

Inhibition of  $\beta$ -Amyloid Fibrillization by Directed Evolution of a  $\beta$ -Sheet Presenting Miniature Protein

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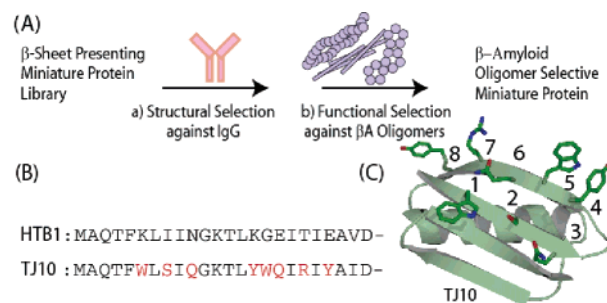
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Human diseases, such as Alzheimer's, Parkinson's, Creutzfeldt-Jakob, and Huntington's, are linked to protein misfolding events. In these diseases, proteins and peptides aggregate and subsequently form fibrils, with a characteristic cross- $\beta$  pattern.<sup>1</sup> In Alzheimer's disease, the primary components of senile plaque associated with neurological toxicity are fibrils of the 39–43 residue  $\beta$ -amyloid ( $A\beta$ ) peptides, which are cleavage products of the amyloid precursor protein.<sup>2</sup> Thus, the prevention of  $A\beta$  peptide oligomerization is a goal for many therapeutic efforts in this area as well as being a possible route to trapping intermediates for understanding the  $A\beta$  aggregation pathway.<sup>3</sup>

Many recent studies have focused on designing inhibitors of protein aggregation, the most successful being the elegant class of molecules designed by Kelly and co-workers that prevents aggregation by stabilizing the native state of transthyretin.<sup>1a,4</sup> However, similar approaches toward many amyloidogenic proteins are problematic as the non-amyloidogenic state is often ill-defined.<sup>1,3</sup> In Alzheimer's research, many efforts in the design of  $A\beta$  fibrillization inhibitors have focused upon mimetics of the parent  $A\beta$  peptide.<sup>5</sup> Truncated analogues of the  $A\beta$  peptide with selective methylation of the amide backbone have been shown to prevent  $A\beta$  aggregation.<sup>5c</sup> Recently, Hammer and co-workers have designed  $\alpha,\alpha$ -disubstituted peptide mimetics of  $A\beta$  that can block  $A\beta$  fibrillization.<sup>5d</sup> Murphy and Kiessling have attached lysine oligomers to an  $A\beta$  fragment that alters the fibrillization pathway resulting in nontoxic aggregates.<sup>6</sup> In a different selection-based approach, Wetzel et al. have identified antibodies against  $A\beta$  that are surprisingly general for all types of amyloid fibrils tested.<sup>7a</sup> Interestingly, Glabe and co-workers have identified an antibody that recognizes soluble  $A\beta$  oligomers but also recognizes oligomers of unrelated amyloidogenic proteins.<sup>7b</sup>

Building on these studies, we posed the question whether it would be possible to generate a new class of structurally well-defined  $\beta$ -sheet epitopes that target  $A\beta$ , that are not based upon the parent  $A\beta$  sequence and do not possess the complexity of an antibody. Toward this goal, we chose a 56-residue hyperthermophilic IgG-binding protein redesigned by Malakauskas and Mayo,<sup>8</sup> termed HTB1, which could potentially provide a structurally matched  $\beta$ -sheet template for targeting intermediates in  $A\beta$  aggregation (Figure 1). We envisioned that evolved variants of HTB1 could provide new structurally defined fibrillization inhibitors. To this end, eight positions on adjacent strands of the  $\beta$ -sheet scaffold, HTB1, were randomized to yield a  $\beta$ -sheet presenting phage-displayed library. Our selection involved two steps: (a) selection against IgG, mediated by the helical region on HTB1, to maintain the native structure of the HTB1 library members;<sup>8b</sup> and (b) selection against  $A\beta_{1-40}$  (25  $\mu$ M) utilizing the  $\beta$ -sheet region of HTB1, to evolve a new binding function.<sup>8b</sup>

We targeted  $A\beta_{1-40}$  as it displays slower aggregation kinetics than  $A\beta_{1-42}$ . The conditions for  $A\beta_{1-40}$  immobilization (48 h in a

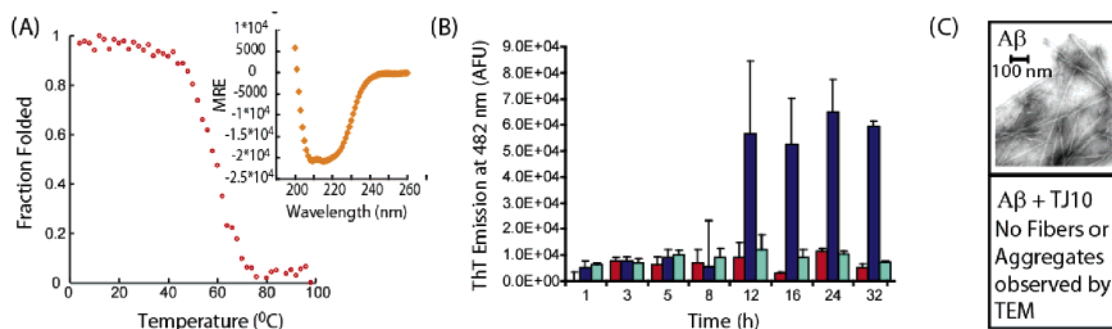


**Figure 1.** (A) Selection strategy for evolution of  $A\beta$  oligomer-specific miniature proteins. (B) Amino acid sequences of the first two  $\beta$ -strands of HTB1 and the selected mini-protein TJ10 (residues in red represent positions that were randomized). (C) Ribbon model of TJ10 based on the NMR structure of the parent HTB1.

96 well plate at 37 °C in PBS) prevented complete  $A\beta$  fibrillization as verified by transmission electron microscopy (TEM). After five rounds of panning, only two specific HTB1 library members were preferentially selected (Supporting Information). We chose to evaluate the properties of one of these miniature proteins, TJ10 (Figure 1), as it contained a large number of aromatic residues (2 Trp and 2 Tyr) that have been postulated to be an important motif in amyloid inhibitors.<sup>10</sup>

After cloning, sequencing, and purification, we wanted to verify that the selected mini-protein, TJ10, with two new Tyr and two new Trp residues maintained its native, primarily  $\beta$ -sheet secondary structure. The circular dichroism (CD) spectrum of TJ10 (Figure 2a inset) was characteristic of proteins with high  $\beta$ -sheet content and very similar to that of HTB1.<sup>8</sup> TJ10 also retained the well-folded, hyperthermophilic characteristics of the parent HTB1, as evidenced by thermal denaturation experiments in the presence of 3 M Gdn·HCl, a chemical denaturant (Figure 2a). TJ10 was very stable, displaying a  $T_m$  of 60 °C, whereas most proteins are completely unfolded in 3 M Gdn·HCl at room temperature. Finally, fluorescence-based competition assays confirmed that TJ10 retains IgG-binding activity comparable to that of the parent HTB1 (Supporting Information, Figure S2).

Having established that we had selected a stable, well-folded,  $\beta$ -sheet-containing miniature protein, we turned to interrogating its effect upon  $A\beta$  aggregation. To this end, we allowed  $A\beta_{1-40}$  (50  $\mu$ M) to aggregate in the presence and absence of TJ10 at equimolar concentration (PBS, 37 °C, with continuous shaking at 250 rpm). The degree of aggregation was measured by changes in Thioflavin T (ThT) fluorescence ( $\lambda_{exc}$  = 450 nm,  $\lambda_{em}$  = 482 nm). The fibrillization assay was repeated three times in duplicate and on separate days to ensure reproducibility. Figure 2b shows the combined results, where TJ10 was observed to completely prevent amyloid aggregation at equimolar concentrations. We also tested the mixture (TJ10: $A\beta_{1-40}$  ratio of 1:1) over a period of 2 weeks



**Figure 2.** (A) Circular dichroism (CD) spectroscopy of TJ10: thermal denaturation profile of TJ10 in 3 M guanidine·HCl at 218 nm. Inset shows the full CD spectrum of TJ10. (B) Thioflavin T assay for Aβ<sub>1-40</sub> fibrillization. TJ10 (50 μM) in red; Aβ<sub>1-40</sub> (50 μM) in blue; Aβ<sub>1-40</sub> + TJ10 (50 μM each) in light blue. (C) TEM images of Aβ<sub>1-40</sub> (50 μM) show typical amyloid fibrils, whereas Aβ<sub>1-40</sub> + TJ10 (50 μM each) showed no fibrils or aggregates by TEM.

and observed no changes in ThT fluorescence and no aggregates were visible by TEM, whereas Aβ<sub>1-40</sub> formed fibrils over the same period (Figure 2c). These results validated our approach for designing well-defined β-sheet inhibitors of amyloid aggregation.

To further characterize the effects of TJ10, we evaluated its effect upon Aβ aggregation at substoichiometric concentrations. At 10 μM of TJ10 (TJ10:Aβ ratio of 1:5), Aβ aggregation was completely retarded for 16 h and was only 50% complete after 24 h. Even at 2 μM of TJ10 (TJ10:Aβ ratio of 1:25), 50% aggregation was only observed after 16 h, whereas in the absence of inhibitor, Aβ aggregation was complete within 12 h. In contrast, HTB1 did not perturb Aβ aggregation (Supporting Information, Figures S4 and S5). In preliminary studies, we have evaluated the size of possible Aβ oligomers stabilized by TJ10 by dynamic light scattering (DLS). We observed that aggregated Aβ showed species >8000 nm, whereas the TJ10:Aβ<sub>1-40</sub> ratio of 2:1 showed a mixture of species in the 10–300 nm regime (Supporting Information, Figure S6). CD was not utilized to gauge direct binding by changes in the secondary structure of the complex, as both TJ10 and Aβ may undergo structural rearrangements making interpretation difficult. Thus, we utilized fluorescence spectroscopy, expecting that complexation of Aβ, containing a single Tyr, with TJ10, containing 3 Trp (1 native) and 4 Tyr (2 native), may lead to change(s) in the aromatic environment. Indeed, we observed a significant decrease in fluorescence intensity in the TJ10:Aβ complex ( $\lambda_{exc}$  = 270 nm,  $\lambda_{em}$  = 340 nm) (Supporting Information, Figure S7), further verifying the direct interaction of TJ10 with Aβ.

In conclusion, we have established that the evolved miniature protein, TJ10, which presents a well-folded, aromatic-residue-enriched, β-sheet can abrogate Aβ fibrillization at stoichiometric concentrations. TJ10 may function by capping intermediate-sized Aβ oligomers,<sup>3a</sup> such as protofibrils that perhaps resemble the final fibrillar state.<sup>11</sup> Interestingly, Hammer and co-workers have very recently observed stoichiometric inhibition utilizing Aβ mimetics.<sup>5d</sup> Future work will test the effect of TJ10 as well as other HTB1 variants in their ability to ameliorate Aβ-mediated toxicity in cells, their effect on other proteins that are known to form amyloid-like aggregates, and their ability to disaggregate preformed Aβ fibrils. Thus, these studies provide a new, structurally well-defined Aβ inhibitor that can be used for testing emerging methods in characterizing Aβ fibrillization.<sup>3d,12</sup> Finally, this strategy may be

generally applicable for evolving β-sheet epitopes to target numerous proteins implicated in amyloidogenic diseases.<sup>1,3</sup>

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**Supporting Information Available:** Experimental details, ThT assay, dynamic light scattering, and fluorescence spectroscopy data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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