Published in final edited form as:
Int J Biochem Cell Biol. 2008 ; 40(5): 954–967. doi:10.1016/j.biocel.2007.10.035.

Interactive sequences in the molecular chaperone, human αB crystallin modulate the fibrillation of amyloidogenic proteins

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Abstract

Multiple interactive domains are involved in the activity of the stress protein, αB crystallin that protects against the unfolding, aggregation, and toxicity of amyloidogenic proteins. Five peptides corresponding to the interactive sequences $_{41}$STSLSPFYLRPPSFLRAP$_{58}$, $_{73}$DRFSVNLDVKHFS$_{85}$, $_{101}$HGKHEERQDE$_{110}$, $_{113}$FISREFHR$_{120}$, $_{131}$LTITSSLSSDGV$_{142}$, and $_{156}$ERTIPITRE$_{164}$ in human αB crystallin were synthesized and evaluated in Thioflavin T fluorescence assays for their effects on the modulation of fibrillation of four disease-related amyloidogenic proteins: amyloid-β, α-synuclein, transthyretin, and β2-microglobulin. The $_{73}$DRFSVNLDVKHFS$_{85}$ and $_{101}$HGKHEERQDE$_{110}$ peptides in the conserved α crystallin core domain of αB crystallin were the most effective fibril inhibitors. $_{73}$DRFSVNLDVKHFS$_{85}$ completely inhibited α-synuclein fibrillation and reduced the fibrillation of amyloid-β, transthyretin, and β2-microglobulin by >50%. $_{101}$HGKHEERQDE$_{110}$ completely inhibited amyloid-β fibrillation and reduced the fibrillation of α-synuclein, transthyretin, and β2-microglobulin by >50%. The peptides FSVN, NLDV, HGKH, and HEER, which are synthetic fragments of $_{73}$DRFSVNLDVKHFS$_{85}$ and $_{101}$HGKHEERQDE$_{110}$, inhibited fibrillation of all four amyloidogenic proteins by >75%. In contrast, the peptides FISREFHR, ERTIPITRE, DRFS, KHFS, and EERQ were the strongest promoters of fibrillation. Molecular modeling of the interactions between transthyretin and β2-microglobulin and the synthetic bioactive peptides determined that residues Phe-75, Ser-76, Val-77, Asn-78, Leu-79, and Asp-80 in $_{73}$DR**FSVNLD**VKHFS$_{85}$ and residues His-101, Lys-103, His-104, Glu-105, and Arg-107 in $_{101}$**H**G**KHE**E**R**QDE$_{110}$ interact with exposed residues in the β strands, F and D of transthyretin and β2-microglobulin respectively to modulate fibrillation. This is the first characterization of specific bioactive peptides synthesized on the basis of interactive domains in the small heat shock protein, αB crystallin that protect against the fibrillation of amyloidogenic proteins.

Keywords

crystallin; chaperone; fibril; amyloid; synuclein; transthyretin; microglobulin; Alzheimer's disease; Parkinson's disease

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Introduction

Protein unfolding and aggregation of cytosolic lens crystallins, desmin filaments, microtubules, and amyloidogenic proteins including amyloid-β (Aβ), α-synuclein, transthyretin, and β2-microglobulin are the hallmarks of aggregation diseases that include cataract, desmin-related myopathy (DRM), Alzheimer's disease (AD), Parkinson's disease (PD), senile systemic amyloidosis (SSA), hemodialysis-related amyloidosis (HRA), age related macular degeneration (AMD), and familial amyloidotic polyneuropathy (FAP) (Dobson, 2001; Gorevic et al., 1985; J. Hardy & Selkoe, 2002; Selkoe, 2003; Sunde et al., 1997; Vicart et al., 1998). While formation of light scattering protein aggregates in the ocular lens results in cataracts, abnormal assembly of filaments in cardiac tissues results in DRM, and formation of oligomers and structured amyloid-like fibrils results in neurodegenerative diseases including AD and PD (Dobson, 2004). Small heat shock proteins (sHSP) are a family of molecular chaperones (molecular weight < 43kDa) that protect against the harmful effects of protein unfolding, misfolding, and aggregation (Hartl, 1996). The sHSPs, human αB crystallin and sHSP27 are ubiquitously expressed in response to cellular stress and protect against protein unfolding, misfolding, and aggregation in the normal unfolding protein response (UPR) (Haslbeck, Franzmann, Weinfurtner, & Buchner, 2005; Winter & Jakob, 2004). Clinically, sHSPs including αB crystallin have been observed in neuritic plaques and Lewy bodies in brain tissues of AD and PD patients, and in Drusen of patients with age-related macular degeneration (Dabir, Trojanowski, Richter-Landsberg, Lee, & Forman, 2004; De et al., 2007; Johnson, Brown, Pulliam, Anderson, & Johnson, 2005; Nakata, Crabb, & Hollyfield, 2005; Renkawek, Bosman, & de Jong, 1994; Renkawek et al., 1992; Renkawek, Stege, & Bosman, 1999; Wilhelmus, Boelens, Otte-Holler, Kamps, de Waal et al., 2006; Wilhelmus, Boelens, Otte-Holler, Kamps, Kusters et al., 2006). A recent study reported a marked increase in the expression of αB crystallin and sHSP25 in transgenic mouse models of familial amyotrophic lateral sclerosis (ALS), PD, dentato-rubral pallido-luysian atrophy, and Huntington's disease (HD) (Wang, Martin, Gonzales, Borchelt, & Lee, 2007). Although, human αB crystallin and other sHSPs interact with and inhibit the fibrillation of $A\beta_{1–42}$, α-synuclein, β2-microglobulin, and insulin *in vitro* (Goldstein et al., 2003; Hatters, Lindner, Carver, & Howlett, 2001; Lee, Carson, Rice-Ficht, & Good, 2005, 2006; Liang, 2000; Raman et al., 2005; Rekas et al., 2004; Santhoshkumar & Sharma, 2004; Wilhelmus, Boelens, Otte-Holler, Kamps, de Waal et al., 2006), it has been reported that αB crystallin enhances the neurotoxicity of Aβ in cultured neuronal cells (Stege et al., 1999). The consistent observation of the expression and localization of sHSPs in association with abnormal protein aggregates in diseased tissues suggests an important biological function for sHSPs in aggregation diseases. Identification of the bioactive sequences in sHSPs that mediate protein-protein interactions with the amyloidogenic proteins Aβ, α-synuclein, transthyretin, and β2-microglobulin will provide a molecular basis for the mechanism of sHSP activity in protein aggregation diseases including AD and PD, cataract, and AMD.

The goal of the current study was the characterization of molecular interactions between human αB crystallin, the archetype of sHSPs and four amyloidogenic proteins ($A\beta_{1–42}$, α-synuclein, transthyretin, and β2-microglobulin) that are known to form toxic aggregates and fibrils. Systematic protein pin array analysis, site-directed mutagenesis, and chaperone assays characterized the $_{41}$STSLSPFYLRPPSFLRAP$_{58}$ sequence in the N-terminal domain, the sequences $_{73}$DRFSVNLDVKHFS$_{85}$, $_{101}$HGKHEERQDE$_{110}$, $_{113}$FISREFHR$_{120}$, and $_{131}$LTITSSLSSDGV$_{142}$ in the exposed interface of the α crystallin core domain, and the sequence $_{156}$ERTIPITRE$_{164}$ in the C-terminal domain of human αB crystallin as interactive sequences responsible for the recognition, binding, and solubilization of unfolding/misfolding substrate proteins including lens crystallins, enzymes, filaments, and growth factors (Bhattacharyya, Padmanabha Udupa, Wang, & Sharma, 2006; Ghosh & Clark, 2005; Ghosh, Estrada, & Clark, 2005, 2006; Ghosh, Estrada, Houck, & Clark, 2006; Ghosh, Houck, & Clark,

2007a, 2007b; Ghosh, Shenoy, & Clark, 2006b; Ghosh, Shenoy, & Clark, 2007a). Synthetic peptides corresponding to these sequences suppressed the aggregation of β/γ crystallins, alcohol dehydrogenase, citrate synthase, actin, FGF, and VEGF, and promoted microtubule assembly (Ghosh & Clark, 2005; Ghosh, Estrada, & Clark, 2005, 2006; Ghosh, Estrada, Houck, & Clark, 2006; Ghosh, Houck, & Clark, 2007a, 2007b; Ghosh, Shenoy, & Clark, 2006b; Ghosh, Shenoy, & Clark, 2007a). In this study, we demonstrated the effectiveness of these five αB crystallin sequences in inhibiting the fibrillation of $A\beta_{1–42}$, α-synuclein, transthyretin, and β2-microglobulin using Thioflavin T fluorescence assays. The synthetic $_{73}$DRFSVNLDVKHFS$_{85}$ and $_{101}$HGKHEERQDE$_{110}$ peptides were the most effective modulators of fibrillation. Systematic truncation of these two bioactive peptides led to the identification of four amino acid long peptides (molecular weight <500Da) that were more effective in modulating the fibrillation of Aβ, α-synuclein, transthyretin, and β2-microglobulin than Tramiprosate, NAP, diflunisal, and meclofenic acid, which are anti-amyloidogenic molecules currently in clinical development (Aisen, 2005; Gervais et al., 2007; Gozes et al., 2005). The results define specific interactions that are important in the protective activity of the sHSP, human αB crystallin, as a molecular chaperone in protein aggregation diseases.

Materials and Methods

Materials

The αB crystallin peptides DRFSVNLDVKHFS (DR) and HGKHEERQDE (HG) were synthesized by Advanced ChemTech (Louisville, KY). The remaining three αB crystallin peptides, STSLSPFYLRPPSFLRAP (ST), FISREFHR, LTITSSLSSDGV (LT) and ERTIPITRE (ER), were synthesized by Genscript (Piscataway, NJ). The peptides, NAPVSIPQ (NAP) and DRVYIHPFHL (Angiotensin 1, AT1) were purchased from Genscript (Piscataway, NJ). $A\beta_{1–42}$ (American Peptide, Sunnyvale, CA), transthyretin (Sigma-Aldrich, St. Louis, MO), β2-microglobulin (MP Biomedical, Solon, OH), α-synuclein (rPeptide, Bogart, GA) were also procured from external vendors as listed above. Tramiprosate (3-aminopropanesulfonic acid, APS), diflunisal, flufenamic acid, and meclofenamic acid were purchased from Sigma-Aldrich (St. Louis, MO). The truncated DR and HG peptides were synthesized by Genscript (Piscataway, NJ). All peptides and small molecules obtained from the suppliers were directly dissolved in 0.25% DMSO at a concentration of 2mM without any additional treatment. For all experiments, fresh solutions were prepared immediately prior to use. Assays were performed in 384-well fluorescence plates in duplicates or triplicates and fluorescence was measured using a Perkin Elmer Victor^3 V plate reader. Two molecules, Tramiprosate and NAP that are known inhibitors of $A\beta_{1–42}$ fibrillation and are currently in clinical trials for AD were included in the $A\beta_{1–42}$ and α-synuclein assays for comparison (Ashur-Fabian et al., 2003; Gervais et al., 2007). Diflunisal, flufenamic acid, and meclofenamic acid which are known inhibitors of transthyretin fibril formation were included in the transthyretin and β2-microglobulin assays. A synthetic neuropeptide Angiotensin 1 (AT1) that has no effect on the fibrillation of Aβ, α-synuclein, transthyretin, and β2-microglobulin was included as a negative control in all assays.

Thioflavin T fluorescence assays

The effect of the αB crystallin peptides on $A\beta_{1–42}$, α-synuclein, transthyretin, and β2-microglobulin fibrillation was measured using the Thioflavin T (ThT) fluorescence assay (Hurshman, White, Powers, & Kelly, 2004; Ivanova, Sawaya, Gingery, Attinger, & Eisenberg, 2004; Naiki, Higuchi, Hosokawa, & Takeda, 1989; Raman et al., 2005; Walsh et al., 2005; Zhu et al., 2004). Thioflavin T is a fluorescent dye that has a high affinity for amyloid fibrils and becomes fluorescent (excitation λ = 450nm, emission λ = 486nm) when bound to fibrils. Fibril formation was verified by fluorescence and bright-field microscopy (data not shown).

For the $A\beta_{1-42}$ 1:1 assays, 10μL of 90μM test peptides dissolved in 0.25%DMSO were added to 7μL of buffer. Finally, 3μL of 1μM ThT and 10μL of 90μM $A\beta_{1-42}$ were added to these solutions to start the experiment. The final buffer composition for the assay was 20mM Tris-Cl, 100mM NaCl, 0.08% DMSO, pH 7.4. For the $A\beta_{1-42}$ 5:1 assays, 10μL of 18μM test peptides dissolved in 0.25%DMSO were added to 7μL of buffer. 3μL of 1mM ThT and 10μL of 90μM $A\beta_{1-42}$ were added to these solutions to start the experiment. The final buffer composition for the assay was 20mM Tris-Cl, 100mM NaCl, 0.08% DMSO, pH 7.4. The fluorescence of all samples was simultaneously measured in a plate reader (excitation λ = 450nm, emission λ = 486nm) immediately after addition of ThT and $A\beta_{1-42,}$ and after 24 and 48hrs of incubation at 37°C. The effect of the peptides and control molecules on fibril formation of $A\beta_{1-42}$ was determined using the calculation: Aβ fibrillation = $\Delta F_{A\beta+peptide}/\Delta F_{A\beta}$, where $\Delta F_{A\beta}$ = ThT fluorescence of Aβ at 48hrs - ThT fluorescence of Aβ at 0hrs and $\Delta F_{A\beta+peptide}$ = ThT fluorescence of Aβ+peptide at 48hrs - ThT fluorescence of Aβ+peptide at 0hrs.

For the α-synuclein 1:1 assays, 5μL of 140μM test peptides dissolved in 0.25%DMSO were added to 17μL of buffer. Finally, 3μL of 1mM ThT and 5μL of 140μM α-synuclein were added to these solutions to start the experiment. The final buffer composition for the assay was 20mM Tris-Cl, 100mM NaCl, 0.04% DMSO, pH 7.4. For the α-synuclein 5:1 assays, 5μL of 28μM test peptides dissolved in 0.25%DMSO were added to 17μL of buffer. Finally, 3μL of 1mM ThT and 5μL of 140μM α-synuclein were added to these solutions to start the experiment. The final buffer composition for the assay was 20mM Tris-Cl, 100mM NaCl, 0.04% DMSO, pH 7.4. The fluorescence of all samples was simultaneously measured in a plate reader (excitation λ = 450nm, emission λ = 486nm) immediately after addition of ThT and α-synuclein and after 24, 48, and 96 hrs of incubation at 37°C. The effect of the peptides and control molecules on α-synuclein fibrillation was determined using the calculation: α-synuclein fibrillation = $\Delta F_{\alpha\text{-synuclein+peptide}}/\Delta F_{\alpha\text{-synuclein}}$, where $\Delta F_{\alpha\text{-synuclein}}$ = ThT fluorescence of α-synuclein at 96hrs - ThT fluorescence of α-synuclein at 0hrs and $\Delta F_{\alpha\text{-synuclein+peptide}}$ = ThT fluorescence of α-synuclein+peptide at 96hrs - ThT fluorescence of α-synuclein+peptide at 0hrs.

For the transthyretin 1:1 assays, 5μL of 64μM test peptides dissolved in 0.25%DMSO were added to 10μL of buffer. Finally, 1μL of 1mM ThT and 10μL of 32μM transthyretin were added to these solutions to start the experiment. The final buffer composition for the assay was 5mM PBS, 200mM KCl, 2mM EDTA, 200mM Na acetate, 0.06% DMSO, pH 4.4. For the transthyretin 5:1 assays, 5μL of 6.4μM test peptides dissolved in 0.25%DMSO were added to 10μL of buffer. Finally, 1μL of 1mM ThT and 10μL of 32μM transthyretin were added to these solutions to start the experiment. The final buffer composition for the assay was 5mM PBS, 200mM KCl, 2mM EDTA, 200mM Na acetate, 0.06% DMSO, pH 4.4. The fluorescence of all samples was simultaneously measured in a plate reader (excitation λ = 450nm, emission λ = 486nm) continuously after addition of ThT for 150 minutes at 37°C. The effect of the peptides and control molecules on transthyretin fibrillation was determined using the calculation: Transthyretin fibrillation = $\Delta F_{transthyretin+peptide}/\Delta F_{transthyretin}$, where $\Delta F_{transthyretin}$ = ThT fluorescence of transthyretin at 2.5hrs - ThT fluorescence of transthyretin at 0hrs and $\Delta F_{transthyretin+peptide}$ = ThT fluorescence of transthyretin +peptide at 2.5hrs - ThT fluorescence of transthyretin+peptide at 0hrs.

For the β2-microglobulin 1:1 assays, 10μL of 86μM test peptides dissolved in 0.25%DMSO were added to 7μL of buffer. Finally, 3μL of 1mM ThT and 10μL of 86μM β2-microglobulin were added to these solutions to start the experiment. The final buffer composition for the assay was 25mM Na acetate, 25mM Na phosphate, 0.08% DMSO, pH 2.4. For the β2-microglobulin 5:1 assays, 10μL of 17.2μM test peptides dissolved in 0.25%DMSO were added to 7μL of buffer. Finally, 3μL of 1mM ThT and 10μL of 86μM β2-microglobulin were added to these solutions to start the experiment. The final buffer composition for the assay was 25mM Na acetate, 25mM Na phosphate, 0.08% DMSO, pH 2.4. The fluorescence of all samples was

simultaneously measured in a plate reader (excitation λ = 450nm, emission λ = 486nm) continuously after addition of ThT for 150 minutes at 37°C. The effect of the peptides and control molecules on β2-microglobulin fibrillation was determined using the calculation: β2-microglobulin fibrillation = $\Delta F_{\beta 2\text{-microglobulin +peptide}}/\Delta F_{\beta 2\text{-microglobulin}}$, where $\Delta F_{\beta 2\text{-microglobulin}}$ = ThT fluorescence of β2-microglobulin at 2.5hrs - ThT fluorescence of β2-microglobulin at 0hrs and $\Delta F_{\beta 2\text{-microglobulin +peptide}}$ = ThT fluorescence of β2-microglobulin +peptide at 2.5hrs - ThT fluorescence of β2-microglobulin+peptide at 0hrs

3D molecular modeling of the interactive site for the bioactive peptides

3D co-ordinates for the αB crystallin peptides were extracted from the computed homology model of human αB crystallin described previously using Molecular Operating Environment (Chemical Computing Group, Montreal, Canada) (Ghosh & Clark, 2005; Ghosh, Estrada, & Clark, 2005). 3D co-ordinates for human transthyretin and human β2-microglobulin were obtained from the crystal structures with PDB IDs 1DVQ and 1LDS respectively (Klabunde et al., 2000; Trinh, Smith, Kalverda, Phillips, & Radford, 2002). Co-ordinates for the peptides and the target proteins were provided to the molecular docking program ClusPro (Comeau, Gatchell, Vajda, & Camacho, 2004a, 2004b), which computed docking models for each protein-peptide combination. It is important to note that only the 3D co-ordinates for the αB crystallin peptides and crystal structures of transthyretin and β2-microglobulin were provided to the ClusPro docking program. The active site for docking the αB crystallin peptides was not specified. The docking was performed 3–5 times for each protein-peptide pair and produced the same results. The Cluspro docking program used a fast algorithm for filtering docked conformations with good surface complementarity, and ranked them based on their clustering properties. The free energy filters selected complexes with lowest desolvation and electrostatic energies. Finally, clustering smoothed the local minima and selected the centers of the most populated clusters as predictions of the unknown complex.

Results

The five full-length human αB crystallin peptides that were synthesized for use in fibril assays corresponded to five previously identified functional sequences in the molecular chaperone, human αB crystallin (Figure 1). The Thioflavin T (ThT) fluorescence assay identified αB crystallin peptides that inhibited or promoted fibril formation of $A\beta_{1–42}$, α-synuclein, transthyretin, and β2-microglobulin *in vitro* (Figures 2 and 3). The effects of the αB crystallin peptides on the fibrillation of $A\beta_{1–42}$ and α-synuclein are summarized in Figure 2 and the effects of the αB crystallin peptides on the fibrillation of transthyretin and β2-microglobulin are summarized in Figure 3. At a concentration of 6μM peptide ($A\beta_{1–42}$:peptide ratio of 5:1), the full-length ST and HG peptides, the truncated DR peptides DRFS, RFSV, FSVN, NLDV, LDVK, DVKH, VKHF, and KHFS, and the truncated HG peptides GKHE, KHEE, HEER, and ERQD had the strongest inhibitory effect on $A\beta_{1–42}$ fibrillation and had ThT fluorescence values between 0.00 and 0.71 of the ThT fluorescence of $A\beta_{1–42}$ alone (Figure 2). At the same concentrations, the full-length DR and ER peptides and the truncated HG peptides, EERQ, and RQDE increased $A\beta_{1–42}$ fibrillation and had ThT fluorescence values between 1.14 and 1.65 of the ThT fluorescence of $A\beta_{1–42}$ alone. The full-length FI and LT peptide, the truncated DR peptides SVNL and VNLD, and the truncated HG peptide HGKH had no effect on $A\beta_{1–42}$ fibrillation and had ThT fluorescence values similar to the control peptide AT1. At a concentration of 30μM peptide ($A\beta_{1–42}$:peptide ratio of 1:1), the full-length ST, DR, and HG peptides, the truncated DR peptides DRFS, RFSV, FSVN, VNLD, NLDV, LDVK, DVKH, VKHF, and KHFS, and the truncated HG peptides GKHE and ERQD had the strongest inhibitory effect on $A\beta_{1–42}$ fibrillation and had ThT fluorescence values between 0.00 and 0.72 of the ThT fluorescence of $A\beta_{1–42}$ alone. At the same concentration, the full-length LT and ER peptides and the truncated HG peptides, HEER, EERQ, and RQDE increased $A\beta_{1–42}$

fibrillation and had ThT fluorescence values between 1.36 and 1.68 of the ThT fluorescence of $A\beta_{1–42}$ alone. The full-length FI peptide, the truncated DR peptide SVNL and the truncated HG peptides HGKH and KHEE had no effect on $A\beta_{1–42}$ fibrillation and had ThT fluorescence values similar to the control peptide AT1.

At a concentration of 9.2μM peptide (α-synuclein:peptide ratio of 5:1), the full-length ST, DR, HG, LT, and ER peptides, the truncated DR peptides DRFS, RFSV, FSVN, SVNL, VNLD, NLDV, DVKH, and KHFS, and the truncated HG peptides, HGKH, GKHE, HEER, EERQ, ERQD, and RQDE had the strongest effect on α-synuclein fibrillation and had ThT fluorescence values between 0.0 and 0.75 of the ThT fluorescence of α-synuclein alone (Figure 2). At the same concentration, the truncated HG peptide increased α-synuclein fibrillation and ThT fluorescence values 1.61 times the ThT fluorescence of α-synuclein alone. The full-length FI peptide, the truncated DR peptides, LDVK and VKHF and the truncated HG peptide KHEE had no effect on α-synuclein fibrillation and had ThT fluorescence values similar to the control peptide AT1. At a concentration of 46μM peptide (α-synuclein:peptide ratio of 1:1), the full-length DR, HG, LT, and ER peptides, the truncated DR peptides DRFS, RFSV, FSVN, SVNL, VNLD, NLDV, LDVK, DVKH, and KHFS, and the truncated HG peptides, HGKH, KHEE, and HEER had the strongest effect on α-synuclein fibrillation and had ThT fluorescence values between 0.00 and 0.49 of the ThT fluorescence of α-synuclein alone. At the same concentration, full-length ST peptide increased α-synuclein fibrillation and a ThT fluorescence value 4.46 times the ThT fluorescence of α-synuclein alone. The full-length FI peptide, the truncated DR peptide VKHF and the truncated HG peptides GKHE, EERQ, ERQD, and RQDE had no effect on α-synuclein fibrillation and had ThT fluorescence values similar to the control peptide AT1.

The full-length ST and HG peptides, the truncated DR peptide FSVN, and the truncated HG peptide ERQD were the strongest inhibitors of $A\beta_{1–42}$ fibrillation. The full-length DR peptide, the truncated DR peptides DRFS, RFSV, FSVN, SVNL, VNLD, NLDV, DVKH, and KHFS, and the truncated HG peptide HGKH were the strongest inhibitors of α-synuclein fibrillation (Figure 2).

At a concentration of 3.2μM peptide (transthyretin:peptide ratio of 5:1), the full-length ST, DR, and HG peptides, the truncated DR peptides DRFS, RFSV, FSVN, SVNL, VNLD, NLDV, LDVK, DVKH, VKHF, and KHFS, and the truncated HG peptides HGKH, GKHE, KHEE, HEER, EERQ, ERQD, and RQDE had the strongest effect on transthyretin fibrillation and had ThT fluorescence values between 0.14 and 0.67 of the ThT fluorescence of transthyretin alone (Figure 3). The full-length FI, LT and ER peptides had little or no effect on transthyretin fibrillation and had ThT fluorescence values similar to the control peptide AT1. At a concentration of 16μM peptide (transthyretin:peptide ratio of 1:1), the full-length DR, HG, and ER peptides, the truncated DR peptides DRFS, RFSV, FSVN, SVNL, VNLD, NLDV, LDVK, DVKH, VKHF, and KHFS, and the truncated HG peptides HGKH, GKHE, KHEE, HEER, EERQ, ERQD, and RQDE had the strongest effect on transthyretin fibrillation and had ThT fluorescence values between 0.0 and 0.63 of the ThT fluorescence of transthyretin alone. The full-length ST, FI, and LT peptides had little or no effect on transthyretin fibrillation and had ThT fluorescence values similar to the control peptide AT1. No αB crystallin peptides increased transthyretin fibrillation at the measured concentrations.

At a concentration of 5.8μM peptide (β2-microglobulin:peptide ratio of 5:1), the full-length ST, DR, HG, LT, and ER peptides, the truncated DR peptides FSVN, SVNL, VNLD, NLDV, LDVK, and DVKH, and the truncated HG peptides HGKH, GKHE, KHEE, HEER, EERQ, and RQDE had the strongest effect on β2-microglobulin fibrillation and had ThT fluorescence values between 0.16 and 0.62 of the ThT fluorescence of β2-microglobulin alone (Figure 3). The full-length FI peptide, the truncated DR peptides, DRFS, VKHF, and KHFS increased β2-microglobulin fibrillation and had ThT fluorescence values between 1.28 and 2.17 of the

ThT fluorescence of β2-microglobulin alone. The truncated DR peptide RFSV and the truncated HG peptide ERQD had no effect on β2-microglobulin fibrillation and had ThT fluorescence values similar to the control peptide AT1. At a concentration of 29μM peptide (β2-microglobulin:peptide ratio of 1:1), the full-length ST, DR, HG, LT, and ER peptides, the truncated DR peptides FSVN and NLDV, and the truncated HG peptides HGKH, GKHE, KHEE, HEER, EERQ, and RQDE had the strongest effect on β2-microglobulin fibrillation and had ThT fluorescence values between 0.0 and 0.65 of the ThT fluorescence of β2-microglobulin alone. The full-length FI peptide, the truncated DR peptides, DRFS, RFSV, SVNL, DVKH, and KHFS increased β2-microglobulin fibrillation and had ThT fluorescence values between 1.22 and 2.29 of the ThT fluorescence of β2-microglobulin alone. The truncated DR peptide VNLD, LDVK, and VKHF, and the truncated HG peptide ERQD had no effect on β2-microglobulin fibrillation and had ThT fluorescence values similar to the control peptide AT1.

The full-length HG peptide and the truncated HG peptides HGKH, EERQ, and RQDE were the strongest inhibitors of transthyretin fibrillation. The full-length DR peptide and the truncated HG peptides HGKH, HEER, and EERQ were the strongest inhibitors of β2-microglobulin fibrillation (Figure 3).

A 3D model of the interactions between the αB crystallin peptides $_{73}$DRFSVNLDVKHFS$_{85}$ which forms the β3 strand and $_{101}$HGKHEERQDE$_{110}$ which forms part of the loop connecting the β5 and β7 strands on the exposed surface of the α crystallin core domain was determined by docking the two peptides with the crystal structures of transthyretin and β2-micrglobulin (Figure 4A). In the transthyretin model, residues Ser-76, Asn-78, and Asp-80 from the $_{73}$DRF**S**V**N**L**D**VKHFS$_{85}$ peptide interacted with residues Glu-83, Val-85, Thr-87, and Asp-90 in transthyretin (Figure 4B). Similarly, residues His-104, Glu-105, and Arg-107 from the $_{101}$HGK**HE**E**R**QDE$_{110}$ peptide interacted with residues His-79, His-81, Glu-83, and Val-85 in transthyretin (Figure 4B). The sequence $_{79}$**H**E**H**A**E**V**V**F**T**AN**D**$_{90}$, which contains these residues forms the amyloidogenic F strand in transthyretin (Figure 4A). In the β2-microglobulin model, residues Phe-75, Val-77, and Leu-79 from the $_{73}$DR**F**S**V**N**L**DVKHFS$_{85}$ peptide interacted with residues Glu-51, Ser-53, Asp-54, Leu-55, and Phe-57 in β2-microglobulin (Figure 4B). Similarly, residues His-101, Lys-103, His-104, Glu-105, and Arg-107 from the $_{101}$**H**G**KHE**E**R**QDE$_{110}$ peptide interacted with residues Glu-51, His-52, Ser-53, and Leu-55 in β2-microglobulin. The sequence $_{50}$V**EHSDL**S**F**SKD$_{60}$, which contains these residues forms the amyloidogenic D strand in β2-microglobulin (Figure 4A). Direct interactions between residues of the αB crystallin peptides and residues of the amyloidogenic fibril forming β strands of transthyretin and β2-microglobulin can be expected to prevent abnormal self-interactions between transthyretin and β2-microglobulin molecules that result in the formation of β strand rich amyloid-like fibrils. The absence of X-ray crystal or NMR structures for Aβ$_{1–42}$ and α-synuclein prevented docking studies with these proteins. Alignment of the primary sequences of Aβ$_{1–42}$ and α-synuclein with the primary sequences of transthyretin and β2-micrglobulin identified the residues $_{12}$VHHQKLVFFAED$_{23}$ and $_{87}$SIAAATGFVKKD$_{98}$ in Aβ$_{1–42}$ and α-synuclein respectively that were similar in primary sequence to the amyloidogenic F and D β strands of transthyretin and β2-microglobulin (Figure 4C). The inhibitory effect of the αB crystallin peptides on the fibrillation of all four amyloid target proteins and the sequence similarity of the interactive amyloidogenic regions of the four target proteins is consistent with a common mechanism of fibril inhibition by αB crystallin peptides.

Discussion

Five bioactive peptides synthesized on the basis of interactive sequences from the sHSP and molecular chaperone human αB crystallin inhibited or promoted the fibrillation of Aβ$_{1–42}$, α-

synuclein, transthyretin, and β2-microglobulin. The inhibitory effects of the αB crystallin peptides on $A\beta_{1–42}$, α-synuclein, and β2-microglobulin fibrillation were consistent with previous studies on the effects of full-length αB crystallin and peptide fragments of αA crystallin on $A\beta_{1–42}$, α-synuclein, and β2-microglobulin fibrillation (Lee, Carson, Rice-Ficht, & Good, 2006; Narayanan, Kamps, Boelens, & Reif, 2006; Raman et al., 2005; Rekas et al., 2004; Santhoshkumar & Sharma, 2004; Stege et al., 1999; Wilhelmus, Boelens, Otte-Holler, Kamps, de Waal et al., 2006). The interactive sequences $_{73}$DRFSVNLDVKHFS$_{85}$ and $_{101}$HGKHEERQDE$_{110}$ in the conserved α crystallin core domain of human αB crystallin reduced the fibrillation of all four target proteins by >50%. The results confirmed previous studies that demonstrated the importance of the $_{73}$DRFSVNLDVKHFS$_{85}$ peptide as a chaperone sequence that inhibited the aggregation of structurally diverse target proteins including lens crystallins, alcohol dehydrogenase, citrate synthase, and FGF (Ghosh, Estrada, & Clark, 2005; Ghosh, Estrada, Houck, & Clark, 2006; Ghosh, Shenoy, & Clark, 2007b). Systematic truncation of the $_{73}$DRFSVNLDVKHFS$_{85}$ and $_{101}$HGKHEERQDE$_{110}$ peptides resulted in the identification of new bioactive peptide inhibitors, FSVN, NLDV, HGKH, and HEER, which reduced fibrillation by >75% as measured by Thioflavin T fluorescence. The identification of these peptides as potent fibril inhibitors was supported by protein-peptide docking studies which identified residues Phe-75, Ser-76, Val-77, Asn-78, Leu-79, and Asp-80 from $_{73}$DR**FSVNLD**VKHFS$_{85}$ and residues His-101, Lys-103, His-104, Glu-105, and Arg-107 from $_{101}$**H**G**KHE**E**R**QDE$_{110}$ as residues that interacted directly with the fibril forming F and D β strands of transthyretin and β2-microglobulin (Figure 4).

The oligomerization and aggregation of amyloidogenic proteins including Aβ proceeds through the formation of various intermediates including dimers, tetramers, soluble oligomers, protofibrils, fibrils, unstructured aggregates, and plaques (Bucciantini et al., 2002; Glabe, 2006; J. Hardy & Selkoe, 2002; J. A. Hardy & Higgins, 1992; Selkoe, 2001). The rate and amount of formation of each intermediate depends on environmental factors including pH, metals, and the presence of stress response proteins like molecular chaperones (Alexandrescu, 2005; Dedmon, Christodoulou, Wilson, & Dobson, 2005; Harper, Wong, Lieber, & Lansbury, 1999). Although, molecular chaperones generally inhibit protein aggregation, abnormal interactions between chaperones and substrate proteins can result in increased aggregation and disease (Liu et al., 2006; Vicart et al., 1998). In the fibril assays reported in this paper, most full-length and truncated αB crystallin peptides inhibited fibrillation. However, the αB crystallin peptides, STSLSPFYLRPPSFLRAP, FISREFHR, LTITSSLSDGV, ERTIPITRE, DRFS, RFSV, DVKH, KHFS, HEER, EERQ, and RQDE increased fibril formation by 14–129%. The fibril promoting effect depended on peptide concentration and the ratio of peptide to target protein used in the assays. The results demonstrated that αB crystallin contains both fibril inhibiting and promoting interactive sequences which may explain the seemingly contradictory effects of αB crystallin on Aβ fibrillation and toxicity reported previously. A previous study demonstrated that the interactive peptides FISREFHR, LTITSSLSDGV, and ERTIPITRE in αB crystallin modulate the assembly of microtubules *in vitro* (Ghosh, Houck, & Clark, 2007a). The ability of αB crystallin to modulate both Aβ fibrillation and microtubule assembly and disassembly suggests that sHSPs may be a link between the Aβ cascade pathway and the tau-tubulin pathway which results in the formation of neurofibrillary tangles in AD (Ghosh, Houck, & Clark, 2007a).

The results illustrate an important finding that sHSPs like αB crystallin may play a more important and direct role in the pathophysiology of AD than previously thought (Lee, Carson, Rice-Ficht, & Good, 2005, 2006; Liang, 2000; Raman et al., 2005; Santhoshkumar & Sharma, 2004; Stege et al., 1999; Wilhelmus, Boelens, Otte-Holler, Kamps, de Waal et al., 2006). Although, self-interactions of amyloidogenic proteins result in fibril formation, smaller, soluble and more toxic oligomeric intermediates may be the pathological factors for aggregation diseases including AD and PD (McParland et al., 2000; Mornon et al., 1998;

Nelson & Eisenberg, 2006; Schormann, Murrell, & Benson, 1998; Serag, Altenbach, Gingery, Hubbell, & Yeates, 2002; Thirumalai, Klimov, & Dima, 2003; Trinh, Smith, Kalverda, Phillips, & Radford, 2002). Our results indicate that αB crystallin peptides modulate the interactions between amyloidogenic proteins to increase or decrease fibrillation. Under conditions in which increasing Aβ fibrillation decreases the levels of more toxic soluble oligomers of Aβ and reduces functional deficits in transgenic AD mice (Cheng et al., 2007; Kirkitadze, Bitan, & Teplow, 2002) peptides of αB crystallin can still have a protective action. Future studies will characterize the interactions between αB crystallin peptides and soluble oligomeric intermediates of amyloidogenic proteins including $A\beta_{1–42}$, α-synuclein, transthyretin, and β2-microglobulin.

sHSPs and amyloidogenic proteins including transthyretin and β2-microglobulin have similar 3D structures consisting of an immunoglobulin-like fold composed of two anti-parallel β sheets arranged in a compact β sandwich (Mornon et al., 1998; Nelson & Eisenberg, 2006; Sunde et al., 1997). Abnormal interactions of β strands F ($_{79}$HEHAEVVFTAND$_{90}$) and D ($_{50}$VEHSDLSFSKD$_{60}$) in transthyretin and β2-microglobulin respectively (Figure 4) result in the formation of β strand rich amyloid-like fibrils (McParland et al., 2000; Mornon et al., 1998; Nelson & Eisenberg, 2006; Schormann, Murrell, & Benson, 1998; Serag, Altenbach, Gingery, Hubbell, & Yeates, 2002; Thirumalai, Klimov, & Dima, 2003; Trinh, Smith, Kalverda, Phillips, & Radford, 2002). When the αB crystallin $_{73}$DRFSVNLDVKHFS$_{85}$ and $_{101}$HGKHEERQDE$_{110}$ peptides were docked with the structures of transthyretin and β2-microglobulin, residues from both αB crystallin peptides interacted with residues in the F ($_{79}$**H**E**H**A**E**V**V**F**T**AN**D**$_{90}$) and D ($_{50}$V**EHSDL**S**F**SKD$_{60}$) strands of transthyretin and β2-microglobulin (Figure 4). Together with the Thioflavin T assays, these results suggest that surface exposed residues in the $_{73}$DR**FSVNLD**VKHFS$_{85}$ and $_{101}$**H**G**KHE**E**R**QDE$_{110}$ sequences in the α crystallin core domain of human αB crystallin interact with surface exposed residues in the F and D β strands of transthyretin and β2-microglobulin respectively to inhibit fibrillation. The absence of crystal structures for $A\beta_{1–42}$ and α-synuclein prevented modeling of the interactions between the αB crystallin peptides and $A\beta_{1–42}$ and α-synuclein. Comparison of the primary sequences of transthyretin and β2-microglobulin with the primary sequences of $A\beta_{1–42}$ and α-synuclein identified residues 14–23 in $A\beta_{1–42}$ and 89–98 in α-synuclein as sites of interaction for the αB crystallin peptides (Figure 4C). The identification of these residues in $A\beta_{1–42}$ and α-synuclein as sites of interaction for the αB crystallin peptide inhibitors was consistent with previous reports that identified these same residues as amyloidogenic residues in $A\beta_{1–42}$ and α-synuclein (Balbach et al., 2000; Miake, Mizusawa, Iwatsubo, & Hasegawa, 2002; Morimoto et al., 2004). Further support for the inhibitory action of the αB crystallin peptides at these sites comes from a recent NMR study that demonstrated direct interactions between human αB crystallin and residues 17–21 of $A\beta_{1–42}$ (Narayanan, Kamps, Boelens, & Reif, 2006). Based on the fibril assays, protein-peptide docking results, and sequence analysis, it is hypothesized that the protective effects of the sHSP αB crystallin results from interactions with $A\beta_{1–42}$, α-synuclein, transthyretin, and β2-microglobulin via residues in the $_{73}$DR**FSVNLD**VKHFS$_{85}$ and $_{101}$**H**G**KHE**E**R**QDE$_{110}$ sequences on the surface interface of the α crystallin core domain.

Development of therapeutics for AD and PD is focused primarily on the amyloid cascade pathway, which proceeds through stages of unfolding, aggregation, oligomerization, and fibrillation of amyloidogenic proteins (Bucciantini et al., 2002; Glabe, 2006; J. Hardy & Selkoe, 2002; J. A. Hardy & Higgins, 1992; Selkoe, 2001). Tramiprosate and NAP, two molecules that disrupt the amyloid cascade pathway (monomer → oligomer → fibril) by preventing the formation of β sheet fibrils have been most successful in clinical trials to date (Gervais et al., 2007; Gozes et al., 2005). The target specificity ($A\beta_{1–42}$ only) of these two molecules limits their potential use as therapeutics in other non-AD amyloidoses. The results of the current study identified interactive sites in bioactive peptides that use similar mechanisms of action to target

the toxic intermediate states of four distinct amyloidogenic proteins and may serve as the basis for a new class of disease modifying therapeutics for a variety of amyloidoses.

In this report, Thioflavin T fluorescence assays, protein-peptide docking, and sequence analysis were conducted with full-length and truncated interactive sequences from the molecular chaperone, human αB crystallin to identify peptides that that interact with and inhibit or promote the fibrillation of the important amyloidogenic proteins: $A\beta_{1–42}$, α-synuclein, transthyretin, and β2-microglobulin. The αB crystallin peptides identified in this report inhibited or promoted fibrillation at low concentrations indicating excellent potential for the rational design of peptidic and peptidomimetic inhibitors targeting the toxic intermediates of amyloidogenic proteins.

Acknowledgments

This work was supported by grant EY04542 from the National Eye Institute.

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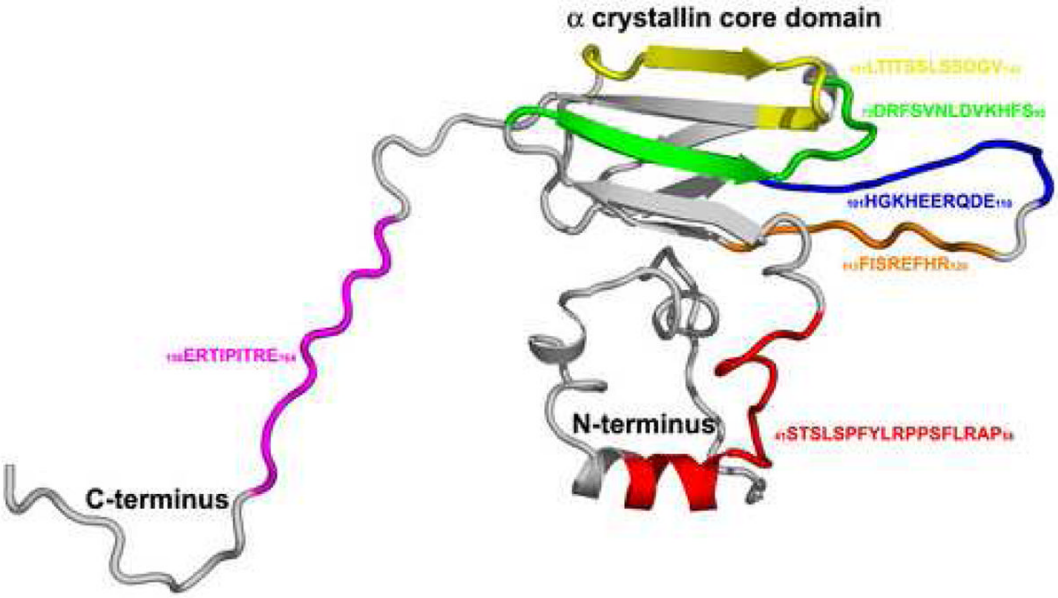

Figure 1.

3D map of the interactive domains in human αB crystallin. The interactive sequences in human αB crystallin were mapped onto the 3D homology model of human αB crystallin (grey). The N-terminal helical sequence $_{41}$STSLSPFYLRPPSFLRAP$_{58}$ (red) is important for substrate recognition and binding (Ghosh, Shenoy, & Clark, 2006). On the exposed surface of the α crystallin core domain, the sequences, $_{73}$DRFSVNLDVKHFS$_{85}$ (green), which forms the β3 strand, $_{101}$HGKHEERQDE$_{110}$ (blue) and $_{113}$FISEFHR$_{120}$ (orange) which form the loop region connecting the β strands 5 and 7, and $_{131}$LTITSSLSSDGV$_{142}$ (yellow) which forms the β8 strand are important for substrate binding (Ghosh, Estrada, & Clark, 2005, 2006; Ghosh, Estrada, Houck, & Clark, 2006). The flexible C-terminal sequence $_{156}$ERTIPITRE$_{164}$ (magenta) is important for solubility of the sHSP-substrate complex (Ghosh, Shenoy, & Clark, 2006). The interactive domains in human αB crystallin have multiple functions and the Thioflavin T fibril assays identified synthetic αB crystallin peptides that inhibit or promote fibril formation of the four amyloidogenic proteins amyloid-β, α-synuclein, transthyretin, and β2-microglobulin.

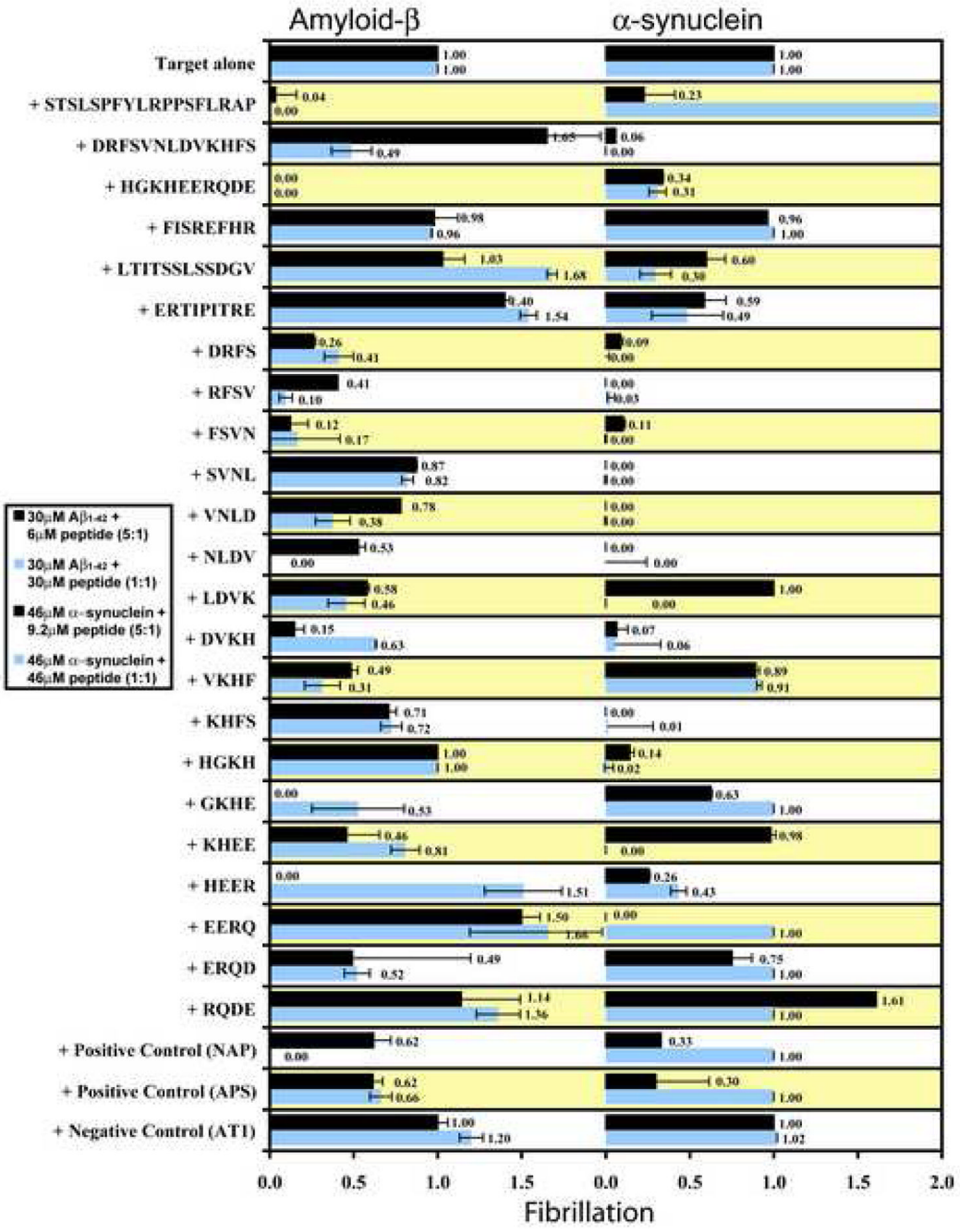

Figure 2.

Bioactive synthetic αB crystallin peptides promoted or inhibited $A\beta_{1–42}$ and α-synuclein fibrillation in Thioflavin T fluorescence assays. The STSLSPFYLRPPSFLRAP, HGKHEERQDE, FSVN, and ERQD peptides had the strongest inhibitory effect and the LTITSSLSDGV, ERTIPITRE, HEER, and EERQ peptides had the strongest promoting effect on $A\beta_{1–42}$ fibrillation. The DRFSVNLDVKHFS, DRFS, RFSV, FSVN, SVNL, VNLD, NLDV, DVKH, KHFS, and HGKH peptides had the strongest inhibitory effect and the STSLSPFYLRPPSFLRAP and RQDE peptides had the strongest promoting effect on α-synuclein fibrillation. The control peptide AT1 had no effect on $A\beta_{1–42}$ or α-synuclein fibrillation, and the two positive control molecules NAP and APS had modest effects on

$A\beta_{1-42}$ and α-synuclein fibrillation. Each assay was done in triplicate and the error bars indicate the standard deviation. The results demonstrated the effectiveness of individual interactive domains in αB crystallin on modulation of fibril formation in vitro.

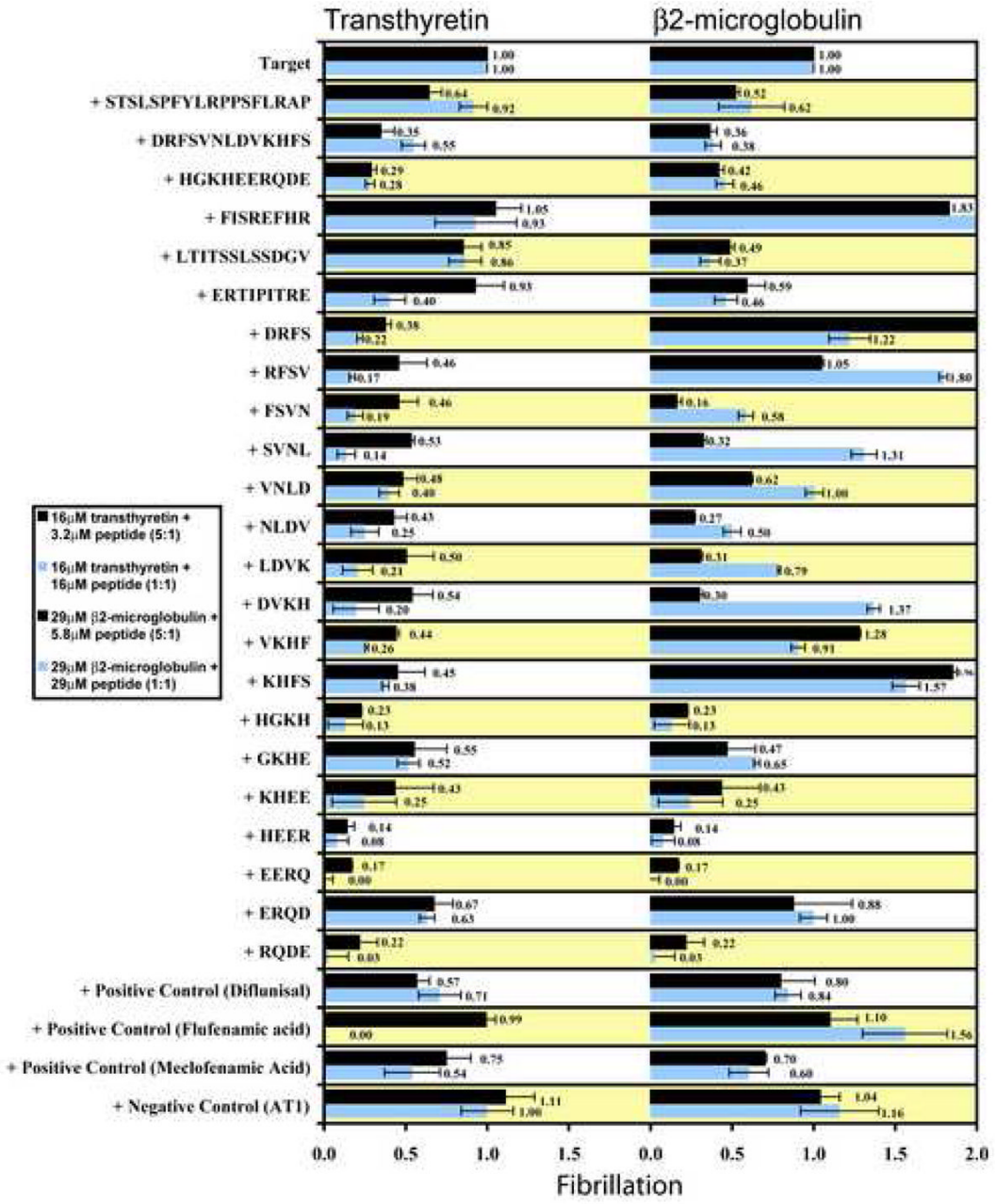

Figure 3.

Bioactive synthetic αB crystallin peptides promoted or inhibited transthyretin and β2-microglobulin fibrillation in Thioflavin T fluorescence assays. The DRFSVNLDVKHFS, HGKHEERQDE, DRFS, HGKH, HEER, EERQ, and RQDE peptides had the strongest inhibitory effect on transthyretin fibrillation. No peptides promoted transthyretin fibrillation. The DRFSVNLDVKHFS, HGKHEERQDE, LTITSSLSDGV, ERTIPITRE, FSVN, HGKH, HEER, EERQ, and RQDE peptides had the strongest inhibitory effect and the FISREFHR, DRFS, RFSV, DVKH, and KHFS peptides had the strongest promoting effect on β2-microglobulin fibrillation. The control peptide AT1 had no effect on transthyretin or β2-microglobulin fibrillation, and the three positive control molecules diflunisal, flufenamic acid, and mecloflenamic acid had modest effects on transthyretin and β2-microglobulin fibrillation.

Each assay was done in triplicate and the error bars indicate the standard deviation. The results demonstrated the effectiveness of individual interactive domains in αB crystallin on modulation of fibril formation in vitro.

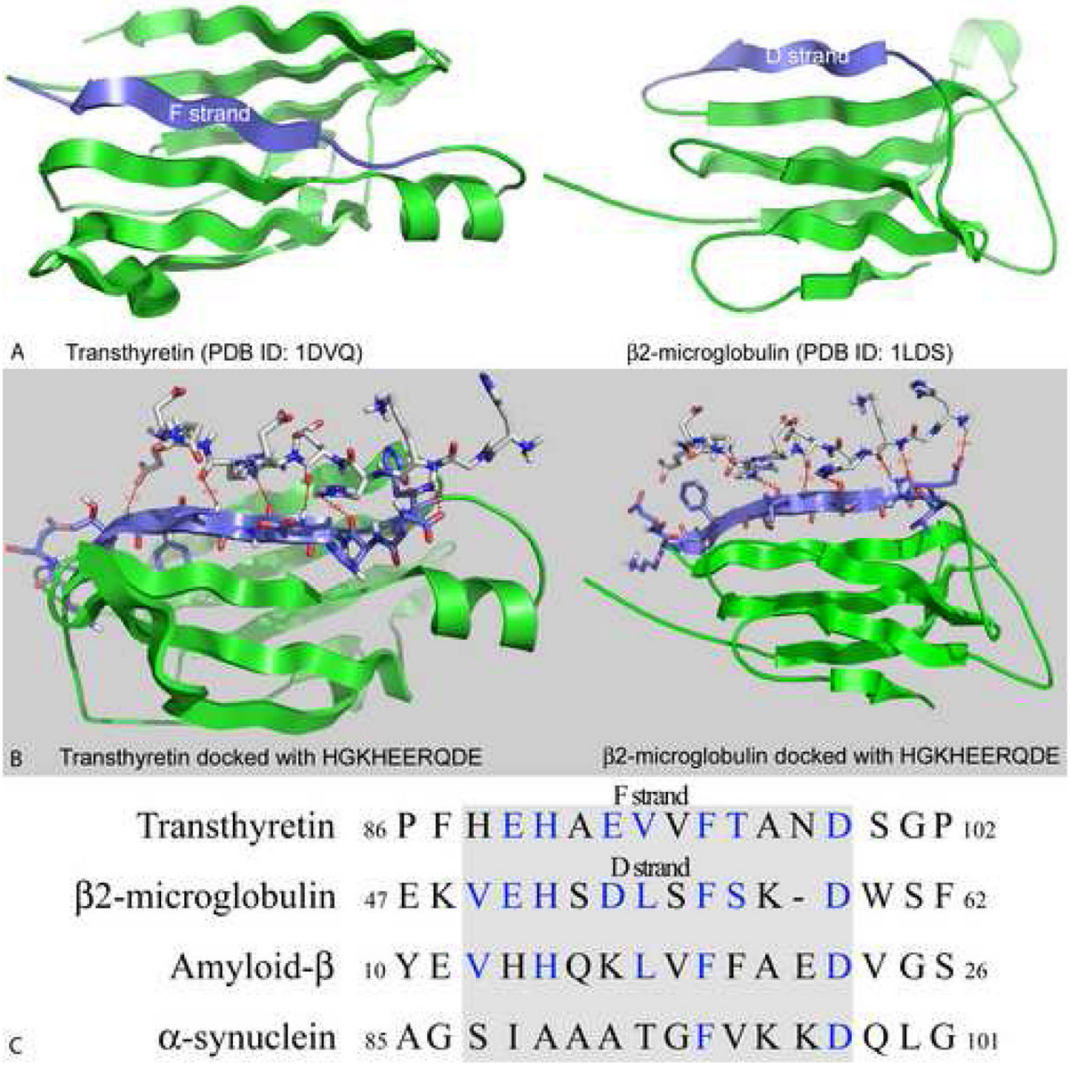

Figure 4.

Structural models of transthyretin and β2-microglobulin, protein-peptide docking models and sequence analysis. A) Ribbon diagrams of the monomeric subunits of transthyretin and β2-microglobulin are shown in green. The amyloidogenic β strands, F and D in transthyretin and β2-microgloublin respectively are in blue (McParland et al., 2000; Serag, Altenbach, Gingery, Hubbell, & Yeates, 2002; Thirumalai, Klimov, & Dima, 2003; Trinh, Smith, Kalverda, Phillips, & Radford, 2002). B) The $_{101}$HGKHEERQDE$_{110}$ peptide from the loop region connecting β5 and β7 in the α crystallin core domain of human αB crystallin was docked with the crystal structures of transthyretin (left) and β2-microglobulin (right) using the ClusPro molecular docking program (Comeau, Gatchell, Vajda, & Camacho, 2004a, 2004b). Residues of the $_{101}$HGKHEERQDE$_{110}$ sequence interact with residues of the F and D β strands of transthyretin and β2-microglobulin respectively. Interactions with the same residues in transthyretin and β2-microglobulin were observed when the $_{73}$DRFSVNLDVKHFS$_{85}$ peptide which forms the β3 strand in the α crystallin core domain of αB crystallin was docked with the crystal structures of transthyretin and β2-microglobulin (data not shown). C) Sequence alignment of the amyloidogenic regions of transthyretin and β2-microglobulin with the primary sequences of Aβ$_{1–42}$ and α-synuclein. The amyloidogenic regions of transthyretin and β2-

microglobulin were aligned with the primary sequences of $A\beta_{1-42}$ and α-synuclein using the DOTTER program (Miake, Mizusawa, Iwatsubo, & Hasegawa, 2002; Serag, Altenbach, Gingery, Hubbell, & Yeates, 2002; Sonnhammer & Durbin, 1995; Thirumalai, Klimov, & Dima, 2003; Trinh, Smith, Kalverda, Phillips, & Radford, 2002). Conserved residues are in blue. The grey box indicates encloses the residues that comprise the F and D β strands in transthyretin and β2-microglobulin. Residues 88–99, 49–59, 12–23, and 87–98 are the amyloidogenic fibril forming regions in transthyretin, β2-microglobulin, $A\beta_{1-42}$, and α-synuclein respectively (Balbach et al., 2000; McParland et al., 2000; Miake, Mizusawa, Iwatsubo, & Hasegawa, 2002; Morimoto et al., 2004; Serag, Altenbach, Gingery, Hubbell, & Yeates, 2002; Thirumalai, Klimov, & Dima, 2003; Trinh, Smith, Kalverda, Phillips, & Radford, 2002). Structural analysis identified key interactions between amyloidogenic proteins and exposed peptides on the surface of the α crystallin core domain of human αB crystallin that account for modulation of fibril formation observed in the Thioflavin T assembly assays.