



Role of α B Crystallin on Serum Amyloid A Fibrils with Systemic Amyloidotic Mice Liver

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ABSTRACT

AlphaB-Crystallin is one of the heat shock proteins and its Chaperonic activity is well established. alphaB-Crystallin forms complexes with denaturing proteins thereby preventing their uncontrolled aggregation. Chaperonine process has been shown to follow a saturation type of complexing, when the ability of the available alphaB-Crystallin to bind the target protein is exceeded, the excess target protein aggregates and eventually precipitates out of solution. The effect of alphaB-Crystallin on SAA fibril formation under systemic amyloidosis in the liver, we have determined they interact with each other. In the present study, in-vitro and in-vivo studies on the role of the interaction of SAA/SAA fibrils with chaperones were investigated by congo red stain, immune fluorescence and radio labeling methods. The results indicated that there was SAA deposits in liver, increases the SAA level in the liver and the alphaB-Crystallin could liver SAA level comes near to control. In conclusion that the implication of SAA in the liver. The significance in-vivo study has shown α B-Crystallin as a therapeutic use for SAA amyloid disease.

Key words: Serum Amyloid A, Systemic amyloidosis, Crystallin, Chaperonine, Aggregate, Amyloid Fibril.

INTRODUCTION

Small heat-shock proteins (sHSPs) are one of four families of heat-shock proteins (HSPs) expressed in response to heat shock and other forms of stress (Parsell and Lindquist 1993). They are a diverse family of proteins that appear to be ubiquitous in nature, being found as surface antigens in eukaryotic parasites, as inclusion body-binding protein in *E. Coli*, and as structural proteins in the vertebrate lens (Caspers et al 1995). Despite their low molecular mass (12-40 kDa), sHSPs are isolated as large oligomeric complexes of 2-40

subunits depending on the physiological state of the cell. Distant members of the family have relatively low sequence similarity except for a highly conserved stretch of 100 amino acids, often called the α -Crystallin domain (Caspers et al 1995).

Mammalian cells express two sHSP: HSP 27 and α B-Crystallin. In addition to their established role in thermotolerance, recent reports indicate that HSP 27 is involved in transduction pathways activated during oncoprotein-mediated neoplasticity (Aoyama et al 1993) in response to

the cytotoxic effects of TNF- α , and in the regulation of apoptosis (Mehlen et al 1996). This protective role is associated with phosphorylation-induced changes in the oligomeric state and intracellular localization of the protein. Both stress and growth stimulation lead to the phosphorylation of specific serine residues in HSP 27, suggesting that phosphorylation provides the coupling mechanism that regulates function via reorganization of the quaternary structure (Mehlen et al 1995). Similar to human HSP 27, *Drosophila* HSP 27 and human α B-Crystallin are able to protect against cell death induced by necrosis or apoptosis. Because the sequence similarity of sHSP is focused in the α -Crystallin domain, it is logical to assume that this domain forms a common structural core (Chothia and Lesk 1986) i.e., a region with similar secondary and tertiary structure, that is the primarily dependant on the oligomeric structure and the common functional features (Wistow 1993). While it has been shown that the α -Crystallin domains of both HSP 27 and α B-Crystallin are able to assemble into large oligomeric aggregates, these separate domains have no chaperone-like activity (Merck et al 1993). The recent isolation of a monomeric sHSP from *C. Elegans* consisting of one domain showing sequence similarity to the α -Crystallin domain suggests a critical role in the N-terminal region in the assembly of the oligomeric structure (Leroux et al 1997).

Even though the *in-vitro* experiments lead to the unclear answer for the role of the HSPs in the stability of folded proteins, every organism responds to a sudden increase in the environmental temperature with the over-expression of HSPs (Lindquist and Craig 1988). However a majority of these Hsps function by assisting protease-degradation. Heat shock proteins act both under normal conditions and under stress conditions, where they are over-expressed to cope with the increased concentration of unfolded proteins (Hendrick and Hartl 1993). They reduce the free concentration of aggregation-sensitive folding intermediates and

effectively prevent aggregation processes, both *in-vivo* and *in-vitro* (Jakob and Buchner 1994).

Whether this chaperone function is active *in-vivo* either in the lens, or, in other tissues, is unknown. To understand the effect of α B-Crystallin on A β fibril formation, we need to determine whether they interact with each other. In the present study, *in-vitro* studies on the role of the interaction of SAA/SAA fibrils with chaperones were investigated. The results indicated that there was interaction between SAA and α -Crystallin when they were incubated together. A possible mechanism for this interaction and its implicated significance *in-vivo* are discussed.

MATERIALS AND METHODS

Production of Systemic Amyloidosis

Colony-bred adult male Swiss White mice (30-35 g) were used. Animals were selected randomly and caged in groups of four at room temperature (25-35°C) and supplied with food (Commercial pelleted animal feed marketed by M/s. Hindustan Lever, Bombay, India under the name "Gold Mohur rat feed") and tap water ad libitum (control n=5, test n=5). All animal procedures were carried out as approved by the Animal Care and Use Committee of Central Leather Research Institute, Chennai. Mice were given 0.5 ml of 10% Vitamin free Casein (1CN Pharmaceuticals, Cleveland, OH, USA) as subcutaneous injection for 66 days to induce systemic amyloidosis (Botto et al 1997). Control animals were treated with saline. After 66 days α B-Crystallin was treated 100 mg/kg-body weight/day for three weeks. At the end of treatment, animals were decapitated. Immediately after decapitation liver was removed in the ice-chilled condition for further analysis. Systemic amyloidosis was confirmed by "ladder formation" in the liver. The presence of amyloid fibrils in the liver is further confirmed by Congo red staining.

Congo red staining of Liver Amyloid Deposits

Frozen tissue sections were analyzed for amyloid deposition with Congo red staining by following the reported procedure (Westermarck et al 1999). In brief, the deparaffinized sections were stained with

Mayer's solution for 1 min and then rinsed in saline solution for few minutes. Sections were treated in solution containing 10 gm NaCl to 100 mL in 80% (v/v) ethanol for 20 min. These sections are transferred directly to a solution of 2gm Congo red and 10 gm NaCl in 1000 mL 80% ethanol and stained for 20 min. The sections were then washed twice with absolute ethanol. The sections were then washed with xylene thrice and mounted under cover glass in a synthetic-mounting medium, DPX. Stained sections were observed with crossed high intensity polarized light in polarization microscopy (EUROMAX, Holland).

Isolation of SAA

SAA was isolated from the plasma of casein injected (Botto et al 1997) mice as reported previously by Lindhorst et al (Lindhorst et al 1997). It was then characterized using 17% SDS-polyacrylamide gels. Isolated SAA was purified by Waters reverse phase-high performance liquid chromatography (Kaplan et al 1999) (RP-HPLC) analytical column 4.6 × 250 mm Spherisorb ODS2 LC₁₈ (Waters, Milford, Massachusetts, USA) and size exclusion chromatography (SEC) columns using a series of 7.8 × 300 mm Ultrahydrogel 250™ and Ultrahydrogel 500™ the molecular weight was determined (Waters, Milford, Massachusetts, USA).

Immunostaining of Liver Sections

The antisera against SAA were prepared as per the following procedure. New Zealand White rabbits were given intradermal injection of 0.1 mg/ mL of mouse SAA weekly once. Prior to the injection, the SAA protein was dissolved in PBS and emulsified in complete and incomplete adjuvant. Three weeks later a fourth intradermal injection was given and the animals were bled in the following week. The antisera were stored for further processing at -20°C. The immunostaining of SAA in the liver was carried out as follows; after blocking nonspecific binding with 2% BSA solution, the liver tissue sections were incubated with anti SAA (1:1000) for 30 min at 4°C. After

washing in PBS, the sections were incubated with FITC conjugated rabbit anti mouse antibody for 30 min at 4°C. After washing in PBS, incubated with FITC conjugated rabbit anti mouse antibody for 30 min at 4°C, the FITC fluorescence was observed by fluorescence microscopy.

Isolation of α B-Crystallin

Bovine lenses (8-10 lenses) were decapsulated and homogenized (Luthra and Balasubramanian 1993) in 0.1 M Tris buffer, pH 7.4 containing 0.5 M NaCl, 1 mM EDTA, and 0.1% NaN₃. The insoluble protein fraction and membrane debris was removed by centrifugation at 30,000g for 30 min. The supernatant was chromatographed on a Sephacryl-S200 (Pharmacia Biotech, Uppsala, Sweden) gel filtration column (0.8 × 90 cm) to separate the α -Crystallin using Gradifrac™ FPLC system (Fig. 1a) (Pharmacia Biotech, Uppsala, Sweden). Each of the α B-Crystallin fractions was dialyzed repeatedly against water using FILTRON concentrating 10000 D. Molecular weight cut off membrane (Gelman Sciences, India) and lyophilized and stored at -20°C. Protein was determined by Lowry et al (Lowry et al 1951). Homogeneous 12% SDS-polyacrylamide gels were run on the mini gel electrophoresis system (Fig. 1b) (Laemmli 1970).

Radio Labeling

The alphaB-Crystallin was labelled with ¹²⁵I (Baba Atomic Research Center, New Mumbai, India) using the method by Hamazaki (Hamazaki 1995) and stored in phosphate buffered saline containing 0.02% NaN₃. Labelled proteins to a final specific activity of approximately 2mci/mM were given to amyliodotic and control mice. ¹²⁵I BSA used as a control. After 24 hrs, animals were sacrificed the organ (liver) and plasma were measured in a 1270 Rack Gamma II γ counter (Pharmacia Biotech (LKB), Uppsala, Sweden). All the groups compared statistically using the Chi-square test, U-Test (Mann-Whitney-Wilcoxon) and analysis of variance (ANOVA) with 2-tailed significance thresholds.

RESULTS

The systemic Amyloidosis results in amyloid deposits in liver as shown in Fig. 2b. The effect of α B-Crystallin on liver of systemic amyloidosis affected mice was examined by analyzing the amyloid deposits by Congo red staining (Westermarck 1999). The brain sections of the α B-Crystallin treated mice with systemic amyloidosis were also examined by this procedure. The Congo red staining of the liver (Fig. 2a) revealed that in control mice there was no fibril deposition. However large amount of fibrils in liver were visible even after discontinuing the casein injections in the control mice (Fig. 2b) indicating the damage caused by the amyloid fibrils that are more difficult to get solubilised. This was already observed by several groups (Baltz et al 1980). However, as seen in the figure, the amyloid burden was considerably reduced in these organs (Fig. 2c) in α B-Crystallin treated mice. This is more evident when the sections were viewed under the fluorescence microscopic picture (Fig. 3a). Similar deposits were also seen in the case of liver and spleen (Fig. 3b). α B-Crystallin (Fig. 3c) treated immuno staining of mice liver. In the case of control liver these dot like structures were more prevalent (Fig. 2a). Previous finding suggests that α -1-antitrypsin (AAT) and α B-Crystallin (also called HSP 27) are down regulated in Huntington's disease (HD). Thus maintaining α B-Crystallin availability during the course of HD might prevent neuronal cell death and therefore suggested being useful in delaying the disease progression (Zabel et al 2002).

Considering the abundance of α B-Crystallin expression in the peripheral tissues and possible therapeutic value of α B-Crystallin combating amyloid burden in the liver, transport of α B-Crystallin to liver tissues was investigated. To find out whether α B-Crystallin could deposit in liver, we injected the radio labeled [125 I] α B-Crystallin in control and casein injected mice (mice with systemic amyloidosis). Fig. 4. indicates that the radiolabeled α B-Crystallin

deposits liver of the mice affected by systemic amyloidosis as well as control mice. The α B-Crystallin incorporation in the liver tissue was also specific to the SAA in the amyloidotic condition, because the amyloidotic mice when injected with radio labeled [125 I] BSA showed negligible accumulation. This observation precludes the possibility of protects the liver cells, which will damage most of the proteins in the inflammatory conditions.

Further, radio labeled [125 I] α -Crystallin and [125 I] BSA to the control shows no significant incorporation in the control liver (Fig. 4). The radio labeling analysis of the α B-Crystallin was also carried out in liver. Most of the α B-Crystallin injected got accumulated in liver, in both control and amyloidotic mice (Fig. 4). It should be mentioned that even in the control mice, the α B-Crystallin got accumulated in the liver to the same extent. This may be due to the general ability of α B-Crystallin retention in liver tissues, which explains their propensity to get involved in the amyloid protein. However, the total clearance in the [125 I] BSA in liver indicates that the protein is totally excreted within 24 hrs from the time of injection. Severe inflammation was observed in systemic amyloidotic mice together with excess production of inflammatory markers and SAA protein. In this work, we have seen that in systemic amyloidotic mice, the SAA level come to normal after treatment with α B-Crystallin. The α B-Crystallin treatment also reduced the amyloid burden in the systemic amyloidotic mice.

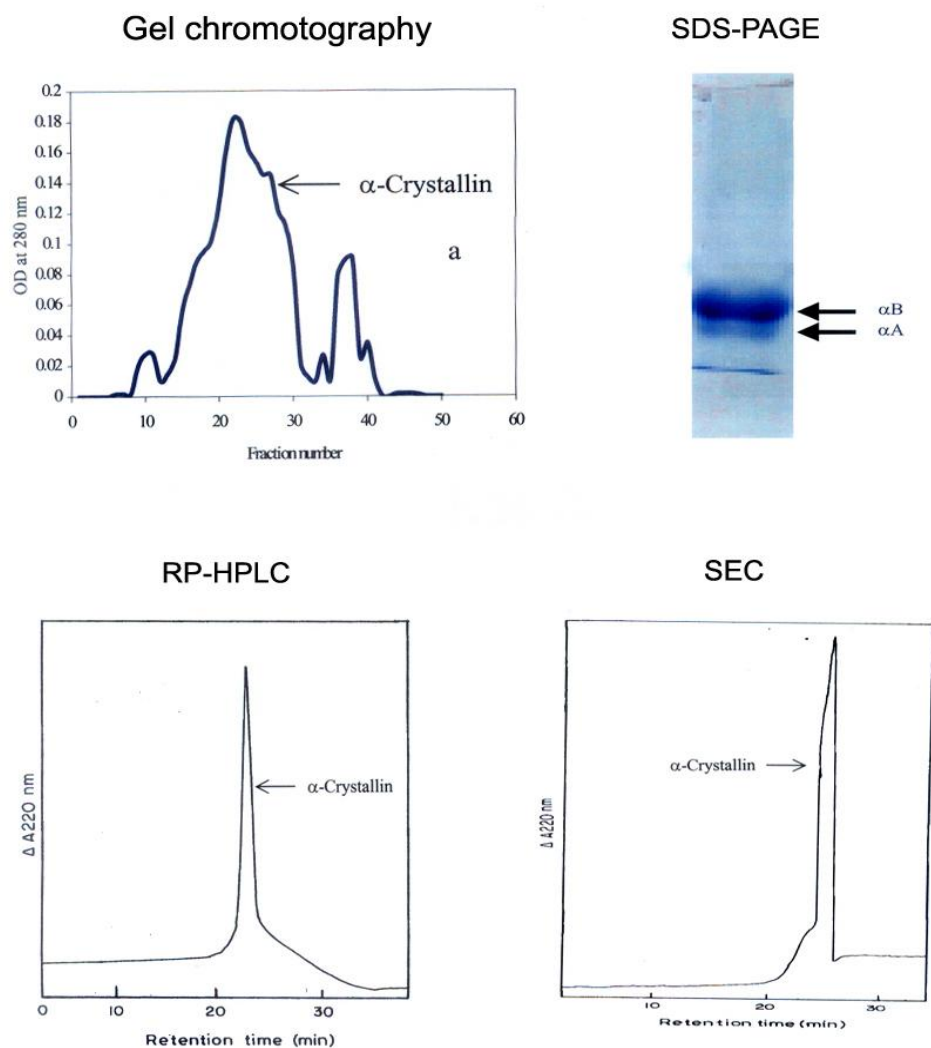


Fig. 1. Gel filtration chromatographic profile of alphaB-Crystallin (a) SDS-PAGE profile of alphaB-Crystallin (b) RP-HPLC profile of the alphaB-Crystallin (c) and SEC profile of the alphaB-Crystallin.

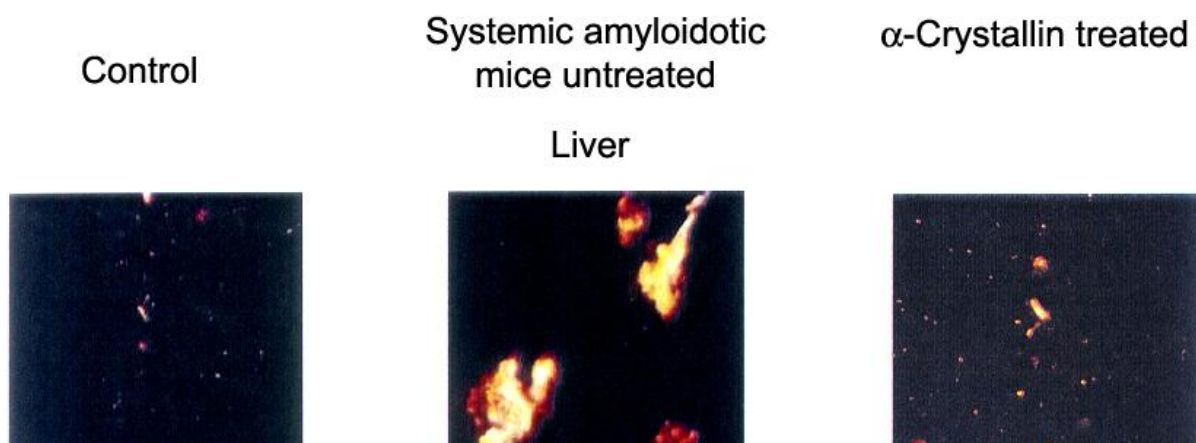


Fig. 2. Congo red stained sections of amyloid deposits with cross-polarized light (a) liver control mice (b) liver section of untreated systemic amyloidotic mice (c) liver of alpha-Crystallin treated systemic amyloidotic mice. Magnification: 40.

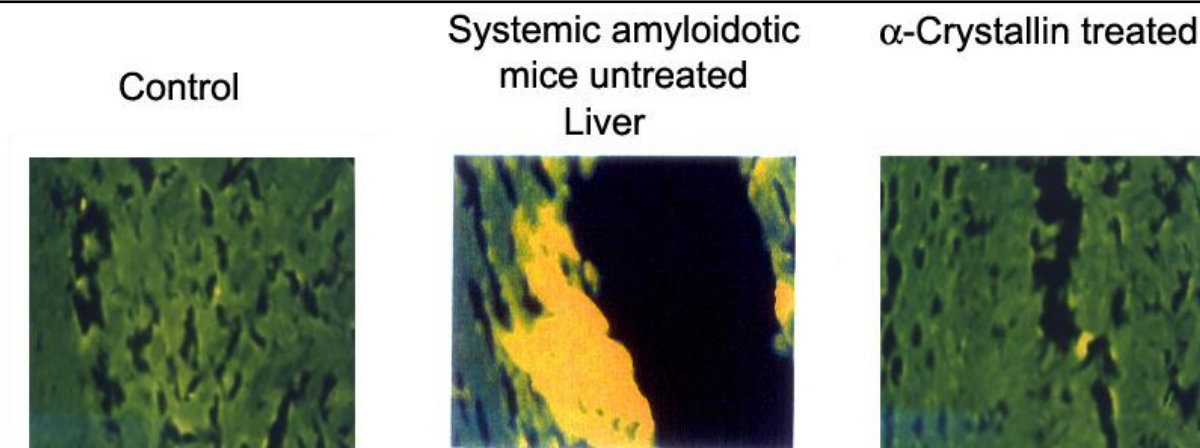


Fig. 3. Fluorescence microscopic picture of amyloid deposits (a) SAA immunostaining of control mice liver (b) SAA immunostained with untreated systemic amyloidotic mice liver (c) SAA immunostaining of α -Crystallin treated mice liver.

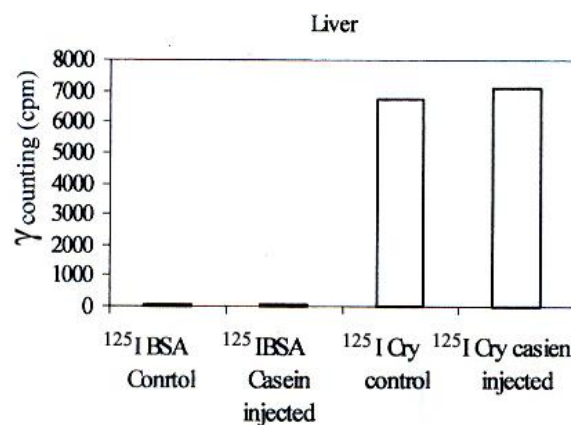


Fig. 4. Incorporation of ^{125}I Labelled alphaB-Crystallin and ^{125}I Labelled BSA in the control and Casein injected mice liver. (a) Accumulation of ^{125}I alphaB-Crystallin in Casein injected mice liver. Note significant accumulation in the control mice. However no significant in BSA incorporation in both control and Casein injected mice. Data show mean γ counting values (\pm SEM) from single, representative experiments. * $p < 0.05$; ** $p < 0.01$: *

** $p < 0.001$, related to control conditions.

DISCUSSION

The results of the experiment present the following conclusions. First, the alphaB-Crystallin protects SAA amyloid burden in the liver of mice with systemic amyloid disease. As in the case of peripheral degenerating tissues, damaged neurons and neurite provide stimuli for inflammation. This will lead to up-regulation of acute phase reactants and cytokines (Song et al 2001). Thus, the elevation in SAA and SAP level may be at least partly attributed to the liver tissue expression of these proteins following inflammatory stimulus.

The result of the present work suggests that peripheral inflammation significantly contributes to SAA aggregate and associated toxic responses.

Under normal conditions, SAA and SAP, which are the normal constituents of blood, will never cross the BBB. However, during the inflammatory condition, SAA level increases considerably in the blood plasma, leading to the possibility of protofibrillar formation, which ultimately deposits in liver. Such a relative interactive motif in the fibrillar proteins/peptides is already known in

amyloid biochemistry. The cellular activation due to SAA fibrils are also implicated to RAGE receptors (Sipe et al 1993; Shroo et al 1998). It was already estimated that RAGE binds SAA1 protein with nanomolar affinity. It was also shown that tissues with amyloid deposition increased expression of RAGE. Recently, apoSAA and mRNA expression have been demonstrated in the Syrian hamster brain following intra-peritoneal injection of the inflammatory stimulant, lipopolysaccharide, as well as in patients with AD (Liang et al 1997) indicating that liver parenchymal cells expressed SAA mRNA in the inflammatory conditions. Since, at high concentration of SAA, amyloid protofibrils formation is possible in the inflammatory condition, there is a possibility of SAA protofibrils crossing the BBB through RAGE that are expressed in the brain endothelial cell capillaries. Further, the binding of SAA protein to the RAGE receptors is known to elicit the cellular oxidative response (Yan et al 2000). RAGE is found in most of the cells known to be affected by amyloid namely, hepatic, endothelial, neuronal, and microglial cells (Giri et al 2000). The consequences of SAA fibril-RAGE interaction include increased cellular oxidative stress (Yan et al 1996 and Yan et al 2000) established the triggering of inflammatory responses with fibrillar protein. They have shown that aged A β fibrils dramatically increased plasma IL-6 levels in mice. IL-6 is a multi functional cytokine involved in the acute phase reactants and overproduction of SAA protein. Thus, these results suggest that systemic amyloidosis and tissue inflammatory responses could be interrelated.

CONCLUSION

In conclusion, the results indicate that SAA fibrils deposits in liver and forms toxic to cells. The presence of SAA in peripheral amyloid deposits is well documented. The present work is consistent with the hypothesis that the SAA, which is found in various inflammatory conditions in the liver, may be due to its amyloidogenic property.

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