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Structural heterogeneity in the pre-amyloid oligomers of β 2-microglobulin

Tyler M. Marcinko^{a,1}, Chungwen Liang^{b,2}, Sergey Savinov^{b,c}, Jianhen Chen^{c,3}, Richard W. Vachet^{a,1}

^aDepartment of Chemistry, University of Massachusetts, Amherst, MA 01003, United States

^bComputational and Modeling Core Facility, Institute for Applied Life Sciences, Amherst, MA 01003

^cDepartment of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003, United States

Abstract

In dialysis patients, the protein β 2-microglobulin (β 2m) forms amyloid fibrils in a condition known as dialysis-related amyloidosis. To understand the early stages of the amyloid assembly process, we have used native electrospray ionization (ESI) together with ion mobility mass spectrometry (IM-MS) to study soluble pre-amyloid oligomers. ESI-IM-MS reveals the presence of multiple conformers for the dimer, tetramer, and hexamer that precede the Cu(II)-induced amyloid assembly process, results which are distinct from β 2m oligomers formed at low pH. Experimental and computational results indicate that the predominant dimer is a Cu(II)-bound structure with an antiparallel side-by-side configuration. In contrast, tetramers exist in solution in both Cu(II)-bound and Cu(II)-free forms. Selective depletion of Cu(II)-bound species results in two primary conformers – one that is compact and another that is more expanded. Molecular modeling and molecular dynamics simulations identify models for these two tetrameric conformers with unique interactions and interfaces that enthalpically compensate for the loss of Cu(II). Unlike with other amyloid systems in which conformational heterogeneity is often associated with different amyloid morphologies or off-pathway events, conformational heterogeneity in the tetramer seems to be a necessary aspect of Cu(II)-induced amyloid formation by β 2m. Moreover, the Cu(II)-free models represent a new advance in our understanding of Cu(II)

Corresponding author: Richard W. Vachet, Department of Chemistry, University of Massachusetts, Amherst, MA 01003. rwvachet@chem.umass.edu Phone (413) 545-2733.

¹Department of Chemistry, University of Massachusetts-Amherst, 374 Lederle Graduate Research Tower A, 710 North Pleasant St., Amherst, MA, 01003

²240 Thatcher Road, Life Science Laboratories, Amherst, MA, 01003

³Department of Biochemistry and Molecular Biology, 912 Lederle Graduate Research Tower, 710 North Pleasant St., Amherst, MA, 01003

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release in Cu(II)-induced amyloid formation, laying a foundation for further mechanistic studies as well as development of new inhibition strategies.

Graphical Abstract

Keywords

Non-covalent complexes; Protein Aggregation; Mass Spectrometry; Ion Mobility Spectrometry; Computational Modeling

Introduction

β 2-microglobulin (β 2m) is a 99-residue structural protein noncovalently associated with major histocompatibility complex I, which is present on the surface of all nucleated cells [1]. β 2m features a 7-membered anti-parallel beta-strand arrangement forming a beta-sandwich, which is connected by a single disulfide bond [2]. In chronic dialysis patients, it is known to form amyloid fibrils that deposit in joints and other organs [3,4]. The long term consequences of these amyloid deposits are joint destruction and organ dysfunction [4].

β 2m is capable of forming amyloid fibrils under a variety of conditions *in vitro* (e.g. incubation at low pH, addition of trifluoroethanol, presence of collagen, truncation of the first six amino acid residues, and incubation with Cu(II)), but the exact physiological mechanism(s) that triggers amyloid formation *in vivo* is unclear [5–12]. Preceding oligomer and amyloid formation, there are a series structural changes that disrupt the native state of β 2m. These changes include the *cis-trans* isomerization of P32 which leads to the repacking of the hydrophobic core of the protein and the repositioning of other residues, such D59, R3, and W60, depending on how amyloid formation is initiated [13–16].

While there are common β 2m structural changes caused by the different amyloid initiating conditions, oligomerization proceeds differently in each case. For example, acid-induced amyloid formation proceeds via the sequential addition of monomeric units [6], whereas Cu(II)-catalyzed oligomerization generally proceeds with the dimer as a building block [11,12]. Moreover, the monomeric subunits in Cu(II)-induced oligomers are thought to be native-like in their structures, while acid-induced oligomerization progresses through partially unfolded intermediates [17]. This apparent complexity and diversity underline the

importance of studying the mechanisms of amyloid formation, and developing models for oligomeric structures generated by different mechanisms.

One particularly interesting feature of amyloid forming proteins that has been recently revealed in other amyloid systems is the presence of different conformational isomers (or conformers) in higher-order oligomers that precede amyloid fibrils [17,18]. In fact, the mature fibrils themselves can be heterogeneous [19], suggesting that different oligomeric conformers might lead to different amyloid morphologies. This heterogeneity can manifest itself in many ways, such as through different oligomeric assembly states that may be off-pathway (i.e. not productive to further assembly and amyloid formation) [17,18]. Our previous study first gathered preliminary evidence of conformational heterogeneity for $\beta 2m$ pre-amyloid oligomers while using ion mobility-mass spectrometry (IM-MS) to understand small molecule inhibitors of $\beta 2m$ amyloid formation [20]. There is little information about such conformational heterogeneity for Cu(II)-induced $\beta 2m$ pre-amyloid oligomers, prompting the study described here.

In this study, we characterize the heterogeneity present in Cu(II)-catalyzed $\beta 2m$ pre-amyloid oligomers and provide insight into the different structural forms that are present during amyloid formation. To do this, we primarily employ IM-MS, which separates protein complex ions based on their collisional cross section (CCS) [21–23]. To relate these measurements to solution-phase structures, the protein complex ions are generated under native-like conditions in which a memory of their solution-phase structures remains [21,24–30]. Computational modeling and other experimental constraints, such as covalent labeling MS data [24,31], are then combined with the IM-MS measurements to derive model structures of the pre-amyloid conformers. From our measurements, we find that oligomers generated in the presence of Cu(II) are structurally distinct when compared to $\beta 2m$ amyloids formed under different conditions (e.g. acid). In addition, we find unique structural heterogeneities in $\beta 2m$ tetramers that are associated with Cu(II) loss, which is a necessary step in amyloid formation. We propose that tetramer heterogeneity is an essential feature of Cu(II)-induced amyloid formation by $\beta 2m$, which contrasts to conformational isomers in other amyloid systems that are typically thought to be associated with off-pathway products [32–34].

Results

Native ESI-IM-MS measurements were performed at several intervals during the early stages (< 7 days) of $\beta 2m$ oligomerization in the presence of stoichiometric amounts of Cu(II) and under conditions that eventually lead to amyloid fibril formation. The resulting mass spectra reveal the presence of soluble even-ordered oligomers, including dimers, tetramers, and hexamers (Figure 1A), whose stoichiometries are the same as measured previously for Cu(II)-induced $\beta 2m$ amyloid formation [11,12,15,35]. The relative population of oligomeric species changes over time, implying that specific assembly steps occur in solution to build the oligomers [12,20,36,37]. For example, dimers are detected after a few hours to 10 days of incubation, while tetramers and hexamers require 1 and 2 days, respectively, before they are detected.

Interestingly, upon examination of the IM data under native MS conditions, we detect multimodal arrival time distributions (ATDs) of the oligomers but not of the monomer (Figure 1B and Figure S1), indicating that the oligomers have conformational isomers. The presence of multiple conformations is particularly striking for the tetramer and hexamer. The monomers with and without Cu(II) bound have identical ATDs, indicating that Cu(II) binding does not itself introduce this conformational heterogeneity at the monomer level (Figure S2). The multiple conformations for each oligomer are present as soon as the oligomer is first detected by MS, and the centroids for these peaks also remain consistent. A summary of the oligomer collision cross sections (CCS) from measurements of different charge states as a function of oligomer stoichiometry is shown in Figure 2. These data demonstrate that the Cu(II)-induced oligomers are all more compact than simple ‘beads on string,’ providing rough insight into the geometry of these species. Moreover, when the CCS values are compared to calculated and measured CCS values for other previously reported $\beta 2m$ and $\beta 2m$ mutant oligomers (Table 1), we find that the Cu(II)-induced oligomers are more compact in almost every case.

The measured monomer CCS value is consistent with the monomeric crystal/NMR structures for the wild-type protein and is more compact and less heterogeneous than the monomers measured upon amyloid initiation at low pH (Table 1). We also find that some of the Cu(II)-induced oligomers are more heterogeneous than corresponding oligomers produced at low pH [6,17]. The measured CCS values for the most abundant conformers for the Cu(II)-induced dimers and tetramers are in good agreement with calculated CCS values for the P32A mutant dimer structure (PDB: 2F8O), as well as dimers and tetramers excised from the Cu(II)-bound H13F hexamer structure (PDB: 3CIQ) (Table 1) [13,38].

To further investigate the dimers measured with ESI-IM-MS, we used computational modeling to generate potential candidate structures for comparison (Figure 3). Because the Cu(II)-induced dimers in our experiments have similar CCS values to the calculated CCS values for the dimeric structures taken from the H13F hexamer (Table 1), we used these structures as starting points for the calculations. The monomeric units in the starting dimer structures are native-like in their conformation, which is consistent with several experimental studies that indicate $\beta 2m$ oligomers are native-like when generated under Cu(II)-catalyzed conditions [11,13,38]. We excised two dimer configurations from the H13F crystal structure, mutated the F13 back to H, subjected the structures to energy minimization, and then performed MD calculations in explicit solvent. For each dimer, we also removed Cu(II) and did separate energy minimization and MD calculations. To assess the possibility of structural changes and/or reorganization of the proteins in the gas phase, we also performed gas-phase MD simulations on the resulting solution phase structures.

Of the four resulting dimer configurations, three were found to be stable during the simulations for up to 1 μs when simulated in solvent and the gas phase. The three stable configurations can be categorized as either having a head-to-head interaction involving the N-terminal region of the protein or a side-by-side interaction involving the four-strand β -sheet (Figure 3A). The side-by-side Cu(II)-free dimer is relatively unstable *in silico*, as it dissociated during the explicit solvent MD simulation. Of the three stable configurations, the Cu(II)-bound side-by-side structure is more consistent with trends from covalent labeling-

MS data (Figure S3) [16]. All of the predicted interfacial residues undergo decreased labeling, and 80% of the residues undergo a labeling change that qualitatively coincides with the SASA change. In contrast, only 30% of the residues in the head-to-head dimer show a positive correlation between the covalent labeling data and the calculated SASA values. The three stable structures in solution were also stable for 1 μ s during the gas-phase MD simulations, and solution and gas-phase structures have a high degree of similarity based on comparisons of the protein backbone (Figure S4).

Moreover, previous work has also shown that Cu(II) remains bound to the dimer, suggesting that the modeling experiments are recapitulating the necessity of Cu(II) for dimer stability [15]. As a way to provide further support for the side-by-side dimer configuration, we then calculated the theoretical CCS values of the three stable configurations (Figure 3B), of which the two head-to-head configurations have identical CCS values. Comparison of these calculated results for dimers with 9 charges to the experimentally measured CCS values for the +9 dimer (Figure 3C) reveals that the main conformer agrees well with the side-by-side dimer structure. The measured +9 dimer charge state was chosen because the lowest charge state ions are typically the most native-like. Critical for the stabilization of the side-by-side dimer are the presence of intermolecular salt bridges (R3-E16, D59-K19) that are found in the solution phase structure only when Cu(II) is present (Figure S5). Overall, these results are consistent with a previously reported model of the dimer [16].

The tetramers seem to consist of several distinct conformers with very different CCS values (Figure 1 and Figure S1). Moreover, the tetramer ATD widths for all charge states narrow over time (Figure 4B) in contrast to the dimers (Figure 4A) and hexamers (Figure 4C) whose ATD widths remain constant during the course of the amyloid formation reaction. The ATD widths for the tetramer ions decrease an average of 45% from day 1 to day 10. Evaluation of peak widths for all the oligomers, including the tetramer, across replicate experiments performed on different days suggests that the ATD variation does not arise from day-to-day variability or from instrumental differences (Figure 4D and Figure S6). The timeframe over which this structural heterogeneity evolves coincides with the emergence of the Cu(II)-free tetramer, which several studies have previously found to be a necessary step for Cu(II)-induced amyloid formation [12,39,40]. Structural information about this Cu(II)-free tetramer is largely absent; however, it is reasonable to hypothesize that formation of this obligatory oligomeric species involves some degree of structural transformation because the Cu(II)-free tetramer is resistant to dissociation upon the addition of EDTA, while the Cu(II)-bound tetramer dissociates into monomers [12]. An intriguing question is whether the Cu(II)-free tetramer is among the measured conformers, and if its CCS value varies significantly.

To address this question, we added an excess concentration of EDTA once dimers, tetramers, and hexamers were present in solution, and then used ion mobility to measure the resulting oligomeric conformers. Consistent with previous observations, the addition of EDTA causes an increase in monomer signal (Figure 5A), dissociation of the dimer (Figure 5B), partial dissociation of the tetramer (Figure 5C), and no effect on the hexamer signal (Figure 5C). Interestingly, the ATDs of the EDTA-treated tetramer ions result in the depletion of the most abundant conformer in each charge state, leaving the more compact and/or expanded conformers unchanged (Figures 5D & E). Given the high affinity of EDTA for Cu(II) and its

excess concentration, we attribute these remaining peaks to the Cu(II)-free tetramer. Evidently, the Cu(II)-free tetramer has multiple conformations and is structurally different than the Cu(II)-bound version. It is possible that the decreased heterogeneity observed for the tetramer over time (i.e. Figure 4A and D) reflects a gradual conversion of different conformers to tetrameric states that are stable in the absence of Cu(II).

To investigate plausible structures of both Cu(II)-bound and Cu(II)-free tetramers, we used computational modeling. Tetramer structures were formed by docking randomly oriented side-by-side dimers, and the resulting 10,000 structures were filtered and constrained via a scoring function that considered previous covalent labeling-MS data [36] (see methods). The filtering yielded five candidate structures, and their CCS values range from 2901 to 3136 Å² (Figure S7). However, these structures are relatively unstable during the subsequent atomistic simulations in explicit solvent, dissociating within 100 ns (Figure S8). While these structures might be consistent with the most abundant conformation of the Cu(II)-bound tetramer (Figure 5D and E), they are not consistent with the full range of CCS values measured by IM-MS, especially for the Cu(II)-free tetramers. Moreover, the instability of the five structures *in silico* suggests that the tetramers probably require very specific interactions not captured in the 10,000 structures from the docking experiments.

Considering the interactions observed in the H13F mutant crystal structure and high predicted stability of head-to-head dimers in MD simulations, we hypothesized that head-to-head contact may be another binding interface in the tetramer, and thus we generated two separate models via crystal structure excision. One model retained Cu(II) (TET1 in Figure 6A) and the other had Cu(II) removed (TET2 in Figure 6B). Both of these structures, which are very similar and have identical interfaces (Figure 6C), remain stable during 1 μs MD simulations in explicit solvent. TET1 and TET2 are also stable for up to 1 μs when simulated in the gas-phase, and their solution- and gas-phase structures are comparable (Figure S4). The interface between the dimer of dimers is consistent with a prior Cu(II)-bound tetramer model obtained from covalent labeling-MS data [36] and features cation-π interactions between the imidazolium of H51 and the phenyl group of F56, as well as a hydrophobic packing between L54 of each dimer subunit (Figure 6C). The calculated CCS value of these two structures with 14 charges is 3002 ± 34 Å² (Figure S9), which has reasonable agreement with the most abundant conformer of the +14 tetramer measured by IM, which has a CCS value of 3080 ± 100 Å² (Figure 5D).

Because this most abundant conformer disappears upon the addition of EDTA, we further sought to identify models for the Cu(II)-free tetramers. Inspired by the *in silico* stability of the Cu(II)-free head-to-head dimer (Figure 3A), we generated tetramer structures by docking two Cu(II)-free monomers to the Cu(II)-free head-to-head dimer. Emerging from these calculations was a structure referred to as TET3 (Figure 7A), which is stable during both solution- and gas-phase MD simulations for 1 μs. Interestingly, TET3 with 14 charges has a calculated CCS values of 2825 ± 32 Å² (Figure S9), which is more compact than TET1 and TET2 and is more consistent with the measured CCS value (2708 ± 77 Å²) of the compact Cu(II)-free +14 tetramer (Figure 5D). Another set of possible tetramers was also generated by docking two Cu(II)-free head-to-head dimers via side-by-side interactions. The structure TET4 (Figure 7B) arises from these docking experiments, and it is stable for 1 μs in

solution- and gas-phase MD simulations. Moreover, with 14 charges it has a calculated CCS value of $3422 \pm 34 \text{ \AA}^2$ (Figure S9), which is more extended than TET1, TET2, and TET3 and is consistent with the measured CCS value ($3547 \pm 129 \text{ \AA}^2$) of the larger +14 Cu(II)-free tetramer (Figure 5D). Both TET3 and TET4 are qualitatively consistent with trends from covalent labeling-mass spectrometry data (i.e. there are more incidences of agreement between SASA change and covalent labeling percentage for dimer-to-tetramer transition assuming heterogeneous tetramers are present) (Figure S10) [36].

Closer examination of the Cu(II)-free tetramer models reveals new interactions that are important for their stability. The central interface of the dimer-of-dimers in TET3 is similar to that of TET1 and 2, where H51-F56, L54-L54, and E50-K58 are key interacting partners (Figure 7C). On the opposing side of the dimer of dimers, salt bridges between K94-E77 and R81-E74 also form an interface (Figure 7C). An important new interaction in TET3 within dimeric units involves the N-terminal amine that upon removal of Cu(II) forms an electrostatic interaction with the sidechain of D59 from the neighboring subunit. H31, which also binds to Cu(II), repositions itself in the absence of Cu(II) by drawing within approximately 5 Å of W60 in a perpendicular orientation (Figure 7D) that is not observed in TET1 (Figure 7E). This non-native interaction is not observed in the monomer structure measured by solution-state NMR (Figure S11). Residues near the Cu(II)-binding site are also repositioned in TET4, and some of them appear to help stabilize the tetramer interface. H31, in particular, forms a salt bridge with D34 from a different subunit, and W60 forms a cation- π interaction with H51 (Figure 7F).

In a manner similar to the tetramer, the hexamer has a multiple conformations, ranging from compact to less compact structures (Figure 2). Simple geometric considerations suggest at least two possible topologies – a ring structure (i.e. blue structure in Figure 2) that is analogous to the H13F mutant hexamer and a more extended structure (i.e. green structure in Figure 2). Modeling the hexamer structures is beyond the scope of the current work due to a lack of additional experimental data other than CCS values, particularly residue-specific covalent labeling-MS results, to guide the modeling. It is worth noting, however, that the hexamer is resistant to dissociation upon EDTA addition (Figure 5C), which suggests that Cu(II) is not necessary for its stability. Its formation is therefore likely dependent on the formation of a Cu(II)-free tetramer.

Discussion

β 2m amyloid formation can be induced under a variety of conditions *in vitro*, and the oligomeric assemblies that precede the amyloids are different [5,12,41–43]. Amyloid formation via Cu(II)-catalysis proceeds through discrete, even-ordered oligomers, ranging from dimers to hexamers, [12], indicating that dimers are the important building block. In contrast, β 2m amyloid formation at low pH proceeds via odd and even-ordered oligomeric states, ranging from dimers to tetradecamers, indicating that assembly occurs via addition of monomers [6].

Another difference between Cu(II)- and acid-induced β 2m amyloid formation is the nature of the conformational heterogeneity. At low pH, the monomer has conformational

heterogeneity, and oligomerization proceeds from partially unfolded states [17]. With Cu(II), however, the monomer is conformationally uniform, and heterogeneity emerges upon oligomer formation, especially for the tetramer and hexamer. The partially unfolded monomer states at low pH cause the acid-induced dimers and tetramers to be 16% (1823 Å² vs. 2180 Å²) and 17% (3080 Å² vs. 3721 Å²) larger than the most abundant Cu(II)-induced dimers and tetramers. This difference is large enough (e.g. 16% corresponds to roughly 32 residues on the dimer) to suggest that the oligomeric intermediates formed under each condition are quite distinct from one another, even though both conditions ultimately result in amyloid fibrils.

Our results indicate that the predominant Cu(II)-bound dimer structure is in a side-by-side configuration that buries significant surface area, rather than a head-to-head or other configuration (Figure 3A). Our IM-MS results are consistent with a significant burial of surface area, as the measured dimer CCS values are substantially smaller than a simple beads-on-a-string model (Figure 2), the previously measured acid-induced dimers, or mutant DIMC constructs (Table 1), which are disulfide-bonded dimers assembled in a lateral (DIMC20) or laterally offset (DIMC50) manner [17,44]. Other orientations are incapable of producing such compact dimers, and other constructs (e.g. DIMC20 and DIMC50) are inconsistent with covalent labeling MS data [16].

The Cu(II)-induced tetramer structures are especially heterogeneous. The mobility distribution of the most abundant tetramer conformer is initially very broad but gradually narrows over time (Figure 4). Systematic factors like instrumental pressure variation, gating timing, and ion diffusion in the IM cell could contribute to day-to-day variability in measured drift times [45], but these factors would impact all oligomer ions and not just the tetramers. We conclude that the breadth of the mobility distribution arises from multiple tetramer conformers that cannot be fully separated. Because this behavior coincides with the emergence of the Cu(II)-free tetramer, we propose that some of these species are structures with different degrees of Cu(II) loading that are transitioning to Cu(II)-free states (e.g. TET1/TET2 → TET3 or TET4).

Measurements of CCS values before and after the addition of EDTA provide the first experimental evidence for differences in the structures of the Cu(II)-bound and Cu(II)-free tetramers. The most striking observation is that Cu(II)-free tetramers exist in both more compact and extended states than the Cu(II)-bound tetramer (Figure 5D and E). Computational efforts to build unbiased tetramer models through randomly oriented dimers were mostly unsuccessful. These results led us to hypothesize that specific contacts and interactions not captured via random docking are critical for tetramer stability.

TET1 was constructed considering the H13F crystal structure, specifically the head-to-head contacts between subunits, involving the cation- π interaction between H51-F56, a van der Waals interaction between L54-L54, and a salt bridge between E50-K58. Removal of Cu(II) from this structure yields TET2, which is stable *in silico* and generally agrees with covalent labeling-MS data (Figure S10). TET3 is a possible structure for the compact conformer, although its calculated CCS value is slightly higher than the experimentally measured CCS value for the compact Cu(II)-free tetramer. New intramolecular and intermolecular

interactions in TET3, like the cation- π interaction between H31-W60, the N-terminal amine-D59 salt bridge, and the interfacial salt bridges of K94-E77 and R81-E74, enthalpically compensate for the release of Cu(II). While TET3 compactness makes it a good candidate for the Cu(II)-free tetramer with the smaller CCS value, it is difficult to envision a structural transformation from TET1 to TET3 as it would necessarily involve an anti-parallel to parallel reconfiguration of the monomeric units in the dimers following Cu(II) removal (Figure S12). Such a reconfiguration would require disruption of key salt bridges that stabilize the side-by-side dimer, but modeling does suggest that these salt bridges are less important in the Cu(II)-free side-by-side dimer.

The other Cu(II)-free tetramer model, TET4, is consistent with the more extended conformer measured with ESI-IM-MS. There are new intermolecular interactions formed that explain its stability without Cu(II), most notably the repositioning of H31 and its involvement in an interfacial salt bridge with D34 from another subunit. We propose that TET4 is good candidate model for the expanded Cu(II)-free tetramer. Importantly, the more expanded conformer assigned to TET4 was found to be a crucial species in previous β 2m amyloid inhibition studies [20]. This tetramer conformer was found to disappear in the presence of molecules that prevented the formation of β 2m amyloids, indicating that it is an important species on the pathway to β 2m amyloid fibrils.

Importantly, TET3 and TET4 feature head-to-head interactions between subunits. Although we do not yet have direct experimental evidence for the presence of head-to-head interactions in Cu(II)-induced β 2m oligomers, there are other examples of β 2m oligomers having such interactions. These include mutant crystal structures [40] and the N6 variant of B2m that is thought to ‘transmit’ its amyloidogenicity via a heterodimeric complex with the wild-type protein through head-to-head interactions [46]. Moreover, in its normal biological context bound to the MHC I receptor, β 2m also interacts through residues located on loops near the head of the molecule [47]. Our computational modeling and IM-MS results suggest that head-to-head interactions could play a role in Cu(II)-induced β 2m amyloid formation.

Conformational heterogeneity in pre-amyloid oligomers is not exclusive to β 2m. Similar structural heterogeneity has been observed for amyloid- β and α -synuclein [48]. Unlike these previous amyloid systems in which heterogeneous structures are hypothesized to be off-pathway (non-productive) oligomers [49], heterogeneity in β 2m oligomers might be a necessary feature of Cu(II) release during fibril formation. Cu(II) is required to initiate β 2m oligomerization, but it is completely released upon formation of amyloid fibrils [12]. Given that Cu(II) is bound at a 1:1 stoichiometry in monomers, dimers, and some forms of the tetramers, release of up to four equivalents of Cu(II) from the tetramer most likely occurs via multiple steps involving several conformers [12,15]. These findings also underline the complexity of this amyloid system.

In summary, we have found that structural heterogeneity is a key feature of Cu(II)-catalyzed amyloid formation with β 2m. This heterogeneity manifests itself through the presence of multiple conformers, which are present in oligomeric states but absent in the monomer. This study represents the first time that these features have been described for the β 2m-Cu(II) system. The conformational heterogeneity increases as the stoichiometry of the oligomers

increase, as the dimer is the least heterogeneous, while the hexamer is the most. The most abundant conformer of the dimer, based on our evidence, is a side-by-side configuration of two anti-parallel monomers. We attribute the heterogeneity observed for the tetrameric species to arise from the transition from Cu(II)-bound to Cu(II)-free states, which is a necessary step in Cu(II)-catalyzed β 2m amyloid formation. We have generated models of these tetramer conformers that are consistent with IM-MS and covalent labeling-MS data and will be validated in future experiments. Because one of the Cu(II)-free tetramers disappears in the presence of β 2m amyloid inhibitors, this tetramer may serve as a target for designing inhibitory molecules [20].

Methods and Materials

β 2m Oligomer Formation

Human, full-length wild type β 2m (Cat #126-11) that is purified from urine was purchased from Lee Biosolutions (Maryland Heights, MO). Non-protein chemicals, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). The solution conditions for the protein samples are similar to our previous work, which included 25 mM MOPS, 150 mM potassium acetate, 500 mM urea at pH 7.4 [12]. Protein concentrations of incubated samples ranged from 50–100 μ M, and copper sulfate concentrations were always kept at a 2:1 Cu: β 2m ratio. Incubation conditions to form β 2m amyloids were carried out at 37 °C, and under these conditions amyloids are fully formed on the order of weeks (<1 month), which is consistent with previous reports [12,20,37]. The pre-amyloid oligomers of interest in this study were sampled during days 1–10.

ESI-IM-MS

Prior to analysis, samples were removed from the incubation chamber and exchanged into 100 mM ammonium acetate through a GE HiTrap desalting column (Cat# 17140801) (Chicago, IL). Desalted fractions were then loaded into gold sputter-coated glass borosilicate nanospray capillaries from Harvard Apparatus (Cat# 30-0035) (Holliston, MA), whose preparation was described previously [50]. Mass spectral data were obtained on a Waters Synapt G2-Si (Milford, MA). Mass calibration of the instrument from m/z 500–8000 was conducted with perfluoroheptanoic acid (PFHA). Our nanospray ESI-IM-MS method was carefully optimized and performed under low energy conditions to ensure the gentle transfer of protein complex ions from the solution phase to the gas phase to minimize any unfolding or dissociation. No evidence of any highly charged monomeric ions, which are a hallmark of oligomer unfolding and dissociation, were noted during these experiments. Briefly, instrumental settings included 1 kV capillary voltage, 20 V cone voltage, 20 V offset, and 30°C source temperature. The ion mobility cell was operated at a wave height of 20 V, while the wave velocity was set to 300 m/s. Drift times were converted to CCS values via a calibration curve using proteins with known CCS values, the details of which are shown in the supplemental methods and is described in theoretical and experimental detail elsewhere [51]. MS data were analyzed and exported with MassLynx and Driftscope. Final plots were made with OriginLab (Northampton, MA). For plotting arrival time distributions (ATDs), OriginLab was used. Here, ATDs were fit using Gaussian distribution functions. If more

than one peak was present, multiple peaks were fit simultaneously. Both the peak centroid as well as the FWHM were determined in this manner.

Cu(II) depletion with EDTA

The introduction of EDTA to deplete Cu(II)-bound species was carried out similar to prior work [12]. Briefly, prior to desalting, EDTA (500 mM) was added to incubated $\beta 2m$ samples to yield a final concentration of 10 mM. The added volume was approximately 2% of the total sample volume, in order to minimize dilution. The EDTA-doped samples were then allowed to remain at ambient room temperature for 10 minutes prior to desalting and ESI-IM-MS analysis. Data were analyzed as described above, but the signals shown in arrival time distribution plots were normalized by dividing the individual ion's signal by the total ion signals of all $\beta 2m$ peaks in the spectra in order to account for oligomer dissociation.

CCS calculations

The CCS value of each MD-calculated protein state was estimated using the IMPACT program that uses the projection approximation (PA) method [52]. For each configuration sampled by MD simulation, ten PA calculations were repeated and the average value was reported. The simulated CCS value for each $\beta 2m$ oligomer was determined by the peak position after fitting the CCS distribution that was calculated from all possible configurations sampled by MD using a Gaussian function. The resulting PA values from the gas-phase MD simulations were then scaled using an empirical factor used previously to facilitate comparison with experimental values [23]. All calculated values from crystal or NMR structures were treated identically.

Structure Excision and Protein–Protein Docking

Initial tetramer models of $\beta 2m$ were generated via protein-protein docking using dimeric subunits from the hexameric structure of the H13F mutant (PDB: 3CIQ). The subunit structures were transformed into protonation-state optimized all-atom models using the Schrödinger Maestro protein preparation wizard (Schrödinger, LLC, New York, NY). For each dimeric subunit, unconstrained protein-protein docking was performed with Schrödinger BioLuminate PIPER algorithm (Schrödinger, LLC, New York, NY). The 'homodimer' mode was implemented, probing 70,000 ligand rotations. The optimal oligomeric models were selected for further analysis via visual inspection and MD stability tests (see below), upon which symmetric docked structures have collapsed onto compact (TET3) or extended (TET4) forms persisting through simulations.

Molecular Dynamics Simulation

Classical MD simulations using atomistic models were performed using the GROMACS 2018 package [53]. The CHARMM36m force field and the TIP3P model were chosen for modeling human $\beta 2m$ and water molecules [54,55], respectively. The protonation states of the titratable amino acid side chains and N-/C-terminus of $\beta 2m$ were chosen to reproduce the physiological condition at pH 7. The detailed treatment of the Cu(II)-binding site is described in the supplemental methods. In short, the binding site was first extracted from the H13F mutant hexamer structure (PDB: 3CIQ) and further modifications were performed

using quantum chemistry calculations. The simulations of β 2m monomer, dimers, and tetramers were then performed to verify that their stability was maintained by crucial interactions. The single disulfide bond between C25 and C80 in all monomers was intact through the simulations for all models. Gas phase simulations were also performed to ensure the stability of the structures in the gas phase and to calculate CCS values for comparison with experimentally measured results. The detailed procedures for constructing different oligomer states and parameters for both types of simulations (solution and gas phase) are summarized in the supplementary material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATD	Arrival Time Distribution
CCS	Collisional Cross Section
EDTA	ethylenediaminetetraacetic acid
FWHM	full width at half maximum
MHC I	Major Histocompatibility Complex I
MD	Molecular Dynamics
ESI-IM-MS	Electrospray Ionization Ion Mobility Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PFHA	perfluoroheptanoic acid
SASA	solvent accessible surface area
SEC-HPLC	Size Exclusion High Performance Liquid Chromatography
TFE	2,2,2-trifluoroethanol
WT	wild type

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Highlights

- Characterizing amyloid formation *in vitro* is complicated by structural heterogeneity
- Multiple conformational isomers of β 2m oligomers have been identified by IM-MS
- Pre-amyloid tetramers are heterogeneous and release Cu(II) for amyloid formation
- Structural models of transformed Cu(II)-free tetramers are derived from modeling
- The structural models allow further studies of mechanisms and inhibitory strategies

Figure 1: Mass spectra and extracted arrival time distributions of B2m oligomers after incubation in the presence of Cu(II) for 6 days. Panel A shows the mass spectrum collected over the full m/z acquisition range, while the inset shows an expanded view of the m/z region where tetramer and hexamer ions are detected. Panel B shows representative ATD plots, extracted from their corresponding mass spectral peaks. Peaks were fit using Gaussian distributions. The collisional cross section for the centroid of each peak is shown in corresponding color. The error values are from estimations of the random error of the CCS calibration curve. Odd charge states are chosen for each oligomer to ensure their unique identity.

Figure 2:

A plot of experimentally determined CCS values for $\beta 2m$ conformers (black) and theoretical models (red & green). The black squares are centroid values from the IM measurements of CCS values. The full list of values can be found in Table S1 in the Supplementary Information. Error bars are from estimated random error of the calibration curve. Red models assume perfect spherical particles assembled as beads on a string. Green models also assume spherical monomers and were chosen to represent a compact configuration of tetramers and hexamers that have dimers as the primary building block. The blue hexamer model also assumes spherical monomers and was chosen to have a hexagonal array of spheres that involves different surface interactions for each monomer unit.

Figure 3:

Panel A shows four different configurations of dimer in both apo- and holo-forms found following structure excision from H13F and MD simulations. Panel B shows calculated CCS centroid values and distributions for both dimer configurations across the MD trajectory of the dimer with 9 charges. These CCS values reflect scaled gas phase structures. Panel C is an extracted ATD for the dimer⁹⁺ ion with a centroid CCS value for the most abundant species. The lowest measured dimer charge state (i.e. +9) was chosen for comparison to the gas-phase calculated values because the lowest charge state ions are the most native-like and because a charge state of +9 was chosen for the gas-phase calculation.

Figure 4: Panels A-C show extracted ATDs for dimer⁹⁺, tetramer¹³⁺, and hexamer¹⁸⁺ ions over the early stages of amyloid formation, respectively. The width of the dashed lines are added to approximate the FWHM of the first day that the ion is detected and are for illustrative purposes only to guide the eye. Panel D shows calculated FWHM values of selected dimer, tetramer, and hexamer ions over the course of the experiment. Error bars are standard deviations from replicate measurements of the corresponding ion ATDs on different days of analysis (see Figure S6 in the Supplementary Information for representative replicate measurements).

Figure 5:

Panels A, B, and C show mass spectra around selected ions from control (black) and EDTA-treated (red) samples of B2m incubated for 6 days in the presence of Cu(II). Panels D and E are extracted ATDs of the control (black) and EDTA-treated (red) for the tetramer $^{14+}$, and tetramer $^{13+}$ charge states, respectively. The associated CCS values are denoted adjacent to the corresponding peak. Ion intensity for panels D and E are normalized by dividing the individual ion intensity by the sum total of B2m intensity in the spectra.

Figure 6:

Panel A shows a Cu(II)-bound tetramer while panel B shows a Cu(II)-free tetramer found following excision and MD simulations in two orientations. Panel C is a stick view of the interface between the dimer of dimers for TET1 and TET2 structures, where important interactions are denoted by residue letter and number.

Figure 7:

Panel A shows the model of a proposed compact Cu(II)-free tetramer (TET3) in two orientations. Panel B shows the model of a proposed extended Cu(II)-free tetramer (TET4) in two orientations. Panel C highlights residues involved at interfaces of TET3. Panel D shows the Cu(II) binding site for TET3, while panel E shows the same region in Cu(II)-bound TET1. Residues highlighted, other than W60, are key interacting partners in Cu(II) binding. Distances that are measured in TET1 reflect the distance to the Cu(II) atom, while the one measured in TET3 reflects the distance to W60. Panel F shows Cu(II) binding site

residues in orange that are now involved in interfacial contact (blue residues) in the TET4 structure.

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Table 1:Survey of calculated and measured CCS values for various monomeric and oligomeric states of $\beta 2m$ ¹

Species	Amyloid inducing agent	CCS (Å ²)	Method	PDB ID	Reference
Monomer (crystal)	None	1142	Calculated	1LDS	[56]
Monomer (2D NMR)	None	1153	Calculated	1JNJ	[57]
Monomer	Cu(II)	1180	Measured	-	this work
Monomer (native-like)	Low pH	1325	Measured	-	[17]
Monomer (partially unfolded)	Low pH	1768	Measured	-	[17]
Monomer (unfolded)	Low pH	2093	Measured	-	[17]
Monomer (reduced)	Low pH	2530	Measured	-	[17]
Dimer	Cu(II)	1823	Measured	-	this work
Dimer	Low pH	2180	Measured	-	[17]
Dimer (edge-to-edge)	Low pH	~2000	Measured	-	[17]
Dimer (end-to-end)	Low pH	~2200	Measured	-	[17]
Dimer P32A	Cu(II)	1829	Calculated	2F8O	[38]
Tetramer P32A	Cu(II)	2997	Calculated	2F8O	[38]
Dimer DIMC20	TFE	2015	Calculated	From 3TLR	[44]
Dimer DIMC50	TFE	2024	Calculated	From 3TM6	[44]
Dimer via H13F (position 1)	Cu(II)	1831	Calculated	From 3CIQ	[13]
Dimer via H13F 2 (position 2)	Cu(II)	1964	Calculated	From 3CIQ	[13]
Tetramer	Cu(II)	3080	Measured	-	this work
Tetramer	Cu(II)	3120	Calculated	From 3CIQ	[13]
Tetramer	Low pH	3721	Measured	-	[17]
Tetramer (DIMC20)	TFE	3278	Calculated	From 3TLR	[44]
Tetramer (DIMC50)	TFE	3059	Calculated	From 3TM6	[44]
H13F Hexamer	Cu(II)	3972	Calculated	3CIQ	[13]
Hexamer	Cu(II)	4050	Measured	-	this work

¹ Only values for most abundant conformers are shown in Table 1. A full listing of experimentally determined CCS values for oligomeric conformers are found in Table S1.