Small heat shock proteins inhibit in vitro $A\beta_{1-42}$ amyloidogenesis

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Abstract We demonstrate that small heat shock proteins (sHsp) inhibit in vitro amyloid formation by the Alzheimer's $A\beta_{1-42}$ polypeptide as detected by a thioflavine T fluorescence assay and electron microscopy. Human sHsp27 (0.50–3.0 μM) inhibited amyloid formation from 20 μM $A\beta_{1-42}$ by 23–75% in 24 h. In contrast, treatment of pre-formed amyloid with 0.5–3.0 μM sHsp27 only reduced the fluorescence signal by 6–36%. The data suggest that ordered fibril formation may represent a form of off-pathway aggregation that can be prevented by chaperone action. The data raise the possibility that age-related changes in chaperone function could contribute toward the pathogenesis of Alzheimer's and other amyloid-associated diseases.

Key words: Heat shock protein; Chaperone; Alzheimer's disease; Amyloidogenesis

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by intravascular or extravascular fibrillar protein deposits in intracellular tangles and extracellular plaques [1]. Extracellular plaques may be either diffuse with limited degeneration of surrounding neurites or neuritic with extensive damage to surrounding neurites and increased numbers of associated microglia and oligodendrocytes [1]. Both plaque types are comprised of a mixture of 39–43 amino acid polypeptides, designated Aβs [2,3], that originate by the processing of several integral plasma membrane proteins (677–770 amino acids) derived from the amyloid precursor protein (APP) by alternative splicing of a single gene on chromosome 21 [4–6]. Aβs spontaneously form amyloid in vitro [7] and are cytotoxic in cell culture [8–10] and in vivo when injected into rat and monkey brain [11,12]. In aged primate cortex, $A\beta_{1-40}$ produced lesions whose size was dose dependent and which closely resembled those in AD [12].

The mechanisms by which amyloid kills cells are unknown, although recent evidence implicates microglial cell-mediated oxidative stress mediated by extracellular amyloid interaction with the receptor for advanced glycation end products (RAGE) [13]. Cell death is commonly associated with all amyloidoses. For example, amyloid formation by the human islet amyloid precursor protein (IAPP) is associated with loss of pancreatic β cells in non-insulin dependent diabetes mellitus (NIDDM). We demonstrated that expression of IAPP in COS-1 cells led to the formation of intracellular amyloid and cell death [14]. COS-1 cells were killed by intracellular amyloid concentrations that were far lower than those required to kill cells by adding IAPP to cultured cells [15], suggesting that intracellular amyloid deposition may represent a primary event in the pathogenesis of many amyloidoses, including NIDDM and AD [14]. A corollary of this hypothesis is that cells have evolved protective mechanisms to either prevent amyloidogenesis or counteract the cytotoxic actions of intracellular amyloid [14] with disease onset being due to an alteration or breakdown in the cell's protective mechanisms.

To begin to test this hypothesis, we investigated an in vitro model of amyloidogenesis by which a number of candidate anti-amyloidogenic mechanisms could be readily evaluated. We examined in vitro amyloidogenesis by $A\beta_{1-42}$, since this system has been extensively characterized [16,17]. As an initial candidate protective mechanism, we chose to study the effects of heat shock proteins (Hsp)/chaperones on amyloidogenesis. These proteins form a large, diverse family of essential proteins that are distributed in virtually all subcellular compartments and whose primary function is to mediate trafficking, folding and assembly of monomeric and oligomeric polypeptides into their physiologic structures [18]. Although the exact mechanisms by which heat shock proteins help to fold proteins is unknown, one of their principal actions is to prevent off-pathway aggregation, a non-productive event which prevents proteins from acquiring their functional conformation. Therefore, we sought to determine if amyloidogenesis represented a form of off-pathway aggregation, that could be intervened through some Hsp/chaperone function.

2. Materials and methods

2.1. Peptide synthesis

$A\beta_{1-42}$ was synthesized (ABI 431A synthesizer, Perkin Elmer/Applied Biosystems) using triple couplings for the R_5 and H_6 residues to prevent generation of peptides lacking one or both of these residues. The sequence of the peptide was verified by direct sequencing. Amino acid analysis revealed that the crude peptide was 72% pure by weight and after purification by reverse phase HPLC on a Vydac C8 column (Separations Group), the peptide content was reduced to 54% by weight. The crude and purified peptides contained 8.9 and 2.4% trifluoroacetate (Galbraith Laboratories) by weight, respectively. Since sequence analysis of the crude peptide indicated that the material was not significantly contaminated with deletion peptides, the difference in peptide composition is most likely due to differing water content. The behavior of the crude and purified peptides in amyloidogenesis assays was comparable, although the HPLC purified peptide formed amyloid at somewhat slower rates. Therefore, the crude material was used for all of the studies reported below.

2.2. In vitro amyloidogenesis

Reactions were performed in 10 mM Tris-HCl (pH 7.4), 100 mM

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NaCl, 5% dimethylsulfoxide (DMSO) in a final volume of 300 μl with constant stirring at 22°C. $A\beta_{1-42}$ in DMSO (100–400 μM) was added to the reaction mixture at a 1:20 dilution to achieve the final concentrations of 5–20 μM. Human sHsp27, murine sHsp25 and α-crystallin, containing both A and B forms, were from StressGen Biotechnologies, Inc. Lactate dehydrogenase (LDH) and bovine serum albumin (BSA) were from Sigma Chemical Co.

2.3. Thioflavine T (ThT) fluorescence assay

Fluorescence was measured on a SLM 8000 spectrofluorometer (Spectronic Instruments, Inc.) at 22°C with excitation and emission wavelengths of 450 and 482 nm, respectively. Fluorescence assays were performed in 50 mM glycine (pH 9.0), 1 μM ThT in a final volume of 1.5 ml according to Naiki et al. [19–21].

2.4. Electron microscopy

Samples were spread on carbon-coated Formvar grids, stained with 1% phosphotungstic acid for 15 min, and washed with distilled water over dental wax. Specimens were examined with a Jeol 1200 electron microscope with an acceleration voltage of 60 kV.

2.5. Data analysis

All experiments were replicated at least three times. Initial rates (arbitrary fluorescence units h^{-1}) were calculated from linear regression slopes of the raw data. Initial rates were analyzed by multivariate ANOVA with post hoc Bonferroni *t*-tests.

3. Results

Amyloid is a highly ordered fibril of varying length with a diameter of ca. 8–12 nm that is rich in β-pleated sheet structure [22]. To examine the influence of heat shock proteins/chaperones on amyloid formation, an assay specific for this ordered structure was required. Although many studies of in vitro amyloid formation have utilized light scattering measurements, non-ordered (amorphous) aggregation may contribute to the signal. Since heat shock proteins can prevent off-pathway aggregation, they may exert differential effects on non-ordered versus ordered aggregation. Thus we employed a fluorometric assay which depends on the altered fluorescent properties of ThT when bound to amyloid [19–21,23] that could distinguish these two types of aggregation. We studied amyloid formation by $A\beta_{1-42}$, since previous in vitro kinetic analyses have indicated that $A\beta_{1-42}$ formed amyloid at much higher rates than shorter Aβs [17].

3.1. Characterization of in vitro amyloid formation by $A\beta_{1-42}$

We characterized the ThT assay for $A\beta_{1-42}$ and found the data comparable in all respects to those of LeVine [23] who studied $A\beta_{1-40}$ with this assay. For this characterization we measured the initial rates of amyloid formation over a 6 h period and studied the effects of varying substrate and salt concentrations, temperature and pH (Table 1). ThT fluorescence increased linearly through 6 h and the initial rates were calculated by linear regression analysis (data not shown). There was a direct relationship between the initial concentration of $A\beta_{1-42}$ and the initial rate of amyloidogenesis (Table 1). The rate of amyloid formation was directly related to the temperature (Table 1), suggesting that this reaction is hydrophobically driven. In agreement with previous studies [23,24], the rate of amyloid formation was minimal at low pH and maximal at pH 6.5–8.5 (Table 1). Electron micrographs of samples of the reactions at pH 6.5–9.5 exhibited prominent fibrils at pH 6.5, 7.4, 8.5 and 9.5 (data not shown). Interestingly, the relative abundance and width (Table 1) of fibrils in a given EM field increased considerably above pH 7.4. These data suggest that fibril structure and ThT binding to fibrils are pH dependent. Na^+ and K^+ (50–100 mM) had only modest effects on amyloid formation, while 200 mM K^+ inhibited the initial rate of amyloidogenesis.

3.2. Effect of small heat shock proteins on amyloid formation

The effects of adding various concentrations (0.5–3.0 μM) of human sHsp27 to a reaction containing 20 μM $A\beta_{1-42}$ as a function of time is shown in Fig. 1A,B. After 24 h substoichiometric amounts of sHsp27 exerted a significant 30% reduction in amyloid formation as assessed by the fluorescence assay at a molar ratio of 0.025. At higher molar ratios of sHsp/$A\beta_{1-42}$ there was a progressive increase in inhibition until at a ratio of 0.15, amyloid formation was inhibited by

Table 1
Effects of substrate concentration, temperature, pH and salt concentration on the initial rates of $A\beta_{1-42}$ amyloidogenesis

$A\beta_{1-42}$ (μM)	Parameter varied	Initial rate[a]	Fibril width (nm)[b]
5	substrate concentration	40.7 ± 13.0*	
10		103.7 ± 20.3*	
20		**200.0 ± 11.1**	
20	temperature = 4°C	14.8 ± 7.4*	
	temperature = 22°C	251.9 ± 18.5*	
	temperature = 37°C	**400.0 ± 7.4**	
20	pH = 3.4	42.9 ± 14.3*	not detected
	pH = 5.5	25.0 ± 8.9*	not detected
	pH = 6.5	200.0 ± 21.4	6.1 ± 0.4*
	pH = 7.4	**162.5 ± 41.1**	**10.5 ± 0.4**
	pH = 8.5	187.5 ± 75.0	9.6 ± 0.4
	pH = 9.5	100.0 ± 16.1*	13.8 ± 0.5*
20	Na^+/K^+ (0 mM)	**200.0 ± 5.7**	
	Na^+ (50 mM)	294.3 ± 5.7	
	Na^+ (100 mM)	256.6 ± 22.6	
	Na^+ (200 mM)	184.9 ± 7.5	
	K^+ (50 mM)	230.2 ± 25.7	
	K^+ (100 mM)	149.1 ± 13.2	
	K^+ (200 mM)	124.5 ± 15.1*	

[a]Average of linear regression slopes for 6 h kinetic data from three independent experiments expressed as arbitrary fluorescence units h^{-1}.
[b]Measured from electron micrographs.
*Significantly different from control group (data in bold type) at the $P < 0.05$ level as described in Section 2.

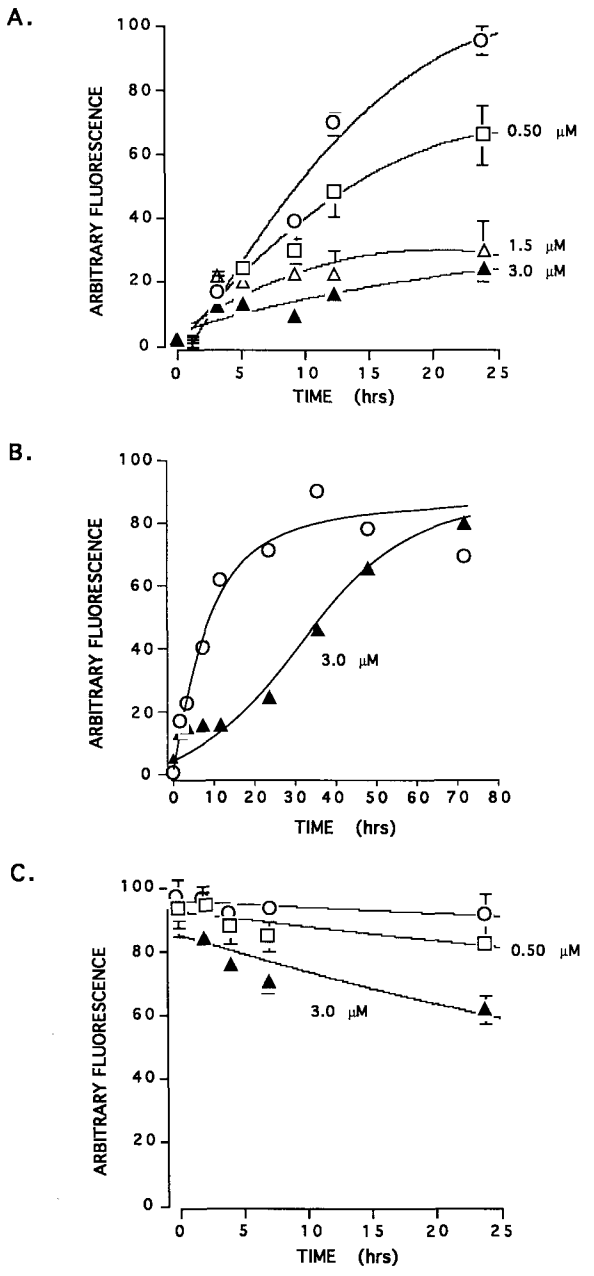

Fig. 1. Kinetics of in vitro amyloid formation (A) and depolymerization (B) by $A\beta_{1-42}$ in the presence of increasing concentrations of human sHsp27. A: Kinetic analysis of ThT fluorescence intensity (arbitrary units) of 20 μM $A\beta_{1-42}$ in the absence (open circles) or presence of varying concentrations of sHsp27 (0.5 μM, closed squares; 1.5 μM, open triangles; 3.0 μM, closed triangles). B: Depolymerization of pre-formed amyloid (from a reaction containing 20 μM $A\beta_{1-42}$) in the absence (open circle) or presence of 0.5 μM (open squares) and 3.0 μM (closed triangles) of sHsp27.

75% as assessed by the fluorescence assay (Fig. 1A). However, by 72 h there was no significant difference in the amount of amyloid in the absence or presence of 3.0 μM sHsp27, indicating that the sHsp27 effect appeared to act on an early step in fibril formation without affecting fibril elongation (Fig. 1B). When sHsp27 (0.5–3.0 μM) was added to pre-formed amyloid and incubated for varying periods of time there was a smaller decrease (6–36%) in the fluorescent signal (Fig. 1C). These data suggest that sHsp can destabilize amyloid fibrils to some extent; however, the dominant effect appears to be to inhibit the initiation of fibril formation.

Fig. 2 shows the appearance of fibrils incubated in the presence (Fig. 2B,C) and absence (Fig. 2A) of 0.5 and 1.5 μM sHsp27 after 24 h. No significant differences in overall length or width of fibrils were apparent from the electron micrographs. These data suggest that sHsps inhibit amyloid formation at an early step rather than exerting a dominant effect on fibril growth.

The sHsps belong to a family of homologous proteins, which also includes the α-crystallins. All members of this family are characterized by the presence of one or more α-crystallin domains, which contain two predicted β-sheet-rich motifs that are connected by a hydrophilic α-helical region. We examined the influence of the α-crystallins and other unrelated proteins on amyloid formation. As shown in Table 2, an α-crystallin preparation containing both A and B α-crystallins lacked the ability to inhibit the initial rate of $A\beta_{1-42}$ amyloid formation at the same concentration at which sHsp27 or sHsp25 completely inhibited amyloidogenesis. Thus the sHsp effect appears to be a relatively specific phenomenon. Also, BSA and LDH had no effect on amyloid formation (Table 2).

4. Discussion

The interaction of ThT with amyloid appears to be specific to the extensive β-pleated sheet structure present in the amyloid polymer [23], providing for the direct assessment of amyloid formation without the complication of signal from amorphous aggregation. Using this assay our data are in good agreement with those of LeVine [23] who utilized ThT fluorescence to study $A\beta_{1-40}$. The initial rates of amyloid formation were directly proportional to the concentration of $A\beta_{1-42}$ and temperature (Table 1). Amyloid formation was maximal over a broad pH range (6.5–8.5) (Table 1). However, electron micrographs indicated dramatic increases in the amount of amyloid at pH 8.5 and 9.5 (data not shown) and significant differences in fibril width at pH 6.5 and 9.5 compared to pH 7.4 (Table 1). Thus unique amyloid structures and/or ThT binding are dependent on pH. Salt (Na^+ and K^+) had only minor effects on the rate of amyloid formation (Table 1). These and earlier data [23] indicate that the ThT assay represents a highly specific amyloidogenesis assay for Aβs.

Addition of substoichiometric levels of human sHsp27 or murine sHsp25 inhibited in vitro amyloid formation (Fig. 1, Table 2). Since the sHsp concentrations were based on monomer molecular weights and sHsps exist as larger aggregates (ca. 200–800 kDa, 8–32 subunits), the sHsp effect occurs at a significantly lower molar ratio than its ability to refold citrate synthase and α-glucosidase in vitro [25]. Fibril formation is thought to involve a nucleation step, a metastable state, that involves the assembly of a 'seed' consisting of several subunits which serves as the 'template' for subsequent fibril growth [17,24]. Thus sHsps may interfere with the nucleation process, a conclusion supported by the findings that sHsps exert their effects in the early phase of the reaction (Fig. 1B), exert lesser effects on amyloid depolymerization (Fig. 1C) and do not significantly affect fibril morphology (Fig. 2).

Whether sHsps inhibit amyloidogenesis in vivo is unknown. The sHsps are normally present in the cytoplasm. Since $A\beta_{1-42}$ is formed by the processing of the integral membrane

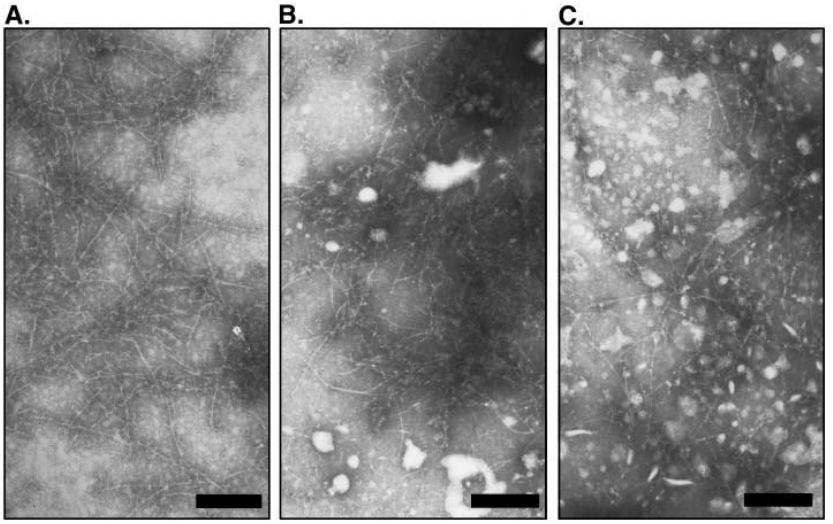

Fig. 2. Electron micrographs of the reaction products of 20 μM $A\beta_{1-42}$ in the absence (A) or presence of 0.5 μM (B) and 3.0 μM (C) of sHsp27. The bars (A–C) represent 200 nm.

protein precursor, APP, most probably within an endosomal-lysosomal structure [26,27], it is unlikely that APP would interact with chaperones that were restricted to the cytoplasm. Since the Hsp/chaperones represent a large family of highly conserved proteins with common functions that are responsible for protein complex assembly, disassembly and subcellular trafficking [18], the sHsp effect on amyloidogenesis may represent a redundant function, shared by other Hsps. Several families of Hsps are known and each family contains several cognate species. For example, the Hsp70 and Hsp60 families are ATPases, whereas sHsps are not [18]. The sHsp family includes the lens A and B α-crystallins; however, these proteins did not inhibit amyloid formation (Table 2). The sHsps are less well understood, but also function as molecular chaperones [25].

The concept that Hsp/chaperones may prevent amyloid formation is attractive. First, amyloidogenesis may represent a unique form of off-pathway aggregation. Prevention of off-pathway aggregation is an important mechanism by which Hsps promote correct protein folding. Second, chaperones have been implicated in the amyloid-associated prion encephalopathies, where they may be involved in the protein conformational changes that accompany the normal prion PrP^{C} to the disease-associated scrapie forms PrP^{Sc} [28]. Hsp70 has been co-localized with PrP^{Sc} in lysosomes where PrP^{Sc} is proposed to be generated [28] and a 73 kDa Hsp70 family member enhances in vitro protein degradation of two different lysosomal systems [29]. Also, Hsp104 plays a central role in modulating the prion-like state associated with the non-Mendelian inherited *[PSI]* phenotype in yeast [30,31]. Finally, most amyloidoses are age-related diseases that are correlated with an aging-associated decrease in the Hsp/chaperone stress response observed in several lymphocytes [32,33], heart [34], and hepatocytes [35]. The post-translational modification of α-crystallin during aging is correlated with the loss of its chaperone functions [36]. Finally, Hsp70 mRNA levels in mononuclear blood cells from Alzheimer patients were significantly lower than those from patients with vascular dementia and non-demented controls [37].

It is important to stress that several other proteins, including apolipoproteins E and J (apoE and apoJ) and transthyretin, have been implicated in modulating amyloid formation or function. Distinct alleles of apoE have been reported to inhibit (apoE3) or promote (apoE4) amyloid formation, possibly by influencing the rate of APP secretion [38]. ApoJ, a widely distributed protein, inhibits toxicity of $A\beta_{1-40}$ in vitro

Table 2
Effect of sHsp27 and other proteins on initial rates of $A\beta_{1-42}$ amyloidogenesis

$A\beta_{1-42}$ concentration	Protein added (μM)	Initial rate[a]
20	none	**200.0 ± 6.9**
	human sHsp27 (0.75)	103.4 ± 1.5*
	human sHsp27 (1.54)	41.3 ± 12.1*
	human sHsp27 (3.08)	27.5 ± 11.5*
	human sHsp27 (6.18)	−41.3 ± 20.7*
20	none	**156.5 ± 6.5**
	mouse sHsp25 (6.18)	−26.1 ± 4.3*
20	none	**210.3 ± 5.7**
	α-crystallin (6.18)	194.3 ± 11.4
	LDH (6.18)	200.0 ± 8.6
	BSA (6.18)	162.5 ± 14.3

[a]Average of regression slopes for 6 h kinetic data from three independent experiments expressed as arbitrary fluoescence units h^{-1}.
*Significantly different from control group (data in bold type) at the $P < 0.05$ level as described in Section 2.

[39]. Transthyretin, a major component of cerebrospinal fluid, has also been shown to prevent amyloid formation in vitro [40]. Whether any of these proteins or the Hsp/chaperone family exert physiologic roles modulating Aβ amyloidogenesis in the central nervous system will require further investigation.

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