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Increased β -Sheet Dynamics and D-E Loop Repositioning are Necessary for Cu(II)-Induced Amyloid Formation by β -2-Microglobulin

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Abstract

β -2-microglobulin (β 2m) forms amyloid fibrils in the joints of patients undergoing dialysis treatment as a result of kidney failure. One of the ways in which β 2m can be induced to form amyloid fibrils *in vitro* is via incubation with stoichiometric amounts of Cu(II). To better understand the structural changes caused by Cu(II) binding that allow β 2m to form amyloid fibrils, we compared the effect of Ni(II) and Zn(II) binding, which are two similarly-sized divalent metal ions that do not induce β 2m amyloid formation. Using hydrogen/deuterium exchange mass spectrometry (HDX/MS) and covalent labeling MS, we find that Ni(II) has little effect on β 2m structure, despite binding in the same region of the protein as Cu(II). This observation indicates that subtle differences in the organization of residues around Cu(II) cause distant changes that are necessary for oligomerization and eventual amyloid formation. One key difference that we find is that only Cu(II), not Ni(II) or Zn(II), is able to cause the *cis-trans* isomerization of Pro32 that is an important conformational switch that initiates β 2m amyloid formation. By comparing HDX/MS data from the three metal- β 2m complexes, we also discover that increased dynamics in the β -sheet formed by the A, B, D, and E β strands of the protein and repositioning of residues in the D-E loop are necessary aspects of β 2m forming an amyloid-competent dimer. Altogether, our results reveal new structural insights into the unique effect of Cu(II) in the metal-induced amyloid formation of β 2m.

TOC image

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Supporting Information

Hydrogen/deuterium exchange (HDX) mass spectrometry (MS) data for β 2m at increasing time points in the absence and presence of Cu(II), Zn(II), and Ni(II); an illustration of how mass spectral data is used to determine HDX at individual residues; HDX/MS data of the β 2m dimer formed upon Zn(II) binding at increasing time points; and size-exclusion chromatography data of the Zn(II)-incubated β 2m samples are all found in the supporting information.

The authors declare no competing financial interest.

β-2-microglobulin (β₂m) is a structural component of the major histocompatibility type 1 complex, and in healthy individuals this protein undergoes normal turnover and is catabolized by the kidney. In patients undergoing dialysis treatment as a result of kidney disease, however, β₂m eventually forms amyloid fibrils, which are the main pathology of dialysis related amyloidosis (DRA).^{1,2} β₂m fibrils are eventually found in the joints of all dialysis patients and induce acute arthropathy.^{3,4} During dialysis treatment, β₂m is not effectively eliminated from circulation, resulting in an increase in serum concentrations from around 0.1 μM up to 6 μM in some cases.^{3,5} While this concentration increase is necessary for amyloid formation *in vivo*, it alone is not sufficient.^{6,7} Other aspects of the dialysis treatment also play a role in the converting soluble monomeric β₂m into insoluble fibrils, but the exact causes are still subject to debate. Research has shown that acidic conditions,^{8,9} certain mutations,^{10,11} cleavage of the six N-terminal amino acids,^{12,13} limited proteolysis,¹⁴ stoichiometric concentrations of Cu(II),^{15–20} and other conditions^{21,22} can induce the β₂m amyloidosis *in vitro*.^{8,23} Our group has been interested in the potential role that Cu(II) could play in inducing the β₂m amyloid formation. Many years ago the switch to using Cu(II)-free dialysis membranes^{24,25} led to delays in the onset of DRA, suggesting a potential role for Cu(II) *in vivo*. More interestingly from a biochemical perspective, Cu(II) has emerged as one of the common motifs for triggering protein amyloid formation.²⁶ Cu(II) is also a convenient means of triggering β₂m amyloid formation *in vitro*, allowing the controlled study of the early stages of this protein's amyloid formation.

In previous work by our group and others, it has been shown that β₂m fibrils are preceded by the formation of soluble di-, tetra-, and hexameric species upon addition of Cu(II).^{20,27} The oligomers, particularly the dimer^{28,29} and tetramer^{29,30} maintain a native-like structure.^{27,29,31,32} Cu(II) has also been shown to play a catalytic role in the formation of β₂m fibrils as it is necessary for oligomer formation but is released before the final fibrils are formed.^{31,32} Cu(II) binding to monomeric β₂m induces several structural changes that are necessary for the formation of the dimer and subsequent aggregated forms. These structural changes include the *cis-trans* isomerization of the His31-Pro32 amide bond, as revealed by X-ray crystallography, that causes a repacking of the hydrophobic core and the repositioning of Arg3 and Asp59 to enable the formation of dimer-stabilizing salt bridges.^{18,29,33}

Recently, we demonstrated that the similar-sized divalent metals Zn(II) and Ni(II) influence β 2m aggregation in very different ways than Cu(II).¹⁸ Zn(II) initiates the formation of β 2m oligomers, yet the types of oligomers that are formed are different than when Cu(II) is present. Moreover, amorphous, rather than amyloid, aggregates are eventually formed, and these aggregates can be readily re-dissolved with sodium dodecyl sulfate (SDS), whereas the amyloid fibrils formed by Cu(II) cannot be re-dissolved by SDS. Part of the explanation for the different effect of Zn(II) on β 2m aggregation is the observation that Zn(II) binds to the protein at a different site than Cu(II).¹⁸ In contrast to Cu(II) and Zn(II), Ni(II) binding to β 2m fails to initiate any oligomerization or aggregation, despite Ni(II) and Cu(II) binding to most of the same residues in the same region of the protein.¹⁸ This failure of Ni(II) to cause amyloid formation is intriguing because it implies that subtle differences in the orientation of residues around Cu(II), particularly Asp59 and the amide between Ile1 and Gln2, are responsible for triggering the structural changes that lead to amyloid formation. In addition, unlike Zn(II) binding, the structural changes caused by Cu(II) binding enable amyloid-competent oligomeric interfaces to be formed. In this work, we set out to identify the unique structural changes caused by Cu(II) binding that are not caused by Ni(II) and Zn(II) binding. To do so, we use hydrogen/deuterium exchange (HDX) and covalent labeling together with mass spectrometry (MS) to compare the structural changes caused by the three metals. Our results identify the essential conformational changes that are necessary to achieve the amyloid-competent state of β 2m upon binding Cu(II).

Experimental Procedures

Materials

Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide (HNSB), deuterium oxide, pepsin, imidazole, 3-morpholinopropanesulfonic acid (MOPS), potassium acetate, potassium bromide, urea, zinc sulfate, deuterium oxide, tris(2-carboxyethyl)phosphine (TCEP), and dithiothreitol (DTT) were obtained from Sigma-Aldrich (St. Louis, MO). Urea was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Trypsin was purchased from Promega (Madison, WI). Tris(hydroxymethyl)-aminomethane (Tris) and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were purchased from EM Science (Gladstone, NJ). Human β 2m that was purified from human urine was purchased from Lee Biosolutions (St. Louis, MO). Ammonium acetate, methanol, acetonitrile, glacial acetic acid, copper sulfate, and nickel sulfate were obtained from Fisher Scientific (Fair Lawn, NJ). Centricon molecular weight cutoff (MWCO) filters were obtained from Millipore (Burlington, MA). Deionized water was prepared from a Millipore (Burlington, MA) Simplicity 185 water purification system.

Sample Preparation

For the HDX experiments a stock of 4.1 mM β 2m was made in 25 mM MOPS and 150 mM potassium acetate at pH 7.4. All stocks were made fresh daily. For the HNSB labeling experiments a 75 μ M solution of β 2m was prepared in 150 mM potassium acetate and 25 mM MOPS (pH 7.4). The following metal-to- β 2m ratios were used when conducting the HDX or covalent labeling experiments (described below): Cu 2:1, Ni 16:1, and Zn 4:1.

These ratios were chosen to ensure that the metal was 95% bound based on previous K_d measurements.¹⁶

Hydrogen/Deuterium exchange (HDX)

The concentrated stocks of $\beta 2m$, potassium acetate, the MOPS buffer, and the desired metal salt were all made and diluted into D_2O simultaneously after 60 minutes of pre-incubation. For all HDX experiments, the resulting concentrations upon dilution in D_2O were 75 μM $\beta 2m$, 25 mM MOPS, 150 mM potassium acetate, and either 300 μM Zn, 1200 μM Ni, or 150 μM Cu. The total volume of the reaction mixture was 55 μL . The samples were then allowed to incubate in D_2O for various times, ranging from 60 s to 180 min, after which the sample was placed on ice for an additional 30 sec. During the pre-incubation period and H/D exchange time, no significant protein oligomerization is observed under the solution conditions that are used, as determined by size-exclusion chromatography. The exchange reaction was then quenched by lowering the solution pH to 2.5 using a solution of formic acid that also contained 100 mM TCEP for disulfide bond reduction. The total volume after the quench was 110 μL . The samples were then allowed to sit on ice for 1.5 min to allow for disulfide bond reduction prior to proteolysis with pepsin.

HDX of the zinc-induced dimer was initiated by diluting 300 μL of a size-exclusion chromatography (SEC) fraction of the dimer by a factor of 10 into the same buffer as described in the previous paragraph. The samples were allowed to react with D_2O for times ranging from 15 s to 60 min before being quenched in the same way as the described above.

Back exchange measurements were conducted using a fully deuterated protein that was prepared by dissolving lyophilized $\beta 2m$ in 99.9% D_2O with 0.1% formic acid and incubating the solution at 37 °C for two weeks. After this time period, the sample was then lyophilized and re-dissolved again in D_2O with 0.1% formic acid and incubated for another 2 h. This process was repeated 2 times to produce a fully deuterated protein. Deuterium levels reported in the manuscript have been corrected for back exchange based on measurements of the peptides from the fully deuterated protein.

The deuterated samples and the undeuterated controls were directly analyzed using HDExaminer (Sierra Analytics, Modesto, CA). The measured isotopic distribution and resulting centroids for each peptide were manually confirmed. The centroid values for the partially deuterated ($m[P]$), fully deuterated ($m[F]$), and undeuterated ($m[N]$) were used to calculate deuteration via the following equation:

$$\text{Deuteration} = \frac{m(P) - m(N)}{m(F) - m(N)}$$

By multiplying this value by the maximum number of deuteration sites, one can calculate the total number of deuteriums incorporated in any individual peptide. The maximum number of deuteration sites was calculated from the total number of residues minus the first two residues and any proline residue. To examine the HDX changes induced by metal binding, the deuteration level of the apo-protein was subtracted from each metal complex.

The HDX/MS measurements for each metal and the apo-protein were repeated five times, and the results are reported as the average with error bars representing the standard deviation of this average. Significant differences in the extents of exchange were identified using a two tailed t test.

Covalent Labeling

Covalent labeling with HNSB was used to modify solvent exposed Trp residues.³⁴ Stock solutions of HNSB were prepared in water. Labeling of β 2m by HNSB was performed for 45 sec and was initiated through the addition of 68.5 μ M of HNSB. The total reaction volume was typically 27 μ L. The HNSB labeling reaction was quenched through the addition of 10 mM tryptophan.

Proteolytic Digestion

β 2m samples that underwent HDX were digested using pepsin. The digestion was initiated through the addition of $\sim$ 1.9 μ M pepsin to the already quenched samples, resulting in a 1:20 pepsin/ β 2m ratio. The digestion was allowed to proceed on ice for 6 min. The digested samples were then immediately analyzed by LC/MS.

HNSB-labeled β 2m samples were cleaned using a 10,000 MWCO filter and reconstituted with 25 mM Tris-HCl (pH 7) and 1 mM CaCl₂ to a final concentration of 300 μ M. Cleaned β 2m samples were first reacted with 10 mM DTT for 45 min to reduce disulfide bonds. The reduced protein samples were then unfolded in 12% acetonitrile at 37 °C for 45 min. Trypsin (1 μ g/ μ L) was then added to the labeled samples to yield a final enzyme/substrate ratio of 1:20. All samples were digested at 37 °C for 16 h before inactivating the enzyme by the addition of 2 μ L of acetic acid. The samples were then immediately analyzed by LC/MS.

HPLC Separation

To analyze the digests from the covalent labeling experiments, an HP1100 (Agilent, Wilmington, DE) HPLC system with a C18 column (15 cm $\times$ 2.1 mm, 5 μ m particle size) from Supelco (St. Louis, MO) was used. A 5 μ L injection loop was used for all replicates. The HNSB-modified proteolytic fragments were separated using a linear gradient of methanol with 0.1% acetic acid that increased from 5 to 70% over 30 min and 70 to 100% over the final 3 min. The remaining percentage of the mobile phase was water with 0.1% acetic acid.

Peptides produced by pepsin digestion of the HDX samples were trapped on a Vanguard BEH C18 trap cartridge (2.1 $\times$ 5 mm) and desalted for 4 min at a flow rate of 100 μ L/min. The HPLC system cooling chamber, which housed all the chromatographic elements, was held at 0.0 ± 0.1 °C for the entire time of the measurements. The peptic peptides were then separated with a 1.0 $\times$ 100.0 mm ACQUITY UPLC C18 BEH column (Waters, Milford, MA) over 12 min at 40 μ L/min, using an acetonitrile gradient from 5%–40% with 0.1% formic acid for 7 min, followed by flushing with 85%:15% acetonitrile–water with 0.1% formic acid for 3 min.

Some of the HNSB-labeled $\beta 2m$ samples were analyzed as intact proteins. To do this, the samples were first desalted using a Thermo Scientific Ultimate 3000 HPLC system (Thermo Scientific, Tewksbury, MA) fitted with a Protein MicroTrap (Michrom, Auburn, CA). A 5 μ L injection loop was used for all replicates. The protein was eluted using an acetonitrile gradient that increased from 1 to 99% over 5 min at a flow rate of 4 μ L/min.

Size-exclusion chromatography (SEC) of $\beta 2m$ oligomers was conducted using a TSK-gel SuperSW2000 column (Tosoh bioscience, Prussia, PA) installed on an HP1100 series high-performance liquid chromatography system (Agilent, Santa Clara, CA). A mobile phase containing 150 mM ammonium acetate at pH 6.8 was used at a 0.35 mL/min flow rate, and a variable-wavelength detector set to 214 nm was used for detection. 20 μ L of the metal- $\beta 2m$ samples were injected at different time points after adding metal sample. A solution containing 5 μ M bovine serum albumin (MW= 66,000), 5 μ M ovalbumin (MW= 45,000), 5 μ M carbonic anhydrase (MW=29,040) and 5 μ M $\beta 2m$ (MW= 11,731) was used for molecular weight calibration.

Mass Spectrometry

Mass spectral analyses of the HPLC separated samples from the HNSB labeling experiments were acquired on a Bruker Amazon ETD (Billerica, MA) quadrupole ion trap mass spectrometer or a Bruker Esquire-LC (Billerica, MA) quadrupole ion trap, both equipped with electrospray ionization sources. The electrospray source conditions, including the voltage and temperature, were chosen to optimize the peptide or protein signal. Tandem mass spectra of peptides were acquired using collision-induced dissociation (CID) with isolation widths of 1.0 Da and excitation voltages between 0.6 and 1.0 V.

Mass spectra of the HDX peptides were obtained with a Waters Synapt G2-si (Waters, Milford, MA) equipped with standard ESI source. The instrument configuration was the following: capillary voltage = 3.0 kV, sampling cone = 40 V, source temperature = 70 °C, and desolvation temperature = 20 °C. Mass spectra were acquired over a m/z range of 100–2000. Identification of the peptic fragments peptides was accomplished through a combination of mass spectral analyses and MS^E using the ProteinLynx Global SERVER (Waters Corporation). MS^E was performed via a series of low to high collision energies ranging from 5 to 30 V, which helped ensured adequate dissociation of all the eluted peptides.

Solvent Accessibility Calculations

To calculate the solvent accessibility of surface residues, the online tool GetArea³⁵ was used on the intact (pdb: 1JNJ) and N-terminal truncated (pdb:2XKU) structures of $\beta 2m$ solved and described by Eichner *et al.*³⁶

Results

When incubated with different metals, $\beta 2m$ undergoes distinct oligomeric changes. The presence of Cu(II) causes $\beta 2m$ to form dimers, tetramers, and hexamers (Figure 1) before forming amyloid aggregates, as we and others have demonstrated previously.^{27,29} In contrast, the presence of Ni(II) causes no change in the oligomeric state of the protein,

whereas Zn(II) causes β 2m to form dimers and hexamers (Figure 1) before amorphous aggregates are formed.¹⁸ These different oligomeric changes are likely caused by distinct structural changes to the protein monomer upon binding each metal ion, as the first oligomers are not measured until at least 10 minutes after metal addition. Previous extended X-ray absorption fine structure (EXAFS) and MS measurements showed that Cu(II) and Ni(II) bind in the same region of the protein near the N-terminus and His31, whereas Zn(II) binds away from the N-terminus near His51.¹⁸

HDX/MS of Metal- β 2m Complexes

To identify the structural changes caused by binding to each metal, we conducted hydrogen/deuterium exchange (HDX) reactions on metal-free β 2m and compared the exchange of the protein in the presence of Cu(II), Ni(II), or Zn(II), using MS as the readout. Exchange was monitored at different time points ranging from 60 s to 3 h. The protein in the absence of metal undergoes HDX in a manner that is consistent with its β -sheet rich structure and is similar to previous HDX/MS studies of the metal-free protein.^{37–39} Regions of the protein that have β -strands undergo slower exchange over time than regions with unstructured loops (Figure S1).

When Cu(II) is bound to β 2m, the protein's exchange pattern changes in several regions (Figure S2). Figure 2A summarizes the differences in exchange between the metal-free protein and the Cu(II)-bound protein at a short exchange time (i.e. 2 min) and at a longer exchange time (i.e. 180 min). At very short exchange times, most of the Cu(II)-bound protein undergoes exchange to the same extent as the metal-free protein, but there are differences observed in the regions spanned by Ile35 and Leu39. Specifically, this region undergoes slightly greater exchange upon Cu(II) binding. Intriguingly, at longer exchange times the protein undergoes a greater extent of exchange in four different regions of the protein, including Tyr10-Cys25, Ile35-Leu39, Leu40-Ser55, and Tyr63-Tyr67; Figure 3A shows the location of these regions in protein's structure. The unique change in exchange only at longer times compared to the metal-free protein suggests that Cu(II) causes the β -strands in these regions to experience more expansive "breathing" motions than in the metal-free protein.

As compared to Cu(II), the presence of Ni(II) affects β 2m exchange very little (Figure S3). At short exchange times, only the region spanned by Tyr26 and Asp34 undergoes any noteworthy statistically significant change in exchange in the presence of Ni(II), and this change is very minor (Figure 2B). At longer exchange times, only Tyr63-Tyr67 experiences a slightly increased level of exchange (Figure 2B), suggesting that the motion of the β -strand that includes these residues undergoes a small increase in its extent of motion. Taken as a whole, Ni(II) binding causes minor changes in the structure of β 2m, which is consistent with the fact that the protein remains monomeric in the presence of Ni(II).

The pattern of exchange from the Zn(II)-bound protein is different than for the Cu(II)- or Ni(II)-bound forms (Figure S4). Most notably several regions of the protein undergo more extensive exchange at the earliest time points (Figure 2C), suggesting that these regions have become more dynamic and/or exposed than in the apo protein. At the longer time points, the same changes persist (Figure 2C). The regions of the protein where the changes occur are

similar to the ones observed with Cu(II) present, but the fact that these changes manifest themselves at the earliest time points suggest these regions are more unstructured when Zn(II) is bound.

HDX/MS of Zn-induced Dimer

While Cu(II) and Zn(II) both cause dimers to form and lead to H/D exchange changes in similar regions of $\beta 2m$, the fact remains that only Cu(II) enables amyloids to form. A partial explanation for this difference is that the metals bind to different sites in the protein.¹⁸ To obtain a deeper understanding of the different effects of the metals, we used HDX/MS to characterize the Zn(II)-induced dimer. The dimer formed upon Cu(II) binding was previously characterized by our group,²⁸ but no structural information exists for the Zn(II)-induced dimer. SEC fractions of the Zn(II)-induced dimer were collected and subjected to HDX/MS to obtain information about the interface of this dimer. In separate experiments, the Zn(II)-induced dimer was found to be stable for at least 4 hours (Figure S5). Upon comparing the exchange patterns of the Zn(II)-bound monomer and dimer (Figure S6), we find that several regions undergo a slight increase in exchange, but the region spanned by Arg45-Leu54 (see Figure 3B) undergoes significantly less extensive exchange (Figure 4), suggesting this region as the interface for the dimer. While HDX/MS does not necessarily reveal protein-protein interfaces, decreased exchange at only Arg45-Leu54, together with previous EXAFS, MS, and fluorescence data that suggest Zn(II) bridges two monomer units via His51,¹⁸ provides compelling evidence for this region as the interface in the Zn(II)-induced dimer. The Cu(II)-induced dimer has a very different interface, as will be discussed below.

Covalent Labeling of Trp60 with HNSB

Several studies have shown that a key initial step in $\beta 2m$ amyloid formation is the *cis-trans* isomerization of the His31-Pro32 amide bond.^{33,40} A direct consequence of this isomerization is the repacking of the hydrophobic core near this region of the protein. Specifically, Phe30 becomes exposed to solvent while Trp60, which is very solvent exposed in the native structure, becomes buried. Cu(II) binding to $\beta 2m$ causes the *cis-trans* isomerization and subsequent Trp60 burial, but it is an open question whether or not Ni(II) and Zn(II) cause the same structural change. To address this question, we used short (i.e. 45 s) reactions with HNSB to monitor the burial of Trp60 immediately after exposing the protein to the Cu(II), Ni(II), or Zn(II). HNSB is a reagent that specifically reacts with solvent exposed Trp residues and gives rise to a 151 Da mass increase that can be readily measured by MS.

Because $\beta 2m$ has two Trp residues (i.e. Trp60 and Trp95), LC-MS after proteolytic digestion of the protein was used to determine the extent of labeling at each Trp residue. Upon reacting the protein for 45 s with HNSB, 1.2 HNSB labels, on average, are added to the protein (Figure 5A). LC-MS data reveal that about 95% of the Trp60 residues are labeled in the protein (Figure 5B), when uncorrected ion abundances are used, which is the typical approach for such measurements.³⁴ In contrast, only about 15% of Trp95 is labeled (Figure 5C). Together, these data indicate that the vast majority of the labeling occurs on Trp60. This result is not surprising, as in the native protein structure Trp60 is exposed with a solvent

accessible surface area of 174 Å², while Trp95 is more buried with a SASA of 12 Å². When HNSB is reacted with β2m at different times after adding Cu(II), Ni(II), or Zn(II), only Cu(II) causes Trp60 labeling to decrease (Figure 6). The extent of labeling decreases over time in the presence of Cu(II), while no decrease in Trp60 labeling is observed with Ni(II), Zn(II) or the apo protein for up to 2 h (data not shown).

Discussion

In previous work we demonstrated that Cu(II), Ni(II), and Zn(II) influence β2m aggregation in different ways, and some of these differences are caused by differences in metal binding sites. Here, we sought to elucidate the protein structural changes caused by Cu(II) that uniquely cause this metal, and not the others, to induce amyloid formation. Through HDX/MS and covalent labeling/MS measurements, several new structural insights have been obtained that further our understanding of how Cu(II) specifically induces β2m amyloid formation. Our data reveal three important new insights. First, Cu(II) binding causes increased dynamics in the A, B, D, and E β-strands of β2m (see Figure 7A for labeling of the β strands). Second, these increased dynamics appear to be important in forming an amyloid-competent dimer interface. Third, Cu(II) binding induces the *cis-trans* isomerization of the His31-Pro32 amide bond, while Ni(II) and Zn(II) do not.

A fascinating observation in the HDX/MS data of the metal-bound forms of β2m is the distinct way in which Cu(II) binding changes protein amide exchange. All three metals cause some increase in exchange at various regions of the protein, but Cu(II) binding causes the greatest change. Moreover, this increased exchange is manifest predominantly at longer exchange times. It is important to recall that Cu(II) and Ni(II) bind in the same region of the protein, but their effects on β2m structure are clearly different. Ni(II) only causes a slight increase in exchange at Tyr63-Tyr67, which is in the E strand, while Cu(II) causes changes in several regions of the protein. Zn(II) binding causes increased exchange at both short and long exchange times, which is consistent with the protein becoming more unstructured when this metal is bound.⁴¹ For Cu(II), the increased exchange, which is only at longer exchange times, is consistent with regions that become more dynamic and not necessarily more unstructured. In other words, these regions in the Cu(II)-bound protein visit an exchange competent state more times during the labeling experiment without being completely unfolded. A good example of this exchange behavior is seen for Tyr63-Tyr67, which are part of the E strand that is highly protected from exchange in the absence of metal (Figure S1 and Figure 2). Interestingly, all the regions that show increased exchange at longer exchange times are found at the known Cu(II)-induced dimer interface (Figure 7B). The peptide Tyr10-Cys25 contains the A and B strand; the peptide Leu40-Ser55 contains the D strand; and the residues Tyr63-Tyr67 are part of the E strand.

Increased exchange at the observed protein regions upon Cu(II) binding is what would be expected if Cu(II) causes β2m to form the intermediate that is known to be highly amyloidogenic.^{10,40,42,43} Eichner et al.³⁶ recently showed using NMR that an N-terminally truncated version of β2m (N6), in which the first six residues are removed, has high structural fidelity to this previously identified intermediate. Moreover, the N6 form has structural similarity to the monomeric units in the Cu(II)-bound hexamer of the H13F

mutant.⁴⁴ These structural connections suggest that the atomically-defined N6 structure might serve as a model for interpreting the HDX/MS data of the full-length protein when Cu(II) is bound. A comparison of the full-length protein structure to the N6 structure immediately reveals several differences. The most notable are the strand displacements at the ends of the A-B and D-E β -sheets in N6 (circled in Figure 7C). These displacements are consistent with β strands that would sample open states more often, and thus would show greater exchange at longer times.

The peptide Tyr10-Cys25 contains the residues Asn21 and Phe22, which show the greatest displacement in the A-B sheets, and this peptide is one of the peptides that shows increased exchange at longer times (Figure 2A). Unfortunately, this peptide also contains Arg12 through Ser20, which are in an unstructured loop and almost fully exchange by the 2 min time point, so the difference in exchange between the Cu(II)-bound and apo forms of β 2m is small, but it is still significant. The residues at the edge of the D-E sheet that undergo notable displacement are Glu50 through Leu54 and Leu64 through Tyr67. The peptide Leu40-Ser55 contains this first set of residues, but it also contains Lys41 through Val49, which are part of an unstructured loop. Consequently, the peptide shows very rapid exchange, and the displacement in residues Glu50-Leu54 leads only to a small additional increase in exchange at longer times. Much more revealing are the peptides Tyr63-Tyr66 and Tyr63-Tyr67, which are part of the E strand. Clearly, these residues undergo a significant increase in exchange (Figure 2a), which is consistent with the displacement observed in the E strand.

The other peptide that undergoes a significant increase in exchange in the Cu(II)-bound form is Ile35-Leu39, but this region shows an increased exchange at the earliest time points, suggesting that it is simply more unstructured than in the apo protein. The reason for the increased exchange in this region is not immediately apparent upon comparing the full-length and N6 proteins. It should be noted, though, that this stretch of the protein is repositioned due to the *cis-trans* isomerization of the His31-Pro32 amide bond in N6, which occurs with Cu(II) present too,⁴⁴ so it is likely that its dynamics or protection are altered as a result of Cu(II) binding.

An important contrast must be made between the behavior of the Cu(II) and Zn(II)-bound forms of the protein, even though many of the same regions undergo increased exchange (i.e. Tyr10-Cys25, Ile35-Leu39, and Tyr63-Tyr67). The underlying cause of their increased exchange is likely different because of the different binding sites of each metal, the different progression of oligomers, and the different nature of the final aggregates. As suggested above, Zn(II) causes these noted regions to be more unstructured, as indicated by increased exchange at the earliest HDX time points. Cu(II) binding increases exchange only at longer time points, suggesting a different type of structural change or perhaps a different frequency of the β strand “breathing motions.” These different effects on structure may explain why different dimers are formed in each case but why only Cu(II) induces the formation of an amyloid-competent dimer. In other words, our HDX/MS data perhaps imply that increased dynamics in these regions are important for formation of a dimer that is on the pathway to the amyloid.

Consistent with the different outcomes for Cu(II) and Zn(II) is the fact that the interface in the Zn(II)-induced dimer is different than the interface in the Cu(II)-induced dimer. HDX/MS of the Zn(II) dimer shows that the peptide Arg45-Leu54 undergoes a major decrease in exchange. This collection of residues span part of the C β -strand, the C-D loop, and the D β -strand. Because Zn(II) binds to His51 and our previous EXAFS data suggested the possibility of Zn(II) bridging two His51 residues in two monomer units, this HDX/MS data helps us reasonably conclude that the Zn(II) dimer interface involves the edge of the D strand, the C-D loop and perhaps part of the C strand from two monomers (Figure 7D). This interface is quite different than in the Cu(II)-induced dimer (Figure 7B) where the interface involves the β -sheet formed by strands A, B, E, and D.²⁸ Moreover, the identified Zn(II)-induced dimer interface is reminiscent of the Cu(II)-induced tetramer interface,³⁰ except that the Zn(II) dimer has D-D strand interactions, whereas the Cu(II) tetramer has D-G strand interactions. The D-G strand interactions from two sets of dimers in the Cu(II) case likely allow β 2m to propagate to form higher order oligomers and eventually amyloid fibrils. The D-D strand interactions from two monomers in the case of the Zn(II) dimer would prevent the same oligomer propagation as with Cu(II) and thus might explain why Zn(II) cannot induce β 2m amyloid formation. Indeed, D-D strand interactions in the dimer of the W60C mutant of β 2m was also shown to prevent amyloid fibril formation.⁴⁵ Similarly, the dimer of the non-amyloidogenic P32A mutant also forms via interactions of D β -strands from two monomer units.³³ Our results with Zn(II) provide additional evidence that formation of this interface does not allow eventual amyloid formation.

An even greater contrast between the metals is the effect that Cu(II) binding has on the repacking of the hydrophobic core of β 2m. Amyloidosis of β 2m in the presence of acid or Cu(II) begins with the *cis-trans* isomerization of the His31-Pro32 amide bond. This isomerization initiates the repositioning of a number of residues,^{23,27,33,40,46,47} including the repacking of the hydrophobic core in which the process exposes Phe30 and buries Trp60. We tested the ability of the different metals to bury Trp60, which is very exposed in the native protein, via covalent labeling with HNSB. Numerous studies have shown that covalent labeling with reagents like HNSB can monitor changes in residue solvent accessibility.³⁴ Figure 5 clearly shows that upon addition of Cu(II) the labeling of Trp60 decreases over time, which is consistent with a decrease in its solvent accessibility. Addition of Zn(II) and Ni(II) do not decrease labeling, indicating that Trp60 is not buried upon the binding of these metals. We interpret these data to mean that Zn(II) and Ni(II) are not capable of causing the *cis-trans* isomerization of Pro32, whereas Cu(II) is already known to be able to do so. These results are also consistent with the fact that only Cu(II) causes amyloid formation. It is important to emphasize that Ni(II) and Cu(II) both bind to His31,¹⁸ so evidently His31 binding alone is not enough to cause isomerization and repacking of the hydrophobic core. A key difference between the Cu(II) and Ni(II) binding to β 2m is that Cu(II) also binds to Asp59, while Ni(II) does not. Asp59 is present in the D-E loop, and numerous studies have demonstrated the influence of this loop on the formation of oligomers and amyloids by β 2m.⁴⁸⁻⁵² It is reasonable to conclude that Cu(II) binding to Asp59 is also an essential aspect of core repacking, and the inability of Ni(II) to bind to this residue might be the reason Ni(II) does not cause any oligomers or amyloids to form. Because Zn(II) binds β 2m at His51, which is quite distant from the Cu(II) binding site, it is perhaps not surprising that

Zn(II) does not cause *cis-trans* isomerization, repacking of the hydrophobic core, or the formation of amyloid-competent oligomers.

Conclusions

In this work we delineate some of the structural consequences of Cu(II) binding that cause only this metal, and not Ni(II) and Zn(II), to transform β 2m into amyloid fibrils. All three metals influence the structure of β 2m, yet our results clearly highlight the fact that specific structural changes are essential for achieving the amyloidogenic state of this protein. This conclusion is evident from a comparison of the structural changes caused by Cu(II), Ni(II), and Zn(II), as revealed by HDX/MS and covalent labeling with MS. While Ni(II) binds the protein in a similar manner as Cu(II), it appears to be unable to cause the *cis-trans* isomerization of the His31-Pro32 amide bond, which is essential for the formation of the amyloidogenic conformer. Moreover, because Ni(II) does not bind Asp59 like Cu(II) does, Ni(II) does not facilitate the repacking of β 2m hydrophobic core, which is important for oligomer and amyloid formation. In contrast, Zn(II) binding causes partial unfolding of β 2m and the formation of dimers and hexamers, but these structural and oligomeric changes do not result in the formation of amyloids. Instead, Zn(II) binding to His51 and the associated structural changes cause β 2m to form a dimer through D-D strand interactions that is unable to propagate to larger amyloid-competent oligomers or to amyloids themselves. Instead, amorphous aggregates are formed in the presence of this metal. Taken as a whole, the results presented here reveal more of the structural changes that Cu(II) binding causes to enable β 2m amyloid formation. These include: (i) increased dynamics in the A, B, D, and E β -strands, which enables amyloid-competent dimer formation; (ii) *cis-trans* isomerization of the His31-Pro32 amide; and (iii) repositioning of the D-E loop upon binding to Asp59, which facilitates repacking of the hydrophobic core. Generally speaking, our work also demonstrates the value of HDX/MS and covalent labeling for understanding structural changes in amyloid-forming proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Example size-exclusion chromatographs of $\beta 2m$ samples incubated without (control, black) and with Cu(II) (blue), Ni(II) (green), and Zn(II) (red) for 2 days, showing the presence of oligomers formed in the presence of Cu(II) and Zn(II) but not Ni(II). Metal concentrations, protein concentrations, and solution conditions are described in the experimental section.

Figure 2.

Peptide level differences in deuterium uptake (ΔD) between $\beta 2m$ in the presence of metal and $\beta 2m$ in the absence of metal for (A) Cu(II), (B), Ni(II), and (C) Zn(II) at representative short and long exchange times. A positive value for ΔD indicates increased exchange in the presence of the metal. Error bars are standard deviations from 5 replicate measurements. Asterisks (*) indicate exchange differences that are significantly different ($p < 0.05$) according to a two-tailed t test.

Figure 3.

(A) Structure of monomeric $\beta 2m$ (PDB: 1JNJ) showing in red the protein regions that undergo increased H/D exchange at longer times (180 min) upon Cu(II) binding. (B) Structure of monomeric $\beta 2m$ (PDB: 1JNJ) showing in blue the protein region that undergoes decreased H/D exchange in the Zn-induced dimer.

Figure 4.

Peptide level differences in deuterium uptake (ΔD) between the $\beta 2m$ dimer formed in the presence of Zn and $\beta 2m$ monomer in the presence of Zn(II) at representative short and long exchange times. A negative value for ΔD indicates decreased exchange in the dimer. Error bars are standard deviations from 5 replicate measurements. Asterisks (*) indicate exchange differences that are significantly different ($p < 0.05$) according to a two-tailed t test. The dimer and monomer were obtained by SEC fraction collection.

Figure 5.

A) Mass spectrum of $\beta 2m$ after a 45 s reaction with HNSB. The inset shows an expanded region around the +13 charge state. The number of asterisks (*) denotes the number of HNSB labels. B) Extracted ion chromatograms from an LC/MS analysis of an HNSB-labeled $\beta 2m$ digest, showing labeled (red) and unlabeled (black) peptides Asp59-Tyr63 (top) and Trp95-Met99 (bottom), which contain Trp60 and Trp95, respectively.

Figure 6. Relative extent of HNSB labeling on $\beta 2m$ at increasing times after adding the indicated metal. At each time point, HNSB was reacted with the protein for 45 s. The extent of labeling at each time point was normalized to the extent of labeling that occurred at time 0. Error bars are standard deviations from 5 replicate measurements.

Figure 7.

A) Structure of monomeric $\beta 2m$ (PDB: 1JNJ) with the A, B, D, and E β -strands labeled. B) Model of $\beta 2m$ dimer that is formed upon copper binding.²⁸ The A, B, D, and E β -strands are colored blue and orange, respectively, in each monomer unit. (C) Structure of N6 $\beta 2m$ variant (PDB: 2XKU) with the A-B and D-E loops circled. D) Alternate view of wild-type $\beta 2m$ (PDB: 1JNJ) with the C and D β -strands indicated.