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Human lysozyme inhibits the *in vitro* aggregation of Aβ peptides, which *in vivo* are associated with Alzheimer's disease†

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Alzheimer's disease is a neurodegenerative disorder characterized by accumulation of Aβ peptide aggregates in the brain. Using ThT fluorescence assays, AFM imaging, NMR and CD spectroscopy, and MD modeling we show that lysozyme – a hydrolytic enzyme abundant in human secretions – completely inhibits the aggregation of Aβ peptides at equimolar lysozyme : Aβ peptide ratios.

Characterization of organ lesions associated with the intra- or extracellular deposition of amyloid protein fibrils is essential for understanding an important group of human protein aggregation diseases, such as Alzheimer's disease (AD) and Parkinson's disease.[1,2] Identification of human macromolecular components preventing amyloid fibrillation will deepen our understanding of this process and may lead to potential therapies. Furthermore, studying the mechanisms by which such molecules inhibit amyloid peptide/protein fibrillation may reveal fundamental aspects of our body's natural defense system against amyloidosis.

The fibrils that characterize Alzeimer's disease are formed by amyloid beta (Aβ) peptides adopting a hairpin conformation, which exposes hydrophobic surfaces allowing the peptides to link up.[3] Chaperones, ubiquitin and clusterin are important elements in the control of extracellular Aβ peptide protein misfolding by interacting with prefibrillar species.[4–6] The ectopic expression of cytosolic heat shock proteins (Hsp72) or the extracellular chaperone clusterin protected against PrPSc- or Aβ-induced toxicity. The protein deposits found in AD, prion disease or Parkinson's disease are associated with cellular heat shock proteins (Hsps) modulating cytotoxic protein aggregation.[7,8]

Human lysozyme is one of the glycoside hydrolases, and functions as an antibacterial agent.[9] Lysozyme is abundant in a number of secretions such as tears, saliva, milk, and mucus and widely distributed in tissues such as the liver.[10] Lysozyme is also present in cerebral fluid, where substantially increased levels have been reported especially during inflammation, but also during other disease processes.[14] The concentration of lysozyme in plasma varies from 4 to 13 mg l^{-1}, and approximately 500 mg of lysozyme is produced per person per day. Approximately 75% of the protein is eluted from the plasma within 1 h.[15] Because of its ability to self-assemble, lysozyme is often used as a model molecule to study protein stability, folding and aggregation.[11]

Amyloidogenic protein fibrillation can be inhibited by cross-amyloid peptides and proteins that bind to the fibril ends, thereby blocking further polymerization.[12] Thus, Aβ peptides modulate the aggregation of various other amyloidogenic proteins, including tau, alpha-synuclein, transthyretin and islet amyloid polypeptide (IAPP).[13–15] Structural and dynamic characterization of cross-amyloid interactions is therefore essential for understanding amyloid formation, and paves the way towards elucidating the link between different types of human amyloidosis.

Here we show that human lysozyme prevents Aβ peptide aggregation. This inhibitory effect was investigated by ThT fluorescence assays and AFM imaging, combined with secondary structure characterisation by circular dichroism (CD) and NMR spectroscopy. Molecular dynamics studies suggested a potential molecular interaction mechanism between lysozyme and the monomeric Aβ peptide.

ThT assays monitoring the amyloid formation of Aβ peptides (10 μM) indicated the effect of lysozyme (Fig. 1a). The lag phases of Aβ aggregation at 40 nM and 200 nM lysozyme were shorter than that of Aβ alone (Table 1). However, for molar ratios of lysozyme to Aβ ranging from 1 : 2 to 10 : 1 the lag times were significantly delayed, showing that lysozyme efficiently interferes with Aβ amyloid formation at these ratios. At higher lysozyme/Aβ ratios, amyloid formation was fully inhibited (Fig. 1 and Table 1). The transition time of the Aβ fibrillation (the duration of the growth phase) generally decreased in the presence of lysozyme – except at lysozyme concentrations sufficiently high for full inhibition. A control experiment confirmed that lysozyme itself did not form fibrils at 37 °C (Fig. S1, ESI†) which is in agreement with previous studies.[16]

Structural transitions during Aβ peptide aggregation (at 5 μM) were studied in the presence and absence of 10 μM lysozyme by CD spectroscopy (Fig. 1b and c). Fig. 1b demonstrates that after the addition of the Aβ peptide, lysozyme retained its alpha-helical

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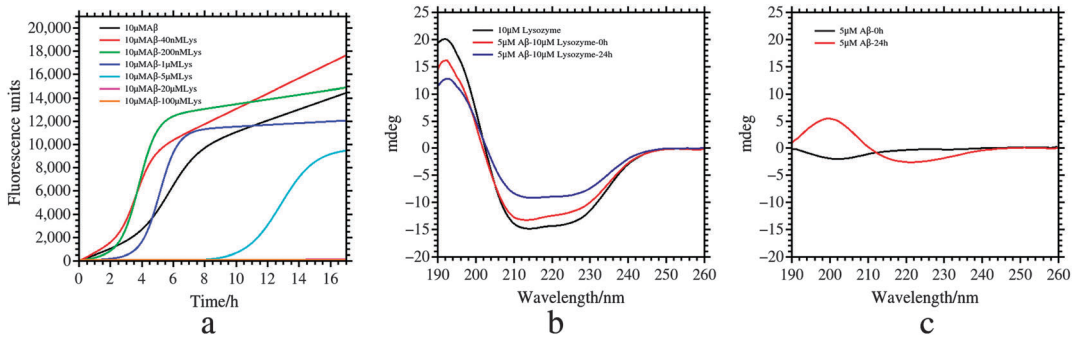

Fig. 1 (a) ThT fluorescence kinetic assays of amyloid formation of 10 μM Aβ(1–40) peptide in the presence of 0 μM, 40 nM, 200 nM, 1 μM, 5 μM, 20 μM and 100 μM lysozyme, measured in 50 mM Tris buffer at pH 7.4 and 37 °C. (b) CD data describing kinetics of secondary structure transitions of 10 μM Aβ(1–40) peptides in 20 mM sodium phosphate buffer at pH 7.3 and 37 °C with and without lysozyme, the CD spectra of free lysozyme (black), the lysozyme–Aβ40 complex after 0 h (red) and 4 h (blue) of incubation. (c) The CD spectra of 5 μM Aβ peptides alone after the incubation of 0 h (black curve) and 24 h (red curve) at 37 °C.

Table 1 Lag time and transition time for amyloid formation kinetics of 10 μM Aβ(1–40) peptides in the presence of 0 nM, 40 nM, 200 nM, 1 μM, 5 μM, 20 μM, 100 μM lysozyme, measured in 50 mM Tris buffer at pH 7.4 and +37 °C

	10 μM Aβ	10 μM Aβ_40 nM lysozyme	10 μM Aβ_200 nM lysozyme	10 μM Aβ_1 μM lysozyme	10 μM Aβ_5 μM lysozyme	10 μM Aβ_20 μM lysozyme	10 μM Aβ_100 μM lysozyme
Lag time (h)	1.43 ± 0.51	0.159 ± 0.27	1.17 ± 0.14	1.75 ± 0.10	10.80 ± 1.25	—	—
Transition time (h)	9.05 ± 1.81	6.44 ± 1.20	4.68 ± 0.12	5.95 ± 0.27	3.13 ± 0.20	—	—

structure with just a small decrease in the overall signal. After 24 h of incubation at 37 °C and shaking at 200 rpm, further signal loss is observed, perhaps as a result of complex formation and precipitation. In the absence of lysozyme, the Aβ peptide undergoes a well-known transition from random coil to beta-sheet structure during 24 h (Fig. 1c). In the presence of lysozyme, however, no structural transitions are observed in the CD spectra during 24 h (Fig. 1b). These results suggest that the largely α-helical lysozyme interacts with the Aβ peptide and inhibits its structural transformation and accompanying aggregation. Furthermore, no aggregates – fibrils or other kinds – were found to be deposited on the mica surface from AFM measurements (Fig. 2b). As a control, the 50 μM Aβ peptide alone was incubated under the same conditions and clear mature fibrils were observed (Fig. 2a).

^{1}H–^{15}N NMR HSQC experiments of ^{15}N-labeled Aβ40 with and without lysozyme further probed the interaction. As shown in Fig. 2c, the liquid phase ^{1}H–^{15}N HSQC spectra for 100 μM Aβ40 show typical random coil resonances in agreement with our previous studies[17] (blue peaks). No significant chemical shift changes appear after addition of 200 μM lysozyme (red peaks), indicating that lysozyme does not alter the random coil structure of the monomeric Aβ peptide in solution.

Then, we first used molecular docking simulations to identify the most likely binding site of lysozyme to the Aβ peptide and molecular dynamics simulations were used to explore the structural stability of this complex. The complex with lowest free binding energy was selected, using the monomeric structure of the Aβ peptide in SDS as determined by NMR, together with the crystal structure of human lysozyme. The N-terminus of the Aβ peptide was found to bind to the lysozyme's substrate binding site, which locates and catalyzes glycoside hydrolysis of the bacterial peptidoglycan cell wall (Fig. S2a, ESI†). In addition to electrostatic interactions between the N-terminus of the Aβ peptide and the cleavage site and the edges of lysozyme (Fig. S2b, ESI†), also some hydrophobic interactions between the Aβ C terminus and lysozyme were indicated by our MD simulations (Fig. S2c, ESI†).

The structure of human lysozyme remained stable throughout the 100 ns simulation (Fig. S3, ESI†). However, a structural transition of the Aβ peptide from α-helix to helical coil was observed. Three different binding sites on lysozyme, *i.e.* A, B and C, were found to interact with the N terminus of the Aβ peptide (Fig. S3b, ESI†),

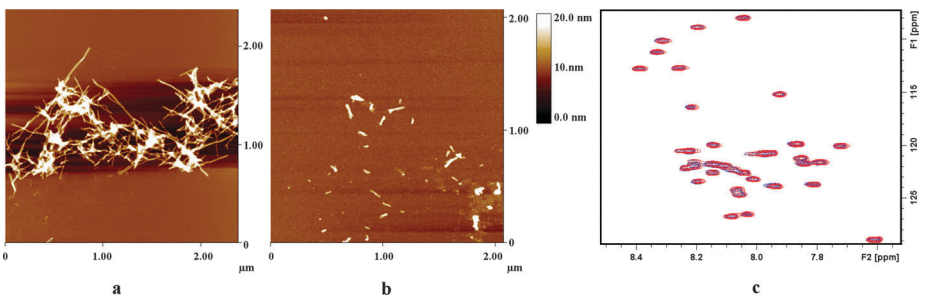

Fig. 2 AFM and NMR measurements of the Aβ peptide in the presence and absence of lysozyme. (a) 50 μM Aβ peptide alone after 12 h of incubation at 30 °C, (b) 50 μM Aβ peptide with 100 μM lysozyme after 12 h of incubation at 30 °C, (c) NMR ^{1}H–^{15}N-HSQC spectra of 100 μM ^{15}N-labeled Aβ(1–40) peptides in 20 mM sodium phosphate buffer at pH 7.3, +5 °C, before (blue) and after (red) addition of 200 μM lysozyme.

its residues Q15–V24 (Fig. S3c, ESI†) and its C terminus (Fig. S3d, ESI†). The interaction at site A was mainly stabilized by hydrogen bonds, electrostatic interactions and aromatic hydrophobic interactions *via* R62, Y63 and W64 of human lysozyme and F4, R5 and H6 of the Aβ40 peptide. In the B site, the helical residues (D102–R107) and the loop residues (Q117–R122) of human lysozyme interacted with residues Q15–V24 and I31–V40 of the Aβ peptide. These peptide residues form the two main hydrophobic areas driving Aβ aggregation. It is noteworthy that residues R62–W64 and D102–R107 (plus S24) located in the cleavage site of lysozyme partake in the binding of the charged natural oligosaccharide substrate. At the C site, a few hydrophobic residues, such as R119 and S24, bind the highly hydrophobic terminus I31–V40 of the Aβ peptide. Taken together, the results suggest that electrostatic interactions between human lysozyme and the Aβ peptide play an important role in the stabilization at the A and B sites, while the interaction at the C site could be more flexible compared to the A and B sites.

To probe how human lysozyme affects the conformational transition of the Aβ peptide, a structural trajectory of a simulation of the complex was analyzed (Fig. S4, ESI†). In general, the structure remained helical even after 100 ns of MD simulations. Particularly, the residues 10–27, a fragment significant because it drives the formation of aggregates, retained their alpha-helical form. Still, the N- and C-termini of Aβ converted into turn and bend structures. For lysozyme there is no significant conformational shift, except for some loop flexibility in the course of the simulation. However, we did notice the brief appearance of a beta strand in residues 15–25 during the simulation (between 35 and 45 ns, Fig. S4, ESI†). This could be a response to the binding of the Aβ peptide. Clearly, in the presence of lysozyme, the Aβ40 peptide remains stable except for its N- and C-terminal structures. This conformational stabilization, which was also observed in the presence of lipids,[18] provides an elegant mechanism explaining how lysozyme prevents Aβ40 peptide aggregation.

The inhibition by chaperones/small aromatic molecules of amyloid aggregation has been suggested as a strategy for the treatment of amyloid disease. For example, the recombinant BRICHOS domains from Bri2 and the pro-SP-C membrane protein significantly inhibit the lag time of fibril formation of amyloid β-peptides (Aβ(40) and Aβ(42)) far below the stoichiometric ratio, probably by interfering with the nucleation process preceding Aβ aggregation.[19] The only molecules so far observed that completely inhibit Aβ aggregation are proteins/peptides that interact with the Aβ peptide in its monomeric form or very early in the aggregation pathway. However, the specific binding of the chaperones to their natural substrates competes with the binding of the amyloid, which may hinder the inhibition of aggregation. Furthermore, the toxicity of small chaperone molecules remains an issue. Therefore, it is necessary to keep searching for human macro-molecules that assist in preventing amyloid formation.

Here, we found that human lysozyme could prevent amyloid aggregation of the Aβ peptide at a 1:1 ratio. Using CD and NMR spectroscopy, we showed that lysozyme does not affect the random coil structure of the monomeric Aβ peptide. Molecular dynamics simulations indicated that human lysozyme stabilizes the N-terminus of the Aβ peptide by electrostatic interactions and interacts with the C-terminus of the Aβ peptide *via* a hydrophobic surface. The binding of the Aβ peptide blocks the substrate binding site of lysozyme by binding to pocket A (Fig. S3b, ESI†). These hydrophobic and the electrostatic interactions therefore seem to be key for human lysozyme to prevent Aβ peptide aggregation. This mechanism is similar to the way in which some chaperones prevent the aggregation. Also, β-2-macroglobulin and clusterin appear to retain the solubility of the Aβ peptide and the PI3–SH3 domain interferes with the prefibrillar phase of aggregation.[20] Furthermore, heat shock protein 70 inhibits alpha-synuclein amyloid aggregation by targeting the prefibrillar species of alpha-synuclein, which is the misfolded monomer.[8]

Human serum albumin was also found to inhibit the formation of Aβ peptide fibrils at micromolar levels in human plasma.[21] The mechanism of albumin inhibition of amyloid fibril formation was recognized as ''monomer–competitor'', scavenging proto-fibrils. In the case of lysozyme, the Aβ peptide appeared to bind as a monomer to monomeric lysozyme, as it stabilised the random coil structure of the amyloid peptide (indicated by NMR).

Lysozyme, just like chaperone proteins and the chaperone-like BRICHOS domain, is small, compared to human albumin. Surprisingly, the amyloid aggregation inhibition by these small molecules appears to have a similar mechanism *via* the interaction with the monomeric substrate.

The ability of lysozyme to prevent misfolding of the Aβ peptide and thus interfere with the formation of the Aβ oligomer may provide a molecular basis for the identification of other human plasma proteins that may play a role in modulating amyloidosis.

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