αB-crystallin, a small heat-shock protein, prevents the amyloid fibril growth of an amyloid β-peptide and β_2-microglobulin

Bakthisaran RAMAN*†, Tadato BAN†, Miyo SAKAI†, Saloni Y. PASTA*, Tangirala RAMAKRISHNA*, Hironobu NAIKI‡, Yuji GOTO†[1] and Ch. Mohan RAO*[1]

*Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India, †Institute for Protein Research, Osaka University, and CREST, Japan Science and Technology Agency, Yamadaoka 3-2, Suita, Osaka 565–0871, Japan, and ‡Faculty of Medical Science, University of Fukui, and CREST, Japan Science and Technology Agency, Matsuoka, Fukui 910-1193, Japan

αB-crystallin, a small heat-shock protein, exhibits molecular chaperone activity. We have studied the effect of αB-crystallin on the fibril growth of the Aβ (amyloid β)-peptides Aβ-(1–40) and Aβ-(1–42). αB-crystallin, but not BSA or hen egg-white lysozyme, prevented the fibril growth of Aβ-(1–40), as revealed by thioflavin T binding, total internal reflection fluorescence microscopy and CD spectroscopy. Comparison of the activity of some mutants and chimaeric α-crystallins in preventing Aβ-(1–40) fibril growth with their previously reported chaperone ability in preventing dithiothreitol-induced aggregation of insulin suggests that there might be both common and distinct sites of interaction on α-crystallin involved in the prevention of amorphous aggregation of insulin and fibril growth of Aβ-(1–40). αB-crystallin also prevents the spontaneous fibril formation (without externally added seeds) of Aβ-(1–42), as well as the fibril growth of Aβ-(1–40) when seeded with the Aβ-(1–42) fibril seed. Sedimentation velocity measurements show that αB-crystallin does not form a stable complex with Aβ-(1–40). The mechanism by which it prevents the fibril growth differs from the known mechanism by which it prevents the amorphous aggregation of proteins. αB-crystallin binds to the amyloid fibrils of Aβ-(1–40), indicating that the preferential interaction of the chaperone with the fibril nucleus, which inhibits nucleation-dependent polymerization of amyloid fibrils, is the mechanism that is predominantly involved. We found that αB-crystallin prevents the fibril growth of β_2-microglobulin under acidic conditions. It also retards the depolymerization of β2-microglobulin fibrils, indicating that it can interact with the fibrils. Our study sheds light on the role of small heat-shock proteins in protein conformational diseases, particularly in Alzheimer's disease.

Key words: Aβ peptide, α-crystallin, amyloid fibril, β2-microglobulin, chaperone activity, heat shock protein.

INTRODUCTION

αB-crystallin, an abundant eye lens protein, is also present in other tissues and is heat- and stress-inducible, whereas αA-crystallin, the other eye lens protein, is not heat-inducible [1–4]. Both αA- and αB-crystallin (subunit molecular mass ≈ 20 kDa) form homo- as well as hetero-multimers of various sizes [5], and exhibit molecular-chaperone-like activity in preventing aggregation of other proteins [6–9], with αB-crystallin being more efficient than αA-crystallin [8,9]. They belong to the sHsp (small heat-shock protein) family. Primary sequence analysis of sHsps divides the sequence into three parts [11]: a highly conserved central region (≈ 80 residues), rich in β-strands, called the 'α-crystallin domain', which is flanked by an N-terminal domain and a C-terminal extension, which vary considerably both in their sequence and length [11]. The N-terminal domain as well as the α-crystallin domain has sites for target protein binding [12]. The C-terminal extensions, previously believed to function as 'solubilizers' of the chaperone–target protein complex, also play an important role in its chaperone-like activity [13].

Aggregation of proteins can be classified into two types: (i) well-ordered amyloid fibril formation with intermolecular β-sheet structure [14]; and (ii) irregular or amorphous aggregation. The molecular-chaperone-like activity of α-crystallin towards the amorphous aggregation and precipitation of other proteins has been the subject of intense research, since this property is crucial for maintaining eye lens transparency [6]. Failure of the activity of α-crystallin, owing to either post-translational age-dependent modifications or mutations, may be involved in cataract formation [2]. However, its effect on well-ordered amyloid fibril formation is not completely understood.

αB-crystallin is present in brain tissues, and its expression is elevated in several neurodegenerative diseases, such as Parkinson's disease, CJD (Creutzfeldt–Jakob disease) and AD (Alzheimer's disease) [15,16]. AD is a progressive neurodegenerative disease characterized by cerebral deposits of extracellular amyloid plaques, intracellular tangles and intra- or extra-vascular deposits [17]. αB-crystallin is found to co-exist in these deposits [18], along with the amyloid fibrils of a mixture of 39–43-amino-acid polypeptides, generally designated as Aβ (amyloid β)-peptides, produced from proteolytic processing of the amyloid precursor protein [19]. One of the Aβ peptides comprising 40-amino-acid residues [Aβ-(1–40)] constitutes approx. 90 % of the total Aβ peptides [20]. The increase in levels of αB-crystallin found in AD reveals an important role of the sHsp(s) in AD. Calf eye lens α-crystallin has been shown to prevent the fibril formation of model systems, such as apolipoprotein [21] and α1-antichymotrypsin [22]. However, Kudva et al. [23] have reported that αB-crystallin has no effect on the amyloid fibril formation of Aβ-(1–42), whereas Hsp27, another sHsp, was shown to prevent amyloid formation. Liang [24] has reported that the interaction between Aβ-(1–40) and αB-crystallin leads to promotion of fibril formation. On the other hand, Stege et al. [25] have concluded that the presence of calf eye lens αB-crystallin does not lead to

Abbreviations used: Aβ, amyloid β; AD, Alzheimer's disease; β2m, β_2-microglobulin; HEWL, hen egg-white lysozyme; (s)Hsp, (small) heat-shock protein; ThT, thioflavin T; TIRFM, total internal reflection fluorescence microscopy.

[1] Correspondence may be addressed to either author (email mohan@ccmb.res.in or ygoto@protein.osaka-u.ac.jp).

fibril but proto-fibril formation of an Aβ peptide. Thus the role of αB-crystallin in the amyloid fibril formation of Aβ peptide still remains elusive.

The mechanism for amyloid fibril formation involves two important steps: nucleation and propagation [26]. We have studied the effect of recombinant human αB- and αA-crystallin, their mutants and engineered α-crystallins on the amyloid fibril propagation of Aβ-(1–40) after providing the required nucleation using the sonicated fibril seed, as well as on the spontaneous fibril formation (without externally added seeds) of Aβ-(1–42). We have also studied fibril growth of a larger polypeptide, β2m (β_2-microglobulin), a constituent of the class I MHC, which is involved in dialysis-related amyloidosis [27]. β2m fibrils form under acidic conditions [28] and depolymerize upon shifting the pH to neutral [29]. Thus β2m may serve as a good model system to study the effect of α-crystallin on such a fibril growth and depolymerization process. Our study demonstrates that the sHsp α-crystallin prevents the amyloid fibril growth of Aβ-(1–40), Aβ-(1–42) and β2m, and also reveals, for the first time, the underlying mechanism.

EXPERIMENTAL

Materials

Recombinant human αA- and αB-crystallin, R120G-αB-crystallin (a mutant that causes desmin-related myopathy and congenital cataract [30]) and the deletion mutant αBdel, in which the conserved SRLFDQFFG residues in the N-terminal region have been deleted, and the chimaeric proteins, αANBC-crystallin (comprising the N-terminal domain of αA- and the C-terminal domain of αB-crystallin) and αBNAC-crystallin (comprising the N-terminal domain of αB- and the C-terminal domain of αA-crystallin), were expressed in *Escherichia coli* and purified to homogeneity, as described in earlier studies by Rao and colleagues [31–33]. Human Aβ-(1–40) was purchased from the Peptide Institute, Inc., Osaka, Japan. Human Aβ-(1–42) was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The monomeric recombinant human β2m was expressed in *E. coli* and purified to homogeneity, as described previously [34]. Fatty acid-free BSA and HEWL (hen egg-white lysozyme) were purchased from Sigma Chemical Co.

Amyloid fibril growth of Aβ-(1–40)

Aβ-(1–40) peptide (50 μM; $\approx$0.22 mg/ml) in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl (referred to hereafter as buffer A), in the absence or in the presence of indicated concentrations of α-crystallins or other proteins, was incubated at 37 °C with 5 μg/ml sonicated, preformed amyloid fibrils (hereafter referred to as 'fibril seed'). Aliquots (5 μl) of the sample were withdrawn at different time points and added to 1 ml of 5 μM Tht (thioflavin T) in 50 mM glycine/NaOH buffer, pH 8.5 (referred to hereafter as ThT solution). Fluorescence intensity of the sample at 485 nm, which is proportional to the extent of amyloid fibril-bound ThT [35] and hence the amyloid fibril growth, was measured using a Hitachi F-4500 fluorescence spectrophotometer with an excitation wavelength of 445 nm.

We have also tested the fibril growth of Aβ-(1–40) using the sonicated fibril seed of Aβ-(1–42) (20 μg/ml) under similar conditions. Aβ-(1–42) fibrils are formed by incubating 50 μM of the peptide in buffer A at 37 °C for 7 h. The effect of α-crystallin on the spontaneous fibril formation (without externally adding seeds) of Aβ-(1–42) was studied by incubating 50 μM ($\approx$0.23 mg/ml) Aβ-(1–42) in the absence and in the presence of indicated concentrations of α-crystallins at 37 °C. Small aliquots of the sample were withdrawn at different time points, and ThT binding was studied as described above. All experiments were repeated three times, and the results were found to be reproducible. Representative data are shown.

Amyloid fibril growth of β2m

The seed-dependent elongation of β2m fibrils was performed essentially following the method described previously [28]. Monomeric β2m (25 μM) in 50 mM sodium citrate buffer, pH 2.5, containing 100 mM NaCl either in the absence or the presence of indicated concentrations of α-crystallins or other proteins, was incubated together with its 5 μg/ml fibril seed at 37 °C. Aliquots (10 μl) of the sample were withdrawn at different time intervals and added to 1 ml of 5 μM ThT solution, and their fluorescence intensities were measured as described above. A similar experiment was performed at pH 5.3 using the buffer system of 25 mM sodium phosphate/citric acid containing 100 mM Na_2SO_4.

TIRFM (total internal reflection fluorescence microscopy)

The Aβ sample (10 μl) was mixed with 10 μl of 10 μM ThT in 100 mM glycine/NaOH buffer, pH 8.5, and the mixture was placed on a glass slide. The TIRFM system to observe ThT-bound amyloid fibrils is developed based on an inverted microscope (IX70; Olympus, Tokyo, Japan) as described previously [36]. ThT was excited by argon laser (Model 185F02-ADM; Spectra Physics, Mountain View, CA, U.S.A.). The fluorescent image was filtered by a band-pass filter (D490/30 Omega Optical; Brattleboro, VT, U.S.A.) and visualized using a digital camera (DP70; Olympus, Tokyo, Japan).

CD spectroscopy

Either samples of 0.22 mg/ml Aβ peptide alone in buffer A or samples of the peptide incubated in the absence and presence of 0.1 mg/ml αB-crystallin or BSA at 37 °C for 80 min were prepared. These samples were diluted 1:1 (v/v) with buffer A, and the far-UV CD spectra were recorded at 37 °C using a Jasco-600 spectropolarimeter equipped with a thermostat-controlled cell holder. A quartz cuvette with 0.1 cm path length was used. The spectral contributions of αB-crystallin or BSA were subtracted from the spectra of the mixture. The data are shown as the mean residue mass ellipticity for the Aβ peptide.

Sedimentation velocity

Sedimentation velocity measurements were performed using an Optima XL-I analytical ultracentrifuge (Beckman Coulter, Fullerton, CA, U.S.A.) with an An-60 rotor and two-channel, charcoal-filled Epon cells. Samples (0.3 ml) of 0.08 mg/ml αB-crystallin and 0.16 mg/ml Aβ-(1–40), and their mixture (0.04 and 0.08 mg/ml respectively), in buffer A were incubated at 37 °C for 15 min, followed by centrifugation at 42 500 ***g***. At these peptide and protein concentrations, the total absorbance at 225 nm was approx. 1.0, with protein and peptide contributing approx. 0.5 each to the mixture. The protein boundary was scanned at 6 min intervals for its absorbance at 225 nm. Boundary curves at 12 min intervals are shown (see Figure 3). The apparent sedimentation coefficient of αB-crystallin in buffer A at 37 °C was calculated using the software Origin 4.1 (Microcal Software, Inc., Northampton, MA, USA).

Binding of α-crystallin to the amyloid fibrils of Aβ-(1–40)

Samples of αB-crystallin or BSA (0.1 mg/ml) in buffer A in the absence or in the presence of 0.11 mg/ml amyloid fibrils of Aβ-(1–40) were incubated at 37 °C for 20 min, and then centrifuged

at 13000 ***g*** for 15 min using a Hitachi Himac CF 15R microcentrifuge. Aliquots (20 μl) of the supernatant of the sample were diluted to 0.3 ml with buffer A, and tryptophan fluorescence spectra (whose intensity is a measure of αB-crystallin or BSA that is not bound to the fibrils) were recorded using a Hitachi F-4500 fluorescence spectrophotometer with an excitation wavelength of 295 nm. The excitation and emission band passes were set at 5 nm. The amount of fibrils in the supernatant after centrifugation was measured from their ThT fluorescence, and found to be less than 2 % in all the samples.

Effect of α-crystallins on β2m amyloid fibril depolymerization

The β2m amyloid fibril formed at pH 2.5 is known to be unstable upon shifting the pH to neutral [29]. β2m amyloid fibrils were prepared following a method described previously [28] by incubating 0.3 mg/ml β2m in 50 mM sodium citrate buffer, pH 2.5, containing 100 mM NaCl together with 5 μg/ml sonicated fibril seed at 37 °C for 4 h. The sample was centrifuged at 13000 ***g*** and resuspended in the same buffer, so that the stock concentration of the fibril was 0.6 mg/ml. To study the effect of α-crystallin on the fibril depolymerization process, buffer A in the absence or in the presence of required concentrations of α-crystallin or other indicated proteins was equilibrated at 37 °C, and then the β2m fibrils were added to a final concentration of 0.06 mg/ml. Aliquots (10 μl) of the samples were withdrawn at indicated time points and added to 1 ml of ThT solution. The amount of fibrils in the sample at various time intervals was measured from their ThT fluorescence. Fractions of the fibrils that were undissociated were calculated on the basis of the ThT fluorescence intensity of the samples with respect to their fluorescence intensity immediately after dilution (zero time).

RESULTS AND DISCUSSION

αB-crystallin prevents the amyloid fibril growth of Aβ-(1–40)

Aβ peptides are known to exhibit nucleation-dependent amyloid fibril formation [26]. Figure 1 shows that incubation of $\approx$0.22 mg/ml synthetic Aβ-(1–40) in buffer A at 37 °C with 5 μg/ml fibril seed leads to progressive amyloid fibril formation, as measured by the binding of ThT (Figure 1, closed circles). We have investigated the role of αB-crystallin on such nucleated fibril growth of Aβ peptide. Figure 1(A) shows that αB-crystallin prevents fibril growth even at the chaperone to Aβ mass ratio of 0.25:1.0. Although αA-crystallin is also able to prevent the fibril growth of the peptide, it is relatively less effective than αB-crystallin at comparable concentrations of the chaperones (cf. Figures 1A and 1B). BSA and HEWL, however, do not prevent the fibril growth of the Aβ peptide significantly (Figures 1C and 1D), indicating that the effect of α-crystallin on the Aβ amyloid fibril growth is specific in nature. We have tested whether αB-crystallin has any defibrillation activity by incubating the fibrils of Aβ-(1–40) (0.1 mg/ml) along with αB-crystallin (0.5 mg/ml) at 37 °C for 4 h. We did not observe a significant decrease in the ThT fluorescence, indicating that αB-crystallin does not exhibit defibrillation activity (results not shown).

TIRFM has been shown to be useful in visualizing ThT-bound amyloid fibrils [36]. We examined the samples of ThT-bound fibril seed, the Aβ amyloid fibrils and the samples of the Aβ peptide and the seed incubated along with αB-crystallin or BSA by TIRFM (Figure 2). Amyloid fibril growth of Aβ-(1–40) occurs to differing lengths of the order of a few micrometres, both in buffer alone and in the presence of BSA: the fibres grown in the presence of BSA seem to be longer than those grown in buffer alone (cf. Figure 2B and 2C). On the other hand, we could see only the added seeds, but no significant fibrils in the samples containing αB-crystallin (Figure 2D). Thus the ThT binding and TIRFM results show that αB-crystallin prevents the growth of Aβ-(1–40) fibrils.

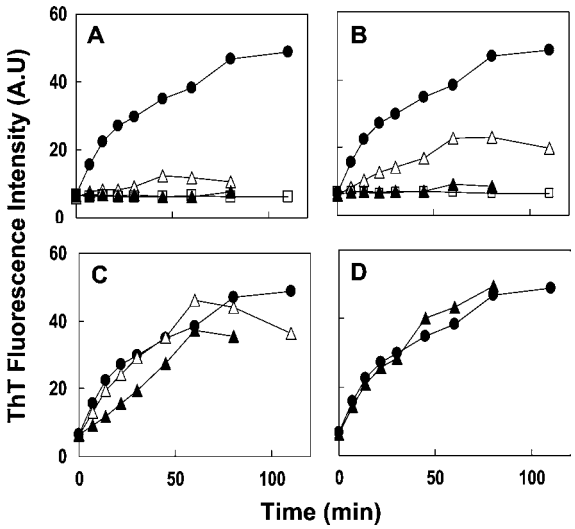

Figure 1 Effect of αB-crystallin (A), αA-crystallin (B), BSA (C) and HEWL (D) on the amyloid fibril growth of Aβ-(1–40)

The fibril growth of Aβ-(1–40) (0.22 mg/ml) in buffer alone (●) and in the presence of 0.05 mg/ml (△), 0.1 mg/ml (▲) or 0.4 mg/ml (□) of the protein additives.

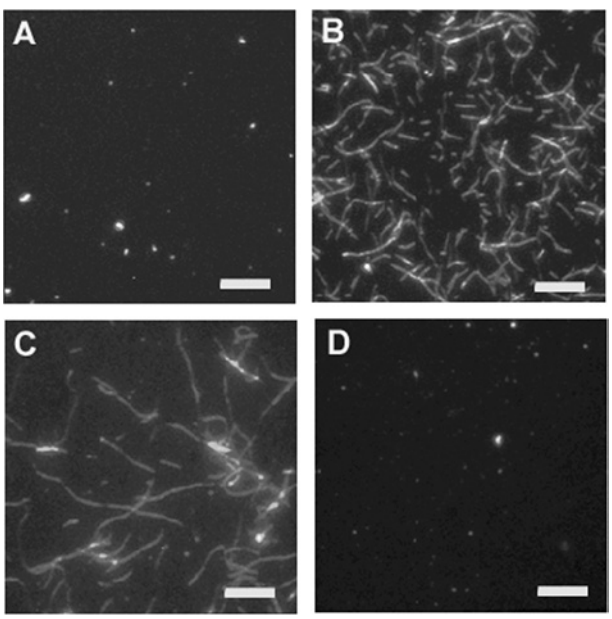

Figure 2 TIRFM images

Shown are ThT-stained samples of 10 μg/ml Aβ-(1–40) fibril seed (**A**), and 0.22 mg/ml Aβ-(1–40) peptide incubated at 37 °C for 2 h with 5 μg/ml fibril seed in the absence (**B**) or in the presence of 0.1 mg/ml BSA (**C**) or αB-crystallin (**D**). In the cases of fibril grown in buffer alone and in the presence of 0.1 mg/ml BSA, the amount of fibril was so high that the samples were diluted four times with buffer A before ThT staining. It should be noted that, in the case of αB-crystallin, no significant fibrils were seen, despite the fact that the sample was used as such for ThT staining. The scale bars represent 5 μm.

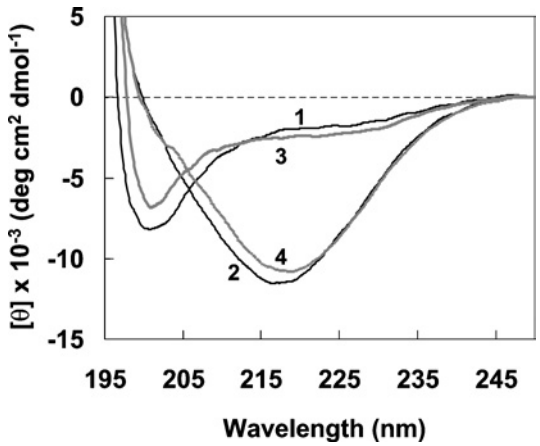

Figure 3 Far-UV CD spectra

Aβ-(1–40) peptide in buffer A (trace 1), and the sample of 0.22 mg/ml Aβ-(1–40) in buffer A incubated at 37 °C for 80 min with 5 μg/ml fibril seed in the absence (trace 2) or in the presence of 0.1 mg/ml αB-crystallin (trace 3) or BSA (trace 4) are shown. The spectral contribution of αB-crystallin or BSA was subtracted from the corresponding spectrum of the sample. [θ] is the mean residue mass ellipticity.

αB-crystallin prevents the induction of β-sheet structure upon fibril growth

Amyloid fibril exhibits characteristic well-ordered, cross-β-sheet structure [14]. Fibril propagation thus leads to association-induced generation of β-sheet structure. We have investigated the effect of αB-crystallin on such association-induced generation of β-sheet structure of the Aβ peptide. Aβ-(1–40) peptide exhibits randomly coiled conformation, as revealed by its far UV-CD spectrum (Figure 3, trace 1). Incubation of the peptide in the presence of fibril seed for 80 min generates a characteristic far-UV CD spectrum for β-sheet structure, with a minimum around 218 nm (Figure 3, trace 2). However, such induction of β-sheet structure does not occur in the presence of αB-crystallin (Figure 3, trace 3). In contrast, induction of β-sheet occurs in the presence of BSA (Figure 3, trace 4). Thus αB-crystallin specifically prevents the amyloid fibril growth of the Aβ peptide and the association-induced generation of β-sheet structure. Monomeric Aβ peptides are not toxic to the cells [37–40]. Earlier studies have shown that amyloid fibrils with induced β-sheet are toxic to the cells [37]. Subsequent studies have shown that protofibrils or prefibrillar oligomers are more toxic to the cells [38–41], whereas the toxicity depends on the size of the aggregates [40,41]. Prevention of amyloid fibril formation by the sHsp, αB-crystallin, may thus serve as one of the protective mechanisms.

αB-crystallin prevents the fibril growth of Aβ-(1–40) seeded by Aβ-(1–42) fibrils and spontaneous fibril formation of Aβ-(1–42)

We have tested whether αB-crystallin also prevents the fibril growth of Aβ-(1–40) if seeded with the Aβ-(1–42) fibrils. Under our experimental conditions, incubation of unseeded solution of Aβ-(1–40) at 37 °C does not lead to significant formation of fibrils, even after several hours. When seeded with the sonicated fibril seeds of Aβ-(1–42), fibril growth occurs progressively, as measured by ThT fluorescence (Figure 4A). Similar to the observation in Figure 1, αB-crystallin inhibits the fibril growth of Aβ-(1–40) seeded with the sonicated Aβ-(1–42) fibrils (Figure 4A). We have also found that αB-crystallin prevents the spontaneous fibril formation of Aβ-(1–42) without externally added seeds (Figure 4B).

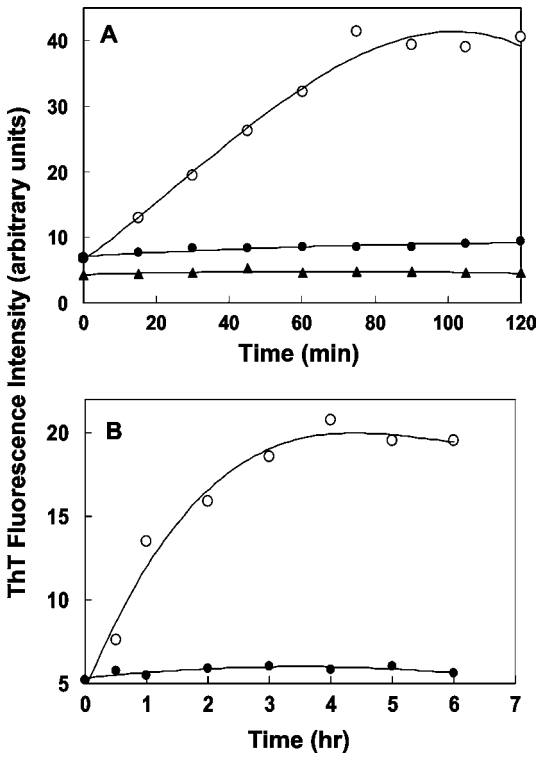

Figure 4 Effect of αB-crystallin on the fibril growth of Aβ-(1–40) seeded with the sonicated fibrils of Aβ-(1–42) (A), and effect of αB-crystallin on the spontaneous fibril formation of Aβ-(1–42) (B)

(**A**) Unseeded 0.22 mg/ml Aβ-(1–40) in buffer A (▲), and the Aβ-(1–40) sample seeded with 20 μg/ml Aβ-(1–42) fibril seed in the absence (○) or in the presence (●) of 0.1 mg/ml αB-crystallin are shown. (**B**) Aβ-(1–42) (≈0.23 mg/ml) in the absence (○) or in the presence (●) of 0.1 mg/ml αB-crystallin.

Effects of mutant and chimaeric α-crystallins

We have compared the relative effects of the wild-type, some mutants and chimaeric proteins of αA- and αB-crystallin in preventing the amyloid fibril growth of Aβ-(1–40) at the concentration where these proteins offer partial protection. As seen in Figure 5, except for the chimaeric protein, αBNAC-crystallin (see the Experimental section), which inhibits completely the fibril growth, other proteins exhibit marginal differences in their ability to prevent the fibril growth. However, earlier studies have shown that they differ drastically in their ability to prevent the amorphous aggregation of target proteins, such as insulin or citrate synthase [32]. For example, the chimaeric protein αANBC-crystallin, which did not prevent the amorphous aggregation of insulin at all [32], prevents the fibril growth of Aβ-(1–40) to an extent comparable with that of αA- or αB-crystallin (Figure 5). Although the deletion mutant, αBdel, exhibits severalfold-increased chaperone-like activity towards the aggregation of insulin or citrate synthase [33], it shows only marginally higher ability than αB-crystallin to prevent the fibril growth of Aβ-(1–40) (Figure 5). The mutation of the conserved arginine residue, R120G, in αB-crystallin leads to significantly decreased chaperone-like activity towards insulin aggregation [31], whereas its ability to prevent the Aβ-(1–40) fibril growth is only marginally lower than that of αB-crystallin. We have also tested αB-crystallin, R120G-αB-crystallin and αBdel-crystallin on the spontaneous fibril formation of Aβ-(1–42) and found that they did not differ drastically in their ability to prevent the fibril formation

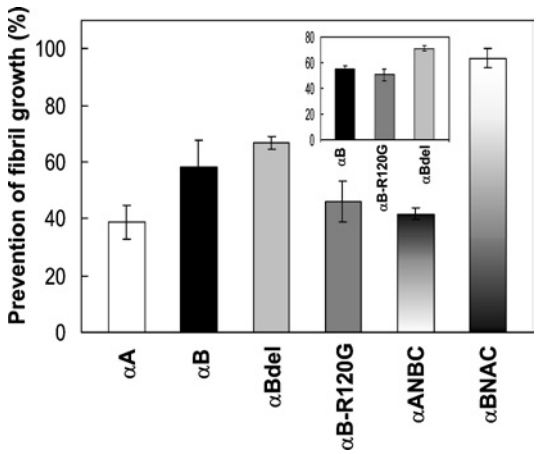

Figure 5 Effect of the wild-type, mutant and engineered α-crystallins in preventing amyloid fibril growth of Aβ-(1–40)

The percentage prevention of the fibril growth was calculated using the formula $[1 - (F_\alpha/F_b)] \times 100$, where F_b and F_α are the ThT fluorescence intensities of samples of 0.22 mg/ml Aβ-(1–40) incubated at 37 °C for 60 min with 10 μg/ml fibril seed in buffer A alone, or in the presence of 0.05 mg/ml α-crystallins, respectively. Error bars derived from the data for three experiments are shown. The inset compares the effect of 25 μg/ml αB-crystallin, R120G αB-crystallin and αBdel-crystallin on the spontaneous fibril formation of Aβ-(1–42) (≈0.23 mg/ml).

(Figure 5, inset). Thus the relative effects of these proteins differ significantly towards the amorphous aggregation of proteins and fibril formation of Aβ peptides. These differences could be because of possible mechanistic differences between the chaperone function in preventing amorphous aggregation and amyloid fibril formation. It is also possible that some common and distinct chaperone sites are involved in preventing the amorphous aggregation and amyloid fibril growth of Aβ peptides.

Mechanism involved in the prevention of amyloid fibril growth by α-crystallin

Our results clearly demonstrate that αB-crystallin prevents the well-ordered amyloid fibril growth. The prevention of Aβ peptide fibrillation by α-crystallin may involve either or both of the two possible mechanisms. (i) α-Crystallin may bind to the amyloidogenic species (monomers) of the Aβ peptide and hence prevent their association. α-Crystallins and other Hsps are known to bind the target proteins to form a stable complex and prevent aggregation [10]. (ii) α-Crystallin may interact with the fibril seed, which nucleates the amyloid fibril growth. Such an interaction may mask the complementary surfaces that are critical for the assembly of fibril growth. Which of the two mechanisms is operative in the case of prevention of well-ordered amyloid fibrils is a pertinent question to be addressed.

We have investigated the possible mechanism of αB-crystallin forming a stable complex with the Aβ peptide by sedimentation velocity measurements, since these two molecules differ drastically in their molecular masses and hence in their sedimentation coefficients. If the chaperone binds to the amyloidogenic peptide to form a stable complex, the slow-sedimenting species of the Aβ peptide should sediment along with the fast-sedimenting αB-crystallin molecules. The concentrations of the protein and the peptide have been selected such that the total absorbance of the sample at 225 nm is approx. 1.0. Figure 6(A) shows the progressive movement of the boundary of αB-crystallin sample alone during the ultracentrifugation. On the other hand, the boundary of the Aβ peptide sample alone does not move

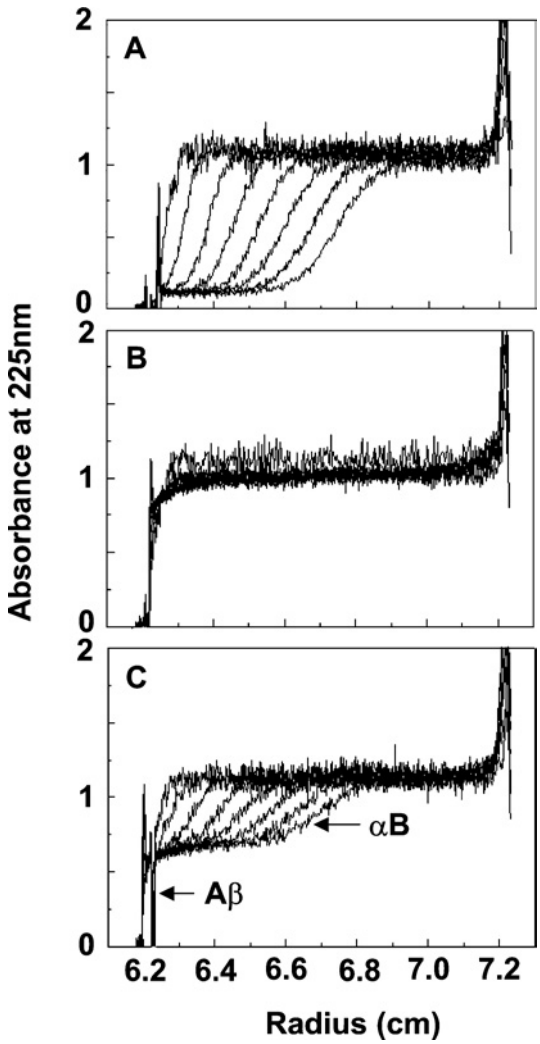

Figure 6 Sedimentation velocity analysis

αB-crystallin (**A**), Aβ-(1–40) peptide (**B**) and the mixture of the two (**C**) in buffer A at 37 °C are shown. The curves represent the radial scan traces corresponding to every 12 min interval (from left to right; see the Experimental section for details).

significantly under the same conditions (Figure 6B). If there is a stable complex formation between these two molecules, we should not distinctly observe the boundary corresponding to the Aβ peptide, as it is expected to move along with that of αB-crystallin in the sample that is a mixture of these two molecules. However, contrary to this expectation, our result (Figure 6C) shows that the boundary corresponding to the peptide is distinctly observed in the mixture of these two molecules; the absorbance value also does not decrease significantly from the expected value of approx. 0.5 for half the concentration of the peptide used in the case of peptide alone (Figure 6B). The apparent sedimentation coefficient of αB-crystallin in the buffer at 37 °C in the absence or in the presence of Aβ-(1–40) was calculated to be approx. ≈18 S. Thus these results rule out the possibility of the formation of a stable complex as a predominant mechanism by which αB-crystallin prevents the amyloid fibril growth of the peptide. However, transient or reversible interaction of the peptide with α-crystallin cannot be ruled out. It should be noted that earlier studies from one of our laboratories showed that α-crystallin binds and forms stable complexes only with the aggregation-prone molten-globule-like states of target proteins [42], and does not form

stable complexes either with compact molten globules with less exposed hydrophobic surfaces [42] or with the randomly coiled conformation of reduced RNase A, for example [43]. Aβ peptide adopts a randomly coiled structure (Figure 3) and it does not form a stable complex with α-crystallin (Figure 6). The present results are consistent with our earlier conclusions.

We have investigated the second possibility of αB-crystallin interacting with the seed fibrils and preventing amyloid propagation. Since the amyloid fibrils sediment at a relatively low centrifugal force, if there is a stable interaction (binding) of the chaperone to the fibrils, the concentration of the chaperone in the supernatant will be decreased, depending on the avidity of the interaction. Since the Aβ peptide does not contain tryptophan residues in its sequence, tryptophan fluorescence can be used to selectively measure the amount of αB-crystallin or BSA (used as a control) in the supernatant. More than 98 % of the amyloid fibrils of the Aβ peptide sediment under our experimental conditions, whereas α-crystallins and BSA alone do not sediment. We have incubated α-crystallin or BSA (0.1 mg/ml) samples in the absence or in the presence of amyloid fibrils of Aβ-(1–40) (0.1 mg/ml) at 37 °C for 20 min, and then centrifuged to remove only the protein bound to the fibrils. The amount of α-crystallin or BSA in the supernatant was measured by its intrinsic tryptophan fluorescence, which represents unbound protein. A remarkable decrease in fluorescence intensity of the supernatant of the αB-crystallin samples incubated with the amyloid fibrils was observed (Figure 7A), indicating that as much as 46 % of the αB-crystallin is bound to the amyloid fibrils of Aβ (Figure 7B, inset). On the other hand, the fluorescence spectra of the supernatant of the samples of BSA in the absence or in the presence of the fibrils differ only marginally (< 10 %) in terms of the fluorescence intensity (Figure 7B), indicating that BSA does not interact with the amyloid fibrils significantly. Corroboration of this result showing the differential ability of αB-crystallin and BSA to bind the amyloid fibrils with the results showing their ability to prevent the amyloid fibril growth (Figure 1) reveals, for the first time, that binding of αB-crystallin to the fibril seed, which nucleates the growth of the fibrils, is the predominant mechanism involved in the prevention of Aβ peptide amyloid propagation by the chaperone molecule. In order to understand the generality of the mechanism, we have studied the effect of α-crystallins on the fibril growth and dissociation of β2m.

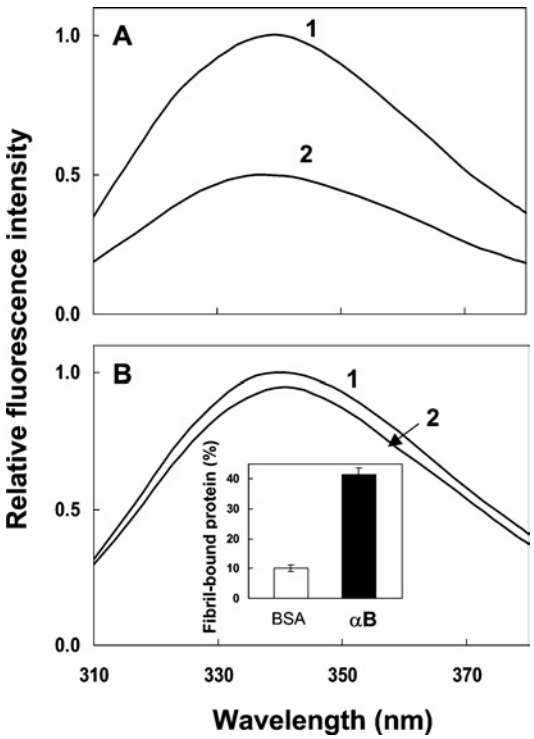

Figure 7 Binding of αB-crystallin to the amyloid fibrils of Aβ-(1–40) measured using the tryptophan fluorescence of αB-crystallin

(**A**) Tryptophan fluorescence spectra of the supernatant of the αB-crystallin samples (0.1 mg/ml) in buffer A incubated at 37 °C in the absence (trace 1) and in the presence of 0.11 mg/ml amyloid fibrils of Aβ-(1–40) (trace 2) after centrifugation to remove the fibrils and bound protein are shown. (**B**) Tryptophan fluorescence spectra of the supernatant of the BSA samples (0.1 mg/ml) in buffer A incubated at 37 °C in the absence (trace 1) or in the presence of the 0.11 mg/ml amyloid fibrils of Aβ-(1–40) (trace 2) after the centrifugation.

Effect of α-crystallin on amyloid fibril growth and dissociation of β2m

β2m is the major component of amyloid deposits found in patients of haemodialysis-related amyloidosis [27]. This protein readily undergoes amyloid formation under acidic conditions below pH 4 [28]. The fibrils of β2m formed under acidic conditions dissociate upon shifting them to neutral pH [29]. Thus β2m serves as another good model system to investigate whether α-crystallin can interact with fibrils.

Although, under the extremely acidic conditions where β2m readily forms fibrils, α-crystallin undergoes denaturation (αB-crystallin has been shown to dissociate into monomers, whereas αA-crystallin forms structurally perturbed small multimers [44]), it was interesting to investigate whether these structurally perturbed species of α-crystallin are capable of preventing amyloid fibril growth. Figure 8(A) shows that α-crystallin is capable of preventing the amyloid fibril growth of β2m, even at pH 2.5. However, the concentrations of α-crystallin required to prevent amyloid fibril growth of β2m almost completely are comparatively higher than those required to prevent the fibrillation of Aβ-(1–40). BSA also seems to prevent the fibril growth to a significant extent, although relatively lower than that of α-crystallin, whereas HEWL shows no effect on the amyloid fibril growth of β2m at pH 2.5 (Figure 8A).

Our recent study on the effect of salts on the amyloid fibril growth of β2m showed that the critical balance of electrostatic and hydrophobic interactions, modulated by preferential co-solute anion interaction, is important in the amyloid propagation process of β2m [45]. We also found that sulphate can promote amyloid fibril growth between pH 5 and 6, just below its isoelectric point [45]. As moderately low pH values can occur physiologically under certain inflammatory conditions, it is probable that circulating β2m may encounter such low-pH conditions [46]. It is not known whether sHsps such as αB-crystallin are found in the amyloid deposits of β2m, as observed in other amyloid deposits involved in several neurodegenerative diseases. However, αB-crystallin could be detected in blood sera using monoclonal antibodies (S. Rao, V. Pasha, S. Mahesh and M. Rao, unpublished work). We have investigated whether α-crystallins can prevent the sulphate-promoted amyloid fibril growth of β2m at pH 5.3 and found that α-crystallin, indeed, prevented fibril growth under such moderately acidic conditions (Figure 8B). Although the implications of these results are not clear at present, the effect of α-crystallin towards this model system shows that it can prevent amyloid growth under acidic conditions as well. Since the amyloid fibril growth of Aβ peptide under acidic conditions is very poor, we could not study the effect of α-crystallin under such conditions. Hence our results on the effect of α-crystallin on the amyloid fibril growth of β2m under acidic conditions can be taken to

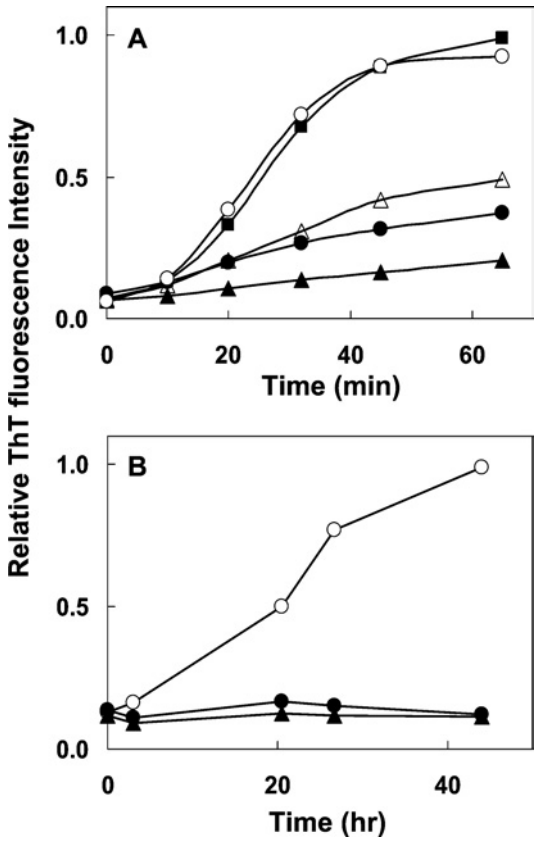

Figure 8 Effect of α-crystallins on the amyloid fibril growth of β2m under acidic conditions

(**A**) Amyloid fibril growth in 50 mM sodium citrate buffer, pH 2.5, containing 100 mM NaCl in the absence (○) or in the presence of 0.4 mg/ml αA-crystallin (●), αB-crystallin (▲), BSA (△) and HEWL (■). (**B**) Sodium sulphate-promoted amyloid fibril growth of β2m at pH 5.3 in 25 mM sodium phosphate–citric acid buffer, pH 5.3, containing 100 mM Na_2SO_4 alone (○), or in the presence of 0.2 mg/ml αA-crystallin (●) or αB-crystallin (▲).

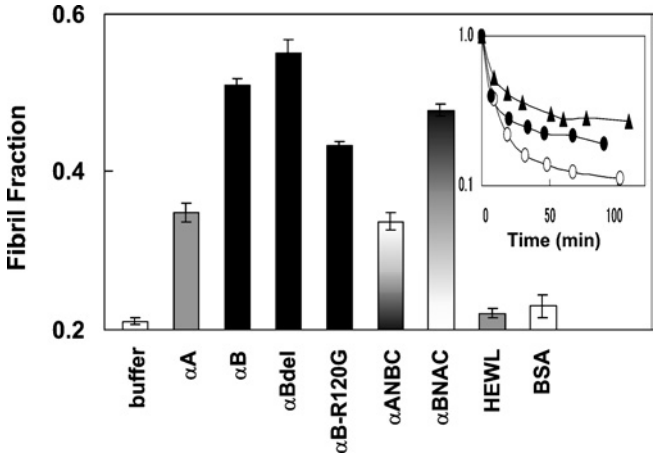

Figure 9 Effect of α-crystallins on the pH-induced dissociation of β2m fibrils

β2m amyloid fibrils (prepared under acidic conditions) were placed in buffer A in the absence or in the presence of 0.1 mg/ml of various α-crystallins and incubated at 37 °C. Fibrils present in the sample at different time intervals were measured by ThT fluorescence. The results shown represent the fraction of the fibril present after 60 min incubation. Error bars are derived from data for three experiments. The inset shows the dissociation of the fibrils as a function of time in the absence (○) or in the presence of 0.05 mg/ml (●) or 0.1 mg/ml (▲) αB-crystallin.

complement our conclusion that αB-crystallin, an sHsp, can prevent the amyloid fibril growth of small peptides (e.g. Aβ) or relatively larger proteins (e.g. β2m) under either normal physiological or acidic pH conditions.

We have exploited the property of the β2m fibrils to dissociate upon shifting them to neutral pH [29] to find out whether α-crystallin can interact with the fibrils of β2m. Interestingly, αB-crystallin retards the dissociation of the β2m amyloid fibril (Figure 9, inset). This effect of αB-crystallin appears to be specific in nature, since BSA and HEWL do not significantly retard β2m amyloid fibril dissociation at comparable concentrations (Figure 9). Moreover, the mutants and the engineered α-crystallins show variations in the extent of retardation, which are generally consistent with their relative effects in preventing the amyloid fibril growth of Aβ (minor variations may partly be due to the assay system and/or the nature of the protein). Thus these results show that α-crystallin can interact with β2m fibrils as well.

The sHsp, αB-crystallin, can act both on the nucleation and propagation processes of amyloid formation

It has earlier been proposed, based on the results with the model system, apolipoprotein C-II, that α-crystallin interacts with partially structured amyloidogenic precursors, inhibiting the amyloid formation at the nucleation, rather than the elongation, phase [21]. Our study on the mechanistic aspects of the prevention of amyloid fibril growth of Aβ-(1–40) and β2m by α-crystallin suggests that binding of the chaperone molecule to the fibril nucleus, and prevention of the propagation process, is the predominant mechanism. This mechanism also suggests that the observed differences in the relative efficiency of mutants and engineered α-crystallin variants towards insulin aggregation and Aβ-(1–40) fibrillation are due to the involvement of aggregation-type specific interactions. Such a phenomenon has not been invoked earlier in the context of the function of α-crystallin. To the best of our knowledge, this is the first demonstration of differential action, providing a new insight into the mechanistic aspects of chaperone action of α-crystallin involving different types of interactions mediating the chaperone process towards amyloid formation and amorphous aggregation (e.g. insulin aggregation) of proteins. Our present study demonstrates that α-crystallins can prevent the amyloid fibril propagation process as well. It appears that, although α-crystallin can act on both nucleation and propagation, its relative involvement in these two phases of amyloid formation may depend on the nature of the amyloidogenic species. If the amyloidogenic species exhibit significant exposed hydrophobic surfaces (molten-globule-like state), α-crystallin binds stably to the species [42,47,48] and thus prevents the nucleation process itself. On the other hand, if the amyloidogenic species has randomly coiled or extended conformation with less or no exposed hydrophobic surfaces [as in the cases of Aβ-(1–40) and β2m], α-crystallins prevent the fibril propagation process by binding to the fibril nucleus, as reported in the present study.

Conclusions

Our study clearly shows that αB-crystallin prevents the amyloid fibril growth of Aβ peptide, indicating that this sHsp has a critical function in preventing the amyloid propagation process. Since αB-crystallin does not form a stable complex with the Aβ peptide, the mechanism of prevention of amyloid fibril growth

of the Aβ peptide appears to be different from the mechanism of prevention of amorphous aggregation of proteins, where α-crystallin is known to form a stable complex. Our study shows that preferential binding of the chaperone molecule to the fibril nucleus is the predominant mechanism involved in its ability to prevent the amyloid propagation of Aβ-(1–40). Thus our study should prove useful in understanding the role of Hsps in protein conformational diseases in general, and AD in particular.

B.R. thanks the JSPS (Japan Society for Promotion of Science), Japan, for the support of a post-doctoral Fellowship.

REFERENCES

1 Dasgupta, S., Hohman, T. C. and Carper, D. (1992) Hypertonic stress induces alpha B-crystallin expression. Exp. Eye Res. **54**, 461–470
2 Clark, J. I. and Muchowski, P. J. (2000) Small heat-shock proteins and their potential role in human disease. Curr. Opin. Struct. Biol. **10**, 52–59
3 Sax, C. M. and Piatigorsky, J. (1994) Expression of the alpha-crystallin/small heat-shock protein/molecular chaperone genes in the lens and other tissues. Adv. Enzymol. Relat. Areas Mol. Biol. **69**, 155–201
4 Bhat, S. P. and Nagineni, C. N. (1989) Alpha B subunit of lens-specific protein alpha-crystallin is present in other ocular and non-ocular tissues. Biochem. Biophys. Res. Commun. **158**, 319–325
5 van den Oetelaar, P. J., van Someren, P. F., Thomson, J. A., Siezen, R. J. and Hoenders, H. J. (1990) A dynamic quaternary structure of bovine alpha-crystallin as indicated from intermolecular exchange of subunits. Biochemistry **29**, 3488–3493
6 Horwitz, J. (1992) Alpha-crystallin can function as a molecular chaperone. Proc. Natl. Acad. Sci. U.S.A. **89**, 10449–10453
7 Sun, T. X., Das, B. K. and Liang, J. J. (1997) Conformational and functional differences between recombinant human lens alphaA- and alphaB-crystallin. J. Biol. Chem. **272**, 6220–6225
8 Raman, B. and Rao, C. M. (1994) Chaperone-like activity and quaternary structure of alpha-crystallin. J. Biol. Chem. **269**, 27264–27268
9 Datta, S. A. and Rao, C. M. (1999) Differential temperature-dependent chaperone-like activity of alphaA- and alphaB-crystallin homoaggregates. J. Biol. Chem. **274**, 34773–34778
10 Van Montfort, R., Slingsby, C. and Vierling, E. (2001) Structure and function of the small heat shock protein/alpha-crystallin family of molecular chaperones. Adv. Protein Chem. **59**, 105–156
11 de Jong, W. W., Caspers, G. J. and Leunissen, J. A. (1998) Genealogy of the alpha-crystallin–small heat-shock protein superfamily. Int. J. Biol. Macromol. **22**, 151–162
12 Sharma, K. K., Kaur, H. and Kester, K. (1997) Functional elements in molecular chaperone alpha-crystallin: identification of binding sites in alpha B-crystallin. Biochem. Biophys. Res. Commun. **239**, 217–222
13 Pasta, S. Y., Raman, B., Ramakrishna, T. and Rao, C. M. (2002) Role of the C-terminal extensions of alpha-crystallins. Swapping the C-terminal extension of alpha-crystallin to alphaB-crystallin results in enhanced chaperone activity. J. Biol. Chem. **277**, 45821–45828
14 Sunde, M. and Blake, C. C. (1998) From the globular to the fibrous state: protein structure and structural conversion in amyloid formation. Q. Rev. Biophys. **31**, 1–39
15 Renkawek, K., de Jong, W. W., Merck, K. B., Frenken, C. W. G. M., van Workum, F. P. A. and Bosman, G. J. (1992) Alpha B-crystallin is present in reactive glia in Creutzfeldt-Jakob disease. Acta Neuropathol. (Berlin) **83**, 324–327
16 Shinohara, H., Inaguma, Y., Goto, S., Inagaki, T. and Kato, K. (1993) Alpha B crystallin and HSP28 are enhanced in the cerebral cortex of patients with Alzheimer's disease. J. Neurol. Sci. **119**, 203–208
17 Selkoe, D. J. (1997) Alzheimer's disease: genotypes, phenotypes, and treatments. Science **275**, 630–631
18 Renkawek, K., Vooter, C. E. M., Bosman, G. J., van Workum, F. P. A. and de Jong, W. W. (1994) Expression of alpha B-crystallin in Alzheimer's disease. Acta Neuropathol. (Berlin) **87**, 155–160
19 Shoji, M., Golde, T., Ghiso, J., Cheung, T., Estus, S., Shaffer, L., Cai, X., McKay, D., Tintner, R., Frangione, B. and Younkin, S. (1992) Production of the Alzheimer amyloid beta protein by normal proteolytic processing. Science **258**, 126–129
20 Ida, N., Hartmann, T., Pantel, J., Schroder, J., Zerfass, R., Forstl, H., Sandbrink, R., Masters, C. L. and Beyreuther, K. (1996) Analysis of heterogeneous A4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive Western blot assay. J. Biol. Chem. **271**, 22908–22914
21 Hatters, D. N., Linder, R. A., Carver, J. A. and Howlett, G. J. (2001) The molecular chaperone, alpha-crystallin, inhibits amyloid formation by apolipoprotein C-II. J. Biol. Chem. **276**, 33755–33761
22 Devlin, G. L., Carver, J. A. and Bottomley, S. P. (2003) The selective inhibition of serpin aggregation by the molecular chaperone, alpha-crystallin, indicates a nucleation-dependent specificity. J. Biol. Chem. **278**, 48644–48650
23 Kudva, C. Y., Hiddinga, J. H., Butler, P. C., Mueske, C. S. and Eberhardt, N. L. (1997) Small heat shock proteins inhibit *in vitro* A beta (1–42) amyloidogenesis. FEBS Lett. **416**, 117–121
24 Liang, J. J. N. (2000) Interaction between beta-amyloid and lens alphaB-crystallin. FEBS Lett. **484**, 98–101
25 Stege, G. J. J., Renkawek, K., Overkamp, P. S., Vershuure, P., van Rijk, A. F., Aalbers, A. R., Boelens, W. C., Bosman, G. J. and de Jong, W. W. (1999) The molecular chaperone alphaB-crystallin enhances amyloid beta neurotoxicity. Biochem. Biophys. Res. Commun. **262**, 152–156
26 Jarrett, J. T. and Lansbury, P. T. (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell **73**, 1055–1058
27 Gejyo, F., Homma, N., Suzuki, Y. and Arakawa, M. (1986) Serum levels of beta 2-microglobulin as a new form of amyloid protein in patients undergoing long-term hemodialysis. N. Engl. J. Med. **314**, 585–586
28 Naiki, H., Hashimoto, N., Suzuki, S., Kimura, H., Nakakuki, K. and Gejyo, F. (1997) Establishment of a kinetic model of dialysis-related amyloid fibril extension *in vitro*. Amyloid **4**, 223–232
29 Yamaguchi, I., Hasegawa, K., Takahashi, N., Gejyo, F. and Naiki, H. (2001) Apolipoprotein E inhibits the depolymerization of beta 2-microglobulin-related amyloid fibrils at a neutral pH. Biochemistry **40**, 8499–8507
30 Vicart, P., Caron, A., Guicheney, P., Li, Z., Prevost, M. C., Faure, A., Chateau, D., Chapon, F., Tome, F., Dupret, J. M. et al. (1998) A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. Nat. Genet. **20**, 92–95
31 Kumar, L. V., Ramakrishna, T. and Rao, C. M. (1999) Structural and functional consequences of the mutation of a conserved arginine residue in alphaA and alphaB crystallins. J. Biol. Chem. **274**, 24137–24141
32 Kumar, L. V. and Rao, C. M. (2000) Domain swapping in human alpha A and alpha B crystallins affects oligomerization and enhances chaperone-like activity. J. Biol. Chem. **275**, 22009–22013
33 Pasta, S. Y., Raman, B., Ramakrishna, T. and Rao, C. M. (2003) Role of the conserved SRLFDQFFG region of alpha-crystallin, a small heat shock protein. Effect on oligomeric size, subunit exchange, and chaperone-like activity. J. Biol. Chem. **278**, 51159–51166
34 Chiba, T., Hagihara, Y., Higurashi, T., Hasegawa, K., Naiki, H. and Goto, Y. (2003) Amyloid fibril formation in the context of full-length protein: effects of proline mutations on the amyloid fibril formation of beta2-microglobulin. J. Biol. Chem. **278**, 47016–47024
35 Naiki, H., Higuchi, K., Hosokawa, M. and Takeda, T. (1989) Fluorometric determination of amyloid fibrils *in vitro* using the fluorescent dye, thioflavin T1. Anal. Biochem. **177**, 244–249
36 Ban, T., Hoshino, M., Takahashi, S., Hamada, D., Hasegawa, K. and Goto, Y. (2004) Direct observation of Abeta amyloid fibril growth and inhibition. J. Mol. Biol. **344**, 757–767
37 Lorenzo, A. and Yankner, B. A. (1994) Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. Proc. Natl. Acad. Sci. U.S.A. **91**, 12243–12247
38 Hartley, D. M., Walsh, D. M., Ye, C. P., Diehl, T., Vasquez, S., Vassilev, P. M., Teplow, D. B. and Selkoe, D. J. (1999) Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. J. Neurosci. **19**, 8876–8884
39 Forloni, G., Lucca, E., Angeretti, N., Della Torre, P. and Salmona, M. (1997) Amidation of beta-amyloid peptide strongly reduced the amyloidogenic activity without alteration of the neurotoxicity. J. Neurochem. **69**, 2048–2054
40 Kayed, R., Head, E., Thompson, J. L., McIntire, T. M., Milton, S. C., Cotman, C. W. and Glabe, C. G. (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science **300**, 486–489
41 Hoshi, M., Sato, M., Matsumoto, S., Noguchi, A., Yasutake, K., Yoshida, N. and Sato, K. (2003) Spherical aggregates of beta-amyloid (amylospheroid) show high neurotoxicity and activate tau protein kinase I/glycogen synthase kinase-3beta. Proc. Natl. Acad. Sci. U.S.A. **100**, 6370–6375
42 Rajaraman, K., Raman, B., Ramakrishna, T. and Rao, C. M. (1998) The chaperone-like alpha-crystallin forms a complex only with the aggregation-prone molten globule state of alpha-lactalbumin. Biochem. Biophys. Res. Commun. **249**, 917–921
43 Raman, B., Ramakrishna, T. and Rao, C. M. (1997) Effect of the chaperone-like alpha-crystallin on the refolding of lysozyme and ribonuclease A. FEBS Lett. **416**, 367–372

44 Augusteyn, R. C., Ellerton, H. D., Putilina, T. and Stevens, A. (1988) Specific dissociation of alpha B subunits from alpha-crystallin. Biochim. Biophys. Acta **957**, 192–201
45 Raman, B., Chatani, E., Kihara, M., Ban, T., Sakai, M., Hasegawa, K., Naiki, H., Rao, C. M. and Goto, Y. (2005) Critical balance of electrostatic and hydrophobic interactions is required for β_2-microglobulin amyloid fibril growth and stability. Biochemistry **44**, 1288–1299
46 Drueke, T. B. (1998) Dialysis-related amyloidosis. Nephrol. Dial. Transplant. **13**, 58–64
47 Rajaraman, K., Raman, B., Ramakrishna, T. and Rao, C. M. (2001) Interaction of human recombinant alphaA- and alphaB-crystallins with early and late unfolding intermediates of citrate synthase on its thermal denaturation. FEBS Lett. **497**, 118–123
48 Goenka, S., Raman, B., Ramakrishna, T. and Rao, C. M. (2001) Unfolding and refolding of a quinone oxidoreductase: alpha-crystallin, a molecular chaperone, assists its reactivation. Biochem. J. **359**, 547–556

Received 24 February 2005/18 July 2005; accepted 1 August 2005
Published as BJ Immediate Publication 1 August 2005, doi:10.1042/BJ20050339