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Structural Insights into the Pre-Amyloid Tetramer of β-2-Microglobulin from Covalent Labeling and Mass Spectrometry

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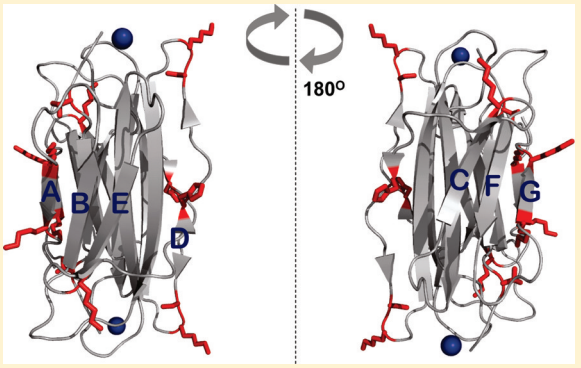
Supporting Information

ABSTRACT: The main pathogenic process underlying dialysis-related amyloidosis is the accumulation of β-2-microglobulin (β2m) as amyloid fibrils in the musculoskeletal system, and some evidence suggests that Cu(II) may play a role in β2m amyloid formation. Cu(II)-induced β2m fibril formation is preceded by the formation of discrete, oligomeric intermediates, including dimers, tetramers, and hexamers. In this work, we use selective covalent labeling reactions combined with mass spectrometry to investigate the amino acids responsible for mediating tetramer formation in wild-type β2m. By comparing the labeling patterns of the monomer, dimer, and tetramer, we find evidence that the tetramer interface is formed by the interaction of D strands from one dimer unit and G strands from another dimer unit. These covalent labeling data along with molecular dynamics calculations allow the construction of a tetramer model that indicates how the protein might proceed to form even higher-order oligomers.



β-2-Microglobulin (β2m) is the noncovalently bound light chain of the class I major histocompatibility complex (MHC-I)[1] and can accumulate as amyloid fibrils in the musculoskeletal system as a complication of long-term hemodialysis, leading to a condition known as dialysis-related amyloidosis (DRA). β2m has 99 residues (~12 kDa) and adopts an immunoglobulin fold with seven β-strands,[2] forming a β-sandwich in its native state (Figure 1). One β-sheet is formed by strands A, B, D, and E, and the other consists of strands C, F, and G. A disulfide bond between Cys25 and Cys80 connects strands B and F in the folded state of the protein.

As part of normal cell turnover, β2m is released from MHC-I and carried to the kidney where it is usually degraded. Upon renal failure, serum levels of β2m increase up to ~60 times above their normal levels of ~0.1 μM, and the protein aggregates into insoluble amyloid deposits.[3,4] An elevated level of β2m, however, is not unique to renal failure patients and is not sufficient to trigger fibrillogenesis.[5,6] β2m amyloid formation must therefore result from factors particular to hemodialysis. These causative factors are not definitively known, but several approaches to generate β2m amyloid fibrils in vitro have been established. These include incubation under acidic conditions (pH <3.6),[7] removal of the first six N-terminal amino acids,[8] mixing with collagen at pH 6.4,[9] sonication with sodium dodecyl sulfate at pH 7.0,[10] and incubation with stoichiometric amounts of Cu(II) under physiological conditions.[11,12]

We have become interested in Cu(II) as a causative factor for several reasons. It has been argued that Cu(II) might initiate β2m fibril formation in vivo because of the elevated Cu(II) concentrations in dialysate.[11] The in vitro conditions necessary to stimulate β2m fibril formation in the presence of Cu(II) are also more similar to physiological conditions than other methods used to stimulate β2m fibril formation. Moreover, a recent study indicates that Cu(II) plays a catalytic role in causing β2m fibril formation.[13] This latter observation is important because large systemic increases in Cu(II) concentrations are therefore not necessary. While these observations do not confirm a role for Cu(II) in vivo, β2m does represent yet another protein system for which Cu(II) can stimulate the formation of amyloid fibrils.[14−18] Finally, from a biochemical perspective, adding Cu(II) is a discrete way to trigger amyloid formation so that the intermediates that precede the fibrils can be more easily studied.

Previous work has shown that Cu(II)-induced β2m amyloid formation is preceded by the formation of discrete, oligomeric intermediates, including dimers, tetramers, and hexamers.[13,19] Several studies have attempted to characterize these pre-amyloid oligomers, but this task is very challenging because the oligomers are present as a mixture of species and, as intermediates, are only transiently populated. One successful approach to obtaining structural information about the oligomers has been to create β2m mutants that are stable enough to crystallize as oligomers.[20,21] In this way, it has been found that a P32A mutant of β2m forms a dimer in the absence

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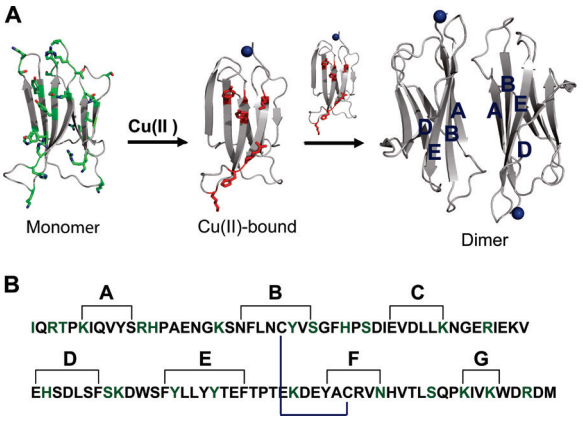

Figure 1. (A) Ribbon representation of monomeric β2m (PDB entry 2D4F), Cu(II)-bound β2m, and the formation of the dimer by stacking of two antiparallel ABED sheets. Amino acids modified by the covalent labels are shown as green sticks. The amino acids found in the dimer interface (ABED β-sheet) are shown as red sticks. (B) Amino acid sequence of β2m showing strand nomenclature.[2] Black lines show amino acids on each β-strand. The internal disulfide bond is shown with a blue line. The amino acids probed by the covalent labels are colored green.

of Cu(II) and an H13F mutant forms a hexamer in the presence of Cu(II). These crystal structures provide high-resolution atomic-level information about possible β2m oligomer structures, but they do not provide a complete picture about oligomer structure because these mutants do not ultimately form amyloid fibrils.

As a complement to these crystallographic studies, we have used a protein surface mapping approach in conjunction with mass spectrometry (MS) to study the oligomeric intermediates of β2m. The method involves covalent modification of amino acid side chains that are exposed to solvent and the identification of the modified residues by MS. Because amino acids are usually buried upon formation of new protein−protein interfaces, residues involved in mediating these interactions are much less reactive and can be identified from differential modification patterns. This approach can be directly applied to wild-type β2m under amyloid-forming conditions, and the specificity of MS allows us to obtain structural information even when a mixture of β2m forms is present. We recently used this method to gain insight into the structure of the dimer formed by wild-type β2m and found that the dimer interface is formed by the antiparallel stacking of ABED β-sheets from two β2m monomers.[22]

In this study, we have applied this covalent labeling−MS approach to characterize the tetrameric form of β2m that follows formation of the dimer. Through these measurements, we find evidence that the tetramer is formed by the interaction of D strands of one dimer unit and G strands of another dimer unit. Using the covalent labeling data along with molecular dynamics calculations, we are then able to build a model of the tetramer that suggests how the protein can continue to form higher-order oligomers. Our results demonstrate the potential power of covalent labeling and MS for studying oligomer-forming proteins, and they help develop a model for the pre-amyloid tetramer of β2m that could be used to design inhibitors of β2m oligomerization and fibril formation.

MATERIALS AND METHODS

Materials. Human β2m was obtained from Fitzgerald Industries International, Inc. (Concord, MA). Diethyl pyrocarbonate (DEPC), 2,3-butanedione (BD), sulfo-*N*-hydroxysuccinimide acetate (NHSA), imidazole, dithiothreitol (DTT), copper(II) sulfate ($CuSO_4$), 3-morpholinopropanesulfonic acid (MOPS), potassium acetate, arginine, ubiquitin from bovine erythrocytes, equine heart cytochrome *c*, equine skeletal muscle myoglobin, chicken egg white ovalbumin, human hemoglobin, and bovine transferrin were purchased from Sigma-Aldrich (St. Louis, MO). Tris(hydroxymethyl)-aminomethane (Tris) was purchased from EM Science (Gladstone, NJ). Urea was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Trypsin was from Promega (Madison, WI), and chymotrypsin was purchased from Roche Diagnostics (Indianapolis, IN). Centricon molecular weight cutoff (MWCO) filters were obtained from Millipore (Burlington, MA). Deionized water was prepared from a Millipore Simplicity 185 water purification system.

Formation of β2m Oligomers and Fibrils. Reports have shown that discrete oligomers precede β2m amyloid fibrils when monomeric β2m is incubated under near-physiological conditions in the presence of Cu(II).[13,19] Amyloid fibrils were formed by incubation of 100 μM β2m in 200 mM potassium acetate, 500 mM urea, and 25 mM MOPS (pH 7.4) with 200 μM $CuSO_4$ at 37 °C. This concentration of protein is higher than that found in dialysis patients but was necessary to ensure the formation of oligomers in an experimentally reasonable time period. All components were equilibrated at 37 °C prior to Cu(II) addition and immediately returned to 37 °C after mixing. The covalent labels were added to aliquots of incubated β2m taken at several time points after the amyloid fibril formation reaction had been initiated.

Carbethoxylation with DEPC. Stock solutions of DEPC were prepared in acetonitrile. The DEPC reactions of β2m were performed for 1 min at 37 °C and were initiated by addition of 0.25 mM DEPC. The total reaction volume for the experiments was 30 μL, and the total amount of acetonitrile added was ~1.5%. The reactions were quenched after 1 min via addition of 10 mM imidazole.

Acetylation with NHSA. Stock solutions of NHSA were prepared in water. The labeling of β2m with 0.30 mM NHSA was conducted for 1 min at 37 °C. The total reaction volume for the experiments was 30 μL. The reactions were quenched by addition of 10 mM Tris.

BD Modification. Stock solutions of BD were prepared in water. The reactions of β2m with 35 mM BD were performed in the dark for 1 min at 37 °C. Reactions were conducted in the dark to avoid possible photoactivation of the label, which could enhance nonspecific reactions with residues other than arginine.[23,24] The total reaction volume for the experiments was 30 μL. The reactions were quenched by addition of 100 mM arginine.

Proteolytic Digestion. Before proteolytic digestion, all modified samples were purified using a 10000 MWCO filter and reconstituted with deionized water to a final concentration of 300 μM. Purified β2m samples in 25 mM Tris-HCl (pH 7) and 1 mM $CaCl_2$ were first reacted with 10 mM DTT at 37 °C for 45 min to reduce the disulfide bonds. This was followed by addition of acetonitrile and incubation at 37 °C for 45 min. Trypsin and chymotrypsin (1 μg/μL) were then added to

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DEPC-modified samples to yield a final enzyme:substrate ratio of 1:20. For the NHSA- and BD-modified β2m samples, only chymotrypsin (1 μg/μL) was added. Although trypsin is a reliable and robust protease, chymotrypsin was used instead because trypsin can no longer cleave proteins after acetylated lysine and modified arginine residues. All samples were digested at 37 °C for 16 h. The enzymes were inactivated by addition of 2 μL of acetic acid, and the samples were immediately analyzed.

Instrumentation. The amount of modification was determined by removing an aliquot of the purified β2m and analyzing the samples using a Bruker Daltonics (Billerica, MA) amaZon quadrupole ion trap mass spectrometer equipped with an electrospray ionization (ESI) source. The ESI source was operated at a spray voltage of 4.5 kV, and the capillary temperature was set at 220 °C. The voltages for the transfer optics between the ESI source and the ion trap were optimized for the maximal signal.

The proteolytic fragments were separated with an Agilent (Wilmington, DE) HP1100 high-performance liquid chromatography (HPLC) system with a C18 column (15 cm × 2.1 mm, 5 μm particle size, Supelco, St. Louis, MO) for online analysis by MS and MS/MS. The fragments of NHSA- and BD-modified β2m were eluted using a linear gradient of methanol that increased from 5 to 70% over 20 min and from 70 to 100% over the final 2 min at a flow rate of 0.25 mL/min. For the fragments of DEPC-modified β2m, a linear gradient of methanol that increased from 5 to 70% over 30 min and from 70 to 100% over the final 3 min was used. For both gradient conditions, water comprised the balance of the solvent, and a total of 0.1% acetic acid was present. The LC effluent was directly fed into the mass spectrometer with ESI source conditions similar to those described above. Tandem mass spectra were recorded using collision-induced dissociation (CID) with isolation widths of 1.0 Da and excitation voltages between 0.6 and 1.0 V. Peptide sequences were determined from the MS/MS data via de novo sequencing or with the help of BioTools (Bruker Daltonics).

To monitor the formation of oligomers, we separated the incubated solutions of β2m by size-exclusion chromatography (SEC) using a Superdex 75 PC 3.2/30 column (Amersham Biosciences) installed on an Agilent HP1100 series HPLC system. Before analysis of the sample, the SEC column was first equilibrated with a 20 mM ammonium acetate mobile phase (pH 7.4) at a flow rate of 0.06 mL/min for 1 h. During the analysis, 5 μL of an incubated sample solution was injected into the sample loop. Either a variable-wavelength detector set to 214 nm or a Bruker Esquire-LC quadrupole ion trap mass spectrometer, equipped with an ESI source, was used for detection. The identity of the separated oligomers was confirmed via comparison to a molecular weight (MW) calibration curve or the m/z ratios measured by the mass spectrometer. For the MW calibration, a solution containing a mixture of the following proteins and peptides was used: 1.5 μM bovine serum albumin (MW = 66000 Da), 3 μM carbonic anhydrase (MW = 29040 Da), 3 μM myoglobin (MW = 16951 Da), and 3 μM β2m (MW = 11731 Da).

Determination of Modification Percentages. The percent modification of each labeled amino acid was determined by comparing the LC−MS abundances of modified and unmodified proteolytic peptide fragments containing the amino acid of interest. Because the ionization efficiencies of the modified and unmodified residues are not identical, our evaluation of the modified residues relies on relative rather than absolute changes in the extents of modification. All peptide fragments containing the modified residue were accounted for, including overlapping peptides that are commonly generated during chymotryptic digestion. For each peptide fragment, the modified form eluted after the unmodified form with the difference in retention times ranging from 1 to 12 min. The ion abundances of both modified and unmodified peptides were determined from extracted ion chromatograms; as examples, see Figures S1−S3 of the Supporting Information. The percent modification was obtained by dividing the total ion abundance of the modified fragment (I_{modified}) by the sum of the total ion abundances for the modified (I_{modified}) and unmodified ($I_{\text{unmodified}}$) fragments as shown in eq 1.

$$\%\ \text{modification} = \frac{I_{\text{modified}}}{I_{\text{modified}} + I_{\text{unmodified}}} \times 100 \quad (1)$$

All the charge states for a given peptide fragment were considered in the determination of the total ion abundances. The errors are reported as the standard deviation of the mean using the data from three separate modification reactions. Because ion intensity ratios were used, low modification levels could be accurately determined. Indeed, reliable covalent labeling approaches typically yield relatively low modification levels to avoid label-induced structural changes (refs 25 and 26 and references cited therein). Even so, these methods have been successfully used to obtain accurate protein structural information.

Molecular Dynamics Simulations. All simulations were initiated using a dimer, the B and C chains, from the H13F mutant hexamer crystal structure (PDB entry 3CIQ),[21] which contains 98 amino acid residues. Met0 was eliminated from the original set of coordinates because this residue is not present in the wild-type protein. Two dimers were added together before the simulations began to produce a tetramer structure that was consistent with the covalent labeling data. The two dimers were added together using the docking simulation program Zdock (version 3.0.1).[27,28] Three descriptors were taken into account for dimer−dimer docking: (i) the electrostatic potential of the dimer surface, (ii) the shape complementarity of the dimer−dimer interface, and (iii) the desolvation energy associated with the attachment process. The resulting tetramer structure was then energy-minimized, using an explicit model of the solvent with the AMBER94 force field[29,30] implemented in GROMACS.[31−33]

During the minimization, hydrogen atoms were explicitly added to the tetramer model and His residues were considered to be neutral, as shown in nuclear magnetic resonance (NMR) studies by Esposito and co-workers.[34] Water molecules were simulated using a TIP3P model,[35] and molecules were added until a cubic simulation cell with a side length of 9.6 nm was achieved.

RESULTS

Structural studies of the oligomeric intermediates that precede β2m amyloid formation are challenging because these oligomers are transient, are present as a mixture, and can dissociate back into the monomer during isolation. Using SEC, dynamic light scattering, and ESI-MS, we found previously that β2m oligomers are formed via the discrete addition of dimer units.[13] The dimer first appears within ∼1 h of addition of

Cu(II). A first form of the tetramer appears within 12−24 h. A second Cu(II)-free form of the tetramer appears after approximately 3 days, and the hexamer appears soon after the Cu(II)-free tetramer. Because complete isolation of the tetramer is very difficult without some dissociation back to the dimer and monomer, covalent labeling experiments were performed at different times before (t = 0 min) and after (t = 2 min, 2 h, 0.5 day, 1 day, 1.5 days, 2 days, and 2.5 days) addition of Cu(II) to initiate the amyloid fibril-forming reaction. At time points between 0.5 and 2.5 days, the mixture contains monomer, dimer, and tetramer as indicated by SEC experiments (Figure 2). The percentage of tetramer at time points

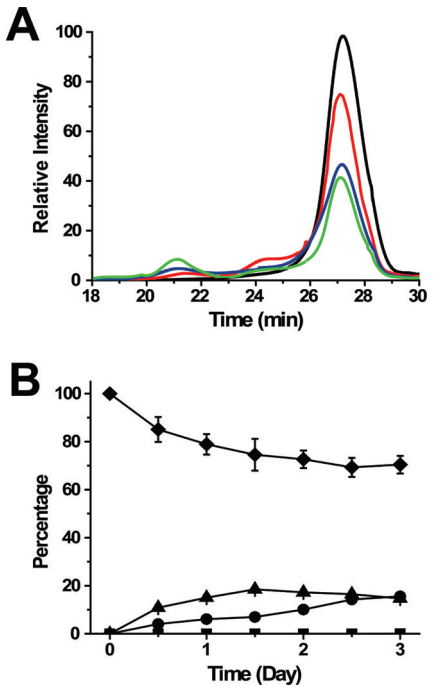

Figure 2. Formation of monomers, dimers, and tetramers as studied by size-exclusion chromatography (SEC). (A) SEC profiles before (black) and 1 day (red), 2 days (blue), and 3 days (green) after the addition of Cu(II). (B) Plot showing the percentage of each oligomer as a function of time: monomer (◆), dimer (▲), tetramer (●), and hexamer (■). The oligomer identities in the size-exclusion chromatogram were confirmed by comparison to a molecular weight calibration curve and by online SEC-ESI-MS.

after the addition of Cu(II) increases from 0 to 15% based on ESI-MS measurements of the desalted sample under amyloid-forming conditions, as described previously,[13] and these results are consistent with the SEC data. Also, the percentage of dimer increases from 0 to ∼30% from 0 to 2.5 days, as indicated by ESI-MS measurements; the ESI-MS and SEC data are also roughly consistent with regard to the dimer as the SEC data indicate an increase in the percentage of dimer from 0 to ∼20% (Figure 2).

In recent covalent labeling studies of the β2m dimer, we identified 20 amino acids that undergo changes in labeling reactivity, relative to the monomer, after Cu(II) binding and dimer formation (Figure 1).[22] In this study, we have sought to identify residues that undergo changes in labeling reactivity only as the tetramer is formed. To do this, the covalent labeling data that are obtained when the tetramer is present (0.5−2.5 days) is compared to the data acquired when only dimer and monomer are present in solution (0.5−2 h). Specifically, we examined the modification trend (i.e., increase, decrease, or leveling off) from the covalent labeling data in the 0.5−2.5 day time period with the modification trend from the covalent labeling data in the 0.5−2 h time period.[22] During the 0.5−2.5 day time period, the rate of tetramer formation is almost twice that of dimer formation and similar to the rate of decrease in the monomer percentage (Figure 2). Indeed, the amount of dimer levels off after 1.5 days. Hence, any residues that undergo a new modification trend (i.e., increase, decrease, or leveling off) after 0.5 day, as compared to the 0.5−2 h time period, are then residues that likely undergo changes in solvent accessibility important for the formation of the tetramer. Furthermore, we have baseline modification data for the monomer [i.e., no Cu(II) added] to ensure that any decreases in the amount of modification are due to tetramer formation. We also performed control experiments in which covalent labeling reactions were conducted with a solution containing only monomeric β2m [i.e., no Cu(II) added] that was incubated under identical conditions for time periods of up to 2 days. No significant changes in the labeling of any residue were observed, indicating that the protein undergoes no significant conformational changes during this time period (Table S1 of the Supporting Information). In addition, both SEC and ESI-MS measurements indicate no oligomer formation over this time period without the addition of Cu(II).

Covalent Labeling with NHSA. Amino groups such as the ε-NH_2 group of lysine residues and the N-terminal α-NH_2 group can react with NHSA, but information from such surface mapping experiments is reliable only if the structural integrity of a protein is preserved during the reaction. Our group recently demonstrated that monitoring the reaction kinetics for individual modification sites is a powerful way to ensure a protein's structural integrity upon modification.[36] This can be done by monitoring the ion abundance of peptide(s) that contain the residue of interest as a function of labeling reagent concentration. Considering these reactions are second-order, such dose−response plots will be linear over the range of reagent concentrations where the protein's structural integrity is maintained and will deviate from linearity when reagent concentrations are reached that perturb the protein's structure (see Figure S4 of the Supporting Information as an example). By generating such plots for all modified peptides, we identify the maximal reagent concentration that can be used to ensure the protein's structure is not perturbed. In the studies here, we found that reaction of β2m with a 3-fold molar excess of NHSA for 1 min at 37 °C does not induce any protein structural changes, which is consistent with our previous work on this protein.[22] The reaction time was kept short (1 min) to minimize tetramer dissociation.

Proteolytic digestion and LC−MS analyses are necessary to identify the amino acids that undergo changes in reactivity as the tetramer concentration increases. Under solution conditions in which β2m forms amyloid fibrils, LC−MS/MS analyses show that the N-terminus, almost all of the lysines (Lys6, Lys19, Lys41, Lys58, Lys75, Lys91, and Lys94), and Asn83 are labeled to different degrees. The unmodified and modified fragments containing these amino acids can be detected at all time points; however, the level of modification for some residues changes over time (vide infra). In our previous study, definitive MS/MS data could not be obtained for the Lys41−Leu54 fragment.[22] Here, the better sensitivity of

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the quadrupole ion trap used in these experiments allowed identification of Lys41 as the modification site.

The reactions with NHSA at different time points after formation of the tetramer reveal that the extents of modification of some residues change as the concentration of the tetramer increases in solution (Figure 3 and Figure S5 of the Supporting Information). The modification percentages for the peptide fragments containing the indicated residues were determined from the LC−MS intensities of the modified and unmodified fragments, as described in Materials and Methods. For each time point, the modification reaction was repeated three times. Because ion intensity ratios of the unmodified and modified peptide fragments are used to determine the extent of modification, small changes in modification levels can be accurately and precisely determined, as we demonstrated in our previous work.[22] Also, it should be noted that the low levels of modification for some residues reflect the need to minimize the overall degree of labeling to prevent the labels from perturbing protein structure.[25]

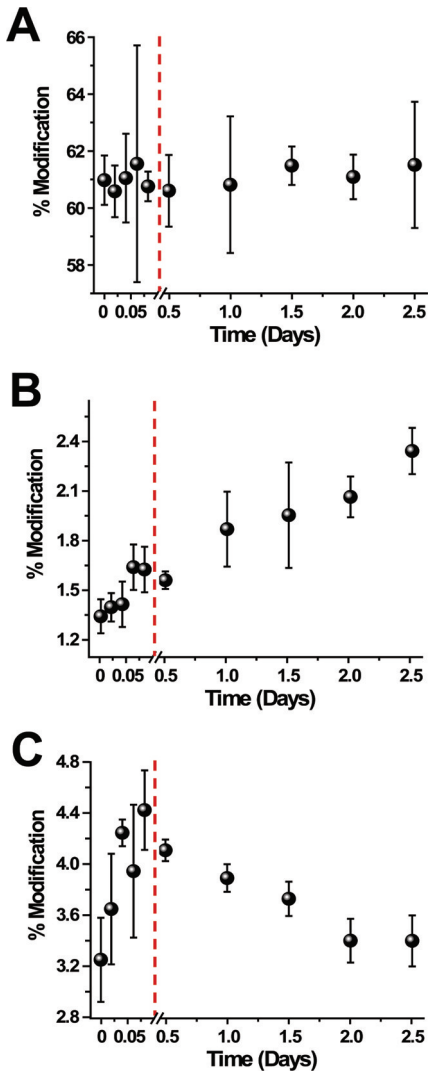

Figure 3. Extent of NHSA modification for selected residues, (A) N-terminus, (B) Lys75, and (C) Lys94, throughout the course of the tetramer formation reaction. The changes in modification during dimer formation are shown (points to the left of the red dashed line) as reference points.

Of all the residues acetylated by NHSA, the N-terminus (Figure 3A) and Asn83 do not significantly change in their extent of modification from 0.5 to 2.5 days after addition of Cu(II). The level of modification of Lys75 (Figure 3B) increases from 0.5 to 2.5 days after Cu(II) is added. In contrast, the reactivities of Lys6, Lys19, Lys41, Lys58, and Lys91 (Figure S5A−D,F of the Supporting Information) and Lys94 (Figure 3C) decrease as the tetramer's concentration increases in solution. The extent of modification of Lys6 decreases slightly within 2 h of addition of Cu(II), and the reactivity of this residue continues to decrease up to 2.5 days. In our previous covalent labeling studies with the dimer, we concluded that Lys6 was part of the dimer interface, so its continual decrease in reactivity is consistent with the dimer's concentration continuing to increase in solution over time.

The rest of the lysine residues, Lys19, Lys41, Lys58, Lys91, and Lys94, behave differently. The reactivity of these five lysine side chains also decreases as the tetramer concentration increases, but their reactivities either do not change or increase prior to tetramer formation. For example, the reactivity of Lys94 increases as the dimer's concentration increases in solution, but as soon as the tetramer begins to significantly populate the solution, the reactivity of Lys94 decreases. On the other hand, Lys41 undergoes no change in reactivity as the dimer is formed, whereas its reactivity decreases as the tetramer's concentration increases in solution.

In summary, our results indicate that the reactivity of NHSA with the N-terminus and Asn83 do not change as the tetramer is formed in solution. In contrast, the reactivity of Lys75 increases as the tetramer is formed, whereas the reactivities of Lys6, Lys19, Lys41, Lys58, Lys91, and Lys94 decrease as the tetramer's concentration increases in solution. Importantly, the extent of modification for Lys6 decreases in the first 2 h after the addition of Cu(II), which suggests that its continuing drop in its level of modification is due to the formation of more dimer in solution rather than the formation of tetramer. Tetramer formation is then likely responsible for the decreased reactivity of the remaining lysine residues (i.e., Lys19, Lys41, Lys58, Lys91, and Lys94).

Covalent Labeling with DEPC. DEPC reacts readily with histidine residues but can also react with amine and hydroxyl groups at neutral pH. Using dose−response plots like that shown in Figure S4 of the Supporting Information, we found that a 2.5-fold molar excess of DEPC is sufficient to label several amino acids in β2m while minimizing any DEPC-induced structural changes to the protein. As was the case with the NHSA reactions, the reaction time was kept short (1 min) to minimize tetramer dissociation but also to minimize DEPC hydrolysis by water.

Proteolytic digestion of the protein and LC−MS/MS analyses indicate that DEPC reacts with the N-terminus, Thr4, Lys6, His13, Lys19, Tyr26, Ser28, His31, Ser33, Lys41, His51, Ser57/Lys58, Tyr63, Tyr67, Lys75, Ser88, and Lys94. These modifications are observed at all time points, but the level of modification for some residues changes over time (vide infra). In all cases but one (Ser57/Lys58), the specific amino acids that are modified could be determined unambiguously from the MS/MS data.

When the covalent labeling reactions with DEPC are performed at different time points after the tetramer begins

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to form, the modification levels of 13 of the 17 labeled residues change as the tetramer's concentration increases in solution (Figure 4 and Figure S6 of the Supporting Information). The

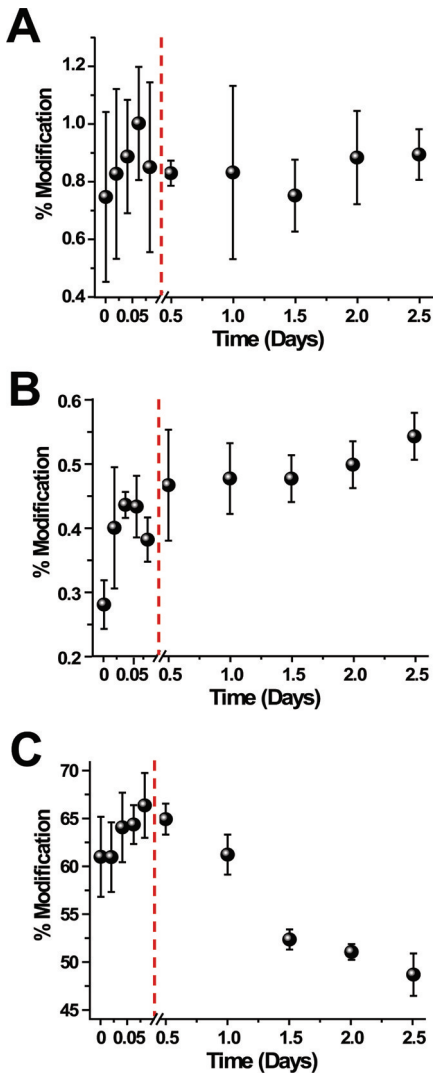

Figure 4. Extents of DEPC modification for selected residues, (A) His31, (B) Lys75, and (C) His51, throughout the course of the tetramer formation reaction. The changes in modification during dimer formation are shown (points to the left of the red dashed line) as reference points.

labeling data reveal that only Lys75 (Figure 4B) shows an increase in its extent of modification upon tetramer formation. The levels of modification of six residues, Lys6, His13, Lys19, Ser28, Tyr63, and Tyr67, initially decrease within 2 h of the addition of Cu(II) and continue to decrease for up to 2.5 days. The extents of modification of five residues, Thr4, Lys41, His51, Ser57/Lys58, and Lys94, increase within 2 h of the addition of Cu(II) but then decrease as the tetramer concentration increases in solution. Five residues, the N-terminus, Tyr26, His31, Ser33, and Ser88, undergo no change in reactivity as the tetramer is formed. In comparing the DEPC and NHSA reactivity for those residues that react with both reagents, we find complete consistency for the N-terminus, Lys6, Lys41, Lys58, Lys75, and Lys94. Only the reactivity of Lys19 is slightly different.

In summary, the reactions with DEPC indicate that 12 residues undergo a notable change in reactivity as the tetramer is formed in solution. The extent of modification of Lys75 increases, whereas the reactivity of the other 11 amino acids decreases. The decrease in the extents of modification for Lys6, His13, Lys19, Ser28, Tyr63, and Tyr67 within 2 h of the addition of Cu(II) suggests that the continual drop in the extents of modification for these residues is due to formation of more dimer in solution rather than the formation of tetramer. Consequently, tetramer formation is then likely responsible for the reactivity changes of Thr4, Lys41, His51, Ser57/Lys58, Lys75, and Lys94.

Covalent Labeling with Butanedione. Unlike DEPC, which reacts with many nucleophilic groups, BD reacts specifically with arginine. The reaction is reversible at pH <9 and is less efficient than the NHSA or DEPC reactions, so larger reagent doses are necessary to improve the product yield. Using dose–response plots like that shown in Figure S4 of the Supporting Information, we determined that a 350-fold molar excess of BD is suitable for preventing any modification-induced structural changes as well as for obtaining readily detectable modifications. The LC–MS data indicate that BD reacts with Arg3, Arg12, Arg45, and Arg97 but not Arg81. The reactions of BD with β2m reveal that Arg3 and Arg12 undergo a very slight decrease in reactivity upon formation of the tetramer (Figure 5 and Figure S7 of the Supporting

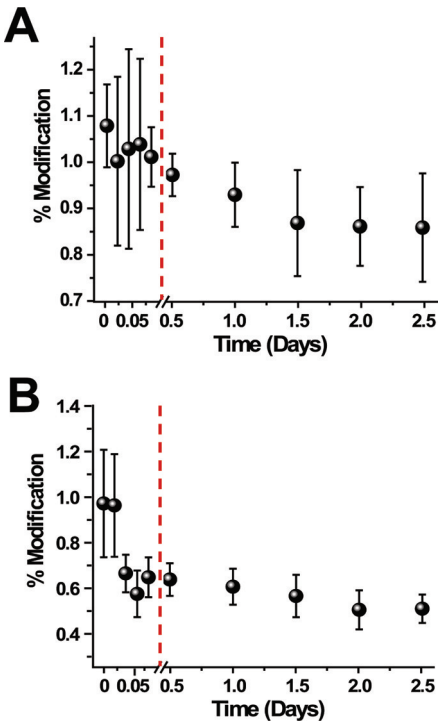

Figure 5. Extents of BD modification for selected residues, (A) Arg3 and (B) Arg12, throughout the course of the tetramer formation reaction. The changes in modification during the dimer formation are shown (points to the left of the red dashed line) as reference points.

Information). The levels of modification of Arg3 and Arg12 decrease slightly as the tetramer's concentration increases in solution, but because the reactivities of these residues also decrease slightly before 0.5 day, their continual decreases in reactivity are likely due to formation of more dimer in solution

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Table 1. Summary of the Modification Percentages for the Modified Amino Acids before the Addition of Cu(II) (t = 0 min) and 2 h (t = 2 h) and 2.5 Days (t = 2.5 days) after the Addition of Cu(II)

residue	t = 0 min	t = 2 h	t = 2.5 days	statistically significant change?[a]
		NHSA		
N-terminus	84 ± 5	61 ± 3	61 ± 3	no
Lys6	18.3 ± 0.9	18 ± 1	14.2 ± 0.7	yes
Lys19	7.1 ± 0.3	7.2 ± 0.3	6.42 ± 0.3	yes
Lys41	13 ± 1	13 ± 1	9.7 ± 0.5	yes
Lys58	16 ± 1	19 ± 1	13.9 ± 0.9	yes
Lys75	1.47 ± 0.1	1.6 ± 0.2	2.3 ± 0.2	yes
Asn83	1.48 ± 0.07	1.46 ± 0.05	1.6 ± 0.2	no
Lys91	27 ± 1	24.1 ± 0.9	22.0 ± 0.8	yes
Lys94	3.2 ± 0.5	4.4 ± 0.5	3.4 ± 0.3	yes
		DEPC		
N-terminus	99 ± 5	68 ± 2	68 ± 3	no
Thr4	87 ± 1	90 ± 4	61 ± 5	yes
Lys6	8.1 ± 0.1	7.64 ± 0.4	5.9 ± 0.6	yes
His13	45 ± 2	40 ± 2	34.2 ± 2	yes
Lys19	13.0 ± 0.9	12 ± 1	10.1 ± 0.5	yes
Tyr26	0.7 ± 0.1	0.4 ± 0.1	0.35 ± 0.04	no
Ser28	0.24 ± 0.03	0.11 ± 0.01	0.078 ± 0.01	yes
His31	1.6 ± 0.3	0.8 ± 0.3	0.9 ± 0.1	no
Ser33	1.7 ± 0.1	1.2 ± 0.2	1.3 ± 0.2	no
Lys41	0.32 ± 0.07	0.34 ± 0.04	0.24 ± 0.02	yes
His51	60 ± 2	66 ± 3	49 ± 4	yes
Ser57/Lys58	39 ± 2	47 ± 5	33 ± 3	yes
Tyr63	6.3 ± 0.3	3.4 ± 0.3	2.7 ± 0.2	yes
Tyr67	2.2 ± 0.2	1.5 ± 0.1	1.2 ± 0.1	yes
Lys75	0.31 ± 0.04	0.38 ± 0.05	0.54 ± 0.06	yes
Ser88	66 ± 2	72 ± 4	69 ± 5	no
Lys94	28 ± 3	36 ± 3	28 ± 2	yes
		BD		
Arg3	0.5 ± 0.2	1.05 ± 0.06	0.8 ± 0.1	yes
Arg12	1.3 ± 0.2	0.64 ± 0.06	0.48 ± 0.07	yes
Arg45	3.8 ± 0.5	6.5 ± 0.8	6.6 ± 0.5	no
Arg97	2.8 ± 0.5	4.9 ± 0.4	5.1 ± 0.3	no

[a]A two-sample unpaired t-test was used to determine if the changes between 2 h and 2.5 days after the addition of Cu(II) are statistically significant. The calculated t-value was compared to the t-value at the 95% confidence level (i.e., t = 2.776). Residues with calculated t-values of >2.776 were considered to undergo a statistically significant change.

rather than the formation of tetramer. The unchanged reactivity of Arg45 and Arg97 is curious because both of these residues undergo increases in reactivity up to 2 h as the dimer's concentration increases in solution. It is likely that the reactivities of these residues slightly decrease as the tetramer is formed, but this decrease is counterbalanced by the increase in reactivity as the dimer's concentration continues to increase in solution for up to 2.5 days. Overall, the BD reactivity suggests that Arg3 and Arg12 undergo no significant changes in reactivity as the tetramer is formed but Arg45 and Arg97 might.

A summary of the reactivity changes for all of the modified amino acids monitored before Cu addition (t = 0 h) and 2 h and 2.5 days after Cu(II) addition is shown in Table 1.

DISCUSSION

The goal of this study is to obtain structural insight into the pre-amyloid tetramer that is formed by β2m after the addition of Cu(II) at near physiological conditions. Our group has previously shown that Cu(II)-induced β2m amyloid fibril formation is preceded by the formation of dimers, tetramers, and hexamers.[13] No odd-numbered oligomers are observed, indicating that the oligomeric intermediates form via the assembly of dimers. In addition, our previous work suggests that the first tetramer species that is formed has Cu(II) bound to it, but as the amyloid fibril-forming reaction proceeds, Cu(II) is lost from this first tetramer before forming Cu(II)-free tetramers, hexamers, and eventually amyloid fibrils. In a recent study, we showed that the pre-amyloid dimer is formed by the interaction of antiparallel ABED β-sheets from two monomers.[22] This dimer structure provides a starting point for gaining insight into the structure of the Cu(II)-bound tetramer.

Covalent modification with detection by MS was selected to probe the tetramer as this method has been effective in mapping protein surfaces, identifying ligand-binding sites, detecting ligand-induced conformational changes, and studying protein−protein complexes,[26,37−39] including the β2m dimer.[23] As described above and in our previous studies,[22] care was taken to select optimal reaction times and stoichiometries for each covalent labeling reagent to ensure that the amount of label added and the resulting modification do not disrupt the protein structure. A total of 24 residues in β2m are probed by NHSA, DEPC, and BD, and these residues are widely distributed along the polypeptide chain and on the surface of the protein, representing ∼30% of the surface amino acids (see

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the monomer in Figure 1A). Changes in the reactivity of amino acid side chains reflect changes in their solvent accessibility. Hence, patterns of modification during the course of the amyloid fibril formation reaction permit us to build a low-resolution map of the protein and identify residues involved in mediating tetramer formation. Having baseline labeling data for the monomer and dimer allows us to readily identify those residues involved in tetramer formation, even though monomer, dimer, and tetramer are present simultaneously. In addition, being able to perform these reactions on wild-type β2m under amyloid fibril-forming conditions makes the resulting structural information relevant for understanding native β2m amyloid assembly.

On the basis of the data listed in Table 1, the amino acid side chains probed by the covalent labels can be grouped into three categories, namely, residues with (1) unchanged reactivity, (2) decreased reactivity, and (3) increased reactivity from 2 h to 2.5 days after the addition of Cu(II) when the first form of the tetramer is present. The covalent labeling experiments reveal that six of the 24 modified amino acids, the N-terminus, His31, Ser33, Arg45, Ser88, and Arg97, maintain the same reactivity with their respective labeling reagents at time points from 2 h to 2.5 days, although the unchanged reactivity of Arg45 and Arg97 is notably different (see below). Five of these residues, the N-terminus, His31, Ser33, Asn83, and Ser88, have the same reactivity in the dimer and tetramer, indicating that these residues retain similar microenvironments in both these oligomers. Therefore, these residues are not likely part of the tetramer interface. The unchanged reactivities of the N-terminus and His31 are consistent with these residues remaining Cu(II) binding sites in the monomer, dimer, and tetramer.[40] The unchanged reactivity of Ser33 is also consistent with steric hindrance caused by nearby Cu(II) binding in the monomer and oligomers. Others have reported that Cu(II) binding induces a *cis*-to-*trans* backbone isomerization of Pro32. In the crystal structures of the P32A mutant dimer and the dimeric unit in the H13F mutant hexamer, which both show this *cis*–*trans* isomerization, Ser33 has a low accessibility to solvent.[20,21] Persistence of this structural feature in the tetramer due to Cu(II) binding would maintain the microenvironment around Ser33 in such a way that its reactivity would not change. The unchanged reactivity of Ser88 suggests that it remains solvent accessible in the monomer, dimer, and tetramer. The reactivity of Arg45 and Arg97 is different than that of the N-terminus, His31, Ser33, and Ser88. These arginine residues undergo increases in reactivity as the dimer is formed, but then their reactivities level off as the tetramer is formed. This behavior suggests that they undergo decreases in reactivity as the tetramer is formed, thereby counterbalancing their increased reactivity as the dimer continues to populate the solution. Thus, we consider Arg45 and Arg97 as residues that decrease in reactivity as the tetramer is formed.

Including Arg45 and Arg97, 18 residues undergo decreases in reactivity as the tetramer's concentration increases. Nine of these residues, Arg3, Lys6, Arg12, His13, Lys19, Tyr26, Ser28, Tyr63, and Tyr67, were found previously to undergo decreases in reactivity as the dimer is formed, and their continued decrease in reactivity is consistent with the dimer's continued increase in concentration over time. The nine remaining residues, Thr4 on the A strand, Lys41 on the C strand, Arg45 on the C–D loop, His51 on the D strand, Ser57/Lys58 on the D–E loop, and Lys91, Lys94, and Arg97 on the G strand, undergo decreases in reactivity only once the tetramer is formed. Because solvent accessibility is the main factor that affects the reactivity of a given amino acid side chain, decreases in reactivity suggest that these latter nine amino acids may mediate interactions in the tetramer.

Even though no crystal structure of a wild-type or mutant β2m tetramer is available to which we can compare our covalent labeling data, several lines of evidence help us arrive at a model for the pre-amyloid tetramer of the wild-type protein. (1) Previous reports have shown that the β2m oligomers are nativelike in structure,[41] indicating that the tetramer will have many of the structural features present in the wild-type monomer for which several crystal structures exist.[42,43] (2) Previous covalent labeling experiments from our group provide insight into the structure of the wild-type β2m dimer, which has essentially the same structural features as the dimer unit formed by the B and C chains of the H13F mutant hexamer of β2m.[22] This dimer structure (see Figure 1) serves as a convenient starting point for constructing a model of the tetramer because the tetramer very likely arises from the assembly of two dimers. (3) The H13F mutant hexamer contains another oligomeric interface (chains C and D) that could represent the interface of the wild-type tetramer, so our labeling data can be directly compared to data for the interface of these two chains. (4) The P32A mutant dimer, which was previously ruled out as a possible dimer interface for wild-type β2m, could represent the interface of the wild-type tetramer, and our labeling data can be compared to this structure, too.

In comparing our labeling data with the oligomeric interface of chains C and D of the H13F hexamer (Figure 6), we find that only three of the nine residues that undergo decreases in reactivity are found at the C–D interface. The interface of these two chains should result in reduced solvent accessibility for His51, Ser57, and Lys58, and we observe experimentally that these residues undergo decreases in reactivity upon tetramer formation. In contrast, the six other residues (i.e., Thr4, Lys41, Arg45, Lys91, Lys94, and Arg97) that undergo decreases in reactivity and thus are likely at the tetramer interface are all solvent accessible when considering the C–D interface of the H13F hexamer (Figure 6C). This collective observation alone strongly suggests that the interface of the C and D chains of the H13F mutant does not reflect the tetramer interface in wild-type β2m. Indeed, no interface in the H13F hexamer is consistent with our covalent labeling data for the wild-type tetramer. This conclusion is not too troublesome, though, as the H13F hexameric mutant cannot form amyloid fibrils, whereas the wild-type protein can form tetramers, hexamers, and eventually amyloid fibrils. Evidently, the wild-type protein forms a tetramer species capable of continuing on to form amyloid fibrils, whereas the H13F mutant cannot form such an interface and therefore stalls at the hexamer and does not progress to form amyloid fibrils.

The P32A mutant also forms an oligomeric interface with which we can compare our labeling data (Figure 7). Like the interactions seen in the C and D chains of the H13F hexamer, the interactions of the antiparallel D strands in P32A span primarily the Glu50–Lys58 amino acids (Figure 7B). These interfaces predict reduced solvent accessibility of His51 on the D strand and Ser57 and Lys58 on the D–E loop. These interactions protect residues His51, Ser57, and Lys58 from solvent, which possibly explains why we see a decrease in the reactivity of these residues. Like the H13F mutant, though,

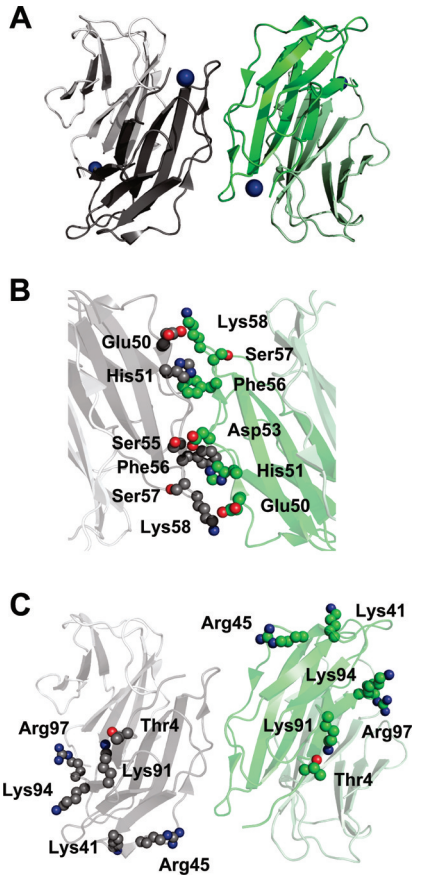

Figure 6. Possible tetramer formed by two dimer units with an antiparallel ABED interface as seen in the H13F hexamer (PDB entry 3CIQ). The side chains involved in the interface are shown as spheres. (A) Possible tetramer formed by D strands and B−C loops from two dimer units in the crystal structure of the H13F hexamer (PDB entry 3CIQ). One dimer unit (chains B and C) is colored gray and the other (chains D and E) green. Chains C and D, which form a possible tetramer interface, are colored dark gray and dark green, respectively. (B) Interaction of adjacent D strands. Interstrand interactions of the side chains of Glu50 and His51 of one monomer with the side chain of Lys58 and the backbone of Phe56, respectively, of another monomer are shown. These interactions make Ser57 less exposed to solvent. (C) Six of the amino acids that undergo decreases in reactivity upon tetramer formation, namely, Thr4, Lys41, Arg45, Lys91, Lys94, and Arg97, are solvent accessible in the H13F structure.

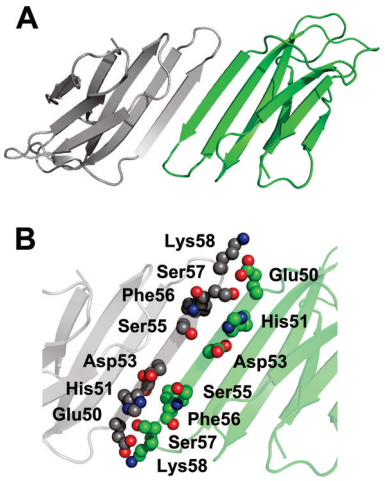

Figure 7. Interface formed by D−D strand interactions in P32A. (A) Crystallographic dimer formed by P32A (PDB entry 2F8O). (B) Amino acids Glu50−Lys58 on the D strands are shown as spheres. Interstrand interactions of the side chains of Glu50 and His51 of one monomer with the side chain of Lys58 and the backbone of Phe56, respectively, of another monomer are shown.

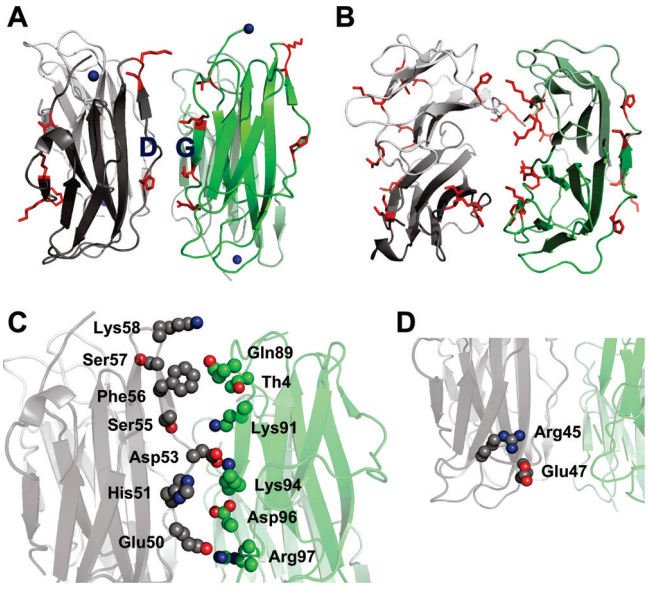

Figure 8. Proposed model for the wild-type β2m tetramer. (A) Tetramer formed by the interaction of two D strands from one dimer unit (dark and light gray) and two G strands of another dimer unit (dark and light). The probed amino acids that undergo decreases in their extents of modification upon tetramer formation (shown as red sticks) are located on edge strands A, C, D, and G. (B) Top view of the tetramer model. (C) Amino acids Thr4 on the N-terminal strand, Glu50−Lys58 on the D strand, and Gln89−Arg97 on the G strand are shown as spheres. Several interactions are shown: salt bridge between Glu50 and Arg97, salt bridge between His51 and Asp96, complex salt bridge among Asp53 and Lys94 and Lys91, and hydrogen bond between Gln89 and Lys58. (D) These interactions alter the orientation of the D strand such that Arg45 can form a salt bridge with Glu47.

Thr4, Lys41, Arg45, Lys91, Lys94, and Arg97 are solvent accessible in the P32A dimer, which is not consistent with the observed decrease in their reactivity upon tetramer formation. This result indicates that an interaction involving D strands from two protein subunits does not represent the tetramer interface in wild-type β2m.

In the development of a viable model for the tetramer, it is helpful to map the amino acids that undergo decreases in reactivity upon tetramer formation. In doing so, it is clear that the residues that undergo decreases in reactivity are localized on the edge strands A, C, D, and G of β2m (Figure 8A). This observation suggests a tetramer interface that involves an antiparallel arrangement of D and G strands from two dimers (Figure 8). In this tetramer model, two D strands from one dimer unit and two G strands from an adjacent dimer form the interface (Figure 8A,B). More specifically, a D strand from one monomer in a dimer and a D strand from the other monomer in the same dimer interact with the G strands from each of the monomers the other dimer. Analysis of such a model, which was computationally constructed and energy minimized as described in Materials and Methods, reveals that seven of the

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nine residues that undergo decreases in reactivity as the tetramer is formed are found at the interface of the D and G strands of adjacent dimers. These seven residues are Thr4, His51, Ser57/Lys58, Lys91, Lys94, and Arg97 (Figure 8C). The amino acid that undergoes an increase in reactivity upon tetramer formation, Lys75, is solvent accessible in the tetramer model. In this model, His51, Lys58, Lys91, Lys94, and Arg97 are involved in mediating the interactions at the tetramer interface whereas Ser57 is protected from solvent by these interactions and Thr4 is enclosed by the D and G strands. Glu50 and His51 form salt bridges with Arg97 and Asp96, respectively. Such electrostatic interactions can substantially enhance the stability of protein−protein complexes,[44,45] and the involvement of His51 in one of these interactions is consistent with a study by Blaho and Miranker in which they found in mutagenesis studies that His51 is a critical residue in mediating the interface.[46] Asp53 is in the proximity of both Lys91 and Lys94, indicating that this residue can form a complex salt bridge with both of these lysine residues. Such complex salt bridges at protein−protein interfaces contribute significantly to protein complex stability.[47−49] In addition, Lys58 is close enough to hydrogen bond with Gln89. Moreover, several hydrophobic residues are buried in this model structure, including Pro32, Leu54, Phe56, Ile92, and Val93 (residues not shown). In this model, Arg45 undergoes a decrease in reactivity but is not buried at the interface. In this case, the orientation of the D strand is altered such that Arg45 can form a salt bridge with Glu47 (Figure 8D), which could explain its decreased reactivity with BD. These interactions might also alter the orientation of the D strand such that Lys41 becomes more protected from solvent than in the dimer and thus undergoes a decrease in reactivity upon tetramer formation.

An interesting feature of our proposed model for the pre-amyloid tetramer is the likely ability of this tetramer to further propagate into higher-order oligomers. In our previous studies, we found that the tetramer can progress to form hexamers. As shown in Figure 8, interaction of D strands of one dimer and the G strands of a second dimer to form the tetramer leaves the G strands of the first dimer and the D strands of the second dimer available for further interaction with another dimer unit. Such unfulfilled interactions would presumably allow formation of a hexamer via another D−G interaction. Covalent labeling studies of the hexamer are beyond the scope of this work. Such studies are ongoing, though, and will be reported in the future.

Finally, it is tempting to compare our tetramer model with the structural information obtained from recent solid-state NMR data of β2m amyloid fibrils produced at low pH,[50−52] especially because cryo-electron microscopy data indicate that the fibrils are assembled as a "dimer of dimers".[53] Such a comparison is challenging, however, because fibrils produced under different conditions can have structural variations at the molecular level.[51,54] Furthermore, NMR data from one study suggest a β-sheet core that preserves β2m's overall native structure,[50] while more recent NMR studies indicate a core structure that is more non-native.[51,52] If the latter is correct, then substantial structural reorganization may occur in going from the oligomeric states to the fibrils. Thus, a thorough comparison of our proposed tetramer with β2m fibril structure will have to await further characterization (e.g., solid-state NMR, labeling) of the fibrils produced by incubation with Cu(II).

SUMMARY AND CONCLUSIONS

Using a combination of selective covalent labeling and mass spectrometric detection, we identify structural features of the pre-amyloid tetramer of β2m, which is generated by incubation with Cu(II) under physiologically relevant conditions. The covalent labeling data allow us to develop a model for the tetramer in which the interface is mediated by interactions between D strands of one dimer unit and the G strands of another dimer unit. Our model, therefore, differs from previous suggestions that the tetramer is mediated by interactions between D strands on separate dimer units. Covalent labeling data that cover ∼30% of the amino acids that are at the interface of the proposed tetramer structure provide strong support for our model. Future studies will further test this model, and more structural information about this pre-amyloid tetramer might be useful for the development of therapeutics against DRA. In broader terms, covalent labeling along with MS detection should be able to be applied to other amyloid-forming systems, especially those in which mixtures of pre-amyloid oligomers are formed. This approach could then become more important because in several amyloid diseases the pre-amyloid oligomers may represent the species responsible for cellular toxicity rather than the amyloid fibrils themselves.[55,56]

ASSOCIATED CONTENT

Supporting Information

Plots illustrating the intensities of unmodified and modified forms of the Val27−Lys41 fragment 2 h and 2.5 days after addition of Cu, intensities of unmodified and modified forms of the Tyr67−Tyr78 fragment 2 h and 2.5 days after addition of Cu, formation of oligomers over time, extent of DEPC modification of a control β2m solution [i.e., no Cu(II)] in the absence of Cu(II) and up to 2 days after the addition of Cu(II), extents of NHSA modification of β2m residues throughout the course of tetramer formation, plots showing the extents of DEPC modification of residues throughout the course of tetramer formation, and plots illustrating the extents of BD modification of Arg residues throughout the course of tetramer formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

β2m, β-2-microglobulin; MS, mass spectrometry; DRA, dialysis-related amyloidosis; MHC-I, class I major histocompatibility complex; NHSA, sulfo-*N*-hydroxysuccinimide acetate; DEPC, diethyl pyrocarbonate; BD, 2,3-butanedione; SEC, size-exclusion chromatography; LC−MS, liquid chromatography−MS; ESI-MS, electrospray ionization MS; PDB, Protein Data Bank.

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