Biochimica et Biophysica Acta 1860 (2016) 2474–2483

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Hsp60, amateur chaperone in amyloid-beta fibrillogenesis

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ARTICLE INFO

Article history:
Received 1 June 2016
Received in revised form 14 July 2016
Accepted 24 July 2016
Available online 26 July 2016

Keywords:
Molecular chaperones
Chaperonin
Amyloid aggregation
Inhibition mechanisms
Alzheimer's disease treatment

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 aggregation through a more complex mechanism than a simple folding chaperone action. The action is specifically directed toward 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

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 behaviour 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

Abbreviations: AD, Alzheimer's disease; AFM, atomic force microscopy; EM, electron microscopy; CD, circular dichroism; SEC, size exclusion chromatography; HSP, heat shock protein; ThT, Thioflavin T.

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http://dx.doi.org/10.1016/j.bbagen.2016.07.019
0304-4165/

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 favour 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 intrinsically 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 and 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, and 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 filtered with two filters in series 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 aseptic 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 h. 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 24 h. 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