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Protein chemistry of amyloid fibrils and chaperones: implications for amyloid formation and disease

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Abstract

Understanding the mechanisms by which amyloid fibrils are formed, both in vivo and in vitro, is vital for developing methods to treat and prevent debilitating deposition diseases such as Alzheimer's disease, Parkinson's disease, type II diabetes and systemic amyloidoses. In recent years, computer modelling and biophysical studies have broadened our understanding of the biochemical mechanisms underpinning protein aggregation. As a result, it is now believed that the ability to form fibrils is an intrinsic property of polypeptide chains and not isolated to disease-related proteins or peptides. Molecular chaperones are a diverse group of functionally related proteins well known for their ability to suppress amyloid formation, and are likely to be important determinants in deciding the fate of protein aggregation prone proteins in vivo. Evidence is presented that suggests that there is striking commonality in the anti-amyloidogenic activity of molecular chaperones regardless of their structural and spatial differences. In this review, we focus on what in vitro biophysical studies tell us about amyloid formation and molecular chaperones, and how investigating the role of chaperones in fibril formation can enhance our understanding of protein misfolding diseases. English Author keywords Aggregation; Amyloid; Chaperone; Oligomer; Protein deposition disease Index Keywords EMTREE drug terms: amyloid; chaperone; polypeptide EMTREE medical terms: Alzheimer disease; amyloidosis; computer model; human; in vitro study; in vivo study; non insulin dependent diabetes mellitus; nonhuman; Parkinson disease; priority journal; protein aggregation; review Chemicals and CAS Registry Numbers amyloid, 11061-24-8

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Protein chemistry of amyloid fibrils and chaperones: Implications for amyloid formation and disease.

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Abstract

Understanding the mechanisms by which amyloid fibrils are formed, both *in vivo* and *in vitro*, is vital for developing methods to treat and prevent debilitating deposition diseases such as Alzheimer's disease, Parkinson's disease, type II diabetes and systemic amyloidoses. In recent years, computer modelling and biophysical studies have broadened our understanding of the biochemical mechanisms underpinning protein aggregation. As a result, it is now believed that the ability to form fibrils is an intrinsic property of polypeptide chains and not isolated to disease-related proteins or peptides. Molecular chaperones are a diverse group of functionally related proteins well known for their ability to suppress amyloid formation, and are likely to be important determinants in deciding the fate of protein aggregation prone proteins *in vivo*. Evidence is presented that suggests that there is striking commonality in the anti-amyloidogenic activity of molecular chaperones regardless of their structural and spatial differences. In this review, we focus on what *in vitro* biophysical studies tell us about amyloid formation and molecular chaperones, and how investigating the role of chaperones in fibril formation can enhance our understanding of protein misfolding diseases.

Introduction

A broad range of human disorders are associated with the formation and deposition of structured fibrillar aggregates [1]. These protein deposits may be extracellular, such as Aβ peptide plaques associated with Alzheimer's disease, or they may be deposited as part of intracellular inclusions such as in the case of α-synuclein in Parkinson's disease [1]. These "protein deposition diseases" encompass neurodegenerative conditions such as Alzheimer's disease, localised amyloidosis such as type II diabetes, and systemic non-neuropathic amyloidoses associated with the proteins transthyretin and lysozyme [2]. Although the diseases associated with protein deposits are commonly sporadic, there is clear evidence that some familial forms are associated with deposition of globular proteins that contain destabilising mutations making them more aggregation prone [3-5]. These inherited variants have been identified as the major components of fibrillar aggregates found in patients suffering from various protein deposition disorders (see [1] for extensive list) strongly suggesting that the aggregation of these proteins is intimately linked with pathology. Indeed, in lysozyme amyloidosis, amyloid deposits can accumulate in extraordinary proportions disrupting the function of whole organs [6]. In other cases, particularly neurodegenerative diseases associated with protein deposits, it is now thought that small soluble aggregates are the most damaging species; however the precise mechanism for toxicity remains unknown [7].

Despite the lack of a common primary sequence or tertiary structure, the various disease associated proteins form fibrils, both *in vivo* and *in vitro*, that are remarkably similar morphologically [1]. Generally, amyloid fibrils share a common cross-β core structure and mature fibrils consist of a number of protofilaments wound around each other to form unbranched rope-like fibres of around 7-13 nm in diameter (as observed by transmission electron microscopy [8]). Amyloid-like fibrils bind the dye Thioflavin T, resulting in an increase in its fluorescence emission [9, 10]; this is thought to result from the binding of the dye to the abundant β-sheet structure running perpendicular to the long axis of the fibrils [11]. In addition, amyloid deposits have the unique property of displaying apple-green birefringence when bound to the dye

Congo Red [6, 12]. Fibrils formed *in vivo* are generally found to be associated with heparin sulfate proteoglycans, glycosaminoglycans, and a range of other proteins such as serum albumin P (SAP) [6, 13, 14] and molecular chaperones [15]. Although there are widely observed, common morphological features that define amyloid fibrils, it is becoming increasingly clear that fibrils generated *in vitro* (even fibrils generated from the same protein or peptide under identical conditions) can be heterogeneous in their diameter, height, lateral association and periodic twisting [16].

In recent years, biophysical studies have helped to broaden our understanding of some of the biochemical mechanisms underpinning this highly structured protein aggregation process [17]. Typically, amyloid formation follows a kinetic pathway similar in nature to protein crystallisation, in which an initial 'lag' or nucleation phase is followed by a rapid exponential 'growth' or polymerisation phase [18, 19]. It is thought that the lag phase represents the amount of time required for the formation of stable soluble oligomers that can act as the functional nuclei required to seed fibril growth. Once a stable nucleus is reached, the formation of mature fibrils is thought to occur by rapid association of either monomers or additional oligomers [20]. Recently, it has been postulated that fibril breakage, producing a rapid increase in the number of growing ends, plays an important role in the rapid kinetics of the growth phase [21, 22].

For decades, monomeric disease related proteins have been used as starting material to produce fibrils *in vitro* which bear close morphological similarities to those found in *in vivo*. Indeed, fibrils were made *in vitro* by proteolytic cleavage of Bence Jones proteins as early as 1971 [23] and fibrils were formed *in vitro* from the synthetic Aβ peptide fragment $A\beta_{1\text{-}28}$ even before the full amino acid sequence was known for $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ [24]. However, more recently, it has become clear that fibrils can also be formed *in vitro* from proteins that have no known association with disease (i.e. SH3-PI3 and acylphosphatase) [25, 26], including some that completely lack any β-sheet structure (i.e. myoglobin) [27]. It has even been possible to design short peptides which can be induced to form fibrils by elevating the incubation temperature (coiled coil β peptide; ccβw peptide) [28]. Given the right experimental conditions, it appears that many proteins can form amyloid-like fibrils *in vitro.* It has

been postulated that the ability of proteins or peptides to form amyloid fibrils is a generic feature of the polypeptide chain and therefore, is common to all proteins [17, 29]. Experiments investigating the effects of point mutations in acylphosphatase on aggregation rates showed that these rates correlated with physicochemical effects such as hydrophobicity, secondary structure propensity and charge [30]. Further studies of these factors led to the development of a computational method for predicting the propensity of polypeptides to form fibrillar aggregates [31-33].

Why don't all proteins form amyloid *in vivo*?

The sequence of any given protein will govern its intrinsic stability and hence its likelihood of being in a partially unfolded state at any given time. Therefore, some proteins are more likely to form amyloid than others. As outlined above, it is thought that hydrophobic residues, net charge and secondary structure propensity all contribute to a protein's ability to form amyloid-like fibrils [1]. Recently, experimental studies in a *Drosophila* model of Alzheimer's disease were carried out to determine whether the calculated intrinsic aggregation propensities (Z_{agg}) could relate to observable effects in living organisms [34]. Indeed, there was a clear relationship between the amount of $A\beta_{1-42}$ aggregated in *Drosophila* and the calculated aggregation propensity of each of the variants (e.g $A\beta_{1-42}$ F20E < $A\beta_{1-42}$ WT < $A\beta_{1-42}$ E22G < $A\beta_{1-42}$ I31E/E22G). A relationship between Z_{agg} of $A\beta_{1-42}$ peptide variants and fly longevity was also observed; however, a number of variants did not follow this trend. Further analysis revealed a better correlation between longevity and the tendency of the variant to form small, soluble aggregates. Therefore, it seems possible to predict the propensity of a protein or peptide to form ordered aggregates, *in vivo*, from its primary structure. However, in addition to these intrinsic factors, the environmental conditions in which a protein finds itself plays an important role in amyloid formation. For example, some proteins, such as β_2-microglobulin, form amyloid fibrils *in vitro* quite rapidly at pH 2-3 but formation of amyloid at neutral pH is extremely inefficient and requires the addition of co-factors, agitation, seeding and extended time frames of aggregation [35]. This illustrates the importance of environmental conditions on stability of a protein and thus its tendency to form fibrils. Consequently, although fibril formation is likely to be a generic

property of proteins, the conditions that promote the partial unfolding and stimulate fibril formation vary greatly from protein to protein. In biological systems there are many complex processes in the intra- and extracellular environment that act to suppress protein aggregation and accumulation.

Molecular chaperones in intracellular and extracellular Quality Control systems.

Since the correct folding of a protein is fundamental to its function and any disturbances in this structure can lead to loss of function and/or inappropriate aggregation (both fibrillar and amorphous), much energy is invested in ensuring that the transfer of structural information from genes to folded proteins is correctly executed. A range of processes including RNA metabolism and processing, protein synthesis and folding, assembly and disassembly, and protein clearance and degradation all contribute to the maintenance of protein homeostasis [36]. One of the most important elements of the ensemble of machinery responsible for protein homeostasis is the molecular chaperones.

Molecular chaperones are a diverse group of proteins that are characterised by an ability to selectively recognise and bind non-natively folded proteins in a non-covalent but stable interaction [37]. They are most well known for their contributions to co- and post-translational folding and for their role in the quality control of partially unfolded or misfolded proteins. In addition, they are also known to assist in assembly and disassembly of large macromolecular complexes and to aid in translocation of proteins to various cellular compartments [38]. There are several families of molecular chaperones, defined by their size, cellular compartment and function that work together to prevent protein aggregation and facilitate the correct folding of non-native proteins [39]. All chaperones fall under two broad, functionally related categories: folding type and holding type chaperones [40]. In most cases, folding type chaperones (e.g Hsp70, Hsp90) facilitate the correct folding of non-native proteins through regulated binding and release. In these cases release of polypeptides is driven by ATP-dependent conformational changes. This permits misfolded proteins to escape their kinetically trapped non-functional conformation and reinitiates the folding process [39]. Holding type chaperones (small Hsps; e.g. α-crystallin, extracellular chaperones; e.g. clusterin) work independently of ATP and offer hydrophobic surfaces for binding of unfolded polypeptides. Their role is

thought to be to protect partially folded/misfolded proteins from aggregation until folding helper chaperones or proteolytic processes are available to either refold or degrade the non-native protein client [40, 41]. Another class of folding machinery, the chaperonins, are thought to offer large cages in which protein molecules can fold in isolation from inappropriate interactions [42]. Although chaperonins are an important part of the protein folding machinery, it is estimated that ~ 9-15% of all cellular proteins require chaperonin assistance for folding [43]; this review does not provide a detailed examination of their involvement in protein aggregation (for a specialist review see [44]).

The components and complexity of the network of chaperones depends on the compartments in which they operate. In the cytosol it appears some chaperones may act as non-native protein scavengers and that binding of cofactors may determine if the protein is to be refolded (when bound to cofactors such as Hip or Hop) or degraded (when bound to cofactors such as CHIP or BAG-1) [45]. These cofactors contain a chaperone binding motif or "adapter" which can bind to a specific site on the C-terminus of Hsp70 and Hsp90. The adapter contains a tandem arrangement of three degenerate 34 amino acid repeats (tetratricopeptide repeats, TPRs) adjacent to a highly charged α-helix [46]. Binding of cofactors CHIP and BAG-1 inhibits the refolding activity of chaperones (Hsp70 & Hsp90) and promotes either ubiquitination or direct loading of non-native protein onto the proteasome, respectively [45]. In contrast, binding of the co-factors Hip and Hop promotes refolding [47, 48].

Other intracellular compartments also have their own distinct chaperone networks. In the mitochondria, chaperones of the Hsp70 and Hsp100 family, together with the chaperonin Hsp60 and ATP-dependent proteases, promote refolding or degradation of misfolded proteins (reviewed in [49]). The ER also has a well characterised chaperone network; including Hsp70 family member BiP, calnexin and calreticulin, cofactors such as SIL1, and proteins that contribute to macromolecular assembly such as protein disulphide isomerase (PDI) which facilitates disulphide bond formation (reviewed in [50]). More recently, a small network of abundant extracellular proteins that can act as chaperones, have been identified. These proteins (including clusterin, haptoglobin and α_2-macroglobulin) are thought to act as holding

type chaperones which can promote the degradation of non-native proteins in the extracellular space via receptor mediated endocytosis and traffic to lysosomes [41, 51, 52]. In this way they are thought to protect against protein misfolding and aggregation in the extracellular space.

Thus, when considering the propensity of a protein to aggregate one must consider that all compartments in living organisms are patrolled by diverse and distinct networks of chaperones that are major and important contributors to maintaining protein homeostasis in the whole organism and thus aid in the prevention of large-scale protein aggregation.

What does studying aggregation and chaperones in vitro tell us?

As mentioned in the previous section, chaperones can prevent aggregation events by blocking intermolecular interactions (by "holding" the substrates) or by assisting in the refolding of aberrant substrates. To understand how chaperones prevent organised aggregation, such as amyloid formation, it is necessary to determine the key aspects of their interactions with the aggregating species. Unfortunately, fibrils formed *in vivo* are amongst countless other molecules in a complex and crowded environment, where individual and synergistic effects of chaperones and other biological co-factors can play a role in preventing or enhancing the process of aggregation. This makes it very difficult to study the exact mechanism by which biological molecules can prevent protein misfolding; therefore, to gain a more in depth understanding of this process, *in vitro* experiments, with purified proteins are of vital importance. Although attempts are made to maintain physiologically relevant conditions for *in vitro* fibril formation, it is not always possible, and destabilising conditions may be required (high temperatures, low pH, denaturants) to allow partial unfolding that will promote amyloid formation. For most studies, researchers try to ensure that the fibrillar products formed *in vitro* are reasonably similar to those isolated *in vivo* by carefully characterising these end products using a variety of biophysical techniques (as described in the introduction). Of course, *in vivo*, chaperones may also interact with other co-factors and/or protein handling processes such as degradative pathways; therefore the knowledge gained from *in vitro* experiments must be considered a snap shot of the processes that occur *in vivo*. The overarching goal of molecular chaperone studies is to help us determine how chaperones behave *in vivo*. Towards this goal, *in*

vitro studies can determine how individual chaperones interact with the various structurally heterogenous species along the fibril forming pathway to affect fibril formation.

A number of research groups have investigated the *in vitro* effects of different chaperones coming from a diverse range of chaperone families, including Hsp70 members [53-57], Hsp40 alone [58], Hsp90 members [57], small heat shock proteins (sHsps) [59-69], apolipoprotein E [70], chaperonins (TRiC) [71], prefoldin [72], Hsp104 [73], and extracellular chaperones [15, 74-80] on the process of fibril formation of a variety of proteins and peptides (Table 1). A striking pattern is apparent from the existing literature with regards to their effects on amyloid formation *in vitro*; regardless of the chaperone tested, there seems to be a remarkable commonality in their mode of action. The remainder of this review will examine in detail various aspects of this functional commonality.

Chaperones efficiently inhibit the formation of amyloid fibrils in vitro

In vitro amyloid formation can be followed by light scattering or by increases in ThT fluorescence. It has been repeatedly demonstrated that *in vitro* incubation of molecular chaperones with amyloid forming proteins inhibits the formation of highly structured amyloid fibrils. Impressively, molecular chaperones have been shown to inhibit the formation of amyloid even when present at extremely substoichiometric ratios of chaperone-to-amyloid forming protein. For example, small heat shock proteins (Hsp20, Hsp 27 and Hsp17.7) have been reported to inhibit the formation of $A\beta_{1-40}$ fibrils when present at an extremely low 1:1000 ratio of chaperone-to-Aβ peptide [65]. While α-crystallin, Hsp70, Hsp90, BiP and clusterin have all been shown to suppress the formation of fibrils by various proteins or peptides at chaperone-to-substrate ratios equal to or lower than 1:50 [53, 57, 61, 66, 79]. Although these molecular chaperones are structurally, compartmentally and functionally distinct, the fact that they can inhibit the formation of amyloid by unrelated proteins and peptides with such efficiency suggests that, at least in these

cases, they are acting via a similar mechanism which may involve interactions with a structurally homologous aggregating species.

In addition to insights gained about the mechanism of chaperone action, it is sometimes possible to obtain information from such assays on the process of amyloid formation itself. Indeed, the fact that chaperones can inhibit fibril formation when present at very low chaperone-to-substrate ratios implies that they interact with a population of amyloid forming species which is present at very low abundance. It follows that only a small proportion of the substrate monomer population contributes to the nucleating units that 'seed' fibril formation. This is consistent with data demonstrating that to initiate fibril formation, only a very small fraction of the population of lysozyme or HypF-N molecules is required to be partially unfolded [81, 82].

Pinpointing the mechanisms of fibril formation prevention

Based on the current knowledge of amyloid formation kinetics, it is possible to identify a number of protein species with which chaperones may interact (Figure 1), including:

1) The monomeric form of the protein or peptide involved in fibril formation; interactions here may stabilise the native state thereby preventing partial unfolding, or small structural changes which can lead to intermolecular interactions.

2) Partially unfolded, monomeric intermediates; interactions with intermediates could prevent further interactions, including oligomer formation, towards fibril formation.

3) Oligomeric species; interactions with oligomers could prevent the creation of stable nuclei required for 'seeding' fibril growth or may inhibit the addition of oligomers to protofibrils during the construction of mature fibrils.

4) Mature fibrils; interactions coupled with disaggregation may shift the equilibrium of the process to favour the smaller, less ordered species.

The remaining sections of the review will draw on the available *in vitro* data to examine the likelihood that interactions with the species identified above mediate the effects of chaperones on fibril formation.

Molecular chaperones show limited binding to native monomer

In general, the process of amyloid formation must involve the partial unfolding and a marked refolding of the native protein to generate intermediate structures with a high proportion of β-structure that can subsequently assemble into mature fibrils. This notion is consistent with the observation that amyloid fibrils are more likely to form under conditions in which the native state is destabilised, such as elevated temperature or low pH [83]. For intrinsically unfolded proteins and peptides, there is evidence to suggest that molecular chaperones not only inhibit fibril formation but may help to maintain the native secondary structure of a population of protein under conditions that would normally see the shifting of its structure to one that is predominantly β-sheet. Monomeric Aβ peptide displays a random-coil like CD spectrum; however under fibril forming conditions, there is a change in overall secondary structure to β-sheet as mature fibrils form. In the presence of αB crystallin, the random-coil like secondary structure of Aβ is maintained and this coincides with inhibition of fibril formation [66]. In addition, the presence of α-crystallin (including both αA and αB crystallins) inhibits the dramatic change in secondary structure of ApoC-II which, in the absence of chaperone, progresses from a random coil to a structure containing 80-85% β-sheet character under fibril forming conditions [61]. Similarly, Hsp70 inhibits the transition of α-synuclein to a structure which is predominantly β-sheet [56]. Due to the low effective concentration of chaperones needed, it is unlikely that this prevention of structural change is due to interactions with the native monomers. Instead, it seems to suggest that the native conformation is in equilibrium with a much less populated, metastable, non-natively structured state, which leads to the formation of the more stable nucleus and eventually to fibrils. By interacting with this small proportion of the protein population, molecular chaperones can prevent large-scale changes in secondary structure and thereby alter the *Native-Intermediate-Unfolded* equilibrium of the substrate.

In many studies, direct interactions with the native monomeric state of the fibril-forming protein and the chaperones of interest have not been investigated. However, a small number of studies have investigated these interactions using techniques such as

sedimentation velocity centrifugation, 2D NMR studies, dot-blot assays, ELISA pull-down assays and immunoprecipitation experiments [15, 54, 56, 61, 78-80]. In these investigations, no specific interaction between the chaperones and the starting native monomeric protein was observed, even when high concentrations of chaperone were present. Together with the observation that chaperones can inhibit amyloid formation at very substoichiometric chaperone to substrate ratios, these findings further confirm that the mechanism of action is not based on the binding of chaperone to native substrate monomer. Of course, in general, interactions between the starting monomeric forms and chaperones are possible considering that some of the substrates are intrinsically unfolded in nature; therefore, weak or non-specific interactions may occur, however these cases are likely to involve the presence of excess amounts of chaperone-to-substrate [54].

Interactions with partially unfolded monomers

In some systems, it is possible to measure the appearance of a population of a partially unfolded monomeric intermediate prior to fibril formation [5, 84]. As this intermediate species is usually very lowly populated, it is possible that chaperones may interact at this stage to prevent fibril formation. In amyloidogenic lysozyme variants, a transient intermediate species, crucial for *in vitro* fibril formation, can be detected using hydrogen/deuterium exchange monitored by mass spectrometry. If an inhibitor binds to the native state of lysozyme, it can abolish or delay the formation of this intermediate species by stabilising the native state. Alternatively, stabilisation of the transient intermediate would change the equilibrium between the native state and the transient intermediate by favouring the formation of the intermediate. Binding of the chaperone to either species would also affect the number of sites protected from H/D exchange. From our studies of the effect of extracellular chaperones on amyloidogenic lysozyme, we observed that while the presence of clusterin can efficiently inhibit fibril formation it had no effect on the number of protected sites (in either native or intermediate species) nor did it have any effect on the rate of intermediate formation as compared to lysozyme alone [79]. Therefore, in the case of fibril formation from human lysozyme, it appears that the mode of action of chaperone inhibition is downstream of the transition from native to non-native monomer.

Interactions with soluble oligomeric species

It is widely accepted that amyloid formation is a nucleation dependent process. Once a "critical nuclei" population is sufficient, fibril elongation proceeds rapidly and leads to the formation of mature fibrils [1]. There is mounting evidence that chaperones affect fibril formation by binding to, or influencing the formation of, functional nuclei [15, 56, 58, 61, 78-80]. Although inhibition of fibril formation can be mediated *in vitro* by substoichiometric amounts of chaperone, the efficiency of the chaperone action is dependent upon the timing of its addition to the aggregation reaction mixture. A number of groups have investigated the inhibitory effects of adding chaperones at various time points along the fibril forming process (i.e. beginning of the assay, mid lag phase, mid-elongation phase) [73, 79, 80, 85]. From these experiments, it is clear that once fibril formation has begun, chaperones are less effective at inhibiting the process. In contrast, when added at the beginning of the reactions, chaperones can completely inhibit fibril formation. In the case of the extracellular chaperones, clusterin shows some inhibitory effect when added mid-elongation phase, however, this is soon overwhelmed and fibril formation proceeds, whereas for haptoglobin and α_2-macroglobulin, addition at this stage has no noticeable effect on lysozyme fibril formation [79, 80]. Similarly, haptoglobin and α_2-macroglobulin had little effect on $A\beta_{1\text{-}42}$ fibril formation when added during the elongation stage [80]. One interpretation of this is that the rapid increase in the number of growing fibril ends, from breakage, overwhelms the chaperones when they are present at concentrations that, if added during lag phase, would normally efficiently suppress fibril formation. If this were the case one would expect that at certain concentrations chaperones would be able to protect against aggregation when added in the elongation phase. Interestingly, in some instances, there are reports that chaperone addition in the elongation phase can cease additional fibril formation. While addition of Hsp70 to α-synuclein at a 1:10 ratio (Hsp70:α-synuclein) efficiently inhibits fibril formation when added at the start of the aggregation reaction, addition of Hsp70 to the growth phase at a higher concentration giving a ratio of 1:2 is able to suppress further elongation [85]. Addition of Hsp104 at the mid-elongation point during $A\beta_{1\text{-}42}$ aggregation results in a slight decrease of Thio-T fluorescence [73, 85]. This suggests that Hsp104 may be acting upon alternate species and is consistent with its disaggregation properties [86].

Taken together, the above data strongly suggest that chaperones are most effective when added early in the lag phase of fibril forming reactions and that their inhibitory effects are drastically reduced once the critical nuclei are formed and fibril elongation begins. This observation suggests that (i) the increasing number of "growing ends" appearing in the growth phase can "overload" molecular chaperones, and/or (ii) molecular chaperones interact preferentially with soluble oligomeric prefibrillar species.

Soluble oligomers of amyloid forming proteins are currently the most intensely researched species on the amyloid forming pathway. This is because they are likely to be both a 'seeding' species for the initial elongation of fibrils (for on-pathway oligomers) and are also thought to be the most toxic of the amyloid species [87]. Interestingly, it appears that there are structural features common to all oligomeric species regardless of the protein or peptide from which they are formed, and that this structural commonality is recognised by the monoclonal antibody known as A11 [88]. However, it is likely that the soluble oligomers that are formed (on or off the amyloid forming pathway), and that are recognised by the A11 antibody, are a heterogeneous mixture of variably sized soluble oligomers rather than one specific oligomer of defined size. *In vitro* studies of various unrelated chaperones indicate that a soluble oligomeric species is the most likely species to interact with chaperones during amyloid formation. Hsp70 was shown to bind to oligomers of $A\beta_{1-42}$ and a prefibrillar species of α-synuclein [56, 57]. In addition, all three extracellular chaperones, α_2-macroglobulin, clusterin and haptoglobin, were shown to bind to intermediate prefibrillar species [15, 79, 80]. Moreover, the chaperonin TRiC has been shown to bind to prefibrillar aggregates of various sizes [71], while prefoldin has been shown to interact with large oligomers of $A\beta_{1-42}$ *in vitro* (up to 250 kDa in size) [72] and Hsp70 and Hsp90 have also been shown to interact with oligomers of $A\beta_{1-42}$ [57] .

In the case of the extracellular chaperones, ELISA pull down assays have been used to detect chaperone/substrate complexes during aggregation reactions of $A\beta_{1-42}$. Interestingly, the time points when these complexes were detected differ for clusterin

and haptoglobin or α_2M [15, 79, 80]. For the latter chaperones, the complex was detected at earlier time points in the fibril inhibition reaction (maximum appearance at 150 min) whereas clusterin appears to interact with species which are populated later on (beyond 300 min). Therefore, although the mechanism of inhibition appears to be similar (i.e. interaction with oligomeric species) there may be some variability in the size of the oligomers recognised by each specific chaperone. This is further supported by the action of prefoldin, which interacts with Aβ oligomers that are larger in size (up to 250 kDa in size) than those usually observed for $A\beta_{1-42}$ interactions *in vitro* [72]. Taken together, the evidence suggests that within the heterogenous pool of oligomeric species present before the critical nuclei are formed, there is a common structural element that can be recognised and bound by a range of unrelated chaperones. It has recently been predicted that these early oligomeric species expose large regions of hydrophobicity relative to their surface area [89] which is of great interest as chaperones are thought to bind to their substrates via exposed hydrophobic regions [37, 90]. Given that exposing hydrophobic regions on proteins is undesirable, both from a protein function and cytotoxicity standpoint, it is not surprising that evolution has driven the quality control mechanisms, such as molecular chaperones, to recognise and prevent this type of exposure [90]. Since hydrophobicity is a key element in the chaperone-substrate interaction, then it makes sense that oligomeric species are bound with the highest affinity, since their larger surface area to volume ratio compared to mature fibrils would make the hydrophobic residues more accessible for interaction.

Interactions of molecular chaperones with mature fibrils

In vivo fibrillar protein deposits generally consist of one dominant aggregated protein, along with a range of other proteins and molecules. Various chaperones are commonly found co-localised with *in vivo* protein deposits depending on the type of deposit and its location [91]. The reason why chaperones are found in protein deposits is unclear and the question still remains as to whether chaperones have bound to the deposits after they have formed or have been incorporated during the aggregation process. It has been observed in solutions containing very low substoichiometric ratios (1:500) of clusterin to aggregating $A\beta_{1-42}$ that clusterin can be co-centrifuged with pellets of insoluble $A\beta_{1-42}$ fibrils [15], yet, in immuno dot blot assays, binding of

extracellular chaperones to mature fibrils was not detected [15, 80]. Lastly, a variety of *in vitro* studies have shown that most chaperones do not disaggregate preformed fibrils, although Hsp104 has been shown to disaggregate fibrils in an ATP dependant fashion [86]. Hsp70 and Hsp90 had no measurable effect on mature $A\beta_{1-42}$ fibrils [57], and neither α_2-macroglobulin or haptoglobin had any effect on mature fibrils formed by $A\beta_{1-42}$, calcitonin or ccβw [80].

It is interesting to note that in some cases chaperones have been observed to promote the formation of fibrils. Recent results suggest that Hsp104 speeds up the formation of NM (Sup35's prion domain) fibrils by contributing to the breakage of long fibrils into shorter fragments, thus providing more "growing ends" [86]. In addition, at very substoichiometric levels (e.g. 1:500 chaperone-to-substrate) the extracellular chaperone clusterin promoted the formation of amyloid fibrils with increased thioflavin T fluorescence properties of calcitonin, α-synuclein and $A\beta_{1-42}$ when compared to controls. Moreover, ApoE and α1-antichymotrypsin (ACT) have also been shown under certain conditions to bind Aβ and promote the formation of β-sheet rich amyloid fibrils, as a consequence both ApoE and ACT have been termed "pathological chaperones" (reviewed in [92]). Regardless of the nature of the association between chaperones and *in vivo* protein deposits, the trapping of chaperones in these deposits may compromise the net chaperone activity of a system. In addition, the co-localisation of chaperones with fibrils *in vivo* leads to the interesting speculation that these chaperones may be labelling fibrils for subsequent degradation, although clear evidence of this has not been reported.

Links between in vitro and in vivo data

Collectively, the above data indicates that chaperones are able to efficiently inhibit the formation of large structured aggregates. Therefore it is not unreasonable to think that chaperones should also efficiently inhibit the formation of amyloid *in vivo*. If one considers this to be true then it follows that in order for protein deposits to occur *in vivo*, chaperone machinery must be either overwhelmed or impaired in some way. This has been proposed previously by Muchowski and Wacker [93] and more recently by Morimoto [36] and is consistent with the increase in protein deposits with age and the "living on the edge theory". The living on the edge theory stems from

recent observations that levels of gene expression are correlated with the *in vitro* aggregation propensity of the respective gene products [94]. The authors conclude that individual proteins have evolved to efficiently function at levels within the cell that border on their respective thresholds for aggregation. This "life on the edge" dictates that any deviation from normal conditions, such as increased expression, oxidative stress, or a mutation that destablilizes the protein, could potentially cause the protein to aggregate and result in disease. This elegant theory may explain why, as human beings age, they are more likely to acquire protein deposition diseases. In fact, recent data from an Amyotrophic Lateral Sclerosis (G93A SOD1) mouse model shows that although mutant SOD1 interacts with chaperones during early presymptomatic stages, insoluble deposits only form in ageing mice. This suggests that cells become progressively less efficient at handling misfolded proteins with age [95]. Moreover, genetic polymorphisms that produce even marginally unstable proteins have been shown to contribute to the overloading of the protein folding network to generate insoluble protein aggregates in *C. elegans* expressing poly Q proteins [96]. This is supported by the observation that increases in the levels of $A\beta_{1-42}$ protein as small as 1.5 fold are enough to result in Aβ pathology (reviewed in [97]). Indeed, inducing the heat-shock response with the small molecule, arimoclomol, delayed disease progression in a mouse model of ALS by rescuing motor neurones from cell death [98]. Similarly, down regulation of small heat shock proteins decreased the lifespan of *C. elegans* expressing Huntington's-like polyglutamine repeat proteins [99], while directed expression of Hsp70 in α-synuclein-expressing *Drosophila* protected against neuron loss [100].

Conclusion

In conclusion, studies of the effects of chaperones on amyloid formation *in vitro* have not only increased our knowledge of chaperone function but have also advanced our understanding of amyloid formation and disease. *In vitro* studies have identified prefibrillar aggregates (oligomers) as a critical stage in fibril formation and the stage at which interactions with chaperones can determine whether the outcome is physiological homeostasis or pathology. It is becoming increasingly clear that while under normal conditions chaperones and other quality control systems efficiently and

rapidly deal with misfolded and partially unfolded proteins, slight variations in the structure or expression levels of a protein may overwhelm these systems and lead to protein deposition and disease. Collectively, current knowledge indicates that soluble protein oligomers are an important target for novel therapeutics in amyloid disease and molecular chaperones present themselves as a broad pallet of tools in this context.

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Figure 1: Typical pathway of *in vitro* fibril formation

A typical pathway of the stages of *in vitro* fibril formation of amyloidogenic human lysozyme variants. The light scattering curve shows sigmoidal kinetics which is representative of a nucleation dependent mechanism. Under physiological conditions, the monomeric protein (1) exists in equilibrium between the native, intermediate and unfolded states. When fibril forming conditions are applied, a shift in the equilibrium increases the population of the intermediate (2) (in the case of human lysozyme this is a transiently populated, partially unfolded species) which can further interact to form a heterogeneous mixture of small oligomeric species (3). As these small oligomers increase in size and stability, they reach a "critical population", which acts as the nucleus for fibril formation. This is followed by a rapid elongation stage, resulting in the identification of mature fibrils by methods such as Thioflavin-T binding and transmission electron microscopy (TEM) (4).

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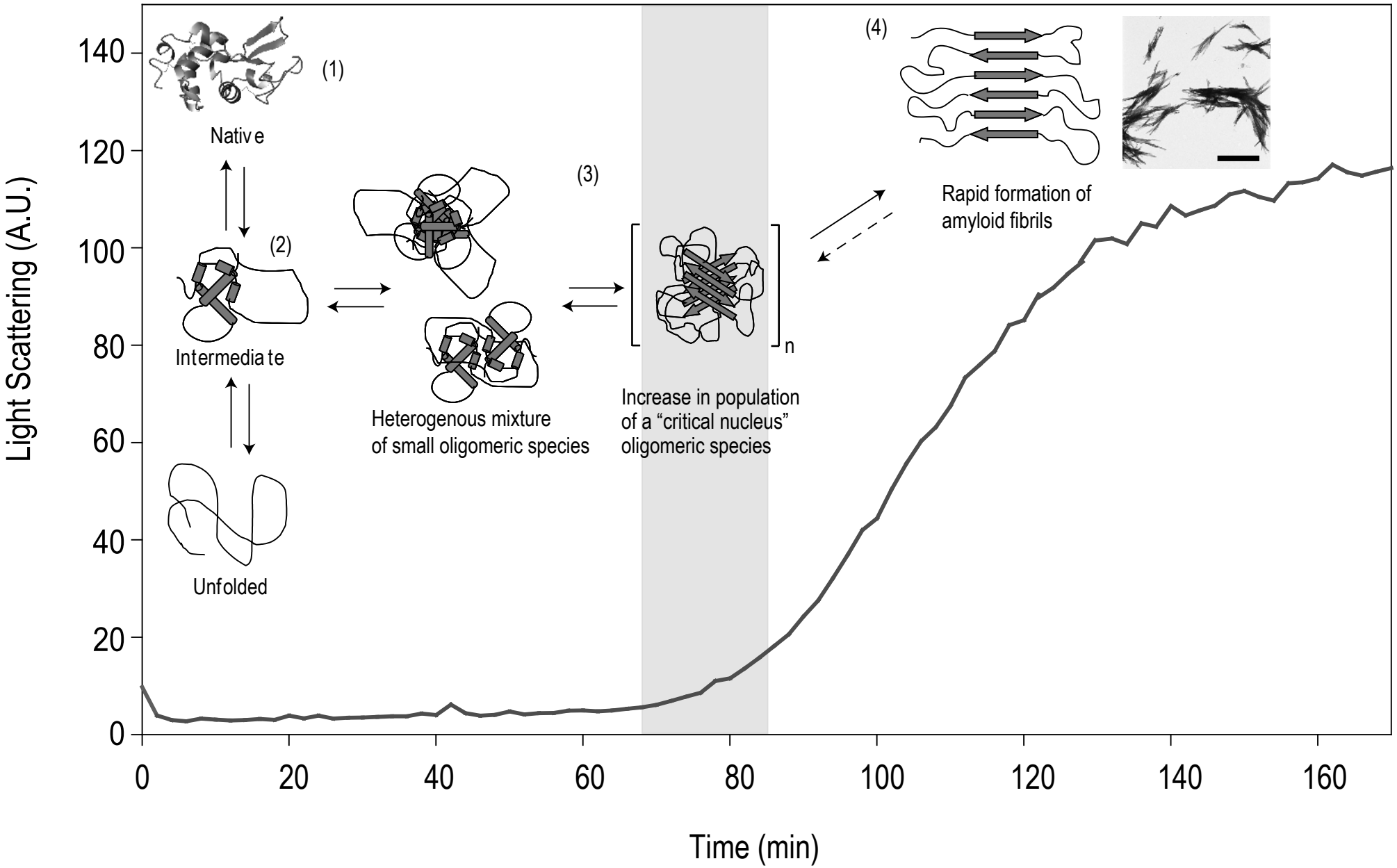
Table 1: Chaperones which affect *in vitro* fibril formation

Chaperone Family	Chaperone Tested	Substrate Peptide or Protein	Effective Ratios tested (chaperone-to-substrate)	References
Hsp40 Members	Hsp40/Hsp70 (1:100)	$A\beta_{1-42}$	1:50, 1:100 (both with ATP)	[57]
	Hsp40/Hsp70 (1:1)	Polyglutamine	2:1 (with ATP)[*]; 1:1 (with and without ATP)[†]	[*][54]; [†][55]
	Ydj1	Prion protein (Ure2)	1:1, 1:2	[58]
Hsp70 Members	BiP	Ig light chain	1:30, 1:100	[53]
	Hsp70	$A\beta_{1-42}$	1:50 (with ATP)	[57]
		α-synuclein	1:10[*†]; 1:2 (with and without ATP)[‡]	[*][56]; [†][101]; [‡][85]
Hsp90 Members	Hsp90	$A\beta_{1-42}$	1:50 (with ATP)	[57]
Hsp100 Members	Hsp104	$A\beta_{1-42}$	1:10, 1:1000 (both with ATP)	[73]
Small heat shock proteins (sHsps)	αB-crystallin	α_1-antichymotrypsin (ACT)	1:2	[62]
		$A\beta_{1-40}$	1:5[*†]; 1:100 (seeded reaction)[‡]	[*][60]; [63]; [†][66]; [‡][67]
		Apolipoprotein C-II	1:67	[61]
		α-synuclein	1:4, 1:2	[102]
		β_2-microglobulin	1:13	[66]

	αA-crystallin	$A\beta_{1-40}$	1:1	[64]
	Hsp17.7	$A\beta_{1-40}$	1:1000	[65]
	Hsp20	$A\beta_{1-40}$	1:1000[*]; 1:5[†]	[*][65]; [†][67]
	Hsp22	αB-crystallin (R120G variant)	-	[68]
	Hsp25	αB-crystallin (R120G variant)	-	[68]
	Hsp27	$A\beta_{1-40}$	1:1000[*]; 1:5[†]	[*][65]; [†][67]
		$A\beta_{1-42}$	1:6, 1:40	[59]
Extracellular Chaperones	α_2-macroglobulin	$A\beta_{1-42}$	1:8[*]; 1:100[†], 1:20[†], 1:10[†]	[*][76]; [†][80]
		Calcitonin	1:30, 1:15	[80]
		ccβw peptide	1:150; 1:30; 1:15	[80]
		Human lysozyme (I59T)	1:10	[80]
	Clusterin	$A\beta_{1-42}$	1:50[*†]; 1:10[‡]	[*][74]; [†][15]
		Apolipoprotein C-II	1:90	[78]
		α-synuclein	1:10, 1:4	[15]
		β_2-microglobulin	1:500, 1:100, 1:50, 1:10	[15]
		Calcitonin	1:10	[15]
		ccβw peptide	1:500, 1:100, 1:50, 1:10	[15]
		Human lysozyme (I56T, I59T)	1:100, 1:40, 1:10	[79]
		κ-casein	1:100, 1:50, 1:10, 1:5	[15]
		Neuropeptide 106-126	1:600	[77]
	Haptoglobin	$A\beta_{1-42}$	1:100, 1:20, 1:10	[80]
		Calcitonin	1:150, 1:30, 1:15	[80]
		ccβw peptide	1:30, 1:15	[80]
		Human lysozyme (I59T)	1:10	[80]
Others	Apoliprotein E	$A\beta_{1-40}$	1:1000	[70]
	Prefoldin	$A\beta_{1-42}$	1:6	[72]


Light Scattering (A.U.)
Time (min)
0
20
40
60
80
100
120
140
160
(1)
Native
(2)
Intermediate
Unfolded
(3)
Heterogenous mixture
of small oligomeric species
Increase in population
of a “critical nucleus”
oligomeric species
n
(4)
Rapid formation of
amyloid fibrils