(REVIEW ARTICLE)

Heparin and β2-microglobulin amyloidogenesis

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Abstract

β2-Microglobulin (β2M) occurs as a precursor protein in dialysis-related amyloidosis (DRA), which is a major complication in lives of patients undergoing dialysis. However, the underlying mechanism by which a native β2M transform into an amyloid β2M remain unclear. This disease has developed exclusively in interstitial tissues, which suggest possible implication of extracellular matrix substances in amyloidogenesis of this precursor protein. By using our monoclonal antibody specific for amyloid β2M, we investigated the function of heparin, that is, one of the glycosaminoglycans, as associated with amyloidogenic conversion of the β2M molecule. We confirmed that heparin induced a dose-dependent and time-dependent unfolding at the C-terminal region of the β2M molecule, which led to amyloidogenic transformation of the β2M molecule and brought about intermolecular interactions between β2M and substances in the interstitial matrix such as GAGs and collagen.

Keywords: β2-microglobulin; Dialysis-related amyloidosis; Heparin; Glycosaminoglycans

1. Introduction

Dialysis-related amyloidosis (DRA) is one of the major complications that is inevitably associated with long-term dialysis treatment used in patients with chronic kidney disease (CKD). Every type of amyloidosis has a specific precursor protein as a causative factor. In 1984, Gejyo et al. identified β2-microglobulin (β2M) as a precursor protein of DRA [1]. This small protein, i.e., with a molecular size of 11,800 Da, exists on membranes of all nucleated cells and is mostly degraded, not excreted, by the kidneys. Amounts produced were estimated to be about 150-220 mg/day/ 60-70 kg body weight, in healthy people [2]; serum β2M levels increase and are found to be extremely high at the end stage of CKD. β2M moves freely through vascular walls and disseminates throughout the extracellular space, where large amounts of glycosaminoglycans (GAGs) and collagen exist. At the end stage of CKD, β2M concentrations in interstitial tissues also increase, supposedly enough to strengthen interactions between β2M and substances in the interstitial matrix such as GAGs and collagen. The general belief is that nonphysiological interplay among these substances contributes to the clinical characteristics of this type of amyloidosis such that it initiates exclusively with arthropathy of the osteoarticular structures [3, 4].

In this review, we first explain the amyloidogenic propensity of β2M molecules, after which we discuss the reason why we focused on heparin, and finally we describe the results of our current work, with implications for the role of heparin in β2M amyloidogenesis.

2. Heparin and Heparan Sulfate

Heparin has been well known as an anticoagulant agent and is routinely used throughout Japan in clinical practices such as hemodialysis, for more than 60 years. Heparin is one of GAGs and highly sulfated form of heparan sulfate (HS), which

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contribute its high negative charge density. On the other hand, heparan sulfate proteoglycan (HSPG) consists of a core protein and another type of GAGs. HSPG is distributed systematically throughout the whole body as a major component of connective tissues, as is collagen. In addition, the number of SO_3 groups per sugar unit of heparan sulfate is close to that of heparin.

In 1987, the first report appeared of the coexistence of HSPG in tissue samples containing amyloid that had been obtained from specimens with experimental systemic amyloidosis that was due to serum amyloid A complicated by chronic inflammation [5, 6]. Subsequent reports were published of the coexistence of HSPG with amyloid β in Alzheimer's disease [7] and with amyloidosis including DRA [8]. This histological evidence led to Snow's concept that GAGs in interstitial tissues dose-dependently promote and stabilize amyloid fibril formation (fibrillogenesis) [9]. With regard to β2M, a binding activity of β2M with heparin was also reported [10]. That such GAG action on fibrillogenesis depends on the number of SO_3 groups has increasingly become clear, but the underlying mechanism by which β2M interacts with GAGs remains uncertain [11]. Among these studies with β2M and GAGs, the report of Yamamoto et al. about treatment of β2M with trifluoroethanol seemed to be intriguing because trifluoroethanol induces unfolding of the β2M molecule as well as guanidine hydrochloride and sodium dodecyl sulfate (SDS) [12]. We believe that unfolding of the β2M molecule, especially unfolding in the C-terminal region, must be a prerequisite for both intermolecular interactions and formation of an amyloid nucleus [13]. Thus, in our study we focused on the actions of heparin in the unfolding of the β2M molecule.

3. β2M and Its Intermediate Conformer

β2M consists of the N- and C-terminal portions and the core. Seven anti-parallel β-strands constitute two β-sheets in the rigid core and acquire a globular configuration by means of approximately 30 inter-strand hydrogen bonds and an S-S bond between Cys25 and Cys80 (B and F strands) at the center. Seven β-strands have been named conventionally as A, B, C, D, E, F, and G, from the N-term to the C-term (Figure 1). The core consists of two β-sheets, i.e., one comprising strands A, B, D, and E, and another comprising strands C, F, and G. Five N-terminal amino acids are temporarily detached from the core surface, even in the native molecule in body fluids. Thus, a native β2M molecule coexists ubiquitously in dynamic equilibrium with a partially unfolded intermediate molecule (conformer), even in the physiological state, because of temporal breaking of hydrogen bond in strand A [14]. The C-terminal portion seem to be folded toward the inside in the global configuration because of the retained hydrogen bond between Asp96 and Met99. In an experimental study, several intermediate conformers with definite, albeit partial, unfolded species were confirmed as occurring in a transitional process from native molecule to amyloid molecule [15]. The unfolding of the β-sheet structure induced by breaking the interstrand hydrogen bonds give rise to changes in the surface charge of the molecule. Thus, we can confirm the unfolding as an alteration of the profile in capillary electrophoresis (CE) [16].
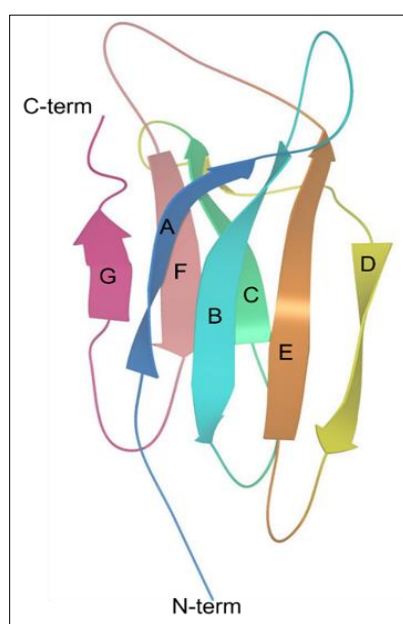
**Figure 1** Seven strands in native β2-microglobulin (β2M) (Protein Data Bank code 2D4F)

We had first used CE to determine the presence of the intermediate β2M conformer in serum from patients undergoing hemodialysis, and we confirmed that the intermediate β2M conformer exists, ubiquitously, in a state of dynamic equilibrium of approximately 1:10, with a native conformer in serum obtained not only from patients undergoing hemodialysis but also from healthy persons [14]. However, a more deformed (destructured) profile was recognized in serum after the hemodialysis procedure in which interstitial fluid was returned (backflow) into circulation from the interstitial space by a rebound phenomenon associated with the hemodialysis procedure itself [17]. A more deformed profile on CE indicates a molecular population of more unfolded conformers, which migrated at a delayed retention time on CE. In contrast, the β2M profile before hemodialysis was constantly like that in a healthy person, which suggested two possibilities: spontaneous refolding into a native conformer in circulation (intravascular space) during dialysis-off days or sequestration in the extravascular space where both GAGs and collagen are abundant. A kinetic study with ^{125}I-labeled β2M by Vincent et al. indicated the latter possibility [18]. They had reported a considerable amount of unrecovered ^{125}I-labeled β2M, which was not exchangeable with β2M in the vascular pool, i.e., no return pool called a capitation compartment existed. We believe that this non-exchangeable compartment must be responsible for the large accumulation of β2M that is retained yearly in the extravascular space during dialysis, even if serum β2M concentrations leveled off. van Ypersele de Strihou et al. estimated an accumulation of β2M in the extravascular space of about 500 g or more during 10 years of hemodialysis [19]. Even with current on-line hemodiafiltration, Canaud et al. estimated a β2M accumulation of 700 mg/body weight/week, which would reach submillimolar or millimolar levels in the extravascular space in several years, which may be comparable with experimental levels [20]. These supraphysiological concentrations are supposedly important for development of DRA, because involvement of the osteoarticular system is lacking in D76N β2M amyloidosis by virtue of no association with renal impairment [21].

One of our original purposes in this study with amyloidogenic β2M was to identify this capitation compartment, as suggested by the kinetic study in patients with dialysis, which we mentioned above. Another purpose concerned finding diagnostic clues about β2M intermediate conformers. Regarding intermediate conformers, two experimental types were proposed as follows: a reversible conformer that can refold into the native conformer and an irreversible conformer, which is an amyloidogenic conformer and cannot refold. McParland et al. reported that the unfolding in the C-terminus may be a critical feature leading to reversibility of the intermediates [22]. However, on CE, we did not directly detect the irreversible conformer in a patient's serum. Moreover, even with the enzyme-linked immunosorbent assay with the monoclonal antibody specific for the C-terminus from Ile92 to Met99, i.e., mAb92-99, we did not detect the amyloidogenic conformer in serum [23]. Therefore, we speculate that the amyloidogenic conformer may be formed exclusively in the extravascular space by interaction with GAGs and may remain there. We believe that once the unfolding process proceeds to form the amyloidogenic conformer, the conformer is immobilized in situ and does not pass through the vascular wall and return to the circulation, except for the limited situation involving the rebound phenomenon at the terminal phase of hemodialysis [17].

Consequently, our data indicated that amyloidogenic intermediates of β2M are not present in circulation, which was confirmed by gas chromatography-mass spectrometry [24].

4. ΔN6β2M

ΔN6β2M is an amyloidogenic variant of β2M, not an intermediate variant, that lacks the 6 N-terminal amino acids that have been found in amyloid tissue from patients undergoing dialysis [25]. Although several artificial fragments of β2M such as the E strand and ΔK56-β2M had proved to be amyloidogenic in experimental studies [26, 27], the ΔN6β2M variant is the natural variant found in amyloid tissues from patients with DRA and is assumed to be a degradation product produced by trypsin from native or aggregated β2M that has accumulated in the interstitial space [28]. Bellotti et al. reported the reversibility (refolding) to the soluble conformer of this variant in an ex vivo study, but a highly fibrillogenic variant had been confirmed experimentally by several researchers including us [29-32]. Although spontaneous fibril formation by this variant was not demonstrated at concentrations of 0.2 mM in the report of Borysik et al. (pH 7.4), our recent study indicated definite fibril formation at 0.1 mM (1 mg/mL) (pH 7.4), a concentration that is supposedly comparable with concentrations in the interstitial space of patients undergoing long-term hemodialysis (Figure 2) [32-34]. Although the C-terminal side strands in ΔN6β2M—E, F, and G strands—have reportedly been mostly conserved, according to nuclear magnetic resonance analysis, our study using mAb92-99 demonstrated the unfolding of the C-terminus of this variant [23]. Thus, 4 hydrogen bonds between the F and G strands may be partially broken. In addition, the isoelectric point of ΔN6β2M should be lower than the 6.4 value of native β2M because of the loss of arginine and lysine in the N-terminus, but the ΔN6β2M peak was found at a delayed retention time by CE analysis, 2 min later than native β2M, and the ΔN6β2M peak on CE differed from that of intermediate β2M found in serum (Figure 3) [23]. As far as we know, this variant has as yet never been reported in serum. The assumption has been that ΔN6β2M is limited to the extravascular space by protease (trypsin) and cannot return to the circulation.
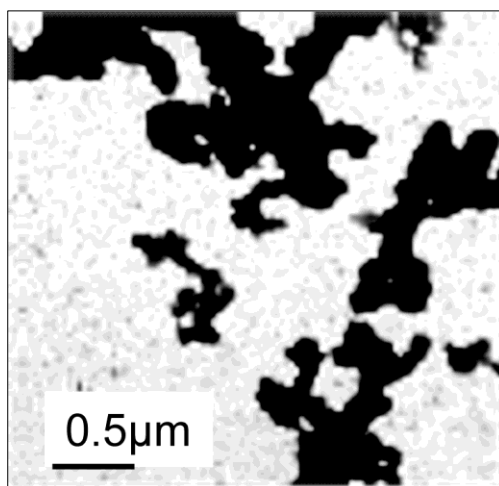
Figure 2 Fibrillogenesis of ΔN6β2M shown by electron microscopy according to the method of Domanska et al [34]. The ΔN6β2M fibrils were prepared by incubating 0.1 mM of the purified protein at 37 °C in 50 mM phosphate buffer and 100 mM NaCl, pH 7.4, in the presence of 20% TFE and 20 μg/mL fibrillar seeds. Fibrils were collected after 1 month by centrifugation
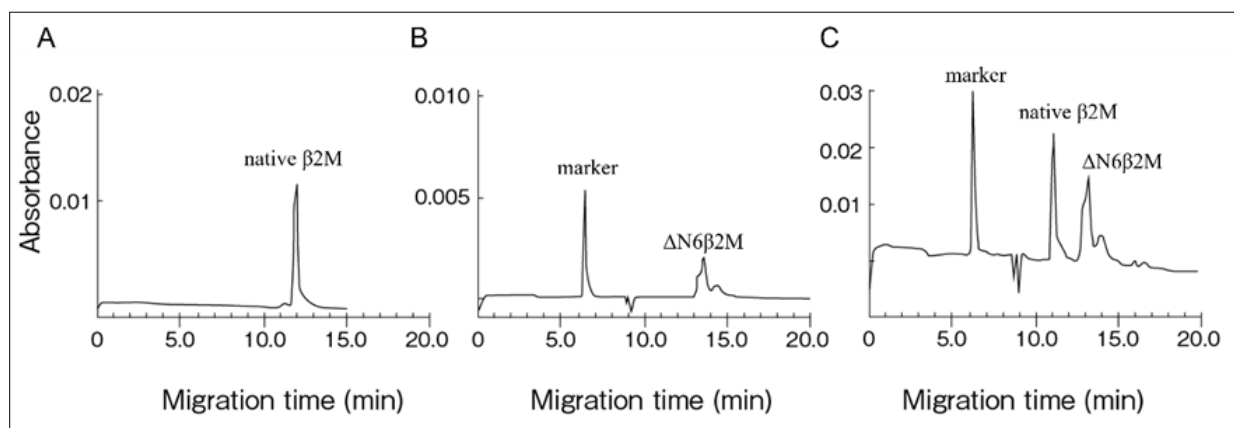

Figure 3 Capillary electrophoresis profiles. (A) Native β2M: a single peak with a sloping shoulder at the retention time of 12 min. (B) ΔN6β2M: a double peak at the retention time of 14 min. (C) Mixture of native β2M and ΔN6β2M. Figure adapted from ref. [23]

As mentioned above, the concentrations of β2M that accumulate in the extravascular space must reach levels as high as submillimolar concentrations in patients receiving maintenance hemodialysis for more than 10 years, which may be sufficient concentrations for amyloidogenic molecules to cause intermolecular interactions and to form aggregations and/or oligomers or to generate ΔN6β2M via protease [35].

5. C-Terminal Unfolding in β2M Induced by Heparin

In the clinical setting of hemodialysis, two types of heparins, i.e., high-molecular-weight heparin (HMH) and low-molecular-weight heparin (LMH), have been used routinely. Although clinical application of LMH has been now approved, HMH is still generally used in hemodialysis [36]. We previously reported the interactions of native β2M and two amyloidogenic β2M variants, i.e., ΔN6β2M and D76N β2M, with HMH and LMH [37]. Our binding study not only confirmed the binding of ΔN6β2M and D76N β2M with HMH but also suggested the binding potential of native β2M with HMH (Figure 4A). The K_D (dissociation constant) values of native β2M, ΔN6β2M, and D76N β2M as related to HMH were 3.71×10^{-6} M, 2.07×10^{-8} M, and 1.72×10^{-7} M, respectively. The K_D value of ΔN6β2M was low enough to be comparable to the K_D value of ΔN6β2M with the specific monoclonal antibody mAb92-99. In contrast, LMH showed no association even with ΔN6β2M (Figure 4B). Because GAGs in the interstitial space are rich in heparin molecules, β2M has been

trapped and accumulated time-dependently in the interstitial space in patients undergoing hemodialysis, and β2M levels are expected to reach values as high as millimolar concentrations in 10 years or more. It is interesting that HMH induced a C-terminal unfolding of native β2M, which indicated production of an amyloidogenic intermediate, i.e., β2M92-99, in a time-dependent manner (Figure 4C). LMH, however, did not cause C-terminal unfolding in native β2M, even after incubation for 24 h [37].
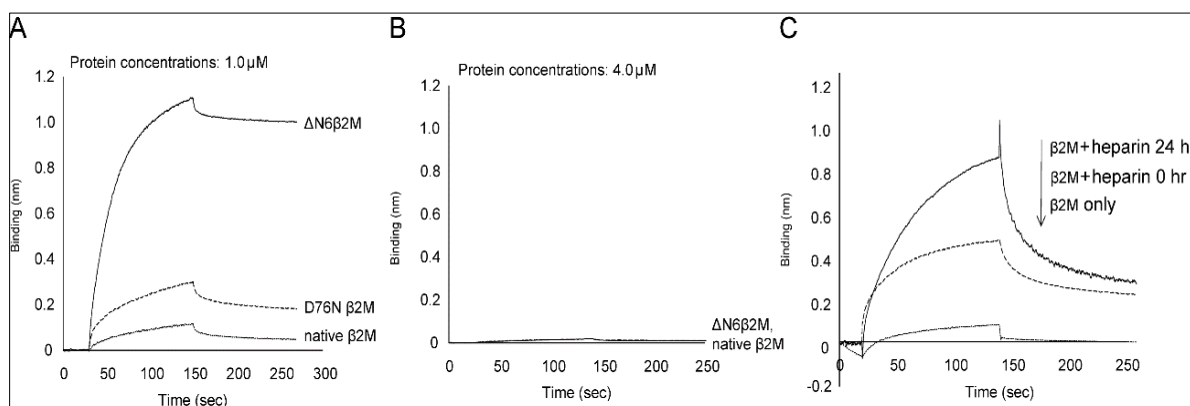
Figure 4 Analysis of the interactions of β2M variants with high-molecular-weight heparin (HMH) (A) and low-molecular-weight heparin (LMH) (B), and heparin-dependent interaction of native β2M with mAb92-99 (C) via a biolayer interferometry technique. β2M variants—ΔN6β2M (solid line, —), D76N β2M (dashed line, ---), and native β2M (dotted line, ····)—were injected onto the HMH-immobilized (A) or LMH-immobilized (B) sensor chip, respectively. Native β2M incubated with HMH for 0 h or 24 h was injected onto the mAb92-99-immobilized sensor chip (C). Figure adapted from ref. [37]

Furthermore, our study showed a distinct difference in the strength of the bond between HMH and the two β2M variants ΔN6β2M and D76N β2M. Valleix et al. first reported the amyloidogenicity of D76N β2M, which is a natural amyloidogenic mutant [21]. However, patients with D76N β2M showed no signs of CKD, and their serum β2M levels continued to be close to normal, that is, approximately 0.1 μM. ΔN6β2M had a moderate response with HMH even at 0.1 μM, compared with the response of D76N β2M, at 1.0 μM, which showed a 10-fold difference in the intensity of the binding response for these two variants. A similar difference in collagen-binding affinity was reported for ΔN6β2M and native β2M [28]. Because the C-terminus of D76N β2M was confirmed as unfolded, as was that of ΔN6β2M, a difference in binding response levels for the two amyloidogenic variants may result from a difference in the populations of molecules with C-terminal unfolding, i.e., molecules with C-terminal unfolding should occur considerably more commonly in ΔN6β2M than in D76N β2M [21, 37].

During amyloidogenesis, an intermediate molecule of a precursor protein with a partially unfolded structure had been generally known to be a key molecule. For β2M, we had verified that the unfolding at the C-terminus from Ile92 to Met99 was the key process for amyloidogenicity of this precursor protein [23]. Our study demonstrated that HMH could cause a C-terminal β2M concentration to change to 1 μM, which is comparable to the serum concentration in patients undergoing hemodialysis.

A majority of SO_3^- groups in GAGs, as in SDSs, has been known to induce conformational changes in proteins. For β2M, several studies indicated amyloidogenic conversion depending on the large number of SO_3^- groups in GAGs [28]. As is well known, heparin is highly negatively charged and highly sulfated (SO_3^- groups). Although we have not studied dose-dependent effects of heparin on the C-terminal unfolding of β2M, we believe that the number of SO_3^- groups in heparin may make the difference in the results for HMH and LMH. The conformation of the β2M molecule is maintained by multiple intramolecular hydrogen bonds, which may be broken, partly by the SO_3^- groups.

Our studies together pointed to two actions of HMH with β2M, i.e., direct binding and induction of C-terminal unfolding. The former may result in an accumulation of β2M in the interstitial space and the latter may induce amyloidogenicity of β2M. LMH, however, showed no definite interaction, even with ΔN6β2M, which indicated a superior clinical availability compared with HMH as an anticoagulant in long-term hemodialysis, in which DRA is a serious complication.

6. Discussion

According to a concept that was based on several experiments, amyloid fibrils form via two serial processes: first, formation of an amyloid nucleus (nucleation) and then subsequent fibril formation [38]. Thus far, since the study of interstitial substances by Snow and Kisilevsky, both GAGs and collagen have proved to induce and stabilize fibrillogenesis [5, 6]., and a scaffolding effect of collagen via electrostatic action was confirmed as a factor contributing to fibrillogenesis of β2M [39]. For GAGs, an association between sulfated GAGs and β2M fibrillogenesis was also proved but a relationship with the nucleation process was not yet fully clarified [12, 33]. The nucleation process is a rate-limiting step in amyloid formation, which suggests that nucleation is unlikely to proceed spontaneously in vivo because of the high energy barrier [38]. However, C-terminal unfolding is believed to lower the energy barrier and quickly facilitate the nucleation process. Nucleation is thought to start by means of formation of highly unfolded intermediate molecules, and such unfolded intermediate molecules are poorly reversible to native molecules or are irreversible [31]. In clinical settings, these kinds of intermediate β2M molecules may be generated and limited to the extravascular space, where reversible intermediates transferred from the vascular space undergo more unfolding by means of interactions with sulfated GAGs. Such intermediates supposedly have extensively unfolded C-termini that differ from the intermediate conformers that occur in serum and are likely to induce intermolecular interactions. Intermolecular interactions involve the generation of aggregates or the formation of oligomers, which leads to nucleation. In addition, β2M trapped by the GAG network is also believed likely to undergo cleavage or truncation via proteases such as trypsin and to generate ΔN6β2M, i.e., the amyloidogenic variant. In the body, protease activation is essentially regulated by protease inhibitors such as α_2-macroglobulin. Thus, as previously reported, circulating levels of the α_2-macroglobin-β2M complex were expected as an indicator of ΔN6β2M generated in the extravascular space in patients undergoing hemodialysis [13]. As shown here, ΔN6β2M showed definite unfolding in the C-terminus. Such a variant with extensive unfolding can contribute directly to formation of the amyloid nucleus (nucleation) on its own. McParland et al. had revealed that the unfolding process proceeds in a specific order, not in a random order, such as beginning with the N-terminus and A strand, and then proceeding to the C-terminus and G strand [22]. The amyloidogenicity of intermediates is thought to depend on the reversibility of their refolding. To our knowledge, no report has as yet clarified the critical point of reversibility. However, as McParland pointed out, we also believe that the highly unfolded state such that both the C-terminus and G strand are unfolded may be necessary for amyloidogenic conversion of β2M, which suggests partial breaking of 4 hydrogen bonds between the G- and F-strands, at least. Such an unfolded state can be detected by mAb92-99 [13, 23].

Eichner et al., however, had demonstrated that trans-isomerization of Pro32 is a critical event for β2M amyloidogenicity [40], and Rennella et al. showed that Pro32 was trans-isomerized in I_2 (kinetic intermediate), as well as in the amyloid variant D76N β2M [41]. However, which of these processes, i.e., Pro32 trans-isomerization or C-terminal unfolding, is responsible for the amyloidogenicity of ΔN6β2M and/or D76N β2M remains unclear.

In our previous histological study with mAb92-99, which is the specific antibody for the C-terminus, we detected this kind of β2M with extensive unfolding in pre-amyloid tissue where Congo red reactions remained negative [13]. A negative Congo red stain indicated the presence of immature fibril structures, i.e., protofibrils, which also suggested premature β2M intermediates with extensive C-terminal unfolding. However, Chiti et al. had reported that their intermediate β2M molecule, termed I_2, showed a definite binding both with Congo red and thioflavin T [42]. However, intermediate I_2 was experimentally identified as in transition from completely unfolded β2M by guanidine hydrochloride, which may be more destructured than intermediate β2M found in clinical settings.

Pro32 in I_2 had reportedly been trans-isomerized, but whether Pro32 or the unfolded C-terminus take an initiative role in amyloidogenesis remained to be determined; nevertheless, we believe that extensive unfolding (destructured) in the C-terminal region may be a prerequisite for intermolecular interactions and subsequent nucleation.

Three kinds of force including hydrogen bonds (H–H), hydrophobic interactions (CH_2–CH_2), and electrostatic interactions (NH_4^+–O^-) supposedly exist among the 7 strands in the β2M molecule, and heparin can have an effect on all of them [43].

Therefore, C-terminal unfolding should be interpreted as being a net effect by heparin. As mentioned earlier, C-terminal unfolding is thought to enable intermolecular interactions, which may be also affected by heparin. We believe that heparin is implicated in multiple aspects of the nucleation process. A recent in silico study also suggested the importance of the unfolding in the C-terminus for β2M amyloidogenicity (Figure 5) [44].
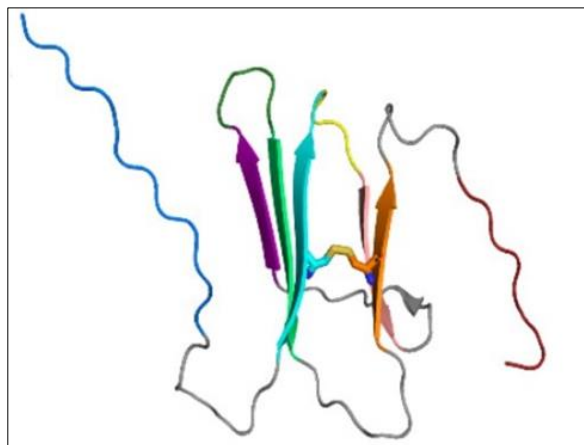
Figure 5 The I$_2$ intermediate populated by D76N β2M, which features a well-preserved core and two unstructured and decoupled termini. The N- and C-termini are blue and red, respectively. The BC- and DE-loops are yellow and green, respectively. Figure adapted from Figure 1C in ref. [44]

Relini et al. had reported that heparin enhanced β2M fibrillogenesis by collagen, which is of great importance from the clinical viewpoint because sulfated GAGs including heparin coexist ubiquitously with collagen in the body [28]. As demonstrated here, heparin gives rise to C-terminal unfolding to react with mAb92-99 in the β2M molecule, which is sufficient to form the nucleus itself. Therefore, in vivo, heparin is expected to have a synergistic effect on nucleation as well as fibrillogenesis.

7. Conclusion

The unfolding in the C-terminal region from Ile92 to Met99 is a primary prerequisite for induction of the amyloidogenicity of β2M. An essential and sufficient condition for this unfolding is regional accumulation of β2M to concentrations as high as submillimolar values that are comparable to laboratory concentrations. By using HMH and LMH, we determined that sulfonated GAGs are critical both for formation of an amyloid nucleus and for this regional accumulation. This idea forms the foundation of our proposed "β2M shuttle hypothesis" in DRA [17]. Given that the nucleation process is a rate-limiting step in amyloid formation, the clinical importance of sulfated GAGs in interstitial tissue should be stressed. Therefore, the interstitial space may be the origin of amyloid β2M. With reference to the amyloidogenicity of β2M, we also described several features of ΔN6β2M, i.e., an amyloidogenic derivative.

Compliance with ethical standards

Acknowledgments

Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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