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Visualization of Transient Protein-Protein Interactions that Promote or Inhibit Amyloid Assembly

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INTRODUCTION

The assembly of proteins into amyloid fibrils is a complex process requiring specific and sequence-dependent polymerization of initially unfolded or partially folded monomers into fibrils with elaborate cross-$\beta$ architectures (Greenwald and Riek, 2010). Despite recent insights into the structural characteristics of the amyloid fold (Eisenberg and Jucker, 2012; Fitzpatrick et al., 2013), the process of amyloid assembly is less well understood in structural terms. Assembly from natively folded precursors is commonly initiated by partial unfolding (Chiti and Dobson, 2009). These nonnative species then combine, generating an array of oligomeric intermediates that are transiently populated and usually heterogeneous in mass and conformation (Cremades et al., 2012; Smith et al., 2010). Although recent advances in structural methods have enabled the conformational properties of rarely populated, partially folded monomers of aggregation-prone proteins to be determined (Eichner et al., 2011; Jahn et al., 2006; Neudecker et al., 2012), the nature of the first protein-protein interactions that initiate amyloid formation remains unclear. Early in assembly, an array of biomolecular interactions arise, some of which may be productive for amyloid formation, whereas others may be unproductive, with the potential to inhibit or retard amyloid assembly (Campioni et al., 2010; Cremades et al., 2012). The course of amyloid assembly thus may depend on the stability and lifetime of the productive interactions versus their unproductive counterparts. From this viewpoint, identifying and characterizing different biomolecular interactions early in amyloid assembly are crucial for a full understanding of the structural, kinetic, and thermodynamic determinants of amyloid formation and for interpreting phenomena such as species barriers in prion formation. Such information could also pave the way toward the design of molecules able to define or control the course of amyloid assembly.

Amyloid formation is highly specific, with only proteins of closely related sequence capable of copolymerization into amyloid fibrils (Sarell et al., 2013a). Copolymerization may occur by cross-seeding, in which monomers of a different sequence are capable of extending preformed fibrils from a related protein (Giasson et al., 2003; Guo et al., 2013). In other cases, copolymerization may occur prior to the critical nucleation step of fibrillation. In this case, monomers or small oligomers coassemble into assembly-competent species in the early stages of amyloid assembly (Middleton et al., 2012; Sarell et al., 2013b). One such example can be found in prions, proteins that possess at least one conformation that is infectious by being able to transmit their structural and pathological properties onto noninfectious prion monomers (Chien and Weissman, 2001; Tessier and Lindquist, 2009). Interestingly, when prion molecules are transferred to different species they can lose their high infectivity, establishing a species barrier (Chien et al., 2003, 2004), or can confer their toxic conformation onto previously innocuous proteins of a related species (Sindi and Serio, 2009). The molecular determinants of species barriers, however, remain unclear.

Here we have explored the nature of protein-protein interactions in the first steps of amyloid formation of $\beta_2$-microglobulin ($\beta_2$m), a 99-residue protein that forms amyloid deposits in dialysis-related amyloidosis (DRA) (Gejyo et al., 1985). Despite being the main constituent of fibrils in DRA, wild-type human $\beta_2$m (h$\beta_2$m) is not capable of forming amyloid-like fibrils on an experimentally tractable timescale in vitro at neutral pH without the addition of
external factors or cosolvents (Calabrese and Miranker, 2009; Eichner and Radford, 2011). By contrast, a truncated variant of $\beta_m$ in which the N-terminal six amino acids are deleted ($\Delta N6$), a species that is found in amyloid fibrils in DRA (Esposito et al., 2000), is able to form amyloid fibrils spontaneously at neutral pH in vitro (Eichner et al., 2011; Esposito et al., 2000). NMR studies have shown this variant to be a close structural mimic of a folding intermediate of full-length $h\beta_m$ that contains a nonnative trans X-prolyl bond at Pro32 ($I_2$) (Figure 1A), the formation of which has been shown to initiate aggregation (Eichner et al., 2011; Jahn et al., 2006). Importantly, $\Delta N6$ is able to convert $h\beta_m$ into an aggregation-competent state at neutral pH when added in a substoichiometric molar ratio (Figure 2A, inset), in a mechanism reminiscent of conformational conversion associated with prions (Eichner et al., 2011). By contrast with the behaviors of $\Delta N6$ and $h\beta_m$, murine $\beta_m$ ($m\beta_m$), which is 70% identical in sequence to $h\beta_m$ (Figure 1B), is unable to form amyloid fibrils at neutral pH (Eichner et al., 2011; Ivanova et al., 2004) and is capable of inhibiting the self-association of $\Delta N6$ into amyloid fibrils when added in a stoichiometric ratio (Eichner et al., 2011).

Such diversity in the outcomes of interactions between $\beta_m$ molecules that lead to the promotion ($\Delta N6-h\beta_m$) or inhibition ($\Delta N6-m\beta_m$) of fibril formation. The results reveal that the surfaces involved in the inhibition and promotion of fibrillation are similar. However, the spatial distribution and chemical properties of the generated ensembles differ in detail, sufficient to alter the affinities of these interactions and the effects of the biomolecular collision on the conformational properties of the monomeric precursors involved. Our findings highlight the complexity of the first steps in amyloid assembly, wherein protein association via similar binding surfaces results in different molecular outcomes. They also reveal information about the origins of species barriers in amyloid formation and identify the surfaces to target by molecular design to enable the course of amyloid assembly to be controlled and/or defined.

**RESULTS**

$m\beta_m$ Kinetically Inhibits $\Delta N6$ Assembly

In previous studies, we have shown that $m\beta_m$ is able to inhibit the assembly of $\Delta N6$ into amyloid-like fibrils when added in a stoichiometric ratio (Eichner et al., 2011), despite the high structural and sequence similarity of the two proteins (sequence identity 70%, sequence homology 90%, root-mean-square deviation 0.91 Å) (Figures 1A and 1B). This phenomenon was further investigated here by measuring the kinetics of fibril formation of $\Delta N6$ at pH 6.2 (the pH optimum for $\Delta N6$ fibril formation in vitro; Eichner et al., 2011) to which $m\beta_m$ had been added in different molar ratios. To account for the effect of protein concentration on the kinetics of amyloid formation, the total protein concentration was maintained at 60 $\mu$M in all experiments. Figure 2A shows that $\Delta N6$ assemblies into fibrils able to bind thioflavin T (ThT) with lag-dependent kinetics typical of $\beta_m$ amyloid formation (Xue et al., 2008), whereas $m\beta_m$ does not form fibrils under the conditions employed. Measured over more than ten replicates, the mean lag time of $\Delta N6$ assembly was 32.7 ± 3.8 hr, after which time long straight fibrils typical of amyloid formed (Figure 2Bi). When $m\beta_m$ was mixed with $\Delta N6$ in substoichiometric molar ratios, 4:1 $\Delta N6:m\beta_m$ or 2:1 $\Delta N6:m\beta_m$, the mean lag time...
increased to 63.2 ± 3.8 and 91.0 ± 6.2 hr, respectively (Figure 2A), although fibrils formed over the 1 week (120 hr) time course of the experiment using a 5:1 molar ratio of the two proteins (Figure 2B).

When the two proteins were mixed in a 1:1 molar ratio, complete inhibition ensued (Figure 2A). The dependence of the lag time on the concentration of \( \text{m} \beta_2 \text{m} \) added (Figure S1A available online) suggests that inhibition of fibrillation is a kinetically determined process. Accordingly, increasing the molar ratio of \( \text{m} \beta_2 \text{m} \) to \( \Delta N6 \) delays, but does not inhibit, the formation of amyloid. In support of this notion, the mixtures of \( \Delta N6:\text{m} \beta_2 \text{m} \) that did not show evidence of fibril formation after 120 hr were incubated for longer periods of time (≥350 hr) and the extent of fibril formation was again measured using ThT fluorescence and negative-stain electron microscopy (EM). These experiments showed that fibrils were able to form after extended incubation times, with the lag time depending on the excess of \( \text{m} \beta_2 \text{m} \) added (Figures S1B–S1D). These findings confirm that the interaction between \( \Delta N6 \) and \( \text{m} \beta_2 \text{m} \) retards fibril assembly but, because fibrils are thermodynamically favored, the kinetic barrier to their formation is eventually overcome.

To identify whether \( \text{m} \beta_2 \text{m} \) is incorporated into fibrils when mixed with \( \Delta N6 \), the aggregates formed in samples containing
different molar ratios of ΔN6:mβ2m after 350 hr were collected by centrifugation, depolymerized by incubation at 100°C in SDS-PAGE loading buffer or by incubation in 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP), and subjected to analysis by SDS-PAGE or electrospray ionization mass spectrometry (ESI-MS) (Experimental Procedures; Supplemental Experimental Procedures). As a control, fibrils were assembled from ΔN6 alone, incubated subsequently with the same concentrations of monomeric mβ2m, and analyzed in a similar manner. The results of these experiments showed that mβ2m associates with the ΔN6 fibrils to a similar extent irrespective of whether the protein was added pre- or postassembly (Figures 2C and 2D). These results indicate that mβ2m is not incorporated into the ΔN6 fibrils but associates with the fibril surface subsequent to assembly. By contrast, hβ2m has been shown to be incorporated into the fibril core when incubated with ΔN6 in a 1:1 ratio at pH 6.2 (Sarell et al., 2013b).

Different Binding Affinities for the Inhibition and Promotion of Fibril Assembly

To investigate the interfaces involved in the inhibition (ΔN6-mβ2m) or promotion (ΔN6-hβ2m) of amyloid assembly, NMR studies were carried out by mixing 15N-labeled ΔN6 with 15N-labeled mβ2m or hβ2m (80 μM) and monitoring the chemical shift perturbations upon binding using 1H-15N HSQC spectra (Experimental Procedures). For both interaction types, the exchange was found to be in the intermediate-to-fast regime (data not shown), giving rise to small, but significant, chemical shift changes upon binding.

In the case of the inhibitory complex (ΔN6-mβ2m), changes in the 1H-15N HSQC spectrum, including chemical shift differences and exchange line broadening, were observed for a subset of resonances, even when the proteins were mixed in substoichiometric ΔN6:mβ2m ratios. Residues that show significantly altered chemical shifts upon binding are localized in the BC and DE loops in the apical region of mβ2m (Figure 3A). By contrast, an excess (~80 μM) of 15N-labeled ΔN6 was required to observe significant chemical shift changes in the spectrum of 15N-labeled hβ2m (Figure 3B). In this case, the residues experiencing significant chemical shift differences include the N-terminal regions, the B strand, and the BC and DE loops (Figure 3B).

Globally fitting the resulting data (Supplemental Experimental Procedures) yielded Kd values of 68 ± 20 μM for the mβ2m-ΔN6 interaction and 494 ± 180 μM for the interaction between ΔN6 and hβ2m (Figures 3C and 3D). Together, these data suggest a larger interface for the ΔN6-hβ2m interaction (more

**Figure 3. Chemical Shift Changes and Binding Affinities for Different Complexes**

(A) Chemical shift differences (1H, cyan; 15N, green) when 15N-labeled mβ2m and 15N-labeled ΔN6 are mixed in a 1:1 ratio (80 μM each; ~41% mβ2m-bound). All residues experiencing significant chemical shift differences (yellow boxes) locate to the top half of the molecule (BC and DE loops; highlighted in yellow on the surface of the molecule; right-hand side). Residues that show large chemical shift differences in the presence of 40 μM ΔN6 but are broadened beyond detection at these protein concentrations are marked with dark blue bars.

(B) As in (A) but for 15N-labeled hβ2m and 15N-labeled ΔN6 mixed in a 1:6 ratio (80 μM hβ2m; 480 μM ΔN6; ~47% hβ2m-bound). Residues with missing assignments are colored gray on the structure of mβ2m. Dotted lines in (A) and (B) represent two standard deviations of the mean over the entire data set for each atom type.

(C) Plots of the chemical shifts of different residues (51, 59, 65, 84) in 15N-labeled mβ2m upon titration with 14N-labeled ΔN6. Solid lines represent global fits to a binding hyperbola. Error bars were calculated using resonances known not to be involved in the binding interface. For these residues the chemical shift was measured in each spectrum, and the error bars represent the standard deviation of the mean of their peak positions (see Experimental Procedures and Supplemental Experimental Procedures).

(D) As in (C) but for 15N-labeled hβ2m upon titration with 14N-labeled ΔN6. Curves for residues 6, 26, 30, and 58 are shown.
residues experience significant chemical shift perturbations) in comparison to its inhibitory ΔN6-mβ2m counterpart, despite an ~7-fold decrease in binding affinity.

**Inhibition and Promotion of Fibril Formation Involve Similar Binding Interfaces**

Although chemical shifts are excellent probes of protein-protein interactions, they can be affected by long-range effects upon binding (Zhuravleva and Gierasch, 2011). Thus, we next sought to investigate the nature of the protein-protein interactions that lead to inhibition (ΔN6-mβ2m) or promotion (ΔN6-hβ2m) of fibril formation in more detail using PRE studies. The PRE depends on the distance between a paramagnet and adjacent nuclei and can provide long-distance (~30 Å) information quantified by the H_N-Γ_s PRE rate (Supplemental Experimental Procedures) for each amide proton (Clore and Iwahara, 2009). The PRE approach is ideally suited to the analysis of weak intermolecular associations (Clore and Iwahara, 2009), providing distance information that can be used to visualize transient and lowly populated (~0.5%) protein states (Tang et al., 2006, 2008) such as those occurring in the early stages of amyloid formation. To enable these experiments, variants containing a solvent-exposed cysteine were created in ΔN6 by mutating either S20 (AB loop), S33 (BC loop), or S61 (DE loop) to cysteine (Figure 1A) while maintaining the disulfide bond involving C25 and C80 (Experimental Procedures). Chemical modification with (1-oxyl-2,2,5,5-tetramethyl-pyrroline-3-methyl) methanethiosulfonate (MTSL) yielded ΔN6 molecules 100% labeled at a single site (Experimental Procedures). These chemically modified molecules were then used in PRE studies to map the interactions between 14N-labeled and MTSL-labeled ΔN6 with 15N-labeled hβ2m or mβ2m, each pair in a stoichiometric ratio (60 M each) at pH 6.2 and 25°C (Experimental Procedures). Under the conditions employed, and in the absence of agitation, fibril formation does not occur for either pair of proteins over the time course of the experiment (<40 hr). Accordingly, the difference in the 1H R2 relaxation rates of the 15N-labeled protein (hβ2m/mβ2m) in the presence of oxidized or reduced MTSL-labeled 14N-ΔN6 was measured (H_N-Γ_s rate) (Experimental Procedures) and used to map the interaction surfaces of the different protein pairs.

The PRE data collected for the inhibitory interaction between 14N-labeled ΔN6 (S61C-MTSL) and 15N-labeled mβ2m are shown in Figure 4Ai and Figure S2A. Backbone assignments for mβ2m at pH 6.2 were obtained using standard triple-resonance NMR experiments and uniformly 15N/13C-labeled protein (Experimental Procedures). The results showed high Γ_s values (Γ_s > 60 s⁻¹) for residues in the BC and DE loops of mβ2m and lower Γ_s values (<60 s⁻¹) for residues in the N-terminal 10 residues and the FG loop. These regions cluster on one side of mβ2m surrounding P32 (Figure 1A; Figure 4A, inset), a residue that undergoes cis-trans isomerization known to be required for amyloid formation from hβ2m (Eichen et al., 2011; Sakata et al., 2008). A similar PRE pattern was obtained when the spin label was attached at position 33 (Figure 4Aii). The results suggest that the region of mβ2m surrounding P32 is involved in the interaction with ΔN6 to create a heterodimer (as supported by analytical ultracentrifugation; see below) that kinetically inhibits amyloid formation. Consistent with this supposition, when the spin label is moved to position 20 on 14N-labeled ΔN6, the Γ_s rates of mβ2m in the BC and DE loops are substantially reduced (<25 s⁻¹) (Figure 4Aiii), suggesting that S20 is distant from the site of interaction (Supplemental Experimental Procedures). These data suggest, therefore, that a head-to-head configuration of the ΔN6-mβ2m heterodimer, involving the BC and DE loops from both monomers, creates the inhibitory complex.

Having identified the protein-protein interactions that lead to the inhibition of ΔN6 fibril formation, we next investigated the interactions that lead to ΔN6-induced promotion of hβ2m fibril assembly. Again, 14N-labeled ΔN6 was spin labeled with MTSL at residues 61, 33, or 20 and PREs to 15N-labeled hβ2m were measured (Figure 4B; Figure S2B). In marked contrast with the results obtained for the ΔN6-mβ2m interaction, the magnitude of the Γ_s values is reduced significantly when the spin-labeled ΔN6 variants are mixed with hβ2m (compare Figures 4Ai and 4Aii with Figures 4Bi and 4Bii), consistent with the ~7-fold lower K_d of the hβ2m-ΔN6 complex (Figures 3C and 3D). Despite the differences in magnitude of the Γ_s rates for the two complexes, the pattern of H_N-Γ_s values obtained is similar to that for the ΔN6-mβ2m interaction, with the largest PREs observed for residues 55–65 in the DE loop and 26–34 in the BC loop when the spin label is attached at position 61 (Figure 4B). Residues in the N-terminal region (residues 2–10) showed increased PRE rates when the spin label is attached at position 33, which were not observed when MTSL was added at position 61 (Figures 4Bi and 4Bii). Again, only very small PREs were observed when MTSL was added at position 20 (Figure 4Biii). These results suggest that the promotion of hβ2m fibril formation also involves a head-to-head association of the two monomers.

**Distinct Conformational Ensembles with Structurally Similar Binding Surfaces**

To obtain more detailed insights into the protein complexes that give rise to the inhibition or promotion of amyloid formation, the PRE data were used in a rigid body/torsion angle simulated annealing approach to generate structural ensembles of the different complexes by minimizing the difference between the observed and calculated Γ_s values. PRE data for each complex obtained using spin labels at positions 33 and 61 in ΔN6 were fitted simultaneously, along with data from chemical shift perturbations upon binding that were treated as ambiguous distance restraints (see below and Experimental Procedures). Data arising from spin-labeled ΔN6 at position 20 were not included (Supplemental Experimental Procedures). The population of the interconverting species was set to 18% in both cases based on the known K_d of each complex.

In a first series of simulated annealing calculations, the interconverting species were represented as a single conformer (N = 1) (Experimental Procedures). The results of this analysis revealed a head-to-head configuration for the association of ΔN6 with mβ2m in which the DE loops from each monomer make the majority of the intermolecular contacts (Figure 5A). Interestingly, the high Q factor (0.54; Figure S2C; Supplemental Experimental Procedures) suggests that multiple conformations are required to satisfy the experimental restraints. In exchanging
Figure 4. Interaction Interfaces in Different Protein Complexes

(A) Per-residue $\Gamma_2$ rates of m$\beta_m$ (60 $\mu$M) when MTSL is attached to S61 (i), S33 (ii), or S20 (iii) on $\Delta$N6 (60 $\mu$M) colored according to their amplitude (blue, not assigned; gray, insignificant; yellow, $>20$ s$^{-1}$; red, $>60$ s$^{-1}$; pH 6.2, 25°C). The structure of m$\beta_m$ as a surface representation colored by the amplitude of the $\Gamma_2$ rates is shown (insets). Red crosses indicate residues for which the $\Gamma_2$ rate is either too large to appear on this scale or resonances broadened beyond detection when the spin label is oxidized and hence the $\Gamma_2$ rate cannot be measured. Blue dots represent proline or overlapping resonances, and blue crosses denote residues for which the assignments are missing. Error bars were calculated from the noise level in the experiment.

(B) As in (A) but for the interaction between $^{14}$N- and MTSL-labeled $\Delta$N6 (60 $\mu$M) and $^{15}$N-labeled h$\beta_m$ (60 $\mu$M). The structure of h$\beta_m$ is colored according to the amplitude of the $\Gamma_2$ rates after extrapolation to the same % bound as in (A) (blue, not assigned; gray, insignificant; yellow, $>9 \times 4$ s$^{-1}$; red, $>16 \times 4$ s$^{-1}$). Note that the scale is expanded in (B).

(C) The distribution of the m$\beta_m$ molecules in the $\Delta$N6-m$\beta_m$ complex, with the m$\beta_m$ ensemble shown as a pink surface around $\Delta$N6 (cartoon). The 50 top-scoring ensembles ($N = 2, 2 \times 50$ structures) were included in the calculation.

(D) As in (C) but for the $\Delta$N6-h$\beta_m$ association. The pose of $\Delta$N6 is identical to (C) and the ensemble of h$\beta_m$ subunits is colored in blue. The BC, DE, and FG loops of $\Delta$N6 are highlighted in green, yellow, and blue, respectively, and the positions of the spin label (S20, S33, and S61) are shown as spheres.
facing the β sheet composed of the A, B, E, and D strands, whereas the second cluster of ΔN6 molecules locates opposite the edge strands D and C (Movie S2). On the other hand, the ΔN6-hβ2m interaction is more heterogeneous, extending to both sides of the apical region of ΔN6 (around P32) (Figure S4D; Figure S3B; Movie S1). The volume of the ΔN6-mβ2m density map is calculated to be 7,157 Å³, whereas that of the ΔN6-hβ2m cluster is almost twice as large (13,670 Å³; a cutoff of 40% was used in both cases; Table S1). Interestingly, the distributions of mβ2m and hβ2m molecules around ΔN6 do not completely overlay. Areas showing high intermolecular contacts unique to the ΔN6-hβ2m complex involve the BC and FG loops (Figure S3D; Movie S2). A correlation between the hydrophobic surface area of mβ2m (shaped mainly by the region surrounding the DE loop) and the distribution of ΔN6 molecules is observed, indicating that this interaction interface is predominantly hydrophobic in nature, with residues F56, W60, and F62 participating in key intermolecular contacts (Figure S3C; Movie S2). By contrast, the apical region of hβ2m (DE, BC, and FG loops) displays less solvent-exposed hydrophobic surface area and a greater predominance of charged residues that reflect the differences in the sequence of the proteins in these regions (Figure 1; Figure S3D; Table S1; Movie S2). Together, the results indicate that inhibition of ΔN6 fibril formation involves a “specific” head-to-head protein association driven by hydrophobic interactions with mβ2m. On the other hand, the ΔN6-hβ2m interaction, although also adopting a head-to-head configuration, is weaker, more heterogeneous, and involves electrostatic interactions. Whether these data reflect the formation of a range of “encounter complexes” systems the observed PRE rate is the weighted population average of the species in solution, as long as those are in the fast exchange regime (Iwahara et al., 2004). In this case, the PRE methodology allows the visualization of the ensemble of the interconverting species. Increasing the number of conformers to two (N = 2) results in a significant decrease in the Q factor for the ΔN6-mβ2m interaction (Q = 0.37), with no further significant decrease (Q = 0.36) when N is increased to three (Figures S2C and S2D). Similar analysis of the ΔN6-hβ2m association revealed that (at least) two conformers are also required to describe the experimentally measured PRE data (Figures S2E and S2F).

The associating monomers in the ΔN6-mβ2m and ΔN6-hβ2m ensembles were visualized as atomic probability density maps as described (Tang et al., 2006) (Figures 4C and 4D). The resulting ensemble for the ΔN6-mβ2m complex shows that mβ2m molecules cluster around the DE loop of ΔN6 (residues 52–63), which makes the majority of contacts with mβ2m (Figure 4C; Figure S3A; Movie S1). ΔN6, by contrast, shows a bimodal distribution around the DE loop of mβ2m, with one cluster of molecules

Figure 5. F56 and W60 in mβ2m Form Interactions Required for Amyloid Inhibition
(A) The lowest-energy calculated structure of the ΔN6-red-mβ2m (green) complex highlighting F56 and W60 in the interface. Interface residues are colored blue on ΔN6 (right).
(B) Representative sample resonances in the 1H-15N HSQC spectrum of 15N-labeled ΔN6 (80 μM; red) that show chemical shift changes upon the addition of 15N-labeled mβ2m (green) but not its F56E/W60E variant (160 μM; blue). Addition of mβ2m shifts the resonances of ΔN6 toward their positions at pH 8.2 (black), where ΔN6 is not amyloidogenic (Eichner et al., 2011) (additional examples are shown in Figure S5A).
(C) Fibrillation kinetics of ΔN6 alone (20 μM; pink) at pH 6.2 and in the presence of a 2-fold molar excess of mβ2m (green) or F56E/W60E mβ2m (blue).
(D) Sedimentation velocity AUC traces of ΔN6 alone (80 μM; ΔN6; 60 μM) mixed with an equimolar concentration of mβ2m (green), or F56E/W60E mβ2m (blue).
between ΔN6 and hβ2m that is not observed for the ΔN6-mβ2m interaction, or whether they report on the transient formation of higher-order oligomers between ΔN6 and hβ2m, remains to be resolved.

**Mutation of Aromatic Residues Prevents Inhibition of ΔN6 Assembly by mβ2m**

To confirm that the head-to-head association of ΔN6 with mβ2m is involved in inhibition of fibril formation, two amino acid substitutions (F56E and W60E) were introduced into mβ2m at sites that were found to participate in the majority of intermolecular contacts between the two molecules (Figure 5A; Figure S4A). The ability of this variant to bind to ΔN6 and to inhibit fibril assembly was then monitored using NMR and ThT fluorescence assays, respectively. When 15N-labeled F56E/W60E mβ2m (160 μM) was mixed with 15N-labeled ΔN6 (80 μM) at pH 6.2, only small changes in the chemical shifts of ΔN6 (~20% in comparison to wild-type mβ2m) were observed in the BC, DE, and FG loops (Figure 5B; Figures S5A and S5B), consistent with the proteins no longer interacting tightly. Consistent with these observations, F56E/W60E mβ2m is unable to inhibit ΔN6 fibril assembly when added in a 2-fold molar excess (Figure 5C; Figures S5C and S5D), conditions under which wild-type mβ2m delays the onset of amyloid for more than 120 hr (Figure 2A; Figure S1C). The interaction of wild-type mβ2m with ΔN6 prevents the formation of oligomeric species by the latter protein as observed by sedimentation velocity analytical ultracentrifugation (AUC) (Figure 5D), resulting in a monomer-dimer (~80:20) equilibrium, consistent with a specific interaction as suggested by the analysis of the PRE data. Notably, under identical conditions, the F56E/W60E variant abolishes the ability of the murine protein to dissociate preformed oligomers of ΔN6 (Figure 5D).

**Binding-Induced Unfolding versus Rigid Body Docking: A Rationale for the Outcome of Biomolecular Collision**

To investigate why biomolecular collision of hβ2m or mβ2m with ΔN6 results in different outcomes of assembly, the effect of ΔN6 binding on the conformational dynamics of each monomer was analyzed using hydrogen-deuterium (H/D) exchange. In each case, the rate of H/D exchange of monomeric (unbound) hβ2m/mβ2m was compared with its ΔN6-bound counterpart at pH 6.2, using samples in which the protein concentrations of hβ2m/mβ2m were adjusted to generate complexes containing a similar percent (~20%) of ΔN6-bound hβ2m/mβ2m monomer. These experiments showed that the H/D exchange rates of mβ2m are unaffected ($k_{ex}$ increases by less than ~1.3-fold).
upon interaction with ΔN6 (Figure S6A; Figure S6A). By contrast, the addition of ΔN6 to hβ2m causes a 2- to 3-fold increase in the H/D exchange rates of residues throughout the sequence of hβ2m (Figure 6B; Figure S6B), consistent with an increase in global dynamics of the protein upon interaction with ΔN6. These results were confirmed using a variety of ΔN6 concentrations for both complexes, ranging from 40 to 320 μM.

Close examination of the chemical shift changes that occur when 15N-labeled ΔN6 is added to 15N-labeled mβ2m reveals that the residues that undergo significant chemical shift changes also experience increased PRE rates (BC and DE loops), confirming that these regions of the protein form the interaction interface (Figure 3A). On the other hand, residues in the N-terminal region including the AB loop of hβ2m (residues 12–13) show significant chemical shift changes upon binding to ΔN6 (Figure S3B) but minor PREs (Figure 4B), consistent with these residues not being involved in the interface of the lowest-energy structures of the ΔN6-hβ2m complex (Figure S3B). These observations suggest that the binding of ΔN6 to hβ2m provides sufficient energy to alter the conformation of the N-terminal 12 residues of hβ2m (observed previously by H/D exchange and relaxation NMR methods; Eichner et al., 2011) such that a more amyloidogenic conformation is adopted. By contrast, the nonamyloidogenic (and thermodynamically less stable) mβ2m (ΔG‡ human = −10.7 kJ/mol, ΔG‡ human = −22.5 kJ/mol; C. Pasley and S.E.R., unpublished data) is not affected significantly by binding. Differences in cooperativity or local stability of the interacting monomers thus dictate the progress of amyloid assembly.

Finally, the consequences of binding on the conformational properties of ΔN6 were investigated by measuring the changes in the chemical shifts of 15N-labeled ΔN6 (80 μM) upon titration with 15N-labeled mβ2m (80 μM) or 15N-labeled hβ2m (480 μM) (~45% ΔN6 bound in each case) (Figures 6C and 6D). Significant chemical shift differences were observed for residues in the BC and DE loops of ΔN6 upon binding to hβ2m and mβ2m, consistent with the head-to-head structure of both complexes. The larger number of ΔN6 residues showing chemical shift differences observed upon binding and the greater Δ observed for the ΔN6-hβ2m complex are consistent with the larger interface of this interaction, but could also suggest that ΔN6 responds to binding hβ2m by undergoing conformational change. The picture that emerges, therefore, is that the promotion of hβ2m fibril formation by ΔN6 involves weak binding that nonetheless leads to conformational changes in one or both of the interacting partners. By contrast, the ΔN6-mβ2m complex, even though employing a similar head-to-head interaction, involves the formation of a relatively specific, tight binding, inhibitory complex with little or no effect on the conformational properties of the interacting partners.

**DISCUSSION**

**Protein Interaction Surfaces and the Molecular Mechanism of β2m Aggregation**

Amyloid fibrils share similar structural features based upon a cross-β core, irrespective of the organism of origin, the protein involved, or the sequence of the protein precursor (Eisenberg and Jucker, 2012). Despite their similarity in structure, amyloid fibrils can be beneficial to the organism concerned, whereas for others amyloid formation is deleterious (Otzen, 2010). For each scenario, mechanisms have evolved that either facilitate assembly or protect against the accumulation of aggregation-competent proteins, depending on whether the fibrils are beneficial or not (Succintini et al., 2002; Otzen, 2010; Maji et al., 2009). One such example can be found in prions, proteins that possess at least one amyloid-competent conformation that is infectious by being able to transmit its structural and pathological properties onto innocuously folded prion monomers (Sindi and Serio, 2009). When prion molecules are transferred between species, they can lose their infectivity or allow propagation depending on the organism involved, establishing a so-called species barrier (Chien et al., 2003; Tessier and Lindquist, 2009; Baskakov, 2014). The precise molecular details of how and why species barriers occur between very similar proteins remain unclear. ΔN6 has been shown to possess prion-like properties in its ability to convert hβ2m in an aggregation-prone conformation by biomolecular collision (although the protein is not infectious) (Eichner et al., 2011). Here we show that the prion-like characteristics of ΔN6 are not only limited to its ability to convert hβ2m into an amyloid-competent conformation but also in its ability to experience species barriers (when the molecule interacts with mβ2m, amyloid assembly is inhibited). The results show that aggregation propensity is not simply related to the kinetic and/or thermodynamic properties of the proteins involved (the least stable β2m variant studied here [mβ2m] inhibits assembly, whereas propagation involves interaction of ΔN6 with the most stable variant [hβ2m]). Instead, the fate of amyloid assembly involves a fine interplay between molecular recognition and protein plasticity, which is governed by the precise location and chemical properties of the interfaces involved in the first biomolecular interaction events.

**Interactions that Result in Inhibition or Promotion of Amyloid Assembly**

Amyloid diseases are usually late-onset disorders, with symptoms appearing many decades into life, even for individuals carrying the most deleterious of mutations (Greenwald and Riek, 2010). Why this is the case remains unclear; possibilities include the time taken to nucleate fibril formation, and/or atrophy or overload of the proteostatic mechanisms that protect cells from protein misfolding and aggregation (Balch et al., 2008). Defining the nature of the complex network of protein-protein interactions that form in the earliest stages of amyloid assembly is of crucial importance, therefore, in our quest to understand the events that initiate protein aggregation at a molecular level. Such knowledge will also open the door to the design of inhibitors able to arrest amyloid formation by targeting specific surfaces that block the formation of fibrils and their toxic precursors, thereby halting the disease process at its outset.

Attempts to identify the intermolecular interactions that form early in amyloid assembly have remained a significant challenge as a consequence of the interactions’ heterogeneity and transient nature (Cremades et al., 2012). By exploiting the power of biomolecular NMR methods and applying them to β2m sequences from different species, we have been able to define
the intermolecular surfaces that determine the course of amyloid assembly. Specifically, we show that the interaction of ΔN6 with mβ2m inhibits aggregation via trapping the amyloidogenic precursor (ΔN6) in kinetically stable dimers (K_d = 68 ± 20 μM). These involve the formation of a relatively well defined interface, stabilized by hydrophobic interactions involving the side chains of residues in the DE and BC loops of both molecules, including F56 and W60 (Figure 7, bottom). Interestingly, mβ2m is the least stable variant of the three β2m homologs studied here, as shown by its increased H/D exchange rates and decreased unfolding free energy relative to ΔN6 and hβ2m (T.K.K., C. Pashley, and S.E.R., unpublished data). Thermodynamically and kinetically unstable proteins, therefore, and not only their stable counterparts (e.g., antibodies or affibodies; Dumoulin et al., 2003; Hoyer et al., 2008), can act as efficient and specific inhibitors of aggregation. Surprisingly, the amyloid-promoting association of ΔN6 with hβ2m also involves a head-to-head interaction similar, but not identical, to that of the inhibitory complex. Consistent with this finding, the folding intermediate I_2 of hβ2m that structurally resembles ΔN6 (Eichner and Radford, 2009; Eichner et al., 2011) was recently shown to form transient oligomers during folding that are also organized in a head-to-head configuration, although the structures formed and their implications for aggregation were not described (Rennella et al., 2013).

We show here that the amyloid-promoting interaction between ΔN6 and hβ2m is thermodynamically weaker than its inhibitory counterpart (K_d = 494 ± 180 μM) and involves multiple interaction sites that involve complementary electrostatic interactions between the interacting molecules that are not utilized in its inhibitory ΔN6-mβ2m counterpart. These differences in the interaction interfaces result in binding-induced conformational changes in hβ2m that are manifested by a 2- to 3-fold increase in its hydrogen exchange rates (Figure 7, top). This interaction also alters the conformation of the AB loop of hβ2m, as shown previously (Eichner et al., 2011). Accordingly, ΔN6 is able to act as protein saboteur, each molecule interacting with numerous copies of hβ2m, destabilizing the native fold of hβ2m and allowing P32 to relax from its native cis isomer to its trans form, which then traps the protein irreversibly in an aggregation-competent state. cis Pro32 in hβ2m, therefore, acts as a key switch in amyloid formation. Accordingly, any event that promotes relaxation of Pro32 to the trans conformer (mutation, formation of ΔN6 or I_2, interaction with Cu^{2+} ions, chaperones, or proline isomerase) promotes formation of amyloid fibrils (reviewed in Eichner and Radford, 2011).

Implications for the Origins of Transmissibility in Amyloid Diseases

The results presented reveal that subtle differences in the nature of protein-protein interactions can give rise to fundamentally different outcomes of amyloid assembly that depend on the affinity of the interaction, the stability of the interacting partners, and the chemical nature of the interacting surfaces. The results have significance that extends beyond the specific case of the β2m variants studied here. The catalytic templating model proposed to explain the conversion of the cellular human prion protein (PrP^C) to its infectious scrapie form (PrP^Sc) is one such case (Aguzzi et al., 2008). Accordingly, mutations that have little effect on the structural and thermodynamic properties of the monomeric PrP precursors (Bae et al., 2009) could alter the surface properties of the protein, influencing the network of intermolecular interactions formed, and hence lead to increased or decreased infectivity. Other amyloid proteins that are intrinsically disordered (such as Aβ40 and α-synuclein) are known to mutually enhance each other’s aggregation (Guo et al., 2013), possibly involving a similar mechanism of binding-induced conformational change. Indeed, heteropolymerization in amyloid assembly seems to be more common than initially anticipated (Sarell et al., 2013a). As shown here, protein association, response to binding, and the effect of transient intermolecular association on the course of assembly are all interlinked. Binding, even to similar surfaces, can cause a different response on the partners
involved and thus lead to a different outcome of assembly. The HET-S/HET-s prion strains in filamentous fungi represent another example (Greenwald et al., 2010). HET-S, even though 97% identical in sequence to HET-s, does not aggregate, and can also inhibit the propagation of the prion form of HET-s by biomolecular interaction, resembling the effect of mβ2m on ΔN6 assembly. A model for prion inhibition by HET-S has been proposed in which HET-S, although able to interact with HET-s and adopt the amyloid β-solenoid fold, is incompetent for further polymerization (Greenwald et al., 2010), further highlighting the observation that collision of similar proteins can result in different outcomes of assembly. Application of the approach taken here for β2m to other proteins involved in human disease, including the classic examples of species barriers in PrP propagation (Baskakov, 2014), prion compatibility in yeast and other fungi (Tessier and Lindquist, 2009), and other proteins purported to be infectious (Brundin et al., 2010), will reveal the similarities and distinctions between ΔN6-induced conformational conversion and amyloid inhibition and the molecular events occurring in other systems.

As well as providing insights into the molecular origins of species barriers in amyloid formation, the results presented provide opportunities for the design of molecules to control amyloid disease by targeting intermolecular contacts in the specific surfaces involved. The design of small molecules able to disrupt protein-protein interactions and the generation of other reagents (antibodies, affibodies, or nucleic acid aptamers [Bunka et al., 2007] selected to bind to a specific surface) are exciting possibilities for future avenues of research. The complexity of amyloid assembly, especially in the cellular environment, may require multiple routes involving different strategies to delay, prevent, or revert disease to be deployed simultaneously (for example by combining interference of protein assembly with small molecules or aptamers in concert with regulation of the cellular mechanisms that recognize protein misfolding events). The ability to target the earliest biomolecular events in the aggregation cascade offers potential for a route toward amyloid therapy that will add to the arsenal of approaches currently being developed to combat these devastating disorders.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**

hβ2m, mβ2m, and ΔN6 (15N- and 18N-labeled) and their variants were expressed and purified as described (Platt et al., 2005).

**Assembly of Amyloid-like Fibrils**

Samples containing 0.6–60 μM protein, 10 mM sodium phosphate buffer (pH 6.2), 83.3 mM NaCl (total ionic strength 100 mM), 0.02% (w/v) sodium azide, and 10 mM ThT were incubated at 37°C in sealed 96-well plates with agitation at 600 rpm (Supplemental Experimental Procedures).

**PRE Experiments**

The ΔN6 variants (15N-labeled) C20S, C33S, and S61C modified with MTSSL (Supplemental Experimental Procedures) were mixed with 15N-labeled hβ2m or mβ2m (60 μM, unless otherwise stated) in 10 mM sodium phosphate buffer (pH 6.2) and H2O-PRE data were measured as described in Supplemental Experimental Procedures.

**Simulated Annealing Calculations**

All structure calculations were performed using a torsion angle-simulated annealing protocol in XPLOR-NIH as described (Iwahara et al., 2004) (Supplemental Experimental Procedures).

**Kd Measurements**

Binding affinities for the complexes of mβ2m and hβ2m with ΔN6 were determined at pH 6.2 and 25°C by titrating 80 μM 15N-labeled mβ2m with 0–320 μM 15N-labeled ΔN6 or 80 μM 15N-labeled hβ2m with 0–480 μM 15N-labeled ΔN6 and measurement of the resulting chemical shift changes using 1H–15N HSQC spectra (Supplemental Experimental Procedures).

**Hydrogen Exchange Experiments**

The rate of H/D exchange of samples of 15N-labeled hβ2m or mβ2m (80 μM) alone or mixed with 15N-labeled ΔN6 (160 or 40 μM, respectively) to produce ~22% bound complexes in each case was measured at pH 6.2. Hydrogen exchange was measured using SOFAST-HMQC NMR methods as previously described (Schanda et al., 2005) (Supplemental Experimental Procedures).

**Additional Procedures and Further Information**

Detailed description of all other methods and protocols can be found in Supplemental Experimental Procedures.

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