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Amyloid-forming Peptides from β_2-Microglobulin—Insights into the Mechanism of Fibril Formation *in Vitro*

Susan Jones, James Manning, Neil M. Kad and Sheena E. Radford*

School of Biochemistry and Molecular Biology, University of Leeds, Leeds, LS2 9JT, UK

β_2-Microglobulin (β_2m) is one of over 20 proteins known to be involved in human amyloid disease. Peptides equivalent to each of the seven β-strands of the native protein, together with an eighth peptide (corresponding to the most stable region in the amyloid precursor conformation formed at pH 3.6, that includes residues in the native strand E plus the eight succeeding residues (named peptide E′)), were synthesised and their ability to form fibrils investigated. Surprisingly, only two sequences, both of which encompass the region that forms strand E in native β_2m, are capable of forming amyloid-like fibrils *in vitro*. These peptides correspond to residues 59–71 (peptide E) and 59–79 (peptide E′) of intact β_2m. The peptides form fibrils under the acidic conditions shown previously to promote amyloid formation from the intact protein (pH <5 at low and high ionic strength), and also associate to form fibrils at neutral pH. Fibrils formed from these two peptides enhance fibrillogenesis of the intact protein. No correlation was found between secondary structure propensity, peptide length, pI or hydrophobicity and the ability of the peptides to associate into amyloid-like fibrils. However, the presence of a relatively high content of aromatic side-chains correlates with the ability of the peptides to form amyloid fibrils. On the basis of these results we propose that residues 59–71 may be important in the self-association of partially folded β_2m into amyloid fibrils and discuss the relevance of these results for the assembly mechanism of the intact protein *in vitro*.

Keywords: peptides; amyloid-like fibrils; lag phase; β_2-microglobulin; haemodialysis-related amyloidosis

*Corresponding author

Introduction

β_2-Microglobulin (β_2m) is the non-covalently bound light-chain of the class I human leukocyte antigen (HLA class 1). As part of its normal catabolic mechanism, β_2m dissociates from the surface of cells displaying the major histocompatability (MHC) class I complex and is transported by the serum to the proximal tubule of the kidney, where it is degraded and excreted. As a result of renal failure the concentration of β_2m in the serum rises up to 60-fold above the normal level.[1] As a consequence, and by an unknown mechanism, full-length unmodified human β_2m forms amyloid fibrils that accumulate predominantly in the musculoskeletal system.[2] Full-length human β_2m has been shown to form amyloid-like fibrils *in vitro* under a variety of conditions including acidification to below pH 5.[3–7] In addition, the protein forms amyloid fibrils by incubation at neutral pH in the presence of β_2m amyloid seeds,[8] copper ions,[9,10] by concentrating the protein on a membrane surface,[11] or by removal of the N-terminal six amino acid residues.[12] All of these conditions involve destabilisation of the native protein and population of one or more partially unfolded states. At pH 3.6 and low ionic strength (50 mM), or from pH 1.5–5.0 at high ionic strength

Present address: N. M. Kad, Department of Molecular Physiology and Biophysics, 115 Health Science Research Facility, University of Vermont, Burlington, VT 05401, USA.

Abbreviations used: β_2m, β_2-microglobulin; HLA class 1, class I human leukocyte antigen; MHC, major histocompatability; DMSO, dimethylsulphoxide; ThT, thioflavin T; EM, electron microscopy.

E-mail address of the corresponding author: s.e.radford@leeds.ac.uk

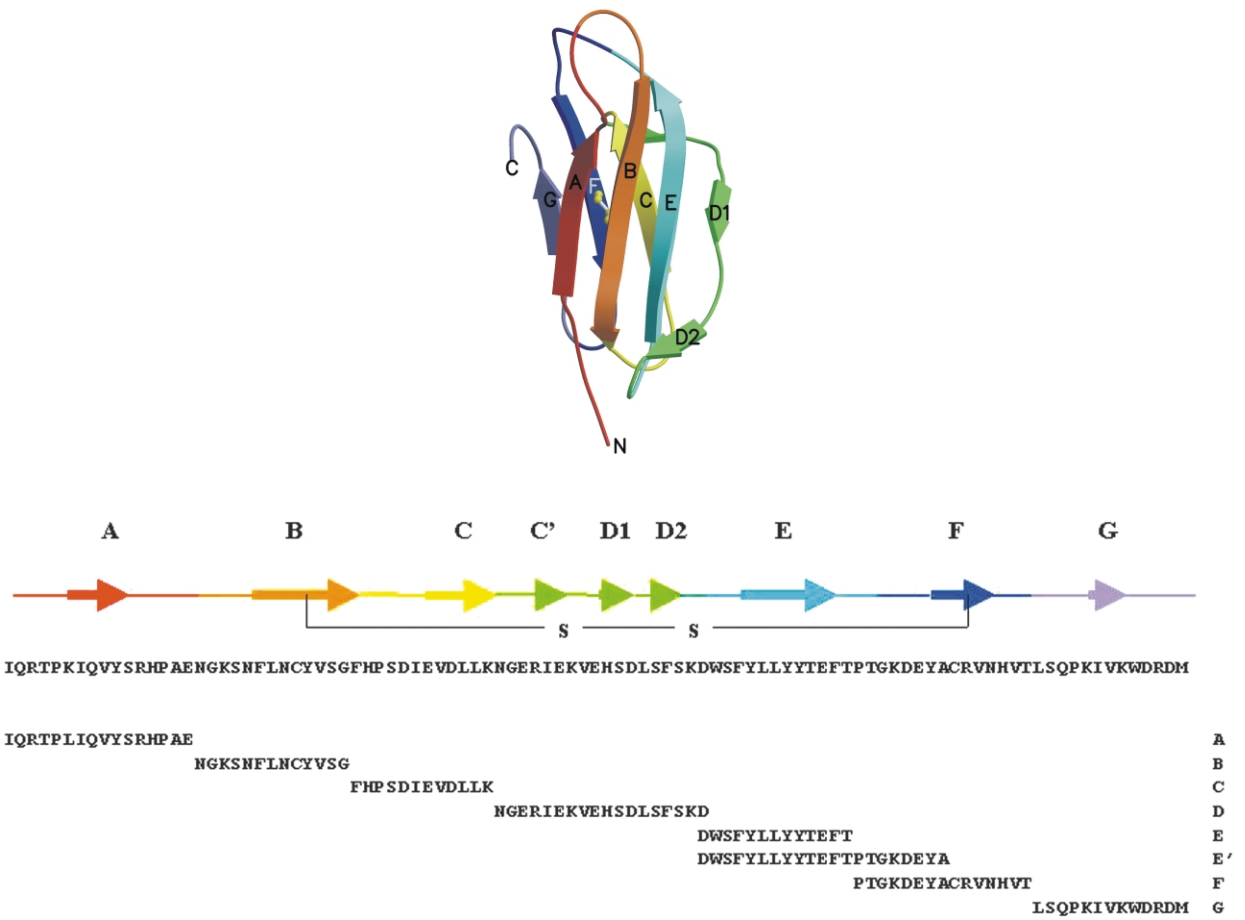

Figure 1. Structure of human β_2m and the location of the peptides analysed in this study. (a) Structure of human β_2m coloured from the N terminus of the protein (red) to the C terminus (violet). The location of elements of secondary structure was determined using DSSP.[46] Individual β-strands are labelled A through G. The disulphide bond that links Cys25 (strand B) and Cys80 (strand F) is shown in yellow. The Figure was made using the coordinates 1DUZ[47] and programmes MOLSCRIPT[48] and Raster 3D.[49] (b) The amino acid sequences of the peptides of β_2m studied here. With the exception of Ile1 and Met99, all peptide sequences were acetylated at their N termini and amidated at their C termini.

(400 mM), β_2m assembles spontaneously *in vitro* to form fibrils with a characteristic curved and nodular morphology that bind Congo red, show red-green birefringence and give rise to a fibre diffraction pattern consistent with a cross-β structure.[5] By contrast, at pH 2.5 and low ionic strength, long, straight amyloid-like fibrils are formed that have well-defined periodicities[3,7] and are more reminiscent of β_2m amyloid *ex vivo*.[2]

In recent studies it has been shown that the monomeric amyloid precursor of β_2m formed at pH 3.6 is a partially folded ensemble that contains stable secondary structure in the B, C, D, E and F strands, whilst the N and C-terminal β-strands (strand A and G) (Figure 1(a)) are substantially destabilised.[5,13] By contrast, the amyloid precursor conformation formed at pH 2.5 is a more highly denatured state.[5,13,14] Interestingly, recent experiments using hydrogen exchange have shown that the A and G strands are also relatively weakly protected from exchange in β_2m amyloid fibrils, suggesting that these strands are not involved in fibril formation *in vitro*.[15] Despite these observations, little is known about the mechanism by which the partially unfolded precursors of β_2m associate to form amyloid fibrils *in vitro*, or the regions of the protein that are important in self-recognition in the assembly process. Previous studies using a peptide fragment equivalent to residues 20–41 in native β_2m suggested, however, that this region could be important in assembly.[16]

Here, we investigate the ability of a range of self-peptides of β_2m to assemble into amyloid-like fibrils *in vitro* and to influence the association of the intact protein into amyloid fibrils. The peptides correspond to each of the seven β-strands of native human β_2m, including several residues from each of the adjacent loop regions (the peptides were named A to G, corresponding to their equivalent β-strands in the full-length protein) (Figure 1(b)). In addition, a peptide corresponding to the most stable region in the amyloid precursor conformation of the intact protein formed at pH 3.6

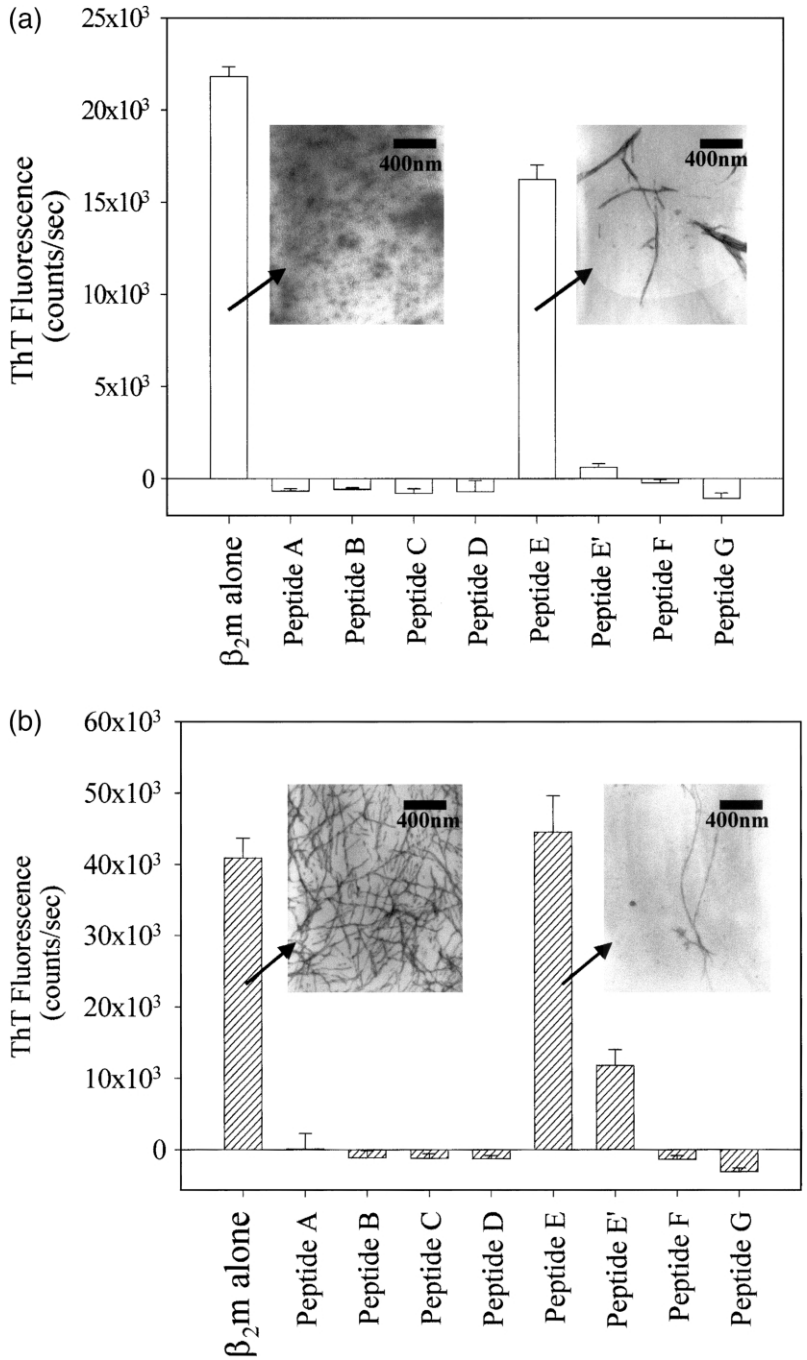

Figure 2. Fibrillogenesis of β_2m peptides. (a) ThT fluorescence of β_2m peptides in buffer A. (b) ThT fluorescence of β_2m peptides in buffer B. Negative stain EM images of fibrils formed from full-length β_2m and peptide E under these conditions are shown as insets. Note that although the ThT fluorescence signal of peptide E′ in buffer A is small, the sample contained long, straight fibrils akin to those observed for peptide E as judged by negative stain EM. Each peptide, supplied as a pure lyophilised powder, was dissolved in 100% DMSO to form a stock solution of 5 mg/ml. The stock solutions were then diluted into the required reaction buffer. The buffers used were buffer A (25 mM each of sodium phosphate, sodium acetate, Tris–HCl and Mes (pH 3.6) to which NaCl was added to give a final ionic strength of 400 mM) or buffer B (25 mM each of sodium phosphate and sodium acetate (pH 2.5), final ionic strength 50 mM). A final β_2m or peptide concentration of 84 μM was used. Samples in buffer A were incubated for four days at 37 °C without agitation and samples in buffer B were incubated for seven days at 37 °C with agitation (220 rpm). All samples were incubated in 96-well microtitre plates. In all experiments a final concentration of ≤5% (v/v) DMSO was included. After incubation for the required period, 10 μl of each reaction was taken and diluted 1:100 into Thio-T buffer (50 mM Tris–HCl containing 10 μM ThT (pH 8.5)) and the fluorescence was measured immediately over a 60 second time-course. The excitation wavelength was 444 nm and emission was measured at 480 nm (3 nm slit widths) using a Photon Technology International QM-1 spectrofluorimeter (PTI, UK) at 25 °C. All measurements were performed in triplicate and values are expressed as the mean ± one standard deviation (plotted as error bars). Samples for EM were prepared and imaged as described.[3]

(residues 59–79 (peptide E′))[13] was synthesised and analysed (Figure 1(b)).

Fibril formation from individual peptides

Peptides were first incubated at the same concentration (84 μM) and conditions used previously to form amyloid fibrils from full-length β_2m *in vitro*.[3,5] Two conditions were employed: pH 3.6 in the presence of NaCl (0.4 M final ionic strength) (buffer A), under which conditions intact β_2m assembles into short (~200 nm), curved, nodular fibrils;[5] and pH 2.5 in the absence of NaCl (50 mM final ionic strength) (buffer B), wherein the intact protein forms long, straight amyloid fibrils.[3,7] All of the experiments also contained ≤5% (v/v) dimethylsulphoxide (DMSO) at 37 °C (see the legend to Figure 2). This solvent was used initially to dissolve the synthetic peptides to ensure that they did not contain pre-assembled material.

In buffer A full-length β_2m assembles rapidly (in three days) into fibrils that bind thioflavin T (ThT) and have a short, curved morphology, identical to fibrils generated previously in the absence of

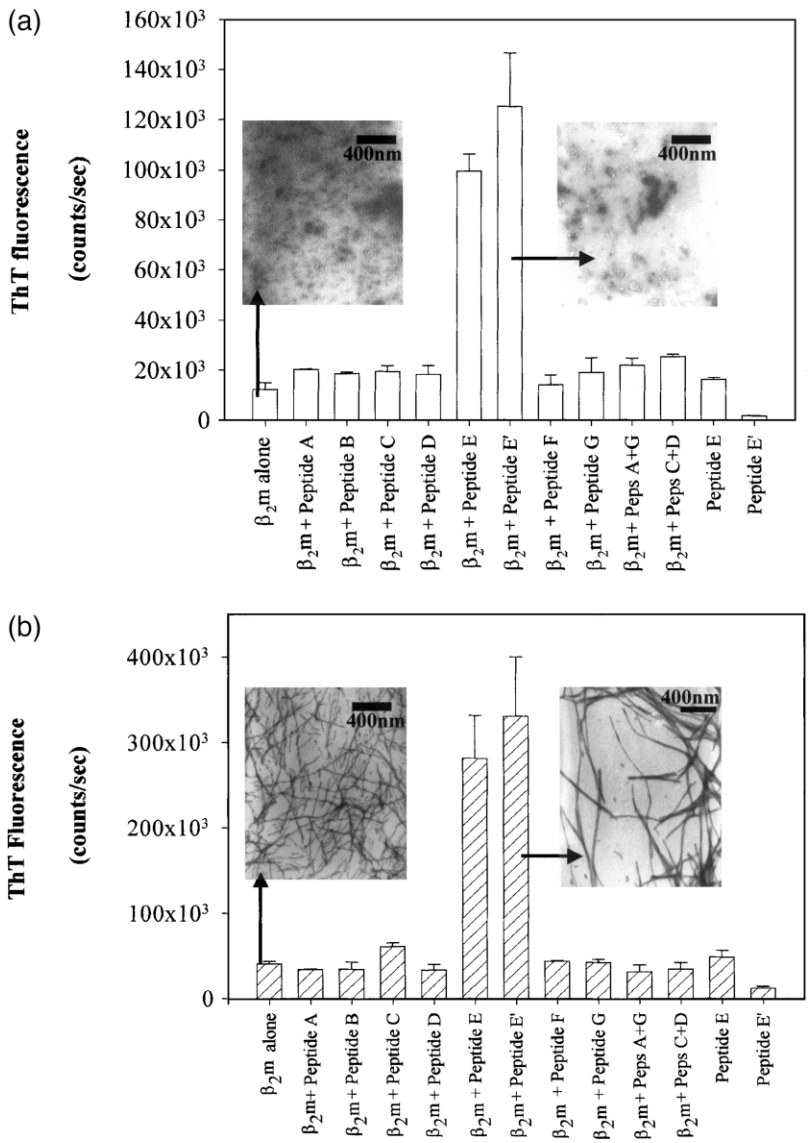

Figure 3. Effect of peptides on fibril formation of intact β_2m. (a) ThT fluorescence of β_2m (34 μM) incubated at pH 3.6 (buffer A) in the presence of different peptides (84 μM). (b) ThT fluorescence of β_2m (84 μM) incubated at pH 2.5 (buffer B) in the presence of different peptides (84 μM). Negative stain EM images of fibrils formed from the full-length protein alone and in the presence of peptide E′ under these conditions are shown as insets. All samples contained a final concentration of 5% (v/v) DMSO. Fresh stock solutions of β_2m were made, filtered (0.2 μm filter) and used immediately. Previous experiments demonstrated that samples treated in this way form amyloid fibrils with the same rate as gel-filtered material, demonstrating that filtration is effective at removing any potential seeds.[3] Samples in buffer A were incubated for four days at 37 °C without agitation and samples in buffer B were incubated for seven days at 37 °C with agitation (220 rpm). All samples were incubated in microtitre plates for the required time period and assayed for ThT fluorescence as described in the legend to Figure 2. All measurements were performed in triplicate and expressed as the mean ± one standard deviation (plotted as error bars). Samples for EM were prepared and imaged as described.[3]

DMSO (Figure 2(a)).[5] By contrast, in buffer B, full-length β_2m assembles more slowly (in seven days) to form fibrils that also bind ThT, have a characteristically large ThT fluorescence signal, and a long, straight morphology, in accord with previous results[3] (Figure 2(b)). Even though each of the synthetic peptides contains the sequence corresponding to a β-strand in native β_2m, only peptides E and E′ form fibrils under the conditions employed (Figure 2(a) and (b)). Peptides A, B, C, D, F and G did not form fibrils or amorphous aggregates, even when incubated for several weeks under these conditions and at higher peptide concentration (336 μM) (as judged both by the lack of an increased ThT fluorescence and by negative stain electron microscopy (EM)). Further pair-wise mixtures of each of the peptides (84 μM each), in all combinations, also failed to produce fibrils, unless the solution contained peptides E or E′ (data not shown).

To determine the full range of conditions under which peptides could form fibrils, 84 μM of each peptide was incubated over a wide range of pH values (between pH 1 and 7), at both high ionic strength (buffer A) and low ionic strength (buffer B) at 37 °C. Again, peptides A, B, C, D, F and G did not form fibrils under any of the conditions explored (as judged by both ThT fluorescence and negative stain EM). By contrast, peptides E and E′ showed rapid and extensive fibril growth across the entire pH range studied at both ionic strengths. Both peptides formed fibrillar material within two minutes of dilution into buffer (at pH 7.0) as judged by EM and by a greater than 100-fold increase in ThT fluorescence (data not shown). The fibrils formed from both peptides E and E′ under all conditions have a long (0.1–1 μm), straight morphology (Figure 2(a) and (b)), and showed red-green birefringence with Congo red, typical of amyloid-like material.[17]

Peptides E and E′ alter fibril formation of intact β_2m

Each peptide was analysed to determine if it influences the formation of amyloid fibrils from

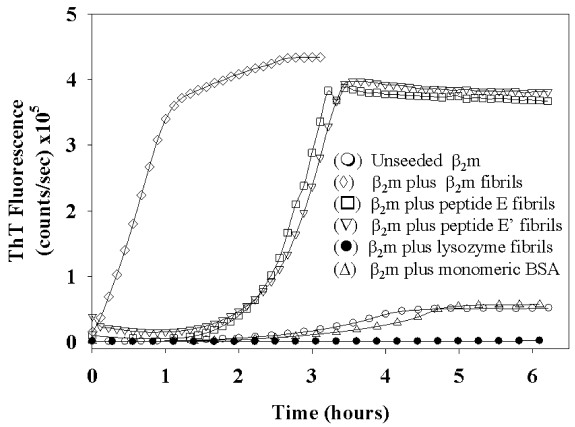

Figure 4. The effect of peptide fibrils on β_2m fibrillogenesis. Fibrillogenesis of β_2m either alone (○) or in the presence of pre-formed fibrils (1%, v/v) of β_2m (◇); peptide E (□); peptide E′ (▽), or hen lysozyme (●). An equivalent concentration of bovine serum albumin was also added as a control (△). Fibrils of β_2m were grown by incubating freshly dissolved and filtered monomeric protein (84 μM) in buffer B containing 10 μM ThT. A 1% (v/v) seed formed by sonicating (five minutes pulse, in a sonicating water bath) pre-formed fibrils of full-length β_2m, was added as a seed. Aliquots of fibrils formed from peptide E, peptide E′ (grown in buffer B) or hen lysozyme (grown according to Krebs *et al.*[21]) were also added to test their ability to seed fibril growth. Replicate experiments showed that the growth kinetics varied from experiment to experiment. Each experiment was thus carried out consecutively, using the same protein stock solution for all reactions. In this way, whilst the growth kinetics were variable, the relative effect of the different fibrils added on fibrillogenesis of the intact protein could be compared directly. Fibril assembly was monitored continuously using ThT fluorescence using a Photon Technology International QM-1 spectrofluorimeter (PTI, UK) at 37 °C as described above. All reactions were carried out in 1 ml cuvettes and stirred at 1400 rpm throughout.

full-length β_2m. In these experiments β_2m was incubated for several days in the presence of each peptide at pH 3.6 (in buffer A) or pH 2.5 (in buffer B) and the extent of fibril formation was measured by ThT fluorescence and negative stain EM (see the legend to Figure 3). When intact β_2m was incubated in the presence of peptide E or E′, the ThT signal obtained was significantly greater than that obtained when β_2m or the peptides were incubated alone (Figure 3(a) and (b)). Moreover, the signal obtained was greater than that expected if fibrils of the intact protein and peptide were to form independently in the peptide/protein mixture. By contrast, none of the other peptides had a significant effect on the extent of fibril formation of the intact protein as judged by ThT fluorescence (Figure 3(a) and (b)) and EM (not shown). This remained the case even when the peptides were added individually in tenfold molar excess, or in pair-wise mixtures in all combinations (in a 2:1 molar ratio of total peptide to protein) (Figure 3(a) and (b), and data not shown). The results indicate that rather than inhibiting fibrillogenesis, as observed in other peptide/protein systems,[18–20] the addition of peptides E or E′ to a solution of intact β_2m facilitates fibril formation, resulting in both a larger ThT signal and more fibrils as judged by EM (Figures 2(b) and 3(b)). In accord with this, the long, straight fibrils generated by incubating the peptides in isolation at pH 3.6 (buffer A; Figure 2(a)) are no longer observed when the peptide is incubated in the presence of the intact protein (Figure 3(a)). Instead, the majority of material visualised by negative stain EM comprises short curved fibrils (~200 nm), together with rarer fibrils that are intermediate in length (~500 nm) between those formed from the intact protein and peptide alone (Figure 3(a)), suggesting that the peptide and proteins may interact to form a new fibrillar form.

The ability of peptides E and E′ to facilitate fibril formation from full-length β_2m was further investigated by monitoring the effect of fibrils from each of the peptides on the rate of fibril growth from the intact protein. Fibrillogenesis of the peptides is very rapid (occurring within minutes) and so the reverse experiment in which the effect of β_2m fibrils on the rate of fibril formation from peptides E and E′ could not be performed. At pH 3.6 and high ionic strength fibril growth of β_2m occurs rapidly without a lag phase.[3,5] By contrast, at pH 2.5 the protein produces fibrils by nucleation-dependent growth.[3,7] Accordingly, fibrils develop after a lag phase, which is eliminated in the presence of a 1% (v/v) seed of pre-formed β_2m fibrils (Figure 4). These self-seeds also reduce the time taken for assembly to reach equilibrium and result in a substantial increase in the final ThT fluorescence (Figure 4). The addition of pre-formed fibrils formed from peptide E or E′ to a growth assay of intact β_2m at pH 2.5 also decreased the time taken to reach equilibrium (from 4.4 hours to ~3.3 hours) and increased the final fluorescence signal considerably (Figure 4). This effect was not observed when fibrils formed from hen lysozyme[21] were added or in the presence of an equivalent concentration of monomeric bovine serum albumin (Figure 4). The data indicate that fibrils generated from the peptides can also facilitate fibrillogenesis of the intact protein. Importantly, the lag time was not abolished in the presence of the peptide fibrils, a phenomenon that has been observed in other assembly reactions when the seed is non-self.[16,22–24] β_2m fibrils formed in the presence of the self-seed have a long, straight morphology and clear periodicity, akin to the fibrils grown in the absence of the seed, in accord with previous results.[3,7] By contrast, in the presence of fibrils formed from either peptide E or E′, long straight fibrils, as well as smaller amounts of shorter (100–600 nm), aperiodic, fibrils were observed (Figure 3(b)). The latter may result from random shearing of fibrils formed from the intact protein, or may reflect the formation of novel fibrils generated by the association of the intact protein and the peptides into a

common fibrillar array. These possibilities cannot be distinguished here.

Discussion

The most remarkable finding here is that despite surveying a wide range of pH and ionic strengths only the peptides containing the sequence corresponding to strand E in wild-type human β_2m are capable of forming amyloid-like fibrils *in vitro*. In order to identify which features of the peptides studied here are responsible for their amyloidogenic properties, a number of their characteristics were compared, including length, hydrophobicity, β-sheet propensity, pI, the arrangement of hydrophobic and hydrophilic residues (binary patterning), charge, and the number of aromatic residues. No correlation was observed between the length of the peptides, their hydrophobicity, secondary structural propensity and their ability to associate into fibrils. Whilst peptides B and E are both 13 residues in length, only the latter forms fibrils *in vitro*. Similarly, peptides E and E′ are 13 and 21 residues in length, respectively, yet both readily form fibrils. The hydrophobic indexes (average per residue per peptide[25]) of peptides C and E are similar (−0.08, −0.06, respectively), but only peptide E forms fibrils in this study. Moreover, whilst peptide E is the most hydrophobic of all the sequences studied, peptide E′ (which still forms fibrils) is one of the most hydrophilic of the peptides studied, as judged by both the total hydrophobicity score, as well as the average hydrophobicity per residue. All of the peptides have similar β-sheet propensities (0.95 ± 0.12 averaged per residue per peptide measured using a number of different scales[26–28]), ruling out a simple correlation between this property and amyloidogenicity. Similarly, peptides E and E′ have the highest helical propensity[29] of all the sequences compared, suggesting that helical structure in the denatured state is not responsible for the inability of the other peptides to form fibrils.[30,31] Whilst peptides E and E′ form fibrils over a wide pH range, the other peptides do not form fibrils under any condition, even when incubated for extended periods of time or at high concentrations (336 μM) at a pH equivalent to their pI, suggesting that pI is not a major determinant of aggregation. Finally, none of the eight peptides possesses a regular pattern of hydrophobic and hydrophilic residues, a feature that has been used successfully for the *de novo* design of amyloid-like fibrils[32,33] and purportedly selected against by nature to avoid amyloidosis.[34] In accord with the results obtained on other peptides and proteins,[35,36] our data demonstrate that rather than being dominated by general properties of the main-chain, amyloid formation depends on the precise amino acid sequence and is a highly specific process.

One of the most notable differences between peptides E and E′ and the other peptides studied here is the unusually high occurrence of aromatic residues in peptides E and E′. Although peptide E is only 13 residues in length, it contains six aromatic amino acid residues, whilst peptide E′ contains seven such residues. By contrast, the other peptides studied here contain three or less Phe, Tyr or Trp residues (Figure 1(b)). Interactions between aromatic residues have recently been proposed to promote ordered assembly of peptides and proteins, by favourable π−π-stacking that align in adjacent β-strands in the polymer.[37–39] Our data suggest that the ability to undergo favourable π−π-stacking may be critically important in determining the ability of the peptides of β_2m studied here to form amyloid fibrils *in vitro*. The second notable feature of peptides E and E′ is that they contain few, if any, positively charged residues at the acidic pH values studied here. Thus, peptide E contains no Arg, Lys or His residues, whilst peptide E′ contains only one Lys (Figure 1(b)). By contrast, the remaining peptides contain between one (peptide B) and four (peptide D) such residues. The data suggest that electrostatic repulsion, even at the high ionic strengths, may play a role in determining the ability of the peptides to self-assemble at low pH, in accord with results for other peptides and proteins.[35,40] This phenomenon alone, however, cannot rationalise the behaviour of the peptides under all conditions. Thus, at pH 7, peptide E is negatively charged (one Glu and one Asp residue) and E′ is highly negatively charged (it contains five Asp or Glu residues and only one lysine), whilst peptide G has no net charge at this pH. Nonetheless, only peptides E and E′ form fibrils at neutral pH. Whether a peptide can form ordered amyloid assemblies thus depends critically on the precise amino acid sequence and not only the electrostatic interactions. Interestingly, a different 22-residue self-peptide of β_2m formed by proteolysis has recently been shown to form fibrils in isolation that also enhance fibrillogenesis of the intact protein, similar to the behaviour of peptides E and E′ described here.[16] This peptide consists of strands B and C (residues 20–41) in full-length β_2m (Figure 1(b)) and contains three aromatic residues and three Lys or Arg residues. Here, we show that shorter synthetic peptides corresponding to each of strands B and C do not form fibrils in isolation *in vitro*, nor do they influence fibril formation of the intact protein when added separately or in pair-wise combination, demonstrating that the fibrillogenic properties of the different sequences involve a subtle interplay between a number of competing factors and are context-dependent.

How could peptides E and E′ facilitate the formation of amyloid in intact β_2m? Interestingly, a number of peptides have been shown to rapidly assemble into amyloid-like fibrils in isolation and promote assembly of the corresponding intact protein, presumably by presenting new recognition surfaces that seed assembly.[21,24,41] Here, we have shown that the sequence corresponding to strand E could represent a recognition surface for human

β_2m. In addition, fibrils formed from a different peptide corresponding to the joined strands B and C[16] facilitate assembly of intact β_2m into amyloid fibrils, suggesting that this region may also be involved in assembly. In both cases, however, the lag phase in assembly of the intact protein is not abolished in the presence of the peptide fibrils, suggesting that the peptides do not directly seed assembly. Various models have been proposed to rationalise these observations, including more complex mechanisms or the requirement for conformational changes in the seed or the monomer prior to elongation.[16,22] Further experiments will be needed to determine which, if any, of these possibilities are responsible for the inability of the β_2m peptides to directly seed assembly of the intact protein. Nonetheless, the data presented here indicate that fibrils formed from peptides E and E′ interact with β_2m during assembly such that fibrillogenesis of the intact protein is enhanced.

Previous NMR studies of the amyloid precursor of β_2m formed at low pH have shown that this species is a non-cooperatively stabilised, partially folded ensemble, that contains a stable core involving the native B, C, D, E and F strands, whilst strands A and G are highly destabilised.[13] The finding that peptides A and G do not form fibrils in isolation, nor do they affect fibril formation of the intact protein, suggests that these regions are not involved directly in self-assembly. Moreover, these regions are also relatively weakly protected from hydrogen exchange in the amyloid fibril, and the N-terminal 6–20 residues are often removed by proteolysis in fibrils formed *in vivo*, consistent with the view that these sequences do not play a direct role in formation of the amyloid core.[12,15] Instead, dissociation of strands A and G from the β-sandwich structure of the native protein appears to be a critical step in fibril formation of this protein.[12,13,42] Indeed, an antibody that binds, in part, to residues 92–99 in native β_2m inhibits fibril formation, presumably by preventing dissociation of this edge strand from the native core.[43] It is likely that further conformational changes that expose new assembly competent sites will also be required to permit assembly of adjacent monomers into amyloid fibrils. A number of different regions of β_2m have been proposed as potentially important in assembly, including strand E (here), strands B/C,[16] as well as the edge strands, including strand D.[44,45] Further experiments will now be needed to determine the importance of each of these regions in different stages in assembly and the role of specific amino acid residues in these and other regions in the self-recognition process. Such experiments could involve the creation of suitable mutants or the synthesis of new peptide fragments and analysis of their effects on the kinetics of assembly. The results presented here provide the framework for such studies and suggest regions of potential interest for targeting future development of inhibitors of β_2m amyloidosis.

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