Collagen Plays an Active Role in the Aggregation of β2-Microglobulin under Physio-Pathological Conditions of Dialysis-Related Amyloidosis*

Annalisa Relini‡1, Claudio Canale‡, Silvia De Stefano‡, Ranieri Rolandi‡, Sofia Giorgetti§, Monica Stoppini§, Antonio Rossi§, Federico Fogolari¶, Alessandra Corazza¶, Gennaro Esposito¶, Alessandra Gliozzi‡, Vittorio Bellotti§

From the ‡Department of Physics, University of Genoa, I-16146 Genoa, Italy, the §Department of Biochemistry, University of Pavia - Laboratori di Biotecnologie IRCCS Policlinico San Matteo - 27100 Pavia, Italy, and the ¶Department of Biomedical Sciences and Technologies, University of Udine, I-33100 Udine, Italy

Running title: Role of Collagen in the Aggregation of β2-Microglobulin

1To whom correspondence should be addressed: Department of Physics, University of Genoa, Via Dodecaneso 33, I-16146 Genova, Italy; tel. +39-010-3536427, fax +39-010-314218, e-mail relini@mail.fisica.unige.it

Dialysis-related amyloidosis (DRA) is characterized by the deposition of insoluble fibrils of β2-microglobulin (β2-m) in the musculo-skeletal system. The AFM inspection of ex-vivo amyloid material reveals the presence of bundles of fibrils often associated to collagen fibrils. Aggregation experiments were undertaken in vitro with the aim of reproducing the physio-pathological fibrillation process. To this purpose, atomic force microscopy, fluorescence techniques and NMR were employed. We found that in temperature and pH conditions similar to those occurring in peri-articular tissues in the presence of flogistic processes, β2-m fibrillogenesis takes place in the presence of fibrillar collagen, while no fibrils are obtained without collagen. Moreover, the morphology of β2-m fibrils obtained in vitro in the presence of collagen is extremely similar to that observed in the ex-vivo sample. This result indicates that collagen plays a crucial role in β2-m amyloid deposition under physio-pathological conditions and suggests an explanation to the strict specificity of DRA for the tissues of the skeletal system. We hypothesize that positively charged regions along the collagen fibre could play a direct role in β2-m fibrillogenesis. This hypothesis is sustained by aggregation experiments performed by replacing collagen with a poly-L-lysine-coated mica surface. As shown by NMR measurements, no similar process occurs when poly-L-lysine is dissolved in solution with β2-m. Overall the findings are consistent with the estimates resulting from a simplified collagen model whereby electrostatic effects can lead to high local concentrations of oppositely charged species, such as β2-m, that decay on moving away from the fibre surface.

The deposition of β2-microglobulin (β2-m) into amyloid fibrils is the hallmark of dialysis-related amyloidosis (DRA), a disease arising as a complication of long term haemodialysis. β2-m is a 99-residue protein (MW 11.7kDa) that represents the light chain of the major histocompatibility complex class I (MHCI), an integral membrane protein involved in the immune response. As a result of normal MHCI catabolism, β2-m is released in the serum from the cell surface and carried to the kidney for clearance. In the presence of kidney failure, the concentration of free circulating β2-m can increase by up to 50-fold; the persistent increase in β2-m concentration results in amyloid deposition, preferentially localized in the musculo-skeletal system. The accumulation of β2-m deposits has been shown to cause arthralgias, destructive osteoarthropathies, and carpal tunnel syndrome (1). Although a high concentration of β2-m is a necessary condition for the onset of the disease, there is not a strict correlation between the disease severity and β2-m levels (2), suggesting that other factors might be involved in β2-m amyloid deposition.
The aggregation process of β2-m has been the object of extensive investigation since many years. Several experiments have been designed to reproduce β2-m amyloid deposition in vitro and understand the molecular conformational changes involved in this process. Spontaneous assembly of β2-m amyloid fibrils was observed in vitro under acidic conditions hardly compatible with the physiologic ones; in this case the morphology of in vitro fibrils is influenced by pH and ionic strength (3,4). It has been reported that, at neutral pH, β2-m can form fibrils in the presence of high concentrations of free copper ions (5). Furthermore, at neutral pH, β2-m can elongate preformed fibrils if the solution contains trifluoroethanol (6), sodium dodecyl sulfate (7) or when the protein populates an intermediate state of the folding pathway (8). The ability of β2-m to elongate preformed fibrils at neutral pH is also enhanced by truncation at the N-terminus (9) as well as by the cleavage of the peptide bond at the carbonyl side of Lys 58 (10).

Although the information resulting from these in vitro experiments can contribute to get insight into the aggregation process of β2-m, it cannot be directly related to in vivo fibrillogenesis. A further step towards the understanding of β2-m fibrillation in vivo is required, by designing aggregation conditions as close as possible to the physio-pathological ones. One of the most surprising properties of DRA is its strict specificity for tissues of the skeletal system, in spite of the fact that β2-m is ubiquitously released throughout the body from every cell expressing the MHCI complex. In all patients, in fact, bones and ligaments are primarily involved. Therefore, it might be speculated that the molecular environment that β2-m encounters in the skeletal system might favour the local protein aggregation and formation of amyloid fibrils.

Two macromolecules highly represented in these tissues, glycosaminoglycans (GAGs) and collagen, have been investigated as a putative target of β2-m deposition. Ohashi et al. (11) have found that β2-m binds GAGs with a K_d of approximately 1x10^-5 and our group has shown that β2-m binds collagen giving an adduct with similar affinity, while with the truncated β2-m form lacking six amino acids at the N-terminus the collagen adduct exhibits a ten fold higher affinity (12).

In this work we have explored the fibrillogenesis of β2-m under physio-pathological conditions by atomic force microscopy, which is one of the most sensitive and specific techniques currently available to study the amyloid aggregate morphology (13-15). We started from the inspection of ex-vivo material, then we settled a procedure for monitoring by AFM the process of β2-m aggregation in vitro in conditions as close as possible to the physio-pathological ones. Thioflavin T assay was employed to check fibril formation in solution, while NMR spectroscopy was used to monitor possible changes of the protein structure in solution. Our experiments reveal a strict association between β2-m fibrils and collagen fibrils and suggest that collagen might act as a catalyst for fibril formation as a consequence of its positive surface charge. To test this hypothesis, we performed aggregation experiments in the presence of artificial positively charged surfaces. Finally, a simplified model of the collagen fibre was developed to analyze direct and/or orientational electrostatic effects which could lead to an accumulation of β2-m at the charged interface.

**EXPERIMENTAL PROCEDURES**

*Extraction and purification of ex-vivo β2-m amyloid material* — Amyloid fibrils were extracted from amyloid deposits isolated from the femoral head of a patient submitted to hip replacement surgery. The material was homogenized in 2 ml of 10 mM Tris/EDTA pH 8.0, containing 1.5 M phenylmethylsulfonyl fluoride (PhMeSO2F)/100 mg of tissue and centrifuged at 50,000 g in an ultracentrifuge (Beckman L8-704; Beckman Instruments) for 30 min and the supernatant removed. After this step was repeated nine times, the absorbance measurement at 280 nm was less than 0.05, the pellet was then homogenized in water in the presence of 1.5 M PhMeSO2F following the procedure of Gejyo et al. (16) and six aqueous fractions were obtained. The yield in fibrils was monitored by microscopic analysis of the extracted material stained with Congo Red.

*Expression and purification of recombinant β2-m* — Expression and purification of recombinant β2-m and ΔN6β2-m was carried out as previously reported (9). The concentration of the protein sample was determined spectrophotometrically at 280 nm by using an extinction coefficient (A 1cm%) of 16.17 for β2-m and 17.22 for ΔN6β2-m.
Preparation of type I fibrillar collagen — Type I collagen was purified from calf skin as previously described (12). The purity of collagen was checked by SDS-PAGE and the concentration of the collagen solutions were determined by the hydroxyproline assay according to Huzar et al (17). Fibrillar collagen was prepared by solubilizing purified collagen in 5mM acetic acid. The solution was diluted 1:1 with PBS 2X and incubated at 37°C for 30'.

Thioflavin T assay — Quantification of amyloid fibril formation was performed with the method described by LeVine (18). Thioflavin T (ThT) concentration was 5 mM and the buffer used was 50 mM glycine NaOH, pH 8.5. Measurements were made using a LS50 Perkin Elmer spectrofluorometer with excitation at 455 nm; emission was collected at 485 nm. The slits were set at 5 nm.

Congo red birefringence — Congo red birefringence of β2-m fibrils grown in the presence of collagen was assayed as described previously (19).

NMR spectroscopy — 1H NMR spectra were obtained at 500.13 MHz with a Bruker Avance spectrometer on 0.1 - 0.63 mM solutions of either pure β2-m or mixed with of poly-L-lysine (hydrobromide, viscosimetric MW av 55,200, Sigma). The average molecular weight of 50 kDa was adopted to calculate poly-L-lysine amounts to be added to β2-m solution. Samples were dissolved in H2O/D2O 90/10 or 95/5 with 50-70 mM phosphate buffer, 50-100 mM NaCl and pH in the range 6.5-6.7, or in 150 mM deuterated ammonium acetate at pH 6.4. Deuterium oxide (D 99.9 %) was from Aldrich, d4-acetic-acid (D 99.5%) was from Cambridge Isotope Laboratories. Samples were filtered using 20 nm pore-size Whatman filters. The studies were carried out at 37 and at 40°C. For the pure protein samples 2D TOCSY (20), spectra were acquired as previously reported (21). Experiment with poly-L-lysine were carried out on filtered samples of protein at pH 6.6 (0.63 mM β2-m, 70 mM phosphate buffer, 100 mM NaCl) and pH 6.4 (0.50 mM β2-m, 150 mM ammonium acetate-d3). The protein samples had always been incubated at those conditions for 24 hours at 37°C, before addition of calculated amounts of poly-L-lysine directly into the NMR tube at final concentrations around 0.04 mM β2-m/poly-L-lysine 14-16/1). The interaction between β2-m and poly-L-lysine was monitored at 37°C over 7-9 days by alternating acquisitions of 1D and 2D-DOSY (Diffusion Ordered SpectroscopY) NMR experiments (22). In order to remove the effects of convective motions, a compensated pulse sequence was used (23) including solvent suppression by excitation sculpting (24). The length of the diffusion time was between 60 and 90 ms, and the duration of the encoding/decoding gradient was 3-4 ms. Using the same 2D acquisition parameter as described for TOCSY, 131 increments (128 scans/increment) were collected in the indirect dimension, with a gradient strength varying from 2 to 95% of the maximum (68 G/cm).

Data processing and analysis was performed using Felix software (Accelrys Inc., San Diego, CA) and spectra were referenced on the L23 C=H3 resonance peak (21). DOSY spectra were analysed by means of the routines of GIFA 4.4 (25), i.e. single-exponential fitting and inverse Laplace transformation with the maximum entropy. Fitting was performed on the most intense peaks from β2-m and poly-L-lysine and final results were estimated from averaging over the values affected by small errors (< 2%).

Mass spectrometry — LC-ESI/MS analysis was carried out with a Q-STAR spectrometer (Applied Biosystems/PE SCIEX), operating in positive electrospray-ionization mode at atmospheric pressure, and coupled to an Agilent 1100 series micro LC pump equipped with a Phenomenex Jupiter 5μ C4 300Å (150 x 0,50 mm) column for standard reverse phase chromatographic elution. Typically, the pellet deposited in the NMR tube was isolated by centrifugation at 14,000×g and dissolved in 5M guanidinium chloride (BDH) to get 0.1 - 0.3 mM concentration of denatured β2-m. Aliquots of treated pellet and of NMR tube surfactant were diluted 20-fold by 0.1% TFA aqueous solution before running LC-MS analysis. The analysis was systematically repeated by direct infusion of TFA-free aqueous solution for the optimal detection of the highly charged poly-L-lysine polypeptide.

Atomic force microscopy — AFM images were acquired in tapping mode using a Multimode Scanning Probe Microscope (Digital Instruments–Veeco, Santa Barbara, California), equipped with a “E” scanning head (maximum scan size 10 μm) and driven by a Nanoscope IV controller. For larger scan sizes, a Dimension 3000 microscope (Digital Instruments–Veeco, Santa Barbara, California), equipped with a ‘G’ scanning head (maximum scan size 100 μm) and driven by a Nanoscope IIIa controller, was...
employed. Single beam uncoated silicon cantilevers (type OMCL-AC, Olympus, and RTESP, Veeco) were used for in air imaging. For imaging in liquid, we used V-shaped gold-coated Si$_3$N$_4$ cantilevers (200 μm length, nominal spring constant 0.06 N/m) with pyramidal tips having nominal curvature radii of about 40 nm. The drive frequency was around 300 kHz in air and 6 kHz in liquid; the scan rate was between 0.3 and 0.8 Hz. Vertical displacements were calibrated measuring the depth of grating notches (180 nm) and the half unit cell steps (1 nm) obtained by treating freshly cleaved mica with hydrofluoric acid. The horizontal displacements of the piezoelectric tubes were calibrated using a 3 μm pitch diffraction grating.

Aggregation of β$_2$-m—Lyophilized β$_2$-m was dissolved in ammonium acetate 50 mM, pH 7.4 at the concentration of 2 mg/ml and centrifuged at 16500×g for 1 h to remove large aggregates. The supernatant was collected and filtered with a 20 nm pore size filter; protein concentration was then determined using a Jasco V-530 spectrophotometer. Aggregation experiments were performed at a protein concentration in the range between 0.2 and 0.4 mg/ml, after acidification of the protein solution to pH 6.4 by using an HCl solution at pH 2. The final ammonium acetate concentration was in the range between 12 and 30 mM. For AFM inspection, 20 μl aliquots of the sample kept in incubator at the chosen temperature were extracted at different times during the aggregation reaction, deposited onto freshly cleaved mica and dried under mild vacuum.

For the experiments in the presence of collagen, fibrillar collagen was sonicated for 10 minutes in a bath sonicator (ACAD, Genoa, Italy), washed with the ammonium acetate buffer and, while keeping it under hydration on a microscope slide, cut into several pieces which were washed again and added to the protein solution. Aggregation was performed in the same conditions described above, except for the protein concentration which was increased to 0.5 or 0.6 mg/ml. For AFM inspection, a piece of collagen with its surrounding protein solution was extracted from the sample, deposited onto freshly cleaved mica and dried under mild vacuum.

Poly-L-lysine-coated mica substrates were prepared by incubating a drop (typically 10 μl) of 1 mg/ml poly-L-lysine solution onto freshly cleaved mica for 2 min; the excess polymer was then washed out with the buffer used for the protein. Aliquots of the protein sample extracted at different aggregation times were deposited onto poly-L-lysine-coated mica, incubated for ten minutes, washed with buffer and then imaged under liquid.

Zeta potential measurements—Zeta potential was measured at 25°C with a Zetasizer Nano ZS (Malvern Instruments) at a protein concentration of 0.3 mg/ml in ammonium acetate 25 mM, pH 6.4. Each measurement was performed on a freshly extracted aliquot of the protein sample incubated at 40°C and pH 6.4.

RESULTS

Analysis of ex-vivo β$_2$-m amyloid material—Ex-vivo fibrillar material was extracted from the amyloid deposits surgically obtained from a patient that after 20 years of chronic haemodialysis suffered from femoral fracture. The AFM analysis of this material reveals the presence of bundles of fibrils of typical length between 0.7 and 3 μm and height between 1.5 and 3 nm. As the sample was dried under mild vacuum to facilitate its adhesion to the mica substrate, this procedure results in a reduction in the size of the imaged objects due to dehydration and/or deformation. Similar results have been obtained also by other groups using tapping mode AFM in air to image amyloid fibrils (26). Previous control measurements yielded a correction factor of about 2.5 for monomeric globular proteins; in the case of amyloid fibrils, however, this factor may be larger (about 5) (14). Taking into account this correction, the measured sizes are compatible with those generally acknowledged as peculiar of amyloid fibrils (27).

Ex-vivo β$_2$-m fibrils are closely packed laterally to generate a kind of planar sheet or plaque (Fig. 1). To obtain a reliable estimate of fibril height, the statistical analysis of the fibril height in cross section was performed considering only measurements obtained on those fibrils which were sufficiently separated from the rest, thus avoiding artifacts possibly induced by fibril close packing. Fibril height results to be 2.4±0.4 nm. Adjacent fibrils are often partially interconnected or supercoiled (Fig. 1a). A very interesting feature of the ex-vivo material is that β$_2$-m fibrils are often found in association to collagen fibrils, as shown in Fig.1b and in the surface reconstructions reported in Fig.2. Collagen fibrils are much thicker than amyloid fibrils and exhibit the characteristic banding pattern, with a periodicity
of 67 nm. The height of the collagen fibril shown in Fig. 2 is 50 nm, while its width is about 300 nm. The latter value is much larger than that expected as a result of broadening effects due to the AFM tip size and indicates that the collagen fibril is flattened on the mica surface. The measured sizes, however, are consistent with the well known diameter of collagen fibrils (28).

**Designing conditions for β₂-m fibrillation in vitro** — To model the conditions occurring in vitro in peri-articular tissues of patients subject to long term haemodialysis, we chose to operate at pH 6.4, a value reported to be plausible in the synovial fluids in the presence of flogistic processes (29). Experiments were carried out both at a temperature of 37°C, mimicking the physiologic state and at 40°C, which could represent the extreme temperature occurring in the presence of fever.

When β₂-m is dissolved at pH 6.4, isolated tangles of fibrils can be occasionally observed already at room temperature (not shown). This scanty formation of fibrils could be due to the presence of small seeds which are not removed by filtration with the 20 nm pore size filter. In order to avoid the presence of this material, all the experiments discussed below were performed by dissolving, first, the protein at pH 7.4, which results in a more stable sample with negligible fibril formation, and then lowering the pH to 6.4 just at the start of the aggregation experiment.

Fig. 3a shows typical patterns observed for β₂-m kept at 40°C and pH 6.4. A relatively low number of globular aggregates and rare filamentous structures are found. The height of the latter is between 0.6 and 1.0 nm; taking into account the dehydration effects discussed above, it is consistent with protofilament rather than fibril size. The number of aggregates of both kind observed over a 30μm×30μm scan area at different aggregation times is reported in the histogram in Fig. 3b. These results show that at the concentrations employed in our experiments (30-50 μM), β₂-m does not exhibit massive aggregation in vitro at 40°C and pH 6.4, although there is a significant increase in the number of aggregates within one hour from the start of the aggregation process. However, no increase in ThT fluorescence was observed, indicating that the amount of cross β-structured protein is too low to provide a significant fluorescence signal. After six days of aggregation neither fibrils nor filaments are observed and only a few globular structures (5±1

over a 30μm×30μm area) are found. The results obtained for β₂-m at 40°C and pH 6.4 are in agreement with those obtained under different ionic strength conditions and slightly lower temperature (37°C) by McParland et al. (30).

The same experiment was repeated with the addition of 30% of the truncated form of the protein ΔN6β₂-m, i.e. the variant devoid of the first six residues at the amino terminus. In this case the sample morphology after 30 min at 40°C was similar to that observed with wild type β₂-m, while after 3 h large globular aggregates 70-300 nm high were found to coexist with straight, flat aggregates 2-3 nm high and a few hundreds of nm wide, resembling two dimensional crystals (Fig. 3c). Interestingly, filament loops (indicated by the arrows in Fig. 4) often detach from the body of the flat aggregates. The filament size is compatible with that measured for β₂-m alone. The histogram in Fig. 3d reports the counts of filamentous and other structures. Compared to the experiment with wild type β₂-m alone, a much larger number of non filamentous aggregates is found, while the number of filaments does not increase.

The aggregate morphologies observed in vitro at 40°C and pH 6.4 for β₂-m and ΔN6β₂-m enriched β₂-m are completely different from those observed in the ex-vivo amyloid material, as non fibrillar morphologies prevail. In addition, no increase in ThT fluorescence is detected during aggregation, indicating that no amyloid formation occurs. From these experiments we have therefore concluded that in these conditions β₂-m was unable to make amyloid fibrils in spite of the formation of various types of other aggregates.

A new set of experiments was then carried out under the same conditions described above but in the presence of fibrillar collagen of type I. After 4 days of aggregation we observed the formation of bundles of fibrils whose morphology was extremely similar to that reported in Fig. 1 and 2 for the ex-vivo sample. Also in this case fibrils are not isolated, but grow with a close lateral packing. Fibril formation was more extensive as the aggregation time increased (Fig. 5a-b). When the protein was pre-filtered with a 0.22 μm instead of 0.02 μm pore-size filter the aggregation kinetics was accelerated, resulting in fibril formation already after two days of aggregation (Fig. 5c-d and 6a). Since these experiments were carried out at 40°C, which is close to denaturation conditions for collagen, control experiments in the absence of
β2-m were also performed to rule out possible temperature-induced modifications and/or rearrangements of collagen which could give rise to fibrillar structures. No fibrillar structures such as those shown in Fig. 5 and 6a were observed in the absence of β2-m. In addition, the experiment of β2-m aggregation in the presence of collagen was repeated at 37°C; at this temperature collagen is stable (31), and the results obtained were very similar to those obtained at 40°C (not shown). The diffuse fibrillar aggregation found for β2-m in the presence of collagen suggests that the latter may act as a catalyzer for fibril formation. Indeed, fibrillation is strictly localized and associated to the presence of the collagen surface; no fibrils were observed by AFM in aliquots of solution extracted from the sample bulk liquid and deposited on mica, while they were present only in close proximity to the collagen network. For the same reason, when performing aggregation experiments of β2-m in the presence of collagen, it was not possible to detect any increase in ThT fluorescence, as this assay is based on the analysis of the bulk solution. The Th assay in solution is quite unreliable in this particular case for the presence of insoluble fibrillar collagen that makes quite problematic the specimen sampling.

However, the presence of fibrils surrounding the collagen is confirmed by microscopic analysis of specimens stained by Congo Red showing the typical green birefringence of amyloid fibrils (Fig. 6b, c).

Since the collagen fibre surface exposes patches of clusters of positively charged residues, we have hypothesized that electrostatic effects could be involved in fibril formation. Therefore, to test the fibrillogenic effect of surface charge arrays, we have exposed β2-m previously incubated at 40°C and pH 6.4 to a poly-L-lysine-coated mica and imaged the sample by AFM in liquid. Deposition of β2-m on the positively charged substrate resulted in widespread fibril formation after 25 hours of incubation at 40°C and pH 6.4 (Fig. 7a-b), but fibrils were already detectable after 4.5 hours of incubation (Fig. 8a). Temperature appeared to have a remarkable effect on fibrillogenesis in these conditions. Actually deposition at pH 6.4 without incubation at 40°C did not generate any fibrils (Fig. 8b). Zeta potential measurements performed as a function of incubation time on β2-m at 40°C and pH 6.4 show that between 2 and 24 hours of incubation in these conditions a decrease of the zeta potential occurs, from the initial value of 10±5 mV to 22±5 mV. Therefore, both this change in surface charge (which becomes more negative) and the presence of a positively charged surface (which helps dipole crowding and alignment) are necessary for fibril formation. As a control experiment, we have tested a negatively charged mica substrate and found that it does not promote beta2-m fibril formation.

NMR spectra — 1H NMR spectra of isolated β2-m solution, typically at pH 6-5-6.7 at either 37 and 40°C, did never exhibit any pattern suggesting partial or total protein unfolding. This conclusion proved always confirmed by spectroscopic monitoring for at least one week after sample preparation when the solution had been filtered with 20 nm pore-size filters prior to being submitted to NMR observation. NMR monitoring of β2-m solutions in the presence of collagen did not appear viable because of phase heterogeneity. Therefore, NMR measurements were carried out only on β2-m/poly-L-lysine mixtures that were checked to be reproducibly homogeneous when solutions were prepared.

β2-m solutions in the presence of poly-L-lysine did not show any detectable changes of the protein NMR chemical shift and/or linewidth for prolonged observation intervals (typically 7-9 days) under either investigated conditions (pH 6.6 in phosphate buffer and pH 6.4 in ammonium acetate). These conclusions could be safely inferred from inspection of those regions of the spectrum that were not populated by poly-L-lysine resonances. Diffusion ordered spectroscopy confirmed that no detectable interactions between β2-m and poly-L-lysine take place under the selected experimental conditions. The calculated self-diffusion coefficient for the protein was \((1.48 ± 0.01) \times 10^{-10} \text{ m}^2 \text{s}^{-1}\) that is not significantly different from the value obtained with isolated β2-m under the same conditions, i.e. \((1.53 ± 0.01) \times 10^{-10} \text{ m}^2 \text{s}^{-1}\). The self-diffusion coefficient obtained for poly-L-lysine was \((0.96 ± 0.01) \times 10^{-10} \text{ m}^2 \text{s}^{-1}\), a value that reflects the MW distribution (40-60 kDa) of the homopolyptide product and is in agreement with expectation for a globular protein of mass around 60-65 kDa. Thus an interaction with poly-L-lysine should affect β2-m diffusion more significantly than the very small effect that emerged from our experimental fitting, although aspecific, transient interactions can not be ruled out. The NMR spectrum quality was not altered...
by the limited amount of precipitation that occurred after some 24-48 hours from poly-L-lysine addition. The overall resonance amplitude was only marginally affected by this precipitation in phosphate buffer (some 3% decrease), whereas in ammonium acetate an overall amplitude decrease by 10% was observed. The precipitate, collected and redissolved in guanidinium chloride, was analysed by electrospray mass spectrometry and shown to contain only β2-m with a contamination from the methionine-oxidised β2-m (at most 30%), but no detectable trace of poly-L-lysine. The same precipitate submitted to AFM analysis proved to be only amorphous material, mainly globular disordered aggregates of varying dimensions (not shown).

Electrostatic Effects in β2-m Collagen Interaction — Modeling has been performed for a simplified model of collagen fibre in order to estimate the possible electrostatic influence on β2-m collagen interaction. Lack of knowledge on the details of collagen fibre assembly prevents any attempt to reach precise quantitative predictions. However, analysis of a the simplified model of the collagen fibre shows that direct and/or orientational electrostatic effects could be substantial and could lead to very high local concentrations of oppositely charged species (such as β2-m) which decay on moving away from the surface. The details of the model are given in the Supplementary Material section. Assuming values of 500 mV/nm, 333 mV/nm, 166 mV/nm for the electric field at the modeled collagen surface, the numerical solution of the Poisson-Boltzmann equation (32,33) gives a set of corresponding potential values. The detailed shape of the fibre could locally magnify or reduce the potential value.

Although qualitative features can not be described by our model, an important conclusion is that the local concentration (c) of the charged species (say with charge number z) at the surface of the cylinder may be significantly different from the bulk (or free) concentration c_f. Based on Boltzmann equation the local concentration is given by:

\[ c = c_f e^{-zF} \]

If the charged species has charge number \( z \gg 1 \) or it has a large dipole moment, electrostatic effects may be substantial. Although these considerations should be regarded with much caution because they are not easily transferable to macromolecules, some qualitative assessment of the electrostatic effects on β2-m collagen interaction can be attempted.

As far as β2-m is concerned, the net charge of the intact protein at neutral pH is \(-2.4\) and it is \(-4.3\) for the ΔN6 mutant (12). The dipole moment is 9.1×10^{-28} C m (2.7×10^2 Debye), which is moderate for proteins of this size, and it is decreased to 3.4×10^{-28} C m (1.0×10^2 Debye) for the ΔN6 mutant. The net effect of deleting the first six residues would be therefore an increase of local concentrations with a possible decrease in orientation effects, although the concept of dipole applies poorly to macromolecules.

The increase in local concentration of protein around the collagen fibre, or other charged surfaces, would thus aid fibrillogenesis, consistent with the experimental observations. β2-m is found at a concentration 0.3 μM in healthy individuals (34, 35). According to the proposed model, its concentration at the collagen cylinder surface, at neutral pH, could be raised by a factor in the range 10^3 to 10^5. This range should increase by 20-50 fold in haemodialysed patients, although the net β2-m charge reduction, due to the pH decrease (12) associated with local inflammation, should downsize the effect. However, the expected values of surface concentration would still remain remarkably high (in the order of mM). These effects are pictorially shown in Fig. 9. On the other hand, the molecular dipole orientation favoured by the electric field at the surface of collagen should be quite significant. If the overall dipole geometry of β2-m were idealized as a point dipole located at the centre of the molecule, in practice all molecules would be favorably oriented at the collagen surface. This exclusive single-level population should prevail also at one Debye length (about 8 Å), even considering the dipole as a charge separation spread over the whole molecular length (corresponding to two 35 Å-spaced, opposite charges of 1.6 elementary units). The dipole energy should become comparable to thermal energy kT, and therefore negligible, at 35 Å away from collagen surface.

DISCUSSION

It is well known that along the collagen fibres there are regions presenting an excess of positive charge (36), and it has been shown that the exposure of other amyloidogenic peptides (37) and proteins (38) to charged surfaces can highly enhance the rate of fibril formation.
In particular, it has been shown that the prototypic kIV immunoglobulin light chain SMA, that has a folding motif similar to β2-m, displays a very high propensity to make fibrils when it is exposed to the charged surface of mica (38). Fibrillogenesis on charged mica is highly productive at pH 5, but occurs also at pH 7.4, though less efficiently. On the contrary, fibrillogenesis is totally abrogated at pH 7.4 and significantly reduced at pH 5 when the light chain is exposed to a hydrophobic surface-modified mica.

It might be speculated that the mechanism by which collagen facilitates β2-m fibrillogenesis is related to its positively charged patches. To test the effect of positive immobilized charges on β2-m fibrillogenesis, we deposited β2-m previously incubated at 40°C and pH 6.4 on a poly-L-lysine-coated mica. As described in the Results section, under those conditions we could ascertain a temperature-dependent formation of fibrils. The two conditions, pH 6.4 and the temperature of 40°C do not appear to have an effect on the 3D structure of β2-m in solution, when considered separately. 2D NMR spectra recorded in very similar conditions (0.1 mM, pH 6.5-6.6, 40°C), exhibit the same pattern as observed at neutral pH. These results indicate that either the onset of partial unfolding transitions of the protein is absent, or partially unfolded intermediates are native-like and thus hard to distinguish by NMR. A possible perturbation of the solvation properties from NMR-silent intermediates could affect protein-protein interactions, but no specifically consistent evidence was ever inferred from extensive and repeated NMR observations on β2-m solutions kept for weeks at 40°C.

It is worth noting that poly-L-lysine in solution has no effect on β2-m fibrillogenesis at pH 6.4 and 37°C, an observation confirmed also by corresponding NMR results. The small extent of deposit obtained in the NMR tube, in fact, did not exhibit any specific morphologic features but those of a globular precipitate.

The necessity of increasing the temperature to 37-40°C to obtain fibrils may suggest that temperature and pH could cooperatively trigger an initiating conformational transition that, however, appears to be productive only in the presence of an immobilised array of positive charges. This interpretation is consistent with the results of our assessment of the role that the electric field at the collagen surface may play in concentrating locally favourably charged counterparts such as β2-m molecules. The ensuing concentration gradient and plausible dipole orientation effects could overcome the unfavourable statistics of productive conformational transitions leading to fibril-competent nuclei (21). This effect could be considered analogous to that recently reported by Ohhashi et al (39) concerning the induction of β2-m fibril formation by ultrasonication. The ultrasound energy should not only increase the frequency of molecular collisions but also destroy the improductive low-energy interactions and select the high-energy ones, i.e. those that are more likely to be fibril-competent. Based on our results, it can be therefore concluded that an immobilised charged surface is required for fibrillogenesis.

The experimental evidence presented in this study show that fibrillar aggregation of β2-m under conditions close to the physiological ones is possible and that collagen could play a fundamental role in local fibrillogenesis, possibly representing an immobilised charged surface where the protein is correctly oriented for priming an ordered polymerization that could generate fibril seeds. This observation might have a fundamental implication in the future therapeutic strategies for DRA, that affects approximately one million people worldwide. In fact, the demonstration that the interaction collagen/β2-m has an effect on fibrillogenesis prompts for a new target for pharmaceutical strategies.

REFERENCES

FOOTNOTES

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2The abbreviations used are: β2-m, β2-microglobulin; DRA, dialysis-related amyloidosis; ThT, thioflavin T; TM-AFM, tapping mode atomic force microscopy.
**FIGURE CAPTIONS**

**Figure 1.** TM-AFM image (height data) of *ex-vivo* β₂-m amyloid material obtained from the femoral head of a patient affected by DRA. a) interconnection and supercoiling between neighbouring fibrils; b) bundles of fibrils close to a collagen fibril. Scan size a) 1.7 µm, b) 2.4 µm; Z range a) 30 nm, b) 50 nm.

**Figure 2.** Surface plots of a TM-AFM image (height data) of β₂-m *ex-vivo* amyloid material obtained from the femoral head of a patient affected by DRA. a) amyloid fibrils surrounding a collagen fibril; b) higher resolution image of a portion of Figure 2 a), showing amyloid fibrils crowding around the collagen fibril.

**Figure 3.** a) TM-AFM image (height data) of β₂-m kept at 40°C and pH 6.4 for 30 min. A relatively low number of fibrils and other aggregates is observed on a background of small globular structures (enlarged in the inset). Scan sizes, 16 µm and 1.4 µm (inset); Z ranges, 15 nm and 24 nm (inset). b) Number of filamentous (black) and globular aggregates (grey) observed at different aggregation times over a 30µm x 30µm scan area (obtained as mean value from at least four images corresponding to different regions of the sample). c) β₂-m enriched with 30% ΔN6β₂-m kept at 40°C and pH 6.4 for 3 h; in the presence of ΔN6β₂-m a larger number of aggregates is formed. Scan size 30µm, Z range 19 nm. d) Number of filamentous (black) and non filamentous aggregates (grey) observed at different aggregation times over a 30µm x 30µm scan area for β₂-m enriched with 30% ΔN6β₂-m; note the different scaling of the y axis with respect to graph b).

**Figure 4.** TM-AFM image (height data) of β₂-m enriched with 30% ΔN6β₂-m, kept at 40°C and pH 6.4 for 3 h. The arrows indicate fibrillar loops which seem to act as a template for the growth of flat aggregates. Scan size 6 µm, Z range 25 nm.

**Figure 5.** TM-AFM images (a,c) height data; b), d), amplitude data) of β₂-m fibrils obtained in vitro at 40°C and pH 6.4 in the presence of collagen. a), b) sample prefILTERED with 20 nm pore size filter and aggregated for four days; the arrow indicates a collagen fibre embedded into fibrillar and amorphous material; c), d) sample prefILTERED with 0.2 µm pore size filter and aggregated for two days. Scan size a), b) 880 nm, c), d) 1.5 µm. Z range a) 12 nm; c) 10 nm.

**Figure 6.** a) TM-AFM image (amplitude data; scan size 0.90 µm) of a branched collagen fibre completely embedded into β₂-m amyloid fibrils obtained from a sample prefILTERED with 0.2 µm pore size filter and aggregated for two days at 40°C and pH 6.4. b), c) Congo red staining of beta2-m prefiltered with 0.2 µm pore size filter and aggregated for four days at 37°C in the presence of collagen. Sample observed in b) bright light; c) cross-polarized light. The typical green birefringence of Congo Red in the presence of amyloid fibrils is clearly evident in the region close to the edge of the sample, as indicated by the arrow.

**Figure 7.** TM-AFM image (a), height data; b), amplitude data) of β₂-m aggregated for 25 h at 40°C and pH 6.4, deposited on a poly-L-lysine coated mica substrate and imaged in liquid. Scan size 4.6 µm, Z range 18 nm.

**Figure 8.** TM-AFM images (height data) of β₂-m deposited on a poly-L-lysine coated mica substrate and imaged in liquid. Deposition was performed a) after 4.5 h of aggregation at 40°C and pH 6.4; b) at the starting of the aggregation process. Scan size a) 3.9 µm, b) 8.8 µm, Z range a) 18 nm, b) 28 nm.

**Figure 9.** The Boltzmann factor (left axis) / electrostatic potential (right axis) for a charged species of valency −2 computed according to the non-linear Poisson-Boltzmann equation around a collagen fibre cylindrical model. On the x-axis *r* is the distance from the axis of the cylinder. The upper and lower curves refer to an assumed electric field at the surface of 500 and 166 mV/nm, respectively.
Figure 4
Figure 6
Figure 7
Figure 8
Figure 9
Collagen plays an active role in the aggregation of β2-microglobulin under physio-pathological conditions of dialysis-related amyloidosis
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