Systemic amyloidosis: lessons from β2-microglobulin*

Monica Stoppini1, and Vittorio Bellotti1,2

1Department of Molecular Medicine, Institute of Biochemistry, University of Pavia, Pavia, Italy

2Wolfson Drug Discovery Unit, Centre for Amyloidosis and Acute Phase Proteins, Division of Medicine, University College London, London NW3 2PF, United Kingdom

*Running title: β2-m amyloidosis

To whom correspondence should be addressed: Vittorio Bellotti, Wolfson Drug Discovery Unit, Centre for Amyloidosis and Acute Phase Proteins, Division of Medicine, University College London, Rowland Hill Street, London NW3 2PF, UK. Tel: +44 20 7433 2773; Fax: +44 20 7433 2803; E-mail: v.bellotti@ucl.ac.uk (or vbellot@unipv.it)

Keywords: β2-microglobulin; amyloid; genetic variant Asp76Asn; fibrillogenesis in vitro; mechanism of amyloidogenesis

ABSTRACT

β2-microglobulin is responsible for systemic amyloidosis affecting patients undergoing long-term hemodialysis. Its genetic variant Asp76Asn causes a very rare form of familial systemic amyloidosis. These two types of amyloidoses differ significantly in terms of the tissue localization of deposits and for major pathological features. Considering how the amyloidogenesis of β2-microglobulin mechanism has been scrutinized in depth for the last three decades, the comparative analysis of molecular and pathological properties of wild type β2-microglobulin and of the Asp76Asn variant offers a unique opportunity to critically reconsider the current understanding of the relation between the protein’s structural properties and its pathologic behavior.

In 1984 George Glenner and Caine Wong (1) provided the first chemical evidence that Alzheimer’s disease amyloid plaques were constituted by Aβ protein. The following year, the paper BBRC (2) by Fumitake Gejyo’s group followed shortly after by the paper JCI by Peter Gorevic’s and Blas Frangione’s team (3) both showed that the constituent of amyloid deposits of patients treated with chronic hemodialysis was the protein β2-microglobulin (β2-m). The further demonstration that the formation of amyloid required a persistently high plasma concentration of β2-m (4) was a clear proof that a critical concentration of a protein precursor is required for priming the formation of amyloid fibrils. Hence, the early biochemical characterization clearly showed that full-length non-mutated β2-m was abundantly present in natural amyloid fibrils (5). Further biochemical studies were carried out by Reynold Linke on different types of tissues which included specimens of the carpal tunnel, as well as specimens derived from bone fractures caused by amyloid deposits and even urinary stones (6). From these studies, emerged that in all natural amyloid deposit the truncated species of β2-m lacking the six N-terminal residues was significantly represented (7). No other major post-translational modifications are apparently present in natural fibrillar β2-m. In amyloid deposits, the presence of the protein precursor’s fragments is quite common. The truncation of extensive portions of the constant region is common in light chains of Aβ.  

"This paper is dedicated to the memory of our unforgettable colleague and mentor, Prof. Giuseppina Ferri.

Copyright 2015 by The American Society for Biochemistry and Molecular Biology, Inc.
AL amyloid deposits. Natural fibrils of Apolipoprotein A-I mainly contain the N-terminal polypeptide corresponding to the first 100 residues and the presence of transthyretin (TTR) fragments can be considered almost a hallmark of the cardiac involvement in TTR amyloidosis (8). The biochemical characterization of β2-m natural amyloid fibrils highlighted the co-deposition of other macromolecules. Some of them, like serum amyloid P component (SAP) and glycosaminoglycans (GAGs), are generic co-constituents of all types of systemic amyloidosis (9, 10), but a few are apparently specifically associated with the β2-m related form. In an ante-litteram proteomic study Campistol and colleagues (11, 12) showed that several anti-proteases are co-deposited in β2-m natural fibrils and that particularly abundant is the presence of α2-macroglobulin (α2-M). It is worth noticing that a specific complex between α2-M and β2-m also circulates in the plasma of hemodialysis patients (13). In 2012, the first natural variant of β2-m was discovered in a French family where all the heterozygous carriers of the mutation presented a multi-visceral amyloid deposit (14). Liver, kidney and heart were all involved, but, unexpectedly, bones and ligaments were not affected. This finding was quite surprising in terms of the known tropism of the wild type (wt) β2-m for the muscle-skeletal system. Another unexpected finding was the absence of wt β2-m in the deposits even though its intrinsic amyloidogenic propensity is well established. Equally surprising was the absence of N-terminal truncated species, which are ubiquitous constituents of β2-m amyloid deposits in Dialysis Related Amyloidosis (DRA).

In the last two decades the molecular characterization of amyloid deposits caused by wt β2-m in patients under hemodialysis and more recently the molecular and pathological features of the familial form of β2-m, have stimulated seminal studies on the molecular basis of the amyloidogenesis of globular proteins in vivo; moving from totally artificial conditions to more bio-compatible methods (Table 1). These studies have provided new insights on the molecular basis of the intrinsic predisposition to amyloid conversion, on the identification of the physical-chemical conditions suitable in vivo to prime the conformational transition, as well as some clues on the mechanism responsible for the selective tissue targeting of amyloid deposits in systemic amyloidosis.

β2-m fibrillogenesis in vitro

The first successful attempt to obtain the fibrillar conversion of native β2-m was achieved by Connors et al. (15) immediately after the identification of β2-m as the causative protein of DRA. This first method was based on the minimization of ion strength and on the maximal increase of β2-m concentration. Although the yield was quite low, the study provided the first demonstration that globular β2-m can be converted into fibrils in vitro and that the concentration represents a crucial condition. A more efficient method of β2-m fibrillogenesis was introduced in 1997 by Naiki and colleagues (16). In this case the massive conversion of β2-m into fibrils was primed by the presence of seeds of natural fibrils and required a very low pH. This method highlighted how fibrillogenesis is accelerated by the presence of fibrillar nuclei and in general how the amyloidogenesis requires both a nucleation and an elongation phase. Moreover the low pH implied that the fibrillogenesis of the wild type required the protein unfolding and was perfectly consistent with similar evidences obtained in the same historical phase with other two globular amyloidogenic proteins such as the lysozyme and TTR variants (17, 18). Other ways to unfold the β2-m were pursued by Japanese groups using sodium dodecyl sulfate (SDS) or trifluoroethanol (TFE) as mild protein denaturants (19, 20). The possibility to transform native β2-m into fibrils led to studies aiming to monitor major conformational changes occurring during the transition and to identify which part of the native molecule is substantially unaltered within the fibrils.
The work carried out by Goto’s (21) and Radford’s (22) teams revealed that low pH fibrillogenesis implies massive conformational changes of the N-terminal and C-terminal portions of β2-m. However, the central core of the native protein constrained through its single disulphide bridge is apparently substantially conserved in the fibrils. These data were consistent with the fact that the disulphide bridge 25-80 is necessary for the formation of fibrils in vitro and when this is reduced, the β2-m can only form amorphous aggregates (23). Moreover, the disulphide bridge is present in natural amyloid fibrils (24). The maintenance of the disulphide bond present in the native protein precursors is common to other types of amyloidosis and it is particularly meaningful for lysozyme fibrils, where the four disulphide bonds of the native enzyme can be found in the natural fibrils (17).

The structure of the natural fibrillar proteins is very informative and represents a profitable guide for designing proper conditions of fibrillogenesis in vitro. This proposition was particularly true in relation to the evidence that truncated species of β2-m were ubiquitously found in all the natural amyloid deposits associated to DRA. The removal of six residues at the N-terminus of β2-m (ΔN6β2-m) has a massive impact on the structure, on the stability and on the fibrillogenic propensity of β2-m (25). ΔN6β2-m rapidly forms fibrils in physiologic conditions that are otherwise not permissive for full-length wt β2-m. This most likely plays a pivotal role in the pathogenesis of the disease and it can be considered a natural metastable conformer suitable for the nucleation of β2-m fibrils. ΔN6β2-m rapidly forms oligomer in solution even at very low concentrations and it catalyses the oligomerization of wt β2-m even in a physiologic environment (26). The Radford’s group has extensively investigated the structural features of ΔN6β2-m and its capacity to recruit the wt into amyloid fibrils (27). The NMR spectra revealed a remarkable overlapping of the structure of ΔN6β2-m with the structure of the Pro32Gly variant that was prepared to investigate the effect of the transition cis-trans of Pro32 on β2-m fibrillogenesis. When Pro32 is in cis conformation, β2-m is protected from self-aggregation, whereas the trans conformation makes the β2-m highly susceptible to a rapid fibrillar conversion (28, 29).

The role of selective proteolytic cleavage in amyloidogenesis has been extensively debated. However there are still doubts on the timeline of the proteolytic cleavage, notably if the event precedes the fibrillar aggregation or if it simply represents a process of trimming of the less protected portion of the polypeptide cemented in the amyloid fibrils. Unequivocal demonstration of a pre-fibrillogenic proteolytic event is still missing, however it is unquestionable that selective proteolytic cleavage mimicking those occurring in vivo has a strong enhancing effect on the kinetics of fibrils formation of β2-m and other proteins such as TTR (30).

Despite the relevance of proteolytic cleavage, some types of amyloid fibrils like those formed in vivo by variants of lysozyme (17) and the natural variant of β2-m Asp76Asn (14) only contain an intact and full-length mature protein.

The discovery that in vitro truncated β2-m can form amyloid fibrils, in a physiologic environment, has moved the methods of in vitro fibrillogenesisistowards more bio-compatible conditions. A successful example of a bridge between the known tropism of β2-m for the muscle skeletal system and an in vitro method was achieved by studying the effect of type I and type II collagen on β2-m amyloidogenesis (31). The effect of the interaction of β2-m with the collagen’s surface is remarkable and Fig. 1 illustrates a representative image of the growth of amyloid fibrils stemming from type I collagen fibres. The presence of β2-m oligomers and GAGs was able to accelerate the process of the amyloid grown on the collagen surface. This confirmed the generic pro-amyloidogenic effect played by the aforementioned components in fibrillogenesis (32). However, despite the growth of fibrils on the collagen surface, in the absence of fluid flow, the
majority of the bulk of wt β2-m in solution was not converted into fibrils on a time scale of several days.

β2-m such as many other globular amyloidogenic proteins displays a strong propensity to spontaneously aggregate into soluble oligomers; in particular β2-m does so in the physiologic buffer (26, 28, 33). It is likely that the oligomerization is mainly primed by a small population of partially folded conformers in equilibrium with the fully folded protein. NMR studies, molecular dynamic simulation, capillary electrophoresis and spectroscopic analysis highlighted the existence of a partially folded intermediate, initially named I2 (34, 35), in which specific regions of the molecule exhibit a very high flexibility and rapid structural fluctuations (36). The fluctuation toward a partially folded state is probably dictated by the peculiar dynamics of the loop interconnecting the strands D and E. This loop is particularly unstable, most likely due to the presence of a Tryptophan (Trp60) in the centre of the loop (37, 38) (Fig. 2).

Remarkably, despite an unfavorable thermodynamic effect, a Trp is present in this position in almost all the vertebrate species (39) and it is crucial for the proper binding of β2-m to the heavy chain of the major histocompatibility complex class I (MHC1). In human β2-m, the Trp60 can therefore play two contrasting roles: a functional physiologic contribution to the correct assembly of the MHC1, and a detrimental destabilizing and pro-amyloidogenic effect when the plasma concentration of β2-m is persistently high.

What did the comparison between the amyloidogenesis mechanism of the wild type and the rare Asp76Asn variant reveal?

Elucidation of the molecular mechanisms of rare diseases can promote crucial progress in the interpretation of more general phenomena as well as illuminating the mechanisms of common diseases. The discovery of the rare natural amyloidogenic variant of β2-m allowed a systematic analysis of its pathological and biochemical features as well as provided the opportunity to correlate specific molecular characteristics of the wt and of the variant with peculiar clinical and pathological different features of the two related types of amyloidosis. The first, associated to hemodialysis, is caused by the persistently high concentration of β2-m in plasma and it is characterized by a selective localization of amyloid in bones and ligaments. The genetic form is not associated with any increase of β2-m in plasma and the amyloid localization is mainly visceral, involving liver, spleen, kidney and heart, whereas, quite surprisingly, bones and ligaments are spared. The original question was: as far as this variant is concerned which theories, hypothesized to explain amyloidogenesis of wt β2-m, do remain applicable? This question became particularly cogent once we discovered the destabilizing effect of the mutation and the possibility to convert the Asp76Asn variant into amyloid fibrils in physiological buffer, by simply exposing the protein to fluid shear forces in the presence of natural or artificial hydrophobic surfaces (40).

In wt β2-m, its intrinsic amyloidogenic propensity, was ascribed to its dynamic properties, particularly evident in the D strand and in the D-E loop region, whose flexibility is propelled by the above mentioned Trp60 (38). The comparative measurement of the folding and unfolding kinetics as well as of the native protein’s unfolding free energy allowed establishing that approximately 5% of wt β2-m physiologically populates a partially folded intermediate state and that the mutation Asp76Asn causes a five-fold enhancement of the concentration of the intermediate at the equilibrium in the physiological buffer (40).

Therefore, a direct correlation exists between the amyloidogenic propensity of β2-m species and the concentration of this partially structured intermediate. The structure of this intermediate was extensively investigated, but it is still elusive due to the high conformational dynamic on a microsecond to millisecond time scale. A
brilliant approach to single out specific characteristics of this intermediate is based on the discovery that several structural and functional features overlap those of ΔN6β2-m. A remarkable feature of both ΔN6β2-m and of the folding intermediate I2 is most certainly the trans conformation of the His-Pro32 peptide bond (27). The cis–trans transition is crucial for the fibrillogenesis and it is a hallmark of the fibrillar state (41, 42).

Although the amyloidogenic potential of the two β2-m species (wt and Asp76Asn) is coherent with the concentration of the partially folded state at the equilibrium, a major and peculiar difference emerges from the clinical/pathological features of the two type of amyloidoses. Notably the localization in bones and ligaments of the wt and the visceral localization of the Asp76Asn. The amyloid deposition in bones is quite rare in other systemic amyloidoses, but it is an unavoidable complication of DRA. The specific localization in bones and ligaments of the amyloidosis caused by hemodialysis was ascribed to a peculiar affinity of β2-m for collagen type I and type II. Nonetheless, the measurement of the affinity constant of β2-m for collagen revealed a weak Kd of 1 x 10–4 M (43). It is plausible to believe that preferential accumulation of β2-m in collagen becomes significant only for the micromolar concentration during hemodialysis whereas it does not become so in physiological sub-micromolar concentrations. Furthermore, it is likely that in DRA the truncated ΔN6β2-m species, which has a ten-fold higher affinity to the full length wt for collagen (43), is a potent promoter of amyloidogenesis on the collagen surface and that this is present only in the amyloid deposits localized in bones and ligaments. The latter suggests that a protease, well represented in the aforementioned tissues could cleave β2-m in its first strand, consequentially accelerating the local accumulation of this conformer which is then rapidly followed by its fibrillar aggregation.

The mechanism by which collagen facilitates the amyloidogenesis of β2-m is uncertain. Collagen offers wide hydrophobic surfaces and it is known that the flow of a physiologic fluid, at the interface between polar and hydrophobic surfaces can generate sufficient forces to partially or totally unfold a globular protein (44). The intensity of these forces expressed as shear stress can be quantified through the equation:

\[ T = F_s / A = \mu \cdot (dv/dx) \]

T represents the shear stress, \( F_s \) is the shear force, A is the cross sectional area of the molecule, \( \mu \) is the dynamic viscosity of the fluid and \( dv/dx \) is the shear rate, that is the fluid velocity gradient.

We hypothesize that, in the extracellular matrix in the very thin fluid space at the interface between hydrophobic surfaces and the interstitial fluid, the amyloidogenic proteins could partially unfold and expose normally buried hydrophobic patches. These partially folded conformers could locally accumulate and reach a condition of supersaturation (Fig. 3). Such a state is extremely unstable and several events can break solubility leading to protein precipitation. Although the shear flow of the fluid is not \textit{per se} sufficient to unfold globular proteins it may play a fundamental role in breaking the condition of super-saturation. In fact, in conditions of supersaturation, the intensity of shear flow inversely correlates to the lag phase of β2-m fibrillogenesis (45). All these data suggest that the concentration of β2-m and its level of thermodynamic stability could direct the amyloid formation in two distinct tissue targets. In conditions of high concentration, but relatively good thermodynamic stability, the amyloid is deposited in bones and ligament. When plasma concentration is low, implying a negligible accumulation of β2-m on the collagen surface, bones and ligaments are spared. If a mutation reduces the stability of β2-m (i.e Asp76Asn mutation), the shear stress in the extracellular matrix of visceral organs like liver, spleen, kidney and heart is sufficient to unfold the unstable variant and prime a cascade of events as represented in Fig. 3. It is worth noticing that the amyloid deposits of heterozygous patients for the
mutation Asp76Asn do not contain the wt β2-m. However, in vitro, once Asp76Asn fibrils are formed, shear stress generated by the dynamics of a physiologic fluid and the exposure to hydrophobic surface of biological molecules, can also prime amyloidogenesis of wt β2-m (40). These findings are apparently incompatible, but let grasp the complexity of amyloidogenesis in vivo. The demonstration that in the presence of a generic extracellular chaperone like αB crystallin, even in a very low molar ratio, the wt β2-m becomes resistant to the fibrillar conversion induced by the Asp76Asn fibrils (40) suggests that in vivo factors like chaperones can modulate the amyloidogenesis of wt proteins.

The existence of natural factors modulating amyloidogenesis

The discovery that forces physiologically harboring the human tissues are sufficient to prime the protein unfolding and fibrillogenesis, is shedding light on the pathophysiology of amyloid formation. However a deep gap still exists between the in vitro and in vivo conditions of protein amyloidogenesis. The in vitro investigation usually concerns the transition of an isolated and homogeneous molecule, but in vivo the amyloidogenic protein interplays with a variety of other chemical entities and peculiar physical environments. In vivo an equilibrium exists between pro-fibrillogenic and anti-fibrillogenic factors. In the interstitial space, where the amyloid deposits are formed, a few proteins could specifically influence the aggregation propensity of amyloidogenic proteins. Albumin per se which is present in the interstitial space at a concentration around 10 mg/ml can partially inhibit the fibrillogenesis by improving the protein’s colloidal stability. However, more specific and more effective inhibitors of protein aggregation are present in the extracellular space where chaperones play a rather important role (46). In β2-m amyloidosis the anti-fibrillogenic properties of some of these chaperones were specifically studied, namely the α2-M (47) and, as mentioned above, αB-crystallin (48). The mechanism by which in vitro α2-M interferes with β2-m amyloidogenesis reveals that the most effective species is a dimeric form of α2-M, resulting from the stress-mediated dissociation of the tetramer. It was demonstrated that α2-M binds the unfolded β2-m more avidly than the folded one (47). These findings suggest that conditions promoting at once the unfolding of the amyloidogenic protein as well as the structural rearrangement of the chaperone could activate the chaperone function of α2-M. Consistent with the in vitro and ex vivo studies it was also demonstrated that although α2-M inhibits β2-m aggregation it is nonetheless unable to disaggregate mature fibrils. Other extracellular chaperones display similar properties. αB-crystallin is capable of inhibiting fibrillogenesis when added in sufficient quantity to compete with the self-assembly of β2-m variant and, as mentioned above, to dissociate the aggregation of wt and variant (48). However once the fibrils are formed, chaperones, such as α2-M, are unable to solubilize the amyloid and co-precipitate with the mature fibrils (unpublished).

General function of extracellular chaperones on amyloidogenesis is well characterized in clusterin. The capacity of clusterin to interfere with amyloid formation was not tested on β2-m, but on Apolipoprotein C-II (49), lysozyme (50), synuclein and other amyloidogenic proteins (51, 52). Similarly to α2-M and crystallin, clusterin too is unable to dissociate preformed fibrils and it is frequently found as a bystander component of natural amyloid fibrils.

The emerging scenario is consistent with the hypothesis that these extracellular chaperones could perform a dual, apparently antithetical function; notably the inhibition of oligomerization and fibrillogenesis acting on the solubility of partially folded intermediates as well as the stabilization of amyloid fibrils once these are formed. The balance between these two functions in the formation of amyloid, in the natural environment, is yet to be determined, but it is most probably crucial for the elucidation of the natural history of the
disease and the possible therapeutic exploitation of these molecules.

**β2-m pharmaceutical interactants and amyloidogenesis inhibition**

Besides extracellular chaperone proteins, other compounds can inhibit β2-m amyloidogenesis through different mechanisms. As a prototype of a generic inhibitor of amyloidogenesis we proved that *in vitro* the antibiotic doxycycline can inhibit the amyloidogenesis of wt β2-m (53) and with a similar dose it can affect the fibrillogenesis of the natural variant Asp76Asn (unpublished). The main obstacle to *in vivo* doxycycline therapeutic efficacy was expected to be the difficulty in obtaining a therapeutic concentration. However, the concentration of doxycycline in the tissue targets proved to be much higher than in plasma (54) and most likely sufficient to inhibit aggregation of oligomers and β2-m toxicity. Preliminary data, obtained in the first three patients affected by DRA and treated with doxycycline, suggest that a therapeutic response can be achieved even with a plasmatic concentration that is apparently insufficient to abrogate fibrillogenesis *in vitro* (55). Small protein's ligands can be therapeutically used, as the ligand-mediated stabilization can be sufficient to protect from the unfolding and aggregation. The best example for this approach is the stabilization of TTR through small analogues of its natural ligands (56). However no specific small ligands with similar properties are available for β2-m. An immunological approach, mainly based on the use of specific antibodies can be probably properly pursued for this amyloidosis. We previously showed that the specific monovalent single chain camelide antibodies can inhibit wild type amyloidogenesis (57) and we are confirming their effect on the Asn76 variant. In absence of other possible therapies and considering the relatively low concentration of circulating β2-m, a treatment based antibody should be pursued in the familial form of β2-m amyloidosis and once its efficacy will be demonstrated it could be extended to other forms of systemic amyloidoses.

**Concluding remarks**

The extensive research carried out on β2-m related amyloidosis has substantially contributed to elucidating the general rules dictating the amyloid conversion of globular proteins in systemic amyloidosis. In order to self aggregate into a cross beta structured fibril, the amyloidogenic globular protein must partially unfolded. The loss of the native structure can be primarily caused by mutations of covalent modifications such as limited proteolytic cleavages. Wt β2-m is a paradigmatic example of how the aggregation risk of some proteins, exposing hydrophobic and flexible regions for functional reasons, is controlled by maintaining very low protein concentrations. The risk of protein aggregation becomes instead extremely high when a condition of super-saturation of the partially folded intermediates occurs in the extracellular space of the target organs. β2-m models investigations have provided unique new insights elucidating the mechanism of selective tissue targeting in other forms of systemic amyloidosis, where the amyloid is deposited in organs away from far synthesis site. The most common forms of systemic amyloidosis like those caused by immunoglobulin light chains, TTR, lysozyme and lipoproteins are prototypic example of instances when the sites of production and of major deposition are totally different. It is now possible to address the crucial and challenging question of the role played by local tissue factors in favoring and contrasting protein aggregation. The equilibrium between these factors probably determines the peculiar natural history of the different types of systemic amyloidosis. It is plausible that even within the same type of amyloid diseases said factors will influence the precise medical and pathologic features of the individual patient.
REFERENCES


flow on protein structure and function. *Biopolymers* **95**, 733-745
triggered by agitation of beta2-microglobulin under acidic and neutral pH conditions. *Biochemistry* **47**, 2650-2660


**FOOTNOTES**

1To whom correspondence should be addressed. Wolfson Drug Discovery Unit, Centre for Amyloidosis and Acute Phase Proteins, Division of Medicine, University College London, Rowland Hill Street, London NW3 2PF, UK. Tel: +44 20 7433 2773; Fax: +44 20 7433 2803; E-mail: v.bellotti@ucl.ac.uk (or vbellot@unipv.it)

2The abbreviations used are: MHCI, the major histocompatibility complex class I; DRA, Dialysis Related Amyloidosis; β2-m, β2-microglobulin; ΔN6β2m, truncated β2-m isoform lacking the 6 N-terminal residues; TTR, transthyretin; α2-M, α2-macroglobulin; SAP, serum amyloid P component; GAGs, glycosaminoglycans; wt, wild type; SDS, sodium dodecyl sulfate; TFE, trifluoroethanol.

3The study was supported by the UK Medical Research Council (MR/K000187/1 to V.B.); the UCL Amyloidosis Research Fund and UCL Wolfson Drug Discovery Unit Funds; the Cariplo Foundation (Project No. 2011-2096 to V.B. and 2013-0964 to M.S.); Telethon (grant n. GG14127 to V.B.).

**FIGURE LEGENDS**


**FIGURE 2.** β2-m structure (PDB entry 2yxf) highlighting the N terminal peptide and three key residues: Pro 32, Trp60 and Asp76.

**FIGURE 3.** Schematic picture of the hypothetical events occurring in the interstitial space where globular soluble proteins undergo fibrillar conversion. The chemical physical characteristics of the interstitial space and forces generated by the fluid flow are well reviewed in Swartz (61).

Native globular proteins flow through a network of fibrous proteins (i.e collagen, elastin) and GAGs. These matrix proteins expose hydrophobic patches with which the native globular proteins
collide. At the interface between the hydrophobic surface and the aqueous fluid, proteins are exposed to forces sufficient to perturb the folded state.

The hydrophobic force \( F_{\text{Hydro}} \) acting on the molecule can be calculated according to Mangione et al. (40) through the equation:

\[
F_{\text{Hydro}} = -\left( \frac{dE_{\text{Hydro}}}{dd} \right) = \left( -2Y(a - a_0) \exp \left( - \frac{d}{D_{\text{hydro}}} \right) \right)/D_{\text{hydro}}
\]

\( E_{\text{Hydro}} \) represents the hydrophobic interaction energies between two apolar surfaces, \( Y \) is the interfacial tension, \( d \) is the distance between the two surfaces, \( a \) is the exposed area of the molecule at distance \( d \), \( a_0 \) is the optimum exposed area of the molecule, which we consider to be equal to the area of one amino acid, and \( D_{\text{hydro}} \) is the hydrophobic decay length.

The exposure of normally buried hydrophobic elements further facilitates the interaction with the hydrophobic matrix, local accumulation of partially folded globular conformers reaching a condition of supersaturation. Supersaturation is the pre-condition for protein aggregation and loss of solubility. Even minimal changes in the intensity of the shear flow can break the very labile soluble state of partially folded proteins when they reach the condition of supersaturation. If supersaturation is not reached the simple unfolding of the proteins does not imply a fibrillar conversion and the protein can properly refold and escape from the aggregation.
Table I: Summary of the different methods reported in literature to generate β2-m amyloid fibrils \textit{in vitro}

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Na citrate pH 2.5-4, 37°C 100 μM β2-m in the presence of seeds</td>
<td>16</td>
</tr>
<tr>
<td>50 mM Na citrate, 100 mM NaCl, pH 2.5, 37°C 100 μM β2-m</td>
<td>58</td>
</tr>
<tr>
<td>50 mM Na citrate pH 6.5, 37°C 100 μM ΔN6β2-m in the presence of seeds</td>
<td>25</td>
</tr>
<tr>
<td>50 mM Na citrate pH 7.3, 37°C 100 μM refolding intermediate in the presence of seeds</td>
<td>35</td>
</tr>
<tr>
<td>50 mM Na phosphate, 100 mM NaCl pH 7.4, 0.5% SDS, 37°C 25 μM β2-m in the presence of seeds</td>
<td>19</td>
</tr>
<tr>
<td>50 mM Na phosphate, 100 mM NaCl pH 7.4, 20% TFE, 37°C 100 μM β2-m in the presence of heparin–stabilized seeds</td>
<td>20</td>
</tr>
<tr>
<td>25 mM sodium phosphate, pH 7.0, 37°C, stirring at 250 rpm 40 μM β2-m in the presence of heparin, SAP, apolipoprotein E–stabilized seeds</td>
<td>28</td>
</tr>
<tr>
<td>50 mM ammonium acetate pH 6.4, 20 μM heparin, fibrillar collagen type I, 37-40°C 40-50 μM β2-m</td>
<td>31, 32</td>
</tr>
<tr>
<td>1 M NaCl, pH 7.5, 37 °C, 24 h stirring, incubation without agitation for 25–45 days 30–60 μM β2-m</td>
<td>59</td>
</tr>
<tr>
<td>1M NaCl, pH 7.5, 60-70°C, 24 h stirring 40-80 μM β2-m</td>
<td>60</td>
</tr>
<tr>
<td>25 mM sodium phosphate pH 7.4, 37°C, stirring at 1500 rpm 40 μM Asp76Asn β2-m</td>
<td>40</td>
</tr>
</tbody>
</table>
FIGURE 1.
FIGURE 2.
FIGURE 3

$F_{\text{hydrophobic}} = -(dE_{\text{hydrophobic}}/dd) = -2\gamma(a - a_s)\exp(-d/D_{\text{hydrophobic}})/D_{\text{hydrophobic}}$

Hydrophobic surface

Interstitial space