

COMMUNICATION

Kidney Dialysis-associated Amyloidosis: A Molecular Role for Copper in Fiber Formation

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In the US alone, more than 250,000 people have impaired renal function that necessitates treatment by dialysis. A debilitating complication of long-term treatment is the deposition of β 2-microglobulin (β 2m) as amyloid fibers within the joint space. However, the intrinsic propensity of isolated β 2m protein to initiate *in vitro* fiber formation is negligible under conditions matched to the neutral pH and ionic conditions of serum. Here, we present evidence for a novel interaction between β 2m and Cu^{2+} at a concentration within institutionally recommended limits for this metal ion in dialysate solution. Mass spectrometry, using electrospray ionization from native conditions, demonstrates that the binding of Cu^{2+} is specific over Ca^{2+} or Zn^{2+} . Despite maintaining a native-like conformation upon Cu^{2+} binding, the folded protein is unusually destabilized against thermal and urea denaturation. We further demonstrate that destabilization by Cu^{2+} uniquely promotes *de novo* fiber formation at 37°C and neutral pH. Since the incidence of amyloidosis is dramatically reduced upon elimination of copper from dialysis membranes, our results provide a molecular understanding for dialysis-associated amyloid formation by β 2m.

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The deposition of normally soluble proteins as amyloid fiber is implicated in more than 15 diseases, including $\text{A}\beta$ in Alzheimer's disease and β 2-microglobulin (β 2m) in dialysis-related amyloidosis (DRA).¹ β 2m, the 12 kDa polypeptide subunit of the class I major histocompatibility complex (MHC),² is necessary for the cell-surface expression of MHC. Its turnover includes release as a soluble globular protein and subsequent catabolism within the kidney. As a consequence, serum levels of β 2m during renal failure are five–50 times the normal level of $\sim 0.1 \mu\text{M}$. Elevated levels may be necessary but are not sufficient for amyloid deposition as the magnitude of β 2m concentration does not correlate with the pathology of DRA.³ Furthermore, concen-

trated solutions of β 2m, for example 1 mM for *in vitro* NMR studies, may be prepared and remain stable for many months at pH 7.⁴ This suggests that other factors directly related to dialysis therapy mediate the conformational switch of β 2m from native state to amyloid fiber. The current paradigm for fibrillogenesis is that these conformational changes are closely linked to the folding pathway of the precursor.⁵ We focused, therefore, on several aspects of renal failure and dialysis that are directly analogous to the perturbations routinely implemented in experimental studies of *in vitro* protein folding. In particular, the effects of elevated urea levels associated with uremia and perturbations in mono- and divalent cation concentrations on the folding of β 2m.

Abbreviations used: DRA, dialysis-related amyloidosis; MHC, major histocompatibility complex; β 2m, β 2-microglobulin; ThT, thioflavin T; PrP, prion protein; AAMI, American Association of Medical Instrumentation.

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Interaction of copper (II) with β 2m

The two tryptophan residues of β 2m facilitate analysis by intrinsic fluorescence. Addition of Cu^{2+} to a solution of β 2m buffered at pH 7.4 (potassium phosphate) results in a blue shift of 4 nm in the

fluorescence emission spectrum (inset, Figure 1). Shifts such as this are commonly observed upon folding of globular proteins as a result of burial of tryptophan within a hydrophobic environment. The presence of Cu^{2+} does not, however, significantly affect the folded conformation of β 2m, judging by the lack of perturbation in 1D ^1H NMR, near and far UV CD spectra (data not shown). More likely, the shift results from metal ion binding and the Cu^{2+} -mediated quenching of a proximal tryptophan residue. The change in fluorescence permits measurement of the affinity of Cu^{2+} for β 2m by computing the average fluorescence emission wavelength, $\langle\lambda\rangle$, as a function of the total copper concentration (Figure 1). Assuming an equimolar stoichiometry of Cu^{2+} for β 2m, we measure a dissociation constant of $2.7 \pm 0.6 \mu\text{M}$.

The specificity of β 2m for copper was assessed by mass spectrometry with protein ionization

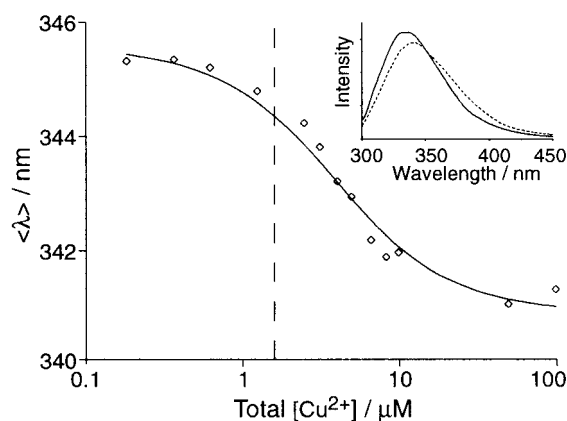
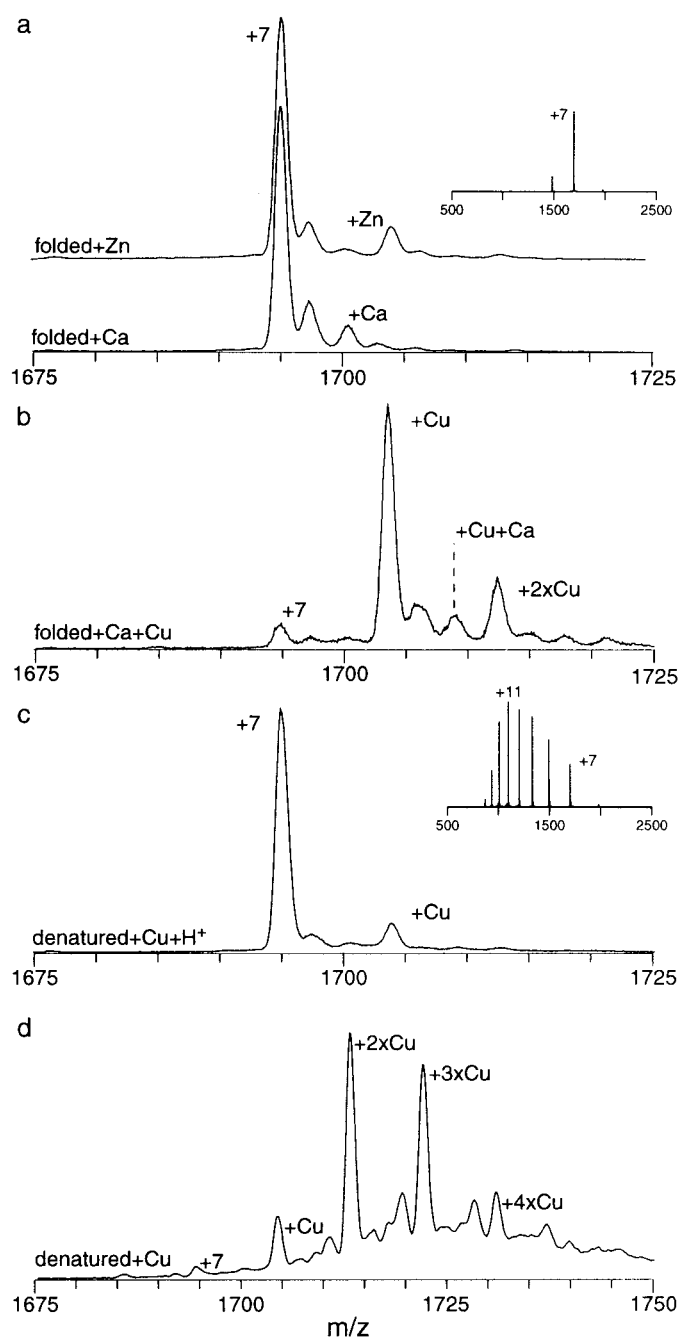


Figure 1. Change in intrinsic fluorescence on binding of Cu^{2+} to β 2m. Average emission wavelength, $\langle\lambda\rangle$, as function of Cu^{2+} concentration demonstrates sigmoidal binding behavior. The continuous line represents a non-linear fit assuming equilibrium dissociation. As stoichiometry is not measured directly by these experiments, we also assume for simplicity that stoichiometry is 1:1. This results in a binding constant of $2.7 \mu\text{M}$ with a confidence interval of $\pm 0.6 \mu\text{M}$. The broken vertical line represents the AAMI limit ($1.6 \mu\text{M}$) for copper in dialysate.²¹ Protein concentration, $2.4 \mu\text{M}$, is within the reported range of serum β 2m levels.³ Inset: Representative spectra showing normalized emission intensity of $2.4 \mu\text{M}$ β 2m, 25 mM potassium phosphate, 150 mM KCl, pH 7.4, 25°C in the presence (continuous line) and absence (broken line) of $100 \mu\text{M}$ CuCl_2 . A PTI Quantamaster C-61 was used, slit widths were 2 nm with excitation at 283 nm, $\langle\lambda\rangle$ was calculated between 290 and 450 nm. Denatured protein was extracted from *Escherichia coli* inclusion bodies, refolded by dialysis³¹ against 25 mM phosphate, 150 mM KCl, 1 mM EDTA (pH 7.4), 4°C and concentrated by centrifugation (Vivascience 5 kDa cutoff). Final purification used a SynChroPak GPC100 size-exclusion column, 25°C , pH 7.4. Protein purity was assessed by electrospray ionisation-mass spectrometry and both SDS- and native PAGE. ESI-MS of recombinant β 2m yielded a mass of 11859.5 (calculated mass 11860.4).

under native conditions, i.e. $20\text{--}25^\circ\text{C}$, buffered at pH 7 with 20 mM ammonium acetate. In the presence of $100 \mu\text{M}$ Ca^{2+} or $100 \mu\text{M}$ Zn^{2+} , three principle peaks are observed (Figure 2(a)). The peak at 1695 m/z dominates and corresponds to a metal-free β 2m ion in which all seven charges are derived from H^+ . Within both the Ca^{2+} and Zn^{2+} spectra, a peak of lower intensity ($\sim 10\text{--}20\%$) is observed at $1695(\pm 2.4)$ m/z and corresponds to an overall mass shift of 17 Da (7×2.4). This reflects a small proportion of charge arising from NH_4^+ rather than H^+ . Peaks of lower intensity ($\sim 16\%$) with mass shifts of 38 Da and 63 Da are observed in the Ca^{2+} and Zn^{2+} spectra, respectively. As with NH_4^+ , these associations are likely the result of non-specific adduct formation during ionization rather than specific binding.

In contrast, mass analysis of β 2m in the presence of $100 \mu\text{M}$ Cu^{2+} reveals at least a tenfold excess of Cu^{2+} -bound protein over the metal-free form. This distinction between Cu^{2+} , Ca^{2+} and Zn^{2+} was consistent over many samples (≥ 3) and on different days. Nevertheless, as non-specific adduct formation can be a variable aspect of ionization, samples were prepared in which Cu^{2+} was mixed with either Zn^{2+} or Ca^{2+} . In the case of β 2m with Cu^{2+} and Ca^{2+} (Figure 2(b)), the dominant peak corresponds in mass to the binding of a single copper (II) ion while association of one calcium (II) ion is virtually undetectable. If binding was non-specific, similar intensities would be expected for the Cu^{2+} and Ca^{2+} adducts. In spectra derived from mixed $\text{Zn}^{2+}/\text{Cu}^{2+}$ solutions, discrete peaks for Zn^{2+} and Cu^{2+} binding cannot be resolved, however, shifts can be determined accurately. In this case, the mass shift due to bound metal is measured as $+61.8(\pm 0.4)$ Da. This compares well to a calculated change of 61.4 Da for the substitution of one Cu^{2+} for two H^+ but compares poorly to the calculated mass shift of 63.4 Da for Zn^{2+} . For β 2m samples containing Cu^{2+} only, we measure a mass shift of $+61.5(\pm 0.2)$ Da. Clearly, native β 2m exhibits preferential binding of Cu^{2+} over Ca^{2+} and Zn^{2+} .

The ability of non-native states to bind copper was assessed by mass spectrometry. Ionization of denatured β 2m (Figure 2(c) and (d)) exhibits a broader distribution of charge states (inset, Figure 2(c)) centered at lower m/z in comparison with native protein (inset, Figure 2(a)). This is a reflection of the greater ability of extended conformations to stabilize charges relative to compact states.⁷ Under denaturing conditions at acidic pH, spectra reveal only a small fraction of protein with a single Cu^{2+} bound (Figure 2(c)). We attribute this to non-specific adduct formation. By contrast, under denaturing conditions at neutral pH, a range of Cu - β 2m complexes are seen (Figure 2(d)). The dominant species is a complex between β 2m and two Cu^{2+} , however protein/metal ratios of 1:1, 1:2, 1:3 and 1:4 are evident. Similar results are observed for β 2m unfolded in the presence of Zn^{2+} (data not shown). The ability of non-native conformations of



source was maintained at 20–25 °C. Protein ion counts of metal-bound and unbound forms of β 2m were determined from the +7 charge state of more than three separately collected spectra.

β 2m to coordinate up to four divalent ions is presumably due to four histidine residues contained within the β 2m primary sequence. Since specificity of copper binding by β 2m occurs only under native solution conditions, and since neither 1D $^1\text{H-NMR}$ nor CD spectra are significantly perturbed in the presence of copper (data not shown), we conclude that the native state of the protein is required for specific Cu^{2+} binding.

An energetically favorable interaction between metal and protein is expected to stabilize the folded state over the unfolded state. Therefore, the

Figure 2. Mass spectra of folded or denatured β 2m in the presence or absence of added divalent metal ions. A stock solution of recombinant β 2m was prepared in volatile buffer (20 mM ammonium acetate (pH 7)) and diluted to 20 μM with 20 mM ammonium acetate \pm metal. (a) Spectra collected in the presence of 100 μM ZnCl_2 or 100 μM CaCl_2 . Neither Zn- β 2m nor Ca- β 2m contribute significantly. Inset: Charge-state distribution of folded β 2m. (b) Spectra of β 2m in the presence of 100 μM CaCl_2 and 100 μM CuCl_2 are dominated by Cu- β 2m. Levels of Ca- β 2m and Ca-Cu- β 2m are very small and therefore are likely to have resulted non-specifically during ionization. (c) The stock solution of β 2m was diluted instead to 20 μM β 2m with 50% CH_3CN , 0.2% formic acid, 100 μM CuCl_2 . The presence of Cu^{2+} under these conditions gives rise to levels of Cu- β 2m comparable to those observed for the non-specific association of β 2m with Ca^{2+} and Zn^{2+} . Inset: Charge state distribution of denatured β 2m. (d) The stock solution of β 2m was diluted to 20 μM β 2m with 50% CH_3CN , 20 mM ammonium acetate (pH 7). Copper binding is seen to a maximum stoichiometry of 4:1. Spectra were acquired using a Micromass LCT electrospray time of flight mass spectrometer from 200 to 5000 m/z with signal averaging for one minute. Atmospheric pressure ionization was performed using borosilicate glass capillaries drawn and sputter-coated in-house. All mass determinations were made using a minimum of three charge states. External calibrations were performed with 20 mM CsI in H_2O ionized under matched instrument conditions. The temperature of the mass spectrometer ionization

effect of Cu^{2+} on both the chemical and thermal denaturation of β 2m was assessed by intrinsic fluorescence measurements. Clearly and unusually, the binding of Cu^{2+} by β 2m results in destabilization rather than stabilization of the protein (Figure 3). The midpoint for urea denaturation of β 2m in Mops buffer at pH 7.4 in the presence of 100 μM CuCl_2 is 1.5 M compared to 5.6 M in the presence of EDTA (Figure 3(a)). Fitting of this data to a model in which the free energy varies linearly with denaturant concentration⁸ yields $\Delta G_{\text{folding}} = -27.9(\pm 2.9)$ kJ mol⁻¹ and $\Delta G_{\text{folding}} = -9.4(\pm 0.3)$ kJ

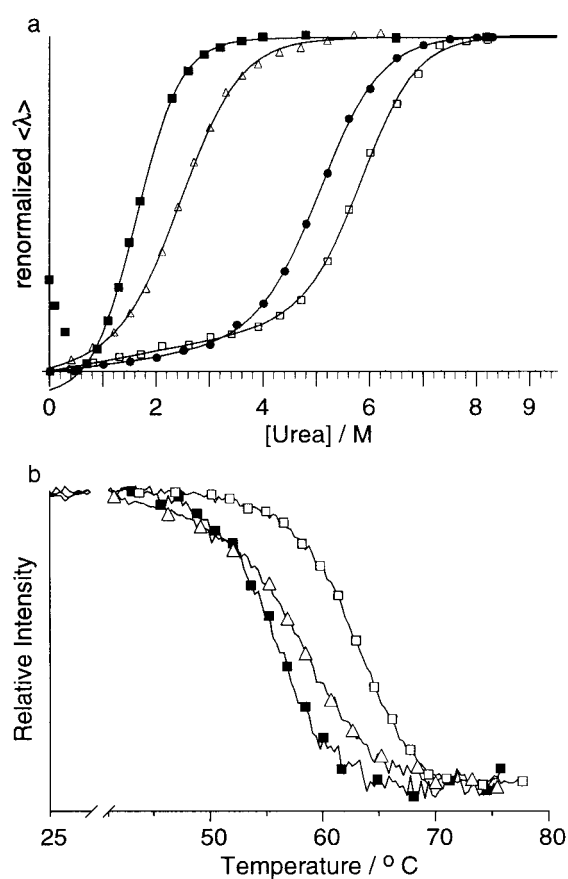


Figure 3. Chemical and thermal stability of β 2m measured by intrinsic fluorescence. (a) Urea denaturation of 5 μ M β 2m, 150 mM KCl, 25 °C, in the presence of 100 μ M CuCl_2 , 25 mM Mops (pH 7.4) (■) or 10 mM EDTA, 25 mM Mops (pH 7.4) (□) or 100 μ M CuCl_2 , 25 mM phosphate (pH 7.4) (●) or 25 mM acetate (pH 5.0) (Δ). Fluorescence spectra were acquired several hours after sample preparation using a PTI Quantamaster C-61, slit widths were 2 nm with excitation at 283 nm. Average emission wavelength was calculated between 290 and 450 nm and renormalized for display. Data were fit (shown as a continuous line) to a cooperative two-state model in which the free energy varies linearly with denaturant concentration.⁸ Data were indistinguishable regardless of whether samples were prepared from folded (0 M urea) or denatured β 2m (9.7 M urea). Furthermore, addition of EDTA to samples containing Cu^{2+} resulted in data indistinguishable from samples that had not been exposed to Cu^{2+} . (b) Thermal denaturation of 2.5 μ M β 2m, 150 mM KCl in the presence of 100 μ M CuCl_2 , 25 mM phosphate (pH 7.4) (■) or 10 mM EDTA, 25 mM phosphate (pH 7.4) (□) or 25 mM Mes (pH 5.8) (Δ). Heating was performed at 0.4 °C min^{-1} with denaturation monitored every minute as the ratio of emission intensity at 325 and 340 nm (PTI Quantamaster C-61, excitation at 283 nm, excitation and emission slit widths of 5 and 2 nm respectively). Degradation due to incident light was minimized by automatic shutter control.

mol^{-1} at pH 7.4, 0 M urea and $\pm 100 \mu\text{M}$ Cu^{2+} , respectively. We also note that stability of β 2m (pH 7.4) is unaffected by the presence or absence of either 150 mM KCl or 10 mM EDTA (data not shown).

The stability of β 2m in the absence of Cu^{2+} is insensitive to the choice of buffer system. The midpoint for urea denaturation across four buffer systems, Pipes, HEPES, potassium phosphate or Mops, is 5.8(± 0.1) M. In contrast, the apparent destabilization by the addition of Cu^{2+} is sensitive to the buffer composition; for example, in the presence of phosphate the midpoint for urea denaturation is 5.3 M compared to 1.5 M in Mops (Figure 3(a)). This presumably results from the weak binding of divalent ions by phosphate ($K_d \sim 20 \text{ mM}$).⁹ This disparity suggests that the protein provides a subset of ligands for copper binding while the remaining coordination sites are occupied by other solutes. For example, when Mops buffer is used in place of phosphate in the copper-binding assay (Figure 1), the intrinsic fluorescence of β 2m is unaffected by copper concentrations below 100 μM . However, in the presence of Mops and 100 μM Cu^{2+} , addition of urea (up to $\sim 1 \text{ M}$), results in a decrease in average emission to shorter wavelengths (Figure 3(a)). Therefore, in the presence of a non-chelating buffer system such as Mops, urea may serve as a coordinating ligand for copper and facilitate binding to the protein. Thermal denaturations of β 2m were conducted in phosphate-containing buffers since the pK_a of phosphate is relatively insensitive to temperature. The midpoint for denaturation in the presence of 100 μM Cu^{2+} is 56 °C compared to 63 °C in the presence of EDTA (Figure 3(b)). In agreement with our observations of specificity by electrospray ionisation-mass spectrometry (Figure 2), neither Ca^{2+} nor Zn^{2+} is destabilizing to β 2m (data not shown). Thermodynamic parameters were not derived from measurements of thermal denaturation, since only partial recovery of fluorescence intensity is observed upon cooling, suggesting that the process is not fully reversible.¹⁰

De novo fiber formation

The predominant form of β 2m extracted from *ex vivo* fibers is unmodified and wild-type, as judged by mass spectrometry.¹⁰ However, *in vitro* amyloid formation by full-length human β 2m is thought to be limited to acidic conditions ($\text{pH} < 6$).¹¹⁻¹³ This requirement for mildly acidic pH is observed in other fiber-forming proteins, notably transthyretin.¹⁴ Since an acidic compartment is available in cellular lysosomes and infiltration of inflammatory cells can accompany dialysis,¹⁵ it is reasonable to suggest that circulating β 2m encounters acidic conditions. However, as these conditions are not unique to dialysis therapy, they are not sufficient for DRA. We investigated the effect of Cu^{2+} on *de novo* fibrillogenesis of human-derived β 2m at normal serum pH and 37 °C. Amyloid formation was assayed by measuring the change in fluorescence

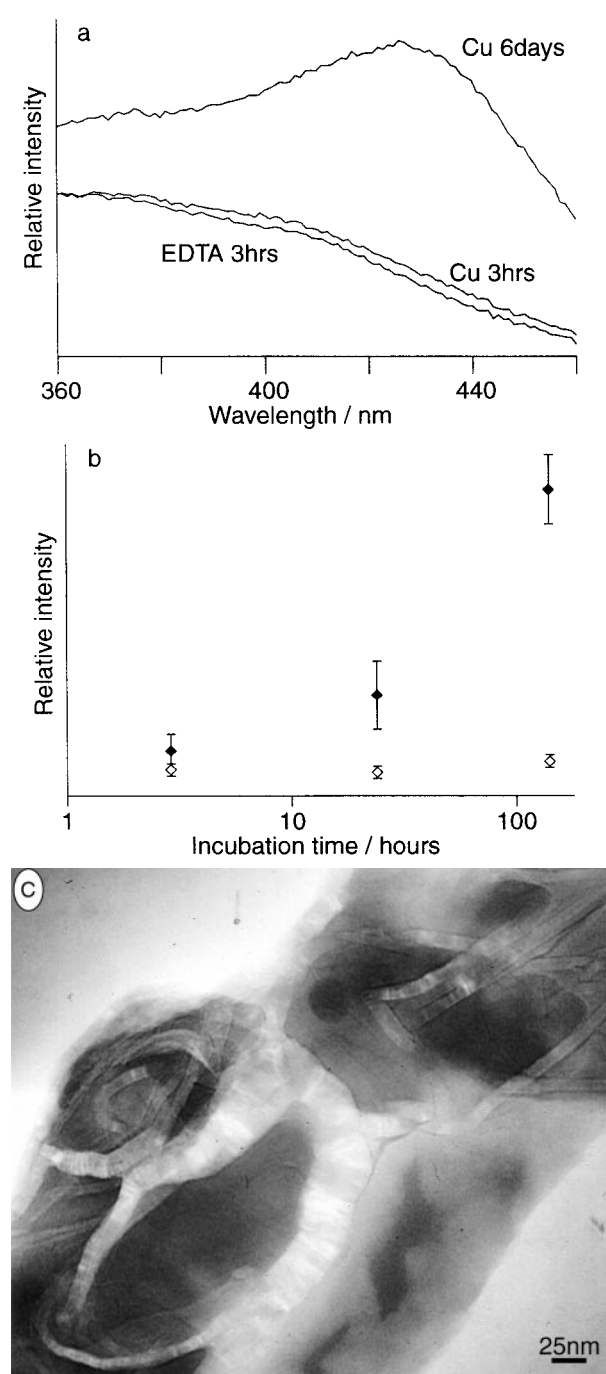


Figure 4. Comparison of fiber formation by β 2m at neutral pH in the presence and absence of Cu^{2+} . (a) Fiber formation of β 2m was assayed in a reaction at 37°C containing 100 μM human β 2m, 25 mM phosphate (pH 7.4), 150 mM KCl, 1 M urea and 200 μM of either CuCl_2 or EDTA. Representative data set of buffer and lamp corrected excitation spectra of the dye, thioflavin T (ThT),¹⁶ following three hours incubation (β 2m with Cu^{2+} or EDTA) and six days incubation with Cu^{2+} . Our observation of copper binding permitted purification of β 2m (unpublished results) by immobilized metal-ion chromatography in a manner similar to that developed for PrP.³² For human derived β 2m, two patients with Dent's disease (excreting 50-100 mg β 2m l^{-1} urine) provided 2-4 l urine in containers containing 50 ml 1 M phosphate (pH 7.4), 0.3 g ampicillin (Yale School of

of the histological dye, thioflavin T (Figure 4(a)).¹⁶ After one day and dramatically after six days, β 2m incubated with Cu^{2+} but not EDTA shows a significant enhancement in ThT fluorescence (Figure 4(a) and (b)). We also examined the six day samples by transmission electron microscopy. Fibrillar and amorphous aggregates were abundant and plainly visible in β 2m samples incubated with Cu^{2+} (Figure 4(c)). The fibers are short and somewhat curved, consistent with the reported morphologies of β 2m fibers derived from *ex vivo* sources.^{17,18} Development of DRA normally occurs on a timescale greater than five years. Our use of elevated urea, 1 M compared to 50 mM for uremic patients¹⁹ and protein concentration (100 μM) in excess of physiological levels accelerates fiber formation to a rate that is accessible on the laboratory timescale. We note, however, that this concentration of urea is significantly less than that required to unfold the protein in phosphate buffer (Figure 3(a)).

The normal copper content of serum (13-25 μM) is significantly higher than our measured dissociation constant (2.7 μM). Since the majority of

Medicine Human Investigation Committee protocol 11522). At 4°C, urine was filtered (0.2 μM) and applied directly to Cu^{2+} -charged NTA-Superflow (Qiagen), washed (three times) with 5 mM phosphate (pH 7.4), 150 mM KCl and five times with no salt and 1 mM imidazole. Protein was fractionated upon elution with 10 mM imidazole. β 2m-containing fractions were pooled, concentrated and further purified by size-exclusion as performed for *E. coli*-derived β 2m (for details, see the legend to Figure 1). Electrospray ionization-mass spectrometry analysis using recombinant β 2m as an internal calibrant yielded a mass of 11,729.6(\pm 0.5) Da for human β 2m (calculated mass 11,729.2 Da). (b) Time-dependence of fiber formation for incubations with CuCl_2 (\blacklozenge) or EDTA (\diamond) monitored by the enhancement of excitation fluorescence (433 nm) of ThT. Error bars represent one standard deviation of triplicate experiments. Reaction conditions for fiber formation were 100 μM human β 2m, 25 mM phosphate (pH 7.4), 150 mM KCl, 1 M urea and 200 μM of either CuCl_2 or EDTA. Reactions of 100 μl were incubated at 37°C in sealed 5 mm \times 20 mm airfuge tubes (Beckman). Insoluble material was sedimented by ten minutes centrifugation at \sim 150,000 g: 80 μl of supernatant was removed and the remainder resuspended with 120 μl of 5 μM thioflavin T, 25 mM phosphate, 150 mM KCl, 1 mM EDTA (pH 7.4). Excitation spectra for ThT (Figure 4(a)) were collected with emission at 482 nm. (c) Negative stain transmission electron micrograph of six days incubation of human β 2m with Cu^{2+} . Straight and curved fibers with widths varying from 6-10 nm can be seen in the context of a large, amorphous aggregate. Electron micrographs were acquired using a Zeiss JEM-A transmission electron microscope, accelerating voltage 80 kV. Protein solution (4 μl) was placed on a formvar/carbon-coated copper grid for five minutes, washed twice with 10 μl of MilliQ-purified water and stained negatively using 10 μl of 1% (w/v) phosphotungstic acid (pH 7.7).

this copper is tightly bound to plasma proteins,²⁰ it is unavailable to β 2m. The dialysis procedure, however, has two potential ways of allowing interaction of β 2m with free Cu^{2+} . The first is *via* the dialysate. One year of dialysis exposes a patient to 15,000-30,000 l of water.²¹ The maximal level of copper in dialysate recommended by the American Association of Medical Instrumentation is 1.6 μM .²¹ This is within a factor of 2 of our measured copper affinity (Figure 1). A second source of copper contamination is the use of $\text{Cu}(\text{N}-\text{H}_3)_4(\text{OH})_2$ solution in the preparation of cellulose membranes such as Cuprophan[®]. These membranes contain ~ 2 mg copper per m^2 (a typical dialysis membrane is 1-2 m^2) and in 1997 were in use by 27% of dialysis centers in the USA.²² Furthermore, these membranes readily release Cu^{2+} upon incubation with plasma at 37 °C, whereas saline solution used as a prewash²³ fails to extract Cu^{2+} . This could provide a high local concentration of Cu^{2+} within the membrane itself and flux of Cu^{2+} towards the plasma. Since β 2m is small (12 kDa) compared with the major Cu^{2+} binding proteins (68 kDa for serum albumin, 132 kDa for ceruloplasmin), it can partition into the membrane to a greater extent. Compelling clinical evidence for the relevance of copper is a >50% lower incidence of DRA symptoms among patients who are treated with synthetic membranes that do not include $\text{Cu}(\text{NH}_3)_4(\text{OH})_2$.^{24,25}

The relevance of copper within the wider context of amyloid formation is supported by studies of $\text{A}\beta$ ²⁶ from Alzheimer's and prion protein (PrP) from scrapie.²⁷ For example PrP₂₉₋₂₃₁²⁸ binds copper ($K_d = 14 \mu\text{M}$) and aggregates without accompanying redox chemistry. Metal binding in PrP has been ascribed to a conserved histidine residue containing an octapeptide repeat of PrP.²⁹ While β 2m possesses two highly conserved histidine residues (H31,H84), no sequence similarity with PrP is apparent.³⁰ Rather, the underlying mechanism for Cu^{2+} -induced fiber formation is the destabilization of folded β 2m. Indeed, reports of acid destabilization and subsequent fiber formation of β 2m^{10,12} further support this view. We note that the urea and thermal denaturation of β 2m with 100 μM Cu^{2+} at neutral pH in Mops (Figure 3(a)) and phosphate (Figure 3(b)) resemble closely denaturation in the presence of EDTA under acidic conditions, pH 5.0 and 5.8, respectively. Indeed, if the ionization state of one or more histidine residues proves central to the stability of β 2m, then binding of Cu^{2+} at a histidine residue may affect β 2m stability in a manner similar to histidine protonation. Nevertheless, destabilization by Cu^{2+} suggests an unusual mechanism, since favorable ligand binding to any conformational state lowers the free energy of that state. We conclude that a non-native state must also bind the metal. Clearly, this can be the unfolded state of β 2m (Figure 2(d)). However, given the specificity of destabilization of β 2m by Cu^{2+} , it is more likely that there exists a conformational state that is neither native nor unfolded,

which acts as a precursor to the formation of β 2m fibers.

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