Supporting Material - Binding of the molecular chaperone αB-crystallin to Aβ amyloid fibrils inhibits fibril elongation

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Materials & Methods

Materials

ThT (purchased from Sigma-Aldrich, Gillingham, UK) was purified prior to use by dissolving the supplied solid in tetrahydrofuran, and recovering by filtration three times. The final solid was dried under reduced pressure. Aβ peptides were purchased from Bachem Ltd (Weil am Rhein, Germany). Stock solutions of Aβ_{42} and Aβ_{42arc} were initially prepared as follows. The peptide (1 mg) was dissolved in 1 mL of 10 mM sodium hydroxide, and the resulting solution placed for 5 min in an ultrasonic bath to remove pre-formed aggregates. The solution was then divided into aliquots and stored at -80°C until required for use. The concentration of the stock peptide solution was determined by quantitative amino acid analysis (Biochemistry Department, University of Cambridge). This stock solution was later used in preparing low molecular weight (LMW) Aβ_{42arc} and in preparing Aβ_{42} and Aβ_{42arc} fibrils (see below). α_B-Crystallin and GFP were expressed and purified according to previously published protocols (1, 2). UCH-L3 was kindly provided by Fredrik Andersson (Department of Chemistry, University of Cambridge).

Preparation of Aβ fibrils for pelleting assays and seeds

Aliquots of frozen Aβ stock solutions were thawed rapidly, and diluted with buffer A (50 mM sodium phosphate pH 7.4) to a final Aβ concentration of 30 μM. The solution was incubated without agitation for 18-96 h at 30°C until fibril formation (as assessed by measuring the β-sheet content in circular dichroism). Fibrils were then stored at room temperature until required for further study. Analytical SEC of supernatants collected after centrifugation of such solutions detected no LMW Aβ. The detection sensitivity (estimated using stocks of known Aβ concentrations) implied that the solutions must be at least 96 % fibrillar.

Preparation of low molecular weight (LMW) Aβ solutions for plate reader assays

LMW Aβ_{42} was prepared by dissolving in trifluoroacetic acid, and sonicating for 30 s on ice. The trifluoroacetic acid was removed by lyophilization, and the peptide was then dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol and divided into aliquots that were dried by rotary evaporation at room temperature. The amount of peptide in the individual aliquots was determined by quantitative amino acid analysis (Biochemistry Department, University of Cambridge). LMW Aβ_{42arc} peptides for the amyloid fibril elongation studies were prepared by diluting a 50 μL aliquot of stock solution directly into buffer A to an Aβ_{42arc} concentration of approximately 30 μM, and filtering through a 10 kDa filter (Centricon-Y10) by centrifugation for 30 min at 14,000 $\times$ g (4°C). The filtrate (LMW Aβ_{42arc}) was stored on ice until use, and its concentration determined by quantitative amino acid analysis (Biochemistry Department, University of Cambridge).

Binding of α_B-crystallin to Aβ fibrils by centrifugation and SDS-PAGE

α_B-Crystallin, UCH-L3 and GFP were incubated at a final concentration of 12 μM for 1 h at room temperature in buffer A, in the presence and absence of 12 μM Aβ_{42} fibrils, and then centrifuged at 16,000 $\times$ g for 30 min. The supernatant was carefully removed and analysed by SDS-PAGE (4-12% Bis-Tris, Invitrogen), together with equivalent α_B-crystallin supernatants (above).

To quantify the strength of the binding interaction, Aβ fibrils were diluted to a final concentration of 15 μM with α_B-crystallin in buffer A, to a range of different α_B-crystallin concentrations. The mixtures were incubated for 1 h at room temperature to facilitate binding, and then spun at 16,000 $\times$ g for 30 min. The supernatant was removed and analysed by fluorescence spectroscopy using a Cary Eclipse spectrophotometer (Santa Clara, USA) to calculate the concentration of unbound α_B-crystallin. α_B-Crystallin solutions were kept on ice until transfer into a 3 mm pathlength fluorescence quartz cuvette. The intrinsic tryptophan fluorescence was then determined by excitation at 280 nm (5 nm slit) and the emission was measured from 300 to 500 nm (5 nm slit) with 0.1 s averaging. The fluorescence intensity at 342 nm was used to indicate the α_B-crystallin concentration. The total concentration of α_B-crystallin was calculated from fluorescence measurements of a similarly prepared set of α_B-crystallin standards (of known concentration), without Aβ fibrils and not subjected to centrifugation. The concentration of α_B-crystallin bound to fibrils was identified as the difference between the α_B-crystallin concentration initially and in the supernatant following centrifugation. The dissociation constant (K_d) was estimated by direct non-linear regression according to Eq. 1:

$$L_B = \frac{S_T \times L_F}{K_d + L_F} \tag{1}$$

where L_B and L_F are the concentrations of bound and free α_B-crystallin respectively, S_T is the concentration of binding sites, and K_d is the dissociation constant of the complex. This approach provides better estimates of K_d and S_T than straight line fitting of conventional Scatchard plots.

In determining the effect of ionic strength upon the process, the procedure used was identical to that above, with initial concentrations of α_B-crystallin and Aβ_{42} fibrils of 15 μM, and the addition of NaCl to the incubation mixture (to final concentrations of 0 M, 0.5 M, 1.0 M and 1.5 M). Control experiments performed with α_B-crystallin but no Aβ_{42} fibrils displayed a small decrease (of around 3 %) in tryptophan fluorescence following the centrifugation, possibly reflecting a change in α_B-crystallin self-association behaviour. Consequently, the α_B-crystallin supernatant concentrations following centrifugation were compared (and are reported) relative to the concentrations found for solutions of the same ionic strength but without Aβ_{42} fibrils. Experiments were performed in triplicate, and errors were calculated by combining the errors (estimated from the standard deviation) in quadrature.

Immunoelectron microscopy

The fibril-chaperone complex for immuno-EM was obtained by the addition of α_B-crystallin to Aβ fibrils in buffer A, with final concentrations of 3.5 μM and 15 μM respectively. The mixture was incubated for 1 h at room temperature and then spun at 16,000 $\times$ g for 30 min. The supernatant was carefully aspirated to remove free α_B-crystallin and the pellet resuspended in one volume of buffer A, prior to dilution for immunogold labelling as required. Charged carbon-coated nickel electron microscopy grids (400 mesh; Agar Scientific, Stansted, UK) were prepared for this study by the addition of 4 μL of a 100-fold dilution of the fibril-chaperone complex in buffer A (final concentrations 150 nM Aβ fibrils and 35 nM α_B-crystallin). Samples were deposited onto grids immediately following dilution. Grids were thrice washed with 10 μL H_2O, blocked with 15 μL 0.1% (w/v) bovine serum albumin (BSA) in buffer B (10 mM sodium phosphate, 100 mM sodium chloride pH 7.4) for 15 min, then incubated for 30 min with the α_B-crystallin monoclonal primary

antibody (100 μg/ml, Assay Designs, Michigan, USA) diluted 250-fold in 0.1% BSA in buffer B. The grids were blotted with filter paper between each washing step. Next, the grids were thrice washed with 50 μL 0.1% BSA in buffer B, for 4 min each time, before a 30 min incubation at room temperature with goat anti-mouse 10 nm immunogold conjugate (GMHL10, BBInternational, Cardiff, UK), diluted 150-fold into 0.1% BSA in buffer B. The grids were then thrice washed with 50 μL 0.1% BSA in buffer B, again for 4 min each time, then thrice with 50 μL H_2O and negatively stained with 20 μL of uranyl acetate (2% (w/v) in H_2O); Agar Scientific, Stansted, UK). Samples were viewed under 20-80k magnifications at 80 kV excitation voltages using a Philips CM100 transmission electron microscope.

Effect of α_B-crystallin on seeded growth of Aβ fibrils

ThT fluorescence measurements were performed in black 96-well half-area plates (Corning) using a FLUOstar fluorescence platereader (BMG Labtech, Offenburg, Germany). To investigate the effect of α_B-crystallin on seeded growth in solution, fibril solutions were pre-incubated with α_B-crystallin (at a molar ratio of 2:1) for 1 h prior to the elongation experiment. This ratio of Aβ:α_B-crystallin was demonstrated to result in near maximal fibril coverage by α_B-crystallin (see Fig. 2). As a control, the same concentrations of all components were also investigated without pre-incubation. LMW Aβ solutions were kept on ice until required. The final concentrations of LMW Aβ_{42} and Aβ_{42arc} were 9.7 μM and 1.8 μM respectively. Where present the concentrations of ThT, α_B-crystallin and Aβ fibril seeds were 18.5 μM, 0.74 μM and 0.38 μM respectively. The concentration of LMW Aβ_{42arc} used is lower than that for LMW Aβ_{42}, because the former is extremely aggregation prone, and lower concentrations were required to observe a clear difference between the aggregation rates of seeded and unseeded solutions. Three replicates were performed for each condition, and the average gradient of the linear fits to the initial 1000 s (for Aβ_{42}) or 200 s (for Aβ_{42arc}) was calculated to report on the initial rate of fibril elongation.

Quartz crystal microbalance measurements of fibril elongation

We probed the inhibitory effect of α_B-crystallin on the Aβ_{42} amyloid fibril elongation rate using an E4 quartz crystal microbalance (QCM)(Q-Sense, Västra Frölunda, Sweden) and QSX 301 gold-coated quartz crystals. The functionalization with Aβ_{42} was performed as described in detail previously (3). Briefly, Aβ_{42} seed fibrils were coupled to a self-assembled monolayer of mercaptoundecanoic acid (MUA) using standard amine coupling. The remaining activated MUA was passivated with a solution of ethanolamine, after which the QCM crystal was rinsed with water and inserted into the flow cell. After a stable baseline was established, the surface-bound seed fibrils were incubated with a solution of 1.67 μM soluble Aβ_{42} in buffer C (100 mM sodium phosphate, pH 7.4). The elongation of the seed fibrils led to a decrease in the resonant frequency (see Fig. 4), indicating elongation of the surface-bound fibrils. To stop the growth, the liquid cell was rinsed with buffer C. Subsequently, the sensor surface was exposed to a solution of 2.5 μM α_B-crystallin and then, after again flushing with buffer C, with the same Aβ_{42} solution as before. Atomic force microscopy images of the QCM sensors were acquired of the functionalized sensors using a Molecular Imaging Pico Plus AFM (Tempe, AZ), using tapping mode in air.

References

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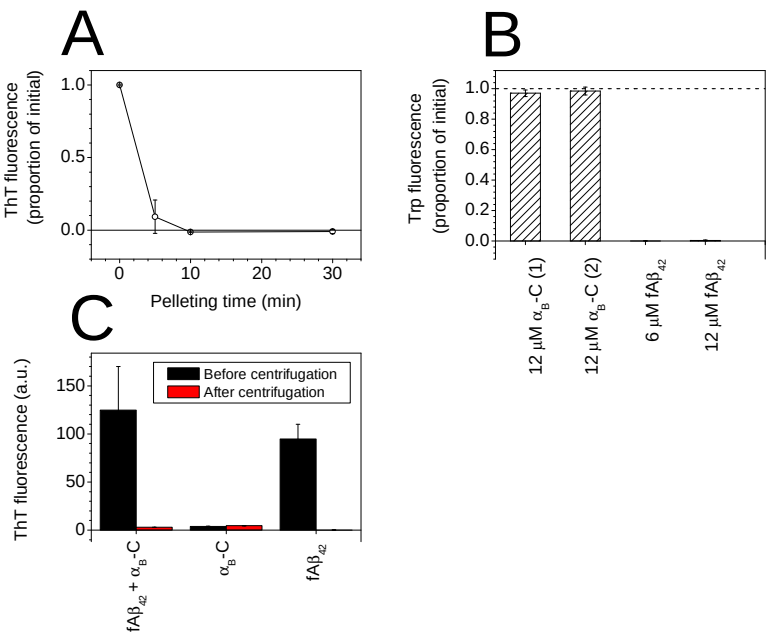

Figure S1.

Centrifugation for 30 min at 16,000 × g is sufficient to pellet Aβ fibrils, but not soluble α_B-crystallin. All error bars represent the standard deviation of triplicate measurements. (A) The concentration of Aβ_{40} fibrils remaining in the supernatant was monitored as a function of centrifugation time through the use of ThT fluorescence measurements. A 30 μM solution of Aβ_{40} fibrils in buffer A was centrifuged at 16,000 × g, and 3 μl aliquots removed at various timepoints. The aliquots were mixed with 20 μM ThT in buffer A and the fluorescence intensity recorded immediately. ThT fluorescence intensity is reported relative to the ThT fluorescence prior to centrifugation. (B) Solutions of 12 μM α_B-crystallin alone (1 and 2 represent independent replicates) and 6 and 12 μM Aβ_{42} fibrils alone were prepared by dilution of stock solutions with buffer A and centrifuged at 16,000 × g for 30 min. The tryptophan fluorescence of the supernatant is reported as a proportion of the intrinsic tryptophan fluorescence of a freshly prepared 12 μM α_B-crystallin solution. Concentrations of soluble α_B-crystallin in the supernatant were the same within error as those prior to centrifugation. Centrifuged Aβ_{42} fibrils did not display significant tryptophan fluorescence. (C) Aβ_{42} fibrils still sediment under the applied centrifugation conditions in the presence of α_B-crystallin. Solutions containing 15 μM Aβ_{42} fibrils, 15 μM α_B-crystallin, and 15 μM Aβ_{42} fibrils with 15 μM α_B-crystallin were incubated at room temperature for one hour, and 2 μl aliquots removed for ThT fluorescence measurements before, and after, centrifugation for 30 min at 16,000 × g. The aliquots were mixed with 20 μM ThT in buffer A and the fluorescence intensity recorded immediately. After centrifugation of the mixture, a small but non-zero fluorescence signal remained. However this was similar to the signal found in the absence of Aβ_{42} fibrils, and therefore reflects ThT binding to alphaCB rather than incomplete pelleting of Aβ_{42} fibrils.

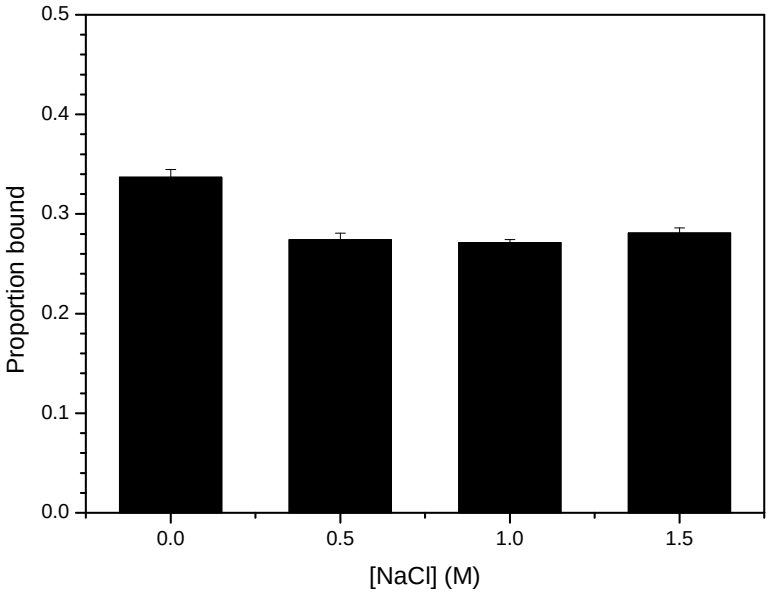

Figure S2.

Ionic strength dependence of the cosedimentation behaviour of α_B-crystallin and Aβ_{42} fibrils. Solutions containing 15 μM Aβ_{42} fibrils with 15 μM α_B-crystallin and NaCl (0 M, 0.5 M, 1.0 M and 1.5 M) in buffer A were incubated at room temperature for one hour, and the supernatant analysed for α_B-crystallin concentration after centrifugation for 30 min at 16,000 $\times$ g. By comparing the α_B-crystallin concentration obtained with that of equivalent solutions without Aβ_{42} fibrils, the proportion of α_B-crystallin bound to the fibrils was calculated. Despite an initial small decrease in amount bound, which could reflect an electrostatic contribution to the binding process, a complex between α_B-crystallin and Aβ_{42} fibrils remained even at 1.5 M NaCl.

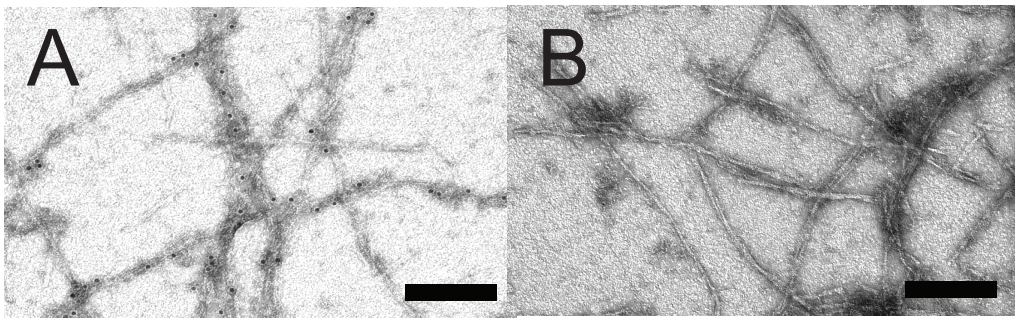

Figure S3.

Immunoelectron microscopy of Aβ_{40} fibrils in (A) the presence, and (B) the absence of α_B-crystallin. Scale bars represent 200 nm. Aβ fibrils were prepared and incubated in the presence or absence of an equal molar concentration of α_B-crystallin for 1 h at room temperature. The mixtures were centrifuged at 16,000 × g for 30 min, and the pellets resuspended and treated as described in the Methods Section.

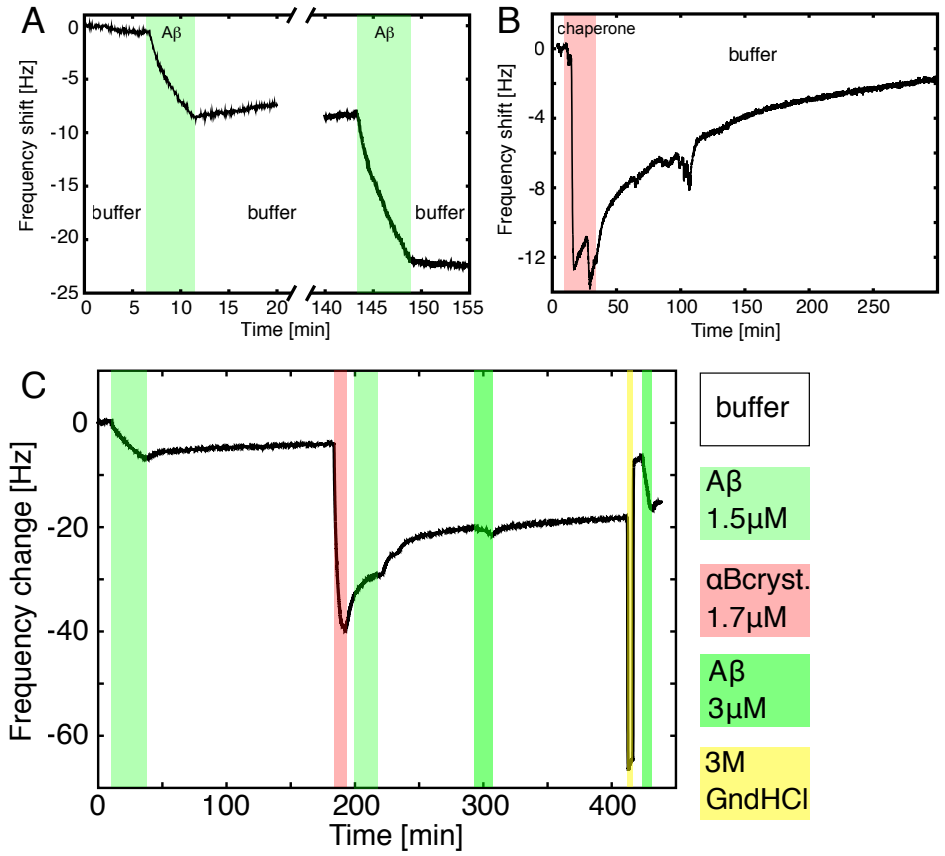

Figure S4.

Supplementary QCM experiments. (A) Continued elongation ability of seed fibrils. Following an intervening buffer wash, and without intermittent contact with chaperone, previously elongation competent seed fibrils may be further elongated by renewed contact with LMW Aβ_{42}. (B) Extent of non-specific interaction of α_B-crystallin with a passivated control surface without bound Aβ_{42} fibrils. Some minor non-specific binding occurs, but the signal is significantly smaller than in the presence of fibrils. In addition, the majority of the induced frequency shift is reversible when the surface is flushed for a prolonged period with buffer. (C) Reversibility of chaperone-binding related fibril elongation inhibition demonstrated through a sequence of incubations. Initially, incubation of surface-bound seed fibrils with LMW Aβ_{42} leads to an increase in mass, demonstrating their elongation competence. Following an incubation with α_B-crystallin, the seed fibrils are less able to extend, even at a higher concentration of LMW Aβ_{42}. Subsequent incubation with 3 M GdnHCl results in a mass decrease as α_B-crystallin is removed from the surface (Aβ_{42} fibrils do not dissociate under these conditions, data not shown), and a final incubation with LMW Aβ_{42} demonstrates a restoration in elongation competence.