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## Crystallin proteins and amyloid fibrils

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### Abstract

Improper protein folding (misfolding) can lead to the formation of disordered (amorphous) or ordered (amyloid fibril) aggregates. The major lens protein, alpha-crystallin, is a member of the small heat-shock protein (sHsp) family of intracellular molecular chaperone proteins that prevent protein aggregation. Whilst the chaperone activity of sHsps against amorphously aggregating proteins has been well studied, its action against fibril-forming proteins has received less attention despite the presence of sHsps in deposits found in fibril-associated diseases (e.g. Alzheimer's and Parkinson's). In this review, the literature on the interaction of alpha B-crystallin and other sHsps with fibril-forming proteins is summarized. In particular, the ability of sHsps to prevent fibril formation, their mechanisms of action and the possible in vivo consequences of such associations are discussed. Finally, the fibril-forming propensity of the crystallin proteins and its implications for cataract formation are described along with the potential use of fibrillar crystallin proteins as bionanomaterials.

### Keywords

proteins, amyloid, crystallin, fibrils, CMMB

### Disciplines

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## **Crystallin proteins and amyloid fibrils**

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### Abbreviations

A $\beta$ , amyloid-beta; Hsp, heat shock protein; sHsp, small heat shock protein; ThT, thioflavin T

### Running title

Crystallin proteins and amyloid fibrils

## Summary

Improper protein folding (misfolding) can lead to the formation of disordered (amorphous) or ordered (amyloid fibril) aggregates. The major lens protein,  $\alpha$ -crystallin, is a member of the small heat-shock protein (sHsp) family of intracellular molecular chaperone proteins that prevent protein aggregation. Whilst the chaperone activity of sHsps against amorphously aggregating proteins has been well studied, its action against fibril-forming proteins has received less attention despite the presence of sHsps in deposits found in fibril-associated diseases (e.g. Alzheimer's and Parkinson's). In this review, the literature on the interaction of  $\alpha$ B-crystallin and other sHsps with fibril-forming proteins is summarized. In particular, the ability of sHsps to prevent fibril formation, their mechanisms of action and the possible *in vivo* consequences of such associations are discussed. Finally, the fibril-forming propensity of the crystallin proteins and its implications for cataract formation are described along with the potential use of fibrillar crystallin proteins as bionanomaterials.

## Keywords

amyloid fibril, protein aggregation, protein folding, molecular chaperone, small heat-shock protein, crystallin, lens, cataract, Alzheimer's disease, Parkinson's disease

## 1. Introduction

Vision is arguably the most important of the senses. Crucial to vision is the ability of the eye lens to focus light on to the retina. Proper lens transparency is highly dependent on the well-ordered arrangement of the crystallin proteins, which are present in high concentration and make up almost the entire composition of the lens. Disruption of crystallin protein arrangement, for example as a result of protein aggregation and precipitation, leads to impairment of lens light refraction, potentially cataract formation and, ultimately, loss of sight.

The nature of the aggregates that are formed by crystallin proteins during cataract formation is a matter of debate, i.e. whether the aggregates are amorphous (unstructured) or highly ordered (amyloid fibril) in form, or a combination of both. So too is the mechanism of crystallin protein aggregation. There is no efficacious drug for cataract and current treatment involves removal of the lens and its replacement with a plastic one. Clearly, determining the fundamental aspects of the processes by which crystallin proteins aggregate will have important consequences for understanding cataract and the development of possible therapeutics.

The major lens protein,  $\alpha$ -crystallin, is a member of the small heat-shock protein (sHsp) family of intracellular molecular chaperone proteins which interact with partially folded proteins to prevent their mutual association and possible precipitation [1-3]. As a result of this ability, in addition to its structural role in the lens,  $\alpha$ -crystallin acts in a chaperone manner to prevent aggregation of the crystallin proteins (including itself).  $\alpha$ -Crystallin is composed of two, closely related subunits,  $\alpha$ A and  $\alpha$ B, and is not lens specific. Apart from the lens, the  $\alpha$ B subunit is expressed extensively in many other tissues, including the brain, lung, and cardiac and skeletal muscle [4] where it functions in a chaperone manner by interacting with a broad range of partially folded target proteins. Thus, *in vitro*,  $\alpha$ B-crystallin (and  $\alpha$ A-crystallin) suppress the aggregation and precipitation of a wide range of target proteins that precipitate amorphously or in a fibrillar form. The interaction of  $\alpha$ B-crystallin with the latter type of aggregating target proteins is of interest because of the presence of amyloid fibrils in the proteinaceous deposits associated with many diseases including Alzheimer's and Parkinson's and type II diabetes.

The interaction of  $\alpha$ -crystallin with amorphously aggregating target proteins has been well described and reviewed (e.g. [3, 5-7]), although specific details of the mechanism by which  $\alpha$ -crystallin (and the sHsps, in general) accomplishes its chaperone action have not been elucidated. In this review, we summarize the literature on the interaction of  $\alpha$ B-crystallin and other sHsps with target proteins that are aggregating to form amyloid fibrils and the possible *in vivo* consequences of such associations. In addition, we describe our recent work investigating the fibril-forming propensity of  $\alpha$ -crystallin and the other crystallin proteins. The implications for cataract formation are discussed along with the possible applications of crystallin amyloid species in the emerging field of bionanomaterials.

## 2. Background to the crystallin proteins

### *Crystallins and the eye lens*

The mammalian lens is a unique organ – it is avascular, it continues to grow throughout the life of an individual and its lens fibre cells have little metabolic activity and are composed almost

entirely of crystallin proteins which are not turned over [5, 8]. As a result, crystallin proteins at the centre (nucleus) of the lens are as old as the individual itself. They are durable proteins, but, due to the destabilizing influences with age of a combination of extensive post-translational modification (for example truncation, phosphorylation, oxidation, deamidation and racemization), as well as changes in cellular conditions, they eventually aggregate and precipitate out of solution. This ultimately leads to the development of cataract [9] and, since the oldest crystallin proteins are in the nucleus, age-related cataract initially occurs in the centre of the lens.

In addition to the  $\alpha$ -crystallin subunits, the mammalian lens contains  $\beta$  and  $\gamma$ -crystallins [8]. Each of these classes is comprised of a variety of closely sequence-related proteins (Table 1). For example, the  $\gamma$ -crystallins encompass seven subunits, A, B, C, D, E, F and S of mass  $\sim 21$  kDa [10]. The  $\beta$ -crystallins encompass four acidic (A) and three basic (B) subunits of mass  $\sim 22$ -28 kDa of which the most populous is  $\beta B2$ . The  $\beta$  and  $\gamma$ -crystallins are homologous  $\beta$ -sheet proteins. At a primary sequence level, the major difference between the  $\beta$  and  $\gamma$ -crystallins is the presence of flexible terminal extensions in the former; the acidic  $\beta$ -crystallin subunits have an N-terminal extension while the basic  $\beta$ -crystallin subunits have N- and C-terminal extensions [11, 12]. X-ray crystal structures are available for representative subunits of both protein classes [10]. They share the same secondary structure comprising two domains each of two Greek Key  $\beta$ -sheet motifs, i.e. a total of 16  $\beta$ -strands. However, due to the different arrangement of the connecting peptides (in the  $\gamma$ -crystallins, the connecting peptide is bent and in  $\beta$ -crystallins, it is extended), the  $\gamma$ -crystallins are monomers whereas the  $\beta$ -crystallins exist as a range of oligomers comprising dimers through to octomers (Fig. 1A). Excellent review articles are available on structural aspects of the  $\beta$ - and  $\gamma$ -crystallins (e.g. [10, 12, 13]).

The two subunits of mammalian  $\alpha$ -crystallins,  $\alpha A$  and  $\alpha B$ , share over 50% sequence similarity and each have a subunit mass of  $\sim 20$  kDa [14-17]. In the human lens, they are present in a ratio of approximately 3:1  $\alpha A$ :  $\alpha B$  with the ratio varying with age. The two subunits co-exist as large, heterogeneous complexes of mass range of approximately 160 to 1000 kDa. No crystal structure is available for any  $\alpha$ -crystallin subunit, although recently structures have become available for three non-mammalian, but well-ordered, sHsp oligomers [18-21]. The building block for these sHsps is a dimer and thus, due to the sequence similarity between sHsp members, the  $\alpha$ -crystallin oligomer is also probably built up from dimeric subunits.  $\alpha$ -Crystallin subunits have three structural regions (Fig. 1B and 1C), of which the C-terminal domain (of around 90 amino acids in length) is relatively well conserved across all sHsps and adopts an immunoglobulin-like  $\beta$ -sheet fold [19, 22]. The C-terminal domain in sHsps is sometimes known as the  $\alpha$ -crystallin domain and most likely comprises one or more of the peptide-binding regions that are involved in interacting and binding to target proteins during chaperone action. Flanking the C-terminal domain are two regions of less sequence similarity: (i) the N-terminal domain which is important in subunit-subunit interactions, i.e. the quaternary arrangement of the protein, and possibly, chaperone interaction [23, 24] and (ii) the C-terminal extension which contains a region of polarity, great mobility and no defined structure at the extreme C-terminus. This flexible region of 10 to 12 amino acids is important in solubilizing the protein and the complexes it makes with target proteins during chaperone action [2, 3, 25]. In addition to flexibility at the C-terminus, there are other regions of mobility in the protein, features which may facilitate their interaction with target proteins during chaperone action. In general, significant conformational flexibility and structural disorder are characteristics of molecular chaperone proteins [26]. Both subunits of

$\alpha$ -crystallin, along with all other sHsps that have been studied, are highly dynamic oligomeric species in which the subunits undergo constant exchange at physiological temperatures, with the rate of exchange increasing at higher temperature [27].

*Protein aggregation pathways and the chaperone action of  $\alpha$ -crystallin against amorphously aggregating target proteins*

The description of the chaperone action of  $\alpha$ -crystallin by Horwitz [1], Bhat's observation that  $\alpha$ B-crystallin was not lens-specific and is found in many other tissues [28], and extensive experimental evidence that  $\alpha$ B-crystallin (and other sHsps) are associated with a wide range of diseases (see below), have led to greatly enhanced research interest in the protein. Over the past ten years, there have been significant advances in understanding the chaperone action of  $\alpha$ -crystallin (and other sHsps) in mitigating target protein unfolding and aggregation. Review articles are available which have extensive discussion of the chaperone action of  $\alpha$ -crystallin towards amorphously aggregating target proteins [3, 5, 6, 19, 25, 29-31], so this topic will not be presented in detail here. However, so that the chaperone action of  $\alpha$ -crystallin can be discussed and understood with relevance to this review, the pathways and mechanisms of protein aggregation must be elaborated upon.

Immediately after synthesis on the ribosome, a protein is in an unfolded state (U) (Fig. 2). It folds into its native, functional form (N) via the folding/unfolding pathway that involves a series of partially folded intermediate states ( $I_1, I_2, I_3, \dots$ ) that have varying degrees of structure, for example molten globule states that have elements of secondary structure but little or no tertiary structure. The intermediate states have their hydrophobic core exposed to solution and are therefore inherently unstable and prone to incorrect folding (misfolding) and mutual association (aggregation). To avoid possible interactions between these intermediates, most proteins fold rapidly (in the order of micro to milliseconds), many without the need for assistance from molecular chaperone proteins that facilitate and encourage correct protein folding, e.g. Hsp70 and Hsp60 [32].

However, if the protein lingers for too long in this partially folded state(s) it may enter the slower off-folding pathways that lead to aggregation and possibly precipitation. This process may also occur if the native protein is destabilized by stress conditions, for example by elevated temperature, which disrupts hydrogen bonding and hydrophobic interactions between some side chains and results in its partial unfolding leading to the intermediately folded species entering one of the off-folding pathways. There are two off-folding pathways that a protein may enter: amorphous and amyloid fibril [33] (see Fig. 2). As the name implies, the amorphous aggregation off-folding pathway gives rise to disordered aggregates that contain the partially folded intermediates in a relatively extended conformation. Via a nucleation-dependent mechanism and when a certain size of the aggregate (a nucleus) is reached, rapid sequestration of other intermediates occurs, leading to a randomly arranged aggregate that exposes significant hydrophobicity to solution, is unstable and therefore irreversibly precipitates. The amyloid fibril pathway also arises from the association of intermediate states via a nucleation-dependent mechanism but, in this case, the aggregates are highly ordered in structure. The aggregation process involves the formation of a prefibrillar nucleus that may be a dimer, trimer or a larger oligomer. Eventually, as with amorphous aggregation, the aggregates precipitate out of solution.

The factors which govern whether a protein enters the amorphous or amyloid fibril off-folding pathway are not entirely clear. However, the rate at which the aggregation occurs is important, with more rapid aggregation favouring the amorphous pathway whereas the fibril pathway is characterized by a slower rate of aggregation which enables the formation of well-structured amyloid fibrils. Other factors which impact on the type of aggregates are the nature of the intermediates from which they are formed and the amino acid sequence of the protein concerned. With regards to the latter, certain amino acid sequences are highly amyloidogenic and there are algorithms available to predict such regions based on amino acid sequence alone [34, 35].

It is clear from a variety of studies that  $\alpha$ -crystallin interacts with amorphously aggregating, intermediately folded target proteins early on their off-folding pathway, i.e. when the former are in a monomeric, destabilized state [36, 37]. In doing so,  $\alpha$ -crystallin binds to the target protein to form a stable complex of large mass.  $\alpha$ -Crystallin exhibits high stoichiometry (i.e. capacity) in binding to target proteins with up to one target protein being bound per subunit of  $\alpha$ -crystallin. Mechanisms have been proposed for the chaperone interaction of  $\alpha$ -crystallin (and sHsps) with target proteins in which the dissociated, dimeric form of  $\alpha$ -crystallin is more chaperone active than the aggregated (oligomeric) species (Fig. 3), implying that subunit exchange involving the dimeric species is important in chaperone action [3, 27, 29, 38]. However, other studies have shown that the chaperone activity and rate of subunit exchange are not correlated (e.g. [39, 40]) and therefore the involvement of subunit exchange in the mechanism of chaperone action of  $\alpha$ -crystallin is somewhat controversial.

$\alpha$ -Crystallin and all other sHsps are ATP-independent molecular chaperones. Their expression is turned on under conditions of cellular stress (e.g. elevated temperature) when ATP levels are low. Under these conditions, sHsps interact and complex to the long-lived, partially folded, destabilized target proteins but have no ability to refold them (Fig. 3) [41]. The interaction between the two proteins is most likely facilitated by the dynamic nature of the  $\alpha$ -crystallin oligomer (i.e. subunit exchange and conformational flexibility and malleability) along with a similar process being undertaken by the target protein during its unfolding and mutual association. The two proteins thereby become co-incorporated into a high molecular weight (HMW) chaperone-target protein complex. In the process of interaction between the two proteins, cryo electron microscopy data suggest that the target protein coats the outside of the  $\alpha$ -crystallin oligomer [7]. When cellular conditions return to normal, the native state of the target protein can be recovered via the action of other molecular chaperones, e.g. Hsp70, coupled with ATP hydrolysis (Fig. 3) [29]. Thus, it is probable that, *in vivo*, these two chaperones,  $\alpha$ -crystallin and Hsp70, function in a synergistic manner.

Recent studies by Mchaourab and colleagues on the interaction of destabilized T4 lysozyme mutants with sHsps have led to a proposed mechanism of sHsp chaperone action in which the equilibrium between the folding and unfolding of the target protein (T4 lysozyme) is coupled to the equilibrium between the dissociated (dimeric) and oligomeric states of  $\alpha$ -crystallin [42-44].  $\alpha$ -Crystallin binds T4 lysozyme in two modes with high and low affinity. In this model, the dimeric form of the sHsp is the activated state which recognizes and binds to the destabilized target protein [44].

In addition, the chaperone action of  $\alpha$ -crystallin is modified by environmental conditions, for example temperature and pH. With increasing temperature, both subunits of  $\alpha$ -crystallin partially unfold which is accompanied by an increase in exposed hydrophobicity to solution and enhanced chaperone ability [45-48]. The increase in  $\alpha$ -crystallin subunit exchange rate with elevated temperature [27] may also contribute to this increase in chaperone ability.

### *sHsps and disease*

There are ten sHsps in the human genome [49, 50]. After  $\alpha$ A- and  $\alpha$ B-crystallin, the most populous sHsp is Hsp27 (Hsp25 in mouse). Other sHsps include Hsp20, HspB2 and HspB3. They are found in varying levels in different organs, depending on the stage of development and the level of stress present and are capable of forming mixed sHsp oligomers, i.e. ones that are composed of two or more sHsps. Plants have a multitude of sHsps [19]. The specific functions of these sHsps in various organisms are far from understood. What is most likely is that all sHsps share the common trait of interacting with partially folded, destabilized states of target proteins. They are involved in a wide variety of cellular tasks such as actin polymerization, regulating cellular redox states and caspase protein inactivation (i.e. as negative regulators of apoptosis).

Of late, it has become apparent that many seemingly unrelated diseases, particularly those of ageing, are characterized by the accumulation and precipitation of specific proteins. They are categorized under the broad umbrella of protein misfolding or conformational diseases and include Alzheimer's, Parkinson's, Creutzfeldt-Jakob and Huntington's and, of course, cataract. In each case, the deposits that are characteristic of the disease contain the predominance of one protein, i.e.  $\beta$  amyloid peptide in Alzheimer's,  $\alpha$ -synuclein in Parkinson's, prion protein in Creutzfeldt-Jakob, huntingtin in Huntington's and crystallin proteins in cataract. In the case of the first four of these diseases, the deposits or plaques contain the proteins in an amyloid fibrillar form. Associated with these deposits are a variety of other proteins, i.e. molecular chaperones including sHsps, particularly  $\alpha$ B-crystallin and Hsp27, which are presumably expressed by the cell in response to the presence of the aggregating fibrillar species (see below). Furthermore, sHsps are implicated in a variety of other diseases including cancers and multiple sclerosis [31, 51].

An amyloid fibril is a generic structure that is accessible to all proteins, under the appropriate conditions [33]. It is comprised of a cross  $\beta$ -sheet structure in which the polypeptide chain is arranged in  $\beta$ -sheets stacked perpendicular to the axis of the fibril forming a steric zipper [52-56]. Amyloid fibrils are composed of two to six protofibrils that plait together to form rope-like structures of 5 to 10 nm in diameter and up to hundreds of nm in length. The structural features of amyloid fibrils are highlighted in Figure 4. Fibrils are highly stable structures and are resistant to proteases and denaturants which may account for the difficulty that cellular degradation mechanisms have in removing them once they have formed.

The mechanism(s) by which amyloid fibrillar species cause disease are a matter of debate but they may involve processes leading to the production of free radicals and/or the formation of pore-like structures which permeabilise the cell membrane. Precursors to amyloid fibres, particularly small oligomeric species, may be toxic, i.e. there is evidence, at least for some proteins, that the fibrillar species is inert [33]. However, for other proteins the mature fibril can also be toxic [57, 58] and, in fact, the cytotoxic species may vary depending on the fibril-forming

protein. By way of a caveat to these discussions, it has recently been recognized that functional amyloid fibrils exist in nature, i.e. not all amyloid fibrillar forms are detrimental to cells [59]. Obviously, to avoid toxic effects, the production of functional amyloid fibres has to be tightly regulated.

In this review, the interaction of  $\alpha$ -crystallin (and other sHsps) with fibril-forming proteins and its possible implications, are major topics for discussion.

### 3. Association of sHsps with amyloid diseases.

The expression of some sHsps is dramatically up-regulated in response to pathological conditions associated with fibril formation. For example, induction of sHsps has been reported in Alexander's disease [60], Creutzfeldt–Jakob disease [61], Alzheimer's disease, [62, 63] and other neurological conditions [64-69]. Moreover, sHsps have been found to co-localize with the protein aggregates associated with a number of these protein misfolding diseases and/or are found at significant levels in neurons that surround the deposits (see below for details). Together, these data suggest that the cell perceives events leading to amyloid fibril formation as a stress and acts accordingly by inducing expression of sHsps. It also implies an important role for sHsps in amyloid diseases, indicating that they are the first line of defence for the cell in its attempt to mitigate amyloid fibril formation by the particular disease-associated protein. In particular, their localization to activated glia cells such as astrocytes [63, 70] implies an important role for sHsps in these cells in neurodegenerative diseases. Despite the correlation between sHsp expression and co-localization with amyloid fibril deposits in various protein conformational diseases, the precise role of sHsps in interacting with the aggregating target protein and the subsequent effect on its toxicity remains controversial and may differ for various target proteins.

An intriguing link between fibril formation and the ability of sHsps to prevent the toxicity associated with this process has been found by studies showing that Hsp27 and  $\alpha$ B-crystallin increase the resistance of cultured cells [71-73] and mice [74, 75] to oxidative stress. In addition, Hsp27 and  $\alpha$ B-crystallin expression has been found to negatively correlate with levels of reactive oxygen species and nitric oxide. This may be significant due to findings indicating that the toxicity of prefibrillar amyloid species is due to the production of reactive oxygen species by the aggregating target protein, which are generated as a consequence of the fibril-forming process [76]. Therefore, sHsps may act both as amyloid inhibitors to stop fibril formation and also as mediators of cell redox resistance.

#### *sHsps and $\alpha$ -synuclein based diseases (synucleinopathies).*

An example of the relationship of sHsps with diseases associated with amyloid fibrils is the synucleinopathies, i.e. diseases that are characterized by fibrils formed by the protein  $\alpha$ -synuclein which subsequently forms intracellular fibrillar inclusions called Lewy bodies. The aggregation of  $\alpha$ -synuclein into these protein deposits is thought to play a central role in the pathogenesis of synucleinopathies [77-79]. The synucleinopathies include both familial and sporadic Parkinson's disease in which  $\alpha$ -synuclein is localized in Lewy neurites in addition to its deposition in Lewy bodies. The immunoreactivity of  $\alpha$ -synuclein in Lewy body deposits is also the characteristic pathological hallmark of dementia with Lewy bodies (DLB) in which Lewy bodies can be found throughout the brain [80]. In addition, multiple system atrophy is a neurodegenerative disease

that is associated with filamentous protein aggregates primarily composed of  $\alpha$ -synuclein that occur as glial cytoplasmic inclusions (GCIs). sHsps are located in neuritic plaques and Lewy bodies in brain tissue from Parkinson's disease patients and related synucleinopathies [81, 82]. For example, several Hsps, including Hsp27 and  $\alpha$ B-crystallin, co-localise with  $\alpha$ -synuclein Lewy body deposits of DLB and GCIs [68, 82]. A recent study found Hsp27 levels were 2.5-fold higher in brains of patients with DLB compared to controls [69] and in a mouse model of Parkinson's disease, the levels of Hsp25 and  $\alpha$ B-crystallin were also found to be significantly higher than controls [83]. These results are suggestive of sHsps playing a role in the disease process and a stress response being invoked by the aggregation of  $\alpha$ -synuclein. In support of this, cell-culture models of  $\alpha$ -synuclein aggregation have shown that Hsp27 perturbs the aggregation process, and in so doing so inhibits the associated toxicity [69, 84]. It appears that Hsp27 inhibits the formation of toxic  $\alpha$ -synuclein aggregates at an early time point in its aggregation.

#### *sHsps and Alzheimer's disease.*

The main histological characteristic of Alzheimer's disease is the deposition of amyloid- $\beta$  ( $A\beta$ ) peptides in extracellular plaques in the central nervous system and the walls of cerebral blood vessels, as well as intracellular tangles of the tau protein in surrounding neurons. The amyloid plaques, besides containing fibrillar forms of the  $A\beta$  peptides, also contain several other proteins including sHsps [62, 70]. In addition, these chaperones are also expressed in neurons that surround these deposits [63, 70]. Quantitative reverse transcription-PCR has also been used to demonstrate increased accumulation of  $\alpha$ B-crystallin mRNA in select regions of post-mortem Alzheimer's disease brains [85] and increased levels of the protein have been reported in the temporal and frontal cortex [62, 86]. In addition, Hsp27 is associated with neurofibrillary tangles of hyperphosphorylated tau protein [87]. With regards to the localization of sHsps in senile plaques associated with Alzheimer's disease, whilst there have been reports of extracellular sHsps [88], they are predominately regarded as intracellular proteins. Thus, their extracellular deposition is most likely as a consequence of their release from surrounding cells due to the toxic effects of the aggregation process.

In a series of elegant papers using transgenic *Caenorhabditis elegans* worms, the expression of human  $A\beta$ (1-42) was found to lead to the induction of Hsp16 proteins (sHsps that are homologs of  $\alpha$ B-crystallin in vertebrates) [89]. Furthermore, the Hsp16 proteins co-localized and co-immunoprecipitated with  $A\beta$ (1-42) in this model [89] and increased expression of Hsp16 partially suppressed the  $A\beta$ -mediated toxicity [90]. It was concluded that sHsps such as Hsp16 may act to reduce  $A\beta$  toxicity by interacting directly with the  $A\beta$  peptide and altering its oligomerization pathways, thereby reducing the formation of some toxic species [90].

#### *sHsps and other diseases associated with amyloid fibrils.*

The poly(Q)protein-based aggregation diseases, which include Huntington's disease, result in intranuclear and cytoplasmic inclusion bodies in which the expanded poly(Q) protein (e.g. huntingtin in Huntington's disease) is present in a fibrillar form. Cell culture models of cell death due to poly(Q) protein-based aggregation have shown that whilst co-expression of Hsp27 reduces cell death, this is not mediated through a reduction in the number of cells with aggregates [91] but correlates with a decrease in free radical production. In a recent study, a mutant huntingtin protein was expressed in the ocular lenses of transgenic mice leading to protein aggregation and cataract formation [92]. Significantly, in mice lacking  $\alpha$ B-crystallin, the onset and severity of

aggregation was markedly accelerated [92]. sHsps have also been found to be highly expressed in astrocytes in the brains of patients with Creutzfeldt–Jakob disease (in which the fibril forming protein is the prion protein) [61] and the expression of Hsp25 and  $\alpha$ B-crystallin is increased in transgenic mouse models of familial amyotrophic lateral sclerosis [83].

#### 4. *In vitro* studies on the effect of sHsps in preventing fibril formation and its associated toxicity

Whilst early studies on the chaperone activity of sHsps were primarily focused on amorphously aggregating target proteins, over the past ten years the role of these chaperone proteins in preventing ordered aggregation leading to amyloid fibril formation *in vitro* has also been well established. The chaperone activity of sHsps against *in vitro* fibril formation by target proteins has to date focused predominately on  $\alpha$ B-crystallin, most likely due to its links with the fibrillar deposits associated with protein misfolding diseases (see above). Also, the chaperone activity of  $\alpha$ B-crystallin can be easily compared with well-established data on its chaperone activity against amorphously aggregating target proteins. Thus, studies have shown that  $\alpha$ B-crystallin inhibits the fibrillation of  $\alpha$ -synuclein [48, 83, 93],  $\beta$ 2-microglobulin [94] (Esposito, Carver, *et al.*, unpublished results),  $\kappa$ -casein [95, 96], the cc $\beta$ -Trp peptide [95], apoC-II [97], and the prion protein (Ecroyd, unpublished results). In doing so,  $\alpha$ B-crystallin acts as a chaperone under a range of solvent conditions, including at pH values as low as 2.5 against  $\beta$ 2-microglobulin fibrillation [94].

##### *sHsps and the A $\beta$ peptides*

The A $\beta$  peptides (i.e. A $\beta$ (1-40) and A $\beta$ (1-42)) have been the most widely studied fibril-forming targets against which the activity of sHsps has been assessed. The most studied sHsp in this context has been  $\alpha$ B-crystallin. However, to date there is no consensus on the overall effect of  $\alpha$ B-crystallin on fibril formation by A $\beta$  peptides and the effect of this chaperone on the toxicity associated with the fibril forming process by A $\beta$ . The variance in the reported results is no doubt due to differences in the experimental methods used. Stege *et al.* [98] reported that a sample containing a 1.0: 0.2 molar ratio of A $\beta$ (1-40):  $\alpha$ B-crystallin led to an increase in toxicity to isolated rat neurons compared to A $\beta$ (1-40) alone (all molar ratios are stated as per monomeric unit of the sHsp). However, these toxicity experiments involved the proteins being applied directly to cells in the culture medium (i.e. the peptide was not incubated previously in the presence and absence of  $\alpha$ B-crystallin to induce its aggregation) and the fibril-forming propensity of the A $\beta$  peptide in the culture medium used was not reported. Therefore, whether the measured toxicity correlates with that involved in the aggregation of A $\beta$ (1-40) is not clear. These authors reported that when the aggregation of A $\beta$ (1-40) was studied in phosphate buffer under physiological conditions (i.e. 37°C and pH 7.4), the presence of  $\alpha$ B-crystallin (at the same molar ratio of A $\beta$ (1-40):  $\alpha$ B-crystallin, i.e. 1.0: 0.2) resulted in an increase in thioflavin T (ThT) fluorescence. However, no fibrils were seen by electron microscopy and this led to the conclusion that  $\alpha$ B-crystallin induces and/or stabilizes a non-fibrillar,  $\beta$ -sheet rich A $\beta$ (1-40) species (protofibril) which is highly toxic to these neuronal cells [98]. An increase in the level of ThT fluorescence of A $\beta$ (1-40) incubated in the presence of  $\alpha$ B-crystallin compared to A $\beta$ (1-40) incubated alone was also reported by Liang [99] using a 1.0: 0.5 molar ratio of A $\beta$ (1-40):  $\alpha$ B-crystallin. Using a fluorescence energy transfer approach, Liang concluded that a stable complex

forms between the chaperone and A $\beta$  peptide which promotes the aggregation of A $\beta$ (1-40) and hence leads to an increase in ThT fluorescence [99].

In contrast to these studies, Kudva *et al.* [100] reported that  $\alpha$ -crystallin (made up of both  $\alpha$ A- and  $\alpha$ B-crystallin subunits) had no effect on the initial rate of fibril formation by A $\beta$ (1-42) when used at a 1.0: 0.3 molar ratio of A $\beta$ (1-42):  $\alpha$ -crystallin. Wilhemus *et al.* [101] showed that  $\alpha$ B-crystallin (at a 1.0: 1.0 molar ratio) completely inhibited the formation of fibrils by DA $\beta$ (1-40) (a Dutch mutation of A $\beta$ (1-40) where Glu22 is replaced by Gln which forms fibrils more rapidly than the wild-type A $\beta$ (1-40) peptide) and delayed the formation of fibrils by A $\beta$ (1-42) (as assessed by TEM). They also found that the toxicity of aggregated DA $\beta$ (1-40) and A $\beta$ (1-42) to human brain pericytes was completely abolished by co-incubation with  $\alpha$ B-crystallin (at a 1.0: 0.2 molar ratio of DA $\beta$ (1-40):  $\alpha$ B-crystallin and a 1.0: 0.02 molar ratio of A $\beta$ (1-42):  $\alpha$ B-crystallin). Raman *et al.* [94] have reported that the increase in ThT fluorescence associated with fibril formation by the A $\beta$  peptides is completely inhibited by a 1.0: 0.05 molar ratio of A $\beta$ (1-40):  $\alpha$ B-crystallin and a 1.0: 0.1 molar ratio of A $\beta$ (1-42):  $\alpha$ B-crystallin ( $\alpha$ B-crystallin was also found to be more effective at preventing the fibril formation of A $\beta$ (1-40) than  $\alpha$ A-crystallin). These authors concluded that no stable complex forms between  $\alpha$ B-crystallin and A $\beta$ (1-40), but instead  $\alpha$ B-crystallin acts primarily by forming a complex with the fibril nucleus to prevent its growth [94].  $\alpha$ A-Crystallin has also been shown to completely inhibit fibril formation by A $\beta$ (1-40) at a 1.0: 0.2 molar ratio (A $\beta$ (1-40):  $\alpha$ A-crystallin) and when  $\alpha$ A-crystallin was added together with A $\beta$ (1-40) to rat neuronal (PC12) cells (at the same molar concentration), it suppressed the toxicity compared to when A $\beta$ (1-40) was added on its own [102].

A number of other sHsps inhibit *in vitro* fibril formation by the A $\beta$  peptides. For example, a study by Wilhemus [101] using five different human sHsps ( $\alpha$ B-crystallin, Hsp20, Hsp27, HspB2 and HspB3) reported that whilst  $\alpha$ B-crystallin, Hsp20 and Hsp27 effectively inhibited fibril formation by DA $\beta$ (1-40), they had no effect on A $\beta$ (1-42) aggregation. However, this study reported that these three sHsps (i.e.  $\alpha$ B-crystallin, Hsp20 and Hsp27) were effective inhibitors of the cerebrovascular toxicity associated with the aggregation of both forms of A $\beta$  (indicating that aggregation and toxicity are not correlated in the case of A $\beta$  fibril formation) [101]. In support of this, a separate study by Lee *et al.* [103] using sHsps from three different sources (Hsp20 from the parasite *Babesia bovis*, Hsp 17.7 from carrot and Hsp27 from humans) found that, whilst sHsps may prevent fibril formation (with Hsp20 from *B. bovis* being the most effective), this did not correlate with a decrease in the toxicity of A $\beta$ (1-40) aggregates to cultured cells. Similar observations have been reported using the *C. elegans* model whereby chaperone suppression of A $\beta$  toxicity is independent of a reduction of toxic protein accumulation *per se* [90].

Due to the conflicting results of these studies, the ability of sHsps, and in particular  $\alpha$ B-crystallin, to prevent the aggregation of A $\beta$  peptides remains to be definitively established. In addition, the results highlight the need for an assessment (using standardized protocols) of both the effect of the chaperone on preventing fibril formation and the toxicity associated with this process. The finding that sHsps inhibit fibril formation, but increase the toxicity of the A $\beta$  aggregation process is intriguing and should also be studied using other target proteins that form fibrils. Moreover, the generic nature of the toxic species formed during fibril formation is yet to be established and

sHsps may help to determine if any structural differences exist between the toxic species from various aggregating proteins.

*The mechanism by which sHsps prevent amyloid fibril formation.*

There appears to be mechanistic differences in the ability of sHsps to prevent amyloid fibril formation compared to amorphous aggregation. This conclusion is based on studies which have compared the chaperone activity of mutant and/or chimaeric forms of  $\alpha$ -crystallin proteins, (i.e. proteins in which the N-terminal domain of  $\alpha$ B-crystallin has been fused with the C-terminal domain of  $\alpha$ A-crystallin and vice-versa) [94] against both amorphous and amyloid fibril forming target proteins. For example, the chaperone activity of the R120G  $\alpha$ B-crystallin mutant (which is linked to desmin-related myopathy and early onset cataract) towards amorphously aggregating target proteins is significantly reduced compared to the wild-type protein [104, 105], however, its ability to prevent fibril formation by A $\beta$ (1-40) is only slightly lower [94]. As well, although a deletion mutant of  $\alpha$ B-crystallin (in which residues 21-29 in the N-terminal region have been deleted) exhibits several fold increase in chaperone activity towards amorphously aggregating insulin and citrate synthase compared to wild-type  $\alpha$ B-crystallin, it shows only a marginal increase in ability over the wild-type protein to prevent fibril growth by A $\beta$ (1-40) [94]. Similarly, we found that a double mutation in the C-terminal hinge region of  $\alpha$ B-crystallin (i.e. I159A/I161A  $\alpha$ B-crystallin) had no effect on its chaperone activity against amorphously aggregating target proteins, but it significantly enhanced its ability to prevent fibril formation by the model proteins  $\kappa$ -casein and cc $\beta$ -Trp [106]. Thus, whilst all sHsps share structural features, such as a conserved  $\alpha$ -crystallin domain, the differences between the proteins play an important role in their interaction with target proteins. Whether these differences are manifested through distinct target protein binding sites or binding modes remains to be determined. Interestingly, this raises the possibility that mutant forms of sHsps may be designed as more effective inhibitors of fibril formation compared to the wild-type protein and therefore may be an avenue for therapeutic potential in the future [106]. In addition, the variation in chaperone activity of sHsps may provide clues as to the different nature of the partially folded intermediate which are the precursor(s) to disordered (amorphous) versus ordered (amyloid fibril) forms of aggregation.

The mechanism of action of sHsps in preventing fibril formation also appears to vary depending on the nature of the target protein (see Fig. 4E). Studies such as those with apolipoprotein C-II (apoC-II) have shown that, in preventing amyloid fibril formation, sHsps can act at very low sub-stoichiometric ratios and do not form stable complexes with partially folded intermediates of the aggregating target protein [97]. Instead the sHsp and target protein are proposed to form a transient complex by which the sHsp stabilizes the aggregating protein before releasing it so that it may refold back to its native state via the reversible on-folding pathway [97]. This is in contrast to its mechanism of action in preventing fibril formation by  $\alpha$ -synuclein and  $\kappa$ -casein, whereby  $\alpha$ B-crystallin forms a stable, soluble chaperone-target protein complex with the partially folded intermediate of these target proteins (i.e. a 'reservoir of intermediates') [48, 93, 95]. This is analogous to the mechanism of chaperone action used by sHsps against amorphously aggregating proteins [2] (Fig. 3). In the case of  $\alpha$ -synuclein,  $\alpha$ B-crystallin redirects the protein from the amyloid fibril forming pathway to the amorphous aggregation pathway (see Fig. 2) [93], a process that would benefit the cell since these amorphous aggregates are non-toxic and more easily degraded through the ubiquitin-proteasome system.

In addition,  $\alpha$ B-crystallin is able to halt the increase in ThT fluorescence associated with fibril formation of  $\alpha$ -synuclein after it has commenced, but has no capacity to disassemble preformed fibrils [48].  $\alpha$ B-crystallin may elicit this effect by directly inhibiting the supply of partially folded intermediate required for fibril elongation and stable nuclei formation, and/or by interacting with the fibril itself to ‘cap’ it and prevent further growth. With regards to  $\alpha$ B-crystallin acting directly with the growing fibril following stable nuclei formation, this is the primary mode by which A $\beta$ (1-40) fibril formation is thought to be inhibited by  $\alpha$ B-crystallin [94]. The differences in the mechanism by which sHsps prevent fibril formation by various target proteins highlight their capacity to influence both the nucleation and propagation processes. The primary mechanism by which sHsps prevent a particular aggregating species from forming fibrils is likely to reflect the relative importance of each phase of fibril formation for that amyloidogenic species.

We have previously shown that for the reduction-induced amorphous aggregation of target proteins, there is a direct correlation between the mass of the target protein and the chaperone ability of  $\alpha$ -crystallin, i.e. as the mass of the target protein increases, a higher subunit molar ratio of  $\alpha$ -crystallin is required to completely suppress aggregation [107]. These results are rationalised by consideration of steric factors in the chaperone ability of sHsps: the sHsps more efficiently bind smaller target proteins on their surface than larger ones. Similarly, steric hindrance is likely to contribute to differences in the chaperone ability of sHsps to inhibit fibril-formation. Furthermore, variation in oligomeric distribution for a particular sHsp (resulting from factors such as mutations, a change in environmental conditions (e.g. heat) and/or post-translational modifications) and between sHsps need to be considered when comparing relative chaperone abilities of sHsps. A systematic study of the size of the fibril-forming target proteins and the chaperone efficiency of sHsps is required to elucidate this relationship.

With respect to the ability of sHsps to inhibit amorphous aggregation, it is well established that the kinetics of the aggregation process are a significant factor in the efficacy of sHsps to prevent this process, i.e. sHsps are most effective against slowly-aggregating target proteins undergoing amorphous aggregation [3, 36, 108]. This is because sHsps interact with intermediately folded states of target proteins and thus the longer these states persist, the better the ability of sHsps to interact with them. Similar logic can be applied to the comparative chaperone activity of sHsps against fibril-forming proteins; however, there has not been a definitive study that has addressed this area. On the basis of aggregation kinetics, one may conclude that sHsps would be better suited to preventing fibril formation as compared to amorphous aggregation since the formation of fibrils is typically a much slower process than amorphous aggregation. In support of this, the limited work that has been done in this area suggests the longer the lag phase of fibril formation (representing the time taken to form stable nuclei), the more effective the chaperone ability of  $\alpha$ B-crystallin. For example,  $\alpha$ B-crystallin is more effective at inhibiting fibril formation by wild-type  $\alpha$ -synuclein compared to A53T $\alpha$ -synuclein which forms fibrils more rapidly (Ecroyd, unpublished data). As well, when the rate of  $\alpha$ -synuclein fibril formation is increased through the addition of dextran (which acts as a molecular crowding agent), the effectiveness of  $\alpha$ B-crystallin as a chaperone decreases [93]. Moreover, we have previously suggested that the intermediate formed during the fibril formation of  $\kappa$ -casein is very short lived compared to other proteins that form fibrils through the typical nucleation-dependent mechanism [109]. This may, at least in part, explain why comparatively high molar ratios of  $\alpha$ B-crystallin are required to inhibit fibril formation by  $\kappa$ -casein compared to other systems which have been studied (i.e. the molar ratio

(target protein:  $\alpha$ B-crystallin) to completely inhibit fibril formation for  $\alpha$ -synuclein is 1.0: 0.25 [93], apoC-II is 1.0: 0.02 [97] and cc $\beta$ -Trp 1.0: 0.1 [95] compared to > 1.0: 1.0 for RCM $\kappa$ -casein [95]). These differences cannot be accounted for by simple comparison of the masses of the target proteins, as discussed above (mass of  $\alpha$ -synuclein is 14.5 kDa, RCM $\kappa$ -casein is 19.0 kDa, apoC-II is 8.8 kDa and cc $\beta$ -Trp is 2.2 kDa).

The effectiveness of  $\alpha$ B-crystallin to act as a chaperone against a particular target protein is also dependent on the various conformational states of the target protein's intermediately folded forms. This is because  $\alpha$ B-crystallin has a differential mode of binding to target proteins that reflects the latter's free-energy of unfolding [43]: the more destabilised and unfolded substrates have higher binding affinities to  $\alpha$ B-crystallin [110]. Thus, each protein's intermediate states may vary in their structure, hydrophobicity and binding affinity for  $\alpha$ B-crystallin leading to differences in the relative effectiveness of  $\alpha$ B-crystallin (and sHsps in general) to prevent fibril formation by different target proteins.

*The effect of post-translational modifications on the chaperone activity of sHsps against fibril-forming target proteins.*

Both inside and outside the lens, the major post-translational modification described for  $\alpha$ B-crystallin and Hsp25 is phosphorylation at three serine residues; in  $\alpha$ B-crystallin these are S19, S45 and S59 (S15, S78 and S82 in Hsp27) [111-114]. Various types of cellular stress, such as heat, oxidation and increased intracellular calcium levels, stimulate the phosphorylation of sHsps [111]. With regard to  $\alpha$ B-crystallin, phosphorylation is mediated by at least two distinct mitogen-activated protein kinase enzymes, mitogen-activated protein kinases associated protein kinase2 (MAPKAPK2) and p42/p44 MAPK [111, 112, 115, 116] and, in the lens, phosphorylation increases with age [117, 118]. However, even in the young lens,  $\alpha$ B-crystallin is extensively phosphorylated [113, 118]. All three sites of serine phosphorylation of Hsp27 are mediated by MAPKAP kinases [119, 120]. In the brain, some of the  $\alpha$ B-crystallin isolated from the proteinaceous aggregates of patients with degenerative diseases [121], amyloid plaques and Lewy bodies is phosphorylated [68]. However, it is not known whether there is a preference for the phosphorylated forms of  $\alpha$ B-crystallin to interact with the proteins associated with these deposits. In fact, the role of phosphorylation of sHsps in their chaperone action is far from understood.

Introducing a negative charge into recombinant sHsps by replacing serine residues with aspartate/glutamate mimics the natural phosphorylation state of the protein. This method has been employed previously for  $\alpha$ B-crystallin and led to similar effects as endogenously phosphorylated  $\alpha$ B-crystallin with regards to its oligomeric distribution [122], subcellular localization [123] and cellular trafficking [124]. Biophysical characterisation studies of these phosphomimics have shown that an increase in the number of sites that are phosphorylated decreases the oligomerization of the protein [122] by disrupting the dimeric substructure within the oligomer [125]. Studies using these phosphomimics against both amorphous and fibril-forming target proteins have shown that the effect of phosphorylation of  $\alpha$ B-crystallin on its chaperone activity is target protein specific [95]. The triple phosphomimic of  $\alpha$ B-crystallin (i.e. S19D, S45D, S59D  $\alpha$ B-crystallin or 3D $\alpha$ B-crystallin) is a more effective chaperone in preventing fibril formation by the natively unstructured target proteins  $\kappa$ -casein and  $\alpha$ -synuclein compared to the wild-type protein, but is less effective against the cc $\beta$ -Trp peptide which adopts a well-

structured coiled-coil configuration in its native state prior to fibril formation [95, 126]. These results emphasise the role of the conformational states of the target protein's intermediately folded forms on the relative chaperone ability of sHsps. Interestingly, 3D $\alpha$ B-crystallin forms mixed oligomers with the wild-type protein and exhibits enhanced subunit exchange which may be responsible for the increase in chaperone activity seen with some target proteins [126] rather than a direct interaction of the phosphorylation site with the target protein..

Cell culture experiments investigating the role of phosphorylation of Hsp27 in protecting against poly(Q)-aggregation and the associated cell toxicity of this aggregation process have shown that unphosphorylated Hsp27, in its large oligomeric state, is more effective at protecting against oxidative stress than the triple phosphomimic of Hsp27 [91]. These results were supported by experiments involving the over-expression of MAPKAP kinase 2 and subsequent increased phosphorylation of Hsp27, in which a decrease in the ability of the phosphorylated chaperone to prevent poly(Q)-mediated aggregation and cell toxicity was observed [91]. Other than phosphorylation, sHsps, and in particular the  $\alpha$ -crystallins undergo a variety of other forms of post-translational modifications including deamidation, oxidation, glycation and C-terminal truncation. However, to date, the impact of these modifications on the chaperone ability of sHsps to prevent amyloid fibril formation has not been investigated. This area of study is significant since these post-translational modifications occur to a significant extent to the crystallin proteins in the eye lens [12, 127-133]. There is increasing evidence that cataract may be an amyloid fibril based disease (see below) and, as such, the impact of such modifications on the chaperone ability of  $\alpha$ -crystallin to prevent this process is highly relevant.

*The region(s) of  $\alpha$ -crystallin responsible for target protein binding and prevention of fibril formation.*

Hydrophobic sites in  $\alpha$ -crystallin are thought to be responsible for its chaperone activity [45, 134-137] and hydrophobic peptides isolated from  $\alpha$ A-crystallin (residues 70-88) [137] and  $\alpha$ B-crystallin (residues 73-92 and 131-141) [24, 138] possess chaperone activity against amorphously aggregating target proteins. The chaperone activity of these peptides results from their ability to bind independently to target proteins [137]. These sequences are within the  $\alpha$ -crystallin core domain that is shared by all members of the sHsp family (Fig. 1B). The same peptide region in  $\alpha$ A-crystallin (i.e. residues 70-88) has also been shown to have anti-fibril forming activity against A $\beta$  peptides and inhibits the toxicity to rat pheochromocytoma cells (PC12) associated with addition of A $\beta$  to the culture medium [102, 139]. In addition, a peptide comprising residues 73-85 of  $\alpha$ B-crystallin has been shown to reduce the increase in ThT fluorescence associated with fibril formation of four disease-related target proteins (A $\beta$ (1-42),  $\alpha$ -synuclein,  $\beta$ 2-microglobulin and transthyretin) by >50% when used at equimolar ratios [140]. Another  $\alpha$ B-crystallin peptide (residues 101-110) was also found to have high anti-fibril activity in this study [140]. Moreover, the anti-fibril activity of these peptides was apparent under a range of solution conditions (including a pH as low as 2.4 to induce fibril formation by  $\beta$ 2-microglobulin). Systematic truncation of these bioactive peptides led to the identification of four tetrapeptides that were highly effective as inhibitors of fibril assembly of these target proteins, i.e. FSVN, NLDV, HGKH and HEER [140].

Interestingly, the N-terminal region of the  $\alpha$ A-crystallin 70-88 peptide (i.e. KFVIF; the corresponding peptide region in  $\alpha$ B-crystallin is RFSVN) has strong sequence similarity to a

region of the fibril-forming core domain of A $\beta$ (1-40), KLVFF [102, 141]. This region in A $\beta$ (1-40) has recently been shown by NMR spectroscopy to be primarily involved in the interaction between A $\beta$ (1-40) and  $\alpha$ B-crystallin [142]. Moreover, KLVFF of A $\beta$ (1-40) facilitates the elongation of fibrils formed from A $\beta$ . Thus, it can be concluded that the isolated peptides from  $\alpha$ A-crystallin and  $\alpha$ B-crystallin inhibit fibril formation by A $\beta$ (1-40) by competing for this binding site with monomeric forms of A $\beta$ (1-40) during its aggregation [102, 140]. Significantly, this same peptide region appears to mediate the chaperone activity of  $\alpha$ B-crystallin against a range of other disease-related, fibril-forming target proteins, including  $\alpha$ -synuclein and  $\beta$ 2-microglobulin [138, 140]. At present, the development of therapeutics for Alzheimer's disease is largely focused on identifying inhibitors of fibril formation by A $\beta$  peptides with some molecules that exhibit high inhibitory activity having progressed to stage III clinical trials [143-145]. The target protein specificity of these molecules (i.e. to A $\beta$  peptides only) precludes their use as therapeutic agents for other fibril-related diseases, but their success highlights the potential of using general inhibitors of fibril formation to treat a range of protein misfolding diseases. As such, the identification of the sites responsible for the ability of sHsps to prevent fibril formation by a range of disease-related target proteins is of great interest and may lead to their development as therapeutic agents, e.g. via the use of peptide mimics.

### 5. *In vitro* and *in vivo* formation of amyloid fibrils by crystallin proteins

Cataract is a disease of principally crystallin protein aggregation and subsequent precipitation that occurs over the timeframe of years. Intuitively, one would anticipate that these slow aggregation conditions would favour the formation of highly ordered aggregates such as amyloid fibrils. Indeed, as discussed above, a long aggregation timeframe is one criterion that contributes to the facilitation of fibril formation. However, the protein aggregates that are present in cataract are generally considered to be amorphous in form with little ordered structure. Accordingly, *in vitro* models of target protein aggregation that have investigated the chaperone action of  $\alpha$ -crystallin have, in the main, utilized target proteins that aggregate amorphously.

Some *in vitro* and *in vivo* work has investigated the possible fibrillar nature of crystallin protein aggregates in normal and cataractous lenses. Frederikse [146] showed that the amyloidophilic dyes, Congo red and ThT, bound readily to the crystallin proteins of normal murine lenses *in situ*. From this, he concluded that the crystallin proteins in organelle-free lens fibre cells were arranged in a supramolecular  $\beta$ -sheet structure that had amyloid-like properties. Previous studies using Raman and infrared spectroscopy had also concluded that  $\beta$ -sheet structure predominates in the crystallin proteins of the normal lens (references 8 to 15 in [146]). All three crystallins ( $\alpha$ ,  $\beta$  and  $\gamma$ ) are highly  $\beta$ -sheet in character. The binding of the amyloidophilic dyes to lens proteins *in situ* is therefore probably not surprising as they interact with the native state of  $\beta$ -sheet proteins, including  $\alpha$ -crystallin (Carver, Ecroyd *et al.*, unpublished results).

Earlier studies by Frederikse and co-workers had shown that amyloidogenic proteins such as the A $\beta$  peptide (and its precursor, the amyloid- $\beta$  precursor protein (APP), and the presenilin enzymes that cleave APP) and the prion protein are present in low concentrations in the eye lens [146-148]. It was hypothesized that species such as A $\beta$  and the prion protein could act as seeds to facilitate fibril formation by other proteins such as the crystallins. In a similar vein, Goldstein *et*

*al.* [149] showed that inclusions present in equatorial supranuclear cataracts from the lenses of Alzheimer's disease patients contained A $\beta$  species which may act to promote crystallin protein aggregation.

The relationship between the aggregation of amyloid species and R120G  $\alpha$ B-crystallin, a mutation which causes desmin-related myopathy (DRM) and cataract, was examined in cardiomyocytes by Sanbe *et al.* [150]. The direct involvement of R120G  $\alpha$ B-crystallin in DRM was demonstrated by halting R120G  $\alpha$ B-crystallin expression in transgenic mice which led to the reduction in amyloid species and improved cardiac function. As a follow-up to these studies, Sanbe *et al.* [151] showed that recombinant R120G  $\alpha$ B-crystallin at pH 7.5 formed toxic amyloid oligomers *in vitro*. The formation of amyloid species was prevented by the presence of two other sHsps, Hsp25 and Hsp22. In transfected cardiomyocytes, the presence of these two sHsps also restored ubiquitin/proteasome activity and cell viability [151]. Our work has also shown that recombinant R120G  $\alpha$ B-crystallin forms amyloid fibrils readily [152]. In a recent innovation, Muchowski *et al.* [92] have developed a transgenic mouse in which the proteins associated with Parkinson's and Huntington's diseases ( $\alpha$ -synuclein and a huntingtin fragment respectively) are expressed in the lens leading to their aggregation and cataract formation. It is intended to use these mice as screens to study Parkinson's and Huntington's diseases, for example in the development of therapeutics to inhibit protein aggregation. Interestingly, in mice lacking  $\alpha$ B-crystallin the huntingtin fragment exhibited markedly enhanced protein aggregation, implying that the molecular chaperone action of  $\alpha$ B-crystallin was important in suppressing huntingtin aggregation *in vivo*.

Our work has shown that each of the crystallin protein classes ( $\alpha$ ,  $\beta$  and  $\gamma$ ) readily forms amyloid fibrils under mildly denaturing conditions [153]. The fibrils have all the generic structural and physical characteristics of amyloid fibrils (Fig. 5). A couple of studies have investigated the fibril-forming propensity of individual  $\gamma$ -crystallin subunits. Quinlan and co-workers [154] showed that a mutant of murine  $\gamma$ B-crystallin with its last (fourth) Greek key motif absent readily forms amyloid fibrils *in vitro*. In contrast, the wild type  $\gamma$ B-crystallin did not form fibrils. *In vivo*, the inherited  $\gamma$ B-crystallin mutant caused cataract in mice and produced inclusions in the lens that had amyloid-like characteristics. Recently, Papankolopoulou *et al.* [155] showed that wild type human  $\gamma$ D-crystallin, along with its individual N- and C-terminal domains, formed well-defined amyloid fibrils when incubated at pH 3. Our recent work [152] has investigated in detail, using a range of biophysical techniques, fibril formation by the individual  $\alpha$ -crystallin subunits and R120G  $\alpha$ B-crystallin. In particular, NMR spectroscopy was used to show that the short and highly mobile C-terminal extension of  $\alpha$ B-crystallin [156] maintains its flexibility in the fibrillar state. Furthermore, NMR measurements determined the diffusion coefficients of the native and fibrillar forms of  $\alpha$ B-crystallin. As expected, this value was significantly larger in the fibrillar state due to the greater size of the aggregate. Morphologically, the structure of  $\alpha$ B-crystallin fibrils can be varied by altering solution conditions (discussed more in the next section). The material properties of  $\alpha$ B-crystallin fibrils have been examined by measuring their bending rigidity, from which it was determined that  $\alpha$ B-crystallin forms relatively flexible fibrillar species compared to other systems [157]. The C-terminal extension of  $\alpha$ B-crystallin may be responsible for this flexibility. Recently, Tanaka *et al.* [139] have examined the fibril-forming propensity and chaperone activity of peptides from  $\alpha$ A-crystallin, specifically those corresponding to the

putative chaperone-binding regions of the protein previously identified by Sharma *et al.* [136, 137]. Tanaka *et al.* [139] found that one of these peptide (70-88) inhibited A $\beta$  fibril formation but promoted the aggregation of insulin. Interestingly, the 71-88 peptide is itself amyloidogenic with F71 being a crucial residue for this property. The implication from this result is that the hydrophobic interactions that facilitate the chaperone activity of sHsps against fibril-forming target proteins are similar to those that mediate fibril formation in the first place.

Thus, it is apparent for the above discussion that all the crystallins, under appropriate conditions, will readily form amyloid fibrils. It is possible, therefore, that some of the aggregates present in cataractous lenses are fibrillar in nature. If present, however, they may be mixed in with amorphously aggregated crystallin proteins. Interestingly,  $\alpha$ A- and  $\alpha$ B-crystallin are as susceptible to fibril formation as the  $\beta$ - and  $\gamma$ -crystallins [152, 153]. Thus, even the molecular chaperones  $\alpha$ A- and  $\alpha$ B-crystallin, which are highly stable proteins (like the other crystallins), are prone to aggregate and form fibrils, despite their role in protein stabilization in the lens.

## 6. Crystallin proteins as bionanomaterials

The mechanism of amyloid fibril formation has obviously attracted much interest because of the intimate association of fibrils with diseases of protein misfolding and aggregation. In addition, the highly structured nature of amyloid fibrils, their ability to self-assemble, their inherent strength and their possibility to be functionalised have elicited research interest of late in their potential use as bionanomaterials, for example as drug delivery agents and in the field of bioelectronics [158]. Nature already produces its own bionanomaterials in the form of functional amyloid, for example as structural components of lower organisms such as bacteria, fungi and insects [59]. From a synthetic point of view, bionanomaterials prepared from amyloid fibrils have advantages over other nanomaterials such as carbon nanotubes in that they are biocompatible and can be readily functionalized.

Of late, we have been investigating the potential of crystallin proteins as ready sources of amyloid fibrils for use as bionanomaterials (Garvey *et al.*, submitted for publication). The advantage of crystallin proteins is their ready availability in significant quantity which is of particular importance if amyloid fibrils are to be used for commercial purposes. As described above, individual  $\alpha$ - and  $\gamma$ -crystallin subunits readily form fibrils under mildly denaturing conditions [152, 155] as do the individual  $\alpha$ -,  $\beta$ - and  $\gamma$ -crystallin classes [153]. Our recent work has shown that semi-pure and crude mixtures of the crystallins from bovine, ovine and deer lenses formed amyloid fibrils under similar conditions employed for the preparation of crystallin fibrils, i.e. the three individual crystallin classes do not need to be purified individually prior to fibril preparation. Thus, fibril formation occurred for these mixtures when the  $\alpha$ -crystallin subunits were present which, when acting as molecular chaperones, would have the potential to prevent fibril formation. The morphology of these fibrils, varying between short and curly and closed loops to straight, can be altered significantly depending on the solvent conditions. Likewise, varying solution conditions dramatically alters the morphology of  $\alpha$ B-crystallin amyloid fibrils with acidic pH and dilution leading to the dissociation of the fibrils into shorter protofibrillar species that include some closed loop structures (Fig. 6).

In summary, crystallin proteins have great potential as bionanomaterials because of the relative ease of obtaining significant quantities and the ability to alter their morphology dramatically depending on the solution conditions for their preparation. Further research in this area will explore these possibilities and the potential applications of crystallin fibrillar species in the emerging field of bionanomaterials.

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Table 1: The human crystallin proteins

Crystallin	SwissProt Accession Number
$\alpha$ -crystallins	
$\alpha$ A-crystallin	P02489
$\alpha$ B-crystallin	P02511
$\beta$ -crystallins	
$\beta$ A-crystallins	
$\beta$ A1-crystallin	P05813
$\beta$ A2-crystallin	P53672
$\beta$ A3-crystallin	P05813
$\beta$ A4-crystallin	P53673
$\beta$ B-crystallins	
$\beta$ B1-crystallin	P53674
$\beta$ B2-crystallin	P43320
$\beta$ B3-crystallin	P26998
$\gamma$ -crystallins	
$\gamma$ S-crystallin	P22914
$\gamma$ A-crystallin	P11844
$\gamma$ B-crystallin	P07316
$\gamma$ C-crystallin	P07315
$\gamma$ D-crystallin	P07320
$\gamma$ E-crystallin*	-
$\gamma$ F-crystallin*	-

\* - genes for these proteins are inactive in human due to a nonsense mutation [159].

## Figure Legends.

**Figure 1. (A) X-ray crystallographic structures of the  $\gamma$ B-crystallin monomer (top) and the  $\beta$ B2-crystallin dimer (bottom) (from [12]).** Both proteins are very similar in secondary structure in having two domains which are each comprised of four  $\beta$ -strands (shown in blue and turquoise in  $\gamma$ B-crystallin) arranged in two Greek key motifs, i.e. a total of eight  $\beta$ -strands per domain. The bent nature of the connecting (linker) peptide between the two domains in the  $\gamma$ B-crystallin structure ensures that the inter-domain contacts are intramolecular and hence the protein is a monomer. By contrast, the connecting peptide is straight in  $\beta$ B2-crystallin which enables intermolecular inter-domain contacts and a dimeric structure results. N and C refer to the N- and C-termini respectively and P to the crystallographic dyad between the two subunits in the  $\beta$ B2-crystallin dimer. **(B) A schematic diagram of the arrangement of the three structural regions in a representative sHsp,  $\alpha$ B-crystallin.** The N-terminal domain comprises residues 1 to 65, the C-terminal ( $\alpha$ -crystallin) domain residues 66 to 149 and the C-terminal extension residues 150 to 175. The last 12 amino acids in  $\alpha$ B-crystallin are highly mobile, solvent exposed and of no ordered structure [2, 3, 11, 20]. **(C) Homology modelling of the monomeric subunit of human  $\alpha$ B-crystallin based on the crystal structure of wheat sHsp 16.9 (from [23]).** The figure shows the superposition of the secondary structures of human  $\alpha$ B-crystallin (red), wheat sHsp16.9 (green) and *Methanococcus jannasschi* sHsp 16.5 (blue). The immunoglobulin-like  $\beta$ -sheet fold of the C-terminal ( $\alpha$ -crystallin) domain is apparent.

**Figure 2. The protein folding/unfolding and off-folding pathways (adapted from [2]).** A native protein (N) unfolds via a variety of intermediate states ( $I_1$ ,  $I_2$ ,  $I_3$ , ...) which can progress to the unfolded state (U). If the intermediate states linger for long enough, they can enter the off-folding pathways, comprising either the disordered (amorphous) aggregation pathway in which interaction ultimately produces irreversibly precipitated, amorphously aggregated species or the amyloid fibril-forming pathway which, via the formation of small, soluble protofibril species, leads to insoluble, highly ordered cross  $\beta$ -sheet fibril arrays.

**Figure 3. Mechanism of sHsp chaperone interaction with amorphously aggregating target proteins (modified from [2]).** sHsps selectively interact with and stabilize slowly aggregating, intermediately folded target proteins on their amorphous off-folding pathway. The intermediates ( $I_2$ ) are relatively disordered in structure with only some elements of secondary structure in place. The dynamic nature of the equilibrium between the monomeric and aggregated  $I_2$  species facilitates interaction with the similarly dynamic sHsp. There is some evidence that the dissociated, dimeric form of the sHsp is the chaperone-active species which interacts with  $I_2$  and is subsequently sequestered into a high-mass complex containing both proteins. It is possible to recover natively folded target protein (N) via the action of another chaperone, for example by Hsp70 action coupled to ATP hydrolysis [6].

**Figure 4. The structure of an amyloid fibril.** (A) Amyloid fibrils formed by  $\alpha$ -lactalbumin as viewed by transmission electron microscopy. The fibrils are typical of amyloid fibrils formed from a range of different proteins in being long, unbranched and rope-like. Scale bar is 500 nm. (B) In the left panel, a magnified electron micrograph view of an amyloid fibril formed by  $\alpha$ -

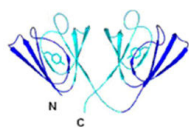
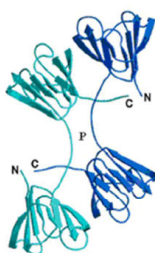
synuclein. Scale bar is 100 nm. In the middle panel, a schematic view of the arrangement of the internal protofilaments within the  $\alpha$ -synuclein fibril. In the right panel, a schematic view of an insulin fibril highlighting the substructure of the fibril core, i.e. the typical cross  $\beta$ -sheet array formed from sheets of  $\beta$ -strands lying perpendicular to the axis of the fibril and the aligning of these  $\beta$ -sheets into individual filaments (from [52]). (C and D) Structural models of amino acids that compromise the steric zipper that forms the core of fibrils from (C) the peptide GNNQQNY [53] and (D) residues 109-122 of the Syrian hamster prion protein [56]. The arrows indicate the direction of the fibril axis. (E) **Mechanisms by which sHsps may act in a chaperone manner to inhibit amyloid fibril formation by target proteins.** The nucleation-dependent mode of amyloid fibril formation commences with the unfolding of a native protein, forming a pool of partially folded intermediates, via a process that is reversible. The partially folded intermediates associate with each other until they reach a critical size/mass at which a stable nucleus is formed. The formation of this nucleus from the partially folded intermediates is slow and rate-limiting in the overall process of fibril formation (lag phase). Fibril elongation then proceeds via the addition of intermediates to the growing nucleus (elongation phase). The mechanism by which sHsps prevent fibril formation varies depending on the target protein. In a manner analogous to preventing amorphous target protein aggregation, sHsps recognise and bind to the partially folded intermediate of target proteins to form a soluble sHsp-target protein complex. This sHsp-target protein complex may be stable leading to a 'reservoir of intermediates' which may be picked-up and refolded by other chaperone proteins (e.g. Hsp60 and Hsp70). In some cases, sHsps only form transient complexes with the aggregating protein, releasing it so that it can spontaneously refold back to its native state via the on-folding pathway. The interaction of sHsps with the partially folded intermediates also prevents them from being available to facilitate fibril elongation. In addition, sHsps may act directly with the fibril/nucleus to 'cap' it and prevent further growth.

**Figure 5. Transmission electron micrographs of amyloid fibrils formed by  $\alpha$ -crystallin (adapted from Fig. 1 of [153]).** Samples of bovine  $\alpha$ -crystallin were incubated at 60 °C for 24 h in varying concentrations of GdnHCl. *A*, 0.001 M GdnHCl. *B*, 0.1 M GdnHCl. *C*, 0.75 M GdnHCl. *D*, 1.0 M GdnHCl. Scale bar is 200 nm.

**Figure 6. Alteration in morphology of  $\alpha$ B-crystallin amyloid fibrils upon varying solvent conditions (from [152]).** Atomic force microscopy images of  $\alpha$ B-crystallin fibrils prepared at (a) pH 7.4 with GdnHCl, then, following a tenfold (b), or 100-fold (c), dilution into a pH 2.0 solution; (d)  $\alpha$ B-crystallin fibrils prepared with trifluoroethanol at pH 2.0. Scale bar is 500 nm.

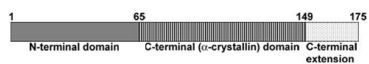
Figure 1

A

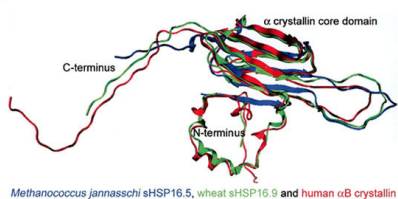
Monomeric  $\gamma$ -crystallin

Dimeric I/B2-crystallin

B



C

*Methanococcus jannaschii* sHSP16.5, wheat sHSP16.9 and human  $\alpha$ B crystallin

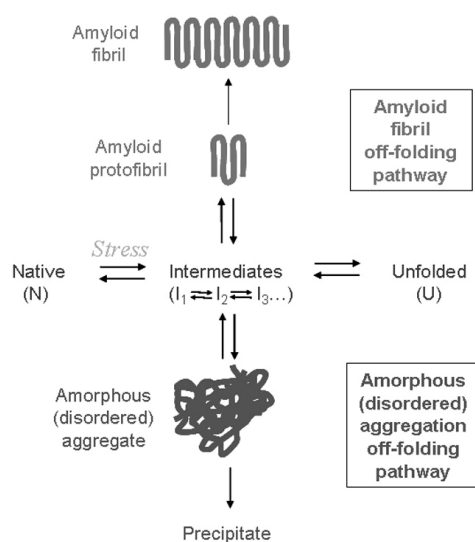
**Figure 2**

Figure 3

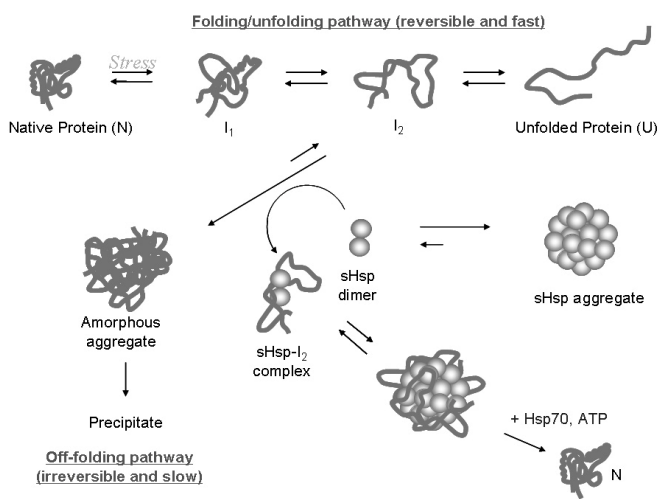
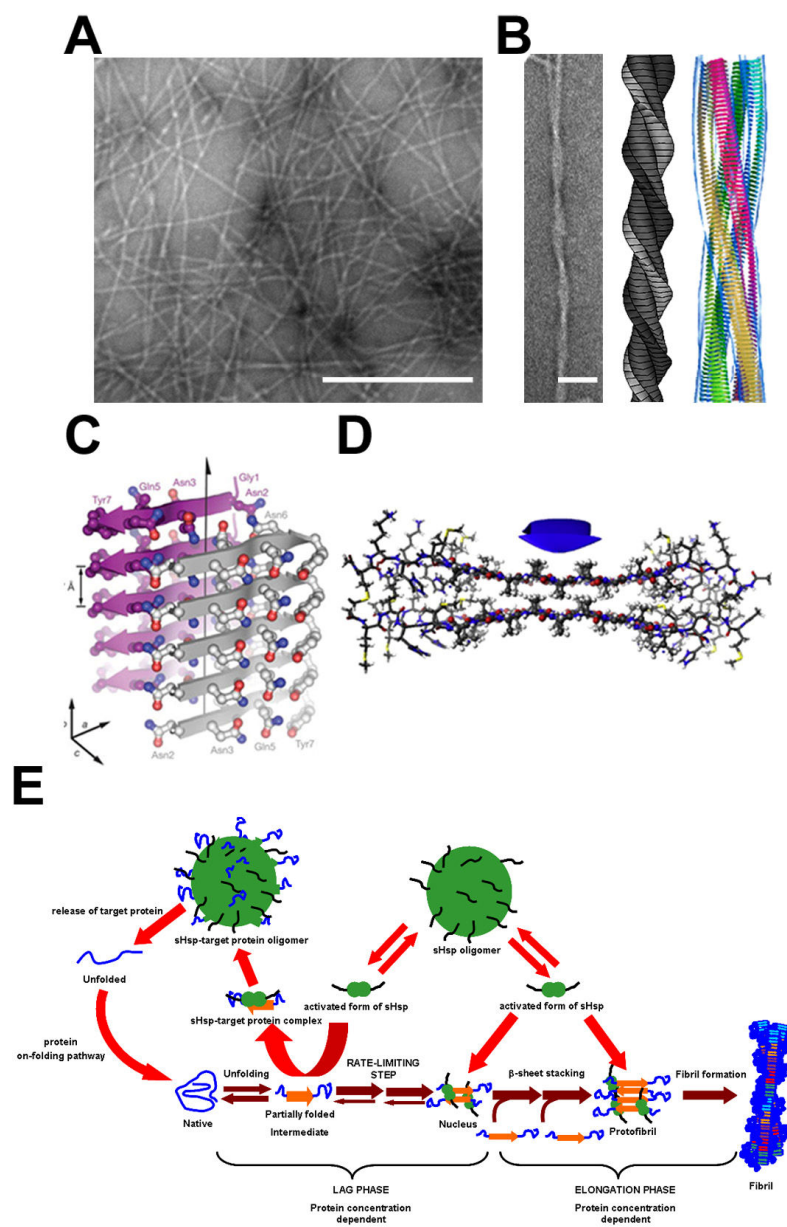
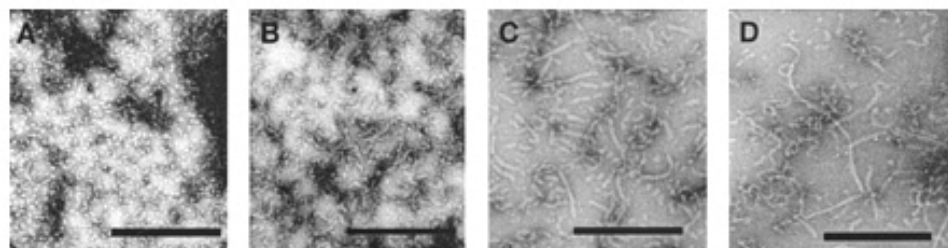


Figure 4



**Figure 5**

**Figure 6**