Molecular variants of β_2-microglobulin in renal insufficiency

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Many patients with renal insufficiency treated by dialysis for more than 10 years have tissue deposits of amyloid material containing polymerized β_2-microglobulin (β_2m). The mechanisms of β_2m polymerization and degradation remain unknown. In biological fluids (serum and urine) from haemodialysis patients and in dialysis fluids from patients treated by chronic ambulatory peritoneal dialysis (CAPD), we have characterized different molecular forms of β_2m, including proteolytic split products. β_2m isoforms of pI 5.7, 5.3 and 4.5–5.0 were isolated from urine and CAPD fluid. The pI 5.3 β_2m, but not the other forms, was recovered both as monomers and as dimers. Such dimers were also detected in serum from patients but not from healthy controls. pI 5.3 and 5.7 β_2m isoforms were found to be nearly identical by mass spectrometry and by their amino acid sequences. The amino acid sequence of the 43 N-terminal amino acids of β_2m of pI 5.0 showed identity with the corresponding region of pI 5.7 β_2m. Fragments recovered from CAPD fluid were similar to proteolytic fragments generated from pure pI 5.7 β_2m by incubation in mouse ascitic fluid at acidic pH. Furthermore, pure pI 5.7 β_2m was converted into more acidic forms of 12 kDa upon incubation in mouse ascitic fluid at acid pH. β_2m dimers found in serum may represent a precursor of amyloid fibrils.

INTRODUCTION

Many uraemic patients treated by long-term haemodialysis present after a few years of treatment with tissue deposits of amyloid material that are responsible for carpal tunnel syndrome, bone cysts and arthropathies [1]. β_2-Microglobulin (β_2m), which occurs as a free monomer or non-covalently bound to major histocompatibility complex class I molecules on cell membranes and in biological fluids [2], is markedly elevated in sera from dialysis patients [3]. Two groups have reported that β_2m molecules are the major component of amyloid fibrils [4,5]. Isolation of material from biopsies and analysis of the extracted proteins revealed that amyloid fibrils observed in uraemic tissues are mainly composed of intact β_2m, but the presence of additional degraded material was also reported [6]. Degraded β_2m molecules were also found in kidney stones of haemodialysis patients [7], and heptapeptide corresponding to residues 13–19 of the β_2m sequence was isolated from the ultrafiltrate of haemodialysis patients [8].

The mechanisms responsible for the local accumulation and polymerization of the molecule in amyloid deposits are not well understood. By gradually decreasing the salt concentration and increasing the β_2m concentration, Connors et al. [9] were able to transform native β_2m into amyloid fibrils *in vitro*. However, their experimental conditions were too far removed from those occurring *in vivo* to exclude other hypotheses. More recently, Campistrol et al. [10] obtained molecules with the appearance of characteristic amyloid fibrils in the supernatant of cultivated mononuclear cells from dialysis patients. β_2m was also reported to bind with low affinity to collagen molecules [11], but we have not been able to reproduce these data in our laboratory. None of these mechanisms of amyloid formation seems satisfactory, and the 10–50-fold elevation of serum β_2m levels in haemodialysis does not account solely for amyloid formation. Therefore the hypothesis that enzymic modifications of the molecule are necessary for β_2m deposition and polymerization should be considered.

The aim of this study was to identify and characterize the different forms of circulating β_2m present in the serum and urine of uraemic patients. We report for the first time the occurrence of dimers of β_2m in serum from haemodialysis patients without amyloidosis, and we describe various proteolytic split products of β_2m which may be generated in these patients.

MATERIALS AND METHODS

Patients and controls

Four patients treated by maintenance haemodialysis for 2–5 years, of whom two continued to have a residual diuresis, were selected for the study. Blood samples were taken at the beginning of dialysis and stored at −40 °C after sampling. Urine from two of these patients was stored at 4 °C after collection in the presence of 10 ml of 3 mM NaN_3/3 M Tris per litre of urine in order to avoid β_2m degradation at low pH. Four patients with renal insufficiency and complete anuria were treated by continuous ambulatory peritoneal dialysis (CAPD). Dialysis fluids from these patients were cooled at 4 °C and 20 ml of a 0.1 M trisodium dihydrate/80 mM NaN_3 solution was added per litre of CAPD fluid in order to prevent the formation of fibrin. CAPD fluids were collected, pooled and processed each day. All patients were seronegative for human immunodeficiency virus (HIV), and had no infectious episode at the time of biological fluid sampling. There was no clinical or radiological evidence for dialysis-related amyloid formation. Control sera samples were provided by three healthy subjects and two HIV-positive patients present with acquired immune deficiency syndrome (AIDS).

Reagents

Peritoneal exudates were induced in Balb/c mice by injection of 0.5 ml of Bayol F oil into the peritoneal cavity. After 48 h, 2 ml of PBS was injected, and the PBS/oil mixture was recovered and centrifuged for 10 min at 2000 *g* at 4 °C. The cell pellet and the upper part containing oil were discarded. Anti-β_2m antibodies

Abbreviations used: β_2m, β_2-microglobulin; 2D-PAGE, 2 dimensional SDS/PAGE; IEF isoelectrofocusing; CAPD, continuous ambulatory peritoneal dialysis; HIV, human immunodeficiency virus; f.a.b., fast-atom bombardment; HLA, human leucocyte antigen.

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(M37) used for immunoabsorption were prepared by hyper-immunization of sheep; specific IgGs were isolated on immobilized β_2m columns and the IgG cross-reacting with human Ig [12] was removed by absorption on human IgG columns.

Protein assays

Protein content was measured by the Bio-Rad protein assay using human albumin as standard. Determination of β_2m was performed either by an e.l.i.s.a. as previously described [13] or in pure β_2m fractions by absorbance at 280 nm using a molar absorption coefficient of $19.4 \times 10^4\ M^{-1} \cdot cm^{-1}$. Soluble human leucocyte antigens (HLAs) were determined by inhibition of lymphocytotoxicity in the presence of rabbit complement and by using specific anti-HLA-B27 or A9 antibodies according to Mittal et al. [14]. The amount of antigen was expressed in arbitrary units; 1 unit is the amount of antigen capable of decreasing by one dilution the 50% cytotoxic titre of the antiserum.

Isolation of β_2m from urine of haemodialysis patients

Urine from each patient was treated individually. Urine was thawed and batches of 2 litres were filtered on Whatman No. 4 filter paper before concentration up to 100 g/litre on hollow fibre (Amicon DC2 with two H1P5 cartridges). The crude material was immunoabsorbed on sheep anti-β_2m columns; 500 mg of immunopurified sheep anti-β_2m IgG was immobilized on CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden), and a column of 2.5 cm × 25 cm was filled with the gel. A desalting column (5 cm × 30 cm) filled with AcA202 Ultrogel (LKB) was connected to the exit side of the previous column by a three-way connector. After injection of the material to be purified and extensive washing with saline, the two columns were connected and the system was equilibrated with phosphate/glycine buffer, pH 7.0 (0.1 M glycine, 0.01 M NaN_3 and 0.1 M NaCl adjusted to pH 7.0 with 1 M sodium phosphate buffer, pH 7.0). The bound material was eluted at acidic pH with the desalting column on line by a gradient between the pH 7.0 glycine buffer and a pH 2.7 buffer (the previous glycine solution adjusted to pH 2.7 with 1 M HCl) and recovered in a fraction collector. Continuous pH monitoring was performed before collection using a flow-through pH electrode. pH controls indicated that β_2m was immediately separated from the front of the pH shift and collected within the pH 7.0 buffer. The eluted β_2m fraction was dialysed against 0.1 M glycine and further purified by isoelectrofocusing (IEF). IEF fractionation was performed by preparative flat-bed electrofocusing in an Ultrodex gel (Multiphor System; Pharmacia) in the presence of 1.5% Servalyte 4–7 and 0.5% Servalyte 3–10 (Serva, Heidelberg, Germany). Ampholytes and high-molecular-mass proteins were separated from β_2m by gel-permeation chromatography on a column (1 cm × 100 cm) of AcA54 Ultrogel (LKB). Elution was performed at 0.5 ml/min with 30 mM Tris/HCl, pH 7.8, supplemented with 0.1 M NaCl.

Isolation of β_2m from CAPD fluids

Fresh CAPD fluids were filtered on Whatman 3M paper, then concentrated on hollow fibres (Amicon, Danvers, MA, U.S.A.) with H1P5 cartridges (cut-off 5 kDa). After concentration, β_2m was extracted by several steps of dilution with water and concentration using H1P50 cartridges (cut-off 50 kDa). The filtrate (5–50 kDa) was concentrated up to 2.5–5 g/litre, dialysed against 0.1 M glycine and fractionated by flat bed electrofocusing as described above.

Immunoaffinity purification of β_2m for SDS/PAGE analysis

Immunoselection was performed with sheep anti-β_2m immobilized on Sepharose. Normal serum (1 ml) or 0.1–0.5 ml of serum from a haemodialysis patient (depending on the serum β_2m concentration) was added to 50 μl of anti-β_2m–Sepharose beads and incubated with vigorous shaking for 30 min. After five wash steps with PBS/20% Tween 20, elution was carried out with 100 μl of 8 M urea. The eluate was desalted by dialysis against PBS and dried with a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY, U.S.A.), then analysed by SDS/PAGE.

Analytical methods

Concentration of fractions was done by ultrafiltration under nitrogen pressure on YM5 membranes (Amicon). Gel chromatography was performed on Sephadex gels with 30 mM Tris/HCl, pH 7.8, supplemented with 0.1 M NaCl; elution speed was 5 ml/min per cm^2. Electrophoretic analysis was performed by SDS/PAGE (20% gels) according to Laemmli [15] or by IEF on ultrathin plates (100 μm) with a pI range of 4–7 (Servalyts). Two-dimensional electrophoresis in a polyacrylamide gel (2D-PAGE) was performed according to the procedure of O'Farrell [16]. In the first dimension, 2% carrier ampholytes were used (Servalyt 3–10) in the presence of 9.2 M urea and 0.5% Triton X-100; the second dimension was run in a 20% polyacrylamide gel as described previously [17]. Gels were stained with Coomassie Blue G-250 or by the silver staining procedure, or were transferred on to nitrocellulose sheets by transverse electrophoresis on a semi-dry gel electroblotter and treated for immunodetection with ^{125}I-labelled sheep anti-β_2m as already described [17]. The nitrocellulose sheet was submitted to autoradiography at −80 °C with Kodak 'direct exposure' film.

Mass spectrometry and microsequencing

β_2m was adjusted at 10 mg/ml in 0.1 M ammonium bicarbonate buffer, pH 8.1. The molecule was reduced by 30 min of incubation at ambient temperature with 0.3 M dithiothreitol and then alkylated for 1 h in the dark under a nitrogen atmosphere by addition of solid iodoacetamide to a final concentration of 1 M. Treated β_2m was desalted by injection on to a Fast Desalting Column HR 10/10 in an f.p.l.c. system (Pharmacia) and eluted with the bicarbonate buffer.

Enzymic cleavage, if required, was carried out in the same buffer supplemented with 10 mM $CaCl_2$ and either Tos-Phe-CH_2Cl-treated trypsin (Worthington; enzyme/substrate ratio of 1:50) for 4 h at 37 °C or protease V8 (EC 3.4.21.19; Boehringer) for 12–20 h at the same temperature and with the same enzyme/substrate ratio. Peptide fragments were separated by reverse-phase h.p.l.c. on a C8 Aquapore RP300 column (22 cm × 0.46 cm; Applied Biosystems). A linear gradient of 4–60% acetonitrile with an aqueous phase of 0.1% trifluoroacetic acid or 20 mM trimethylamine/formic acid, pH 3.0, was used at a flow rate of 1 ml/min. Peaks were detected by u.v. absorption at 214 nm.

Sequence determinations were performed by Edman degradation on a 470A Applied Biosystems gas phase sequenator using the O3RPTH program. Phenylthiohydantoin amino acids were identified on line with a 120A PTH analyser (Applied Biosystems).

Fast-atom bombardment (f.a.b.) mass spectrometry was performed on alkylated and reduced proteins, after a desalting step by reverse-phase h.p.l.c. Positive-ion f.a.b. spectra were obtained using a VG Analytical ZAB-2 SEQ instrument and were the accumulation of 10 scans performed over the mass range 4000–13 500 Da.

RESULTS

Presence of high-molecular-mass β_2m-reactive material in sera

Serum from two normal subjects and two haemodialysis patients was chromatographed on Sephadex G200 gel columns, and β_2m and HLA were assayed in each fraction (Figures 1a and 1b). A major peak corresponding to free monomers of β_2m was observed in the 12 kDa region. In addition, high-molecular-mass peaks above the ovalbumin marker were detected in both the β_2m and HLA assays, and therefore contained Class I-associated β_2m. In the sera from the haemodialysis patients, an additional β_2m fraction was observed in a region where no HLA was detected (fractions 24–28). As this peak may represent free β_2m molecules associated in a dimeric form, a further characterization of free β_2m forms in such patients was carried out by immunopurification of β_2m. The eluate was analysed by SDS/PAGE, and β_2m bands were amplified by binding with polyclonal ^{125}I-labelled anti-β_2m antibodies after transfer to nitrocellulose blots (Figure 1c). The major band at 12 kDa was present in all sera, but an additional band at 24 kDa was detected only in the sera from the haemodialysis patients.

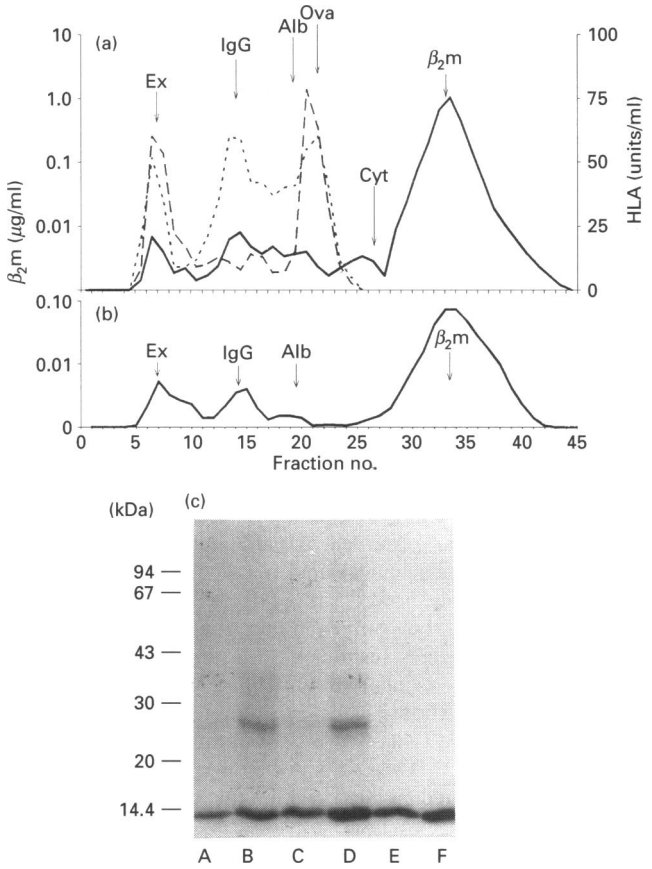

Figure 1 Chromatography on Sephadex G200 of serum from a uraemic patient (a) and normal serum (b)

Fractions were assayed for their β_2m (——), HLA-A9 (------) and HLA-B27 (– – –) content. Molecular mass standards are indicated by arrows [Ex, excluded volume (250 kDa); IgG (160 kDa); BSA (67 kDa); Ova, ovalbumin (45 kDa); Cyt, cytochrome *c* (12.5 kDa)]. (c) Autoradiography of β_2m-reactive material isolated from uraemic patients (lanes A–D), from normal serum (lane E) or serum from an HIV patient (lane F). Serum β_2m concentrations were 40, 21, 57, 29, 1.7 and 3.2 μg/ml for lanes A, B, C, D, E and F respectively, and the volumes used were 0.1, 0.5, 0.1, 0.3, 1 and 1 ml. β_2m was identified using specific ^{125}I-labelled antibodies on nitrocellulose transfer after SDS/PAGE fractionation.

Characterization of β_2m isolated from urine and CAPD fluids

Fractionation of β_2m from the urine of the two haemodialysis patients gave the same results. Three fractions with β_2m activity were isolated by preparative electrofocusing: one in the pI 5.7 region, another in the pI 5.3 region and a third in the pI 4.5–5.0 region. The β_2m recovered in the pI 5.7 zone represented 80–90 % of the total, whereas the pI 4.5–5.0 β_2m never exceeded 1 % of the total. Upon chromatography on an AcA54 gel, the pI 5.7 β_2m was eluted in the 12 kDa zone, as was the pI 4.5–5.0 β_2m. However, β_2m of pI 5.3 was recovered mainly as monomers, but partly in a 24 kDa position and in the void volume (Figure 2a). Isolation of the 24 kDa peak and re-chromatography on the same gel did not modify the chromatographic profile. When chromatography of the 24 kDa peak was performed in the presence of zwitterionic ions such as betaine, a proportion of the β_2m molecules was recovered in the 12 kDa zone while the remaining β_2m was still in the 24 kDa position. SDS/PAGE analysis under reducing conditions of the pI 5.3 fractions confirmed the presence of stable dimers, which indicates a probable covalent link (Figure 2b). Analytical IEF of the different fractions isolated by preparative IEF followed by AcA54 chromatography showed that pI 4.5–5.0 β_2m was a mixture of various forms, whereas the pI 5.7 and 5.3 fractions were homogeneous (Figure 2c). The results with β_2m from CAPD fluid were identical to those obtained with urine from haemodialysis patients (results not shown).

Mass spectra and amino acid sequences

β_2m samples of pI 5.3 and 5.7 isolated from the urine of one haemodialysis patient were analysed by mass spectrometry (Figure 3). The molecular mass for the pI 5.7 form from urine was measured using both reduced and unreduced β_2m. A mass of 11727 Da was measured for the unreduced molecule; after reduction it was 11 840 Da. Both values corresponded to values calculated from the amino acid sequence. The molecular mass of pI 5.3 monomeric β_2m was 11 860 Da. The amount of the stable dimeric form was too low to allow a mass spectrum analysis. The results obtained with β_2m preparations from the other patient were identical (results not shown). With β_2m isolated from CAPD fluids, the molecular mass values for pI 5.7 and 5.3 β_2m were 11 844 and 11 869 Da respectively. Mass spectra profiles (not shown) were similar to those for the urine forms presented in Figure 3.

β_2m forms isolated from one sample of urine and one sample of CAPD fluid were sequenced. The amino acid sequence of pI 5.7 β_2m was obtained using the intact protein, and the first 46 amino acids of the N-terminal part of the molecule were identified (Figure 4). The whole sequence was determined using tryptic or protease V8 peptides. The sequence corresponds to the sequence deduced from the gene [18]; in particular, amino acids in positions 17 and 42 are Asn residues. pI 5.7 β_2m isolated from CAPD fluids had an identical sequence. By sequencing intact pI 5.3 β_2m, the first 43 amino acids were determined in β_2m from urine and the first 45 amino acids in β_2m from CAPD fluid. These sequences were found to be those of the native molecule. The sequences of tryptic and V8 peptides confirmed the conservation of asparagine and glutamine residues in the whole pI 5.3 molecule. The low amount of pI 5.0 material available allowed us to perform only a single determination on the intact molecule from pooled dialytic fluid and urine. The 43 N-terminal amino acids were

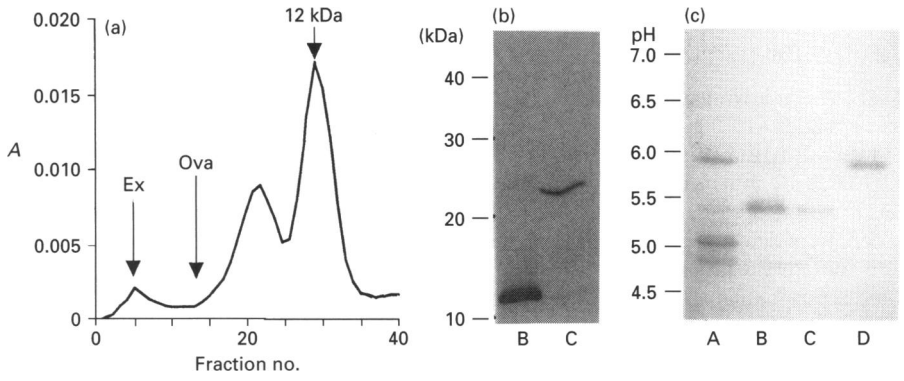

Figure 2 Analysis of β_2m fractions isolated from urine

(**a**) Chromatography on an AcA54 gel of the pI 5.3 fraction from preparative IEF. Molecular masses were calculated after chromatography of standard molecular mass markers on the same column (Ex, excluded volume; Ova, ovalbumin). (**b**) SDS/PAGE analysis of pI 5.3 fractions isolated from AcA54 chromatography: lane B, monomers; lane C, dimers. (**c**) IEF analysis of fractions isolated by preparative IEF and AcA54 chromatography, lane A, pI 4.5–5.0 fraction; lanes B and C, as in (**b**); lane D, pI 5.7 monomers.

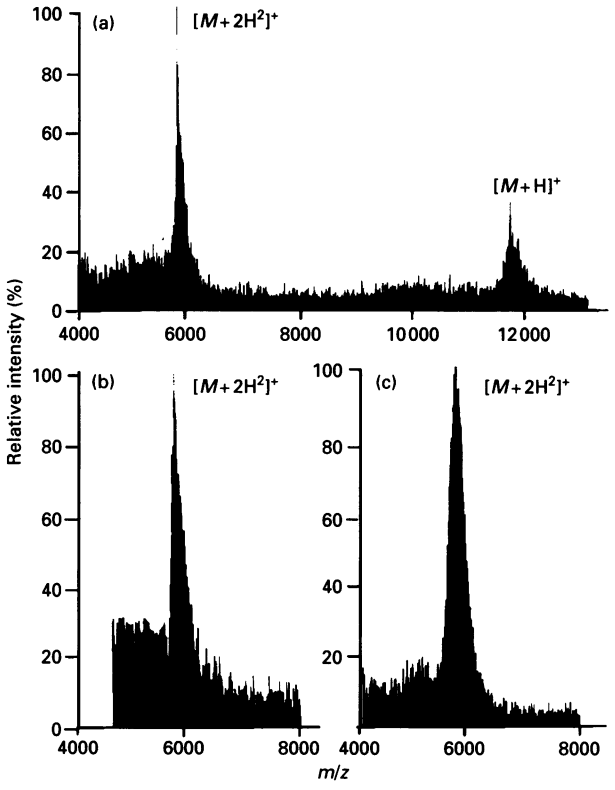

Figure 3 F.a.b. mass spectra of pI 5.7 β_2m in native (a) and reduced (b) forms, and of pI 5.3 β_2m in reduced form (c)

identified, and were again identical to the corresponding amino acids of pI 5.7 β_2m.

Stability of the molecule

pI 5.7 β_2m was diluted in PBS and incubated for 30 min at various temperatures, and then cooled on ice and tested by e.l.i.s.a. As indicated in Table 1, at concentrations of 0.5 mg/l or above the antigenic activity of β_2m remained unmodified at 37 or 60 °C, but only 40–50 % of the initial activity was recovered after heating to 80 °C. The loss of β_2m below 0.04 mg/l may be the consequence of non-specific absorption on to the tube walls, as the addition of 1 % BSA prevented this phenomenon.

Purified pI 5.7 β_2m diluted in PBS or in various sera and urine samples was adjusted to low pH values and then incubated for 2 h at 37 °C and assayed by e.l.i.s.a. after neutralization. Most of the β_2m antigenic activity was preserved in pure β_2m solution and in normal sera, but in all urine samples only a small part of the β_2m remained antigenically active after this treatment (Table 2).

As β_2m seemed to be quite resistant to a pH shift in pure solution or in serum, the inactivation observed in normal urine could be attributed to urinary enzymes. In order to test the possibility of a pH-dependent enzymic activity, we tested the effect of normal urine and of mouse peritoneal fluid on pure pI 5.7 β_2m. After 2 h of incubation at 37 °C and pH 5.0, a loss of immunoreactivity occurred (Table 3), and this phenomenon was lessened either by incubation at 4 °C or by pre-heating the biological fluids for 3 min at 100 °C. No decrease in immunoreactivity of β_2m was observed at pH 7.2, indicating that the enzymes were more efficient at acid pH. Kinetics studies using mouse peritoneal fluid showed a rapid decrease of β_2m immunoreactivity at pH 5.0, but only a minor diminution at pH 7.2 (Figure 5). The enzymes responsible may originate from peritoneal macrophages, as incubation of 20 μg of β_2m with 20×10^6 mouse peritoneal exudate cells in RPMI 1640 cell culture medium at pH 7.2 led to a 50 % loss of immunoreactivity of the molecule in 24 h at 37 °C (results not shown).

β_2m samples treated at acid pH in the presence of mouse peritoneal fluid were analysed by 2D-PAGE, and numerous spots were observed (Figure 6). Pure pI 5.7 β_2m migrated in the system as a single spot with an apparent pI of 6.6 (spot 3). Such a pI shift was observed with other molecules and was the consequence of the presence of urea [16]. Upon acid treatment, the intensity of the spot decreased without an apparent pI change (Figure 6c). Incubation of pI 5.7 β_2m with mouse peritoneal fluid at pH 5.0 induced the production of three additional spots that were visible after silver staining (Figure 6a) in the 12 kDa region, at pI 5.9 (spot 1), 6.2 (spot 2) and 7.0 (spot 4). Three other spots, representing degradation products, were observed at pI 5.8 (spot 5), 5.9 (spot 6) and 6.8 (spot 7), and corresponded to 9.7, 2.8–3.0 and 5 kDa fragments respectively. After transfer on to nitro-

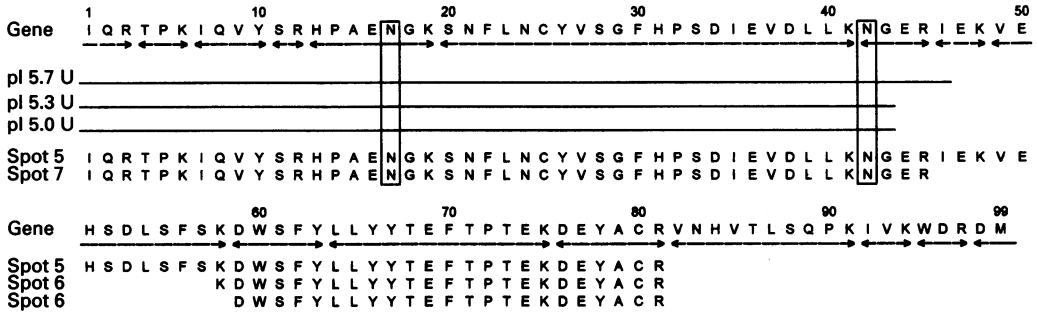

Figure 4 Amino acid sequences of β_2m

The sequence deduced from the gene is presented complete with the trypsin cleavage points, indicated by arrows. The N-terminal sequences of β_2m forms of pI 5.7, 5.3 and 5.0 were obtained on intact molecules from urine. Spots 5, 6 and 7 were fragments isolated from CAPD fluids.

Table 1 Stability of β_2m antigenic activity at different temperatures

Samples were incubated for 30 min at the given temperatures, then cooled and assayed by e.l.i.s.a. Values in parentheses represent percentage retention of immunoreactivity compared with the value at 18 °C.

β_2m added (μg/ml)	β_2m antigenic activity (μg/ml)			
Temp (°C)...	18	37	60	80
2	1.68	1.67 (99)	1.80 (107)	0.95 (57)
1	1.01	0.99 (98)	0.88 (88)	0.45 (45)
0.5	0.51	0.54 (106)	0.50 (98)	0.24 (47)
0.04	0.039	0.037 (95)		
0.01	0.011	0.007 (65)		
0.0012	0.0012	0.0006 (47)		

Table 2 Stability of β_2m antigenic activity in biological fluids upon 2 h of incubation at 37 °C in acid pH conditions

β_2m was assayed by e.l.i.s.a. Values in parentheses represent percentage retention of immunoreactivity compared with the value before treatment.

Sample	pH	β_2m (μg/ml)	
		Before treatment	After treatment
Pure β_2m	5.0	100	91.5 (91.5)
Pure β_2m	3.2	100	79.2 (79.2)
Pure β_2m	1.2	100	72.0 (72.0)
Serum 1	5.0	76.0	54.0 (71.0)
Serum 2	5.0	104	92.0 (88.5)
Urine 1	5.0	0.62	0.21 (33.9)
Urine 2	5.0	4.57	1.29 (28.2)
Urine 3	5.0	5.67	1.14 (20.1)
Urine 4	5.0	0.024	0.008 (33.8)
Urine 5	5.0	0.220	0.042 (19.2)

cellulose and immunodetection with ^{125}I-labelled sheep anti-β_2m (Figure 6b), spots 1–7 were readily identified and an additional spot appeared close to spot 7. The same spots 1–7 were identified by silver staining of β_2m isolated from CAPD fluids concentrated with the one-step immunoselection procedure (Figure 6d).

Sequencing experiments were done using fractionated fragments bound after transfer on a PVDF membrane (Millipore).

Table 3 Effects of pH and incubation temperature on β_2m antigenic activity in the presence of normal urine or mouse peritoneal exudate fluid

Pure β_2m was diluted (1:200) in biological fluids before incubation. β_2m was assayed by e.l.i.s.a. The results are expressed as percentage retention of immunoreactivity by reference to the value at pH 7.2 in PBS (40.8 μg/ml). n.d., not determined.

	β_2m (%)			
Temp (°C)...	4		37	
pH...	7.2	5.0	7.2	5.0
PBS	100	75.0	100	74.3
Urine*	104	89.2	92.1	32.8
Urine†	n.d.	n.d.	n.d.	93.1
Peritoneal fluid*	104	100	92.6	29.9
Peritoneal fluid†	n.d.	n.d.	n.d.	79.4

* β_2m concentration was below 0.02 μg/ml in urine and below 0.6 μg/ml in peritoneal fluid before addition of purified pI 5.7 β_2m.

† Urine or peritoneal fluid was preheated for 3 min at 100 °C.

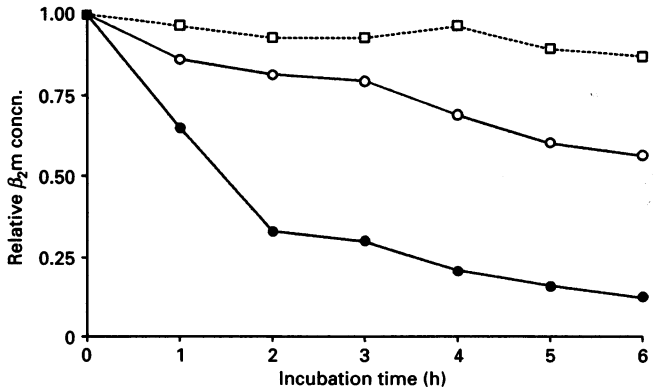

Figure 5 Kinetics of β_2m degradation at 37 °C by enzymic activity in mouse peritoneal exudate fluid

β_2m was in PBS (□), exudate fluid at pH 7.2 (○) or exudate fluid at pH 5.0 (●). β_2m was assayed by e.l.i.s.a.

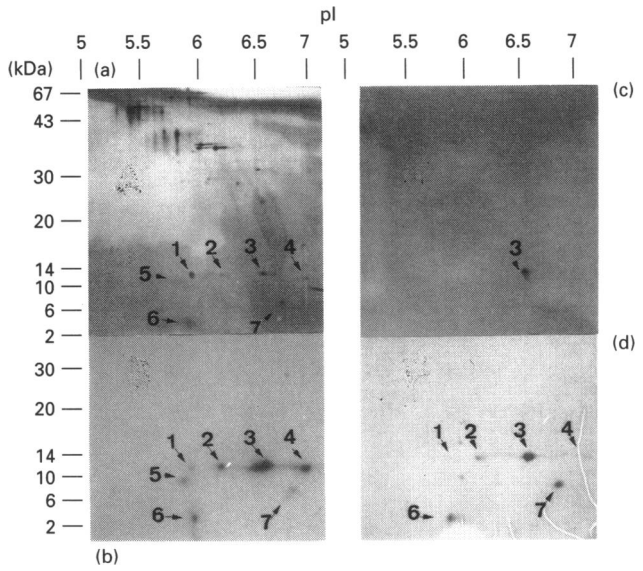

Figure 6 2D analysis of β_2m incubated with mouse ascitic fluid for 4 h at pH 5.0

(**a**) Silver staining; (**b**) immunodetection with ^{125}I-labelled sheep anti-β_2m; (**c**) silver staining of β_2m incubated in the same conditions but without ascitic fluid; (**d**) silver staining of β_2m immunopurified from human dialytic peritoneal fluids.

In spot 6, 90% of the molecules presented the sequence KDWSFYLLYYT... and 10% had the sequence DWSFYLLYYTE.... The length of the fragments calculated from the SDS/PAGE standards corresponded to 22–24 amino acids. For spots 5 and 7 the sequence corresponded to the intact N-terminal part of the molecule, and the fragment lengths were compatible with cleavage between amino acids 81–82 and 45–46 respectively (Figure 4).

DISCUSSION

The major β_2m form found in healthy individuals is the monomer of 12 kDa with a pI of 5.7 [2]. The molecule is either free or associated with HLA Class I molecules. It corresponds to the native form of the molecule as indicated by *in vitro* synthesis in the rabbit reticulocyte system [19]. A unique gene has been described for β_2m in humans [18,20] and there are no known allelic variations. Conversely, true β_2m alleles have been described in mice, with a polymorphism in position 85 [21,22]. A genetic polymorphism has also been suggested in monkeys but the two forms have not been sequenced [23]. In guinea pigs [24], two forms of β_2m proteins have been described, the more acidic one being derived from the other by proteolytic cleavage between residues 98 and 99. In 1977, Hall et al. [25] reported for the first time the presence of pI 5.3 material in the urine of transplant patients and healthy subjects. Using extracts of amyloid material from patients presenting with end-stage renal disease, Gorevic et al. [5,26] isolated β_2m that was heterogeneous in charge, with a predominance of acidic forms. Part of this β_2m material was made of non-covalently linked dimers or polymers. More recently, Odani et al. [27] and Argilés et al. [28] have isolated and sequenced the acidic forms of monomeric β_2m isolated from dialysis fluid and serum respectively of haemodialysis patients.

We demonstrate for the first time in this paper the presence of a circulating dimeric form of β_2m in uraemic sera; some of these dimers are covalently linked. We have not found such dimers in sera from healthy individuals or in sera from HIV-positive patients. Our patients did not present any clinical or radiological sign of amyloidoses, and they had been on dialysis for 2–5 years, whereas clinical disease usually appears after 10–15 years of treatment. Therefore the present results indicate that β_2m may polymerize before the occurrence of massive tissue deposition.

β_2m isolated from the serum and urine of dialysis patients and from CAPD fluids is made of intact molecules recovered in three pI zones, i.e. 5.7, 5.3 and 5.0. These three forms of β_2m are not artefacts of preparation, as they are produced by lymphoid cells in culture [17]. Such acidic forms have been recently reported [28], but the pI 5.0 form recovered from the urine in the present study was not found by those authors. The conservation of the antigenic sites was demonstrated by the binding of sheep anti-β_2m antibodies. In comparison with pI 5.7 β_2m, the low-pI spots appear more intense after silver staining (Figure 6a) than on immunoblotting with ^{125}I-labelled polyclonal antibodies (Figure 6b), suggesting a lower affinity of these peptides for the antibodies.

By chromatography on an AcA54 gel, the pI 5.7 and 5.0 β_2m forms were always recovered as monomers, whereas the pI 5.3 β_2m must be considered as an heterogeneous fraction containing free monomers, monomers with a tendency to aggregation and stable dimers. Trace amounts of higher-pI β_2m as well as fragments are present in CAPD fluid (Figure 6d). Molecular masses calculated from mass spectra indicated only minor differences between pI 5.3 and 5.7 $\beta_2$ms, but the peak was broader in pI 5.3 β_2m, suggesting a more heterogeneous protein. Consequently, pI 5.3 β_2m may be derived from native β_2m by minimal changes such as methylation, deamination, acetylation or other processes, probably on various sites. Mass spectra of β_2m isolated from CAPD fluids led to the same conclusion.

Amino acid sequence determination by Edman degradation confirmed the absence of a major difference between pI 5.7 and 5.3 β_2m forms, such as the loss or substitution of an amino acid. In particular, the modification of Asn-17 found by Odani et al. [27] was not confirmed. In low-pI forms of β_2m, amino acid 42 is Asn and not Asp as found by Argilès et al. [28]. At position 42 of pI 5.7 β_2m we identified Asn, as can be deduced from the sequence of the gene and in agreement with the sequence reported by Nissen et al. [29], Argilès et al. [28] and Odani et al. [27]. Conversely Cunningham et al. [30] and Gorevic et al. [5] identified an Asp residue at this position. If the modifications of amino acids 17 and 42 are not sequencing artefacts, as residues 17 and 42 are directly accessible on the intact molecule [31], deamination during the preparation steps remains possible.

In order to identify the possible mechanism that induces the polymerization of the β_2m molecule, we have studied the stability of the native molecule. In pure solution, β_2m loses only a part of its antigenic activity at acidic pH and no degradation fragments can be identified. Most of the antigenic activity is destroyed at acidic pH in normal urine, but not in serum (Table 2). The loss of immunoreactivity can be attributed to proteolysis by enzymes, as suggested by Davey and Gosling [32]; this is supported by the following arguments: (i) for a constant volume of urine, the loss of β_2m is inversely proportional to the amount of β_2m added, (ii) the loss is proportional to the time of incubation, and (iii) heating for 3 min at 100 °C inhibits the activity in urine. The enzymes responsible have a higher potency at acidic pH than at neutral pH, and enzymes with identical specificity are found in mouse peritoneal fluid. In CAPD fluids, fragmented β_2m was recovered (Figure 6d) that had a 2D-PAGE profile superimposable with that of native pI 5.7 β_2m treated at acidic pH in the presence of mouse peritoneal exudate, indicating the action of a comparable

set of enzymes. One may conclude that β_2m has characteristic cleavage zones and that acidic forms of apparently intact β_2m may be obtained by enzymic processes.

Linke et al. [7] described the presence of truncated β_2m material extracted from kidney stones and proposed the hypothesis of limited degradation of the N-terminal part of β_2m as a prerequisite for polymerization. None of the fragments that we have obtained correspond to the cleavage zones of Linke. The heptapeptide described by Abiko et al. [8] has not been identified, but the SDS/PAGE gel used was not adapted to the detection of small fragments. The cleavage site observed with one of the 2.8–3.0 kDa fragments is probably related to the observation of Nissen et al. [29,33], who isolated in sera from patients with small-cell lung cancer a serine proteinase which cleaves β_2m at amino acid 58.

The 9.5 kDa polypeptide isolated from uraemic serum by Haag-Weber and Hörl [34] called granulocyte-inhibitory protein, which corresponds to the N-terminal part of β_2m, may be similar to our 9.7 kDa fragment, but the exact cleavage zone remains to be identified.

The presence of such enzymes and their probable macrophagic origin may explain the extrarenal catabolism of the molecule in anephric patients [35].

The presence of circulating dimers of β_2m and of modified monomeric forms of the molecule in biological fluids from uraemic patients without apparent signs of amyloid tissue deposition suggests that the entrapping of circulating modified β_2m may contribute to its tissue deposition. The structural modification responsible for the tendency towards aggregation and dimerization of pI 5.3 β_2m remains to be determined.

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