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Non-chaperone Proteins Can Inhibit Aggregation and Cytotoxicity of Alzheimer Amyloid β Peptide

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Background: A β amyloid formation is associated with Alzheimer disease.

Results: Non-chaperone proteins prevent amyloid formation and reduce the cytotoxicity of the A β peptide.

Conclusion: Non-chaperone proteins may affect the onset and development of Alzheimer disease by interfering with A β peptide aggregation.

Significance: Non-chaperone proteins can function as a chaperone protein to regulate the pathway of the A β fibrillation in proteostasis providing a new strategy in the treatment of Alzheimer disease.

Many factors are known to influence the oligomerization, fibrillation, and amyloid formation of the A β peptide that is associated with Alzheimer disease. Other proteins that are present when A β peptides deposit *in vivo* are likely to have an effect on these aggregation processes. To separate specific *versus* broad spectrum effects of proteins on A β aggregation, we tested a series of proteins not reported to have chaperone activity: catalase, pyruvate kinase, albumin, lysozyme, α -lactalbumin, and β -lactoglobulin. All tested proteins suppressed the fibrillation of Alzheimer A β (1–40) peptide at substoichiometric ratios, albeit some more effectively than others. All proteins bound non-specifically to A β , stabilized its random coils, and reduced its cytotoxicity. Surprisingly, pyruvate kinase and catalase were at least as effective as known chaperones in inhibiting A β aggregation. We propose general mechanisms for the broad-spectrum inhibition A β fibrillation by proteins. The mechanisms we discuss are significant for prognostics and perhaps even for prevention and treatment of Alzheimer disease.

The correlation between Alzheimer disease (AD)² and the occurrence of extracellular amyloid β (A β) peptide plaques (oligomers/fibrils) in brain has been well established since the pioneering work of Glenner and Wong in 1984 (1). A β oligomers that precede the formation of fibrillar oligomers (FOs), fibrils, and amyloid have been shown to be the most toxic form of A β . These prefibrillar oligomers (PFOs) are now considered to be a prime link between cognitive impairment/neurodegeneration and A β plaques (2). Although the A β fibrils have been recognized as a less toxic structural variant of A β , these fibrils may drive the formation of oligomers by secondary nucleation (3).

Several small molecules are known to interfere with oligomerization and fibrillation of A β , for instance meclocycline sul-

fosalicylate, hemin, and hematin (4). Also, certain specifically engineered cyclic peptides have similar inhibitory effects on fibrillation (5–7). However, these organic compounds are potentially toxic. Even more effective was an engineered affibody that ceased the aggregation of A β (8) and stabilized the structure of the A β oligomers (9). Also, chaperone proteins, which are the body's own defense against protein misfolding, have been recently found to interfere with A β aggregation. α B-crystallin (10), clusterin (11), and heat shock protein (Hsp)B8 (12) have all been associated with pathological lesions of AD. They also inhibit fibrillation and reduce cytotoxicity of A β *in vitro* (13).

Amyloid aggregates can sequester other proteins, which may disrupt essential cellular functions (14). As a promiscuous binder of (disordered) proteins, A β in its aggregated state may, therefore, also function as a molecular hub in protein interaction networks (15). For instance, A β binds the enzyme catalase with high affinity and inhibits its hydrogen peroxide breakdown (16). Hence catalase is associated with senile plaques. A β also inhibits key enzymes of mitochondrial respiration (17). These results indicate that besides chaperone proteins, other proteins interact with A β and may, therefore, interfere with its fibrillation. Indeed, *in vitro* studies revealed that albumin, membrane-related Brichos domains, and lysozyme efficiently delay the fibrillation of A β (18–20). It is unknown to what extent these aggregation-inhibiting activities are specific functionalities of these proteins or whether there is a more generic, broad-spectrum mechanism at work.

Here we report that proteins not known for chaperone activity can inhibit A β (1–40) aggregation with surprisingly high efficiency. Catalase and pyruvate kinase completely suppressed A β fibrillation at a molar ratio of 1:100 (protein:A β). But also albumin was effective, albeit at a lower molecular ratio of 1:10 (protein:A β). Even the whey proteins β -lactoglobulin and α -lactalbumin inhibited A β aggregation (but at a 1:1 molar ratio). As these whey proteins are not present in brain, they cannot have a biological function in inhibiting A β aggregation. In the presence of all tested proteins, the A β peptides retained their secondary random coiled structure. In an earlier, preliminary study, we already reported that lysozyme inhibits fibrilla-

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² The abbreviations used are: AD, Alzheimer disease; A β , amyloid β ; FO, fibrillar oligomer; PFO, prefibrillar oligomers; HSQC, heteronuclear single quantum correlation; ThT, thioflavin T; ANS, 8-anilino-1-naphthalenesulfonic acid.

tion at a 1:1 molar ratio (18). The tested proteins also suppressed the toxicity of A β aggregates. HSQC NMR spectroscopy indicated that the interactions between the tested proteins and monomeric A β peptide were nonspecific. These results prompt us to propose common, broad-spectrum, protein-based inhibition mechanisms of A β aggregation.

EXPERIMENTAL PROCEDURES

Materials—Human lysozyme (catalog no. L1667), human serum albumin (catalog no. A3782), α -lactalbumin from bovine milk (catalog no. L6010), catalase from bovine liver (catalog no. C9327), and β -lactoglobulin from bovine milk (catalog no. L3908) were purchased from Sigma. Pyruvate kinase (catalog no. 10836821) from rabbit muscle was bought from Roche Diagnostics. The A β (1–40) peptides (either unlabeled or ^{15}N -labeled) were purchased from AlexoTech AB (Umeå, Sweden) and prepared according to previously described protocols; the A β peptides were dissolved in 10 mM NaOH to a peptide concentration of 2 mg/ml and then sonicated for 1 min in an ice bath before dilution in the appropriate buffer. The preparations were kept on ice.

ThT Fluorescence Assay—A 10 mM ThT stock solution was prepared in distilled H₂O. The proteins were added in the desired amounts to aliquots of this solution. Then freshly prepared A β (1–40) peptide was added, yielding final samples containing 5 μM ThT, 10 μM A β (1–40), 50 mM Tris buffer (pH 7.4), and protein in the desired concentrations. All buffers and samples were prepared on ice to avoid the aggregation. The samples were pipetted into a well plate with 384 wells holding 45 μl each. Fluorescence measurements were recorded with an Infinite M1000 PRO microplate reader every 15 min using excitation and emission wavelengths of 446 and 490 nm, respectively. The plate was thermostated at +37 °C, and the wells were automatically shaken 30 s before each measurement.

Each sample was prepared in duplicate, and average fluorescence signals were calculated after subtracting the base-line fluorescence of control samples without A β (1–40) peptide. The fluorescence intensity (I) data were fitted to a sigmoid curve with a sloping base line using the equation, $I(t) = k_1 t + A/(1 + \exp(-k_2(t - t_{1/2})))$, where the parameter k_1 describes the sloping base line, A is the amplitude, k_2 is the elongation rate constant, and $t_{1/2}$ is the time of half-completion of the aggregation process (21).

For the disaggregation assay, a stock of A β (1–40) peptide aggregates was obtained by incubating 50 μM A β (1–40) peptide in 50 mM Tris (pH 7.4) at 200 rpm for 18 h at 30 °C. Then, 5 μM aggregates were diluted and mixed with the test proteins and ThT for the fluorescence assay. The protocol and buffer conditions were the same as described above. Fluorescence was measured after 0, 5, 10, and 15 h. For the fibrillation control assay, we prepared 10 μM A β and 10 μM ThT in Tris buffer (pH 7.4) in the presence and absence of non-chaperone proteins and then incubated them in Eppendorf tubes in a 37 °C shaker at 200 rpm.

CD Spectroscopy—A Chirascan CD unit from Applied Photophysics was used to monitor the kinetics of a 10 μM solution of A β (1–40) peptide dissolved in 5 mM sodium phosphate buffer at pH 7.3 in the presence and absence of test proteins. CD

spectra at 30-min intervals were recorded between 190 and 260 nm using a step size of 2 nm and a slit size providing 1-nm resolution. The sample was thermostated at 37 °C in a 10-mm path length quartz cuvette (lot: 119.004-QS, Hellma Analytics) and mechanical rotary stirring (22 rpm) with an 0.7-cm magnet was used to speed up aggregation.

Cell Viability Assay—Neuroblastoma SH-SY5Y cells were used with a maximum passage number of 15. Cells were cultured in Dulbecco's modified Eagle's medium, a 1:1 mixture of DMEM and Ham's F-12 medium, and 10% supplemental fetal bovine serum containing 1% (v/v) penicillin/streptomycin at 37 °C, 5% CO₂ in a 75-cm² flask (Greiner Bio-one, catalog no. 658170). To avoid using trypsin, cells were detached by 5 mM EDTA, PBS for 5 min in 37 °C. Then cells were resuspended at a concentration of 200,000 cells/ml in DMEM/F-12 containing 1% (v/v) penicillin/streptomycin. The resuspended cells were plated at a volume of 50 μl and a cell density of 20,000 cells/well in a 96-well plate. The plated cells were incubated for 48 h at 37 °C at 5% CO₂. A β 40 oligomer-enriched fractions were prepared at a concentration of 100 μM in the presence of the test proteins at 25 °C for 100 min in PBS with 1 mM EDTA. The A β oligomer solutions were diluted to final concentrations of 30 μM in wells in the presence of the different proteins. As a control, the PBS-dissolved test proteins in 50 μl of medium were added to control wells and incubated for 48 h. After 48 h the plate was equilibrated at room temperature for ~30 min. Cell-Titer-Glo[®] Luminescent Cell Viability Assay (Promega, catalog no. G7571) compound was added to each well, and then the contents in the plate were mixed using an orbital shaker for 2 min to induce cell lysis (22). Luminescent intensity was measured (1000-ms integration time) with the Infinite M1000 PRO 384-well microplate reader. Measurements from three independent experiments were analyzed statistically to calculate average values and S.D.

ANS Fluorescence Assay—A β oligomers were prepared in a similar fashion as for the cell viability assay. 100 μM A β (1–40) peptide was incubated with PBS at room temperature for 2 h in an Eppendorf tube. Aliquots of 20 μl oligomeric A β and 0.4 μl of proteins (500 μM in PBS) were pipetted into a 384-well plate (OptiPlate 384, PerkinElmer Life Sciences), and then 1 μl of 50 mM ANS was added. ANS fluorescence spectra between 400 and 600 nm were recorded with an Infinite M1000 PRO microplate reader (excitation at 350 nm). ANS fluorescence intensity of A β oligomers was calculated after subtracting the intensity of non-chaperone proteins or buffers, which were handled similarly.

NMR Spectroscopy—A Bruker Avance 500-MHz spectrometer was used to record ^1H , ^{15}N HSQC spectra at +5 °C of 100 μM ^{15}N -labeled A β (1–40) peptide in 20 mM sodium phosphate buffer at pH 7.3 (90/10 H₂O/D₂O) both in the absence and presence of the different proteins. The spectrometer was equipped with a triple-resonance cryogenically cooled probe head, and the spectra were referenced to the water signal. All NMR measurements were done at +5 °C to slow down the aggregation process. The assignment of the amide peaks for the A β (1–40) peptide is known from previous work.

Bioinformatics—The molecular weight, theoretical PI, and amyloidogenic regions of proteins were calculated using the

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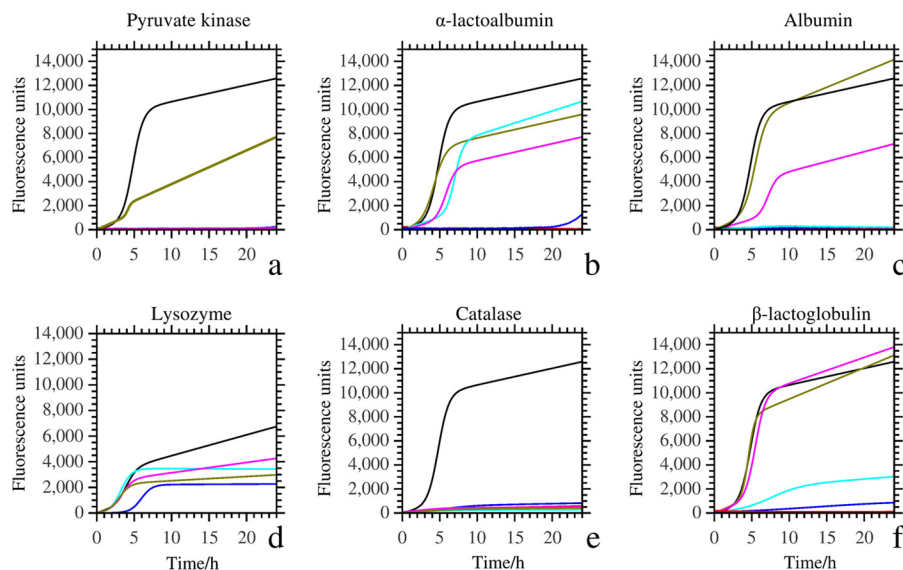


FIGURE 1. ThT aggregation kinetics of 10 μ M A β peptide in the presence of the test proteins pyruvate kinase (a), α -lactalbumin (b), albumin (c), lysozyme (d), catalase (e), and β -lactoglobulin (f). For pyruvate kinase (panel a), which was most active in preventing fibrillation, we tested five different concentrations: 0 nM (black), 25 nM (olive), 100 nM (magenta), 500 nM (cyan), and 1000 nM (blue). For the other test proteins we used five slightly higher concentrations: 0 nM (black), 100 nM (olive), 500 nM (magenta), 1 μ M (cyan), and 10 μ M (blue). Red curves (all coinciding with the horizontal axis) are controls in which no A β was added to 20 μ M test proteins. The curves show the average of three independent experiments.

TABLE 1

Lag time and transition time for amyloid formation kinetics of 10 μ M A β (1–40) peptides in the presence of 0, 0.1 μ M, 0.5 μ M, and 1 μ M proteins, measured in 50 mM Tris buffer at pH 7.4 and +37 $^{\circ}$ C

	Control	Pyruvate kinase	α -Lactalbumin	Albumin	β -Lactoglobulin
t_{lag}/h					
0.1 μ M	3.5	3.7	2.7	4.0	3.7
0.5 μ M			4.5	6.0	4.1
1 μ M			5.9		3.7
t_{trans}/h					
0.1 μ M	2.8	0.9	3.1	2.9	1.7
0.5 μ M			2.6	2.4	2.7
1 μ M			2.3		8.0

ExPASy server and Waltz software (23), respectively. Total solvent-excluded surface area and total solvent-accessible surface area were calculated using the Chimera software suite (24).

RESULTS

All Test Proteins Suppress A β Peptide Fibrillation—We monitored the effect of various concentrations of catalase, pyruvate kinase, albumin, lysozyme, α -lactalbumin, and β -lactoglobulin on the fibrillation of 10 μ M A β using a ThT assay (Fig. 1, Table 1). All test proteins inhibited A β fibrillation. The fluorescence increase characteristic of A β fibrillation significantly dropped in the presence of 25 nM pyruvate kinase and did not occur at all in 100 nM pyruvate kinase (Fig. 1a). Fibrillation of A β was hardly observed in 100 nM catalase (Fig. 1e).

Although α -lactalbumin, albumin, and β -lactoglobulin delayed the fibrillation of A β at low concentrations, their inhibition efficiencies were weaker compared with catalase and pyruvate kinase. Albumin and α -lactalbumin completely prevented A β fibrillation >24 h at 1 and 10 μ M, respectively. And β -lactoglobulin decreased fibrillation of A β at concentrations >1 μ M. Low concentrations of α -lactalbumin somewhat enhanced nucleation (as witnessed by a reduction in the lag time), but at higher concentrations α -lactalbumin also delayed fibrillation. We included the results we obtained earlier with

lysozyme (using a different batch of A β) in Fig. 1d (17). As a control we ascertained that ThT fluorescence was not affected by the test proteins in the absence of A β (red curves in Fig. 1).

We investigated whether the test proteins could redissolve A β -aggregates by incubating them with preformed A β fibrils. Only catalase reduced the fluorescence intensity of A β (Fig. 2), suggesting that catalase interacts with A β fibrils and reverses the amyloid fibrillation. In contrast, pyruvate kinase and α -lactalbumin slightly increased the fluorescence intensity of preprepared A β fibrils. This might indicate that these proteins could also condense and (partially) unfold onto A β fibrils. Alternatively, the A β fibrils might nucleate the fibrillation of these test proteins. Neither lysozyme, albumin, nor β -lactoglobulin affected the fluorescence intensity of preformed A β fibrils. These results also indicate that the reduction in ThT signal observed when the non-chaperone proteins were incubated with soluble A β was not due to interference of these proteins (either through quenching or absorption) with the ThT assay. Catalase could even redissolve preformed fibrils, indicating that its protective effect cannot solely be explained by interference with nucleation events.

We tested if the delayed nucleation could be caused by A β preferentially nucleating on surfaces (air-liquid interface or the

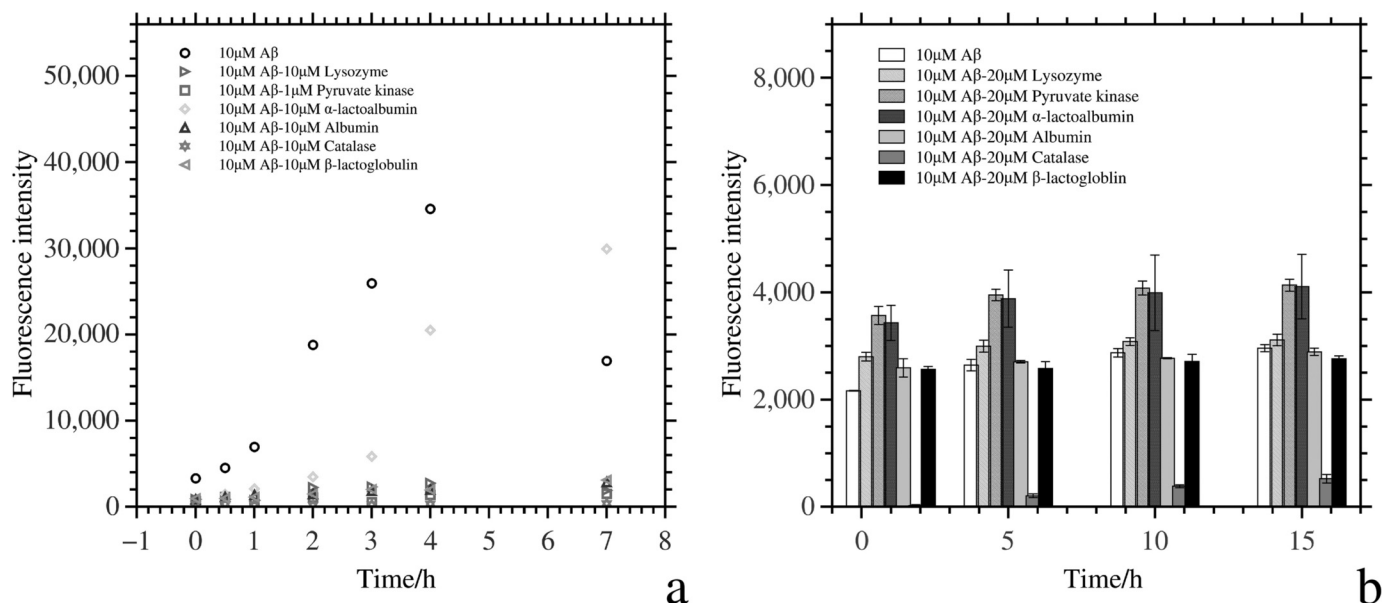


FIGURE 2. *a*, ThT aggregation kinetics of 10 μ M A β peptide in the presence of the test proteins pyruvate kinase, α -lactalbumin, albumin, lysozyme, catalase, and β -lactoglobulin incubated in an Eppendorf tube at 37 $^{\circ}$ C placed in a shaker at 200 rpm. The reaction vessel and the incubation conditions were different from those in Fig. 1, yet the results were very similar, suggesting that surface effects could not explain our results. *b*, ThT disaggregation assay of A β peptide in the presence of α -helical proteins. 50 μ M A β was incubated for 18 h at 30 $^{\circ}$ C in a 200 rpm shaker (50 mM Tris buffer (pH 7.4)), and then 5 μ M aggregates (after diluting the 50 μ M stock in 50 mM Tris buffer (pH 7.4)) were incubated with different test proteins at 37 $^{\circ}$ C. The protocol for ThT disaggregation assay is same as for the ThT aggregation assay.

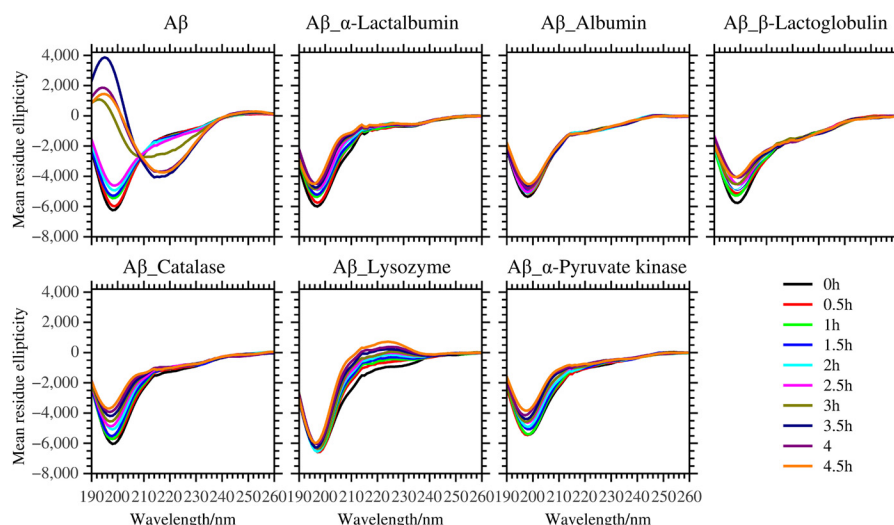


FIGURE 3. The secondary structure transition of 10 μ M A β in the absence and presence of the test proteins: 0.5 μ M α -lactalbumin, 1.25 μ M albumin, 1 μ M β -lactoglobulin, 0.25 μ M catalase, 1 μ M lysozyme, and 0.05 μ M pyruvate kinase by CD spectroscopy. In the assay, we recorded the kinetics of structural transition in the time interval of 0.5 h, black (0 h), red (0.5 h), green (1 h), blue (1.5 h), cyan (2 h), magenta (2.5 h), dark yellow (3 h), navy (3.5 h), purple (4 h), and orange (4.5 h).

surface of the reaction vessel), the nature of which could be affected by the non-chaperone proteins, for instance by coating. To exclude such surface effects, we also incubated A β peptides with and without proteins in an Eppendorf tube in a 37 $^{\circ}$ C, 200 rpm shaker, which has a different geometry, air-liquid interface, and chemical composition compared with the 384-well plates. All of the proteins could also prevent or delay the fibrillation of A β in these different incubation conditions (Fig. 2*a*). The kinetics of A β fibrillation in Eppendorf tubes did differ slightly from fibrillation in well plates. This might be caused by differences in the composition (25) or geometry of the reaction vessels (26). However, the effects were relatively minor. The marked drop in ThT fluorescence observed after 7 h of incubation when no

extra proteins were added to the A β peptide was caused by precipitation of the fibrils as mature amyloid.

The Test Proteins Stabilized the Random Coil Structure of the Monomeric A β Peptide—We investigated the effect of the test proteins on the structural transition of A β by CD spectroscopy. The mean residue ellipticity of A β was obtained after subtracting the signal of the test protein from the measured spectra (Fig. 3). As expected, A β in the absence of the test proteins converted its secondary structure from random coil to β -sheet in 3 h (Fig. 3). However, in the presence of substoichiometric concentrations of test protein, the A β peptide retained its random coil secondary structure (Fig. 3). We observed subtle differences in secondary structure changes of A β in the presence of the

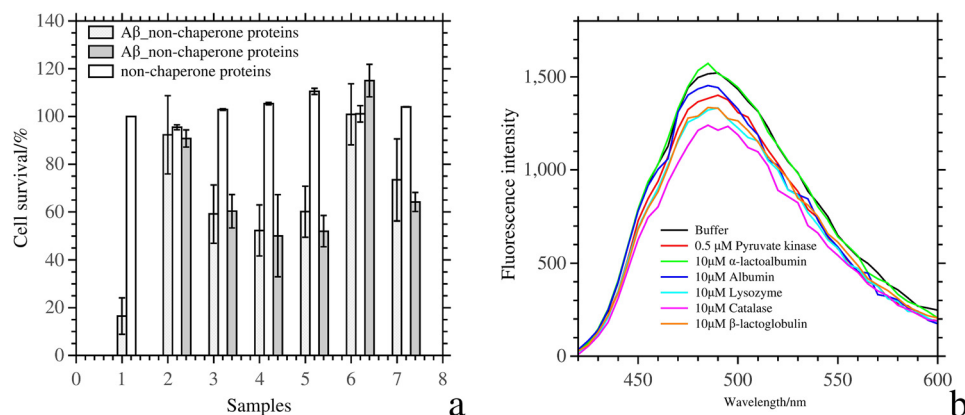


FIGURE 4. *a*, cell survival after incubation with A β aggregates that had formed in the presence and absence of the test proteins. In *sample 1*, the cell survival of 30 μ M A β aggregates that had formed in the absence of test proteins is shown as *light gray columns*, and the cell survival of the control is in *white*. *Samples 2, 3, 4, 5, 6, and 7* are in the presence of pyruvate kinase, α -lactalbumin, albumin, lysozyme, catalase, and β -lactoglobulin, respectively. The *white column* represents cell survival of 1 μ M protein without A β aggregates in *sample 2* and 2.5 μ M protein in the *samples 3–7*. The *dark gray columns* are cell survival after incubation with 1 μ M protein and 30 μ M A β aggregates in *sample 2* and 3 μ M test protein and 30 μ M A β aggregates in *samples 3–7*. The *light gray columns* are cell survival in the presence of 0.2 μ M test protein and 30 μ M A β aggregates in *sample 2* and 0.6 μ M test protein and 30 μ M A β in *samples 3–7*. *b*, ANS fluorescence intensity of 100 μ M A β oligomers with and without proteins. A β oligomers were obtained after a 2-h incubation at room temperature in an Eppendorf tube.

different proteins. The CD amplitudes at 200 nm increased slightly during incubation with albumin, α -albumin, β -lactoglobulin, catalase, and pyruvate kinase. In the presence of lysozyme, there was a CD up-shift at 220 nm during the incubation (Fig. 3).

The changes in lag and transition times are all correlated with the concentration of the non-chaperone proteins in a unimodal fashion (Table 1), but the correlation between the maximal fluorescence can be biphasic. See for instance Fig. 1, *panel b*, where the maximum fluorescence at 100 nm α -lactalbumin is lower than at 500 nm. This is consistent with our disaggregation experiment (see above and Fig. 2) and could indicate that α -lactalbumin could also condense and (partially) unfold onto A β fibrils.

All Test Proteins Reduce Cytotoxicity of A β Aggregates—The effect of the test proteins on the cytotoxicity of the aggregates of A β was measured in a cell assay (Fig. 4). We first incubated 100 μ M A β with the proteins at various concentrations for 2 h and added the aggregates to neuronal SH-SY5Y cells up to a concentration of 30 μ M. After 2 days of incubation with A β aggregates, 20% of the cells survived, but survival significantly increased when A β had been allowed to aggregate in the presence of the test proteins. Cell survival was $\sim$ 100% in the aggregates of A β and pyruvate kinase/catalase, which was much higher than the cell survival rate after incubation with the aggregates in the presence of A β and α -lactalbumin/albumin/lysozyme/ β -lactoglobulin. But even for the latter test proteins, a positive effect on cell survival was observed.

The Hydrophobic Nature of the Interaction between A β and the Test Proteins—We performed ANS fluorescence assays to examine if the hydrophobic surface of A β oligomers changes after adding the non-chaperone proteins. When A β and protein were both present, ANS fluorescence was reduced compared with identical concentrations of A β protein, except for α -lactalbumin (Fig. 4*b*). The shielding of hydrophobic surfaces that this assay indicates strongly suggests that the interaction between A β and the non-chaperone proteins at the very least had a hydrophobic contribution. Our hydrophobic interaction

assay correlated with our toxicity assay: higher ANS fluorescence intensities went with higher cytotoxicity (27). The fluorescence assay did not allow us to ascertain whether the proteins interacted with monomeric A β , with A β oligomers, or with both.

The Test Proteins Bind Monomeric A β in a Nonspecific Fashion—To explore the molecular interaction between monomeric A β and proteins, we used NMR ^1H , ^{15}N HSQC spectroscopy (Fig. 5). Interactions between larger aggregates of A β and the test proteins would not be visible in this assay. The results from NMR spectroscopy allow us to identify the chemical shifts of each detectable residue after the addition of the test proteins. The amide chemical shifts of A β moved slightly after the addition of α -lactalbumin (Fig. 5*a*) accompanied with a decreased of the cross-peak intensity (Fig. 6*a*). Although no significant amide chemical shift of A β was observed after the addition of albumin or lysozyme (Fig. 5, *b* and *c*), the cross-peak intensities of A β increased significantly (Fig. 6, *b* and *c*). Upon the addition of β -lactoglobulin or catalase, there were no noticeable changes in chemical shifts (Fig. 5, *d* and *e*), and the cross-peak intensity of most A β residues was reduced (Fig. 6, *d* and *e*). Lysozyme and pyruvate kinase increased the cross-peak intensity of A β by a factor of 2 (Fig. 6, *c* and *f*), but the chemical shifts did not change (Fig. 5, *c* and *f*). These results suggest that the protein interacted with monomeric A β in a nonspecific fashion and that the interaction was in fast exchange, at least for those proteins where the cross-peak intensities increased (albumin, lysozyme, pyruvate kinase).

DISCUSSION

Our ThT and CD data indicate that the non-chaperone proteins that we tested suppressed A β fibrillation by stabilizing the random coil structure of A β . Pyruvate kinase and catalase were the most efficient inhibitors of A β fibrillation, suppressing A β fibrillation effectively at a molar ratio of 1:100. Catalase was such an effective inhibitor that it could even reverse A β fibrillation. ANS fluorescence indicated that the test proteins reduced the hydrophobicity of A β (-oligomers), suggesting a

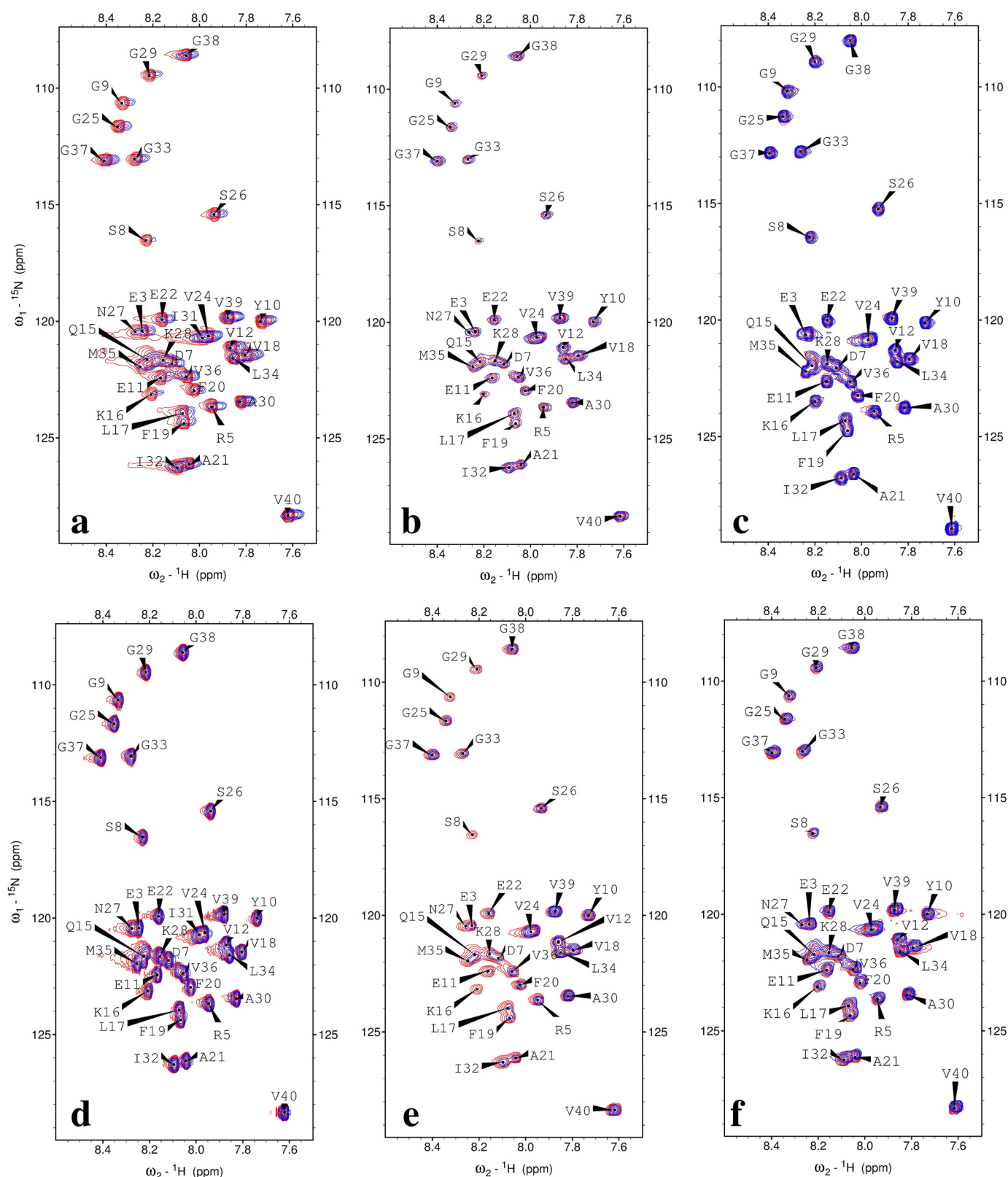


FIGURE 5. NMR ^1H , ^{15}N HSQC spectra of $90\ \mu\text{M}$ ^{15}N -labeled A β (1–40) peptides in 20 mM sodium phosphate buffer at pH 7.3, +5 $^{\circ}\text{C}$, before (red) and after (blue) the addition of $18\ \mu\text{M}$ α -lactalbumin (a), $5.4\ \mu\text{M}$ albumin (b), $200\ \mu\text{M}$ lysozyme (c), $50\ \mu\text{M}$ β -lactoglobulin (d), $9\ \mu\text{M}$ catalase (e), and $4.5\ \mu\text{M}$ pyruvate kinase (f).

(partially) hydrophobic interaction between A β and the proteins. All test proteins reduced the cytotoxicity of A β oligomeric aggregates. This suggests that they either prevented the

formation of PFOs (the most toxic oligomeric state of A β) or interfered with the toxic pathways, for instance by binding and neutralizing the PFOs. Again, pyruvate kinase and catalase had

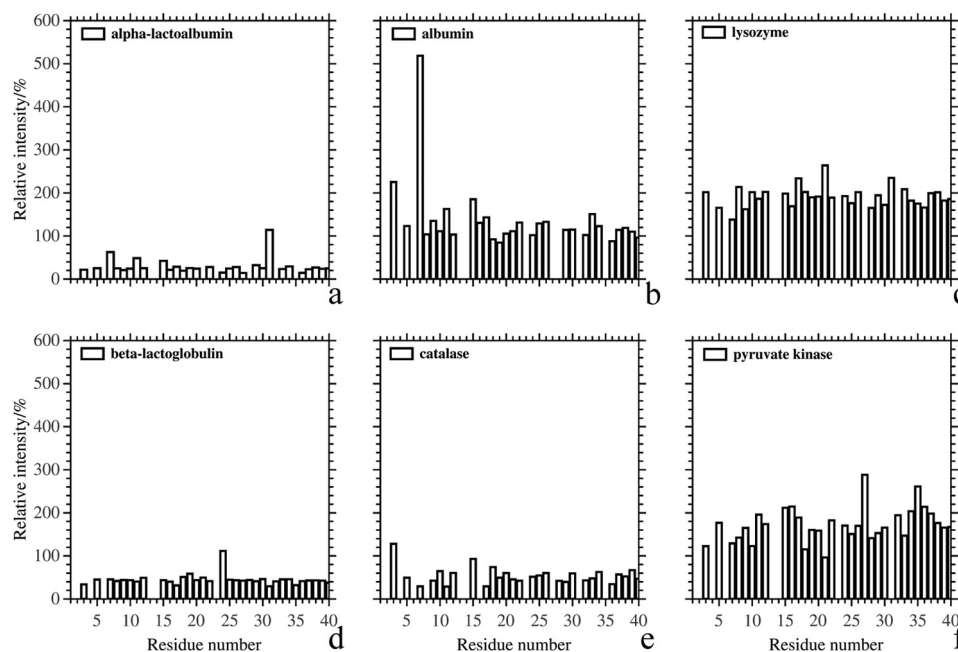


FIGURE 6. The relative change in intensity of the HSQC-NMR cross-peaks induced by the addition of the test proteins was calculated using the spectra in Fig. 5.

the strongest effect. Pyruvate kinase increased the NMR cross-peak intensity of A β residues on average by a factor of about 2, but catalase reduced the cross-peak intensity.

A Model for Broad Spectrum Inhibition of A β Toxicity and Fibrillation—It is intriguing that through apparently a-specific interactions with proteins present in substoichiometric amounts, the formation of amyloid can be inhibited. We can envisage several mechanisms (which do not exclude each other): (i) monomeric peptides that spontaneously convert to a β -sheet conformation are scavenged by the globular, non-chaperone proteins and are released if they convert back to random coil, thus reducing the number of nucleation sites and reducing the number of PFOs, (ii) PFOs or other types of non-fibrillar oligomers are stabilized by the non-chaperone proteins, thus slowing down the conversion from PFO to FO that precedes fibrillation (28), (iii) the non-chaperone proteins bind to (the ends of) less toxic fibrillar oligomers, somehow preventing their growth into fibrils. If mechanism (iii) were solely responsible for the observed inhibition, we would not have observed the reduction of cytotoxicity, as the PFOs are the most toxic species. Therefore, we will focus on mechanisms (i) and (ii) (Fig. 7).

Mechanism (i) has parallels with the mode of action of chaperone proteins like Hsp90 and trigger factor. Such proteins function as holdases and unfoldases by binding to hydrophobic, aggregation-prone sequences of unfolded proteins, thus protecting them from aggregation (29, 30). Our NMR data indicated that the relative intensity of A β HSQC cross-peaks increased in the presence of albumin, lysozyme, or pyruvate kinase. This suggests that these proteins solubilized A β in its unfolded state. Furthermore, our ANS data indicated that the total hydrophobic surface area decreased upon co-incubating A β with these proteins. A likely explanation of these observations is that albumin, lysozyme, and pyruvate kinase weakly bind A β molecules with exposed hydrophobic surfaces, release

them when they convert back to a less hydrophobic conformation, and thereby reduce the number of aggregation-prone hydrophobic monomers in the solution.

However, α -lactalbumin, β -lactoglobulin, and catalase reduced the intensity of A β HSQC cross-peaks, indicating that a substantial amount of A β was no longer present in its monomeric state. This suggests that mechanism (ii) is also employed. The CD measurements show that the A β molecules had not adopted a β -sheet conformation in the aggregates. Perhaps these three proteins induced a dead-end aggregate of A β , which was neither toxic nor able to convert into toxic PFOs or fibril inducing FOs. Alternatively, α -lactalbumin, β -lactoglobulin, and catalase had an enhanced affinity for toxic PFOs and scavenged these from solution.

It is not clear what the structural details are of mechanism (ii) *in vivo*. In any case we have to consider the possibility that PFO and FO formation is reduced indirectly in which the non-chaperone proteins could inhibit PFO and FO formation for instance by binding copper, a metal known to stimulate A β fibrillation. Catalase, α -lactalbumin (31), and β -lactoglobulin (32) are all known to bind copper, so this indirect mechanism has to be considered. Thus, some of non-chaperone proteins may act as mediators to inhibit the formation of copper and the A β complex and reduce the production of reactive oxygen species *in vivo*.

Here we discuss a possible structural interaction underlying mechanism (ii). It is based on the hypothesis that the structure of PFOs is similar to that of cylindricins (33). One of the hypotheses is that once such an oligomer contacts the cell membrane, its hydrophilic regions may interact with the head group of lipids, and then hydrophobic residues disrupt the tail groups, inducing weakening of the membrane, potential leaking of the cell, and eventually apoptosis. We investigated the hydrophobic and hydrophilic surface of the test proteins to test this cylindrin

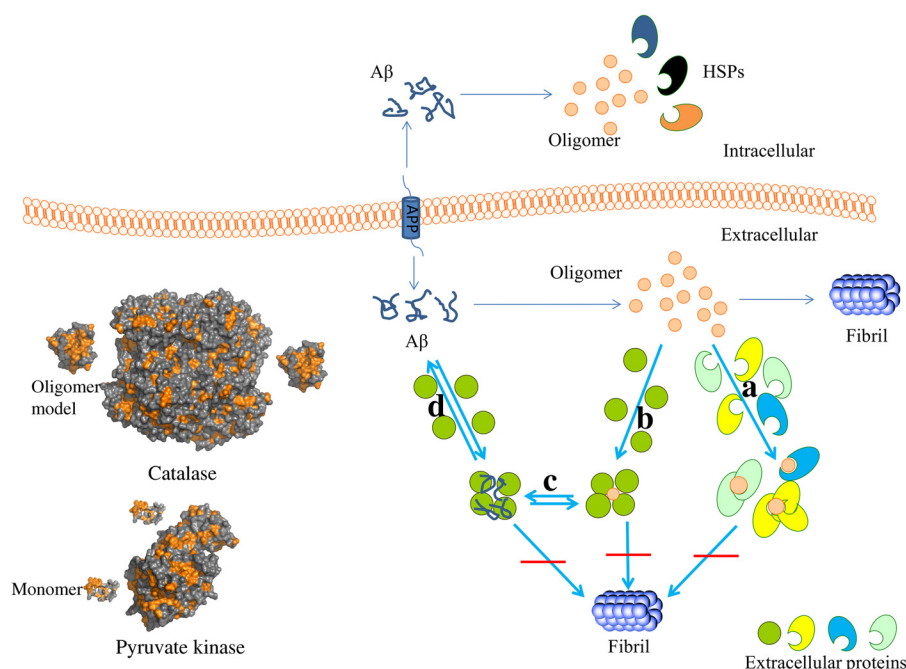


FIGURE 7. **Potential inhibition mechanism of intracellular chaperones and extracellular non-chaperone proteins.** The hydrophobic and hydrophilic surface regions of the proteins are represented in orange and gray, respectively. A β is produced from APP by γ -secretase. Toxic oligomers of A β can be scavenged and potentially redissolved by intracellular chaperones and extracellular non-chaperone proteins. a–d, non-chaperone proteins can bind to A β oligomers directly and then prevent the nucleation of fibrils (a). Alternatively, A β oligomers can be converted into monomer after binding to non-chaperone proteins (b and c), or A β is stabilized in its monomeric state due to the interactions with non-chaperone proteins. HSP, heat shock protein

model (Fig. 7). Based on the surface charge distribution, we propose that once the proteins interact with A β oligomers, the hydrophobic and hydrophilic groups in the protein may act as the tail and head groups of cellular lipids, respectively. However, unlike the lipidic cell membrane or the amyloid oligomers, the proteins are relatively stable. The proteins may, therefore, retain their structure, but the toxic amyloid oligomer may be scavenged or even be disrupted. If the PFOs form the prime source of FOs or if FOs are captured by the proteins in a similar fashion as PFOs, such interactions could effectively reduce the number of FO nucleation seeds required for fast fibrillation. Thus, toxicity and amyloid formation would be reduced.

The Correlation between AD and the Test Proteins—Catalase, pyruvate kinase, and albumin can be found in the brain. To some extent they are related to the progression of AD. As a peroxidase enzyme, catalase may mediate the cellular levels of reactive oxygen species, which is relevant for the hypothesis of oxidative stress in AD. The activity of pyruvate kinase is significantly increased in frontal and temporal cortex of AD brains (34), but direct interaction *in vivo* between pyruvate kinase and A β remains to be investigated. The level of A β -albumin complex in serum is decreased in the AD brain (35). There is no direct relationship between lysozyme and AD, but lysozyme increases upon inflammation reactions, which are also triggered in the Alzheimer brain.

A Comparison with Chaperones against the A β Fibrillation—Extracellular chaperones and chaperone-like proteins have been reported to act as disposal mediators in the proteostasis of A β aggregation (36, 37). For instance, clusterin, a potent sHsp-like chaperone, sequesters prefibrillar oligomers and influences A β fibrillation; at a molecular ratio of 1:10 (clusterin:A β), suppression of A β fibrillation was reported (11). However, clus-

terin *in vitro* does not affect the cytotoxicity of A β aggregates at a molar ratio of 1:500 and increases the cytotoxicity of A β aggregates at a molar ratio of 1:10 (38). Like clusterin, haptoglobin and α 2-macroglobulin (found in extracellular fluid), completely suppressed A β fibrillation at a molecular ratio of 1:10 by interacting with A β PFOs (39). In addition to extracellular proteins, intracellular chaperones, like heat shock proteins hsp70 and hsp90, inhibit phenotypes related to A β aggregation in a model of Alzheimer disease (40). Hsp70 and hsp90 interact with oligomeric aggregates and suppress A β fibrillation effectively at substoichiometric molecular ratio ($\sim$ 1:50) (13). Similarly, hsp104 targets oligomeric intermediates and inhibits the elongation of A β fibrils (41).

Besides extracellular and intracellular chaperones, lysozyme and albumin have been shown to bind to A β peptide and suppress A β fibrillation. Human serum albumin binds to A β oligomers/protofibrils in prevailing stoichiometry and affinity (1–100 nM range) and inhibits A β (1–42) fibril growth at substoichiometric concentrations (molecular ratio 1:18) (42, 43). Human lysozyme inhibits A β fibrillation at a molecular ratio of 1:1. Furthermore, the BRICHOS domain, associated with familial British dementia, interferes with A β aggregation before the formation of fibrils at a molecular ratio of $\sim$ 1:10 (BRICHOS domain:A β) (19).

Size and Surface-dependent Interaction between A β and Folded Proteins—To identify which aspects of these proteins and the test protein on which we report here might contribute to their apparent biological activity, we summarize some of their important biophysical characteristics in Table 2. Table 2 does suggest a positive correlation between molecular weight and inhibition efficiency. For instance, catalase, pyruvate kinase, and Hsps with molecular masses >50 kDa inhibit the

TABLE 2

The comparison of properties of non-chaperone and chaperone proteins on the inhibition of the A β fibrillation

Protein	M_r	State	PI	Molar effective inhibition ^a	Weight effective inhibition ^b	Amylogenic regions ^c	SESA/SASA ^d
Catalase	60	Hexamer	6.90	~1:100	1:7.1	53–58; 81–86; 132–137; 194–202; 265–270; 277–295; 321–329; 459–464; 469–474	1.25
Pyruvate kinase	237	Monomer	7.96	~1:100	1:1.8	144–148; 391–395; 506–514	1.00
Lysozyme	14	Monomer	9.38	~1:1	1:0.3	73–79	0.89
α -Lactalbumin	14	Dimer	4.92	~1:1	1:0.3	70–78; 90–95; 119–125	0.84
β -Lactoglobulin	20	Monomer	4.93	~2:1	1:0.1	14–21; 96–101; 117–122	0.91
Human serum albumin	67	Monomer	5.85	~1:10	1:0.6	9–18; 45–57; 177–182; 352–357; 574–578	0.99
Pro-SP-C BRICHOS domain	19	Monomer	6.19	~1:30	1:6.7	42–51; 100–114; 119–127	1.00
Haptoglobin 2–1	200	Monomer		~1:10	1:0.2	13–19; 188–209; 220–225	
α 2-Macroglobulin	73	Dimer	6.24	~1:10	1:0.59		
Clusterin	70	Dimer	5.88	~1:10	1:0.61	4–16; 364–370	0.93
HSP 70	70	Monomer or Dimer	5.47	~1:50	1:3.1	25–30; 40–46; 103–108; 141–153; 169–174; 179–184; 193–199; 205–210; 367–372; 375–381; 424–432; 439–445	0.98
HSP 90	90	Dimer	4.94	~1:50	1:2.4	30–53; 135–144; 338–342; 360–365; 490–496; 516–523; 663–674	0.90
HSP 104	100	Hexamer	5.31	~1:100	1:4.3	107–133; 278–284; 347–352; 420–429; 608–615; 628–633; 730–737; 768–775	
Affibody Z _{Aβ3}	10	Monomer	6.01	1:100	1:43		
α -Synuclein	14	Disordered	4.67	Promotes A β fibrillation		35–40	

^a Molecular ratio of the effective inhibition of the A β fibrillation in 24 h (protein:A β).

^b Weight ratio (mg/mg) of the effective inhibition of the A β fibrillation in 24 h (protein:A β).

^c Amylogenic regions were predicted by the Waltz program (24).

^d Total solvent-excluded surface area/total solvent-accessible surface area.

fibrillation of A β at a molecular ratio of 1:50. We also investigated the effect of the hydrophobicity/hydrophilicity of the accessible surface area and found that the most effective inhibition of the A β fibrillation can be observed for proteins with a ratio of solvent-excluded surface area:solvent-accessible surface area >0.9. However, there is not a very clear biophysical, molecular property that the most effective inhibitors of fibrillation and toxicity have in common.

Potential Amyloid Cross-interactions between A β and (Non-) folded Proteins—Amyloid proteins also affect A β fibrillation and toxicity. For instance, α -synuclein oligomers interact with A β monomers and/or oligomers and affect A β fibrillation (44). Such (amyloid protein:A β) cross-interactions may promote the progression of AD (45). This interactions may result from conformational transition of A β and α -synuclein (46) where the flexible amylogenic regions with more hydrophobic residues interact with each other, and electrostatic interactions form in the flexible regions. To investigate if such interactions between A β and the test proteins could occur, we searched for their potential amylogenic regions using the Waltz algorithm (Table 2). Despite a cluster of amylogenic regions predicted on the surface of proteins, the folded proteins, lacking a flexible hydrophobic surface, interacted with the A β monomer only weakly as we observed by NMR (which is in agreement with a previous report (19)). So potential amyloid cross-interactions cannot explain the effects on A β fibrillation that we report.

In summary, we discovered that a series of non-chaperone proteins hinder the A β fibrillation and reduce the toxicity of A β aggregates. We propose that the proteins interfere with the stability of the A β oligomer by hydrophobic and hydrophilic interactions of their surface. There is a positive correlation between protein surface size and A β -neutralizing activity, but this does not fully explain why some proteins are more active in inhibiting A β than others. Our results would for instance predict that

high levels of catalase in the brain would not only reduce damage by neutralizing reactive oxygen species but also by reducing the toxicity of A β oligomers. There may be other proteins in extracellular cerebral fluids that similarly reduce A β toxicity to a significant extent, and monitoring or even influencing their levels in AD-prone individuals may be relevant for preventive strategies.

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