

An amyloid-forming segment of β 2-microglobulin suggests a molecular model for the fibril

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In humans suffering from dialysis-related amyloidosis, the protein β 2-microglobulin (β 2M) is deposited as an amyloid; however, an amyloid of β 2M is unknown in mice. β 2M sequences from human and mouse are 70% identical, but there is a seven-residue peptide in which six residues differ. This peptide from human β 2M forms amyloid *in vitro*, whereas the mouse peptide does not. Substitution of the human peptide for its counterpart in the mouse sequence results in the formation of amyloid *in vitro*. These results show that a seven-residue segment of human β 2M is sufficient to convert β 2M to the amyloid state, and that specific residue interactions are crucial to the conversion. These observations are consistent with a proposed zipper-spine model for β 2M amyloid, in which the spine of the fibril consists of an anhydrous β -sheet.

More than 20 proteins have been found to aggregate into amyloids, elongated unbranched fibrils that bind the aromatic dyes Congo red and ThioflavinT (ThT) and have a common cross β x-ray diffraction pattern (1, 2). The proteins that form amyloids differ in size, function, sequence, and native structure, but all form aggregates similar in structure and properties (3–5). It has long been recognized from the cross- β diffraction pattern that amyloids are formed from β -sheets \approx 10–12 Å apart, each made up of extended strands stacked \approx 4.7 Å apart (6, 7). There is evidence that in some amyloids, the β -strands run parallel to each other (8–10), and in others they may run antiparallel (11, 12).

Some models for amyloid structure depict the entire native protein as refolding into the amyloid (13–16); we term these Entire-refolding models. Other models depict the interactions of amyloid to be formed from only a small segment of the protein, with the rest retaining a native-like structure (17–20). Entire-refolding models are based in part on the idea that amyloid formation is an inexorable tendency of all proteins, and that variations in rate of achieving the amyloid state are mainly a matter of amino acid composition (21). In contrast, models that depict amyloid formation as having its basis in a “gain of interaction” (18) focus on the formation of a new intermolecular bond contributed by a segment of the entire protein. The formation of these intermolecular bonds would in principle depend on the amino acid sequence, not just the composition. In this paper, we focus on a particular gain-of-interaction model, called the Zipper-spine model, in which the new interaction is a spine of β -sheet (17).

One of the most intensively studied amyloid-forming proteins is β 2-microglobulin (β 2M), a normally soluble protein that aggregates into pathogenic fibrils either at low pH (22) or under physiological conditions when divalent copper is present (23). The Entire-refolding view of amyloid depicts dialysis-related amyloidosis pathogenesis as destabilization of the native structure of β 2M followed by formation of a nucleating β 2M species that forms amyloid fibrils (24–26). However, there is accumulating evidence that specific sequences play a dominant role in amyloidogenesis of β 2M. For example three segments of β 2M were found to form fibrils in isolation: Ser 20 to Lys 41 (27), Asp 59 to Thr 71 (28), Asp 59 to Ala 79 (28), and Pro 72 to Met 99

(29). Examples of amyloid-forming peptides from other amyloidogenic proteins include segments from yeast and human prion (8, 30, 31). Short designed peptides can also form amyloids (32). Also, hydrogen/deuterium exchange studies (25, 26) suggest that some segments of β 2M in the amyloid state are protected from exchange but others are not.

The observation that short peptides form amyloids implies that exposure of short segments of proteins can nucleate native proteins into the amyloid state and suggests that fibril formation is sequence specific. In this paper, we identify a heptapeptide from human β 2M (h β 2M) that forms an amyloid in isolation, and that does not form amyloid when its sequence is scrambled. When swapped into mouse β 2M (m β 2M), this normally stable protein now forms amyloid. We take this finding to support the idea that amyloid forms by a gain in interaction acquired by a short protein loop that becomes a strand in a new β -sheet, and we present a model of β 2M amyloid that is consistent with this idea.

Materials and Methods

Plasmid Construction and Protein Expression. See *Supporting Materials and Methods*, which is published as supporting information on PNAS web site.

Immunoblotting. See *Supporting Materials and Methods*.

Polymerization and ThT-Binding Assays. The assays shown in Fig. 1C were performed by incubating 60 μ M protein in 1.5 M NaCl/25 mM phosphate, pH 2.0, at 37°C without shaking. The assays shown in Fig. 3A and D were performed by incubating 8 μ M protein in the reaction buffer, 0.2 M NaCl/25 mM phosphate, pH 2.0, at 37°C without shaking. Five-microliter aliquots were then added to 200 μ l of 5 μ M ThT/10 mM Tris, pH 8.0. Fluorescence was measured immediately over a 60-sec time course on a Spex Fluorolog spectrofluorimeter (Jobin Yvon., Edison, NJ) set at 444 nm (excitation; 2-nm slit width) and 482 nm (emission; 2-nm slit width). The signal was corrected for the background by subtracting the measured fluorescence for 5 μ l of reaction buffer in 200 μ l of 5 μ M ThT/10 mM Tris, pH 8.0. Measurements were performed in triplicate, and values are expressed as the mean \pm 1 SD (plotted as error bars).

Electron Microscopy (EM). See *Supporting Materials and Methods*.

Peptide Fibril Formation. The h β 2M residues 83–89 (h β 2M7mer) and m β 2M residues 83–89 (m β 2M7mer) peptides were synthesized by California Peptide Research, Napa, CA. The “scram-

Abbreviations: ThT, thioflavinT; EM, electron microscopy; β 2M, β 2-microglobulin; h β 2M, human β 2M; m β 2M, mouse β 2M; h β 2M7mer, h β 2M residues 83–89; m β 2M7mer, m β 2M residues 83–89; m β 2M/h, residues 83–89 substituted with the corresponding seven-residue segment from h β 2M; h β 2M/m, residues 83–89 substituted with the corresponding seven-residue segment from m β 2M; m β 2M/wt, m β 2M wild type; h β 2M/wt, h β 2M wild type.

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is published as supporting information on the PNAS web site) and have fibrillar morphology (Fig. 2C). In contrast, m β 2M7mer does not form fibrils, and only amorphous aggregates are seen in the EM micrographs of m β 2M7mer sample under the same conditions (Fig. 6B).

To determine whether it is the sequence (NHVTLNQ) or the composition of the h β 2M7mer peptide that causes amyloid fibril formation, we studied the solution behavior of a scrambled version of this peptide, QVLHTSN. We chose to reshuffle the residues in the amyloid-forming peptide (NHVTLNQ) so that the charged H84 is located at the center of the peptide, thus breaking the four-apolar residue stretch. When the “scrambled” peptide (QVLHTSN) was dissolved at 35 mM in a solution (2.0 M NaCl/25 mM phosphate, pH 2.0) that drives NHVTLNQ into amyloid fibrils, the solution remains clear for 14 days. Fibrils are absent when the sample was examined by EM. Also, QVLHTSN did not form fibrils when lower salt concentrations were used (0.2 M NaCl/25 mM phosphate buffer, pH 2.0 or 1 M NaCl/25 mM phosphate buffer, pH 2.0). We conclude that the sequence, not just the composition, of the human peptide is important for amyloid formation.

Design and Characterization of Chimeric β 2M Proteins. Two chimeric β 2M molecules were designed in which the seven-residue segments (residues 83–89) from m β 2M and h β 2M are interchanged (Fig. 7, which is published as supporting information on the PNAS web site).

m β 2M/h Forms Fibrils, but m β 2M Wild Type (m β 2M/wt) Does Not Form Fibrils. m β 2M/wt does not bind ThT (Fig. 3A, filled circles). In contrast, residues 83–89 substituted with the corresponding seven-residue segment from h β 2M (m β 2M/h) aggregates start to bind ThT (Fig. 3A, open circles) \approx 4 days after the beginning of the reaction. When examined by EM, the m β 2M/h sample consisted of many straight long fibrils with morphology typical for amyloids (Fig. 3B). There were no fibrils in the samples of m β 2M/wt when viewed by EM (Fig. 3C). Thus, the replacement of the nonamyloid-forming segment from m β 2M with the amyloid-forming segment from h β 2M was sufficient to promote fibril formation of m β 2M.

Fibril Formation of h β 2M Is Disrupted When a Seven-Residue Segment from m β 2M Is Swapped into the h β 2M Scaffold. Aggregation of h β 2M/m (the human scaffold with inserted m β 2M7mer; Fig. 3D, open circles) lags behind that of h β 2M-wt (Fig. 3D, filled circles). We note that there are variations among the lag times in identical experiments; however, despite the variations, we invariably observed that h β 2M/wt aggregates \approx 3–24 h earlier than does h β 2M/m. After reaching the aggregated phase, the fluorescence of the two proteins is indistinguishable. The h β 2M/wt sample displayed a homogenous population of long straight fibrils when viewed by EM (Fig. 3E), typical for amyloid. In contrast, h β 2M/m consisted of equal amounts of short (black arrow) and long (white arrow) fibrils (Fig. 3F). The swapping of the m β 2M7mer into h β 2M does not completely impede the fibril formation, and two populations of fibrils with different morphologies are present in the h β 2M/m sample (Fig. 3F). The long fibrils of h β 2M/m have the same diameter as the h β 2M/wt and at this resolution, we cannot distinguish morphological differences between them. The presence of two different populations of fibrils (long and short) in the h β 2M/m sample may be due to a different mode of fibril formation than the wild-type protein. This is consistent with the observation that other segments of β 2M can form fibrils in isolation (27–29). We conclude swapping of the mouse amyloid-forming h β 2M7mer into h β 2M diminishes its tendency to form amyloid.

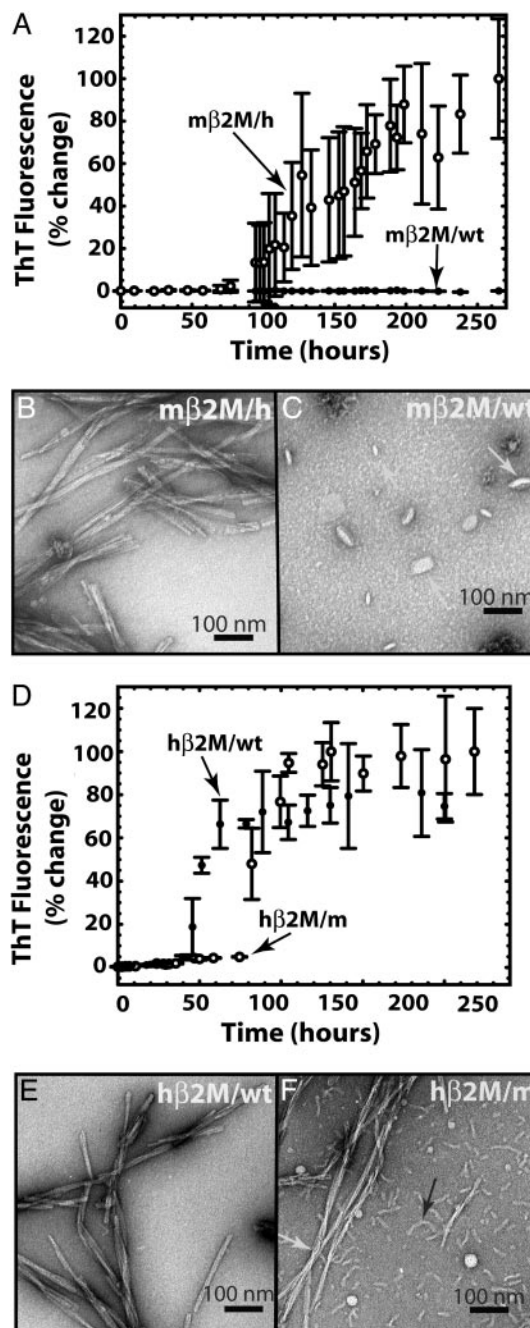


Fig. 3. Amyloid fibril formation of wild-type and chimeric β 2M. (A) m β 2M/wt (filled circles) does not bind ThT. In contrast, the m β 2M/h aggregates (open circles) bind ThT starting \approx 4 days after initiating the reaction. This demonstrates the ability of the human heptamer to promote m β 2M to form amyloid-like aggregates. (B) The m β 2M/h sample consists of long straight fibrils typical for amyloids. (C) No fibrils were observed in the m β 2M/wt sample, and the largest aggregates observed appear globular, as indicated by the white arrows. Micrographs shown in B and C were taken 14 days after the initiation of the aggregation reaction. (D) Fibril formation of h β 2M is disrupted when the m β 2M7mer is swapped into the h β 2M scaffold. Aggregation of the h β 2M/m chimera (open circles) lags behind that of the h β 2M/wt (filled circles) fibril formation. h β 2M/wt reaches the aggregated state \approx 3–24 h earlier than h β 2M/m. The fluorescence of the two proteins after the aggregated state is reached is indistinguishable. (E) h β 2M/wt sample had a homogenous population of long and straight fibrils, typical for amyloid, and no amorphous aggregates were observed in the sample. (F) In contrast, h β 2M/m sample displayed equal populations of long (white arrow) and short fibrils (black arrow). The electron micrographs of samples shown in E and F were taken 10 days after the initiation of the aggregation reaction.

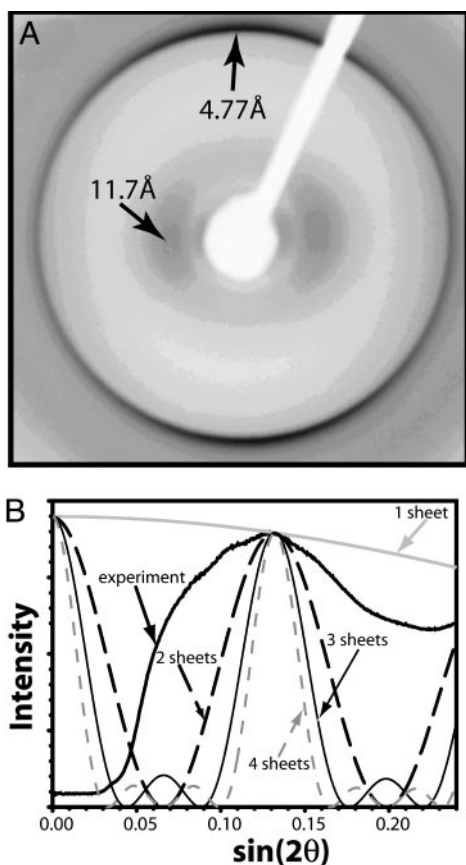


Fig. 4. X-ray diffraction cross β pattern of β 2M/wt fibrils. (A) The characteristic reflections for amyloid fibrils are indicated by arrows. (B) Comparison of the observed profile of the ≈ 11 -Å diffuse reflection with profiles calculated from models representing the Zipper-spine as one to four slits (β -sheets).

X-Ray Diffraction of β 2M. The reflections characteristic of amyloids at ≈ 11 and 4.8 Å are present in the x-ray diffraction pattern of β 2M fibrils (Fig. 4A). The large circular spread of the 4.8 -Å reflection arises from the wide range of orientations of β 2M fibrils. Still, the meridional intensity of the 4.8 -Å arc is strong, which is consistent with β -strands in the fibril being oriented perpendicular to the fibril axis. The width of the diffuse equatorial reflection centered at 11 Å offers a clue as to the number of β -sheets per fibril. The agreement of the observed width with that calculated from a slit model (Fig. 4B) appears closest for two packed β -sheets, and accordingly we model the amyloid spine of β 2M fibrils as two β -sheets in Fig. 5. However, we note that our model for calculating the width of the 11 -Å reflection does not take into account sample polymorphism or x-ray beam divergence, which could broaden the reflection. Until these factors are better understood for the β 2M amyloid, we cannot conclude with certainty that the spine is best modeled as a two- β -sheet structure.

Discussion

Residues 83–89 Are Significant in the Formation of Amyloid by h β 2M.

In *Results*, we note that amyloid deposits are not observed in mice even though the concentration of β 2M in mouse plasma can be 100 times higher than in human plasma. Moreover, h β 2M forms amyloid *in vitro* under conditions at which m β 2M is soluble. The principal difference in amino acid sequence between these two β 2M molecules is the seven-residue segment, residues 83–89. This peptide from h β 2M forms amyloid *in vitro*, under solution conditions in which the corresponding mouse

peptide is soluble. A scrambled version of this seven-residue sequence from h β 2M also fails to form amyloid under conditions that we have studied. Reinforcing the importance of this segment for amyloid formation, we find that when it is swapped for its counterpart in m β 2M, formerly soluble, the chimera forms amyloid. All of this points to the segment of sequence, NH-VTLSQ from residues 83–89 in h β 2M, as being a significant determinant of amyloid formation.

This segment may not be the only determinant of amyloid formation in β 2M. Suggesting that other segments may also be involved is the observation that two other segments of the h β 2M sequence form amyloids *in vitro* (27–29). This can explain the observation that the chimera h β 2M/m forms fibrils. In addition, our observation of two populations of fibrils in the h β 2M/m sample, short and long, indicates that the heptamer substitution affects fibril elongation.

Which Segments of β 2M Participate in Amyloid Structure? There are at least two plausible models for β 2M amyloid formation: (i) The Entire-refolding model. Conditions that destabilize the native structure expose the h β 2M7mer, which has a high propensity to form the amyloid structure, and this segment nucleates the entire protein to fold into an amyloid-like β -sheet. This Entire-refolding model is widely accepted and is illustrated in the papers of Jimenez *et al.* (13), Perutz *et al.* (14), Williams *et al.* (34), Kishimoto *et al.* (16), and others. However, this model does not include a means for β -sheets to pack together and so does not provide an explanation for the appearance of an 11 -Å reflection that is characteristic of amyloid fiber diffraction. (ii) The Gain-of-interaction model (18), of which the Zipper-spine model (17) is a special case. In the Zipper-spine model, the segment containing h β 2M7mer binds to the same segment in other β 2M molecules, forming the β -sheet spine of the amyloid, but the rest of the β 2M molecule remains in its native structure, stabilized by its native disulfide bond, and decorates the periphery of the spine. This model for β 2M is depicted in Fig. 5. The Entire-refolding and Zipper-spine models are extreme cases; the actual β -sheet spine could incorporate several segments of the β 2M.

A Zipper-Spine Model for β 2M Amyloid. We have constructed a speculative molecular model of the Zipper-spine type for the β 2M protofilament, based on four assumptions. (i) The C-terminal segment (residues 83–99) forms the spine of the fibril. This can be achieved if the h β 2M7mer segment forms an extended β -strand (F') followed by a type I β -turn at residues Pro 90 and Lys 91. This permits the β -strand G (residues 92–99) to hydrogen bond to β -strand F' (Fig. 5A and B). (ii) The hairpins from different β 2M molecules stack hydrogen bonded to each other along the axis of the protofilament (Fig. 5C), forming a β -sheet. This β -sheet faces a second twisted β -sheet with alternate side chains of the two sheets interacting. Zipper-spine models with more than two sheets would appear implausible due to severe steric overlap among globular domains flanking the central spine. Two sheets running parallel to the fibril axis is also suggested by comparing the radial profile of the observed 11 -Å reflection with the calculated radial profile for two packed β -sheets (Fig. 4B). (iii) Other than the separation of β -strand G from the core domain of the protein, only a minor rearrangement in β 2M occurs upon fibril formation (Fig. 5C and D). That is, most of the native structure of β 2M is retained in the fibril, which is in accordance with the observation that most of β 2M amino acid residues of β -strands B, C, D', D'', and F are solvent inaccessible in the fibril (25, 26). This large core domain decorates the spine and protects it from the solvent. The width of the fibril in our β 2M model varies between 60 and 85 Å, which is about half the fibril diameter (110 ± 30 Å) measured from the electron micrograph

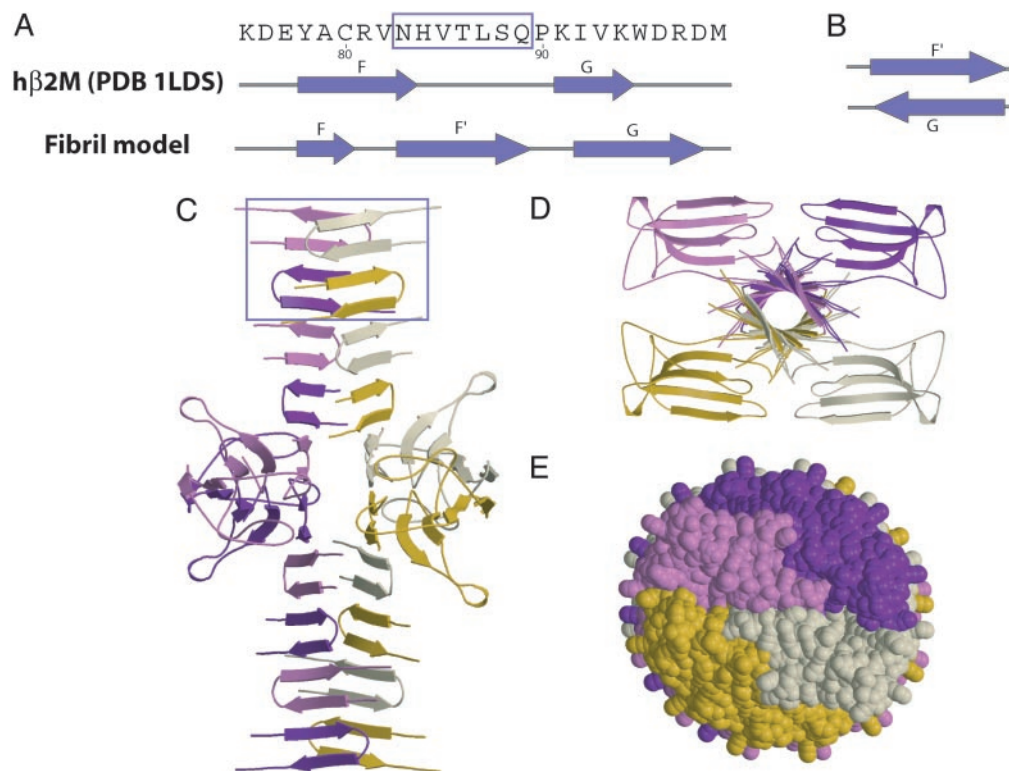


Fig. 5. Speculative Zipper-spine model for the $\beta 2M$ protofilament. (A) The C-terminal segment of $\beta 2M$ is the portion of the structure to rearrange during fibril formation and the amyloid-forming h $\beta 2M$ 7mer (boxed) forms a new β -strand F'. (B) In the fibril model, residues Pro 90 and Lys 91 form a type I β -turn. Thus the β -strand G is hydrogen bonded with the new β -strand F' rather than with the β -strand F of the native structure. (C) Two such F'-G hairpins stack to form the asymmetric unit of one of the sheets of the spine. Each sheet is built with a small (7°) twist between β -strands, stacked 4.7 Å apart, so that the spine has a pitch of ≈ 242 Å. We expect these parameters will be refined as structural data become available. The sheets are separated by ≈ 11 Å. β -Strands A-E and part of F retain their native structure with the disulfide bond between β -strands B and F intact. These native-like core domains decorate the periphery of the double β -sheet spine, with only four molecules shown for clarity. (D) The view down the fiber axis shows the double β -sheet spine. The rest of the $\beta 2M$ molecules, which remain folded, are packed closely around the β -sheet spine. (E) A space-filling model (view of the fibril down its axis) shows that each of the four molecules, represented with different colors, is tightly packed within the fibril, with no space for solvent. Thus, the core domains shield the spine from solvent.

(Fig. 3E). Conceivably the $\beta 2M$ fibrils observed are formed from two protofilaments.

Table 1 examines the compatibility with experimental observations of both the Entire-refolding and Zipper-spine models.

The Zipper-spine model for $\beta 2M$ has been constructed to be compatible with observations of $\beta 2M$ amyloid (Table 1), so it is not decisive that the majority of observations listed in Table 1 are less compatible with the Entire-refolding model than with the

Table 1. Compatibility of the Zipper-spine and Entire-refolding amyloid models with observations of $\beta 2M$

Observation	Zipper-spine model	Entire-refolding model
X-ray diffraction pattern (Fig. 4)		
4.7-Å reflection	Compatible	Compatible
11-Å reflection	Compatible*	Not compatible*
S—S bond between Cys 20 and Cys 85	The native S—S bond is preserved in the model	The S—S bond could interfere with refolding
An antibody to $\beta 2M$ residues 92–97 inhibits fibril formation but antibodies to residues 20–40 and 63–75 do not (36)	Compatible with all three antibody results	Inconsistent with the two antibodies that do not prevent refolding
Residue replacement V82 to P disrupts fibril formation (37)	Compatible with spine formed from residues 83–94	Possibly compatible
Segments 20–41 (27), 59–71 (28), and 72–99 (29) in isolation form amyloid	Compatible with 72–99 (29) forming amyloid, but not necessarily with segments 20–41 (27) and 59–71 (28)	Compatible with all three segments forming amyloid
Amyloids are exceptionally stable	The anhydrous hydrogen-bonded Zipper-spine offers stability	No obvious reason that a parallel β -helix would impart special stability
Specificity: intermolecular bonding between $\beta 2M$ molecules	h $\beta 2M$ 7mer forms strong β -sheet interactions with the same segment from other molecules	No obvious reason for bonding between different $\beta 2M$ molecules

*The recent paper by Kishimoto *et al.* (16) argues that the absence of an observed ≈ 11 -Å reflection in hydrated microfibrils of amyloid from the yeast prion Sup35 is decisive evidence for complete refolding of Sup35 into a β -helix. An alternative explanation for the lack of an observed ≈ 11 -Å reflection is a lack of regular stacking of one sheet against the other in the protofilaments.

Zipper-spine model. Perhaps the observation most incompatible with the Entire-refolding model is that numerous proteins known to form amyloids contain intact disulfide bonds stabilizing the native structure. For example β 2M has one [Protein Data Bank (PDB) 1LDS]; lysozyme has four (PDB 1LYS), insulin has 2 inter- and 1 intrachain (PDB 1ZEI); and human prion protein has one (PDB 1HJM). These crosslinked molecules must retain some semblance of their native structures in the absence of disulfide bond breakage, and hence must retain some native-like structure in their amyloids that form under oxidizing conditions that would keep the disulfide bonds intact. Complete refolding into a β -helix of these crosslinked proteins is not easily explained. That highly crosslinked proteins can form amyloids and that short protein segments can form amyloids are both consistent with an amyloid spine that is formed from only a short segment of each protein. Another observation compatible with the Zipper-spine model is that in electron micrographs, the fibrils formed from entire β 2M appear to have a rougher surface (Fig.

3E) than the regularly twisted fibrils of β 2M7mer (Fig. 2C). This supports the model of the main domain decorating the spine, because the β 2M7mer fibril is nothing more than the Zipper-spine.

Our main conclusions are: (i) A seven-residue segment of β 2M appears to be an important determinant of β 2M amyloid formation, perhaps constituting the spine of the amyloid; and (ii) the Zipper-spine model, with its anhydrous β -sheet spine, is sufficiently compatible with observations on β 2M that it should be given consideration with the Entire-refolding model in interpreting future experiments on amyloids.

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