Cleaved $\beta_2$-Microglobulin Partially Attains a Conformation That Has Amyloidogenic Features*

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$\beta_2$-Microglobulin, a small protein localized in serum and on cell surfaces, can adopt specific aggregating conformations that generate amyloid in tissues and joints as a complication to long-term hemodialysis. We characterize a proteolytic variant of $\beta_2$-microglobulin (cleaved after Lys$^{58}$) that as a trimmed form (Lys$^{58}$ is removed) can be demonstrated in the circulation in patients with chronic disease. An unexpected electrophoretic heterogeneity of these two cleaved variants was demonstrated by capillary electrophoresis under physiological conditions. Each separated into a fast and a slow component while appearing homogeneous, except for a fraction of oxidized species detected by other techniques. The two components had different binding affinities for heparin and for the amyloid-specific dye Congo red, and the equilibrium between the two forms was dependent on solvent conditions. Together with analysis of the differences in circular dichroism, the results suggest that $\beta_2$-microglobulin cleaved after Lys$^{58}$ readily adopts two equilibrium conformations under native conditions. In the cleaved and trimmed $\beta_2$-microglobulin that appears in vivo, the less populated conformation is characterized by an increased affinity for Congo red. These observations may help elucidate why $\beta_2$-microglobulin polymerizes as amyloid in chronic hemodialysis and facilitate the search for means to inhibit this process.

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† The abbreviations used are: $\beta_2m$, $\beta_2$-microglobulin; CE, capillary electrophoresis; CR, Congo red; LMW, low molecular weight; HPLC, high pressure liquid chromatography; RP, reverse-phase; MS, mass spectrometry.

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other groups support the notion that this folding intermediate may be on the pathway to amyloid formation (25). In addition, we observed by CE that purified Lys58β2m is also heterogeneous in the absence of organic solvent (27). In the present study, we characterize the Lys58β2m and des-Lys58β2m molecules using circular dichroism, CE, and affinity CE with heparin and Congo red. We find evidence for the existence of two conformers in cleaved β2m and demonstrate that the variant, less populated conformation has an increased affinity for heparin and Congo red. We conclude that Lys58β2m is less conformationally constrained than wild-type β2m and exists in two distinct and stable conformations under physiological conditions. The variant conformation resembles the organic solvent-inducible conformation of wild-type β2m that may be an intermediate on the folding pathway to amyloid.

EXPERIMENTAL PROCEDURES

Reagents—The sodium salt of low molecular weight (LMW) heparin from porcine intestinal mucosa (average molecular weight, 5,000) was from Calbiochem. Dextran sulfate, heparan sulfate, galactose 6-sulfate (all sodium salts), and tricine [N-(trishydroxymethyl)methyl]glycine), electrophoresis grade, were from Sigma. HPLC-grade water and acetonitrile were from Merck. Mouse monoclonal IgG antibodies against β2m, BBM.1 (HB28) and L368 (HB149), were from ATCC (Manassas, VA) and were purified by protein A-Sepharose affinity chromatography. A marker peptide (M; acetyl-Pro-Ser-Lys-Asp-OH) was synthesized by Schafer-N (Copenhagen, Denmark). Precast polyacrylamide gels from Novex (San Diego, CA) were used for SDS-PAGE.

β2m—β2m was purified from a pool of urine from uremic patients as described previously (7). β2m cleaved at Lys58 (Lys58β2m) and Lys58β2m with a deleted Lys58 (des-Lys58β2m; cf. Fig. 1A) were generated by treating purified β2m with activated complex C1s in the presence or absence of the protease inhibitor (2, 30). Total protein concentrations were estimated using a bichinonic acid protein assay method from Pierce with bovine serum albumin as a standard. The purified proteins in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 6.5 mM Na2HPO4, pH 7.4) were kept at −20°C until use.

Analytical and Preparative Capillary Electrophoresis—A Beckman P/ACE 2000 instrument equipped with a 20 cm × 75 μm capillary was used. CE. Electrophoresis buffer was 0.2% tricine/NaOH, pH 7.65, unless noted otherwise. Detection was by UV absorbance at 200 nm, and the separation tube was a 50-μm-inner diameter, uncoated, fused silica capillary of 37- or 57-cm total length with 30 or 50 cm to the detector window. Separations were carried out at 10 kV. Data were collected and processed by the Beckman system Gold software. The capillary cooling fluid was thermostatted at 25°C. All the capillaries were rinsed with electrophoresis buffer for 1 min with each of the following: 0.1 M NaOH, water, and electrophoresis buffer. The β2m samples and the marker peptide were analyzed in the dilutions given in the figure legends. In affinity experiments, various concentrations of ligands (heparin, other glycosaminoglycans, or Congo red) were added to the electrophoresis buffer from stock solutions of 5–10 mg/ml glycosaminoglycans or 0.2 mg/ml Congo red in electrophoresis buffer. In some experiments, NaCl was added to the electrophoresis buffer from stock solutions of 5–10 mg/ml glycosaminoglycans or Congo red in electrophoresis buffer. In some experiments, NaCl was added at various concentrations to assess the influence of ionic strength on the binding interactions. Preparative affinity CE followed by immunodetection was performed with 1 mg/ml LMW heparin present in the electrophoresis buffer. A sample consisting of 0.3 mg/ml Lys58β2m was injected for 12 s (corresponding to a sample volume of about 11 nl), and the injection program was changed to change outlet vial when the two peaks of Lys58β2m that were separated well in the presence of heparin were found to emerge at the capillary end. Outlet collecting vials contained 25 μl of electrophoresis buffer or 25 mm ammonium bicarbonate (when collecting samples for mass spectrometry). The material was processed for immunodetection after 10 runs by dotting 8 μl of each sample onto nitrocellulose. After air drying, the nitrocellulose was incubated with 2% Tween 20 for 20 min and then washed 5 times with water. Rabbit anti-β2m (Dako, Glostrup, Denmark; code no. A0072) at a dilution of 1:500 for 2 h at room temperature. After washing, bound primary antibody was visualized using an alkaline phosphatase-labeled swine anti-rabbit IgG (Dako; code no. D3036) at 1:1,000 for 1 h at room temperature followed by nitro blue tetrazolium staining.

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RESULTS

Probing β2m and Cleaved β2m Conformation by Circular Dichroism—The structure of β2m-microglobulin and variants thereof generated by limited proteolysis is shown in Fig. 1A. The three purified peptides were analyzed by CE, and the results (Fig. 1B) indicate that significant differences in the conformation of β2m are induced in response to cleavage of the molecule. The differences in the spectra in the near-UV region (240–325 nm) suggest changes of the environment of aromatic side chains, i.e. changes in the tertiary folding of the β2m chain. The two cleaved forms clearly deviate from the fingerprint of the wild-type β2m structure in this region of the spectrum. The far-UV region (180–250 nm) provides quantitative information on the overall secondary structure content of the protein (34), and differences in the spectra in this region for the three forms are also observed (Fig. 5). The characteristic spectrum of wild-type β2m is compatible with that of β2m and suggests that cleavage deviates significantly from the wild-type structure.
with ~50% β-structures (strands and turns), 50% random structure, and practically no helix structures as reported previously (4, 27). The changes in the far-UV spectrum of the cleaved forms, most pronounced for the Lys58-βm species, are similar to the changes in the CD spectra of wild-type βm obtained in 10–15% trifluoroethanol or 20–30% acetonitrile where there is a mixture of the native fold and a solvent-induced species with less β-structure and more helix structure (27). Thus the far-UV spectra of Fig. 5 are compatible with the presence of a mixture of differently folded species in the Lys58-βm preparation. Although it is not possible to quantitatively correct for their individual contributions to the spectra, the observations indicate that the two structures are substantially different.

Electrophoretic Separation of βm and Its Cleaved Variants—When the three purified βm species were separated by CE, more than one peak was observed in both the Lys58-βm and the des-Lys58-βm preparations (Fig. 2B), i.e. a smaller and more slowly migrating peak appeared after the main peaks. This was most pronounced in the Lys58-βm preparation (Fig. 2B, 2), where this slow fraction constituted ~25% of the total sample independently on the sample dilution from 1.3 to 0.13 mg/ml (data not shown). Wild-type βm (Fig. 2B, I) appeared homogenous in the CE analysis, and none of the samples were heterogeneous by SDS-PAGE except for faint bands around M\(^{+}\), 25,000 (Fig. 2A). However, it was noted that the cleaved forms migrated more slowly in SDS-PAGE than wild-type βm.

Characterization of the Components of Lys58-βm Resolved by CE—Two different monoclonal antibodies against human βm (L368 and BBM.1) immobilized on protein G-Sepharose removed the two peaks of des-Lys58-βm and Lys58-βm as well as the single peak representing wild-type βm when added to samples before CE (results not shown). This argued against the possibility that impurities or other 200 nm-absorbing components in the sample aside from βm molecules contributed to the observed peaks. Also, both peaks in both the des-Lys58-βm and the Lys58-βm samples absorbed at 280 nm and were degraded by trypsin treatment (results not shown). Masses of the proteins measured by MALDI-MS were within 0.02% of the theoretical masses of the βm forms based on their amino acid sequences. No evidence of dimers or higher order polymers was seen in the mass spectra. However, two species in both the βm and the Lys58-βm preparations differing by ~16 mass units (16.8 and 18.5) were separated by RP-HPLC using a shallow

![Fig. 1. Solution structure by CD of wild-type and modified βm. A, schematic drawing of structures generated by limited proteolysis of βm (2, 7). B, CD spectra of wild-type and modified forms of βm at 80 μM in 0.1 M phosphate buffer, pH 7.1. Left panel, 180–250 nm; right panel, 225–325 nm. The inset in the right panel represents a close-up of the 220–250 nm range. Signature explanation: —, wild-type; ——, Lys58-βm; ———, des-Lys58-βm.](image)

![Fig. 2. Electrophoretic analysis of βm-microglobulin proteolytic variants. The structures (cf. Fig. 1) are as follows: 1, wild-type βm; 2, Lys58-βm; and 3, des-Lys58-βm. A, SDS-PAGE of purified βm samples 1–3 (samples were 6 μg of total protein each boiled in nonreducing sample buffer, separated on a 4–20% gel, and silver-stained); molecular mass standards (from the bottom): 14.4, 20.1, 30.0, 43.0, 67.0, and 94.0 kDa. B, CE analysis of βm samples 1–3. 8-s injection of a mixture of (1) 6.5 μl of βm (0.07 mg/ml), (2) 4.5 μl of Lys58-βm (1.3 mg/ml), (3) 10 μl des-Lys58-βm (0.25 mg/ml), and (M) 1 μl of 1 mg/ml marker peptide. Electrophoresis buffer was 0.2 M tricine/NaOH, pH 7.65. Separation was accomplished at 10 kV with detection at 200 nm in a 50-μm-diameter, 37-cm capillary (detector at 30 cm).](image)
Lys$^{58}$-m Components Bind Heparin Differently and Are Immunologically Identical—To collect and immunologically characterize each of the CE-resolved fast (f) and slow (s) peaks of Lys$^{58}$-m, it was necessary to enhance the selectivity of the separation. We found that addition to the electrophoresis buffer of heparin or heparan sulfate, highly sulfated glucosaminoglycans that are found associated with all types of amyloid (16, 17), substantially increased the selectivity of the separation (Fig. 4). Heparin prolonged the appearance time of the s peak, indicating complex formation between heparin and the Lys$^{58}$-m s species. Using this approach for preparative CE, it was possible to separately collect enough of the components of Lys$^{58}$-m to probe them with antibody to β-m antibodies. As shown in Fig. 4, material from both peaks subsequently reacted with a polyclonal anti-β-m antibody. This confirmed that β-m was present in both peaks; thus, the apparent heterogeneity of the sample was not due to contaminating, unrelated proteins. When collecting the two peaks together in CE runs without a buffer modifier, the only components that were detectable by MS were the unmodified and the oxidized Lys$^{58}$-m species that were observed in the RP-HPLC analyses (Fig. 3).

The heparin binding activity of the two Lys$^{58}$-m species was quantitated by a series of affinity CE experiments (Fig. 5). As noted in the preparative affinity CE experiments (Fig. 4), the changes in the migration profile with heparin in the electrophoresis buffer were most pronounced for the s component of Lys$^{58}$-m that shifted to longer appearance times as compared with the f component and with the marker peptide (Fig. 5A, M). The longer peak appearance times in the presence of heparin indicate that the s component of Lys$^{58}$-m reversibly binds to the anionic heparin ligand during electrophoresis and thereby get a net increase in electrophoretic mobility, leading to a retardation of this peak. The predominant peak (f) was affected in the same way, but only at the highest concentration of heparin (Fig. 5A). The consistent shift of the whole s peak with increasing heparin additions to the buffer shows that it represents a homogeneous population of molecules with respect to heparin binding affinity.

The migration shifts observed after the addition of LMW heparin and heparan sulfate in similar affinity CE experiments were comparable, whereas smaller shifts were seen with corresponding milligram/milliliter concentrations of chondroitin sulfate (data not shown). No changes in the peak patterns were observed with additions of up to 0.5 mg/ml (1.8 mm) galactose-6-sulfate (data not shown) or 0.5 mg/ml dextran sulfate (data not shown). Decreasing magnitudes of migration shifts were observed with increasing ionic strength of the electrophoresis buffer (data not shown).

Migration shift data are summarized in Fig. 5B. The total LMW heparin concentration was used for the value of c, the concentration of ligand. The des-Lys$^{58}$-m sample showed binding, especially of the minor s peak, but showed negligible migration shifts with heparin (data not shown) and is not included in the data. The data obtained with β-m and Lys$^{58}$-m (Fig. 5B) could be fitted ($r^2 > 0.99$) to a one site binding hyperbola. The inset of Fig. 5B shows the linearized data. The slopes of the lines are a measure of heparin affinity. Whereas the affinities for heparin of wild-type β-m and the fast component of Lys$^{58}$-m are very similar ($K_d = 1.2$ and 2.2 mm, respectively), the slow Lys$^{58}$-m component has a higher affinity ($K_d = 0.6$ mm).

Shift of Lys$^{58}$-m Peak Equilibrium in Organic Solvent—We found that Lys$^{58}$-m samples that had been subjected to RP-HPLC purification and were resolubilized in water almost completely converted to the slow component detected by CE. Over time, this distribution slowly reverted to the normal distribution of the two forms with the fast peak as the major species (Fig. 6).

Slow Component of des-Lys$^{58}$-m Binds Congo Red—With addition of the amyloid-specific dye CR to the electrophoresis buffer, we used CE to assess the binding to the cleaved β-m variant des-Lys$^{58}$-m that circulates in chronic disease (Fig. 7). Whereas both components of this molecule bind heparin poorly, it was readily observed that the s component of des-Lys$^{58}$-m interacted more strongly with CR than the f species. In addition, a late peak (marked with an asterisk, Fig. 7), which we interpret as representing the CR-des-Lys$^{58}$-m complex, increased proportionally with the concentration of CR. This late complex was absent in Congo red affinity CE analyses of marker or wild-type β-m alone (data not shown). Because of the stable complex formation, it was not possible to calculate a binding constant for the interaction between the s conformation of des-Lys$^{58}$-m and Congo red based on peak migration...
shifts, but complex formation was clearly already detectable at 1–2 μM CR (Fig. 7).

**DISCUSSION**

The CE and CD analyses of β2m variants generated by limited proteolysis strongly suggest that these molecules exist in two distinct conformations that are in equilibrium at neutral pH in physiological buffer. Although the β2m variants (as well as wild-type β2m) contain both unmodified and modified molecules (probably oxidized on the C-terminal Met residue), the heterogeneity demonstrated by CE was not caused by a fraction of oxidized molecules. Due to the resolution of the CE analysis and the higher affinity of one of the conformations for heparin, the distribution between the two conformations was readily quantitated under different conditions, and we found that the less abundant s conformation is favored under more hydrophobic conditions. We recently found that such conditions induce a loss of native conformation and the emergence of a specific conformational variant or a partly structured intermediate of wild-type β2m as well (27).

Studies of wild-type human β2m in solution using NMR
techniques (4) and three-dimensional structure elucidation based on x-ray crystallography of β₂m in complex with major histocompatibility complex class I molecules (35) show that β₂m at pH 5.4–8.0 is a pure β-sheet protein that assumes a defined, moderately tightly packed structure of two antiparallel β-sheets formed around the Cys²⁵, Cys⁸⁰ disulfide bridge. Lys⁵⁸ is part of a 5-residue loop between β-strands that is either very rigid or relatively slowly fluctuating between two or more structures (4). β₂m contains no obvious linear consensus sequences (clusters of basic amino acids) (36) for heparin binding, but it is conceivable that a break in the strained loop between Lys⁵⁸ and Asp⁴⁹ with the possibility of rotation around the disulfide bond may facilitate alternative conformations, one of which has an increased binding affinity for heparin. The consequences of non-native conformations for β₂m-microglobulin function in cellular immunity remain to be elucidated, but a changed conformation is likely to influence protein-protein interactions between β₂m-microglobulin and the major histocompatibility complex class I heavy chain and thereby influence the presentation of major histocompatibility complex-associated peptides. Conformational changes are supported by the observations of CE heterogeneity and by the changes in the CD spectra as well as by the overall different mobility in SDS-PAGE of the variants as compared with native β₂m (Refs. 2 and 7; Fig. 2A). Only by CE, however, is it possible to quantitatively separate the two species. The discovery that β₂m and its pro-teleovit variant Lys⁵⁸–β₂m are able to bind heparin is interesting because many of the peptides and proteins that have been identified as major components of different types of amyloid have been shown to bind heparan sulfate or chondroitin sulfate (37–40). Amyloid-like material has been observed in vitro after simply mixing wild-type β₂m with heparan sulfate and/or serum amyloid P component (41). The Kₑ values for the interactions with heparin estimated in the present study are maximum values because the total LMW heparin concentration was used in the plots. It is not known whether a specific subfraction of heparin (heparin is heterogeneous with respect to chain length, sulfation, and disaccharide composition (29, 42)) is responsible for binding. However, the binding is weak and ionic strength-dependent, i.e., involves electrostatic interactions. The presence of glycosaminoglycans in β₂m amyloid is an indication of the abnormal conformation and/or polymerization of the deposited wild-type β₂m (14, 19). A key finding of the present study is that Congo red binds specifically and strongly to the conformational variant existing under native conditions in des-Lys⁵⁸–β₂m. CR resembles heparin/heparan sulfate in being a sulfated molecule with known affinity for amyloid. Taken together, however, our results indicate that anionic groups are not responsible for the binding of CR to the des-Lys⁵⁸–β₂m s conformer. The CE and CD data agree with the notion that the des-Lys⁵⁸–β₂m variant conformation is similar to the organic solvent-induced variant conformation of wild-type β₂m that also displays considerably increased affinity for CR and that may be an intermediate on the pathway to insoluble amyloid formation (27–29). A fraction of cleaved β₂m may thus be able to present differently structured intermediates and thereby instruct wild-type β₂m to malfold and precipitate as amyloid. Although many steps in this process remain to be elucidated, the approach presented here will be helpful in further analyses of the conformational intermediates and oligo-molecular complexes that together form amyloid or amyloid precursors and thus in the discovery of means to inhibit these processes.

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