Hsp60, amateur chaperone in amyloid-beta fibrillogenesis

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Abbreviations: Alzheimer's disease (AD), Atomic Force Microscopy (AFM), Electron Microscopy (EM), Circular Dichroism (CD), Size Exclusion Chromatography (SEC), Heat Schock Protein (HSP), Thioflavin T (ThT)

ABSTRACT

Background: Molecular chaperones are a very special class of proteins that play essential roles in many cellular processes like folding, targeting and transport of proteins. Moreover, recent evidence indicates that chaperones can act as potentially strong suppressor agents in Alzheimer's disease (AD). Indeed, *in vitro* experiments demonstrate that several chaperones are able to significantly slow down or suppress aggregation of Aβ peptide and *in vivo* studies reveal that treatment with specific chaperones or their overexpression can ameliorate some distinct pathological signs characterizing AD.

Methods: Here we investigate using a biophysical approach (fluorescence, circular dichroism (CD), transmission electron (TEM) and atomic force (AFM) microscopy, size exclusion chromatography (SEC)) the effect of the human chaperonin Hsp60 on Aβ fibrillogenesis.

Results: We found that Hsp60 powerfully inhibits Aβ amyloid aggregation, by closing molecular pathways leading to peptide fibrillogenesis.

Conclusions: We observe that Hsp60 inhibits Aβ through a more complex mechanism than a simple folding chaperone action. The action is specifically directed towards the early oligomeric species behaving as aggregation seeds for on-pathway amyloid fibrillogenesis.

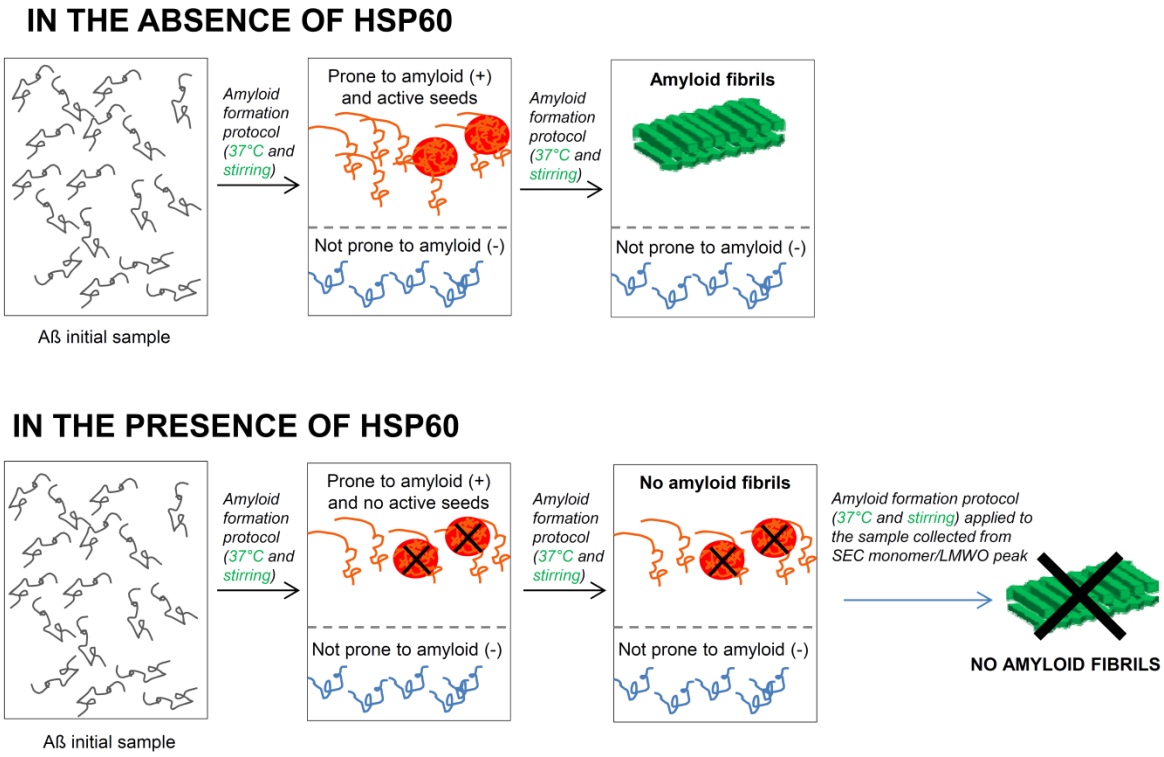
General significance: Understanding the specificity of the molecular interactions of Hsp60 with amyloid Aβ peptide allowed us to emphasize the important aspects to be taken into consideration when considering the recent promising therapeutic strategies for neurodegeneration

Keywords: Molecular chaperones, chaperonin, amyloid aggregation, inhibition mechanisms, Alzheimer's disease treatment

HIGHLIGHTS

- The human chaperonin Hsp60 effect on Aβ amyloid peptide aggregation is studied.
- Aβ amyloid aggregation is inhibited in the presence of Hsp60.
- Hsp60 selectively acts on the species acting as seeds for fibrillogenesis
- Once active seeds are recruited from Hsp60, Aβ peptide is unable to fibrillate.
- Our study contributes to basic knowledge for chaperones-based AD therapies.

GRAPHICAL ABSTRACT



1. Introduction

Molecular chaperones play essential roles in many physiologically relevant cellular processes, including folding, targeting and transport of other proteins [1-3]. Moreover, these proteins are highly expressed under stress conditions, for example thermal stress (so to be defined “heat shock proteins”, HSPs), contributing to guarantee cell proteostasis by avoiding protein misfolding and aggregation and providing protein degradation and disaggregation of toxic aggregates by clearance mechanisms [4-8]. Therefore, it is not surprising that recent evidence indicates that chaperones are potentially strong suppressors of neurodegeneration. Indeed, the protective nature of many HSPs in several experimental models of neurodegeneration, *in vitro* and *in vivo*, supports this hypothesis. Noteworthy, cell free experiments demonstrate that several HSPs, alone or in synergistic action, are able to significantly slow down or eventually suppress protein aggregation involved in severe amyloid diseases, like Alzheimer’s Disease (AD) caused by the accumulation of the 40-42 aa Amyloid β (Aβ) peptide [6, 9-15]. *In vivo* studies, using various animal and cellular models reveal that treatment with specific chaperones or their overexpression can ameliorate pathological behavior dysfunction characterizing AD pathology [11, 16-19]. Current research on the development of therapeutic approaches of intervention in amyloid diseases bases on at least four broad approaches: i) block the production of the amyloidogenic peptide or protein, ii) block its ‘‘misfolding" or transformation from a nonpathogenic monomer or low-oligomer to toxic oligomers and polymers, iii) block the toxic effects of amyloid, or iv) modulate an auxiliary cellular pathway that affects beneficially one or more of the foregoing approaches [20-22]. Molecular chaperones act at all the above mentioned levels and, considering their functional complexity [23], it is hard to imagine other molecules, synthetic or natural, able to exert the same action with the same effectiveness.

All these considerations strongly suggest that the pharmacological activation and induction of specific chaperones can be an effective therapeutic approach [24-26]. Indeed, these strategies are rapidly emerging as promising treatments for cancer intervention [26, 27], but, in the neurodegeneration field and particularly in AD, a careful evaluation of potential pitfalls is necessary. In fact, Aβ oligomers are characterized by a very broad structural polymorphism with

different epitope exposure, antibody-binding properties and peculiar toxicity features [28, 29]. To be considered for a promising therapeutic approach, chaperones should be able to block monomer aggregation or to favor the formation of nontoxic Aβ oligomeric variants.

Molecular chaperones are classified in three functional categories based on their different mechanism [4, 8, 30, 31]. In each mechanism, a crucial role is assumed by the presence of intrinsic disorder regions (IDRs) along the protein sequence [8]. "Folding" chaperones (e.g., DnaK and GroEL in prokaryotes, and Hsp60 and Hsp70 as well as the HspB group of Hsps including Hsp27 and HspB1 in eukaryotes) induce refolding/unfolding of their substrates, with conformational changes depending on adenosine triphosphate (ATP)-binding [8]. "Holding" chaperones (e.g., Hsp33 and Hsp31) bind partially folded proteins and, by keeping them on their surface, allow the subsequent action of "folding" chaperones [8]. Finally, "disaggregating" chaperones (e.g., ClpB in prokaryotes and Hsp104 in eukaryotes) act by solubilizing proteins that exist in an aggregated state [8, 31]. However, the real picture in this field is much more nuanced and it could involve the coexistence of several and more complex mechanisms related to the specific nature of the molecular interactions between chaperone and protein aggregates. Increasing evidence highlights an intriguing feature of the activity of a natural molecular chaperone against misfolding events and associated reactions, suggesting that chaperones activity is not limited to sequestrate single unfolded monomers, but they can selectively interact with specific aggregated species [32, 33]. This, similarly to what occurs with investigations involving antiaggregating agents, intrinsically leads to carefully consider the toxicity of the protein conformations stabilized by the chaperone action [28, 34]. Therefore, it becomes fundamental to study the specific mechanisms of interaction of these chaperones with pathogenic amyloid-forming proteins.

In this study, we analyze the effect of a human chaperonin Hsp60, homologous to the bacterial GroEL, on the aggregation process of $A\beta_{1-40}$ peptide involved in AD. Differently from GroEL, that is found only as a tetradecameric conformation organized in two heptameric rings each formed by seven 57 kDa monomers, Hsp60 seems to exists in dynamic heptamer/tetradecamer equilibrium [35-37]. Also monomers are found under certain conditions for mitochondrial Hsp60 [38, 39]. The major flexibility and richer functional conformational ensemble have evolutionary occurred at the cost of protein stability and unfolding cooperativity [35]. Hsp60, in its naïve and mitochondrial form assumes a crucial role in several carcinogenic and inflammatory processes

[40, 41]. Strong interactions between Hsp60 and amyloid precursor protein (APP) [42], as well as hamster prion protein PrPc [15], have been recently revealed. Moreover, it has been found by NMR measurements that GroEL suppresses $A\beta_{1-40}$ amyloid formation by interacting with its two hydrophobic segments Leu17-Ala21 and Ala30-Val36, key residues in fibril formation [14]. Furthermore, GroEL inhibits the formation of toxic alpha-synuclein aggregates and it is capable of inhibiting the fibrillization of other amyloidogenic proteins such as β2 microglobulin [12]. Here, by using several techniques (fluorescence, circular dichroism (CD), transmission electron (TEM) and atomic force (AFM) microscopy, Size Exclusion Chromatography (SEC)), we show for the first time that human Hsp60, even in the absence of its cochaperonin Hsp10 and ATP, inhibits the fibrillogenesis of $A\beta_{1-40}$ peptide leaving the peptide in an unordered conformation. We suggest a possible mechanism underlying this inhibitory action that could constitute a basic building block in the research field of therapies based on human molecular chaperones for AD and other neurodegenerative diseases.

2. Materials and Methods

2.1 Sample preparation

60 kDa mitochondrial heat shock protein (Hsp60), was purchased from ATGen in 20 mM Tris-HCl buffer (pH 8.0) 10% glycerol containing 0.1 M NaCl. Recombinant human Hsp60 protein, fused to a His-tag sequence at N-terminus, was expressed in E. *coli* and purified by using conventional chromatography techniques. The purity of the protein was tested by SDS–PAGE was > 90%. Sample was stored at -80 °C before use. Prior to each experiment, the protein was thawed at 4 °C in a cold room and the buffer change was operated by appropriate dilution and concentration cycles using centrifugal filter device with a cut-off of 30 kDa (Millipore Amicon – Ultra 4), in order to obtain the protein in 20 mM Tris-HCl buffer (pH 7.7) 3% glycerol and 30 mM NaCl. The stability of the protein and the absence of exogenous growth in time were monitored in time by Static Light Scattering measurements (data not shown) and the concentration was determined by HPLC measurements.

The synthetic peptide $A\beta_{1-40}$ (Anaspec) was solubilized in NaOH 5 mM (Sigma-Aldrich), pH 10, lyophilized according to Fezoui et al. protocol [43]. The lyophilized peptide was then dissolved in 20 mM Tris pH 7.7, 3% glycerol, 30 mM NaCl and then filtered with two filters in series having diameter of 0.20 μm (Whatman) and 0.02 μm (Millex-Lg) respectively, in order to eliminate large aggregates. The sample preparation was operated in asepsis using a cold room at 4 °C. Aβ concentration was determined by tyrosine absorption at 276 nm using an extinction coefficient of 1390 $cm^{-1}M^{-1}$.

Final samples containing Aβ and Hsp60 were obtained by appropriate aseptically mixing of the protein solutions and placed in closed cuvettes in a cold room at 4 °C, before incubation at higher temperatures. The aggregation kinetics was followed at controlled temperature (37 °C) and under controlled stirring (200 rpm) for 24 hours. Aβ samples recovered after SEC separations were prepared at appropriate concentrations using Millipore centrifugal filters with a cut-off of 3 kDa on a centrifuge Thermo (Heraeus Multifuge X3R) at 6000 g speed. AFM acquisitions on 50 μM Aβ with or without 2 μM Hsp60 samples were performed on aliquots freshly prepared and incubated at 37 °C and 200 rpm for 24h. AFM and TEM images on 30 μM Aβ samples obtained after SEC treatments were performed on samples aged 3 days at 37 °C after the usual kinetics at 37 °C and 200 rpm. This procedure was applied to enhance the fibrillation for more diluted samples.

2.2 ThT Spettrofluorometric measurements

ThT fluorescence emission was monitored by using a JASCO FP-6500 spectrometer. The excitation and emission wavelengths were 450 and 485 nm, respectively, with slit widths of 3 nm. ThT final concentration was 12 μM. The sample was placed at 37 °C in the thermostated cell compartment (10 mm). When required, a magnetic stirrer at 200 rpm (mod. 300, Rank Brothers Ltd., Cambridge) was used.

2.3 Circular Dichroism spectroscopy

CD measurements were acquired by using a JASCO J-815 CD Spectrometer. Particularly, during the aggregation kinetics, withdrawals of samples at appropriate time were

observed. Spectra were recorded at 20 °C using a quartz cell with 0.2 mm path length. Each spectrum measurement was obtained by averaging over eight scans and subtracting the blank solvent contribution.

2.4 Size Exclusion Chromatography (SEC)

Chromatographic separations were performed with a modular Prominence Shimadzu HPLC device equipped with a mobile phase degasser (DGU-20As), a quaternary pump (LC-2010 AT) a photodiode array detector (SPD-M20A). A 20 µL sample loop was used for analytical measurements and a 500 µL sample loop was employed to design collection of Aβ. For both analytical and preparative measurements, samples were separated with a Superdex 200 increase (10 300 GE Healthcare), at room temperature and eluted with a flow of 1 ml min^{-1} in the sample buffer (20 mM Tris - HCl pH 7.7 with 3% glycerol and 30 mM NaCl) degassed by an in-line degasser filter (DGU 20A5). The chromatographic profiles were reported at 280 nm. Analytical measurements were used to determine the Hsp60 concentration [36]. The column was calibrated using two specific calibration kits for both low and high molecular weights globular proteins (low code n 28-4038-41; High code n 28-4038-42 - GE Healthcare Life Science). The protein used are: Aprotinin MW 6500 Da, Ribonuclease A MW 13700 Da , Stoke Radius (SR) 1.64 nm; Carbonic anhydrase MW 29.000; Ovalbumin 44000 Da, SR 3.05 nm; Conalbumin MW 75000 Da; Aldolase MW 158000 Da, SR 4.81 nm, Ferritin 440000 Da, SR 6.1 nm; Tyroglobulin 669000 Da SR 8.5 nm.

2.5 Atomic Force Microscope measurements

AFM measurements were performed by using a Nanowizard III (JPK Instruments, Germany) mounted on an Axio Observer D1 (Carl Zeiss, Germany) or on an Eclips Ti (Nikon, Japan) inverted optical microscope. Aliquots of protein solutions were deposited onto freshly cleaved mica surfaces (Agar Scientific, Assing, Italy) and incubated for up to 20 min before rinsing with deionized water and drying under a low pressure nitrogen flow. Imaging of the protein was carried out in intermittent contact mode in air by using NCHR silicon cantilever (Nanoworld,

Switzerland) with nominal spring constant ranging from 21 to 78 N/m and typical resonance frequency ranging from 250 to 390 kHz.

2.6 TEM experiments

TEM acquisition was performed by using a JEOL JEM 1220 TEM at 120 kV. Aliquots of protein solutions were placed on Copper grids for electron microscopy. Before observation the sample were contrasted with uranyl-acetate in methanol solution (0.7g UranylAcetate in 10ml of methanol) and Reynolds's Lead Citrate (1,33g of Pb(NO3)2 and 1,76g NaC6H5O7 x H2O and 8ml of NaOH 1N in 50 ml of distilled water at pH 12). Surplus of both solutions were removed with washes in Methanol and Distilled Water respectively. The pictures were acquired at 50k and 8K.

3. Results

3.1 Analysis of the Hsp60 effect on $A\beta_{1-40}$ amyloid aggregation

In order to investigate the effect of Hsp60 on the $A\beta_{1-40}$ peptide fibrillation, a standardized protocol for the formation of fibres was applied. Briefly, samples of $A\beta_{1-40}$ either alone or in the presence of Hsp60 were incubated under pro-aggregating conditions (stirring and 37 °C) [44, 45]. Different experimental techniques were used to investigate the evolution of the structural organization of aggregates formed in the presence and in the absence of Hsp60. The stability of Hsp60 chaperone in the same conditions was monitored by chromatographic experiments (refer to S.M. for details). On the first, ThT fluorescence assay, a widely accepted method to reveal β-sheet formation was applied. Figure 1 shows a quick and sharp increase in ThT emission intensity for Aβ sample in the absence of Hsp60 that indicates the appearance of cross-β-structure.

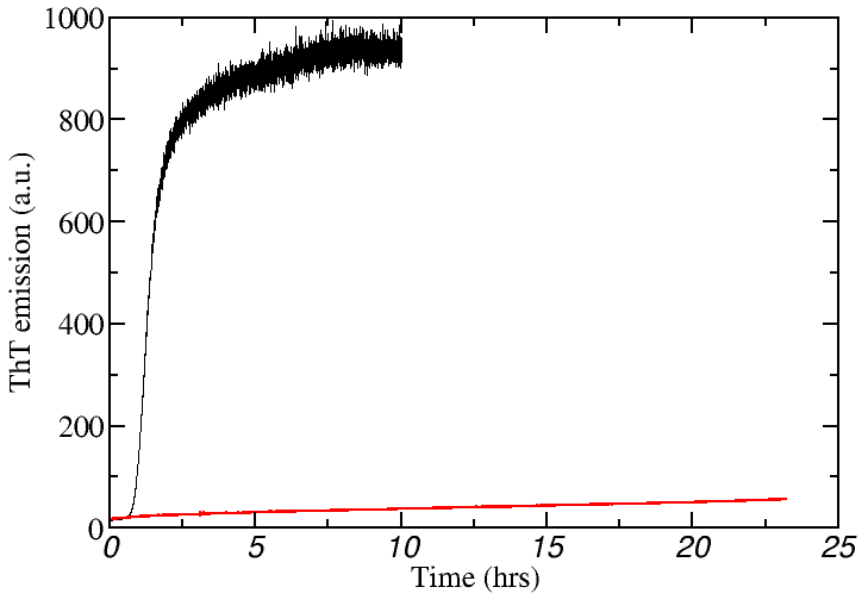


Figure 1: Influence of Hsp60 on Aβ aggregation kinetics. Kinetics of 50 µM Aβ (black line) and 50 µM Aβ incubated with 2 µM Hsp60 (red line) at 37 °C and under 200 rpm stirring monitored by ThT fluorescence assay. [ThT]=12 µM.

On the contrary, no significant change of ThT fluorescence can be observed for the $A\beta_{1-40}$ sample treated with Hsp60 for up to 24 hours, suggesting that the amyloid fibrillation is inhibited by chaperone presence. As the ThT test may be affected by typical pitfalls, due, for example, to probe sequestration by the chaperonin [46], further analyses with other experimental tools were conducted to corroborate ThT test reliability. Insights into the secondary structure of samples were obtained by CD spectroscopy measurements. Figure 2 shows the dichroic spectra of $A\beta_{1-40}$ samples in the presence or in the absence of the chaperonin, recorded at the initial time and after 24 hrs from the beginning of the process. To obtain a clearer comparison of the results, Hsp60 contribution to CD spectra was subtracted by considering the signals recorded for a sample of 2 µM Hsp60. The CD spectra show a typical evolution of $A\beta_{1-40}$ peptide from random coil to β-sheet structure.

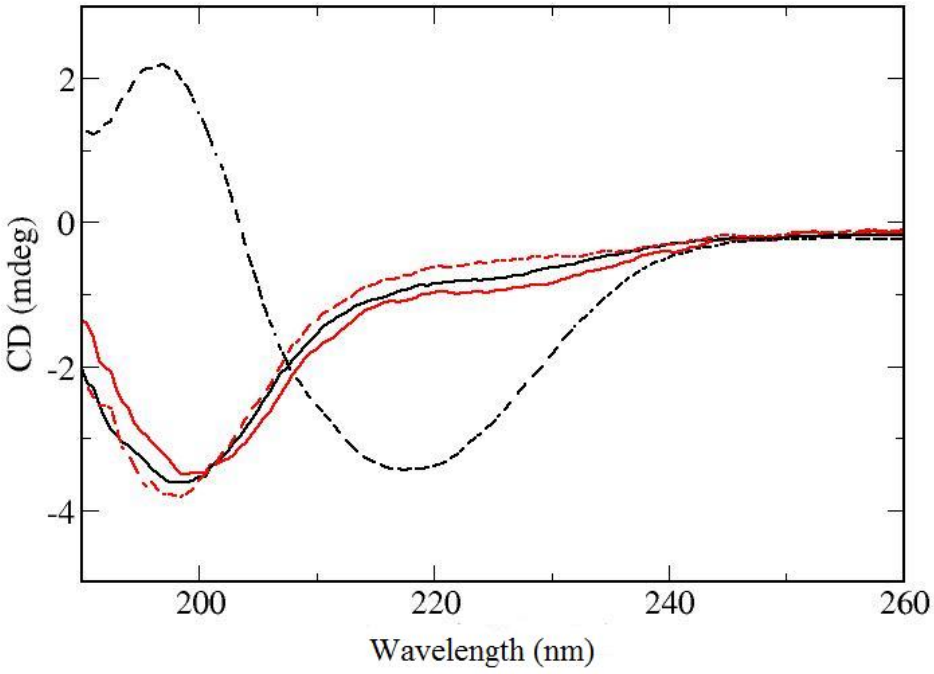


Figure 2: Influence of Hsp60 on Aβ aggregation secondary structure variations during fibrillogenesis. CD spectra recorded at 20 °C for 50 µM $A\beta_{1-40}$ at initial time (black line) and after 24 hrs at 37 °C and 200 rpm (black dotted line), compared with spectra of $A\beta_{1-40}$ incubated with 2 µM Hsp60 at the initial time (red line) and after 24 hrs at 37 °C and 200 rpm (red dotted line). The CD contribution of Hsp60 was subtracted.

On the other hand, the sample incubated with Hsp60 retains a typical random coil profile until the end of the kinetics. Therefore, CD experiments agree with ThT assays, confirming that Hsp60 exercises an inhibitory role on the onset of $A\beta_{1-40}$ cross-ß-structure formation that typically accompanies the peptide assembly toward more ordered structures. Other details on the structural organization of these samples were obtained by AFM imaging. Figure 3 shows images acquired at initial and final state for both samples incubated with or without Hsp60. It is noteworthy that the fibrillation is evident for the sample of $A\beta_{1-40}$ alone, whereas the peptide treated with Hsp60 does not show fibres, and no significant differences between the initial and the final step of the kinetics are detected.

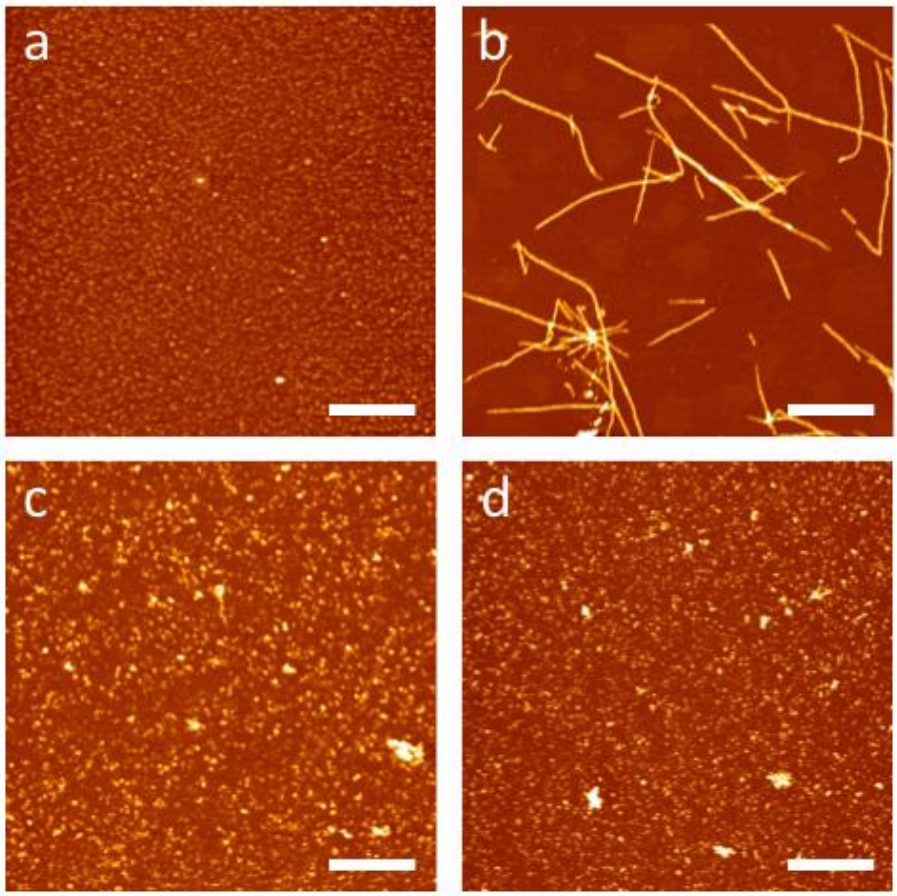


Figure 3: Morphology of Aβ species formed in the presence of Hsp60 under amyloid aggregation conditions. AFM images acquired for: 50 µM $A\beta_{1-40}$ at the initial time (a) and after 24 hrs incubation at 37 °C and 200 rpm (b) compared with 50 µM $A\beta_{1-40}$ incubated with 2 µM Hsp60 at the initial time (c) and after 24 hrs of incubation at 37 °C and 200 rpm (d). Scale bars: 1 µm, Z-range: *(a,b,c)* 7 nm; *(d)* 9.6 nm.

3.2 Investigation into structural features and amyloid propensities of the $A\beta_{1-40}$ species isolated after incubation with Hsp60

Hydrodynamic size and oligomeric organization of $A\beta_{1-40}$ samples incubated for 24 hrs with or without Hsp60 were analysed by SEC using a Superdex 200 increase column. The chromatographic profiles, registered at 280 nm, are shown in Figure 4 and compared with the Aβ chromatographic profile registered at the initial state of the aggregation kinetics. The early part of the chromatogram (7−10 min) accounts for Hsp60 in tetradecameric and heptameric structures. In fact, the column used in these experiments allows us to separate the two oligomeric species that, in the naïve version of the same protein and by using two different columns in series, appeared under the same chromatographic peak [36]. Hsp60 monomers and their fragments fall under the region of the chromatogram between 10 and 14 min (refer to Fig. S1 S.M.), whereas $A\beta_{1-40}$ peptide peak is observed in the region between 16 and 18 minutes. Although, according to the protocol followed (see Materials and Methods paragraph for further information), this Aβ peak should be homogenous, we are aware that the technique used cannot distinguish between monomers or very low molecular weight Aβ peptide oligomers, such as dimers or trimers. Therefore, in the following text, we will refer to the Aβ fraction as a monomers/low molecular weight oligomers (LMWO) peak. If we focus our attention on the last part of the chromatogram where $A\beta_{1-40}$ elutes, we note that the Aβ chromatographic area under monomers/LMWO peak results notably reduced for the sample incubated at 37 °C for 24 hrs. This result is in agreement with the experimental results presented before. The missing sample, which is equivalent to the 70% of the sample injected before the 37 °C/24hrs incubation, is the component of Aß molecules consumed in the formation of amyloid large prefibrillar structures and mature fibres, even though they are not detected in the chromatogram because of their large dimensions, as evidenced by AFM experiments. This was corroborated by the column calibration, which confirmed that permeation inside the column beads is hindered for molecules larger than 1000 kDa (the equivalent of 240 Aβ molecules). Moreover, SEC calibration is performed by using globular proteins (more details in the Materials and Methods session). Therefore, any disorder in Aß fibrils resulting in a higher Stokes radius value, as well as the influence of their oblate shape on the friction coefficient, make large aggregates hydrodynamically not comparable to globular proteins [47], thus suggesting that also fibrils with

molecular weight lower than 1000 kDa may be prevented from permeating the column. The residual part of the sample (30%) corresponding to the monomers/LMWO peak, which did not undergo fibril formation within 24 hours, recovered at the end of its incubation when incubated again under the amyloid formation protocol (stirring and 37 °C resulted in the sample only being abe to form a low/small amount of fibrils (data not shown). Probably, this part of the sample is constituted mainly by species which are off-pathway with respect to amyloid formation, otherwise it would be difficult to explain why they have not been incorporated into the amyloid aggregates.

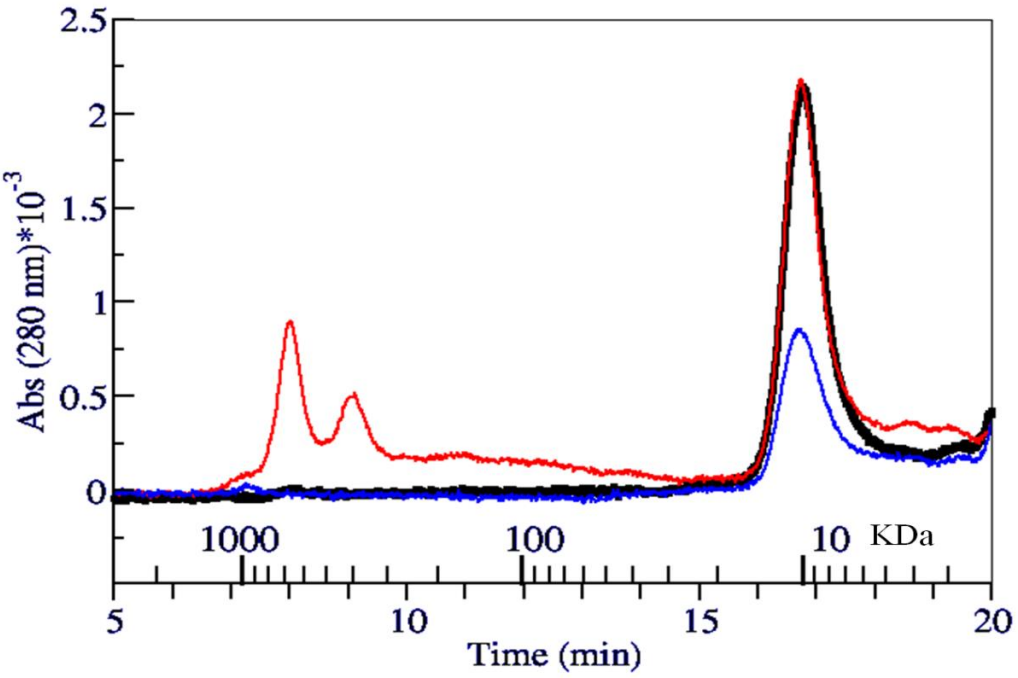


Figure 4: Investigation into the influence of Hsp60 on oligomeric distribution of Aβ after incubation under amyloid aggregation conditions. Analytical chromatographic profiles recorded at 280 nm for 50 µM $A\beta_{1-40}$ freshly dissolved (black line) and after 24 hrs of incubation at 37 °C and 200 rpm with or without 2 µM Hsp60 (red line and blue line, respectively). The column was calibrated using globular protein standards.

By contrast, when we compare the elution profile of $A\beta_{1-40}$ treated with Hsp60 under stirring at 37 °C with a sample of freshly prepared Aβ, no significant difference between the two areas referred to the monomeric/LMWO peaks is observed (red and black lines in Fig. 4). This suggests that the Aß species that participate to the amyloid formation cascade are not recruited anymore when Hsp60 is present, thus confirming the amyloid inhibitory action exerted by the

chaperonin. Since the area under that peak is essentially unaffected by the incubation with Hsp60, the inhibition of amyloid aggregation cannot be due to a sequestering action exerted by the chaperonin of a significant part of Aß peptide sample. In order to clarify which mechanism governs the anti-fibrillogenic action of Hsp60 and the nature of interaction between Aβ and Hsp60, our next goal was to understand whether Aβ exposed to Hsp60, that is almost 100% of the corresponding initial sample, retains its ability to form amyloid fibrils once Hsp60 is removed. Therefore, we collected and further investigated the peptide fraction corresponding to the sample eluted between minute 16.2 and 18.0 (Fig. 4). In particular, we focused our attention on the propensity of the amyloid to fibrillate when incubated at 37 °C and 200 rpm. For this purpose, the fraction of Aβ recovered after SEC-HPLC preparative injection was concentrated and analysed in its amyloid behaviour. Quite interestingly, ThT fluorescence value did not present any increase during the 24 hr kinetics of Aβ separated from Hsp60 by HPLC-SEC. In agreement with fluorometric data, Figure 5 shows that both SEC-eluted Aβ samples, at 24 hrs and after 3 days of aggregation kinetics at 37 °C, are characterized by the same random coil CD spectra. Moreover, the spectra are essentially undistinguishable from the one relating to freshly dissolved Aβ. In addition, no aggregates could be detected by AFM or TEM.

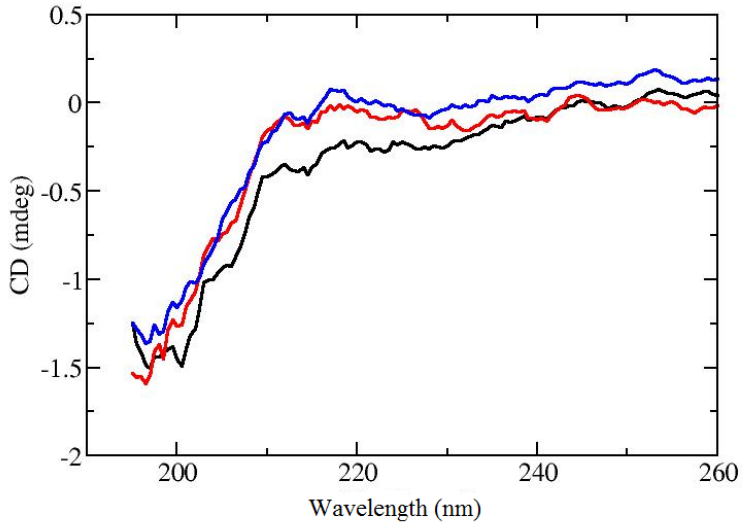


Figure 5: Analysis of amyloidogenic behaviour of Aβ after incubation with Hsp60 under amyloid aggregation conditions. CD spectra of 30 µM $A\beta_{1-40}$ recovered by SEC injection of 50 µM $A\beta_{1-40}$ incubated for 24 hrs at 37 °C under stirring in the presence of 2 µM Hsp60 at the beginning (black line), after 24 hrs at 37 °C under stirring (red line) and aged 3 days at 37 °C (blue line).

Finally, although the purification of Aβ peptide by size exclusion chromatography is an ordinary practice that does not influence the amyloidogenic nature [48], to exclude any possible chromatographic effect on the fibrillation, the same experimental procedure was applied to a 50 μM Aβ sample freshly dissolved in sample buffer. After collecting the fraction corresponding to the peak in the region between 16.2 and 18.0 minutes (black line Fig. 4), the peptide was concentrated and incubated at 37 °C and 200 rpm. Both ThT assay and CD spectra recorded at the beginning and after 24 hrs incubation clearly show that the sample maintains the propensity to fibrillate (refer to Fig. S2 Supplementary Material for details).

3.3 Experimental evaluation of Hsp60 action mechanisms

The overall results reported so far allow the conclusion that the fraction of Aβ sample corresponding to on-pathway amyloid species if incubated with Hsp60 lose their tendency to fibrillate. Several possible scenarios for Hsp60 inhibitory mechanism can be envisaged. The first hypothesis we tested was whether Hsp60 was able to carry out a 'folding' action on the peptide. This hypothesis would attribute to Hsp60 the role of a 'professional chaperone' exerting a 'folding' action [8] by inducing a conformational change of Aβ peptide upon binding. The new conformation of Aβ peptide, induced by Hsp60 interaction, would prevent amyloid fibril formation. In order to test the 'folding hypothesis', we adopted a different protocol to evaluate the effect of the molecular interaction between the two proteins. A sample of Aβ peptide was kept with Hsp60 at 4 °C for 24 hrs without stirring, a condition in which the nucleation process, essential for amyloid fibrillation, is not favoured. In fact, it has been reported that stirring or shaking combined with high temperature promotes amyloid polymerization by shortening the lag phase and speeding up the fibrillation process. Indeed, additional mixing accelerates polymerization by breaking up large complexes and increasing the collision rate of reactive species with each other and with fibre ends. Furthermore, a higher temperature favours hydrophobic interactions of the structures lowering energy barriers for β-sheet amyloid formation and that act as seeds for the other on-pathway species [44, 45]. Thus the stirring-temperature effect would be similar to a 'cross-seeding' effect in which seeds would act as catalytic sites that induce conformational changes in the protein and accelerate the reaction rates

('nucleated conformational conversion') [29, 44, 45]. We previously showed that, when incubated at 37 °C and in the absence of stirring, Aβ forms amorphous, off-pathway species [49]. At low temperature and without stirring, the nucleation phenomenon is very slow and the chaperonin would exert its action on peptide conformation under non-destabilizing conditions. A sample of 50 μM Aβ incubated with 2 μM Hsp60 was kept at 4 °C for 24 hrs, and separated by SEC. The fraction of Aβ peptide was concentrated and the peptide's ability to form amyloid fibrils was tested by applying the protocol previously used (incubation at 37 °C under stirring at 200 rpm rate). Panel a) of Figure 6 shows the time course measurement of ThT fluorescence, and, in the inset, the CD spectra recorded at initial time, after 24 hrs at 37 °C and stirred at 200 rpm, and after aging for 3 days at 37 °C. The increase in ThT fluorescence and the change in the CD spectra from random coil to β-sheet structure indicate the formation of amyloid structures. Furthermore, after 3 days of incubation long fibrillar structures have been observed by TEM and AFM imaging of the same sample (panel b and c, respectively). These results altogether unequivocally show that the Aβ sample recovered after interaction with Hsp60 at 4 °C undergoes amyloid aggregation once incubated at 37 °C and 200 rpm.

This evidence suggests that the inhibition of the Aβ aggregation process and fibril formation observed in the sample incubated with Hsp at 37 °C cannot be simply ascribed to a conformational change in the peptide structure as a result of Aβ−Hsp60 interaction. Our results suggest that the chaperone is able to exert its inhibitory action only under stress conditions and, in particular, in the presence of combined factors (high temperature and stirring) that favour the formation of on-pathway seeding species.

3.4 Study of Hsp60 effect dose dependence

In order to get an insight into the possible mechanisms of Hsp60-induced amyloid inhibition, the effect on Aβ fibrillogenesis of different stoichiometric ratios of Aβ:Hsp60 peptide was investigated. The amyloid aggregation kinetics was followed by ThT assay. Figure 7 shows the results obtained for four different Aβ:Hsp60 molar ratios (100:1, 75:1, 50:1, 25:1) compared to the kinetic profile recorded in absence of chaperonin.

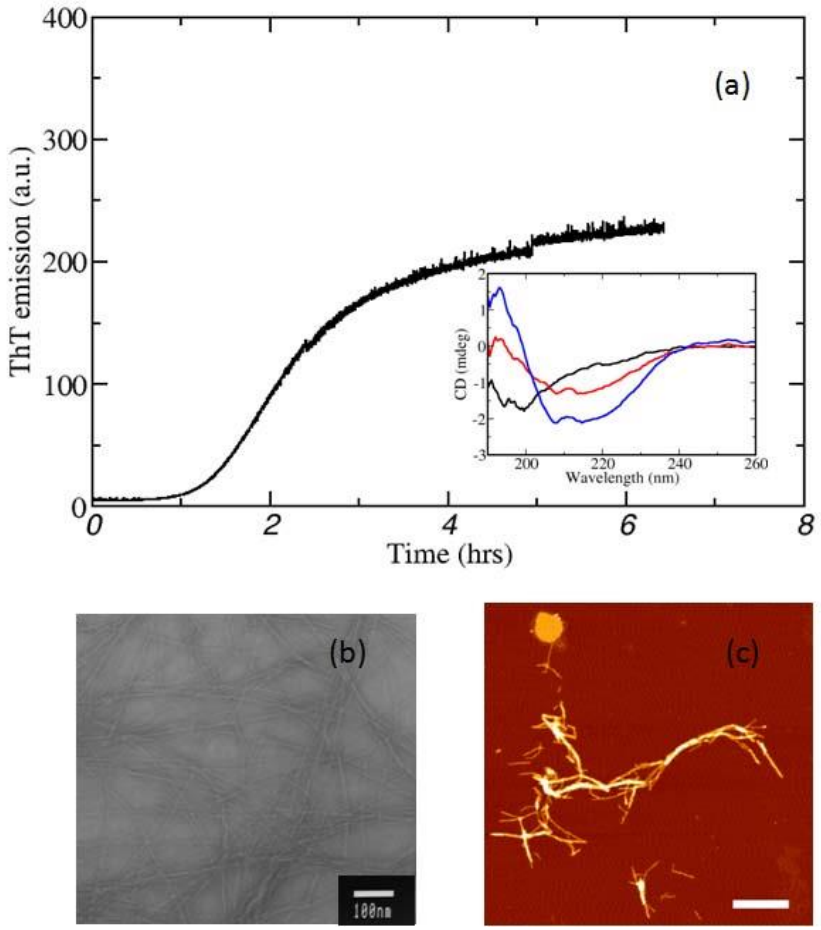


Figure 6: Analysis of amyloidogenic behaviour of Aβ after incubation with Hsp60 under non-destabilizing conditions (4 °C and no stirring). Kinetics of 30 µM Aβ recovered by SEC injection of 50 µM $A\beta_{1\text{-}40}$ incubated with 2 µM Hsp60 at 4 °C for 24hrs: a) 12 µM ThT fluorescence assay during incubation at 37 °C and 200 rpm. In the inset CD spectra recorded at 20 °C for sample at the beginning (black line), after 24 hrs at 37 °C under stirring (red line) and aged 3 days at 37 °C (blue line); TEM (b) and AFM (Scale bar: 500 nm, Z-range: 7.4 nm) (c) images respectively for aged sample.

AFM images showing the morphology of all samples at the end of the process are also reported. It can be noted that the lag phase preceding fibrillation increases with increasing concentrations of Hsp60. A sub-stoichiometric dose dependence in the inhibition of amyloid formation has already been observed for other inhibitors [32, 33], even if without the apparent strong discontinuity observed in our case when going from 75:1 to 50:1 ratio. The dose dependence presents mechanistic similarities to the nucleation process of different amyloid-forming proteins, at varying protein concentrations: the lower the protein concentration, the higher the lag-phase

[50]. In this perspective, the presence of Hsp60 seems to reduce the effective Aβ concentration leading to the formation of initial nuclei, thus interfering with the lag-phase.

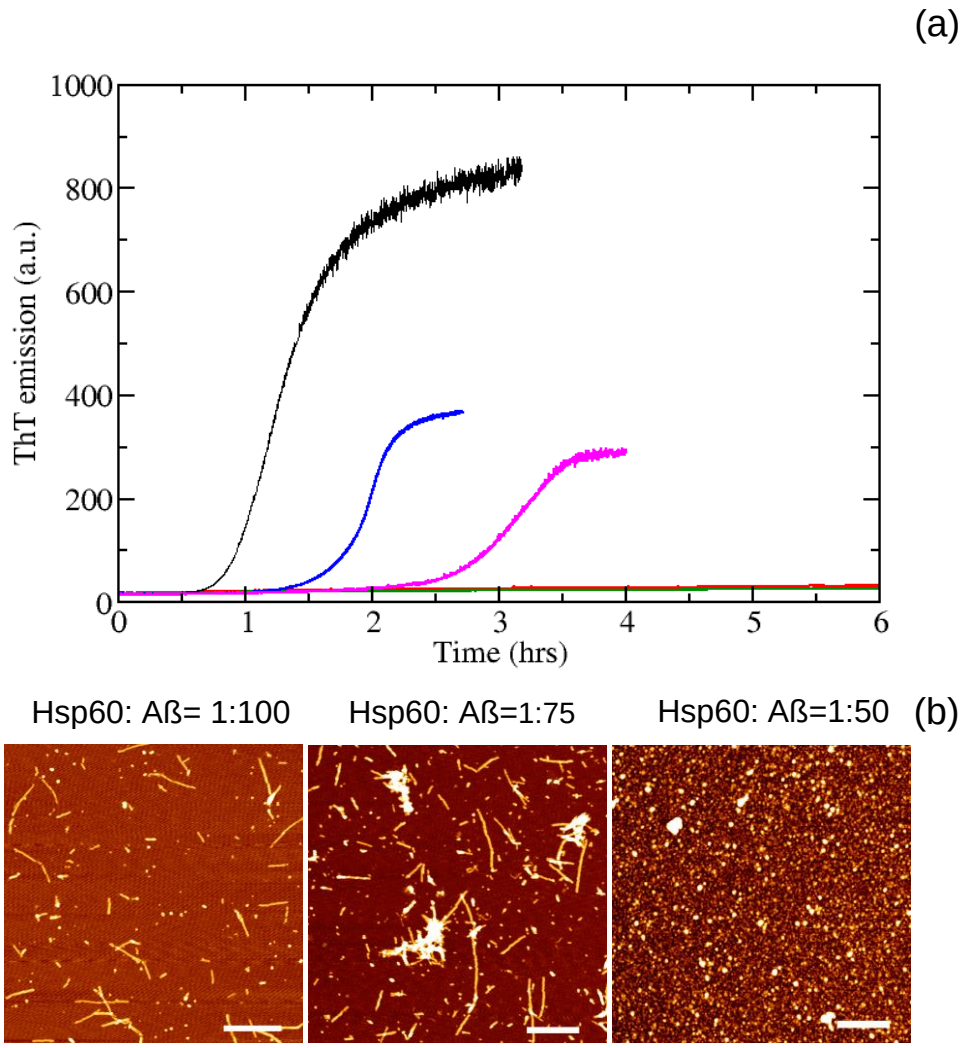


Figure 7: Study of the dose-dependent effect of Hsp60 (a) Dose-dependent effect of Hsp60 on the amyloid aggregation time course of 50 μM $Aß_{1-40}$, monitored by ThT fluorescence spectroscopy. Kinetics were performed at the following Hsp60:Aβ molar ratios: 1:100 (blue), 1:75 (pink), 1:50 (green). The kinetics relative to peptide in the absence of chaperonin (black) and at the molar ratio 1:25 (red) are also reported in the figure for comparison. (b) Samples at the end of the kinetics have been visualized by AFM (Scale bar: 500 nm, Z-range: 12 nm for 1:100 molar ratio; Scale bar: 500 nm, Z-range: 12.3 nm for 1:75 molar ratio; Scale bar: 500 nm, Z-scale: 8.0 nm for 1:50 molar ratio).

Therefore, the specific mechanism of action of Hsp60 as $A\beta_{1-40}$ inhibitor would be a stabilizing effect exerted on the on-pathway seeding species involved in ß-sheet formation and monomers' amyloid assembly [51]. It can be further hypothesized that Hsp60 influences these reactive species formation, or, more probably, interacts with them by sequestering them through a holding action, or favouring their conversion from pathological strains to different off-pathway conformers. The latter mechanism hypothesized, if clarified by further evidences, could also be invoked to explain the strong discontinuity observed in the dose dependence (see above) and the incapability of a sample treated with Hsp60 to form amyloid fibrils even after the removal of chaperonin (see Fig. 5 and the relative discussion).

4. Discussion

Recent studies investigating direct interactions between chaperones and proteins involved in amyloid diseases confirm that molecular chaperones are characterized by an extraordinary protective nature that can be extremely attractive in the field of neurodegeneration, in particular for AD [4, 9, 10, 12, 13, 52]. Based on the Aβ cascade hypothesis, several therapies designed for AD therapy or prevention are aimed at discovering agents or molecules able to directly inhibit Aβ peptide aggregation or disrupt preformed aggregates [53]. The effectiveness of such approaches dramatically depends on several factors, such as the ability to cross the blood−brain barrier, and the time gap between the onset of molecular events triggering the pathology and the appearance of early symptoms that can be diagnosed [53]. All these aspects need to be taken into consideration in the perspective of the chaperone-therapies design based on the pharmacological delivery or overexpression of chaperones. However, a critical step toward the development of any chaperone-based therapeutic strategy and prior to clinical trials is the understanding of molecular mechanisms governing the interaction between these proteins and Aβ species, and how this interaction prevents the amyloid-driven neurotoxic effect. In this study, we investigated the effect of mitochondrial the Hsp60, the human homologue of bacterial chaperonin GroEL, on the fibrillogenesis of $A\beta_{1-40}$ involved in AD. Although Hsp60 and $A\beta_{1-40}$ have different physiological localizations, in pathological situations they can co-localize and directly interact. In fact, it has already been shown that Aβ accumulates in the mitochondria of AD patients [54] and it has been suggested that

intracellular and mitochondrial accumulation of Aβ precedes extracellular Aβ deposition [55]. Aβ can be imported into mitochondria via the TOM import machinery, independently of the mitochondrial membrane potential, and can affect the functional mitochondrial state [56] and Hsp60 provides differential protection against intracellular Aβ-amyloid stress even with mechanisms yet to be understood [57]. Very interestingly, recent studies show that Aβ and APP interaction with Hsp60 may disrupt its physiological role inside the mitochondria leading to organelle malfunction, but through mechanisms to be further clarified [42]. In addition, Hsp60 and Aß peptide can in general directly interact in cell compartments other than mitochondria as well as in the extracellular space, thus influencing the Aß amyloid aggregation process. It is tempting to speculate that, in response to the generation of intracellular Aß oligomers, a small fraction of cytosolic chaperones could be targeted to lipid rafts and become associated with Aß oligomers before their eventual secretion into the extracellular space [58]. In the case of Hsp60, particularly in pathological situations, the human chaperonin accumulates in the cytosol with or without mitochondrial export release [40, 59], and from the cytosol Hsp60 may reach other cellular compartments, such as the Golgi, secretory vesicles, and plasma membrane [60-63].

In this paper we tested the direct effect of Hsp60 on the $A\beta_{1-40}$ amyloid aggregation process. Our results show that in the presence of Hsp60, during the incubation of Thioflavin T with $A\beta_{1-40}$ under pro-aggregating conditions, i.e. stirring and 37 °C [44, 45], there is no variation in the fluorescence of the probe (Fig. 1), thus revealing that the chaperonin completely inhibits the formation of ß-sheet-rich amyloid aggregates. Moreover, at the end of the kinetics, the secondary structural conversion that accompanies amyloid formation for Aβ peptide is not observed in the presence of the chaperonin (Fig. 2) and no fibrils are observed by AFM (Fig. 3). Moreover, we elucidated that the effect is dose-dependent (Fig. 7), with a strong discontinuity when going from 75:1 to 50:1 molar ratio. The overall results indicate that Hsp60 is a powerful inhibitor of the process leading to amyloid fibril formation for $A\beta_{1-40}$.

Based on our evidence, several hypotheses about possible molecular mechanisms of Hsp60 could be formulated to explain the phenomena observed. The first, strongly suggested by the absence of ATP, would attribute to Hsp60 having a holding' role. In other words, Hsp60 in this case would act as a non-catalytic inhibitor of polypeptide aggregation, by sequestering single unfolded conformations of monomers [8]. SEC experiments helped us to gain more insight into this hypothesis. One of the greatest problems that it is possible to encounter during preparations of Aβ is that it typically contains a mixture of species ranging from dimers up to particles consisting of hundreds of monomers [43]. The approach followed by our preparation protocol

(see Materials and Methods paragraph for details) ensures the production of an Aβ homogenous sample that, when analysed by SEC, presents a relatively sharp peak; however, the technique cannot distinguish between monomers or very low molecular weight Aβ peptide oligomers, such as dimers or trimers, neither is it able to distinguish between on-pathway and off-pathway species (Fig. 4). We called this the monomers/LMWO peak. After incubation at 37 °C under stirring, the Aβ sample, as a consequence of fibril formation, presents a significant reduction in the low molecular weight oligomeric peak (70% of the initial sample) (see scheme reported in Fig. 8a). The residual part of this peak, not involved in amyloid aggregation, is formed by off-pathway species that, when incubated again under amyloid formation conditions, result only in being able to form very low amounts of amyloid fibrils. In the presence of Hsp60 under the same conditions, no large difference in the chromatographic profile is noticeable in the Aβ sample other than peaks corresponding to chaperone oligomers. In particular, the low molecular weight oligomeric peak seems to be almost unvaried compared to the freshly dissolved Aβ sample. This also means that the fraction of Aβ molecules that in the absence of Hsp60 would be involved in amyloid fibril formation, in the presence of the chaperonin is unaffected both in hydrodynamic size and amount compared to the freshly dissolved sample. The Aβ molecules normally involved in the aggregation pathway leading to fibres are no longer recruited in the presence of Hsp60. Moreover, this evidence strongly excludes the possibility that the molecular chaperone sequesters the total Aβ sample, or a substantial part of it, by exerting a global holding action on Aβ molecules. In addition, for this sample, even after the removal of the chaperonin and a second incubation under amyloid-promoting conditions (37 °C and stirring) no amyloid formation was observed (Fig. 5 and (see scheme reported in Fig. 8b). These results lead naturally to the hypothesis that Hsp60, following its professional activity but in the absence of ATP, induces a conformational change in Aβ peptide that irreversibly impairs any further aggregation. Differently from GroEL, whose chaperone molecular actions are widely studied and known to involve co-chaperonin GroES and ATP in an allosteric communication between the two heptameric rings [64, 65], the functional mechanisms of the mitochondrial human homologue Hsp60 are very poorly investigated. Differences in the key residues involved in the double-ring ring formations reflect the existence of a dynamic equilibrium in solution between monomers, heptamers and tetradecamers conformations [36, 39, 66]. Indeed a single ring seems to be sufficient for folding assistance *in vivo* [66], even though potential mechanisms involving the

interaction with co-chaperonins should be further clarified. Certainly, similarly to GroEL, the apical domain of Hsp60 contains hydrophobic residues that could be the most involved in the misfolded proteins recruitment [67]. However, our data exclude any active influence of Hsp60 influence on Aβ folding, either monomers or low molecular weight oligomers present in the sample after the preparation protocol but before incubation with the chaperone. In fact, no variation in amyloid aggregation behaviour is detectable in the Aβ sample isolated after incubation with the chaperone under non-destabilizing conditions (4 °C without stirring) (Fig. 6). The only acceptable conclusion is that Hsp60 might act selectively on the specific Aβ amyloid species activated by simultaneous stirring and temperature increase, or by preventing their formation, or, as is more probable, by inhibiting their activity by a folding or a holding action (see scheme reported in Fig. 8). These seeds although very low in number, have the proper molecular determinants to constitute productive steps in the fibrillation pathway and to determine the behaviour and amyloid aggregation in the highest fraction of the whole Aβ population [68]. It is well known, in fact, that a given protein can self-assemble into various aggregated forms, depending on the peculiarities of its environment. The presence of active seeds paves the way among the heterogeneous routes leading to the various aggregated forms, of the amyloid on-pathway fibrillization [68]. In the presence of Hsp60, the recruitment or/and inactivation of these catalytic seeds close the route of on-pathway amyloid fibrillogenesis. Actually, as recently shown, a strong inhibition of aggregation can be observed, even at extremely low doses of inhibitors, for systems such as the Aβ peptide, where secondary nucleation mechanisms play a key role. In fact, in these cases, the whole aggregation process can be prevented by the sequestration of small amounts of aggregates before these seeds can trigger the autocatalytic proliferation of aggregates brought about by secondary nucleation [32, 33, 69, 70]. This shows an intriguing feature recently proposed for the activity of a natural molecular chaperone against misfolding events and associated reactions, indicating that the roles of chaperones are not limited to the sequestration of single monomeric unfolded conformations, but can involve multiple interactions with aggregated species [32, 33]. Our hypothesis is that the chaperone selectively exerts a selective action against the Aβ aggregated species that specifically form during on-pathway aggregation under environmental stress conditions, probably by means of interactions involving peculiar hydrophobicity and exposure to the solvent properties of these Aβ aggregates conformations. The structural polymorphism of Aβ oligomers is a widely accepted aspect of Aβ

amyloid fibrillogenesis with important implications in the analysis of structure-toxicity correlation and in the search of molecular therapeutic inhibitors [28, 68, 49]. In fact, even if similar in size and morphology, Aβ oligomers can represent distinct structural variants, with different epitopes for specific antibodies, different toxicity potential and specific pathophysiological transmission features. Indeed, very recently studies on transgenic micesuggested that specific Aβ aggregates are capable of self-propagation. These prion-like Aβ conformations could represent novel targets for interrupting the spread of Aβ deposition in AD patients [71].

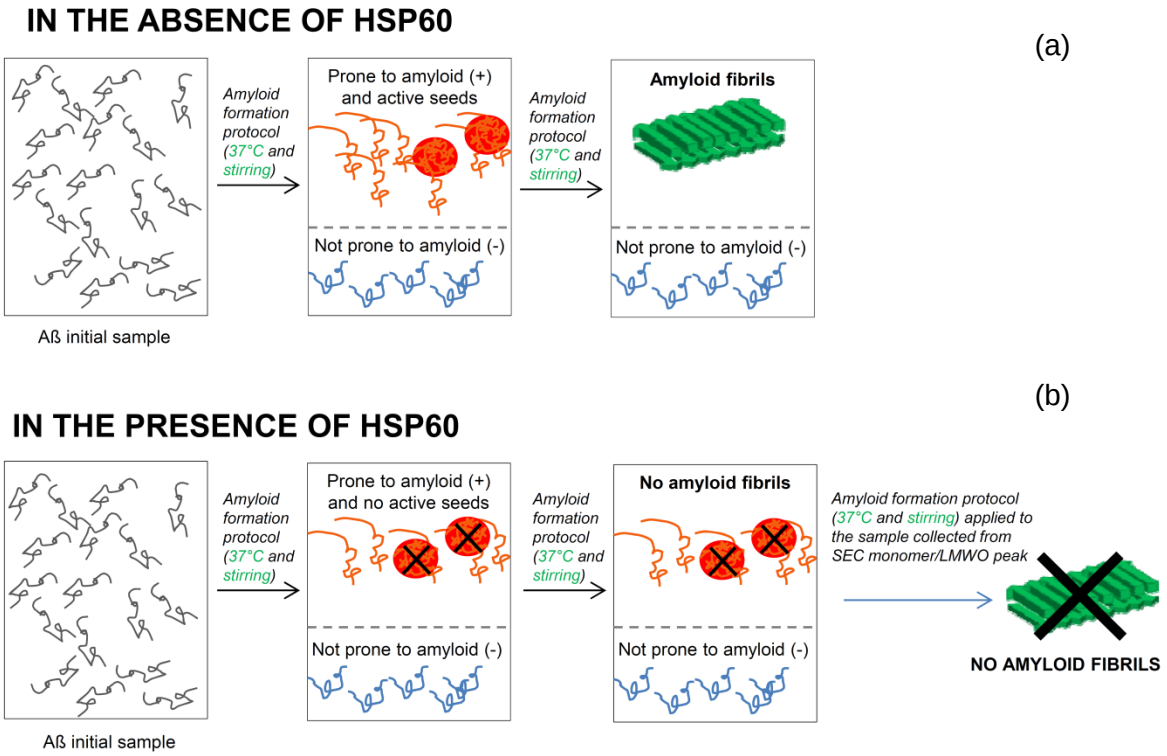


Figure 8: **Simplified scheme of Aß$_{1-40}$ amyloid aggregation in the absence (a) and in the presence (b) of Hsp60.** The initial sample is heterogeneously formed by generically described on-pathway and off off-pathway species (dark blue) with different amyloid molecular fates. The on-pathway species are comprised of seeding oligomers (red circles) whose structural determinants render them able to catalyze productive steps in the fibrillation pathway and of monomers that enter the fibrillogenesis route only in the presence of seeds (orange). The remaining off-pathway species are those not able to form fibrils. In the presence of Hsp60, the occurrence of active seeds is prevented and, in this condition and in the presence of off-pathway species, the pathway for new seeds and amyloid fibrils formation is blocked.

5. Conclusion

Our results demonstrate that Hsp60 is able to target the Aβ species responsible for the induction of amyloid protein assembly. Although, the specific mechanisms by which the presence of the chaperone neutralizes these types of Aβ protein aggregates, preventing their formation or inhibiting them, remain to be elucidated, our results further confirm the extraordinary potentiality of molecular chaperones in interfering with the crucial molecular steps leading to amyloid aggregation in neurodegeneration. Due to the high level of flexibility characterizing some regions of the protein structure, chaperones can interact with a very wide spectrum of biological molecules and protein conformations and with a high versatility of mechanisms of action [8] These molecular machines are evolutionally programmed to catch the more reactive species or protein conformations, and if required, as in the case of Hsp60 and Aβ oligomers, by exploiting amateur, non-classical mechanisms [30]. The most likely involved candidate forces driving the Hsp60−Aβ interaction are hydrophobic in nature, both for the greater molecular size of the chaperonin in respect to the peptide (favouring large non-polar surface patches exposition) and for the presence of hydrophobic clusters inside the chaperonins' cavity that are the most involved in recruitment of client proteins. Hydrophobic interactions are also responsible for Aβ (1-40) aggregation suppression exerted by GroEL as revealed by NMR [14]. However, very recently, protein−protein interactions between Hsp60 and Aß (1-42) have been characterized by a computational approach and strong interactions involving hydrogen bonding between Hsp60 and Aß residues have been found [72]. Once the Aβ active seeds are recruited through these interactions in the presence of only off-pathway species, also the highly prone to aggregate Aβ peptide becomes unable to form new seeds and subsequently to fibrillate.

Our study is still far from a deep comprehension of the molecular basis of a therapy that targets specific amyloid aggregation by treatment with or overexpression of chaperonins, and chaperones in general, as well as being far from an exhaustive evaluation of the potential pitfalls to be verified with specific clinical trials. However, in the last years, chaperones appear to be an emerging and hopeful therapeutic strategy for the field of neurodegeneration, and also suggest the potential for futuristic genetic engineering treatments. In this respect, we believe that the investigation into the biophysical features of the interactions between specific chaperones and

the specific protein structures involved in disease is a crucial basic step towards the development of effective chaperone-based approaches.

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Figure legends

Figure 1: Influence of Hsp60 on Aβ aggregation kinetics. Kinetics of 50 µM Aβ (black line) and 50 µM Aβ incubated with 2 µM Hsp60 (red line) at 37 °C and under 200 rpm stirring monitored by ThT fluorescence assay. [ThT]=12 µM.

Figure 2: Influence of Hsp60 on Aβ aggregation secondary structure variations during fibrillogenesis. CD spectra recorded at 20 °C for 50 µM $A\beta_{1-40}$ at initial time (black line) and after 24 hrs at 37 °C and 200 rpm (black dotted line), compared with spectra of $A\beta_{1-40}$ incubated with 2 µM Hsp60 at the initial time (red line) and after 24 hrs at 37 °C and 200 rpm (red dotted line). The CD contribution of Hsp60 was subtracted.

Figure 3: Morphology of Aβ species formed in the presence of Hsp60 under amyloid aggregation conditions. AFM images acquired for: 50 µM $A\beta_{1-40}$ at the initial time (a) and after 24 hrs incubation at 37 °C and 200 rpm (b) compared with 50 µM $A\ß_{1-40}$ incubated with 2 µM Hsp60 at the initial time (c) and after 24 hrs of incubation at 37 °C and 200 rpm (d). Scale bars: 1 µm, Z-range: *(a,b,c)* 7 nm; *(d)* 9.6 nm.

Figure 4: Investigation into the influence of Hsp60 on oligomeric distribution of Aβ after incubation under amyloid aggregation conditions. Analytical chromatographic profiles recorded at 280 nm for 50 µM $A\beta_{1-40}$ freshly dissolved (black line) and after 24 hrs of incubation at 37 °C and 200 rpm with or without 2 µM Hsp60 (red line and blue line, respectively). The column was calibrated using globular protein standards.

Figure 5: Analysis of amyloidogenic behaviour of Aβ after incubation with Hsp60 under amyloid aggregation conditions. CD spectra of 30 µM $A\beta_{1-40}$ recovered by SEC injection of 50 µM $A\beta_{1-40}$ incubated for 24 hrs at 37 °C under stirring in the presence of 2 µM Hsp60 at the beginning (black line), after 24 hrs at 37 °C under stirring (red line) and aged 3 days at 37 °C (blue line).

Figure 6: Analysis of amyloidogenic behaviour of Aβ after incubation with Hsp60 under non-destabilizing conditions (4 °C and no stirring). Kinetics of 30 µM Aβ recovered by SEC injection of 50 µM $A\beta_{1-40}$ incubated with 2 µM Hsp60 at 4 °C for 24hrs: a) 12 µM ThT fluorescence assay during incubation at 37 °C and 200 rpm. In the inset CD spectra recorded at 20 °C for sample at the beginning (black line), after 24 hrs at 37 °C under stirring (red line) and aged 3 days at 37 °C (blue line); TEM (b) and AFM (Scale bar: 500 nm, Z-range: 7.4 nm) (c) images respectively for aged sample.

Figure 7: Study of the dose-dependent effect of Hsp60 (a) Dose-dependent effect of Hsp60 on the amyloid aggregation time course of 50 µM $A\ß_{1-40}$, monitored by ThT fluorescence spectroscopy. Kinetics were performed at the following Hsp60:Aβ molar ratios: 1:100 (blue), 1:75 (pink), 1:50 (green). The kinetics relative to peptide in the absence of chaperonin (black) and at the molar ratio 1:25 (red)

are also reported in the figure for comparison. (b) Samples at the end of the kinetics have been visualized by AFM (Scale bar: 500 nm, Z-range: 12 nm for 1:100 molar ratio; Scale bar: 500 nm, Z-range: 12.3 nm for 1:75 molar ratio; Scale bar: 500 nm, Z-scale: 8.0 nm for 1:50 molar ratio).

Figure 8: **Simplified scheme of Aß$_{1-40}$ amyloid aggregation in the absence (a) and in the presence (b) of Hsp60.** The initial sample is heterogeneously formed by generically described on-pathway and off off-pathway species (dark blue) with different amyloid molecular fates. The on-pathway species are comprised of seeding oligomers (red circles) whose structural determinants render them able to catalyze productive steps in the fibrillation pathway and of monomers that enter the fibrillogenesis route only in the presence of seeds (orange). The remaining off-pathway species are those not able to form fibrils. In the presence of Hsp60, the occurrence of active seeds is prevented and, in this condition and in the presence of off-pathway species, the pathway for new seeds and amyloid fibrils formation is blocked.