

Review

From chance to frequent encounters: Origins of β 2-microglobulin fibrillogenesis

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Abstract

It is generally accepted that amyloid formation requires partial, but not complete unfolding of a polypeptide chain. Amyloid formation by β -2 microglobulin (β 2m), however, readily occurs under strongly native conditions provided that there is exposure to specific transition metal cations. In this review, we discuss transition metal catalyzed conformational changes in several amyloidogenic systems including prion protein, Alzheimer's and Parkinson's diseases. For some systems, including β 2m from dialysis related amyloidosis (DRA), catalysis overcomes an entropic barrier to protein aggregation. Recent data suggest that β 2m samples conformations that are under thermodynamic control, resulting in local or partial unfolding under native conditions. Furthermore, exposure to transition metal cations stabilizes these partially unfolded states and promotes the formation of small oligomers, whose structures are simultaneously near-native and amyloid-like. By serving as a tether, Cu^{2+} enables the encounter of amyloidogenic conformations to occur on time scales which are significantly more rapid than would occur between freely diffusing monomeric protein. Once amyloid formation occurs, the requirement for Cu^{2+} is lost. We assert that β 2m amyloid fiber formation at neutral pH may be facilitated by rearrangements catalyzed by the transient and pair wise tethering of β 2m at the blood/dialysate interface present during therapeutic hemodialysis.

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1. Protein folding, misfolding and pathological misassembly

It is well known that proteins fold and adopt well-defined three-dimensional structures required for function [1]. Studies aimed at understanding protein folding have characterized the folding process as traversing a funnel-shaped multidimensional landscape [2]. This includes the captivating aspect that most proteins fold, without assistance, to a unique structure representing the energetically most stable conformation [3]. The funnel-shaped landscape of protein folding allows proteins to access a broad ensemble of conformational states. Since conformational sampling occurs under thermodynamic control, even a solution of stably folded protein will transiently adopt both partially and incorrectly or misfolded states [2,4]. The

assembly of these states into insoluble aggregates is associated with an increasing number of human diseases. Notable examples include Alzheimer's disease, Creutzfeldt–Jakob disease, dialysis related amyloidosis (DRA), and type II diabetes. Although each disease has a different clinical presentation, a central component is the formation and deposition of aggregated protein in cells and tissues, resulting in loss of function, physical obstruction, and/or gain of toxic function leading to cell death [5–7].

Although amyloid precursor proteins differ significantly in secondary and tertiary structure, all amyloid fibers share a common cross- β structure. In the cross- β structure, β -strands are aligned perpendicular to the long axis of the fiber. These strands are arranged into sheets in which the backbone hydrogen bonding runs parallel to the fiber axis [8–10]. The formation of similar structures from vastly different protein precursors strongly suggests a common mechanism for all amyloid assembly. As all amyloid fibers are β -sheet rich in structure, global rearrangements and conformational changes are often required prior to or concomitant with assembly. The

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tightly packed environment of a native protein, however, prohibits such changes from readily occurring. Therefore, experiments have suggested amyloid formation most likely occurs from the partially structured or non-natives states naturally sampled by proteins [11]. This has led to partial unfolding of globular proteins as an accepted prerequisite to amyloid assembly.

A variety of protein features are emerging as central to the assembly of disparate primary sequences into closely similar fibrillar structures. These include the presence of polyglutamine rich repeats such as those observed in the huntingtin protein of Huntington's disease [12], and charge neutralization [13]. In our own work, we recently identified that $\beta 2m$ from DRA is a Cu^{2+} specific binding protein [14] and this binding can promote amyloid assembly under near physiological solution conditions [15]. Divalent cation interaction, particularly with Cu^{2+} , is emerging as a feature of several amyloid systems. To date, divalent cation interactions have been implicated in formation or alteration of ordered aggregates in a number of different diseases including: Alzheimer's, Creutzfeldt–Jakob, light chain amyloidosis, Parkinson's, and DRA [16–20]. In each of these diseases, Cu^{2+} associates with a different and unrelated protein resulting in aggregates and amyloid. Thus, it is of critical importance to identify the structural basis of transition metal cation associated conformational changes resulting in assembly.

2. Transition metal cations in amyloid disease

Protein interactions with transition metal cations have long been the subject of investigation, particularly in the neurodegenerative amyloids [21,22]. In vitro and in vivo studies have shown that transition metal cations can initiate or modulate aggregation assembly through a variety of complex mechanisms. For example, divalent cations such as Cu^{2+} can give rise to one or more interrelated effects, such as inducing structure in unstructured regions, free radical mediated oxidation, and stabilization of partially and globally unfolded conformations (Fig. 1). The involvement of Cu^{2+} in amyloid assembly can be further divided into two categories. The first are Cu^{2+} interactions with amyloid precursor proteins as part of an in vivo function. These include amyloid β peptide ($A\beta$) of Alzheimer's disease and prion protein (PrP) from the spongiform encephalopathies [21–23]. The second category involves opportunistic interactions with Cu^{2+} , where such interactions are regarded as strictly pathological. This category includes $\beta 2m$ from DRA, α -synuclein of Parkinson's disease, and immunoglobulin light chains of light chain amyloidosis [14,18,24]. The interaction of Cu^{2+} with these precursor proteins promote aggregation, however, a functional role for these interactions has not yet been identified.

Amyloidogenic proteins in both categories bind divalent cations and undergo similar modifications resulting in inter-

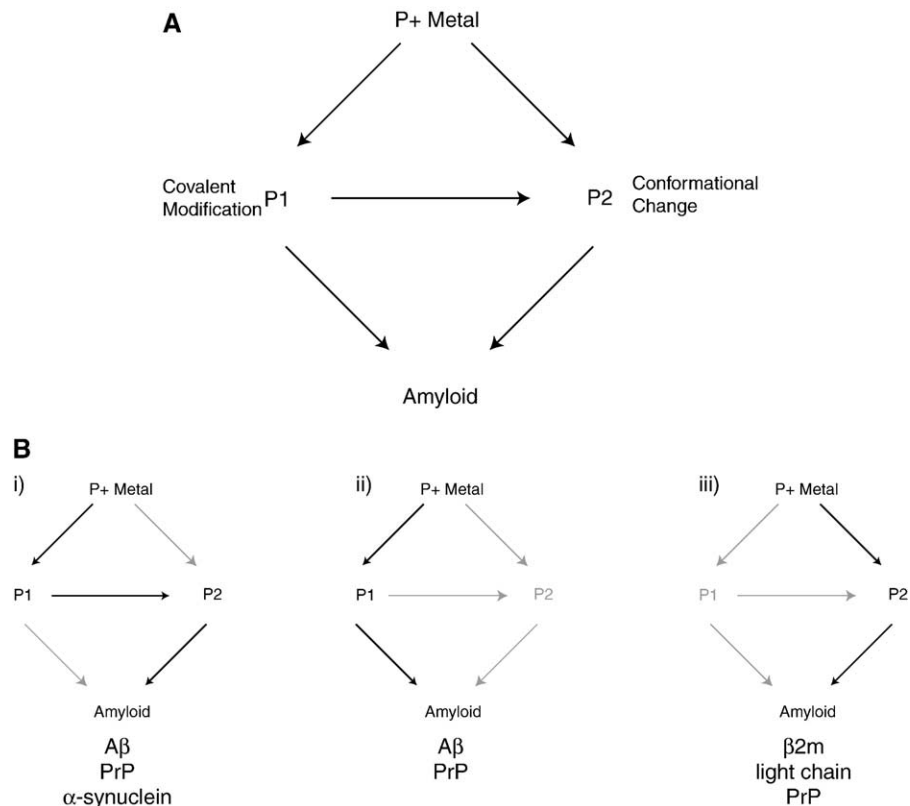


Fig. 1. (A) Simplified model of metal ion associated protein modifications leading to aggregation. Interactions of protein (P) and divalent metal, particularly Cu^{2+} , can result in covalent (P1) and conformational (P2) modifications. The most common covalent modification is protein oxidation as a result of the Cu^{2+} redox chemistry. Cu^{2+} induced conformational changes include the formation of well-defined substructures in unstructured regions and protein oligomerization. (B) Three pathways of metal ion associated modifications promoting amyloidosis are highlighted in black. Amyloidogenic proteins that undergo the indicated modifications to form aggregates are listed below the mechanism.

mediate and mature amyloidogenic states. These are shown schematically in Fig. 1 and include metal cation associated covalent and conformational changes. In Fig. 1A, a simplified model of metal cation associated modifications leading to amyloidosis is shown. Protein (P) interacts with metal cations leading to either covalent modification (P1) or conformational change (P2). The most common covalent modification induced by transition metal cations is protein oxidation. Such oxidation reactions result predominantly in the addition of oxygen as hydroxide, but can also result in backbone cleavage [25]. Interactions with cations can also alter the folding landscape of a protein through the formation and population of well-defined substructures and oligomerization [26,27]. While each of these events has been shown individually to lead to amyloidosis a combination of covalent modification and conformational changes can also result in pathological assembly.

In vivo, the capacity of transition metal cations to mediate redox reactions makes them essential for life. This same activity, however, can also lead to toxicity. If allowed to freely diffuse, transition metal cations can catalyze the production of free radicals causing oxidative damage to both proteins and lipids. As a result, transition metal cations are tightly regulated by chaperones and other metalloproteins. A proposed function of the A β peptide is to trap excess extracellular Cu²⁺ and prevent it from adversely generating such radicals. This results in protection from metal ion mediated oxidation [22,23]. It has been suggested, that as a result of aging, metal ion concentrations increase and A β becomes hypermetalated. This causes a switch in A β activity from protecting against oxidative damage to becoming oxidized itself (Fig. 1Bii). Once oxidized, there is an increase in cell stress and Zn²⁺ present in the synaptic cleft is then thought to induce A β aggregation. This suggests the interactions of A β and Cu²⁺ that promote amyloidosis involve covalent modification with subsequent Cu²⁺ and Zn²⁺ associated conformational changes (Fig. 1Bi). Importantly, therapeutic approaches which chelate Cu²⁺ and Zn²⁺ have proven effective in mouse models [28]. These biological findings are the result, in part, of in vitro analyses of these systems. For example, the strong affinity of A β for divalent cations enables this peptide to sequester copper, iron, and zinc which can be present in trace quantities ($\leq 0.8 \mu\text{M}$) in laboratory buffers [29]. The levels of trace metal in laboratory solutions indeed proved sufficient to initiate oligomer and amyloid assembly in A β [30]. Therefore, it is of fundamental importance when working with cation sensitive proteins to control for trace metal contamination, for example, by inclusion of divalent cation chelating reagents.

The cellular prion protein (PrP^c) also has a proposed functional role involving Cu²⁺ binding. PrP^c is predominantly α -helical protein tethered to cell membranes via a glycoinositol anchor [31]. Binding of metal cations by PrP^c (particularly Cu²⁺ and Zn²⁺) increases the rate of PrP^c endocytosis. It has therefore been suggested that cations regulate PrP^c trafficking. Cu²⁺ binding to a repeated octapeptide sequence in the unstructured N-terminal domain of the protein mediates the endocytosis of PrP^c [21]. In most species, the unstructured N-terminus has four to five copies of the sequence: PHGGGWGQ. This repeat has fM to μM affinity for Cu²⁺ as a result of coordination by

backbone amides, and the sidechains of Trp and His residues [32–34]. A high-resolution crystal structure of this region [26] demonstrates that Cu²⁺ coordination creates well-defined substructure in an otherwise unstructured sequence (Fig. 1Biii). Deletion of the N-terminal region abolishes PrP^c endocytosis, however, it does not completely abolish the susceptibility of PrP^c to convert to the protease resistant form, PrP^{Sc} [21]. Additional complexity in this system comes from the presence of a second binding site. This second site has nM affinity for Cu²⁺. Furthermore, it involves residues more proximal to the structured region of the protein than the octapeptide repeats, yet is still within the otherwise unstructured region required for prion propagation [32,35,36]. This suggests that Cu²⁺ binding in the N-terminal unstructured domain of PrP^c is one of the factors in the pathological conversion of PrP^c to the scrapie conformer, PrP^{Sc}.

The amyloidogenic protein in Parkinson's disease is α -synuclein. This protein has no known native function for Cu²⁺ binding. Nevertheless, α -synuclein binds Cu²⁺ resulting in the induction of a well-defined structure in a predominantly unfolded protein followed by amyloidogenic self-assembly (Fig. 1Bi) [17]. In addition, oxidative damage of α -synuclein is suggested to increase aggregated assembly. The oxidation of α -synuclein likely occurs through interactions with Cu²⁺ released from superoxide dismutase [24]. Upon Cu²⁺ coordination and oxidation the native function of α -synuclein is altered and amyloid assembly can occur. Together, these results suggest the mechanism of Cu²⁺ associated α -synuclein amyloidosis involves both covalent and conformational changes.

3. $\beta 2\text{m}$ amyloidosis

DRA occurs in patients suffering from renal failure whose treatment includes long-term hemodialysis. This disease is characterized by the deposition of amyloid fibers primarily in the tenosynovium, ligaments, and liver, resulting in carpal tunnel syndrome, bone destruction, and spondylarthropathy [37]. Protein deposition in these patients likely begins immediately upon initiation of dialysis treatment with symptoms developing after approximately 5 years. After this time, the number of patients with symptoms increases in a near linear fashion. The penetration of this disorder is $\sim 20\%$ in as little as 2 years and reaches 100% after approximately 15 years of dialysis treatment [38–40]. Worldwide, there are over a million patients on hemodialysis, therefore, understanding the basis of amyloid deposition in DRA is clearly vital.

The predominant protein in DRA amyloid deposits is $\beta 2\text{m}$. $\beta 2\text{m}$ is the 12-kDa polypeptide subunit necessary for the cell-surface expression of class-I major histocompatibility complex (MHC). $\beta 2\text{m}$ has a β -sandwich fold typical of the immunoglobulin superfamily, and contains seven β -strands (Fig. 2A). Three β -strands form one side of the sandwich and four form the other. An internal disulfide bond tethers strands 2 and 6 in the folded protein [41,42]. Extraction of $\beta 2\text{m}$ from ex vivo aggregates indicates amyloid is predominantly formed from full length, wild type $\beta 2\text{m}$. Although up to $\sim 30\%$ of $\beta 2\text{m}$ fibers have been suggested to contain an N-terminal six amino

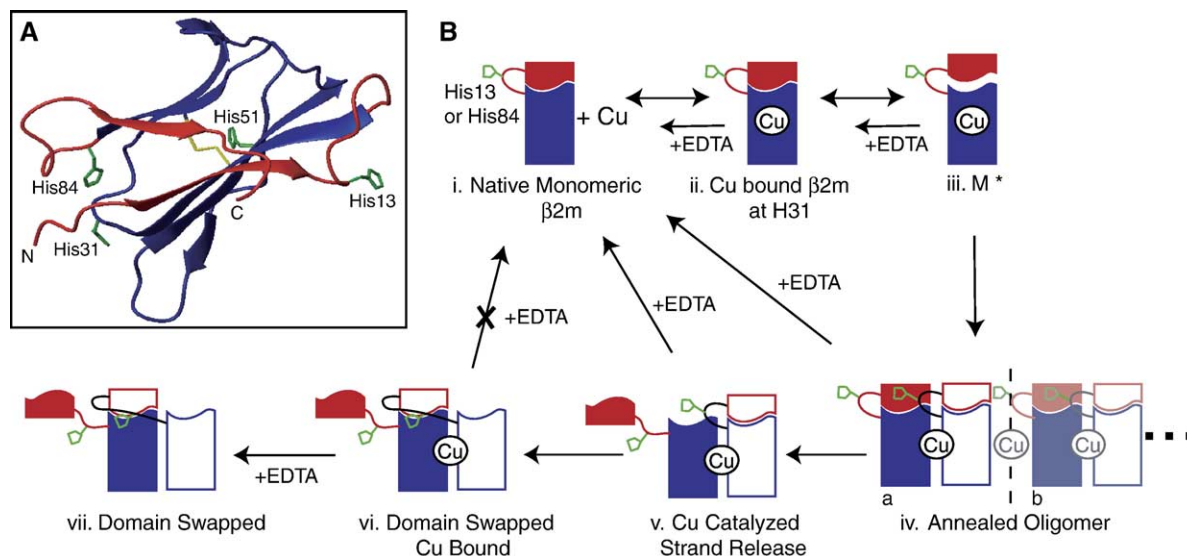


Fig. 2. Cartoon of proposed $\beta 2m$ oligomerization pathways. (A) Ribbon structure of $\beta 2m$ (2CLR) [41]. The histidine residues are shown in green and the disulfide bond in gold. The N- and C-terminal β -strands are shown in red. Note the location of His13 and His84 on the loop prior to the N- and C-terminal strand. (B) A proposed mechanism for $\beta 2m$ oligomerization. Cu^{2+} binds the apo-protein (i) at His31 (ii) and catalyzes the formation of a monomeric rearrangement to M^* (iii). Annealed oligomers of M^* form in discrete units of two (iv). This is followed by a Cu^{2+} catalyzed strand release (v), likely of the N- or C-terminal strand due to interactions of His31 and His13 or His84 with Cu^{2+} . After strand release $\beta 2m$ oligomers can domain swap (vi). Domain swapped structures no longer require Cu^{2+} coordination (vii) and can elongate to amyloid.

acid cleavage [43], it is possible this cleavage results either from the extraction process, or from the long retention time of the aggregated protein in the body. Formation of fibers from full length, wild type protein is in marked contrast to diseases such as immunoglobulin light chain amyloidosis and senile systemic amyloidosis, where aggregation occurs from mutated proteins [44,45].

The formation of $\beta 2m$ amyloid in DRA must result from features unique to renal failure treated by hemodialysis. In vivo, $\beta 2m$ is released extracellularly as a normal part of MHC turnover. Upon release, $\beta 2m$ diffuses to the serum and is principally catabolized by the kidney. In patients with kidney failure, however, $\beta 2m$ is no longer efficiently degraded. This results in a ~ 10 -fold increase in the circulating $\beta 2m$ concentrations above the normal level of $0.1 \mu\text{M}$ [37,38]. As amyloid formation is an oligomerization reaction, it is reasonable to consider elevated concentrations of $\beta 2m$ as causal in DRA. This has given rise to development of large pore hemodialysis membranes which permit significant extraction of $\beta 2m$ as well as affinity approaches for direct extraction of $\beta 2m$ in conjunction with dialysis therapy [46].

Elevated protein concentration, however, is not the cause of DRA. While it is clear that therapeutic clearance of $\beta 2m$ improves the prognosis of patients, elevated $\beta 2m$ concentrations are not unique to renal failure patients. For example, in diseases such as hepatitis C and chronic leukemia, increased $\beta 2m$ serum concentrations can persist for years [47]. Nevertheless, we are not aware of any reports of amyloid deposition in these patients, which are not ascribed to dialysis therapy. Comparable arguments can be made based on biophysical analyses. In vitro, $\beta 2m$ is monomeric and stable at a pH and temperature that is comparable with human serum. This

includes analyses, e.g., by NMR [48], at concentrations which are ~ 1000 -fold that found in serum. As amyloid initiates from a minimum of a bimolecular collision, a significant increase in concentration should dramatically increase the rate of intrinsic amyloid formation. The absence of observable amyloid formation under these conditions, however, strongly suggests that elevated concentrations alone are not sufficient to cause $\beta 2m$ fiber formation in DRA.

We have recently shown that $\beta 2m$ is a metal cation binding protein with a strong preference for Cu^{2+} . Importantly, in vitro interactions of $\beta 2m$ and Cu^{2+} under biomedically relevant conditions gives rise to fibrous $\beta 2m$ aggregates [15]. Such interactions may be causal to $\beta 2m$ amyloidosis for the following reasons. First, only dialysis patients are simultaneously exposed to elevated levels of $\beta 2m$ and transition metal cations. The latter occurs as a result of dialysis treatment. In 1 year of dialysis, a patient is exposed to 15,000–30,000 L of water. The maximum level of copper allowed in the dialysate is $1.6 \mu\text{M}$ [49]. This is within a factor of two of the measured affinity of Cu^{2+} and $\beta 2m$ [15]. A second potential source of free Cu^{2+} is from the use of $\text{Cu}^{2+}(\text{NH}_3)_4(\text{OH})_2$ solution in preparation of some dialysis membranes, such as Cuprophane®. These membranes contain $\sim 2 \text{ mg}$ of copper per m^2 , where a typical dialysis membrane is $1\text{--}2 \text{ m}^2$. Prewashing these membranes with saline solution fails to extract the Cu^{2+} , however, Cu^{2+} is readily released from the membrane upon incubation with serum at 37°C [50]. These membranes, therefore, provide a high local concentration of Cu^{2+} at the site of the membrane. Clinical evidence also indicates that patients treated with membranes not prepared with $\text{Cu}^{2+}(\text{NH}_3)_4(\text{OH})_2$ have $>50\%$ lower incidence of symptoms related to DRA [51].

4. Cu^{2+} -associated amyloidosis of $\beta 2\text{m}$

$\beta 2\text{m}$ forms amyloid fibers on the week timescale when incubated in the presence of Cu^{2+} under conditions close to physiological with aqueous buffer, at pH 7.4, isotonic with serum, and 37 °C. Incubation of $\beta 2\text{m}$ at high molar ratios of Cu^{2+} /protein (36:1) results in protein destabilization. This demonstrated the existence of a Cu^{2+} binding site in a non-native protein conformation [14,15,27]. As protein destabilization has been suggested as a requirement for amyloid formation [11], the molecular basis of Cu^{2+} binding and destabilization of $\beta 2\text{m}$ has been investigated by a number of groups [15,48,52]. Interestingly, Cu^{2+} binds to native and non-native states of $\beta 2\text{m}$ with distinct residues. Our group and others identified His31 as a central residue for native state binding [15,48]. In addition, we identified Trp60 as also being important to native state binding. For residues His 13 and 51, solvent exposure permits transient intersection with Cu^{2+} in the native state as observed by paramagnetic broadening [52]. Our own mutational and thermodynamic analyses, however, indicate that these residues as well as His 84 predominantly form interactions with Cu^{2+} in non-native states of the protein. Specificity measurements indicate that Cu^{2+} binds to the non-native state with both a stronger affinity and greater specificity than the native state. For example, 2.5 μM $\beta 2\text{m}$ is measurably destabilized by 20 μM Cu^{2+} , however, 100 fold more Ni^{2+} does not detectably destabilize $\beta 2\text{m}$ [15]. Importantly, $\beta 2\text{m}$ is also not detectably destabilized under the concentrations of Cu^{2+} (2:1 molar ratio of $\text{Cu}^{2+}/\beta 2\text{m}$) required to initiate amyloid formation [14,27]. This strongly suggests that the non-native state relevant to $\beta 2\text{m}$ amyloid assembly is not wholly unfolded. Furthermore, these observations also suggest that the $\beta 2\text{m}$ structures involved in amyloid formation have considerable specificity and are therefore likely to be highly structured.

Metal cation associated $\beta 2\text{m}$ fibrillogenesis is preceded by the formation of discrete, long-lived oligomeric species [27]. These oligomeric intermediates form rapidly, are reversible with EDTA, and occur under conditions where $\beta 2\text{m}$ is not globally destabilized. We concluded that the observed oligomeric states assemble as a consequence of Cu^{2+} binding to the native conformation. Prior to oligomerization $\beta 2\text{m}$ undergoes Cu^{2+} dependent local rearrangements on the 1 h timescale. We have identified this state as M^* , as its structure is closely similar to the folded monomeric protein [27]. The transient M^* state is essential to the formation of oligomers and fibers, therefore, a detailed molecular description of M^* is highly desirable. If we conjecture that our kinetically identified intermediate is also sampled at equilibrium under conditions used in recent NMR studies, we can obtain clues as to the location of possible changes. This includes paramagnetic broadening of the solvent exposed residue, His 13, which led to speculation of N-terminal strand release [48]. A more detailed, residue specific analysis of Cu^{2+} effects, however, shows increased relaxation notably in the immediate vicinity of the solvent exposed residues His 13 and His 31 and ~15 contiguous residues C-terminal to His 51 [52]. The M^* state is

likely less stable than the native state, but is masked by conventional stability measurement methods, such as denaturant titrations, because such approaches can yield an apparent two state transition even in three state systems. Other reported methods of in vitro $\beta 2\text{m}$ amyloid formation require destabilization of the protein. This includes the use of acidic pH [53], and N-terminal truncation [43]. For Cu^{2+} associated $\beta 2\text{m}$ amyloid assembly we conjecture that global destabilization is not required, rather Cu^{2+} mediates catalysis to a thermodynamically accessible state, even under native solution conditions.

The assembly of protein into amyloid fibers necessarily requires two or more partially folded molecules to encounter each other. The various methods to promote amyloid assembly typically results in significantly increasing the population of such partially folded states. For many amyloid proteins in vitro, amyloidogenic conformations are achieved by methods including heat, surfactants, rapid stirring, structure inducing solvents such as trifluoroethanol, acidification and chaotropes. Non-native sampling can also be enhanced by protein mutations such as in human lysozyme [54] and transthyretin [55]. For $\beta 2\text{m}$, population of these states has been increased through the use of proteolytic truncation, and acidic pH [43,53]. With both truncation of the six N-terminal $\beta 2\text{m}$ amino acids and exposure to acid pH, $\beta 2\text{m}$ becomes less stable relative to the full length protein resulting in molten globule-like states. In these cases, the entire $\beta 2\text{m}$ population is partially folded which greatly increases the collision frequency of amyloidogenic molecules and amyloid assembly.

$\beta 2\text{m}$, like all proteins whose conformational changes are under thermodynamic control, continually samples less stable states in solution. Therefore, under native solution conditions, the encounter of two non-native molecules is extremely rare and thus amyloid fibers do not form. Interactions of $\beta 2\text{m}$ and Cu^{2+} , however, result in the formation and stabilization of the near native state, M^* . $\beta 2\text{m}$ amyloid assembly proceeds from the M^* state when two or more M^* proteins collide and are annealed together through further Cu^{2+} interactions [27]. Formation of M^* occurs on the hour timescale via interactions with Cu^{2+} and is followed by rapid, Cu^{2+} dependent annealing to form discrete oligomeric intermediates. These oligomers form in defined units of two, resulting in dimer-, tetra-, and hexameric states (Fig. 2). While these initial annealed oligomers require Cu^{2+} for stability, over the time course of amyloid formation, oligomeric intermediates rearrange to a state that no longer requires Cu^{2+} for stability. This is evident as initial oligomeric intermediates are fully reversed by the addition of EDTA, while amyloid fibers are not. We assert, therefore, that the energetic requirement of Cu^{2+} for stability is lost between intermediate and amyloid formation. Cu^{2+} plays a dual role in initiating amyloidosis by promoting both a conformational change in the monomer and facilitating oligomer assembly [27]. The requirement of Cu^{2+} to initiate $\beta 2\text{m}$ amyloid assembly is relevant to hemodialysis, since patients are likely only transiently exposed to free Cu^{2+} at the membrane interface during treatment.

The oligomeric intermediates formed by $\beta 2m$ in the presence of Cu^{2+} have a native-like conformation suggesting that the mechanism of $\beta 2m$ fiber formation is either through subunit annealing and/or domain swapping. The domain swap model for amyloid assembly is based on studies of intertwined dimers observed crystallographically. This model postulates that a chain can be formed by each monomer swapping a strand or subdomain into the same environment of an identical monomer [56]. Importantly, however, all structural evidence of domain swapping demonstrates completely swapped or closed structures, resulting in defined oligomeric units rather than open-ended chains. Nevertheless, structural domain swapping forming closed structures has been observed experimentally in several amyloid systems including prion protein [57] and cystatin C [58]. Domain swapping is a particularly attractive possibility for $\beta 2m$ amyloidosis given the all- β structure of the $\beta 2m$ precursor [27,59]. Several of our observations of Cu^{2+} associated $\beta 2m$ amyloid assembly are consistent with this mechanism. These include: (1) Amyloid and intermediates are formed without the requirement for significant destabilization of the protein. (2) Oligomeric intermediates are composed of domains with native-like structure. (3) ThT fluorescence enhancement of $\beta 2m$ amyloid is closely similar to that of its intermediates. (4) Incubation on the week timescale results in the loss for the requirement of Cu^{2+} for oligomer stability. Taken together, these observations form the core basis of our assertion that the mechanism of $\beta 2m$ amyloid formation is structural domain swapping.

Domain swapping typically results in dimeric structures in which terminal strands have exchanged [60]. To date, no structural evidence exists for open domain swapping as central to the structure of an amyloid. However, biochemical evidence in the case of cystatins [58,61], and the simplistic appeal of such a mechanism warrant comparison to $\beta 2m$ assembly. In $\beta 2m$, all four of the histidine residues in $\beta 2m$ appear relevant to either native, or non-native state binding [15,52]. This includes two residues located on the loops prior to both the N- (His13), and C- (His84) terminal β -strands (Fig. 2). These particular residues have been shown to coordinate Cu^{2+} only in non-native states [15]. The terminal strands of $\beta 2m$ have also been implicated in protecting monomeric $\beta 2m$ against amyloid assembly [43,62], and the edge strands of β sheet proteins have been observed to contain protective features preventing aggregation [63]. Furthermore, specific point mutations in the N- and C-terminal strands of $\beta 2m$ results in protein destabilization and fiber formation at neutral pH [59]. We also believe strand release by $\beta 2m$ would most likely occur at either the C- or N- terminus, because the Cys25–Cys80 disulfide bond tethers the second and penultimate strands [41]. Therefore, we conjecture that Cu^{2+} is coordinated in native $\beta 2m$ by His31 which then promotes a monomeric structural rearrangement within $\beta 2m$ to allow Cu^{2+} interaction with either His13 or His84. This subsequently frees one of the termini allowing the formation a domain swapped structure (Fig. 2). Once such a structure is formed, the requirement for Cu^{2+} is lost.

Proteins in native solution conditions continuously sample, although do not significantly populate, non-native states. In the

formation of amyloid fibers, the frequency of interaction between non-native or destabilized states must be increased in order to initiate aggregation. Most in vitro approaches to amyloid formation achieve this by increasing the population of destabilized states. In contrast, Cu^{2+} acts on $\beta 2m$ by specifically drawing near-native states into proximity under strongly native solution conditions [27]. Amyloid assembly of $\beta 2m$ in the presence of Cu^{2+} is of intense interest, since Cu^{2+} initiates $\beta 2m$ amyloid assembly under biomedically relevant conditions and to date is the only known de novo mechanism in which fibers form from wild-type $\beta 2m$ in wholly aqueous buffer at neutral pH [14,27]. As Cu^{2+} binding is a generic feature of several amyloid systems including Alzheimer's and Parkinson's disease, Cu^{2+} induced generation of non-native states followed by tethered association may be a general mechanism for accessing amyloidogenic assembly.

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