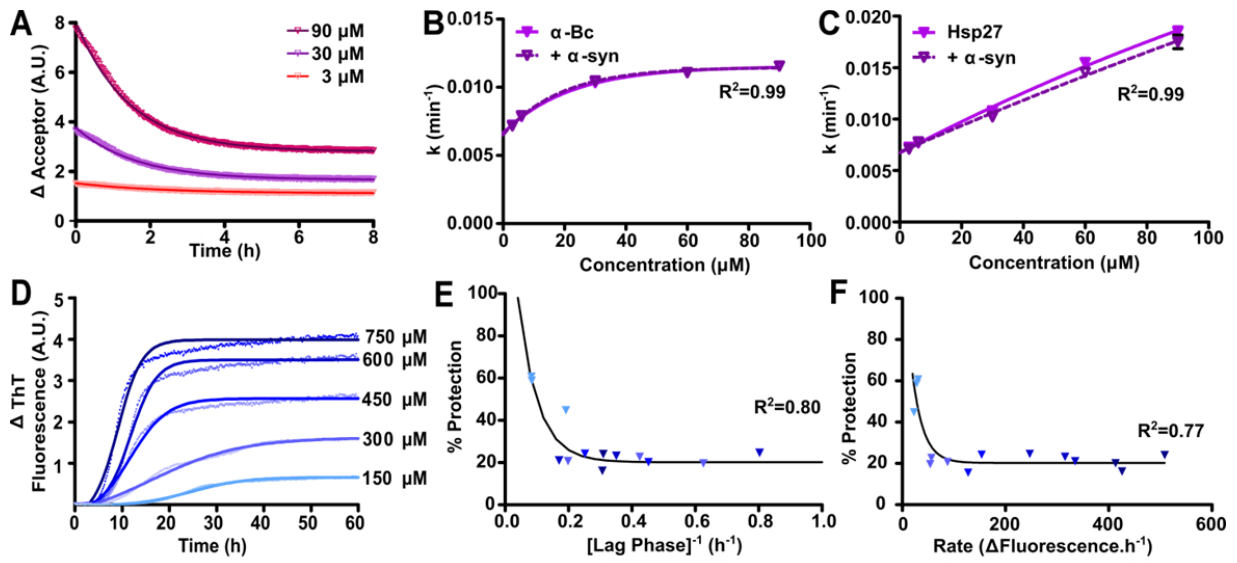


Figure 6

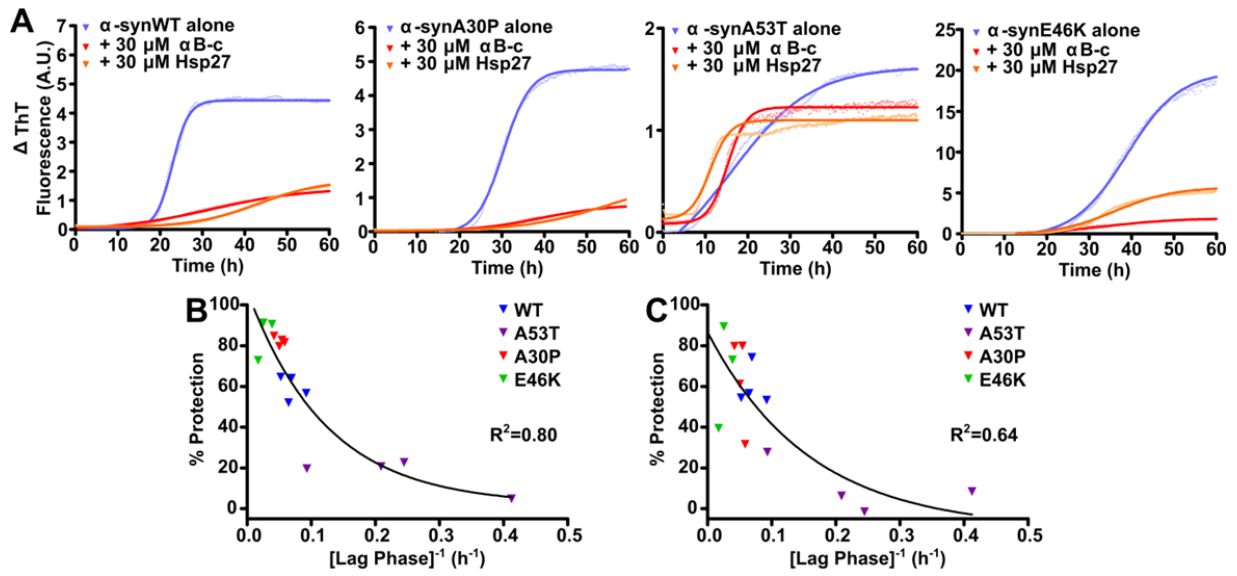


Figure 7

