Protein Misfolding Diseases

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Abstract: Diseases caused by protein misfolding are an emerging pathologic category that are thought to share some basic common mechanisms and display impressive heterogeneity in terms of tissue involvement, age of onset and clinical features. The growing recognition of the impact that protein misfolding has on human diseases is certainly related to the phenomenon of population aging and the expansion of the population in which these diseases are more frequent, but it is also based on a scientific revolution that looks at protein dynamics and relates these data to their potential pathologic implications. The multidisciplinary exchange of knowledge between experts in apparently unrelated diseases, such as sickle cell anemia and Alzheimer's disease, has helped clarify the pathogenesis of these and many other diseases. The quick expansion of knowledge on the mechanisms of these diseases is priming pharmaceutical research that is now providing the first prototype drugs.

Keywords: Protein misfolding disease, amyloid disease, pathogenesis, protein aggregation and cytotoxicity.

INTRODUCTION

Protein misfolding diseases belong to an emerging category of diseases that are determined by abnormalities regarding the folding parameters inscribed in what is recognized as a secondary level of the genetic code. Folding abnormalities can be associated with diseases caused by the decreased presence of a specific protein that never achieves a functional folded structure or can be at the basis of diseases caused by intracellular or extracellular accumulation of insoluble, abnormally aggregated proteins. Therefore, these two types of mechanisms are responsible respectively for the lack of function and the gain of function detectable in "protein misfolding diseases" [1]. The diseases caused by protein misfolding, i.e. incorrectly folded and rapidly degraded proteins, are extremely heterogeneous and their phenotype strongly depends on the availability of molecules potentially capable of recuperating the function of the affected protein. Diseases based on protein misfolding and loss of function are distributed over each phase of life and are associated with genetic diseases such as cystic fibrosis in the pediatric age or diseases of adult life when, for example, somatic mutations in proteins controlling the cell cycle, i.e. p53, can cause cancer. Diseases induced by protein aggregation, on the contrary, are predominantly associated with aging and a long lag phase is observed before the first deposition occurs. However, regarding the latter there exist two emblematic exceptions of protein aggregation diseases: sickle cell anemia and Z alpha-1 antitrypsin (Z A1AT) deficiency, which are associated with protein deposition at the level of synthesizing cells and cause disease in the pediatric population. It is worth noting that both in alpha-1 antitrypsin deficiency and sickle cell anemia, the polymerization of the pathogenic protein does not require a massive conformational change and the folding motif of the protein in the monomeric/globular state is recognizable also in the polymer. Whereas in the majority of age-associated cases, or late onset, diseases caused by protein aggregation, protein polymerization requires an extensive conformational change.

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PROTEIN MISFOLDING DISEASES CAUSED BY LOSS OF FUNCTION

On the basis of the growing number of pathological processes in which folding abnormalities are involved, it has been proposed that at least half of human diseases are somehow associated with protein misfolding [2]. An extensive review of all the diseases in which protein misfolding is involved has not been published, but there are prototypic diseases caused by misfolding of well characterized proteins like cystic fibrosis transmembrane regulator (CFTR) and p53 which *in se* represent the essential molecular features of this disease category.

CFTR MISFOLDING AND CYSTIC FIBROSIS

Cystic fibrosis, a classic pediatric disease, was pathologically and clinically first described at the beginning of the 17th century [3], but its molecular basis was only established in 1989, when the gene coding for the transporter involved was identified [4]. In the cell, the folding of non-pathologic CFTR is spatially associated with the lipid bilayer of the endoplasmic reticulum (ER) and several molecules that assist folding are involved in the process. The folding pathway for CFTR is extremely complex considering that it is a single polypeptide that creates both membrane and cytosolic domains and the kinetics of folding for the nucleotide binding and transmembrane domains are still debated [5]; but certainly folding is essential for the transfer of the protein from the ER to the plasma membrane [6]. After

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identifying the gene coding for CFTR, the disease was shown to be associated with approximately 1500 mutations. Among these mutations, the Phe508 deletion is certainly the best studied at the molecular level. In the presence of this mutation, CFTR is not released from the ER and only a few molecules reach the membrane. However in cell culture models, it has been shown that lowering the incubation temperature [7] or treatment with chemical chaperons is able to stabilize the folded conformation [8] and the membrane localization of the protein is rescued at what is considered an acceptable therapeutic level. In the absence of high resolution data for the 3D structure of CFTR, the structural impact of mutations is not readily evaluable. However the increased sensitivity to proteolytic digestion, i.e. in the case of the Asp508Phe variant [9], suggests an altered folding stability, which is confirmed by evidence of dramatically reduced thermostability of the variant in comparison with the wild type [10]. This destabilization was partially, but significantly rescued by the chaperone effect of glycerol. Extensive work carried out on the misfolding propensity of mutated CFTR, which is undoubtedly a very difficult protein to study, has facilitated the development of a new drug targeting the misfolding propensity of CFTR mutants [11]. This drug, that will be evaluated in a forthcoming phase II clinical trial, represents the fruitful product of joint activities between a pharmaceutical company and the Cystic Fibrosis Foundation Therapeutics (http://www.vpharm.com/current-projects/drug-candidates/VX-809.html).

P53 MISFOLDING AND CANCER

The possibility that destabilization of a specific protein could be related to cancer susceptibility first came from the discovery that p53 mutations strongly affect protein stability. The protein p53 is considered a tumor suppressor protein, it acts as a transcription factor and regulates the expression of genes involved in DNA repair, cell cycle arrest and apoptosis [12]. The association of p53 mutations and cancer was first established for a familial, early-onset, form of cancer susceptibility [13, 14]. However, pathogenic somatic mutations in the p53 gene have been also identified in approximately 50% of epithelial cancers. Pathogenic mutations of p53 are classified as *contact* or *structural* mutants on the basis of molecular data and level of function. *Contact* mutations affect DNA binding affinity, whereas *structural* mutants affect the protein architecture. A few mutations that perturb zinc binding sites destabilize both the structure and interaction with DNA. The large majority of all pathogenic p53 mutations reduce the thermodynamic stability of this protein that is already intrinsically quite unstable under physiologic conditions. Degradation of p53 is facilitated by its low thermostability and by the presence of the negative regulator MDM2 through which E3 ubiquitin ligase sequesters p53 for proteosomal degradation [15]. The MDM2/p53 system represents a paradigmatic example of how nature controls the level of key proteins by controlling their degradation. DNA damage, in fact, is associated with p53 phosphorylation which in turn induces a dissociation of p53 from MDM2 thus blocking p53 ubiquitination and degradation. DNA damage is also associated with increased MDM2 degradation and consequently better preservation of p53. Considering the intrinsic instability of p53 even when enhanced by a pathogenic mutation we would expect very low levels of p53 in the cell. On the contrary, p53 and especially the mutated forms are over-represented in cancer cells through a mechanism that has long challenged cancer biologists [16]. In pathologic cells, the mutated and misfolded p53 forms multiple chaperon complexes. These heterocomplexes stabilize the protein, sequester p53 [17] and block the degradation mediated by the proteasome [18], but p53 function is frozen and unable to prime apoptosis of the neoplastic cells. The elucidation of the p53 mutation effect on the thermodynamic stability, misfolding propensity and vain interaction with chaperones has promoted several new strategies for rescuing the function of p53. Prototypes of innovative pharmaceutical targets include regulators of MDM2, for example Nutlin 3a, which efficiently disrupts the MDM2-p53 complex [19] and minimizes protein ubiquitination. Stabilization of p53 with small ligands could also prevent the misfolding and consequent degradation/sequestration of the protein. The most promising compound from this category is CP-31398, a molecule capable of stabilizing the native conformation of p53 [20]. Even though the *in vivo* mechanism of CP-31398 is still controversial [21] this compound has already shown significant clinical effects in an animal model [22].

PROTEIN MISFOLDING, SELF AGGREGATION AND GAIN OF PATHOLOGICAL FUNCTION

The number of diseases caused by intracellular or extracellular accumulation of protein aggregates has grown exponentially and now the process is recognized at the basis of several diseases mainly associated with the process of aging and a late clinical onset. However a few protein aggregation diseases have an early onset. Sickle cell anemia and Z alpha-1 antitrypsin deficiency are both characterized by the intracellular, pathologic polymerization of globular proteins that maintain the major part of the native structure in the insoluble polymer. Sickle cell anemia is clinically evident in patients homozygous for the mutation Glu6Val in the hemoglobin (Hb) β chain. Early hematologic signs of the disease are already detectable at the age of ten weeks, but the symptoms appear at the age of 6-12 months concurrently with a decrease in circulating fetal hemoglobin that naturally protects hemoglobin S (HbS) from aggregation. The kinetics and thermodynamics of HbS polymerization have now been extensively described. If deoxygenated HbS is above its critical solubility it starts polymerizing into fibers that are rigid enough to distort the flexible red blood cells. The metastability of deoxy HbS has been demonstrated in systems in which compartimentalization was obtained by partitioning with oil but also in uniform bulk solutions [23]. Polymerization is initiated by a nucleation step that can be primed by homogeneous and heterogeneous nuclei. The activation of polymerization is strongly influenced by molecular crowding. The effect of crowding agents on the kinetics of deoxy HbS aggregation has been calculated: a 50% increase in crowding agents can exponentially increase the growth rate of the polymer by 10^4-10^5 fold [24].

A metastable state is also naturally adopted by the protease inhibitors, serpins which limit cleavage after interaction with the enzyme, this mechanism is facilitated by an ester bond that forms between the serpin and the protease.

These covalent modifications induce extensive conformational changes that increase the β-structure, stabilize the acylated form of the enzyme and dramatically increase the thermostability of the serpin. These protease inhibitors have therefore evolved toward a structure compatible with large conformational modifications and flexibility of functionally important domains. The serpin family contains proteins such as alpha-1 antitrypsin and neuroserpin that, in mutated forms, are responsible of prototypic "protein misfolding diseases" [25]. The spectrum of the mutational effects is particularly broad for alpha-1 antitrypsin. In this case some mutations, for example Glu264Val (S A1AT), are mildly pathogenic and only in association with concomitant pathogenic elements (i.e. cigarette smoking) through a loss of function, because in the homozygous state the A1AT is reduced by 40%. On the contrary, patients homozygous for Glu342Lys (Z A1AT), in which the reduction of circulating A1AT is around 90%, are severely ill due to the concomitant reduction of circulating A1AT and the hepatocyte accumulation of aggregated protein. The structural mechanism for the polymerization of the Z form of A1AT has been described and is very similar to the process of aggregation for other serpins including the neuroserpin responsible for "Familial encephalopathy with neuroserpin inclusion bodies" (FENIB). In their seminal study in Nature, Lomas *et al*. [26] demonstrated that polymerization was generated through the interaction of the reactive centre loop of one molecule and a gap in the A-sheet of another. The polymerization chain reaction was clearly sensitive to the shift in temperature from 37° to 41°C and was blocked by the insertion of a specific peptide in the A-sheet. There is evidence that serpin variants that self polymerize in the endoplasmic reticulum have abnormal folding kinetics and the intermediate M* that transiently populates the folding pathway is more stable and persistent in the presence of these mutations [27] and there is a strong correlation between the persistence of partially folded intermediates and the rate of polymerization [28]. Intracellular serpin polymerization typical of Z A1AT and neuroserpin associated with FENIB occurs at the level of the endosplasmic reticulum. Apparently the intracellular aggregate does not activate the unfolded protein response [29], but, through a calcium dependent pathway, triggers the expression of NF-kB. Such a response is typical of the ER to nucleus signaling associated with ER protein overload [30].

PROTEIN DESTABILIZATION, EXTENSIVE STRUCTURAL MODIFICATION AND AGGREGATION OF PROTEINS IN AMYLOID DISEASES

The misfolding, conformational changes and aggregation described for serpin variants are quite similar to the processes that occur in amyloid diseases. However the molecular process of amyloid deposition is significantly different when entire globular proteins are converted into fibrils. The main structural difference between amyloidosis and other protein aggregation diseases is that the secondary and tertiary structure of the fibrillar polymers are extremely different from that of the precursor protein. This phenomenon follows a certain level of graduation from protein to protein, in fact the structural distance existing between the globular amyloidogenic lysozyme variants and their fibrils is certainly greater than that of globular β2-microglobulin (β2-m) and its fibrils. However, the structural transition always involves a large part of the entire molecule. The clinical features of the amyloidoses are extremely heterogeneous and even if we consider just "canonical amyloidoses" those in which the fibrils are deposited in the extracellular space and not inside the cell, we have clinical features that configure osteodegenerative diseases (as in β2-m associated amyloidosis), peripheral neuropathy (as in certain transthyretin amyloidosis), cardiomyopathy (as in amyloid light chain, apolipoprotein A-I and transthyretin associated amyloidoses), neurodegeneration (as in Aβ deposition) and many other clinical features associated with these and other proteins. The large majority of amyloidoses occur later in life and even those genetically transmitted belong to the category of late onset disease. The only *pediatric amyloidosis* is that associated with the lipoprotein SAA (serum amyloid A protein), which is highly increased in the acute phase response and in genetically transmitted auto-inflammatory diseases [31]. This is an acute form of amyloidosis and the formation of fibrils in this case is made possible by the contemporary fulfillment of at least three conditions necessary for fibril formation: 1) a high level of protein precursor (hundred fold higher than physiological levels), 2) proteolytic processing that releases a peptide in amyloidogenic conformation, 3) over expression of specific pro-amyloidogenic molecules such as glycosaminoglycans (GAGs).

The protective role of elements such as protein degradation mechanisms and chaperone assisted solubilization are not well characterized as the three prior mentioned elements, but it is likely that they are weakened during disease. Also in late onset amyloidoses, the three favoring key elements (i.e. concentration of protein precursor, destabilization of native structure or release of an amyloidogenic peptide and pro-amyloidogenic tissue factors) are hallmark characteristics of amyloidogenesis. The weakening of protective elements against amyloidogenesis are certainly important in late-onset amyloid diseases and can be responsible for the variability in the onset of disease even in families where the members are carriers of the same pathogenic mutation. Many of the molecular mechanisms of amyloidogenesis are shared by all the amyloidoses, but every single disease has peculiar features that are mostly related to structure, function and metabolism of the single amyloidogenic protein. We have concentrated our research in the last few years on β2-m, an amyloidogenic globular protein which best illuminates the prototypic aspects of all the amyloid diseases. In particular, the three key factors mentioned above are well characterized in this amyloidosis.

ROLE OF CRITICAL PROTEIN CONCENTRATION

The protein concentration is particularly important, in fact in amyloidosis resulting from hemodialysis, β2-m can reach and maintain a serum concentration ranging between 10 and 20 μg/ml (10-20 fold higher than normal). Persistent high protein concentration, as well as, a long latency of at least ten years is necessary for *in vivo* amyloid deposition. What is progressively changing in the system during this relatively long "lag phase" in unknown. In this amyloidosis as in many other hereditary forms of the disease such as transthyretin amyloidosis, depending on the type of muta-

tion, the lag phase before the onset of amyloid deposition can last from 40 to 60 years. As in the case of many other amyloidogenic globular proteins, the conversion of native β2-m into fibrils apparently requires relevant conformational modifications. In fact, *in vitro* fibrillogenesis is obtained at a very low pH or in the presence of a denaturing concentration of trifluoroethanol [32]. *In vivo*, β2-m accumulates along the surface of collagen types I and II before the fibrillar transition, due to the intrinsic affinity of β2-m for this fibrous protein [33]. Therefore a margination effect probably facilitates the creation of protein crowding along the collagen surface. We have shown *in vitro*, under conditions mimicking the pathophysiology of hemodialysis, that concentrated β2-m can self aggregate into a net of amyloid fibrils which adhere to collagen fibers [34, 35]. The formation of amyloid fibrils on the collagen surface is a rapid phenomenon *in vitro*, it is appreciable after one week of incubation, whereas fibrillar conversion of the protein in bulk solution is extremely slow even if nuclei of collagen-amyloid complexes are present. It can be hypothesized, that also *in vivo*, the formation of small nuclei of amyloid fibrils adhering to collagen could be an early event, whereas the massive formation of deposits require further events with slower kinetics.

ROLE OF PROTEOLYSIS IN AMYLOIDOGENESIS

The kinetics of amyloid deposition can be accelerated by the formation of an N-terminal truncated species of β2-m. Classical chemical analysis of natural β2-m fibrils as well as more sophisticated proteomic approaches have established that in all natural β2-m amyloid fibrils [36-38], but not in the amorphous aggregate [39] there is always present a truncated species lacking the first six N-terminal residues (ΔN6β2-m). It has been shown that this β2-m conformer has the capacity to generate fibrils at neutral pH [40, 41], and accelerate the fibrillar conversion of soluble β2-m in the presence of nuclei of collagen-amyloid complexes [35]. The putative protease responsible for the Lys6-Val7 cleavage is unknown, but it should be sought probably among the various proteases that

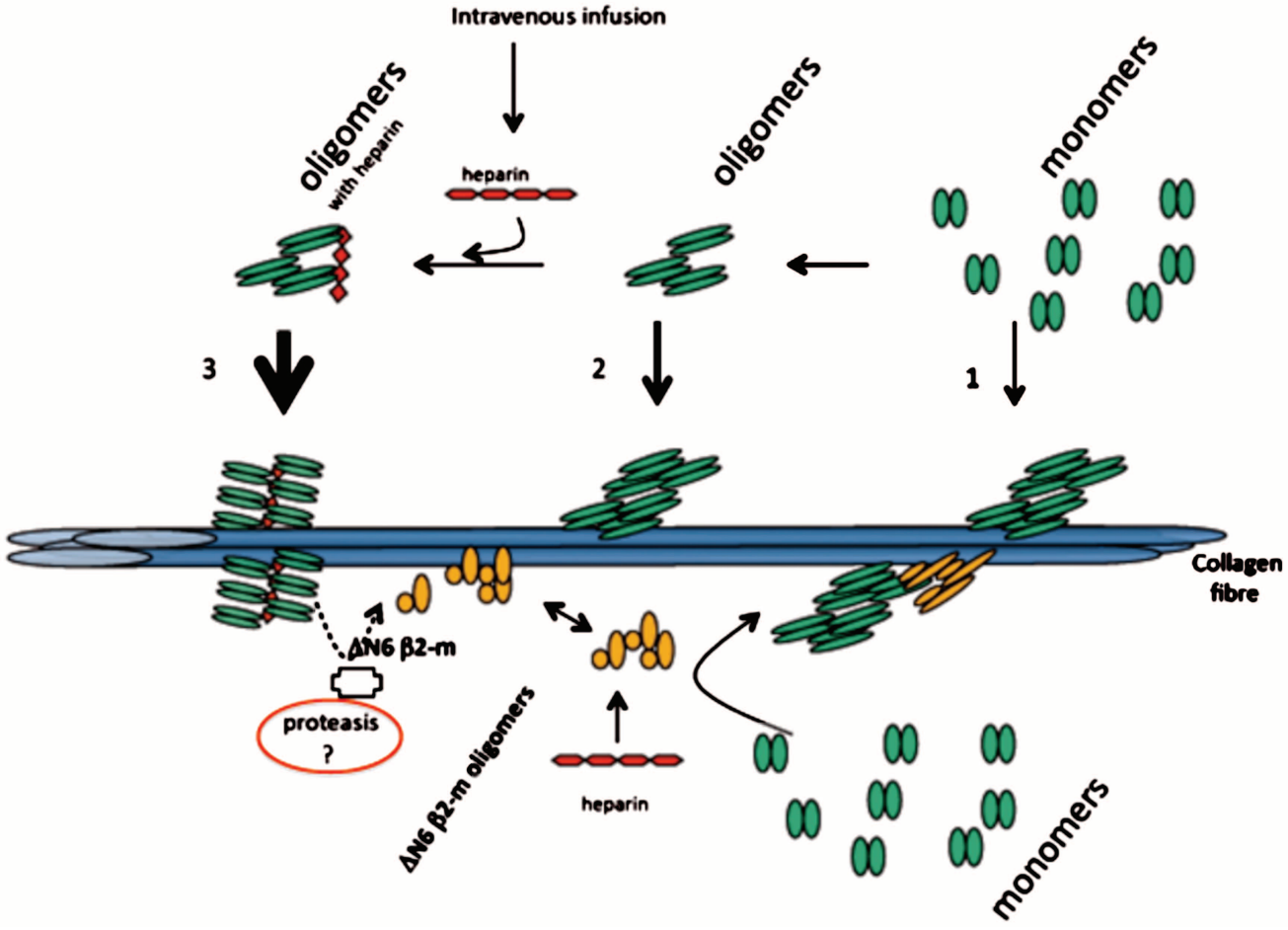

Fig. (1). Proposed mechanism of β2-m amyloidogenesis occurring at the surface of a collagen fiber. Monomers of β2-m can form fibrils at a slow rate (arrow 1) but the kinetics of fibril formation are faster in the presence of β2-m oligomers (arrow 2). Heparin facilitates the formation of β2-m oligomers and further accelerates the kinetics of fibrillogenesis (arrow 3). Once fibrils are formed, β2-m becomes sensitive to proteolytic cleavage in the first strand and the truncated species ΔN6β2-m is formed. ΔN6β2-m quickly oligomerizes and can recruit full length β2-m into new oligomers that are quickly adsorbed on collagen. The adsorption of ΔN6β2-m and heterogeneous oligomers on collagen is particularly facilitated by the high affinity of the truncated β2-m for collagen. The ΔN6β2-m species, in the presence of GAGs, can *per se* generate fibrils in a physiological environment.

continuously remodel collagen. However we have shown that trypsin itself can selectively cleave β2-m at the C-terminal of Lys, but only if β2-m is in the fibrillar conformation [42] or attached to the collagen [35]. The limited proteolytic cleavage of amyloid proteins which subsequently aggregate into natural fibrils is a common but enigmatic observation in the large majority of the amyloidogenic proteins with the well known exception of lysozyme variants [43]. The pathogenic impact of selective cleavage has been recently highlighted [44] by the observation that some proteolytic cleavages of transtyretin are associated with selective heart deposition and late age onset of the disease [45].

OLIGOMERISATION PRECEDES THE FORMATION OF FIBRILS

The truncated ΔN6β2-m can be a key player in the formation and stabilization of fibrils *in vivo* due to its tendency to oligomerize and generate polymerization seeds. Nucleation is an essential process of amyloidogenesis and can dictate the expansion of deposits *in vivo*. Small aggregates of ΔN6β2-m even at a very low concentration and in physiologic buffer can recruit full length β2-m and generate heterogeneous polymers [46]. We have shown that the affinity of the trimeric/tetrameric ΔN6β2-m for collagen is 10^{-6}M, ten fold higher than that of the monomeric full length protein. Oligomerisation *per se* can explain the increase in affinity of β2-m for the collagen surface [47]. The formation of β2-m oligomers, which is facilitated by the N-terminal esapeptide truncation, can have a pathological impact on at least three levels. As proposed in the scheme of (Fig. **1**) the formation of oligomers *in situ* can dramatically accelerate the local deposition of fibrils. A second possible effect of stable, but soluble oligomers, for example those created by ΔN6β2-m, might consist in their putative role in colonization of amyloid in contiguous tissues as a recent clinical report has suggested [38]. Amyloid "metastatization" has been proposed based on several clinical observations, but the precise mechanism by which the amyloid or "amyloid primers" diffuse during the disease is uncertain. However, the Westermark group has recently discovered, that blood monocytes, isolated from animals affected by experimental AA amyloidosis (associated with serum amyloid protein) contain amyloid-seeds capable of priming amyloid formation in other, amyloid-free tissues of the same animal or even in non affected animals [48]. Finally, the oligomerisation of β2-m can influence the β2-m proteotoxicity which is potentially responsible for several symptoms of the disease. It is worth noting that these symptoms require both the presence of amyloid deposits and an high concentration of β2-m [49]. This clinical observation might be explained by the pro-oligomerizing effect produced by the extracellular amyloid deposits in the presence of high concentrations of circulating β2-m [50].

We have recently confirmed that β2-m in solution is toxic for certain cells [51] and this toxicity can be abrogated by the simple removal of β2-m oligomers [52]. β2-m is capable of activating the osteoclast, which has a potent effect on bone erosion and amyloid deposition, however it is unclear whether this effect can be attributed to the oligomeric forms of β2-m [53].

CHARGED GLYCOSAMINOGLYCANS ARE POTENT PRO-AMYLOIDOGENIC NATURAL FACTORS

Glycosaminoglycans are ubiquitous [54] and pathogenically important [55] constituents of all the amyloidoses including the form caused by β2-m [56]. In the case of β2-m, Yamamoto *et al.* [32] first demonstrated that heparin strongly accelerates the extension of amyloid seeds *in vitro*, through a mechanism of binding that stabilizes the fibrils and oligomers. We have confirmed this finding utilizing a method of β2-m fibrillogenesis that closely mimics *in vivo* conditions. The pro-amyloidogenic mechanism is however uncertain. It has been demonstrated that heparin binds monomeric β2-m with very low affinity [57], thus suggesting that the main target of GAGs are probably not the monomers, but the oligomeric forms of the amyloidogenic protein as proposed by Calamai *et al.* [58]. Stabilization of oligomers by GAGs has an immediate effect, shifting the equilibrium monomer-oligomer toward the oligomer species as experimentally shown in our β2-m model [35] (Fig. **1**). All the most active GAGs in amyloidogenesis are highly sulfated [59] and it is plausible that the interaction with negatively charged GAGs neutralizes the oppositely charged area of the protein surface and orients proteins in an array where the intermolecular attraction prevails over repulsive forces. The investigation of the effects of GAGs on amyloidogenesis is extremely promising, with the possibility of translating this basic knowledge into the preparation of GAG function inhibitors. Some of these compounds have been used with limited, but encouraging results, in clinical trials [60]. However, the discovery that heparin promotes the amyloidogenesis of β2-m is particularly relevant, because heparin is commonly used as an anticoagulant in the therapeutic treatment of patients undergoing hemodialysis [61] and this procedure could increase the risk of amyloid deposition.

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