A Systematic Study of the Effect of Physiological Factors on β_2-Microglobulin Amyloid Formation at Neutral pH[†]

Sarah L. Myers,[‡] Susan Jones,[‡,§] Thomas R. Jahn,[‡] Isobel J. Morten,[‡] Glenys A. Tennent,[||] Eric W. Hewitt,[‡] and Sheena E. Radford*[,‡]

Astbury Centre for Structural Molecular Biology, Garstang Building, University of Leeds, Leeds LS2 9JT, United Kingdom, and Centre for Amyloidosis and Acute Phase Proteins, Royal Free and University College Medical School, London NW3 2PF, United Kingdom

Received November 29, 2005; Revised Manuscript Received December 19, 2005

ABSTRACT: β_2-microglobulin (β_2m) forms amyloid fibrils that deposit in the musculo-skeletal system in patients undergoing long-term hemodialysis. How β_2m self-assembles in vivo is not understood, since the monomeric wild-type protein is incapable of forming fibrils in isolation in vitro at neutral pH, while elongation of fibril-seeds made from recombinant protein has only been achieved at low pH or at neutral pH in the presence of detergents or cosolvents. Here we describe a systematic study of the effect of 11 physiologically relevant factors on β_2m fibrillogenesis at pH 7.0 without denaturants. By comparing the results obtained for the wild-type protein with those of two variants (ΔN6 and V37A), the role of protein stability in fibrillogenesis is explored. We show that ΔN6 forms low yields of amyloid-like fibrils at pH 7.0 in the absence of seeds, suggesting that this species could initiate fibrillogenesis in vivo. By contrast, high yields of amyloid-like fibrils are observed for all proteins when assembly is seeded with fibril-seeds formed from recombinant protein at pH 2.5 stabilized by the addition of heparin, serum amyloid P component (SAP), apolipoprotein E (apoE), uremic serum, or synovial fluid. The results suggest that the conditions within the synovium facilitate fibrillogenesis of β_2m and show that different physiological factors may act synergistically to promote fibril formation. By comparing the behavior of wild-type β_2m with that of ΔN6 and V37A, we show that the physiologically relevant factors enhance fibrillogenesis by stabilizing fibril-seeds, thereby allowing fibril extension by rare assembly competent species formed by local unfolding of native monomers.

β_2-microglobulin (β_2m[1]) is the noncovalently bound, nonpolymorphic light chain of the class I major histocompatibility complex (MHC-I) and is one of over 20 proteins currently known to be involved in human amyloid disease (*1*). In healthy subjects β_2m (M_r 11,860) dissociates from the heavy chain of the MHC-I complex and is transported in the plasma to the kidneys where the majority (∼95%) of the protein is degraded (*2*). However, in the event of renal failure the plasma clearance of β_2m is disrupted, causing an increase in the circulating concentration of the protein by up to 60-fold which leads to the formation of amyloid fibrils that deposit predominantly in the joints and connective tissues of the musculo-articular system (*2*, *3*). Clinical symptoms are rare until 5 years after the onset of hemodialysis, but the incidence of β_2m amyloid disease increases to nearly 100% prevalence after 15 years of dialysis (*4*, *5*).

Despite being first identified as the aggregating protein in dialysis-related amyloidosis (DRA) 20 years ago (*6*, *7*), the mechanism by which β_2m self-associates to form amyloid fibrils in vivo is poorly understood (*8*). At acidic pH (≤pH 2.5) wild-type β_2m readily forms amyloid-like fibrils in vitro (*9*−*11*). Fibrillogenesis under these conditions proceeds with nucleation-dependent kinetics (*9*, *12*) that can be accelerated by the addition of fibril-seeds formed from recombinant β_2m fibrils (*12*) or with isolated ex vivo β_2m fibrils (*9*). By contrast, at neutral pH, amyloid fibrils do not form in the absence of seeds, unless the protein is incubated in the presence of copper ions and urea (*13*), or the sequence of β-strands A or G, but not elsewhere in the molecule, is altered (*10*, *14*). Amyloid-like fibrils have also been formed from wild-type β_2m at neutral pH following addition of trifluoroethanol (TFE) in the presence of fibril-seeds formed from recombinant protein at pH 2.5 in the presence of glycosaminoglycans (GAGs) or proteoglycans (PGs), while no fibrils were formed in the absence of TFE (*15*). Stabilization of β_2m fibril seeds and partial unfolding of the monomer by sodium dodecyl sulfate (SDS) also allows fibril formation to proceed at neutral pH (*16*). However, production of amyloid-like fibrils in the absence of detergents, organic

[†] This work was supported by the BBSRC to S.L.M and S.E.R., The Wellcome Trust to T.R.J., Kidney Research UK to I.J.M, and the University of Leeds.

* Correspondence should be addressed to: Sheena E. Radford, Astbury Centre for Structural Molecular Biology, Garstang Building, University of Leeds, Leeds, LS2 9JT, UK; Telephone: +44 113 343 3170. Fax: +44 113 343 3167. E-mail: S. E. Radford@leeds.ac.uk.

[‡] University of Leeds.

[||] Royal Free and University College Medical School.

[§] Current Address: Department of Biomedical Sciences, School of Life Sciences, University of Bradford, Bradford BD7 1DP, UK.

[1] Abbreviations: apoE, apolipoprotein E; β_2m, beta-2-microglobulin; CD, circular dichroism; DRA, dialysis-related amyloidosis; EM, electron microscopy; GAGs, glucosaminoglycans; PG, proteoglycan; SAP, serum amyloid P component; ThT, thioflavin T.

10.1021/bi052434i CCC: $33.50
Published on Web 01/31/2006

solvents or high urea concentrations (i.e. under conditions that are entirely physiological) has not been achieved to date, raising important questions about the identity of factors that facilitate amyloid formation in vivo. Such factors may involve one or more components in the plasma, synovial fluid, and/or synovial joint environment. The identity of such factors, their mechanism of action, and whether they act independently or synergistically remain unresolved.

Here we describe a systematic study of the influence of 11 different, physiologically relevant factors, in isolation and in combination, upon fibrillogenesis of β_2m at neutral pH under entirely native conditions in vitro. These include components commonly found in amyloid deposits of β_2m and other proteins in vivo that are known to affect fibrillogenesis in vitro (GAGs, PGs, apolipoprotein E (apoE), both in the presence and absence of physiological lipids, and serum amyloid P (SAP) component) (*17*, *18*). Components of the joint environment (type II collagen, synovial fluid), and whole uremic serum from a patient with DRA undergoing dialysis for end-stage renal failure were also tested. In addition, we compare the behavior of the wild-type protein with a variant lacking the N-terminal 6-amino acids (ΔN6), a species known to constitute up to 30% of β_2m present in amyloid deposits in vivo (*19*, *20*), as well as the variant V37A, a protein that is highly destabilized yet does not form amyloid-like fibrils spontaneously at neutral also pH (*10*). By standardizing the assay for fibril formation and comparing the behavior of wild-type β_2m with these variants in the presence or absence of different additives, we identify factors that could facilitate formation of β_2m fibrils in and around the sites of amyloid deposition and propose a mechanism by which these factors act.

MATERIALS AND METHODS

Proteins. ΔN6 was produced by PCR using a 5′ primer to encode an N-terminal methionine residue and to delete the N-terminal 6 residues of the protein and a 3′ primer complementary to the gene sequence of the C-terminal 3 residues of the protein and the multiple cloning site. The PCR product was then cloned into plasmid pET23a using *Hin*d III and *Nde* I. Wild-type β_2m and the variant proteins ΔN6 and V37A were expressed and purified to homogeneity (>99% pure by SDS-PAGE) and their correct molecular masses confirmed by electrospray ionization mass spectrometry, as previously described (*10*). A yield of 10−30 mg of pure protein/L of culture was obtained. All proteins were stored at −20 °C as a lyophilized powder. Protein concentrations were calculated from the A_{280} of the denatured state using extinction coefficients determined by the method of Gill and von Hippel (*21*).

De Novo Fibril Formation (Unseeded Reactions). Wild-type and variant β_2m were dissolved in deionized water, filtered (0.2 μm cellulose acetate filter, Sartorius) to remove aggregates, and diluted to a final concentration of 1 mg/mL in 25 mM sodium phosphate, 25 mM sodium acetate containing 0.02% (w/w) sodium azide (buffer A) at pH 2.5 or 7.0. The samples were then incubated at 37 °C with agitation at 200 rpm for up to 42 days. The resulting suspensions were analyzed by negative stain transmission electron microscopy (EM), thioflavin T (ThT) fluorescence, Congo red binding, and dot-immunoblot assays (see below). The presence of typical amyloid fibrils was confirmed by red−green dichroism after staining with Congo red, and binding to ^{125}I-labeled SAP (^{125}I-SAP; see below).

Formation of Seeds. Fibrils formed by incubating β_2m (1 mg/mL) at 37 °C for 7 days in buffer A (pH 2.5) with agitation (200 rpm), were fragmented by three sequential 5-min cycles of freezing on dry ice and thawing in a water bath at 21 °C, before storage in aliquots at −20 °C for use as unstabilized seeds. This procedure fragments long, straight fibrils (see below) (*11*). Alternatively, the following reagents (obtained from Sigma-Aldrich Co. Ltd., Dorset, UK, unless otherwise stated) were added to the fibrils in buffer A (pH 7.0) prior to their fragmentation by freeze/thaw cycles and were used subsequently as stabilized seeds. For fibrils stabilized with GAGs and PGs, 60 μg of low molecular weight heparin (porcine, average mass 5 kDa), heparan sulfate (porcine, average mass 48 kDa), decorin (bovine, average mass 100 kDa), or aggrecan (bovine, average mass 250 kDa) was added per mg of fibrils (quantified as described below) in 1 mL of buffer A, pH 7.0. Alternatively, 2 μL of synovial fluid (porcine; kindly supplied by Eileen Ingham), 100 μL of uremic serum (kindly supplied by Fiona Karet), 100 μL of pooled normal human serum, 100 μg of type II collagen (chicken), 14 μg of pure human apoE3 or 14 μg of a mixture of different apoE isoforms isolated, in lipidated form, from human serum (kindly supplied by John Fryer and David Holtzman) (lipidated apoE) were added. The samples were incubated at 4 °C for 10 min without agitation to permit additive binding, and seeds were produced by freeze−thawing as described above. In separate experiments, the fibril seeds were incubated with increasing concentrations of heparin (0−60 μg/mg of fibrils). Negative stain EM showed that no long fibrils persisted after fragmentation in all cases (see Figure 1D (inset)). The amount of insoluble material in the fibril seeds was quantified as described below, and the results were used to ensure that an equal quantity of seed was added in each experiment. For experiments involving addition of SAP, fibrils were sedimented by three sequential cycles of repeated centrifugation (13,000 rpm for 10 min) and equilibration in 10 mM Tris, 140 mM NaCl, and 0.1% (w/v) sodium azide, pH 8.0 (TN buffer). SAP in TN buffer (47 or 94 μg of human SAP, isolated to >99% purity; kindly supplied by M. B. Pepys) was then added per mg of fibrils, followed by $CaCl_2$ to 2 mM final (or EDTA to 10 mM) (*22*), and the seeds produced by freeze−thawing before quantification and storage as described above. Control experiments included analysis of the effect of sulfate ions (Na_2SO_4) or poly(ethylene glycol) (PEG) ($M_r \approx 5000$, 12 or 120 μM) on fibril assembly at pH 7.0 so that the specific effect of the additive over the physical or chemical properties of the additive could be discerned. A seed (5 μL) comprising a suspension of synovial tissue rich in β_2m amyloid deposits (obtained at autopsy, with informed consent, from a patient with DRA; kindly supplied by M. B. Pepys), in 10 mM Tris, 138 mM NaCl, 2 mM $CaCl_2$, 0.1% (w/v) of sodium azide, pH 8.0 (TC buffer) was also used (without freeze−thawing). Finally, seeds were prepared as described above in the presence of SAP but, in addition, were incubated simultaneously with heparin (60 μg/mg of fibril seeds), lipidated apoE (14 μg/mg of fibril seed) (mix A), and where noted, type II collagen (100 μg/mg of fibril seeds) (mix A + collagen).

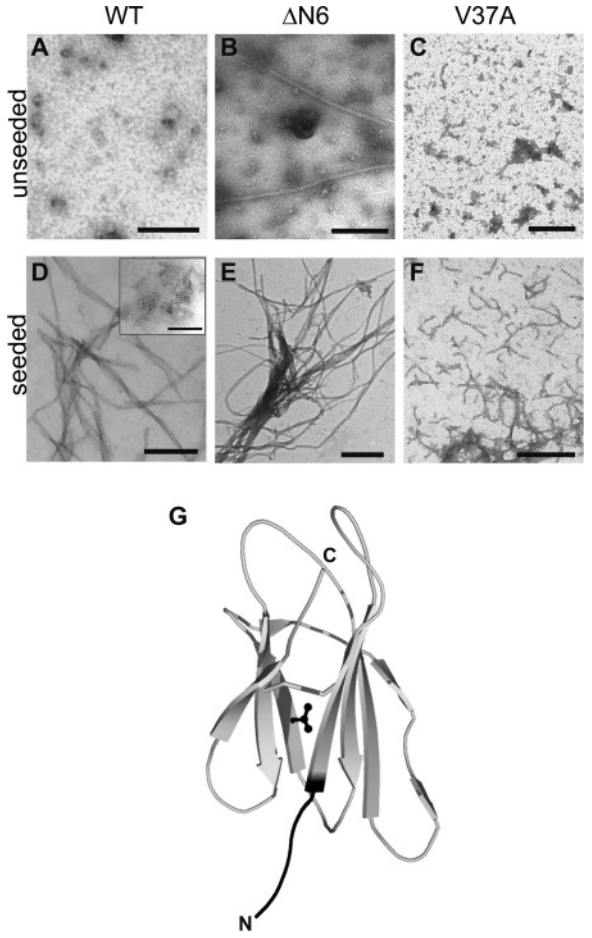

FIGURE 1: Negative stain EM images of samples of wild-type β_2m and the variants ΔN6 and V37A incubated at pH 7.0 in the absence of seed (A−C) or in the presence of heparin-stabilized seeds (D−F). Images were taken after 6 weeks incubation at 37 °C with agitation (200 rpm). Inset in D: Negative stain image of the initial fibril seeds. Scale bar represents 100 nm. (G) Ribbon diagram of monomeric β_2m (1JNJ) (*66*). The disulfide bridge linking strands B and F, the position of Val 37 (ball-and-stick) as well as the region corresponding to the ΔN6 deletion (black) are shown. Figure was drawn using PyMol (*67*).

Seed Elongation. For samples incubated in the absence of fibril seeds, monomeric β_2m (wild-type or variants) was diluted in buffer A pH 7.0 to give a final protein concentration of 0.5 mg/mL. The solutions (1 mL) were then incubated for 0−42 days at 37 °C with agitation (200 rpm). Three separate samples for each experiment were set up, each analyzed in triplicate. First, β_2m was incubated in the absence of seeds, but in the presence of different additives, to ensure that the additives had no effect on fibril growth of the monomer alone. In all samples the w/w ratio of additive to β_2m was the same as that in the presence of seeds (assuming that the additive dissociates completely from the seeds during growth). Second, seeds (50 μg) were incubated in buffer alone in the presence or absence of additive, so that newly elongated fibrils could be discerned from the starting material. Finally, β_2m (500 μg wild-type or variants) was incubated with seeds (50 μg) in a total volume of 1 mL in buffer A, pH 7.0, or for experiments using SAP, TN buffer, pH 8, with subsequently added $CaCl_2$ (2 mM final) or EDTA (10 mM final). Each sample was incubated at 37 °C with agitation (200 rpm) in a 1.5-mL microcentrifuge tube (Eppendorf) for up to 42 days. At different time points, 10-μL aliquots were withdrawn to monitor fibril growth by ThT fluorescence. Analysis of samples at the end of the experiment by SDS-PAGE demonstrated that no visible degradation of β_2m occurred during the experiment. Only wild-type protein seeds were used in the data presented here. Control experiments demonstrated that similar results were obtained in the presence of self-seeds (data not shown).

Thioflavin-T Fluorescence. A discontinuous assay was used to monitor fibril formation by ThT fluorescence, as previously described (*10*). The fluorescence intensity of each sample was normalized to that of buffer containing ThT alone, and the mean ± standard deviation (SD) values of the nine measurements (three replicate samples, each measured in triplicate) for each condition were plotted. The experiment was repeated twice, and the data were highly reproducible (within ±15%).

Fibril Quantification. Samples (100 μL) of each fibril suspension were centrifuged at 13,000 rpm for 5 min at room temperature. The supernatants containing soluble protein were retained, and the pellets were resuspended in 100 μL of buffer A pH 7.0. To each sample, 6 M guanidinium chloride (GdnCl), pH 7.0, was added (900 μL), and the mixtures were incubated at room temperature with agitation for 2 h to denature and resolubilize all material. The percentages of protein in the supernatant and fibril pellet were calculated by comparison of the A_{280}'s of the pellet and supernatant with that of a control solution (1 mg/mL) of monomeric wild-type β_2m. The data shown are the mean ± SD of triplicate measurements.

Fibril Depolymerization Assays. β_2m fibril-seeds (2 μM) formed at pH 2.5 were added to buffer A, pH 7.0, at 37 °C in the presence or absence of heparin (0−100 μM), and the depolymerization of fibrils, under conditions of vigorous agitation, was monitored in real time using the fluorescence of ThT (see above). Depolymerization transients were fitted to single-exponential transitions. ThT fluorescence of these samples after 24 h incubation at 37 °C was measured. All experiments were done in triplicate, and the mean ± SD values are plotted.

Thermodynamic Stability. Samples of wild-type or variant β_2m (4 μM protein), in the presence or absence of heparin (0.29 μg/mL) in 25 mM sodium phosphate buffer, pH 7.0, were incubated for 24 h at 37 °C in the presence of different concentrations of urea (0−10 M). Unfolding was monitored by tryptophan fluorescence using a Quantamaster C-61 spectrofluorimeter (Photon Technologies Inc., Texas, U.S.A.) with excitation and emission wavelengths set to 280 and 325 nm, respectively. Data were fitted to an equation describing a two-state transition (*23*) using Sigma-Plot (Jandel Scientific, UK).

Other Analytical Procedures. Circular dichroism, negative stain EM, Congo red binding assays, Congo red birefringence, and fluid-phase ^{125}I-SAP binding assays were performed as described (*10*, *24*, *25*). Binding by ^{125}I-SAP to fibrils formed at pH 2.5 and 7.0 from wild-type β_2m (in the presence and absence of heparin), was compared with known amyloid fibrils extracted ex vivo from human β_2m (Aβ_2m) amyloidotic tissue and prepared in vitro from Aβ 1−42 peptide (F. Hoffmann-La Roche, Basel, Switzerland). Control incubations were performed in the presence of 10 mM EDTA, and background counts obtained in the absence of fibrils were subtracted from all results. Two separate

Table 1: Properties of Wild-Type β_2m and the Variants ΔN6 and V37A

variant	insoluble material (unseeded) (%)[a]	insoluble material (seeded with heparin-stabilized seeds) (%)[a]	equilibrium denaturation midpoint (M urea)[b]		apparent equilibrium $\Delta G°_{UN}$ (kJ/mol)[b]	
			protein only	plus heparin	protein only	plus heparin
wild-type	3.0 (± 1.0)	40 (± 6.0)	4.8	4.7	17.3 (± 0.4)	16.8 (± 1.2)
ΔN6	5.0 (± 2.0)	50 (± 2.5)	4.0	3.8	14.1 (± 1.1)	12.2 (± 1.1)
V37A	8.0 (± 3.0)	29 (± 4.0)	1.8	2.0	4.5 (± 1.2)	5.7 (± 0.9)

[a] % insoluble material after 42 days incubation at pH 7 (see Materials and Methods). [b] Determined by urea denaturation at pH 7.0, 37 °C (see Materials and Methods).

experiments gave consistent results. Dot-immunoblots were carried out as described previously (*11*, *26*).

RESULTS

Specific Destabilization of the N-Terminal Region Promotes Fibril Formation from β_2m at Neutral pH in the Absence of Seeds. In an initial experiment, the ability of wild-type β_2m and the variants ΔN6 and V37A to form fibrils in vitro at neutral pH in the absence of seeds was monitored using ThT fluorescence and negative stain EM (Figure 1A−C). Previous studies have shown that β_2m variants containing sequence substitutions in the N- or C-terminal regions (I7A, V9A, or V93A) can form fibrils spontaneously in vitro at pH 7.0, albeit with low yield (<10%) (*14*), suggesting that destabilization of one or both of the terminal strands increases the amyloidogenicity of the protein (*14*, *27*). Consistent with these results, ΔN6 is able to form fibrils spontaneously at pH 7.0 in the absence of fibrillar seeds (Figure 1B). The fibrils formed did not give rise to a significant increase in ThT fluorescence, presumably because the yield of fibrils was too low (Figure 3A and Table 1), and had a distinctive morphology that lacks obvious periodicity (Figure 1B), suggesting that they differ from fibrils formed at acidic pH (*10*, *12*, *14*). Interestingly, the fibrils resemble those formed at pH 7.0 by the β_2m variants I7A, V9A, and V93A (*14*), suggesting that this fibril type may be characteristic of unseeded self-assembly at neutral pH. The low yield of fibrillar material was not increased by seeding assembly with self-seeds formed at pH 2.5 or with larger amounts of seed formed from wild-type β_2m at pH 2.5 (up to a 1:1 molar ratio free monomeric protein:monomer in seeds) (data not shown). Wild-type β_2m and V37A were also unable to form fibrils de novo in vitro under the conditions tested (Figure 1A, C), despite the fact that V37A is significantly destabilized compared with the wild-type protein (Table 1). Consistent with previous results (*10*), these data demonstrate that specific destabilization of the N-terminal region of β_2m promotes de novo fibril formation in vitro, while global stability appears to play little role in determining the amyloidogenicity of this protein at neutral pH.

GAGs and PGs Increase Fibril Formation at Neutral pH. GAGs and PGs are associated with amyloid deposits in vivo and, importantly, are present in high concentrations in the joints, known to be sites of β_2m deposition in DRA (*28*, *29*). Therefore, we next analyzed whether fibril yield could be increased at neutral pH by incubation with seeds formed from wild-type β_2m at pH 2.5 that were stabilized against depolymerization at pH 7.0 by these physiologically relevant additives. Building on procedures developed by Naiki and colleagues which involve fibril elongation in the presence of TFE or SDS (*15*, *30*), we developed a fibril elongation assay at neutral pH which can be performed without addition of denaturants. Here, fibril seeds formed from wild-type β_2m at pH 2.5 were stabilized at pH 7.0 by the addition of heparin, heparan sulfate, decorin, or aggrecan (see Materials and Methods), and elongation with monomeric wild-type β_2m, ΔN6, or V37A at pH 7.0 was analyzed. In parallel, these components were added to each monomeric β_2m variant in the absence of seeds (to test the ability of these additives to facilitate nucleation), as well as to seeds formed from the wild-type protein in the absence of monomer (so that fibrils elongated with monomer could be differentiated from the fibril seeds) (Figure 2). The w/w ratio of added PG or GAG in each experiment was maintained, irrespective of their carbohydrate content or molecular weight. After incubation with agitation at 37 °C, the formation of fibrils was monitored as a function of time over a 6-week period using ThT fluorescence. The resulting time course of fibrillogenesis for wild-type β_2m is shown in Figure 2. The addition of these additives to monomeric β_2m in the absence of seeds did not result in an enhanced ThT signal, and no fibrils were detected in these samples by negative stain EM even after incubation for 6 weeks at this pH (data not shown). Similar results were obtained for the variants ΔN6 and V37A (Figure 3A and data not shown), demonstrating that even when destabilized substantially, as in the case of V37A (Table 1), monomeric β_2m is not able to form fibrils spontaneously in unseeded

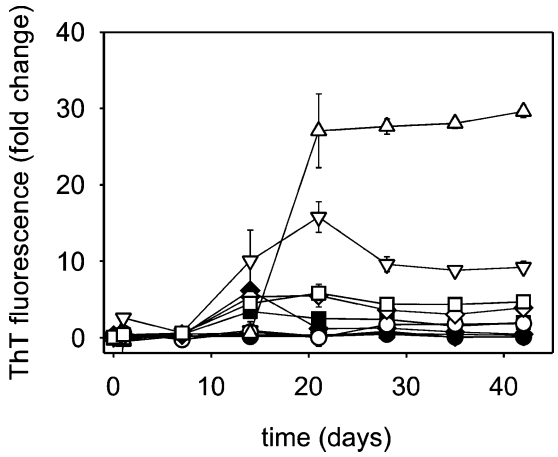

FIGURE 2: Discontinuous time course of fibril formation, monitored by ThT fluorescence, from wild-type β_2m under different conditions. Seeds only (closed symbols), seeds plus monomeric β_2m (open symbols). Unstabilized seed (circle), heparin-stabilized seed (upward triangle), heparin-sulfate stabilized seed (diamond), decorin-stabilized seed (downward triangle) and aggrecan-stabilized seed (square). Triplicate measurements were taken of three replicate samples, and the error bars are standard deviations of the mean.

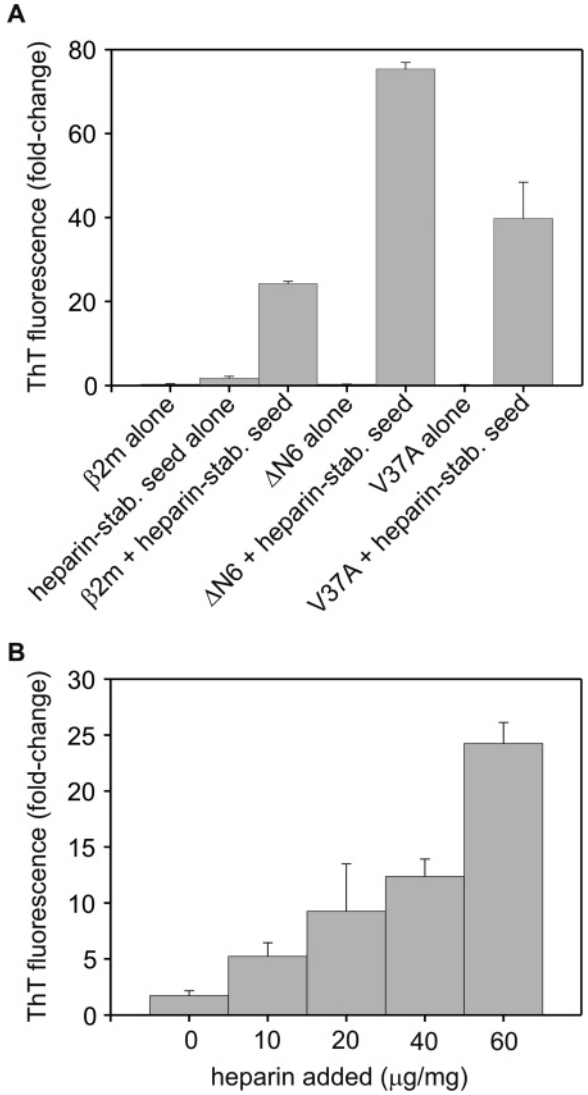

FIGURE 3: Fibril formation monitored by ThT fluorescence. (A) Fibril formation from wild-type β_2m and the variants ΔN6 and V37A in the presence or absence of heparin-stabilized seeds. In this experiment, 60 μg of heparin/mg fibril-seeds were used. (B) Dependence of fibril formation of wild-type β_2m seeds with different concentrations of heparin stabilized seeds. The amount of heparin used to stabilize 1 mg of fibrils is shown. In both (A) and (B) 50 μg of seeds were used to initiate fibrillogenesis of 500 μg of β_2m in a final volume of 1 mL of buffer A pH 7.0. All samples were incubated for 6 weeks at 37 °C with agitation (200 rpm). Triplicate ThT measurements were taken, and the error bars depict the standard deviation of the mean.

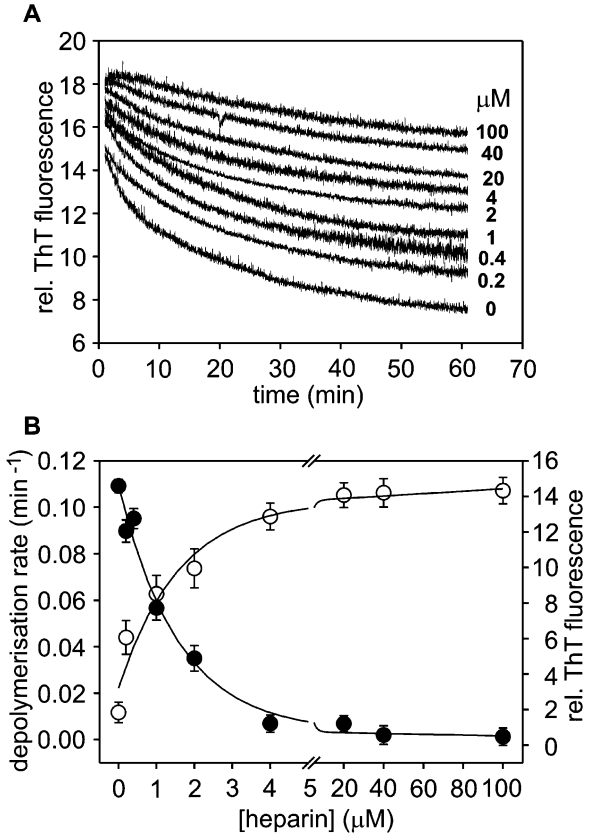

FIGURE 4: Depolymerization of fibrils formed from β_2m at pH 2.5 monitored by ThT fluorescence. (A) Effect of different concentrations of heparin added to the buffer on the rate of fibril depolymerization. (B) Comparison of the measured rates of depolymerization (filled circles) and the final amplitude 48 h after initiating depolymerization (open circles) for different concentrations of added heparin. Each point represents the average and standard deviation for the first-order rate constant over three replicate experiments.

reactions at neutral pH in the absence or presence of these additives. Most dramatically, however, a >25-fold increase in ThT fluorescence resulted when wild-type β_2m was incubated in the presence of heparin-stabilized seeds (Figures 2 and 3A), while incubation of V37A and ΔN6 resulted in increases in ThT signals of >40-fold under these conditions (Figure 3A). A less dramatic effect was observed with decorin-stabilized seeds, while heparan sulfate and aggrecan-stabilized seeds were relatively ineffective in initiating fibrillogenesis of wild-type β_2m (Figure 2). Similar results were obtained for V37A and ΔN6 with these additives (data not shown). Increasing concentrations of heparin result in an increase in fibrillar material obtained (Figure 3B). Measurements analyzing seed stability at pH 7.0 in the presence of different concentrations of heparin confirmed that this GAG protects fibrils formed from wild-type β_2m in acid against depolymerization at pH 7.0 in a dose-dependent manner (Figure 4A, B). The results identify conditions under which amyloid-like fibrils from β_2m can be formed in vitro under entirely native conditions and demonstrate that heparin, a reagent commonly administered during hemodialysis (*31*), enhances the amyloidogenicity of β_2m significantly.

Fibrils Formed from Wild-Type β_2m, V37A, and ΔN6 *at pH 7.0 Are Amyloid-Like.* Fibrils formed at pH 7.0 from wild-type and variant β_2m in the presence of heparin-stabilized seeds show a classical amyloid-like fibril morphology when imaged by EM (Figure 1D−F). Fibrils formed at pH 7.0 by wild-type β_2m and ΔN6 show long, straight fibrils (Figure 1D, E), while fibrils of V37A appear shorter and more flexible (Figure 1F). Fibrils formed from all variants bind Congo red, displaying changes in the absorbance spectrum of the dye characteristic of binding to amyloid fibrils (*32*) (Figure 5A). Samples also exhibited a predominance of apple-green birefringence, pathognomic of amyloid, after staining with Congo red, when visualized by cross-polarized light microscopy (Figure 5B). They were also recognized by the monoclonal antibody WO1 that binds to an epitope displayed generically on all amyloid fibrils (*33*) (Figure 5C), but not by an anti-oligomer antibody (*26*) that recognizes a generic epitope present on prefibrillar

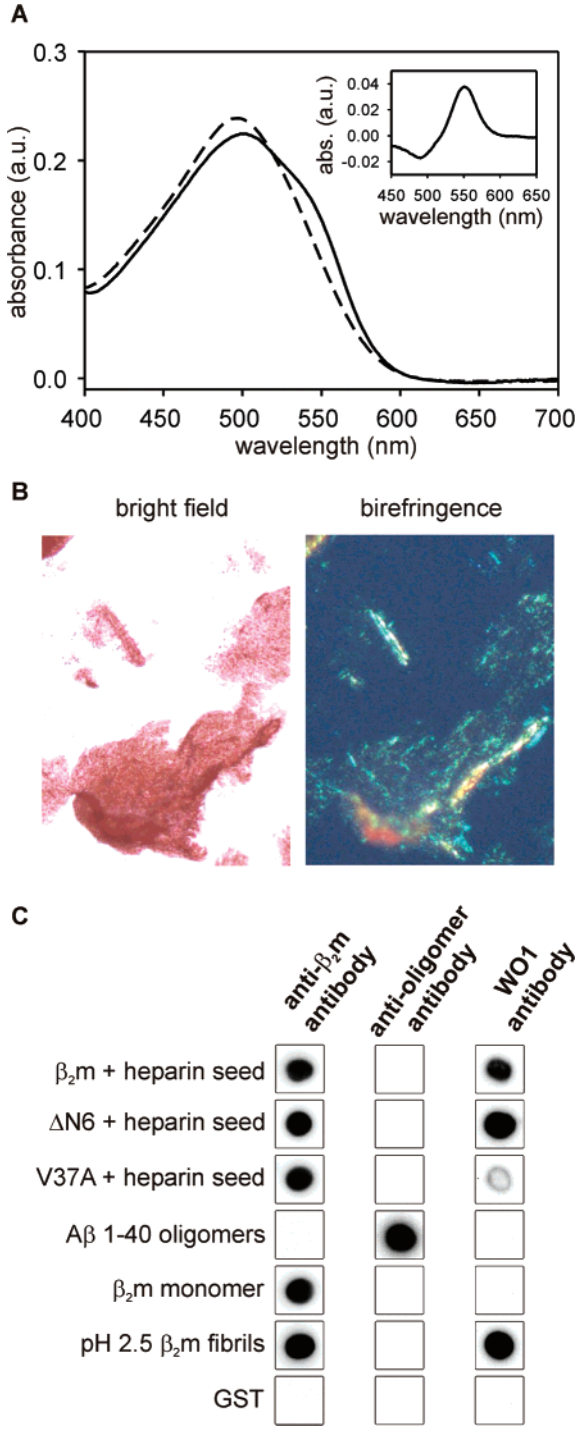

FIGURE 5: Fibrils formed from wild-type β_2m by extension of heparin-stabilized seeds at pH 7.0 are amyloid-like. (A) Absorbance spectrum of Congo red bound to β_2m fibrils (solid line) and free in solution (dotted line) as well as the difference spectrum (inset). (B) (Left) Congophilic uptake in bright light and (Right) typical green birefringence of Congo red in cross-polarized light of fibrils formed from wild-type β_2m at pH 7.0. (C) Binding of anti-β_2m, anti-fibrillar (WO1) (*33*) and anti-oligomer specific antibodies (*26*) to β_2m and its variants. Aβ 1−40 oligomers act as a positive control for the anti-oligomer antibody. Glutathione-S-transferase (GST) is a negative control for all antibodies.

species (Figure 5C). Finally, fibrils formed by the wild-type protein at pH 7.0 (and at pH 2.5) display calcium-dependent binding by SAP (Supporting Information; Table 1). Together, these results demonstrate that the fibrils formed by the elongation of heparin-stabilized seeds with monomeric β_2m

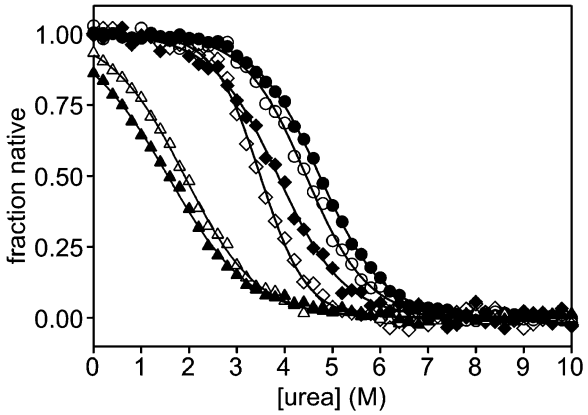

FIGURE 6: Urea-induced denaturation of β_2m and the variants ΔN6 and V37A at pH 7.0, 37 °C. Wild-type β_2m (circles), ΔN6 (diamonds), and V37A (triangles) in the absence (closed symbols) or presence of heparin (open symbols) are shown. The data are shown in terms of the fraction of native protein. Solid lines represent fits to a two-state unfolding transition.

under physiologically relevant conditions at pH 7.0 are amyloid-like.

Heparin Does Not Affect the Stability of Monomeric Wild-Type β_2m, ΔN6, *or V37A.* In addition to increasing fibril stability, heparin may also enhance the ability of wild-type β_2m and the variants ΔN6 and V37A to form amyloid-like fibrils at pH 7.0 by altering the conformational properties of these proteins. To assess whether this was the case, heparin was added to wild-type β_2m and the variants ΔN6 and V37A before examination at pH 7.0 by far- and near-UV CD. In all experiments a final heparin concentration equivalent to that used in the elongation assays (see Materials and Methods) was used. The results revealed that the presence of heparin at this concentration had no effect on the secondary structure of the proteins analyzed (data not shown). All three proteins showed small differences in their CD spectra in the near-UV compared with their heparin-free counterparts, possibly as a consequence of local structural changes by the known ability of heparin to bind to the region encompassing the N-terminal 24 residues of the protein (*34*) (data not shown).

The effect of heparin on the stability of wild-type β_2m and the two variants was also analyzed by urea denaturation (Figure 6). The results confirmed that V37A is destabilized significantly relative to wild-type β_2m, while ΔN6 shows only minor destabilization (midpoints for denaturation 4.8 M (wild-type), 4.0 M (ΔN6), and 1.8 M urea (V37A), respectively; Figure 6 and Table 1). The addition of heparin had little effect on the stability of all three proteins (Figure 6, open circles) (Table 1). Addition of heparin to V37A increased the stability of the protein slightly (Figure 6, open triangles), while a small destabilization of wild-type β_2m and ΔN6 was observed. While the molecular origins of these effects remain to be resolved, the effect of heparin on protein stability was small (relative to the effect of the substitution of Val37 for Ala in V37A, for example), demonstrating that even if the heparin dissociates from the seeds during fibril elongation, the released GAG would have little effect on structure or stability of the monomeric proteins. The development of a denaturant-free fibril formation assay at neutral pH allows determination of whether the additive affects the stability of the fibril, monomer, or both, and demonstrates

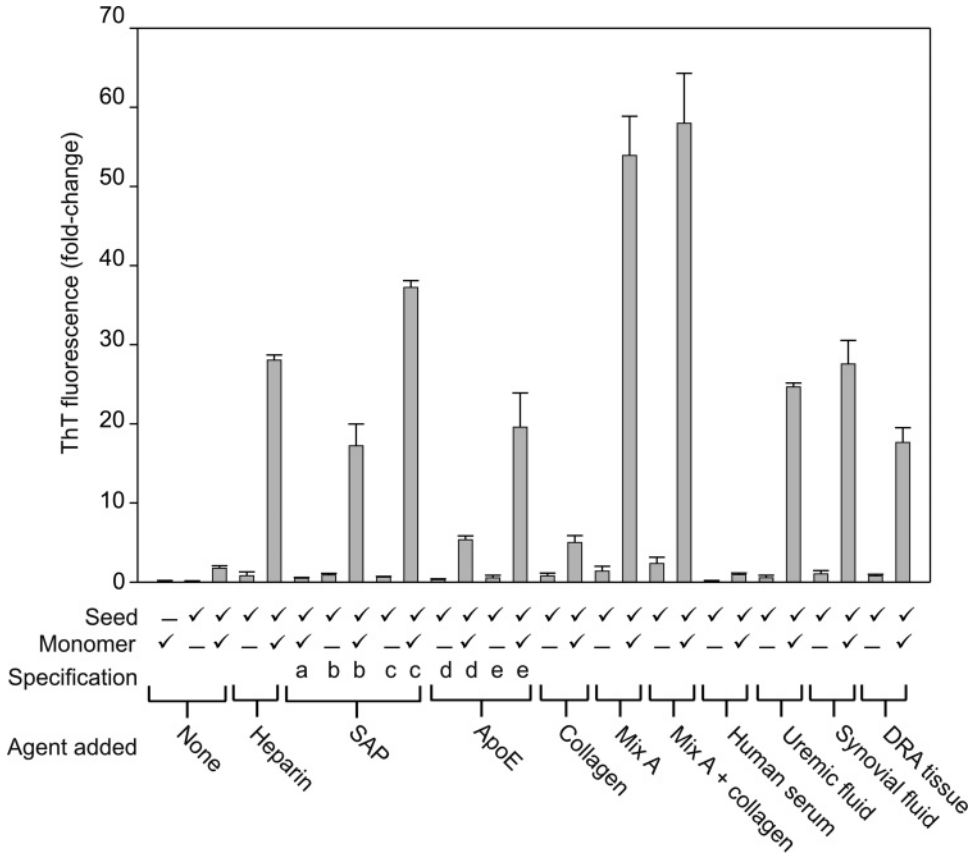

FIGURE 7: End points of incubation of wild-type β_2m in the presence or absence of different physiologically relevant factors, monitored by ThT fluorescence. Samples were incubated for 6 weeks at pH 7.0, 37 °C with agitation (200 rpm). Triplicate samples were set up under each condition and the ThT signal of each was measured three times. The mean of these readings is shown, with error bars depicting the standard deviation. The concentrations of biological factors added are given in the Materials and Methods (specifications: a, SAP plus EDTA; b, 47 μg/mg SAP; c, 94 μg/mg SAP; d, unlipidated apoE; e, lipidated apoE).

that the increase in amyloid fibril formation observed in the presence of heparin results from the effect of the additive on seed stability alone.

Other Physiologically Relevant Factors Promote Fibril Formation of β_2m at Neutral pH. The effect of heparin in promoting fibril formation of β_2m at neutral pH is consistent with the notion that this, and other GAGs, could play an important role in facilitating β_2m amyloidosis in vivo by stabilizing fibrillar seeds at neutral pH (*30*). To determine whether other factors may also facilitate β_2m fibrillogenesis in vivo, and whether such factors have a synergistic role with heparin in promoting fibril formation, we extended our analysis to include other potentially relevant factors in seeding assembly of the wild-type protein into amyloid fibrils (similar results were obtained with ΔN6, data not shown). Based on literature precedents and the known specific deposition of β_2m in cartilaginous joints (*35*−*39*), the effects of type II collagen, apoE (pure protein and mixtures of apoE isotypes within liposomes), and SAP on the rates and amplitudes of fibril formation at pH 7.0 were assessed. Previous studies have shown that these components bind to β_2m fibrils and, in the case of pure apoE, stabilize fibrils against depolymerization at pH 7.0 (*35*−*37*, *39*−*41*). In parallel, the effects of whole normal human serum, uremic serum, and synovial fluid were also assessed (see Materials and Methods). Control experiments included analysis of the effect of sulfate ions and poly(ethylene glycol) (PEG) so that the specific effect of the biological factors over the physical or chemical properties of the additives could be discerned. In total more than 400 assays were performed in two parallel experiments. The results were highly reproducible both within and between experiments (Figure 7 and data not shown).

Consistent with the results above, all experiments in which monomeric β_2m was incubated in the absence of seeds, both in the presence or absence of additive, resulted in no significant increase in ThT signal (Figure 7). Likewise, incubation of monomer in the presence of unstabilized seeds, sulfate anions, or PEG also failed to produce a significantly enhanced ThT signal (Figure 7 and data not shown). By contrast, however, incubation of monomeric β_2m with seeds incubated in the presence of a number of physiologically relevant additives produced a substantial increase in ThT fluorescence (Figure 7). Thus, a significant enhancement in ThT fluorescence was observed in the presence of SAP, pure apoE3, or a mixture of lipid-bound apoE isotypes, the latter experiments demonstrating the increased effectiveness of lipid-bound apoE (Figure 7). Control experiments demonstrated that the effect of SAP is dependent on the presence of Ca^{2+} and on the concentration of SAP added, in line with its known calcium-dependent ligand binding properties (*22*). Addition of type II collagen also had a small effect on promoting fibrillogenesis of wild-type β_2m under the conditions used. In all cases, the fibrils formed showed amyloid-like characteristics, as judged by EM imaging and antibody WO1 binding (data not shown).

Since all of the physiologically relevant factors tested above increased the yield of fibrils (albeit to different extents)

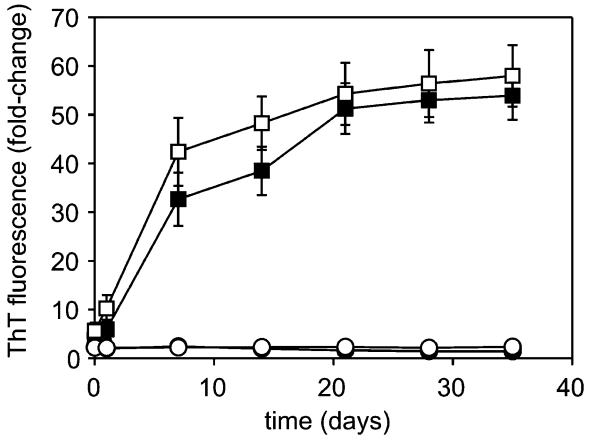

FIGURE 8: Time course of fibril formation in the presence of combined biological factors, monitored by ThT fluorescence. Seeds incubated in the presence of mix A (containing heparin, apoE and SAP) (open symbols) and seeds incubated with mix A plus collagen (closed symbols) (see Materials and Methods). Seeds alone (circles) and seeds elongated with wild-type β_2m (squares).

when seeds were extended with wild-type β_2m, different reagents were then combined to determine whether the effect of the different components was synergistic or competitive. Thus, seeds were formed in which amyloid fibrils of β_2m made at pH 2.5 were stabilized with lipidated apoE, SAP, and heparin (mix A) in the presence or absence of collagen prior to their fragmentation, and these were used in turn to seed elongation of monomeric wild-type β_2m (Figure 7). In parallel, the effect of these seeds in promoting fibril elongation was compared with seeds incubated with either whole normal human serum, uremic serum, or synovial fluid, or by using an amyloidotic tissue homogenate rich in β_2m amyloid fibrils as seed (see Materials and Methods) (Figure 7). The results showed that incubation of wild-type β_2m with a combination of biological factors resulted in a higher ThT signal compared with that obtained with each component alone. The results suggest, therefore, that the different biological factors may have distinct, specific binding sites on the fibril seeds. On the other hand, the concentration of agent added may have been insufficient to saturate a common binding site, as shown in the case of heparin (compare Figures 3B and 4B). Seeds incubated with uremic serum or synovial fluid were also able to promote fibril formation at pH 7.0, while normal human serum was ineffective in facilitating fibrillogenesis (Figure 7). In addition to an enhanced increase in ThT fluorescence, seeds stabilized by multiple factors had a significant effect on the kinetics of fibril assembly, to the extent that the 2−3 week lag period typically observed in fibril elongation using seeds stabilized by heparin was no longer observed (compare Figures 2 and 8). Together, these results suggest that different biological factors may act in concert to facilitate elongation at neutral pH, therefore promoting the joint-specific deposition of β_2m amyloid fibrils in vivo.

DISCUSSION

The Role of Biological Factors in Promoting β_2m Fibrillogenesis at Neutral pH in Vitro. Despite the known ability of β_2m to self-assemble spontaneously into amyloid-like fibrils with high yield (>95%) at acidic pH in vitro (*9*, *12*, *42*−*44*), the mechanism(s) by which β_2m forms amyloid fibrils under physiologically relevant conditions remains obscure. Previous attempts to form amyloid-like fibrils from wild-type β_2m in vitro at neutral pH have demonstrated that the native protein is highly intransigent to self-assembly at this pH, even at concentrations of up to 1000-times higher than that found in DRA patients undergoing dialysis for end-stage renal failure (*42*). Despite lacking several of the protective features often found in all-β-sheet proteins to prevent intermolecular assembly through edge-strand association (*45*), the native β-sandwich of monomeric β_2m is apparently unable to nucleate fibril assembly in vitro. Akin to the behavior of other amyloidogenic proteins (see (*46*−*48*) and (*49*, *50*) for recent reviews), transient unfolding of β_2m is required to initiate fibrillogenesis, presumably by the exposure of new assembly competent sites. For β_2m this may be accomplished by mild acidification of the solution which results in unfolding of the N- and C-terminal regions of the polypeptide chain, while the central region involving residues 25−80 remains stably structured (*51*). Alternatively, more highly unfolded states may initiate fibrillogenesis (*52*, *53*). Fibrils of β_2m have been produced in unseeded reactions in vitro at pH 7.0 by incubating the protein with Cu^{2+} ions in the presence of 1 M urea (*54*), dialysis of β_2m into low ionic strength buffer followed by drying of the protein onto a membrane surface (*55*), mutation of residues in the N- or C-terminal regions of the protein (*14*) or, as we demonstrate here, by deletion of the N-terminal six residues. These results suggest that transient local unfolding of the N- and/or C-terminal region is a critical first step in the nucleation of fibril formation for this protein, while decreasing the global stability (as in the variant V37A) has no effect. By contrast with other proteins for which amyloidogenicity has been shown to correlate with global destabilization of the native protein (*56*−*59*), there is no relationship between global stability and fibril formation in the case of β_2m (*10*, 60). Instead, partially folded species that lack one or more of the native edge β-strands appear to be required to nucleate fibril assembly.

Despite the difficulties of nucleating fibril formation from β_2m in vitro at neutral pH, a number of reports have recently described conditions under which wild-type β_2m is able to form fibrils by the elongation of fibrillar seeds at this pH, hence by-passing the need for nucleation. Initial studies utilized amyloid fibrils extracted ex vivo from amyloidotic tissues of DRA patients that are stable at neutral pH (presumably as a consequence of the binding of apoE, SAP, GAGs, PGs, and/or other factors) and capable of seeding assembly (*9*, *61*). More recent experiments have shown that seeds formed at pH 2.5 in vitro that are stabilized by the addition of GAGs or PGs are also capable of seeding elongation with wild-type β_2m at acidic pH, while fibril elongation at neutral pH in the presence of these additives is only possible in the presence of TFE or SDS (*15*, *36*, *41*, *62*). Here, we have demonstrated that fibril seeds incubated with SAP, lipidated or lipid-free apoE, collagen, synovial fluid, or uremic serum are able to seed fibril formation of β_2m at pH 7.0 in the absence of organic solvent or detergent. We have also demonstrated that these physiologically relevant factors may act in concert with heparin, generating fibrils in vitro that display morphological, tinctorial, and protein−ligand binding characteristics of amyloid fibrils ex vivo. The ability of synovial fluid to stabilize seeds at neutral

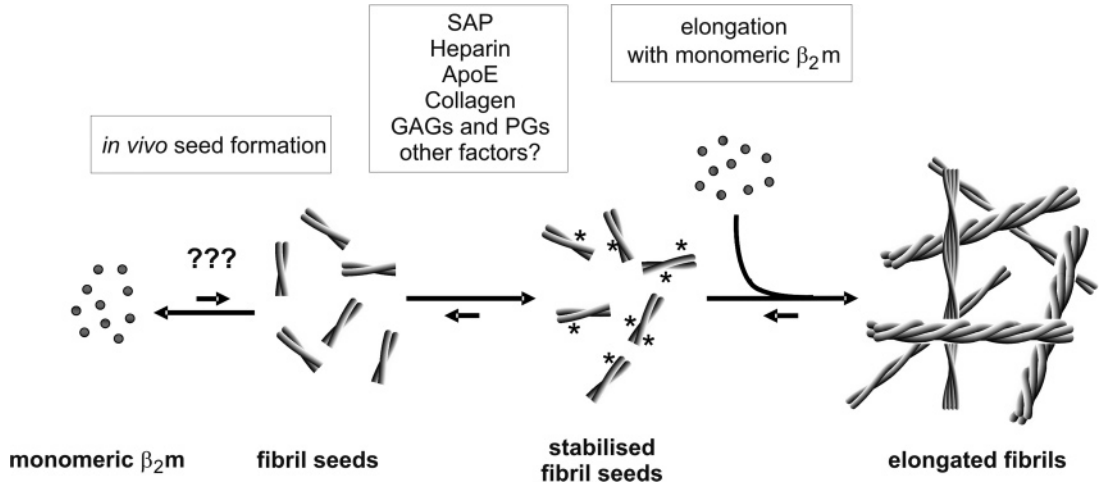

FIGURE 9: Schematic diagram showing the potential influence of different biological factors on in vivo amyloid fibril formation from wild-type β_2m at neutral pH. The diagram highlights the central importance of biological factors in β_2m amyloidogenesis, by their ability to bind and stabilize seeds, providing a surface for elongation by rarely populated assembly competent species. How the seeds are initially formed in vivo remains unknown.

pH and to promote fibril formation is consistent with the synovium being the site for β_2m amyloid deposition in vivo. Furthermore, the observation that uremic serum promotes fibril elongation, while normal serum does not, indicates that dialysis-associated factors may also enhance fibril formation. Given the difficulty in comparing the concentration of individual components added with those in biological fluids, the efficiency of the individual compounds and the biological fluids cannot be directly compared. By contrast with the very rapid elongation of fibrils under acidic conditions (*9*, *12*), however, fibril elongation at neutral pH occurs more slowly, taking weeks to reach completion, even at the high concentrations of monomer used here. This suggests that the slow rate of elongation is limited by the population of assembly-competent species formed by transient local unfolding of the native protein. At least for the proteins studied here, such species must be rare (see also (*63*)).

The Mechanism of Fibrillogenesis of β_2m at Neutral pH. The results presented above can be summarized by the general scheme shown in Figure 9. Thus, monomeric β_2m and small oligomeric species that are populated in solution are unable to self-assemble into amyloid-like fibrils at neutral pH, presumably since the concentration of assembly-competent species is too low to initiate fibrillogenesis. In addition, in the absence of stabilizing agents, elongation of any seeds that do form will be unlikely to occur, since these species will rapidly depolymerize, regenerating monomers and smaller oligomeric forms. By contrast, in the presence of SAP, GAGs, PGs, apoE or collagen, or other physiologically relevant factors present in vivo, seeds are stabilized, providing a surface onto which assembly-competent molecules can add. Further binding of factors onto the growing fibril then provides additional stabilization, effectively driving fibril formation to completion. Our results demonstrate that a stable seed is more critical to fibril formation from β_2m at neutral pH than the stability of the monomeric protein, since wild-type protein and V37A are similarly able to form fibrils in the presence of such seeds. In addition, the results rationalize the inability of V37A to form fibrils in the absence of seeds, as well as the enhanced amyloidogenicity of ΔN6 in the absence of seeds (Figure 1B and (30)) since these species presumably populate assembly competent state(s) to different extents.

Implications for DRA. Despite the increasing knowledge of the mechanisms of β_2m amyloid formation in vitro, key questions remain unresolved, most importantly how and where nuclei are formed and why β_2m amyloid specifically deposits in joints. The environment within the synovium is ideal for β_2m fibrillogenesis, since synovial fluid is rich in GAGs that could stabilize fibrils or fibrillar seeds once formed. The joint cartilage is constructed from a matrix of type II collagen, GAGs, and aggrecan (*64*), factors known to influence fibrillogenesis of many proteins, including tau, prions, and α-synuclein in vitro (*17*, *18*) and which also enhance seed elongation with wild-type β_2m. Circulating SAP may also enhance fibril stability and thereby facilitate elongation. The removal of the six N-terminal residues of β_2m promotes de novo fibril formation in the absence of protein seeds and provides one possible source of the first fibril seeds that could initiate the amyloid cascade, whereas other, currently unidentified, reagents within the synovium or uremic serum that bind to and specifically destabilize the N- and/or C-terminal regions of the polypeptide chain in vivo could also facilitate the initiation of fibrillogenesis. Our experimental findings provide the first insight into the effect of additives on β_2m amyloid fibril formation at neutral pH in the absence of chemical denaturants, detergents, or cosolvents and will allow further systematic analysis of the effect of additives that enhance this process as well as the search for small molecules that inhibit fibrillogenesis. Furthermore, our results suggest several strategies for therapeutic intervention in DRA, including the prevention of cofactor binding to early aggregated states or fully assembled fibrils, or by preventing the local unfolding of the N- and C-terminal strands to reduce the probability of seed elongation. While low molecular mass drugs that inhibit binding of SAP or GAGs to amyloid fibrils are currently undergoing clinical trials in patients with amyloidosis that may result in a generic therapeutic for many, if not all, amyloid diseases (*28*, *65*), the development of molecules that specifically suppress local fluctuations of the polypeptide chain or prevent nucleation remains a significant challenge.

ACKNOWLEDGMENT

We thank Alison Ashcroft for performing ESI-MS and members of the S.E.R. group, particularly Toni Borysik, for helpful discussions. We thank Professor M. B. Pepys FRS (Centre for Amyloidosis and Acute Phase Proteins, Royal Free and University College Medical School, London) for the supply of SAP and DRA tissue. We also thank Ron Wetzel (Graduate School of Medicine, University of Tennessee) for WO1 antibody and Charlie Glabe (University of California, Irvine) for anti-oligomer antibody and Aβ oligomers, Fiona Karet (Cambridge Institute for Medical Research) for providing human uremic serum, Eileen Ingham (University of Leeds) for providing porcine synovial fluid, and John Fryer and David Holtzman (Washington University, School of Medicine) for kindly providing lipidated apoE. S.E.R. is a BBSRC Professorial Fellow.

SUPPORTING INFORMATION AVAILABLE

Binding of ^{125}I-SAP to β_2m and control amyloid fibrils. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Westermark, P., Benson, M. D., Buxbaum, J. N., Cohen, A. S., Frangione, B., Ikeda, S., Masters, C. L., Merlini, G., Saraiva, M. J., and Sipe, J. D. (2005) Amyloid: toward terminology clarification. Report from the Nomenclature Committee of the International Society of Amyloidosis. *Amyloid 12*, 1−4.
2. Floege, J., and Ketteler, M. (2001) Beta-2-microglobulin derived amyloidosis: an update. *Kidney Int. 59*, 164−171.
3. Inoue, S., Kuroiwa, M., Ohashi, K., Hara, M., and Kisilevsky, R. (1997) Ultrastructural organization of hemodialysis-associated beta-2-microglobulin amyloid fibrils. *Kidney Int. 52*, 1543−1549.
4. Miyata, T., Jadoul, M., Kurokawa, K., and Van Ypersele de Strihou, C. (1998) Beta-2-microglobulin in renal disease. *J. Am. Soc. Nephrol. 9*, 1723−1735.
5. Floege, J., and Ehlerding, G. (1996) Beta-2-microglobulin associated amyloidosis. *Nephron 72*, 9−26.
6. Gejyo, F., Yamada, T., Odani, S., Nakagawa, Y., Arakawa, M., Kunitomo, T., Kataoka, H., Suzuki, M., Hirasawa, Y., Shirahama, T., Cohen, A. S., and Schmid, K. (1985) A new form of amyloid protein associated with chronic hemodialysis was identified as beta-2-microglobulin. *Biochem. Biophys. Res. Commun. 129*, 701−706.
7. Gorevic, P. D., Munoz, P. C., Casey, T. T., DiRaimondo, C. R., Stone, W. J., Prelli, F. C., Rodrigues, M. M., Poulik, M. D., and Frangione, B. (1986) Polymerization of intact beta-2-microglobulin in tissue causes amyloidosis in patients on chronic hemodialysis. *Proc. Natl. Acad. Sci. U.S.A. 83*, 7908−7912.
8. Jahn, T. R., and Radford, S. E. (2005) Beta-2-microglobulin. in *Amyloid Proteins: The Beta Sheet Conformation and Diseases*, (Sipe, J. D., Ed.) Wiley-VCH, Weinheim.
9. Naiki, H., Hashimoto, N., Suzuki, S., Kimura, H., Nakakuki, K., and Gejyo, F. (1997) Establishment of a kinetic model of dialysis-related amyloid fibril extension *in vitro*. *Amyloid Int. J. Exp. Clin. Invest. 4*, 223−232.
10. Smith, D. P., Jones, S., Serpell, L. C., Sunde, M., and Radford, S. E. (2003) A systematic investigation into the effect of protein destabilisation on beta-2-microglobulin amyloid formation. *J. Mol. Biol. 330*, 943−954.
11. Gosal, W. S., Morten, I. J., Hewitt, E. W., Smith, D. A., Thomson, N. H., and Radford, S. E. (2005) Competing pathways determine fibril morphology in the self-assembly of beta-2-microglobulin into amyloid. *J. Mol. Biol. 351*, 850−864.
12. Kad, N. M., Thomson, N. H., Smith, D. P., Smith, D. A., and Radford, S. E. (2001) Beta-2-microglobulin and its deamidated variant, N17D form amyloid fibrils with a range of morphologies *in vitro*. *J. Mol. Biol. 313*, 559−571.
13. Morgan, C. J., Gelfand, M., Atreya, C., and Miranker, A. D. (2001) Kidney dialysis-associated amyloidosis: a molecular role for copper in fibre formation. *J. Mol. Biol. 309*, 339−345.
14. Jones, S., Smith, D. P., and Radford, S. E. (2003) Destabilisation in the N- and C-terminal strands of beta-2-microglobulin results in amyloid formation at neutral pH. *J. Mol. Biol. 330*, 935−941.
15. Yamamoto, S., Yamaguchi, I., Hasegawa, K., Tsutsumi, S., Goto, Y., Gejyo, F., and Naiki, H. (2004) Glycosaminoglycans enhance the trifluoroethanol-induced extension of beta-2-microglobulin related amyloid fibrils at neutral pH. *J. Am. Soc. Nephrol. 15*, 126−133.
16. Yamamoto, S., Hasegawa, K., Yamaguchi, I., Tsutsumi, S., Kardos, J., Goto, Y., Gejyo, F., and Naiki, H. (2004) Low concentrations of sodium dodecyl sulphate induce the extension of beta-2-microglobulin related amyloid fibrils at neutral pH. *Biochemistry 43*, 11075−11082.
17. Alexandrescu, A. T. (2005) Amyloid accomplices and enforcers. *Protein Sci. 14*, 1−12.
18. van Horssen, J., Wesseling, P., van den Heuvel, L. P., de Waal, R. M., and Verbeek, M. M. (2003) Heparan sulphate proteoglycans in Alzheimer's disease and amyloid-related disorders. *Lancet Neurol. 2*, 482−492.
19. Linke, R. P., Hampl, H., Bartel-Schwarze, S., and Eulitz, M. (1987) Beta-2-microglobulin, different fragments and polymers thereof in synovial amyloid in long-term hemodialysis. *Biol. Chem. Hoppe-Seyler 368*, 137−144.
20. Stoppini, M., Mangione, P., Monti, M., Giorgetti, S., Marchese, L., Arcidiaco, P., Verga, L., Segagni, S., Pucci, P., Merlini, G., and Bellotti, V. (2005) Proteomics of beta-2-microglobulin amyloid fibrils. *Biochim. Biophys. Acta 1751*, 23−33.
21. Gill, S., and Von Hippel, P. H. (1989) Calculation of protein extinction coeffients from amino acid sequence data. *Anal. Biochem. 182*, 319−326.
22. Pepys, M. B., Booth, D. R., Hutchinson, W. L., Gallimore, J. R., Collins, P. M., and Hohenester, E. (1997) Amyloid P component. A critical review. *Amyloid: Int. J. Exp. Clin. Invest. 4*, 274−295.
23. Friel, C. T., Beddard, G. S., and Radford, S. E. (2004) Switching two-state to three-state kinetics in the helical protein Im9 via the optimization of stabilizing non-native interactions by design. *J. Mol. Biol. 342*, 261−273.
24. Nilsson, M. R. (2004) Techniques to study amyloid fibril formation in vitro. *Methods 34*, 151−160.
25. Tennent, G. A. (1999) Isolation and characterization of amyloid fibrils from tissue. *Methods Enzymol. 309*, 26−47.
26. Kayed, R. H. E., Thompson, J. L., McIntire, T. M., Milton, S. C., Cotman, C. W., and Glabe, C. G. (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science 300*, 486−489.
27. Esposito, G., Michelutti, R., Verdone, G., Viglino, P., Hernandez, H., Robinson, C. V., Amoresano, A., Dal Piaz, F., Monti, M., Pucci, P., Mangione, P., Stoppini, M., Merlini, G., Ferri, G., and Bellotti, V. (2000) Removal of the N-terminal hexapeptide from human beta-2-microglobulin facilitates protein aggregation and fibril formation. *Protein Sci. 9*, 831−845.
28. Kisilevsky, R., Szarek, W. A., Ancsin, J., Vohra, R., Li, Z., and Marone, S. (2004) Novel glycosaminoglycan precursors as anti-amyloid agents: Part IV. *J. Mol. Neurosci. 24*, 167−172.
29. Nishi, S., Ogino, S., Maruyama, Y., Honma, N., Gejyo, F., Morita, T., and Arakawa, M. (1990) Electron-microscopic and immunohistochemical study of beta-2-microglobulin-related amyloidosis. *Nephron 56*, 357−363.
30. Yamaguchi, I., Suda, H., Tsuzuike, N., Seto, K., Seki, M., Yamaguchi, Y., Hasegawa, K., Takahashi, N., Yamamotot, S., Gejyo, F., and Naiki, H. (2003) Glycosaminoglycan and proteoglycan inhibit the deoplymerisation of beta-2-microgloulin amyloid fibrils in vitro. *Kidney Int. 64*, 1080−1088.
31. Webb, A. R., Mythen, M. G., Jacobson, D., and Mackie, I. J. (1995) Maintaining blood flow in the extracorporeal circuit: hemostasis and anticoagulation. *Intensive Care Med. 21*, 84−93.
32. Klunk, W. E., Jacob, R. F., and Mason, R. P. (1999) Quantifying amyloid beta-peptide (A-beta) aggregation using the Congo red A-beta (CR-A beta) spectrophotometric assay. *Anal. Biochem. 266*, 66−76.
33. O'Nuallain, B., and Wetzel, R. (2002) Conformational Abs recognizing a generic amyloid fibril epitope. *Proc. Natl. Acad. Sci. U.S.A. 99*, 1485−1490.
34. Ohashi, Y., Kisilevsky, R., and Yanagishita, M. (2002) Affinity binding of glycosaminoglycans with beta-2-microglobulin. *Nephron 90*, 158−168.
35. Tan, S., Baillod, R., Brown, E., Farrington, K., Soper, C., Percy, M., Clutterbuck, E., Madhoo, S., Pepys, M., and Hawkins, P.

(1999) Clinical, radiological and serum amyloid P component scintigraphic features of beta-2-microglobulin amylidosis associated with continuous ambulatory peritoneal dialysis. *Nephrol. Dial. Transplant. 14*, 1467−1471.
36. Yamaguchi, I., Hasegawa, K., Takahashi, N., Yamamoto, S., Gejyo, F., and Naiki, H. (2001) Apolipoprotein E inhibits the depolymerisation of beta-2-microglobulin-related amyloid fibrils at a neutral pH. *Biochemistry 40*, 8499−8507.
37. Homma, N., Gejyo, F., Isemura, M., and Arakawa, M. (1989) Collagen-binding affinity of beta-2-microglobulin, a preprotein of hemodialysis-associated amyloidosis. *Nephron 53*, 37−40.
38. Moe, S. M., and Chen, N. X. (2001) The role of the synovium and cartilage in the pathogenesis of beta-2-microglobulin amyloidosis. *Semin. Dial. 14*, 127−130.
39. Giorgetti, S., Rossi, A., Mangione, P., Raimondi, S., Marini, S., Stoppini, M., Corazza, A., Viglino, P., Esposito, G., Cetta, G., Merlini, G., and Bellotti, V. (2005) Beta-2-microglobulin isoforms display an heterogeneous affinity for type I collagen. *Protein Sci. 14*, 1−7.
40. Gejyo, F., Suzuki, S., Kimura, H., Imura, T., Ei, I., Hasegawa, H., and Arakawa, M. (1997) Increased risk of dialysis-related amyloidosis in patients with the apolipoprotein E4 allele. *Amyloid Int. J. Exp. Clin. Invest. 4*, 13−17.
41. Ono, K., and Uchino, F. (1994) Formation of amyloid-like substance from beta-2-microglobulin in vitro. Role of serum amyloid P component: a preliminary study. *Nephron 66*, 404−407.
42. McParland, V. J., Kad, N. M., Kalverda, A. P., Brown, A., Kirwin-Jones, P., Hunter, M. G., Sunde, M., and Radford, S. E. (2000) Partially unfolded states of beta-2-microglobulin and amyloid formation in vitro. *Biochemistry 39*, 8735−8746.
43. Yamaguchi, K., Katou, H., Hoshino, M., Hasegawa, K., Naiki, H., and Goto, Y. (2004) Core and heterogeneity of beta-2-microglobulin amyloid fibrils as revealed by H/D exchange. *J. Mol. Biol. 338*, 559−571.
44. Ivanova, M. I., Sawaya, M. R., Gingery, M., Attinger, A., and Eisenberg, D. (2004) An amyloid-forming segment of beta-2-microglobulin suggests a molecular model for the fibril. *Proc. Natl. Acad. Sci. U.S.A. 101*, 10584−10589.
45. Richardson, J. S., and Richardson, D. C. (2002) Natural beta-sheet proteins use negative design to avoid edge-to-edge aggregation. *Proc. Natl. Acad. Sci. U.S.A. 99*, 2754−2759.
46. Litvinovich, S. V., Brew, S. A., Aota, S., Akiyama, S. K., Haudenschild, C., and Ingham, K. C. (1998) Formation of amyloid-like fibrils by self-association of a partially unfolded fibronectin type III module. *J. Mol. Biol. 280*, 245−258.
47. Canet, D., Sunde, M., Last, A. M., Miranker, A., Spencer, A., Robinson, C. V., and Dobson, C. M. (1999) Mechanistic studies of the folding of human lysozyme and the origin of amyloidogenic behavior in its disease-related variants. *Biochemistry 38*, 6419−6427.
48. Khurana, R., Gillespie, J. R., Talapatra, A., Minert, L. J., Ionescu-Zanetti, C., Millett, I., and Fink, A. L. (2001) Partially folded intermediates as critical precursors of light chain amyloid fibrils and amorphous aggregates. *Biochemistry 40*, 3525−3535.
49. Ohnishi, S., and Takano, K. (2004) Amyloid fibrils from the viewpoint of protein folding. *Cell. Mol. Life. Sci. 61*, 511−524.
50. Uversky, V. N., and Fink, A. L. (2004) Conformational constraints for amyloid fibrillation: the importance of being unfolded. *Biochim. Biophys. Acta 1698*, 131−53.
51. McParland, V. J., Kalverda, A. P., Homans, S. W., and Radford, S. E. (2002) Structural properties of an amyloid precursor of beta-2-microglobulin. *Nat. Struct. Biol. 9*, 326−331.
52. Platt, G. W., McParland, V. J., Kalverda, A. P., Homans, S. W., and Radford, S. E. (2005) Dynamics in the unfolded state of beta-2-microglobulin studied by NMR. *J. Mol. Biol. 346*, 279−294.
53. Katou, H., Kanno, T., Hoshino, M., Hagihara, Y., Tanaka, H., Kawai, T., Hasegawa, K., Naiki, H., and Goto, Y. (2002) The role of disulfide bond in the amyloidogenic state of beta-2-microglobulin studied by heteronuclear NMR. *Protein Sci. 11*, 2218−2229.
54. Eakin, C. M., Knight, J. D., Morgan, C. J., Gelfand, M. A., and Miranker, A. D. (2002) Formation of a copper specific binding site in non-native states of beta-2-microglobulin. *Biochemistry 41*, 10646−10656.
55. Connors, L. H., Shirahama, T., Skinner, M., Fenves, A., and Cohen, A. S. (1985) In vitro formation of amyloid fibrils from intact beta-2-microglobulin. *Biochem. Biophys. Res. Commun. 131*, 1063−1068.
56. Colon, W., and Kelly, J. W. (1992) Partial denaturation of transthyretin is sufficient for amyloid fibril formation in vitro. *Biochemistry 31*, 8654−8660.
57. Lai, Z. H., Colon, W., and Kelly, J. W. (1996) The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. *Biochemistry 35*, 6470−6482.
58. Hurle, M. R., Helms, L. R., Li, L., Chan, W. N., and Wetzel, R. (1994) A role for destabilizing amino acid replacements in light-chain amyloidosis. *Proc. Natl. Acad. Sci. U.S.A. 91*, 5446−5450.
59. Nielsen, L., Frokjaer, S., Brange, J., Uversky, V. N., and Fink, A. L. (2001) Probing the mechanism of insulin fibril formation with insulin mutants. *Biochemistry 40*, 8397−8409.
60. Chiba, T., Hagihara, Y., Higurashi, T., Hasegawa, K., Naiki, H., and Goto, Y. (2003) Amyloid fibril formation in the context of full-length protein. *J. Biol. Chem. 278*, 47016−47024.
61. Chiti, F., De Lorenzi, E., Grossi, S., Mangione, P., Giorgetti, S., Caccialanza, G., Dobson, C. M., Merlini, G., Ramponi, G., and Bellotti, V. (2001) A partially structured species of beta-2-microglobulin is significantly populated under physiological conditions and involved in fibrillogenesis. *J. Biol. Chem. 276*, 46714−46721.
62. Gejyo, F., Kimura, H., Suzuki, S., Miyazaki, R., Naiki, H., and Nakakuki, K. (1997) Apolipoprotein E and alpha (1)-antichymotrypsin in dialysis-related amyloidosis. *Kidney Int.g 52*, S75−S78.
63. Jahn, T. R., Parker, M. J., Homans, S. W., and Radford, S. E. (2006) Amyloid Formation under physiological conditions proceeds via a nativelike folding intermediate. *Nature Struct. Mol. Biol*. In press.
64. Morten, I. J., Hewitt, E. W., and Radford, S. E. (2005) Beta-2-microglobulin and dialysis-related amyloidosis. In *Protein Misfolding, aggregation and conformational diseases*, (Uversky, V. N. and Fink, A. L., Eds.) Kluwer Academic/Plenum Publishers, Dordrecht, The Netherlands.
65. Pepys, M. B., Herbert, J., Hutchinson, W. L., Tennent, G. A., Lachmann, H. J., Gallimore, J. R., Lovat, L. B., Bartfai, T., Alanine, A., Hertel, C., Hoffmann, T., Jakob-Roetne, R., Norcross, R. D., Kemp, J. A., Yamamura, K., Suzuki, M., Taylor, G. W., Murray, S., Thompson, D., Purvis, A., Kolstoe, S., Wood, S. P., and Hawkins, P. N. (2002) Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis. *Nature 417*, 254−259.
66. Verdone, G., Corazza, A., Viglino, P., Pettirossi, F., Giorgetti, S., Mangione, P., Andreola, A., Stoppini, M., Bellotti, V., and Esposito, G. (2002) The solution structure of human beta-2-microglobulin reveals the prodromes of its amyloid transition. *Protein Sci. 11*, 487−499.
67. DeLano, W. (2002) *The PyMOL Molecular Graphics System*. DeLano Scientific, San Carlos, CA.

BI052434I