Glycation of Human β_2-Microglobulin in Patients with Hemodialysis-Associated Amyloidosis: Identification of the Glycated Sites

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ABSTRACT: β_2-Microglobulin (β_2M) is a major component forming amyloid deposits in patients with hemodialysis-associated amyloidosis (HAA), a serious complication of long-term hemodialysis. Recently, we demonstrated that β_2M modified with the Maillard reaction is a definite constituent of amyloid deposits in patients with HAA. Our further study demonstrated that this modified β_2M induces not only chemotaxis of monocytes but also secretion of tumor necrosis factor-α, interleukin-1β, and interleukin-6 from macrophages, suggesting the potential link of glycation of β_2M by the Maillard reaction to the pathogenesis of HAA. The present study was undertaken to identify the glycated site(s) of β_2M purified from long-term hemodialysis patients as well as β_2M incubated with glucose *in vitro*. Borotritide-treated β_2M was cleaved by endoproteinase Lys-C, and peptides were isolated by reverse-phase high-performance liquid chromatography, followed by amino acid sequence analysis and fast atom bombardment mass spectrometry to identify the glycated site. The glycated sites of β_2M formed *in vivo* were found to be almost the same as those of glycated β_2M *in vitro*. The primary glycated site was the α-amino group of the amino terminal isoleucine. Other minor sites were the ϵ-amino groups of Lys-19, -41, -48, -58, -91, and -94. Computer graphics of the three-dimensional structure of β_2M suggested that the high specificity for the glycated site at Ile-1 may be explained by its high solvent accessibility and the nearby imidazole group of His-31 as an acid–base catalyst of the Amadori rearrangement.

In patients on long-term hemodialysis, the appearance of β_2-microglobulin (β_2M)[1]-derived amyloid deposits has recently been recognized as hemodialysis-associated amyloidosis (HAA) (Gejyo et al., 1985; Shirahama et al., 1985; Gorevic et al., 1986). The prevalence of amyloid deposits increases continuously with the duration of hemodialysis (Drüeke, 1991). Deposits are mainly located in joint structures especially in periarticular bones, leading to joint and bone destruction (Drüeke, 1991).

It is well known that the serum β_2M level is markedly higher (usually >30-fold) in these patients as compared to healthy individuals, but there is no statistical correlation between its serum concentration and the occurrence of HAA (Gejyo et al., 1986). The pathogenesis of HAA is therefore not accounted for merely by an increase in the serum β_2M level. Several lines of evidence have emphasized the molecular heterogeneity of β_2M forming amyloid fibrils, e.g., polymerization (Gorevic et al., 1986), deamidation (Odani et al., 1990), and truncation (Linke et al., 1989) of this molecule. However, the pathological role of β_2M with these modifications remains to be determined.

Recently, we observed that β_2M isolated from amyloid deposits of patients with HAA was electrophoresed much more acidic than a normal counterpart and that this isoform (referred to hereinafter as acidic β_2M) was also detectable in a small fraction of β_2M in the serum and urine of these patients (Miyata et al., 1993). We purified acidic β_2M as well as normal β_2M from urine of long-term hemodialysis patients. Physicochemical and immunochemical analyses with anti-Amadori product and anti-AGE (advanced glycation end products of the Maillard reaction) antibodies (Horiuchi et al., 1991) showed that acidic β_2M, a dominant constituent of amyloid deposits in HAA, contained both the Amadori products and AGEs of the Maillard reaction (Miyata et al., 1993). Our further study demonstrated that acidic β_2M induces not only chemotaxis of monocytes but also secretion of tumor necrosis factor-α, interleukin-1β, and interleukin-6 from macrophages (Miyata et al., 1994; Iida et al., 1994). It is thus possible that acidic β_2M in amyloid deposits may function as a pathogenic factor that recruits monocyte/macrophage *in situ* and initiates the inflammatory response.

A variety of proteins are known to undergo nonenzymatic modification by forming covalent linkages with reducing sugars (Brownlee et al., 1988; Baynes & Monnier, 1989). During this reaction, called the Maillard reaction, the sugar aldehyde group reacts with the amino group of proteins to form a labile Schiff base, which slowly isomerizes to the more stable ketoamine adduct via the Amadori rearrangement. Through a series of chemical rearrangements and dehydration reactions, some Amadori products are further converted to AGEs that accumulate on long-lived proteins.

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[1] Abbreviations: AGEs, advanced glycation end products; β_2M, β_2-microglobulin; Endo Lys-C, endoproteinase Lys-C; ESI, electrospray ionization; FAB, fast atom bombardment; HAA, hemodialysis-associated amyloidosis; HPLC, high-performance liquid chromatography; MS, mass spectrometry.

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To understand the functional link of β_2M modified with the Maillard reaction to the pathogenesis of HAA, a structural analysis of this glycated β_2M is essential. In the present study, we attempted to determine the glycated site(s) of β_2M purified from long-term hemodialysis patients. Factors that affect the specificity of glycation of amino groups in this protein are also examined by computer graphics analysis of the X-ray crystallographic structure of β_2M.

MATERIALS AND METHODS

Purification of Normal β_2M and Acidic β_2M. We purified normal β_2M and acidic β_2M from urine of two long-term hemodialysis patients (56-year-old male undergoing hemodialysis for 8 years and 44-year-old female undergoing hemodialysis for 5 years). These patients had been on regular hemodialysis using a cuprophane dialyzer and a dialysate containing 30 mequiv/L of bicarbonate and 8 mequiv/L of acetate. The purification procedure and the physicochemical and immunochemical properties of normal β_2M and acidic β_2M were described in detail (Miyata et al., 1993). The purified normal β_2M electrophoresed as a single band, but acidic β_2M electrophoresed as a few acidic bands on the isoelectric focusing. The acidic β_2M, but not normal β_2M, was brown in color and fluoresced. The color and fluorescence are characteristics of advanced glycation end products (AGEs) of the Maillard reaction (Brownlee et al., 1988; Baynes & Monnier, 1989). The acidic β_2M also showed an immunoreactivity to anti-AGE antibody (Horiuchi et al., 1991) as well as anti-Amadori product antibody (Miyata et al., 1993).

In Vitro Glycation of Normal β_2M with Glucose. According to our previous method (Miyata et al., 1993), 2 mg/mL of the purified normal β_2M was incubated at 37 °C for 42 days with 0.1 M D-glucose (Wako Pure Chemicals, Osaka, Japan) in 100 mM phosphate buffer (pH 7.4) containing 200 units/mL penicillin, 80 μg/mL gentamicin, and 1.5 mM phenylmethanesulfonyl fluoride (PMSF). When the extent of modification was estimated by two-dimensional polyacrylamide gel electrophoresis and fluorospectrometry, a major portion of *in vitro* glycated β_2M shifted its electrophoretic mobility to the position similar to that of acidic β_2M, and it showed a similar fluorescence spectrum with that of acidic β_2M (Miyata et al., 1993).

Borotritide Reduction and Digestion with Endoproteinase Lys-C (Endo Lys-C) of β_2M. Hexoses attached to ϵ-amino groups of lysyl residues of β_2M were converted into 1-deoxyhexitolyl groups by reduction with sodium borotritide, stabilizing carbohydrate–protein linkages and conferring a radioactive label on the site of glycation. A solution of sodium borotritide (340 μCi/μmol; 5 μmol) in 10 mM NaOH was added to a 1 mL solution of β_2M (50 μg) in 0.1 M phosphate buffer (pH 8.5). The resulting solution was allowed to stand at room temperature for 4 h. The solution was dialyzed against 50 mM acetic acid to destroy excess sodium borotritide and was then dialyzed against 50 mM Tris-HCl buffer (pH 9.0). In some experiments, the same experimental procedure was performed with β_2M (250 μg) using the same concentration of sodium borohydride (Nakalai Tesque, Kyoto, Japan) instead of radioactive sodium borotritide.

Borotritide-treated β_2M was reacted with 5 μg of Endo Lys-C (Acromobacter Protease I, Wako Pure Chemicals) at 37 °C for 8 h. The digested peptides were separated by high-performance liquid chromatography (HPLC) (Model LC-10A; Shimadzu, Kyoto, Japan) on a C18 reverse-phase column (4.6 × 250 mm; Waters, Tokyo, Japan). The column was operated at a flow rate of 0.5 mL/min. A gradient system formed between buffer A (0.1% trifluoroacetic acid in distilled water) and buffer B (0.08% trifluoroacetic acid in 80% acetonitrile) was used. For the first 10 min, the column was washed isocratically with 5% buffer B, followed by linear gradient elution up to 60% of buffer B for 75 min, and finally up to 100% of buffer B for 10 min. The radioactive digested peptides were first applied to the above HPLC column, the radioactivity was measured with a liquid scintillation counter, and then the nonradioactive peptides were applied to the same column. The peptides were detected by their absorbance at 214 nm. The fractions of nonradioactive peptides, which corresponded to the radioactive peptides, were collected and then subjected to peptide sequencing and mass spectrometry.

Peptide Sequencing. Amino acid sequence analysis of the isolated nonradioactive peptides was determined using an automatic protein sequencer (Model 473A gas-phase sequencer; Applied Biosystems, Foster City, CA).

Mass Spectrometry (MS). MS was carried out using a JMS SX102A double-focusing mass spectrometer with the DA7000 data system (JOEL, Akishima, Japan). Fast atom bombardment (FAB) ionization mode was employed for the analysis of peptides. Purified peptides were ionized by bombardment with a xenon beam of 6 keV, and the secondary ions were accelerated by 10 kV. Glycerol was used as the sample matrix. For the analysis of undigested β_2M, the electrospray ionization (ESI) mode was used (Fenn et al., 1989; Loo et al., 1989; Smith et al., 1990; Wada et al., 1992). The samples dissolved in a solution of 49/49/2 methanol/H_2O/acetic acid (v/v/v) at a concentration of 10 μM were directly infused into the electrospray ion source of the mass spectrometer at a flow rate of 0.7 μL/min by a syringe pump (Harvard Apparatus, MA). The accelerating voltage was 7 kV. Molecular weight was expressed by monoisotopic for peptides and by chemical mass for undigested proteins, respectively.

RESULTS

Mapping of Endo Lys-C Peptides from Normal β_2M and Acidic β_2M. Human β_2M contains eight lysine residues and has an intramolecular disulfide bridge between Cys-25 and Cys-80. Therefore, digestion of β_2M with Endo Lys-C yields eight peptides including a covalently linked one from sequences 20–41 and 76–91 (Figure 1A). Peptides mapping of the nonradioactive normal β_2M on reverse-phase HPLC revealed nine major peaks (Figure 1B). After amino acid sequence analysis and FAB-MS, these nine peaks were identified as shown in Table 1. Peak IV was an oxidized molecule of peak VI and had a methionine sulfoxide residue instead of the carboxyl terminal methionine residue. When normal β_2M in 10 mM sodium phosphate buffer (pH 7.2) was left at room temperature in the presence of atmospheric oxygen, peak IV increased with time compared to peak VI, indicating that the oxidation at the carboxyl terminal methionine was derived from the experimental artifact during the purification procedure.

Peptide mapping of the nonradioactive and radioactive acidic β_2M on HPLC is shown in Figure 2A,B, respectively. The incorporation of radioactivity was observed as one major and several minor peaks in acidic β_2M (closed circle in Figure 2B), whereas no incorporation was observed in normal β_2M (open circle), supporting the notion that acidic β_2M but not normal β_2M is modified with the Maillard reaction. The major radioactive peak corresponded to fraction A in Figure 2A. Amino acid sequence analysis revealed that the fraction A

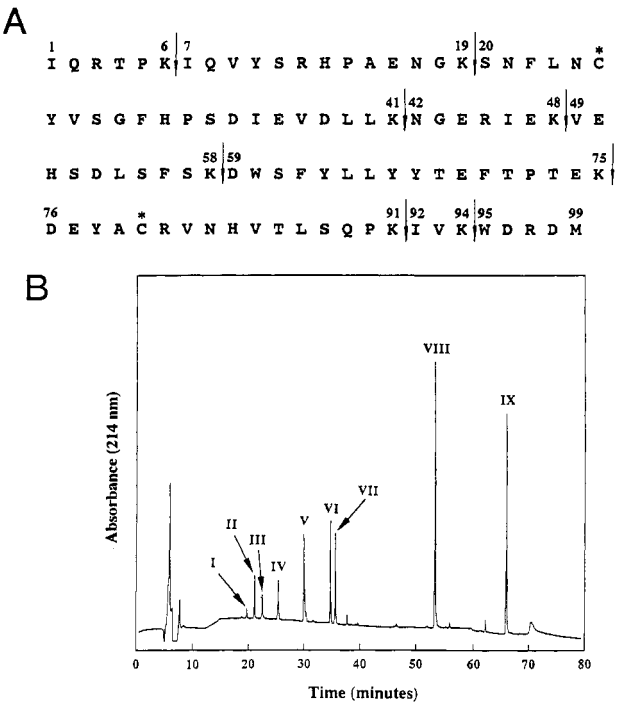

FIGURE 1: Mapping of Endo Lys-C peptides from normal β_2M. (A) Amino acid sequence (Güssow et al., 1987) and sites of Endo Lys-C cleavage of human β_2M. Vertical arrows indicate the cleavage sites. Two cysteine residues forming a disulfide linkage are indicated by asterisks over the sequence. (B) Elution profile of digested nonradioactive peptides derived from normal β_2M on reverse-phase HPLC. For the details, see Materials and Methods. The eluent was monitored for peptide by absorbance at 214 nm. Peptide fractions I–IX were collected and subjected to amino acid sequence analysis and FAB-MS as described under Materials and Methods.

Table 1: Assignment of Endo Lys-C Peptides of Normal β_2M and Acidic β_2M

peak[a]	amino acid sequence	peptide assignment[b]
I/I′	IVK	92–94
II/II′	IQRTPK	1–6
III/III′	N[c]GERIEK	42–48
IV/IV′	WDRDRM[d]	95–99
V/V′	IQVYSRHPAEN[c]GK	7–19
VI/VI′	WDRDM	95–99
VII/VII′	VEHSDLSFSK	49–58
VIII/VIII′	SNFLNC[e]YVSGFHPSDIEVDLLK	20–41
	DEYAC[e]RVNHVTLSQPK	76–91
IX/IX′	DWSFYLLYYTEFTPTEK	59–75
A	( )[f]QRTPK	1–6

[a] Numbers and letters of the alphabet assigned to each peptide (I–IX, I′–IX′, or A) are those of the corresponding fractions on HPLC in Figures 1B and 2A. [b] Number of amino acid residue. [c] Deamidation was observed in some fractions of this asparagine (Y. Wada *et al.*, unpublished observations). [d] Methionine sulfoxide. [e] Cys-25 and Cys-80 are linked with a disulfide bond. [f] A "blank" result shown in parentheses indicates no phenylthiohydantoin derivative was detected.

peptide corresponded to peptide 1–6 (Ile-Gln-Arg-Thr-Pro-Lys), except for the absence of a signal for isoleucine at the first Edman cycle (Table 1). On the other hand, isoleucine was detected as the first residue for fractions II and II′ peptides, corresponding to the authentic peptide 1–6 from normal β_2M and acidic β_2M, respectively. These findings indicated that the primary glycated site of β_2M in long-term hemodialysis patients was the α-amino group of the amino terminal isoleucine. Because of the limited amount of the purified acidic β_2M, we could not collect the nonradioactive peptides corresponding to the minor radioactive peaks.

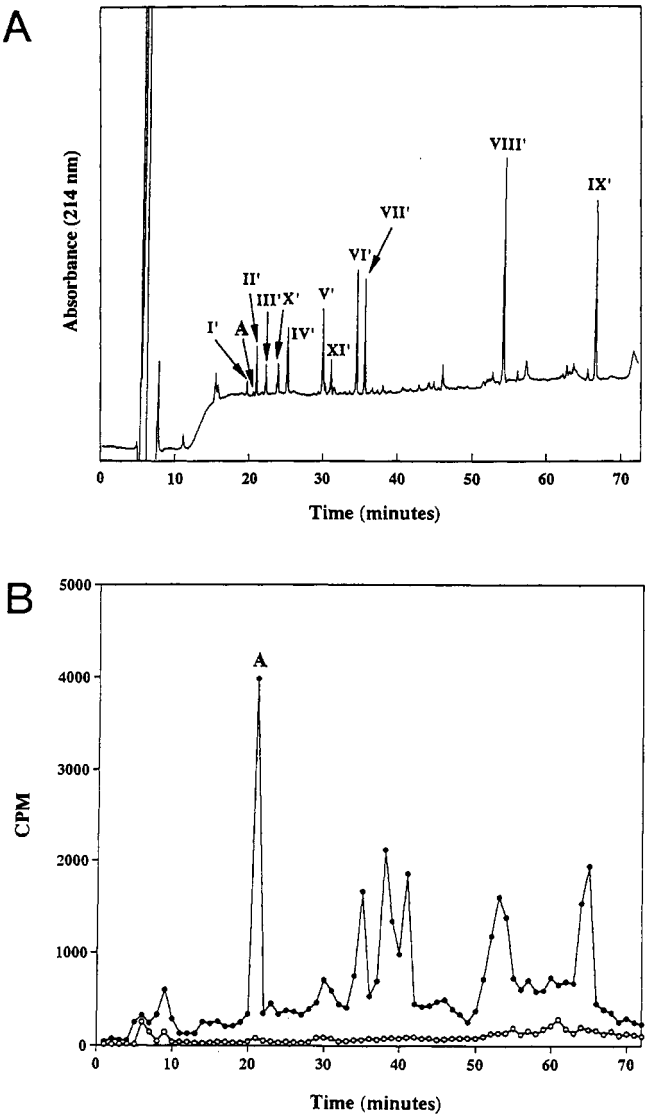

FIGURE 2: Mapping of Endo Lys-C peptides from acidic β_2M. (A) Elution profile of digested nonradioactive peptides derived from acidic β_2M on reverse-phase HPLC. The eluent was monitored for peptide by absorbance at 214 nm. Peptide fractions I′–IX′ and the fraction A, which corresponded to the prominent radioactive peak indicated as A in panel B, were collected and subjected to amino acid sequence analysis and FAB-MS. Fractions X′ and XI′ contained no peptide by the chemical analyses (amino acid sequence analysis, amino acid composition analysis, and FAB-MS). (B) Elution profiles of digested radioactive peptides derived from normal β_2M (open circle) and acidic β_2M (closed circle) on reverse-phase HPLC. For the details, see Materials and Methods. The eluent was monitored for radioactivity by liquid scintillation counting. The most prominent radioactive peak is indicated as A.

Mapping of Endo Lys-C Peptides from Normal β_2M Incubated with Glucose in Vitro. Since glucose is thought to represent a major reducing sugar of the Maillard reaction *in vivo*, normal β_2M was incubated with glucose *in vitro*. Then, we examined a potential glycated site(s) of β_2M after incubation with glucose *in vitro*. Peptide mapping of the nonradioactive and radioactive *in vitro* glycated β_2M on HPLC is shown in Figure 3A (exemplified with normal β_2M after a 35-day incubation) and B, respectively. The intensities of radioactivity increased with time of incubation. The HPLC profile of incorporation of radioactivity (Figure 3B), one major and several minor peaks, was almost identical with that observed in acidic β_2M (Figure 2B). The radioactive peaks detected in *in vitro* glycated β_2M (A′–G in Figure 3B) could also be detected with the same retention times in acidic β_2M.

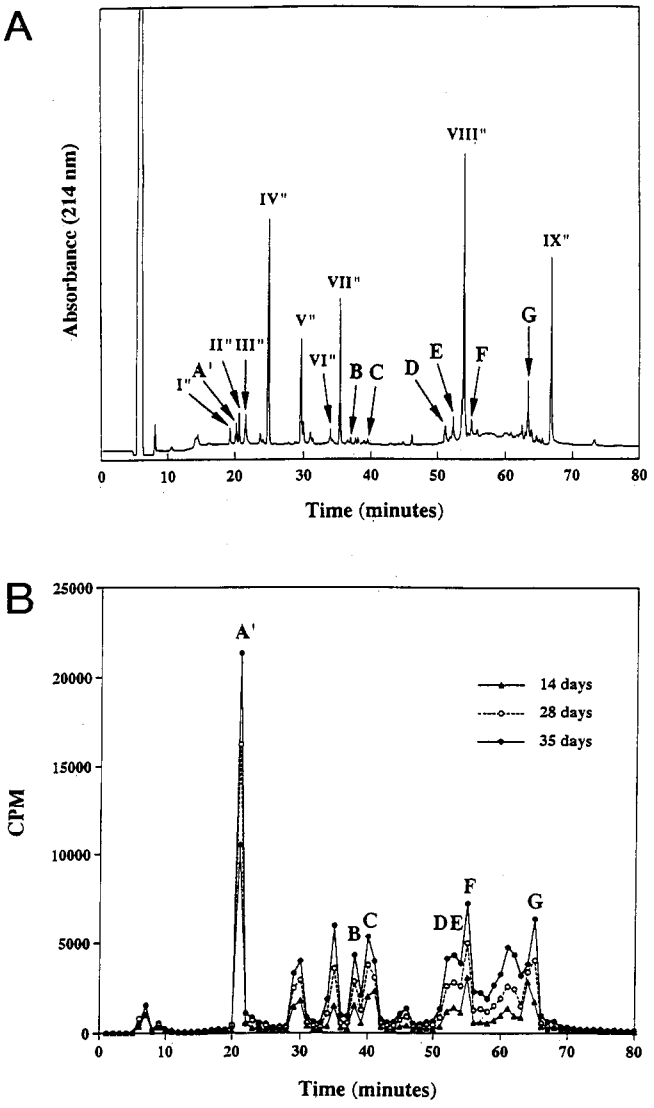

FIGURE 3: Mapping of Endo Lys-C peptides from glycated β_2M *in vitro*. (A) Elution profile of digested nonradioactive peptides derived from glycated β_2M *in vitro* after a 35-day incubation with glucose on reverse-phase HPLC. The eluent was monitored for peptide by absorbance at 214 nm. The fractions indicated by I"–IX" and A'–G, which corresponded to the prominent radioactive peaks in panel B, were collected and subjected to amino acid sequence analysis and FAB-MS. (B) Elution profiles on reverse-phase HPLC of digested radioactive peptides derived from glycated β_2M *in vitro* after a 14-day (closed triangle), a 28-day (open circle), and a 35-day (closed circle) incubation with glucose. The eluent was monitored for radioactivity by liquid scintillation counting. Several radioactive peaks are indicated as A'–G.

This indicates that the glycated sites occurred in acidic β_2M formed *in vivo* and in glycated β_2M *in vitro* are the same.

The results of amino acid sequence analysis and FAB-MS of peptides from *in vitro* glycated β_2M are summarized in Table 2. Amino acid sequence analysis revealed that the fraction A' peptide corresponded to peptide 1–6 except for the absence of a signal for isoleucine at the first Edman cycle, indicating that the major glycated site of β_2M *in vitro* was also the α-amino group of Ile-1. Figure 4 (lower panel) shows the FAB mass spectrum of the fraction A' peptide. The molecular mass for this peptide was found to be 905.5 Da, whereas that for peptide 1–6 in normal β_2M was 741.5 Da (Figure 4, upper panel). The latter molecular mass corresponded to the theoretical molecular mass (741.450 Da). It was thus indicated that the fraction A' peptide from *in vitro* glycated β_2M contained an additional molecule which had

Table 2: Assignment of Endo Lys-C Peptides of Glycated β_2M in Vitro[a]

peak[b]	amino acid sequence	peptide assignment[c]
A'	( )[d]QRTPK	1–6
B	NGERIE( )VEHSDLSF- -	42–56
C	IV( )WDRDM	92–99
D	IQVYSRHPAENG( )SNFLNC[e]YVSG- -	7–29
	DEYAC[e]RVNHVTLSQPK	76–91
E	SNFLNC[e]YVSGFHPSDIEVDLL( )NGERIEK	20–48
	DEYAC[e]RVNHVTLSQPK	76–91
F	SNFLNC[e]YVSGFHPSDIEVDLLK	20–41
	DEYAC[e]RVNHVTLSQP( )IVK	76–94
G	VEHSDLSFS( )DWSFYLLYY- -	49–67

[a] The results of fractions I"–IX" were completely identical with those of fractions I–IX in Table 1, respectively. [b] Letters of the alphabet assigned to each peptide are those of the corresponding fraction on HPLC in Figure 3A. [c] Number of amino acid residue. [d] A "blank" result shown in parentheses indicates no phenylthiohydantoin derivative was detected. [e] Cys-25 and Cys-80 are linked with a disulfide bond.

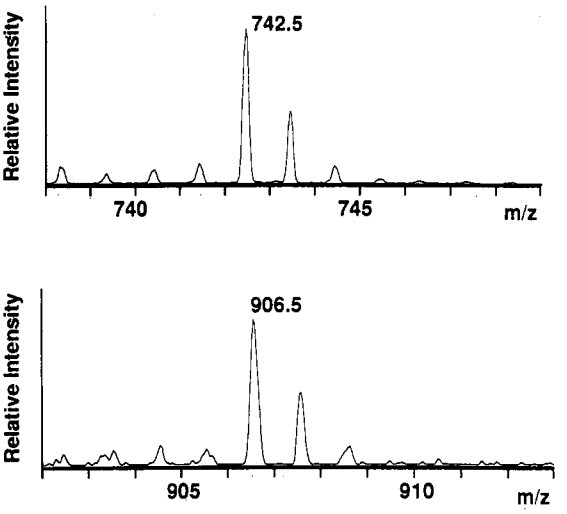

FIGURE 4: FAB mass spectra of Endo Lys-C peptides. The protonated molecular ions for the peptides of fraction II in Figure 1B and fraction A' in Figure 3A were detected at m/z 742.5 (upper panel) and at m/z 906.5 (lower panel), respectively.

the molecular mass of 164.0 Da. Since the increase in the molecular mass by a reduced Amadori product of glucose is calculated to be 164.068 Da, the adduction was attributed to one reduced Amadori product.

Six minor glycated sites of β_2M were identified at the ϵ-amino groups of Lys-19, Lys-41, Lys-48, Lys-58, Lys-91, and Lys-94 (Table 2). It is not definite whether or not two other ϵ-amino groups of lysine (Lys-6 and Lys-75) are glycated. However, even if glycation occurs in these sites, it is unlikely that these are major glycated sites of β_2M.

Time Course of Glycation of β_2M in Vitro. Glycation of β_2M with glucose *in vitro* was also examined by ESI-MS. ESI mass spectrum of normal β_2M showed multiply charged ions for a single major component with a molecular mass of 11 729 Da (peak X in Figure 5A). The calculated mass was identical with the theoretical value (11 729.175 Da). A minor component (indicated by the arrow in the same figure), which was about 16 Da larger than the normal one in the molecular mass, was an oxidized β_2M at the carboxyl terminal methionine residue by FAB-MS of the peptide.

When *in vitro* glycated β_2M was analyzed by ESI-MS, several additional components were observed (exemplified by normal β_2M after a 14-day incubation in Figure 5B and after a 42-day incubation in Figure 5C). The smallest component

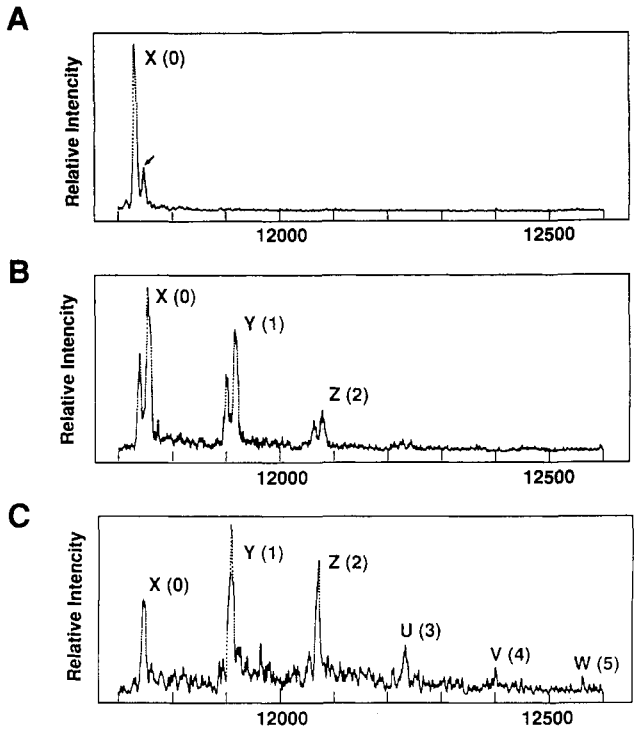

FIGURE 5: Transformed ESI mass spectra of undigested glycated β_2M *in vitro*. Normal β_2M (A); glycated β_2M *in vitro* with glucose for 14 days (B); glycated β_2M *in vitro* with glucose for 42 days (C). The arrow indicates the oxidized β_2M at the carboxyl terminal methionine residue. The number of Amadori products contained are shown in parentheses.

(peak X) was normal β_2M. The molecular mass for the larger components was calculated to be 11 891 Da (peak Y), 12 053 Da (peak Z), 12 215 Da (peak U), 12 377 Da (peak V), and 12 539 Da (peak W), respectively. The difference in the molecular mass from one peak to the next was exactly 162 Da, indicating that each of these materials contained one or more Amadori products. The peak of normal β_2M gradually decreased with time of incubation, whereas the peaks of normal β_2M modified with one or more Amadori products increased. These results indicate that glycation does occur at several sites in β_2M.

DISCUSSION

Hemodialysis-associated amyloidosis (HAA) is a serious complication of long-term hemodialysis that leads to joint and bone destruction (Drüeke, 1991). Recently, we demonstrated that β_2M modified with the early products (the Amadori products) or the advanced glycation end products (AGEs) of the Maillard reaction is a definite constituent of amyloid deposits in patients with HAA and referred to it as acidic β_2M because this isoform migrated much more acidic than normal β_2M on two-dimensional polyacrylamide gel electrophoresis (Miyata et al., 1993). Our further study demonstrated that acidic β_2M in amyloid deposits may function as a pathogenic factor that recruits monocyte/macrophage *in situ* and initiates the inflammatory response (Miyata et al., 1994; Iida et al., 1994). These findings suggest the potential link of the modification of β_2M with the Maillard reaction to the pathogenesis of HAA.

In the present study, we identified the glycated sites of acidic β_2M purified from long-term hemodialysis patients. Sites of glycation were identified by amino acid sequence analysis of peptides generated by digestion of borotritide-treated β_2M with Endo Lys-C. This method was similar to those used previously to identify modified lysine residues, in that the residue involved was identified by obtaining a blank result in the appropriate step of gas-phase sequencing (Rao et al., 1988; Morjana et al., 1989; Shilton & Walton, 1991). In addition, we analyzed the molecular mass of peptides by FAB-MS. The results indicated that the primary glycated site of β_2M in long-term hemodialysis patients was the α-amino group of the amino terminal isoleucine. From the finding that the six radioactive peaks detected in *in vitro* glycated β_2M on HPLC could also be detected with the same retention times in acidic β_2M, it was strongly suggested that the ϵ-amino groups of Lys-19, Lys-41, Lys-48, Lys-58, Lys-91, and Lys-94 were also minor glycated sites of β_2M in these patients. Whether or not two other sites (Lys-6 and Lys-75) are virtually glycated is uncertain, but even so, these are minor glycated sites.

Table 3: Solvent Accessibility of Residues Possible for Glycation and Interatomic Distances within Vicinity of Glycated Sites[a]

residue	accessibility (%)[b]	nearest histidine residue[c]	distance (Å)[d]
**Ile-1	85.4	His-31	6.7
Lys-6	63.8	His-84	12.3
*Lys-19	52.8	His-13	11.8
*Lys-41	34.2	His-13	19.6
*Lys-48	96.9	His-13	17.5
*Lys-58	90.6	His-31	18.0
Lys-75	72.7	His-13	21.4
*Lys-91	47.4	His-84	11.4
*Lys-94	51.2	His-84	23.4

[a] Data are taken from Protein Data Bank file of human β_2M (ID code:3HLA). [b] Solvent accessibility was calculated by the method of Go and Miyazawa (1980). Accessible surface area of a residue is obtained by summing the accessible surface area of its component atoms. Solvent accessibility of a residue is defined by the accessible surface area of the residue in native conformation divided by that in an extended state. [c] Number of the nearest histidine residue. [d] Distance is shown in angstroms from CE1 of a histidine to the amino terminal nitrogen atom of an isoleucine residue or the ϵ-nitrogen of a lysine residue. Although acid–base catalysis by an imidazole group depends upon its nitrogen atoms, distances from CE1 are shown here to obviate the need to specify π or τ nitrogen in each case. Major and minor glycated sites are indicated by ** and *, respectively.

The glycated sites of proteins have been identified in a small number of cases, such as hemoglobin A (Shapiro et al., 1980; Acharya et al., 1983), serum albumin (Day et al., 1979; Garlick & Mazer, 1983; Iberg & Flcükiger, 1986), and alcohol dehydrogenase (Tsai & White, 1983; Shilton & Walton, 1991) both *in vivo* and *in vitro*; osteocalcin (Gundberg et al., 1986) *in vivo*; and Cu,Zn–superoxide dismutase (Arai et al., 1987) and ribonuclease A (Watkins et al., 1985) *in vitro*. It was found that glycation of hemoglobin (Shapiro et al., 1980) and alcohol dehydrogenase (Tsai & White, 1983; Shilton & Walton, 1991) affects different sites *in vivo* and *in vitro*, whereas it occurs at almost the same sites in β_2M modified *in vivo* and *in vitro*.

A computer graphics analysis of the X-ray crystallographic structure of β_2M yields some potential explanations for the fact that the primary glycated site is the α-amino group of Ile-1. First, Ile-1 protrudes markedly from the surface of the moiety, facilitating glucose to access this residue in the aqueous phase. The solvent accessibility (Go & Miyazawa, 1980) of this residue is calculated as 85.4% (Table 3), which seems quite high for residues possible for glycation. A second and more plausible explanation may be that the α-amino group of Ile-1 is uniquely positioned close to an imidazole of a histidine residue. The distance between the ϵ-nitrogen atom of Ile-1 and the nearest nitrogen of the imidazole of His-31 is 6–7 Å

(Table 3). It is generally considered that the Amadori rearrangement is an acid–base catalysis reaction. Several lines of evidence demonstrated that the interatomic distance between a histidine imidazole group and an amino group may play an important role in the glycation reaction, because an imidazole group, which has the capacity to act as an acid–base catalyst, accelerates glycation of a nearby amino group by catalyzing the subsequent Amadori rearrangement (Acharya et al., 1983; Watkins et al., 1985; Shilton & Walton, 1991). There are several cases in which a histidine imidazole group is close to an amino group that is preferentially glycated, such as hemoglobin (Acharya et al., 1983; Shilton & Walton, 1991), alcohol dehydrogenase (Shilton & Walton, 1991), and Cu,Zn–superoxide dismutase (Ookawara et al., 1992). Based on these observations, neither Lys-48 nor Lys-58 could be major glycated sites despite their high solvent accessibility (96.9% and 90.6%, respectively), because the ϵ-nitrogen atom of Lys-48 or Lys-58 is very far (~20 Å) from the nearest nitrogen of the imidazole of His-13 or His-31, respectively.

We previously demonstrated that acidic β_2M contained both the Amadori products and AGEs of the Maillard reaction since it was recognized by both anti-Amadori product antibody and anti-AGE antibody (Miyata et al., 1993). The anti-AGE antibody we used does not cross-react with the early products of the Maillard reaction such as Schiff base and the Amadori product but specifically reacts with AGEs (Horiuchi et al., 1991), indicating that the AGEs moiety is structurally distinct from the Amadori product. In the present study, we identified the sites of the Amadori products in β_2M. Although the Amadori product is the intermediate product leading to AGEs, it is not conclusive at present whether or not the sites of AGEs are identical with those of the Amadori products. Further study will be necessary to clarify this issue.

We estimated the extent of glycation in acidic β_2M and *in vitro* glycated β_2M from the results of peptide mapping on HPLC (Figures 2A and 3A). On the basis of the area ratio of peak II′ to peak A in Figure 2A, there appears to only 5–10% glycation at the amino terminal isoleucine of the purified acidic β_2M. On the other hand, the area ratio of peak II″ to peak A′ in Figure 3A suggests ~45% glycation at this site of *in vitro* glycated β_2M. As the specific radioactivities of sodium borotritide were almost the same between these experiments, these findings indicate that there is clearly much less radiolabeled peptide in acidic β_2M compared to *in vitro* glycated β_2M. If we take the involvement of the other minor glycated sites of *in vitro* glycated β_2M into account, the reason for the acidic behavior of *in vitro* glycated β_2M on the electrophoretic mobility could be explained mainly by glycation. In contrast, even if we take the other minor glycated sites into account, the extent of glycation is at most ~20% in acidic β_2M, suggesting the presence of the other modification in acidic β_2M that has an effect on the electrophoretic or chromatographic mobility. Recently, we found that our acidic β_2M preparations contained β_2M with deamidation at Asn-17 and/or Asn-42. The chemical analysis estimated that the deamidated β_2M were 60–70% in acidic β_2M preparations (our unpublished observation). Since the deamidation of protein is a potent modification causing the electrophoretic shift of protein to an acidic position, the reason for the acidic behavior of acidic β_2M could be explained by not only glycation but also deamidation. However, because β_2M with deamidation does not show the biological activities reported with purified acidic β_2M as well as *in vitro* glycated β_2M (Miyata et al., 1994; Iida et al., 1994), we suggest that the modification of the Maillard reaction but not deamidation might be pathologically significant modification of β_2M in HAA (Miyata et al., manuscript in preparation).

Determination of the structure of β_2M modified with the Maillard reaction will help us to understand the mechanism for amyloid fibril formation of β_2M modified with the Maillard reaction and further understand the functional link of this modified β_2M to the pathogenesis of HAA. In addition, determination of the glycated site may help us to raise a glycated site-specific antibody against β_2M modified with the Maillard reaction, which should be useful not only for immunochemical and immunohistochemical examination but also for radionuclide imaging by administrating radiolabeled antibody intravenously into patients on long-term hemodialysis in order to detect small-sized amyloid deposits for the diagnosis of HAA in the early stage, when a plain X-ray radiography cannot detect the amyloid deposit yet.

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