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Review

Detection and identification of protein variants and adducts in blood and tissues: an application of soft ionization mass spectrometry to clinical diagnosis

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Abstract

The detection and identification of protein variants and abnormally increased modified proteins are important for clinical diagnosis. We applied soft ionization mass spectrometry (MS) to analyze proteins in blood and tissues from various patients. Over the past 8 years, we diagnosed 132 cases (55 kinds) of variant proteins including hemoglobin (Hb), transthyretin (TTR), and Cu/Zn-superoxide dismutase (SOD-1), using MS as the leading technology. Of these variants, eight were new, and nine were the first cases in Japan. Some abnormal Hb cause diseases, and most of them cause erroneous levels of glycated Hb, HbA1c, i.e., a popular index of diabetes. Most of the variant TTR causes amyloidotic polyneuropathy. Variant SOD-1 causes amyotrophic lateral sclerosis. We first showed that immunoprecipitation by a specific antiserum is a reliable and simple method to prepare protein from sera and tissues for analysis by matrix-assisted laser desorption time-of-flight MS, and liquid chromatography–electrospray ionization MS (LC–ESI-MS). The use of this technology has become widespread. Using an immunoprecipitated target protein and LC–ESI-MS, we showed that the ratios of tetra-, di- and a-sialo-transferrin from two cases of congenital glycoprotein deficient syndrome were clearly distinguishable from those of control samples. We first reported a unique modified form of TTR, that is, S-sulfonated TTR, which increased markedly and specifically in three cases with molibdenum cofactor deficiency. We proposed that S-sulfonated TTR is a useful marker for screening this disease. ESI-MS was successfully used for the accurate determination of HbA1c, and we clarified the extent of discrepancies between the HbA1c value measured by conventional methods and the accurate values for samples containing various Hb variants determined by the MS method.

Keywords: Reviews; Clinical diagnosis; Protein variants; Protein adducts

Contents

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1. Introduction

Since Pauling et al. established that sickle cell anemia is caused by a single amino acid substitution of hemoglobin (Hb) in 1949, more than 840 abnormal Hbs have been identified [1]. New variants have been reported still in recent years. About 20% of them show pathological symptoms [1]. Samples containing abnormal Hb may cause erroneous results in measurement of glycated Hb (HbA1c), which serves as an index in the evaluation and management of patients with diabetes. The error is popularly observed for high-performance liquid chromatography (HPLC) ion-exchange/electrophoresis methods, and rarely observed for immunological methods. No erroneous result has been reported for the methods based on the boric acid affinity principle, which has not been commonly used recently. In addition, glycation rate may be different among normal and particular cases of abnormal Hb. Worldwide, an estimated 150 million people carry Hb variants [2]. Therefore, the detection and characterization of the Hb variants are indispensable as a clinical laboratory test.

The variant Hbs have been studied most extensively among variant proteins, and have been a model for studies of various protein abnormalities caused by inborn errors. Amino acid substitution of transthyretin (TTR) was first identified in plasma from a patient with inherited systemic amyloidosis [3], and in an extract from an amyloid deposit in a kidney with the disease [4]. Over 80 variants of TTR have been reported, and most of them cause amyloidosis [5]. A linkage study revealed that mutations in the Cu/Zn-binding superoxide dismutase (SOD-1) gene are associated with about 10% of familial amyotrophic lateral sclerosis cases [6]. Over 90 different mutations of the SOD-1 gene have been reported [7].

Post-translational modifications of abnormal Hb have been reported, e.g., acetylation at an NH_2-terminal substituted amino acid residue [1], oxidation of substituted methionine (β67) to aspartic acid [1], oxidation of leucine (β141) to hydroxyleucine in the Hb Atlanta-Coventry (β75Leu→ Pro) [1], and deamidation of a substituted aspartyl residue [1]. Many more new variants and new types of modified protein will be discovered, and the new findings will give important information for protein chemistry and clinical medicine. Molecular biology, e.g., polymerase chain reaction and DNA sequencing techniques, have revolutionized the identification of variant proteins. In addition, more recently, to analyze variants and abnormally modified proteins, modern mass spectrometry (MS) has played an expanding role in their identification. MS technology is indispensable to elucidate the modified protein structures, which cannot be substituted by DNA technology.

It was Wada et al. in Osaka, Japan, who first applied MS to study variant Hbs, in 1980 [8]. They used field desorption MS, and showed excellent peptide maps of normal and abnormal Hbs. In the nearly 20 years since then, matrix-assisted laser desorption time-of-flight (MALDI-TOF) MS [9], and electrospray ionization (ESI) MS [10], have become popular soft ionization techniques. Analyses of intact protein with most variants of Hb, TTR, and SOD-1 by ESI-MS showed clearly separated doublet ion peaks of normal and abnormal intact components. Furthermore, tandem MS helped to determine amino acid substitutions of these variants using enzyme-digested protein. We applied these methods, and over the last 8 years, diagnosed 75 cases of 38 types of abnormal Hbs using ESI-MS [11–20]. We analyzed also variants of 52 cases, 12 abnormal TTR types [21,22], and confirmed the structure of five cases, and five SOD-1 types using MS [23]. Both cause neurodegenerative diseases. Some of these were new variants.

In contrast to Hb, which is contained in red blood cells in high concentrations, most proteins in plasma

and cells must be subjected to a series of procedures in preparation for the application of MS. We proposed that immunoprecipitated protein could be analyzed by MALDI-TOF-MS, and by LC–ESI-MS [25], in 1994, not long after the presentation of these excellent soft ionization methods [9,10]. Test serum was mixed with specific antiserum, and the generated precipitate was washed with saline and deionized water successively [24–26]. The washed precipitate was then dissolved in a solvent for MS analysis. The antibody and the protein to be tested were separated under an acidic loading solvent, and showed separate signals by MALDI-TOF-MS and HPLC–ESI-MS. Using this procedure, the isolation, concentration and desalting of the protein to be tested could be achieved simultaneously. This procedure has been improved and used widely [27–32]. We applied this technique, and showed the ratio of tetra-, di- and a-sialo-transferrin (Trf) from two cases of congenital glycoprotein deficient syndrome, a unique modified form of TTR, i.e., S-sulfonated TTR [33], and various variants of TTR and SOD-1.

ESI-MS was used to determine the content of the modified protein in mixtures. The values of HbA1c were successfully measured by Kobold et al. [34] and by us [20,35,36]. The ratio of glycated and non-glycated hexapeptides released by enzyme (Glu-C) digestion was measured using ESI-MS. This can be a reference method to standardize the conventional method for HbA1c measurement. We clarified the extent of discrepancies between the values measured by a HPLC ion-exchange method, an immunological method, and an ESI-MS method for samples containing various Hb variants determined using the MS method [20].

In the present review, we describe the procedures and new findings regarding the identification and characterization of variants and modified proteins, mainly by ESI-MS.

2. Experimental

The preparation methods of Hb, and globin were described previously [11–19]. Proteins other than Hb were prepared by immunoprecipitation as described previously [24–26]. MS analysis was performed with a TSQ-7000 triple-stage quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a conventional electrospray ion source, and with an LCQDeca (ThermoQuest) equipped with Magic C18. The details for the procedure were written in original papers, and are briefly described in the following sections.

3. Results

3.1. Hemoglobin variants

Direct examination of hemolysate by ESI-MS may well lead to the rapid ascertainment of a variant Hb, provided that the mass difference between the normal and abnormal chain is larger than the resolution power of standard instruments (i.e., =10 Da). Fig. 1 shows the transformed ESI mass spectra derived from a normal control and a heterozygote with Hb M Boston (α58His→Tyr). Peaks representing the normal β-subunit are seen at masses 15 868.6±0.7 u

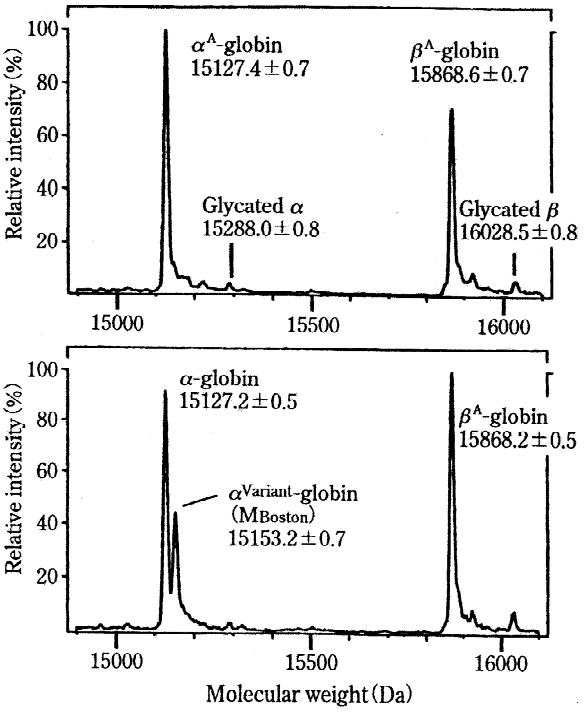

Fig. 1. The transformed ESI mass spectra of hemoglobin derived from a normal control (upper panel) and a heterozygote with Hb M Boston (α58His→Tyr) (bottom panel).

(normal control, upper panel) and 15 868.2±0.5 u (patient, bottom), which correspond to the molecular mass of a normal β-subunit. The ions of an α-subunit from the patient separated into two components: one was 15 127.2±0.5 u and the other was 15 153.2±0.7 u. The former, showing almost the same value as the control, 15 127.4±0.7 u, corresponded to the molecular mass of the normal α-subunit. The latter was 26.0 u larger than the value of the normal β-chain, which corresponds to the difference in the mass between His and Tyr. The structure of this abnormal Hb had been determined using methods of conventional protein chemistry [37]. The ratio of ion peak height of normal and abnormal α-subunits was approximately 3:1, which was equivalent to the value obtained by ion-exchange column chromatography [37]. This was the first experiment in our laboratory using ESI-MS in 1994, and we were convinced that ESI-MS could be used to detect abnormal Hb and quantify the content of the components. Since then, we have analyzed various samples for diagnosis of hemolytic anemia and elucidation of unexpected HbA1c values.

Table 1 shows a list of abnormal Hb detected and identified in our laboratory using mainly MS. We diagnosed 75 cases, 38 types of abnormal Hbs, and most were subjected to structural study because of an unexpectedly low or high HbA1c value by routine measurement with HPLC.

Hb Sagami (β139Asn→Thr) was not detected by conventional methods, since it was clinically and electrophoretically silent [17,19]; it was first detected by ESI-MS. Hb Hokusetu (β52Asp→Gly) was also a new variant [18]. The transformed mass spectra of globin from the two new cases are shown in Fig. 2. These spectra show doublet ion peaks corresponding to the normal and abnormal β-subunits, which show the difference in the molecular mass, (β-subunit of Hb Hokusetsu, −58 Da, and that of Hb Sagami, −13 Da), and the ratio of content between the normal and abnormal subunits. The peak height of the abnormal β-subunit was comparable to that of the normal β-subunit in the case of Hb Hokusetu. However, the spectrum with Hb Sagami showed that the peak height of the abnormal β-subunit was about twice as high as that of the normal β-subunit. This suggested coexistence of thalassemia. We clarified that the case was a compound heterozygosity for β^+thalassemia [−31(A→G)], and a new variant Hb Sagami with low oxygen affinity [19]. Hb Niigata (β1Val→Leu) is a new variant found in Japan [15], and MS technology contributed to determine the acetylation of substituted Met at N-terminal of its β-subunit. We revealed three different amino acids on β67 in one case, Val, Met, and Asp in a patient formally reported as Hb Bristol (β67Val→Asp). There was post-translational change of methionine to aspartyl residue. The β67 residue is in a hydrophobic region and is one of the heme contact points, so an oxy–heme complex may be involved in the oxidation conversion [13].

Here, we show an example of sequence analysis by LC–ESI-MS using enzyme digests. Fig. 3 shows reconstructed selected ion chromatograms and a base peak ion chromatogram made by HPLC–ESI-MS to determine the amino acid substitution of Hb Sagami [17,19]. From reconstructed selected ion chromatograms, the ions corresponding to all normal expected peptides from the β-subunit and the ions ~13 Da less than all normal counterparts were plotted. An abnormal peptide was found in the analysis of the digests, 13 Da smaller than β-T14, and was designated β*-T14. In Fig. 3, chromatograms for a doubly charged ion of an abnormal peptide, β*-T14, 568.5–569.5, and a doubly charged ion of a normal peptide, β-T14, 575.5–576.5, are shown. Fig. 4 shows collision induced dissociation (CID) mass spectra of normal and abnormal β*-T14. Doubly charged ions of the normal β-T14, m/z 575.5 and the abnormal β*-T14, m/z 569.0 were used for CID spectrometry analysis. The CID spectra of normal and abnormal peptide included the common y series ions (from y2 to y5), which coincided with the y ions of the sequence 140–144 of the β-chain, that is, Ala–Leu–Ala–His. The ions y6 to y11 in the CID spectrum of the normal β-T14 (Fig. 4a) were not observed in the spectrum of the abnormal β*-T14 (Fig. 4b). Instead, a series of y ions, y6 to y11, was observed in the abnormal β*-T14, and the mass difference of ions y6 to y11 between normal β-T14 and abnormal β*-T14 was 13 Da. These results suggested that the asparagine at the position 139 of the normal β-chain was substituted by threonine. The sequence was confirmed by a protein sequencer using peptides

Table 1
Hemoglobin variants analyzed in our group (1994–2001)

Hb name	Substitution and mass difference	Case No.	Ref.
α-Subunit			
Tatras	α7Lys→Asn (−14 Da)	1**	
I-Interlaken	α15Gly→Asp (+58 Da)	1**	
Le Lamentin	α20His→Gln (−9 Da)	2	
Shaare Zedek	α56Lys→Glu (+1 Da)	1**	
M Boston	α58His→Tyr (+26 Da)	1**	[37]***
J-Meerut	α120Ala→Glu (+58 Da)	1	
β-Subunit			
Raleigh	β1Val→acAla (+14 Da)	1	
Niigata	β1Val→Met–Leu (+145 Da) & acMet–Leu (+187 Da)	3*	[15]
Okayama	β2His→Gln (−9 Da)	1	
S homozygosity	β6Glu→Val (−30 Da)	1	
SC compound heterozygosity	β6Glu→Val (−30 Da) & β6Glu→Lys (−1 Da)	2	[11]
G-Coushatta	β22Glu→Ala (−58 Da)	1	
E-Saskatoon	β22Glu→Lys (−1 Da)	1	
E homozygosity	β26Glu→Lys (−1 Da)	3	
E heterozygosity	β26Glu→Lys (−1 Da)	2	
Hoshida	β43Glu→Gln (−1 Da)	3	
Hokusetsu	β52Asp→Gly (−58 Da)	1*	[18]
Hamadan	β56Gly→Arg (+99 Da)	12	
J-Lome	β59Lys→Asn (−14 Da)	3	
Hikari	β61Lys→Asn (−14 Da)	2	
Bristol	β67Val→Met (+32 Da)→Asp (+16 Da)	2	[13]
Tigraye	β79Asp→His (+22 Da)	1**	
G-Szuhu	β80Asn→Lys (+14 Da)	6	
Hb Providence	β82Lys→Asn (−14 Da)→Asp (−13 Da)	2	
Santa Ana	β88Leu→Pro (−16 Da)	1**	[12,14]
Agenogi	β90Glu→Lys (−1 Da)	1	
Moriguchi	β97His→Tyr (+26 Da)	2	
Yoshizuka	β108Asn→Asp (+1 Da)	1	
Shizuoka	β108Asn→His (+23 Da)	1	
Peterborough	β111Val→Phe (+48 Da)	1**	[16]
Masuda	β114Leu→Met (+18 Da) & 119Gly→Asp (+58 Da)	1	
Riyadh	β120Lys→Asn (−14 Da)	7	
Takamatsu	β120Lys→Gln (0 Da)	1	
Montfermeil	β130Tyr→Cys (−60 Da)	1**	
Camden	β131Gln→Glu (+1 Da)	1	
Yamagata	β132Lys→Asn (−14 Da)	1	
Sagami	β139Asn→Thr (−13 Da)	1*≠	[17,18]
Mito	β144Lys→Glu (+1 Da)	1	
Thalassemia			
β0Type thalassemia	βcodon17 (AgT); AAG (Lys)→TAG (stop codon)	1	

*First case in the world.
**First case in Japan.
***Detected and sequenced by a conventional method in 1964 [37]; an MS analysis was performed in 1981 [8] by FDMS and in 1994 by ESI-MS.
≠Compound heterozygosity for β^{+}thalassemia and the variant.

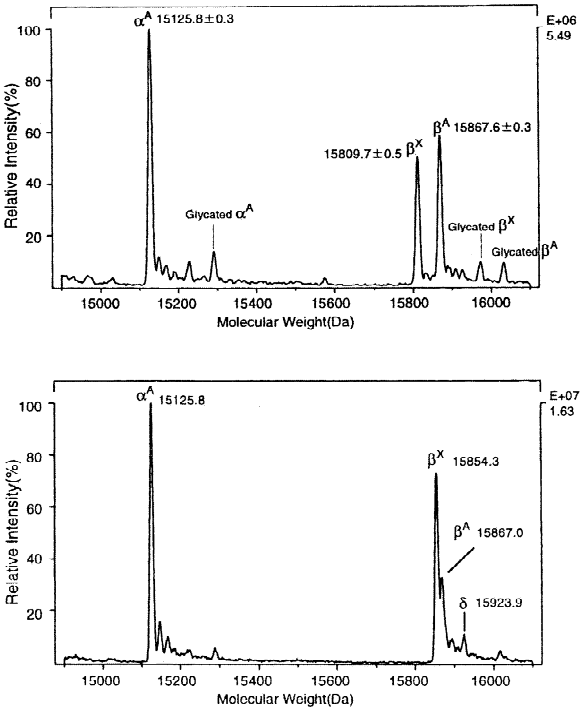

Fig. 2. The transformed ESI mass spectra derived from a patient with Hb Hokusetsu (upper panel) and Hb Sagami (bottom panel). Taken from Nakanishi and co-workers [17,18] with permission.

isolated by reversed-phase HPLC, and by nucleotide sequencing.

3.2. Hemoglobin variant interference in HbA1c measurement

Eighty percent of 840 abnormal Hbs show no pathological symptoms. However, almost all abnormal Hbs cause erroneous estimation of HbA1c by HPLC, and some abnormal Hbs also cause measurement errors in immunoassays [20]. However, it is difficult to know the extent of the error because no method of measuring the true value of HbA1c has been established. We expect that the values with the least error compared to the true value (the highest accuracy) can be obtained by the MS method. Currently, two MS methods are available. One is the measurement of intact hemoglobin by ESI-MS proposed by a UK group [39,40], and by us [41]. Dr. Michael Morris in the UK group conceived the idea of HbA1c measurement using intact Hb, when he visited our laboratory in the summer of 1996, where was conducting abnormal Hb research by MS. Whole blood samples were diluted 500 times with a 0.2% formic acid–50% acetonitrile solution, and 5 μl of the diluted solution was injected into the ESI-MS

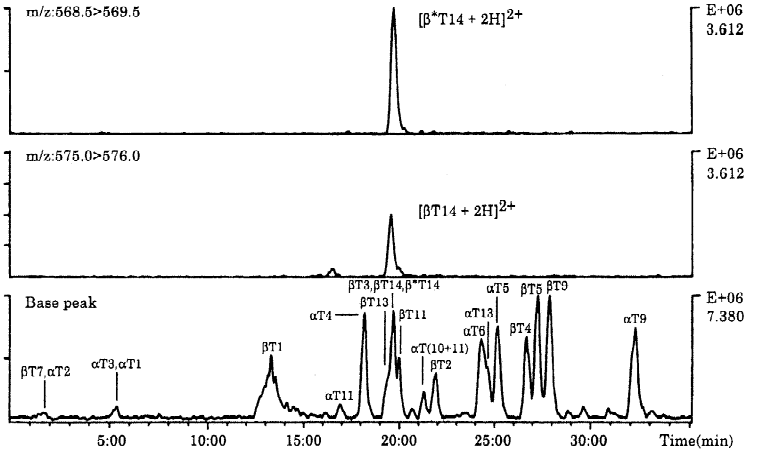

Fig. 3. Reconstructed selected ion chromatograms and base ion chromatogram obtained by LC–ESI-MS of peptides digested with trypsin from a β-subunit (Hb Sagami): upper panel, *m*/*z* 568.5–569.5, doubly charged ion of abnormal βT*14; middle panel *m*/*z* 575.0–576.0, doubly charged ion of normal βT14; lower panel, base ion chromatography. Taken from Nakanishi et al. [17] with permission.

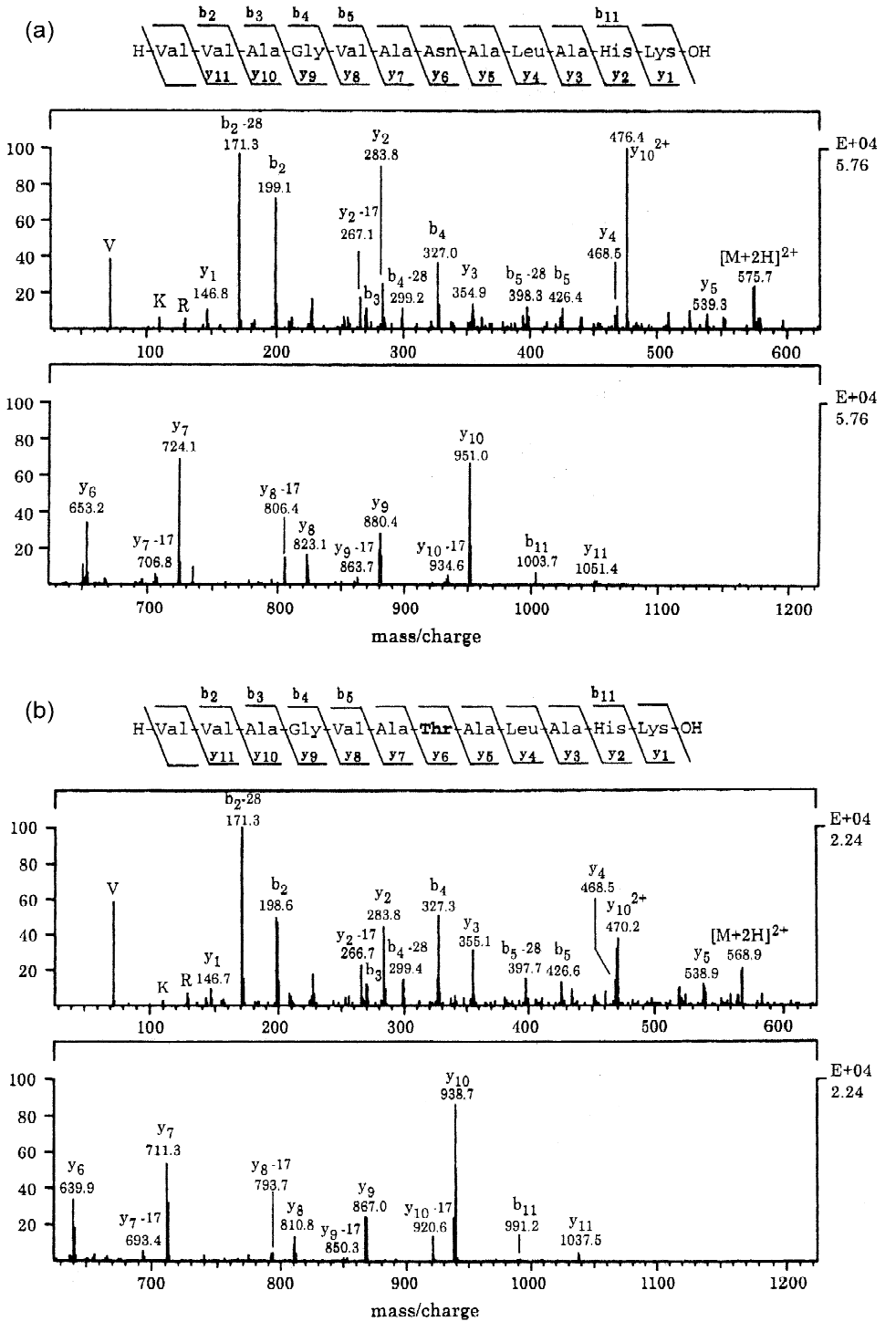

Fig. 4. CID mass spectra of a normal (a) and an abnormal peptide (b) to determine the amino acid substitution of Hb Sagami. Taken from Nakanishi et al. [17] with permission.

(TSQ 7000) system via a sample loop. The ratio of the glycated β-chain to total β-chain was calculated by the formula, glycated hemoglobin (%)=100× {(peak height of glycated-β)/(peak height of glycated-β+peak height of non-glycated-β)}. The within-run and between-run relative standard deviations of the ratio of glycated and non-glycated β-chains were 3.9~4.9%. The correlation between the ESI-MS method with intact globin and the conventional HPLC method was 0.980, and that between the ESI-MS method and the immuno-latex agglutination method was 0.961. Sample preparation was completed in 2 min and MS analysis in 1 min [41]. Using this method, the glycated-β-chain includes β-chains glycated on α-NH_2–valine and on ε-NH_2–lysine, and the ratio calculated by the above equation is not the value of HbA1c, which was designated as a stable adduct of glucose to the NH_2-terminal amino group of the β-chain of hemoglobin A_0 (N-1-deoxyfructosyl hemoglobin). However, the fluctuation of HbA1c in each individual is comparable to that of the% of total (α- and ε-NH_2–) glycated β-chain.

The other promising method for measuring HbA1c was proposed by Kobold et al. [34], followed by us [20,35,36], based on the ESI-MS of the N-terminal residues of the Hb β-chains, which are released by enzymatic cleavage of the intact Hb molecule with endoproteinase Glu-C. The correlations among the percentages measured by the MS-peptide method, and by conventional HPLC and the latex agglutination methods were good. The MS-peptide method is more complicated than the MS-globin method, but the reproducibility by the former (1.4~2.7%) is better than the latter (3.9~4.9%). The MS-globin method is expected to be the best standard method after improvement of MS instruments for quantification, including a deconvolution program. We measured HbA1c in samples with various Hb variants using two commercial systems and the MS-peptide method [20]. A total of 81 samples, normal and diabetic, were analyzed, of which 45 were homozygous for Hb A, 36 were heterozygous for various Hb variants, and one was compound heterozygosity for β^+thalassemia and Hb Sagami. Immunoassays were performed using kits, DCA2000 (immunoassay using intact Hb, Bayer Diagnostics). For HPLC, a Hi-AUTOA1c HA-8150 HbA1c analyzer (Kyoto Daiichi, Kyoto, Japan) was used. For the MS-peptide method, we prepared a calibrator by mixing synthetic peptides of non-glycated and glycated hexapeptide, VHLTPE, and 1-deoxyfructosyl-VHLTPE [20,35,36], instead of mixing HbA1c and HbA_0, which were purified by cation-exchange chromatography, and affinity chromatography [38]. The MS system was a TSQ 7000 triple stage quadrupole mass spectrometer with a conventional electrospray ion source (Finnigan MAT, San Jose, CA). The relative standard deviation (RSD) of intra-assay by our method was 1.4% for a control sample with high level HbA1c (10.2%) (n=5), and 2.7% for a low level sample (4.8%) (n=5). The inter-assay RSD was 1.9% for the high level (9.9%) (n=5), and 2.6% for the low level (5.0%) (n=5). As shown in Fig. 5a and b, all three methods produced similar results for samples without variants, although values by both HPLC and immunoassay were considerably higher than those by MS. Regression line slopes were considerably smaller than 1, and intercepts were slightly less than 0. The correlation between the values obtained by HPLC and MS was better than that between immunoassay and MS. Cases with abnormal Hb, which are marked by circles, are dislocated from the regression line. We believe that the values obtained by MS measurement are closer to the true values. In cases of Hb Niigata (β1Val→Leu, with complete retention of the initiator Met and about 20% acetylation of the amino terminal Met), the HbA1c value obtained by the routine HPLC method was much higher than that by MS, as the acetylated variant component superimposed on the HbA1c peak. In the case of Hb Okayama (β2His→Gln), the main fraction of the variant co-migrated with the HbA1c fraction of HPLC. In both cases, the HbA1c values obtained by HPLC were highly elevated. It is interesting that the variants gained one acidic charge by amino acid substitution on the N-terminal region of β-chains (β1 and 2) co-migrated with HbA1c on ion-exchange column chromatography. The other variants gaining one acidic charge, e.g., Hb Riyahd (β120Lys→Asn), Hb Yoshizuka (β108Asn→Asp), and Hb Camden (β131Gln→Glu), did not co-migrate with HbA1c, but co-migrated with HbA_0.

On HPLC, in most samples containing variant Hb, glycated Hb divided into two fractions, glycated HbA and glycated variant Hb, and this resulted in the underestimation of HbA1c in the samples containing

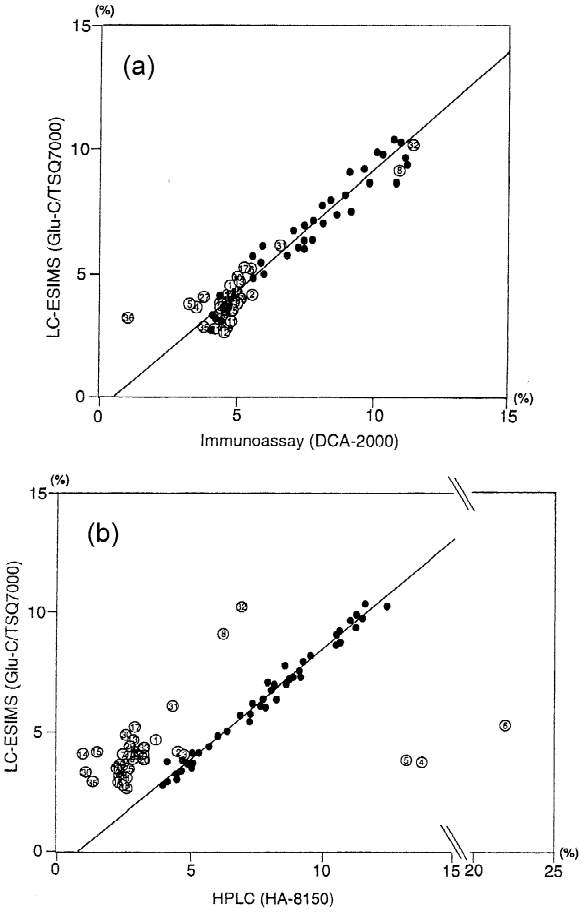

Fig. 5. The effect of hemoglobin variants on routine HbA1c measurements: an assessment by a mass spectrometric method. HbA homozygous (samples without abnormal Hb) results shown by closed circles were compared using Deming regression analysis (———). Open circles: heterozygous Hb variants. (a) Comparison between the percentage obtained by MS and that by DCA2000. $n=45$; slope$=0.959\pm0.033$; intercept$=-0.551\pm0.246$; $r=0.975$; $S_{yx}=0.510$. (b) Comparison between the percentage obtained by MS and that by HPLC. $n=45$; slope$=0.914\pm0.017$; intercept$=-0.744\pm0.138$; $r=0.993$; $S_{yx}=0.306$. Taken from Nakanishi et al. [20] with permission.

variant Hb. In these cases, the range of the difference between the values measured by HPLC and those by MS depends on the content of the variants. In heterozygotes with most β-chain variants, HbA1c values by HPLC were almost half of those by MS. In cases with α-chain variants it was ca. 3/4, as the variant content is usually ca. 1/4. In a β-chain variant case with a minor thalassemia [−31 (A→G)], the content of the variant component was 70%, and HbA1c values were 1.1% by HPLC and 3.9% by MS.

Some variants also gave considerably different values on immunoassay from these obtained by ESI-MS. Samples containing Hb Niigata gave lower values on immunoassay than those ESI-MS, perhaps due to the low antibody reactivity, which was prepared against the glycated N-terminal part of normal β-chain, to the glycated N-terminal part with the variant sequence. Unexpectedly, samples with Hb Okayama (β2His→Gln) did not show a significant discrepancy between values on DCA 2000 (immunoassay with intact Hb) HbA1c and ESI-MS analysis. This sample also gave nearly equal values on Unimate (immunoassay with enzyme digests) (5.6%). The antibody used for these assays may react to the same degree with 1-deoxyfructosyl-Val−His−Leu− and 1-deoxyfructosyl-Val−Gln−Leu− despite the second amino acids of both peptides being different. In Hb Sagami, values of 1.1, obtained by repeated immunoassay (DCA2000) were much lower than those by ESI-MS. This sample was from a compound heterozygote of minor thalassemia and a variant (β139Asn→Thr). The ratio of content of Hb Sagami to HbA was about 7:3 as determined by high resolution HPLC. It is possible that the reactivity of the antibody against a glycated epitope in intact globin was significantly low in Hb Sagami. The value obtained with Unimate, an immunoassay with peptides released by pepsin digestion were reasonable, i.e., 4.6%. The mutation is in the central cavity near the 2,3-DPG pocket, and there are molecular contacts between this site and β82Lys and β134Val. As we reported previously, the oxygen affinity of this variant is 20% lower than that of normal Hb [19], and the conformation of this variant may differ largely from that of normal Hb. The glycated epitope on the variant Hb in the intact molecule may be buried in the molecule.

Each available measurement system gives some erroneous values for specimens containing abnormal Hb. To obtain more reliable values, it may be necessary to use high resolution HPLC to separate the main fraction of Hb (i.e., normal and abnormal Hb) and to calculate the ratio, $HbA1c/HbA_0$ instead of HbA1c/total Hb. Immunoassays may be more likely to misdiagnose abnormal Hb-containing sam-

ples than the HPLC method, because this method does not suggest the existence of abnormal Hb in any cases. The best way may be the routine use of MS as the UK group proposed [39,40], although the MS equipment is more expensive than the other methods. Another strategy is routine checks for abnormal Hb as the first test of routine HbA1c in each individual.

3.3. Carbohydrate-deficient transferrin

We first tried to analyze immunoprecipitated transferrin (Trf) by MALDI-TOF-MS and ESI-MS in 1994 [24,25], not long after the presentation of these excellent soft ionization MS. Fig. 6 shows deconvoluted spectra of serum Trf prepared by immunoprecipitation from a normal individual and from a patient with carbohydrate-deficient glycoprotein (CDG) syndrome, a general disorder of the nervous system. Normal Trf mainly has a single isoform that contains two asparagine N-linked disialylated biantennary carbohydrate chains. The spectrum of Trf from CDG patients (Fig. 6, bottom) shows extra ion peaks between multiply charged normal ion peaks (top). Trf from the patient revealed a molecular species 2200 Da smaller than that from a normal control, which is the size of a disialylated biantennary carbohydrate chain. Using this technique, we showed that the ratios of tetra-, di- and a-sialotransferrin (Trf) from two cases of congenital glycoprotein deficient syndrome was clearly distinguishable from those of control samples (data will be published elsewhere with more material).

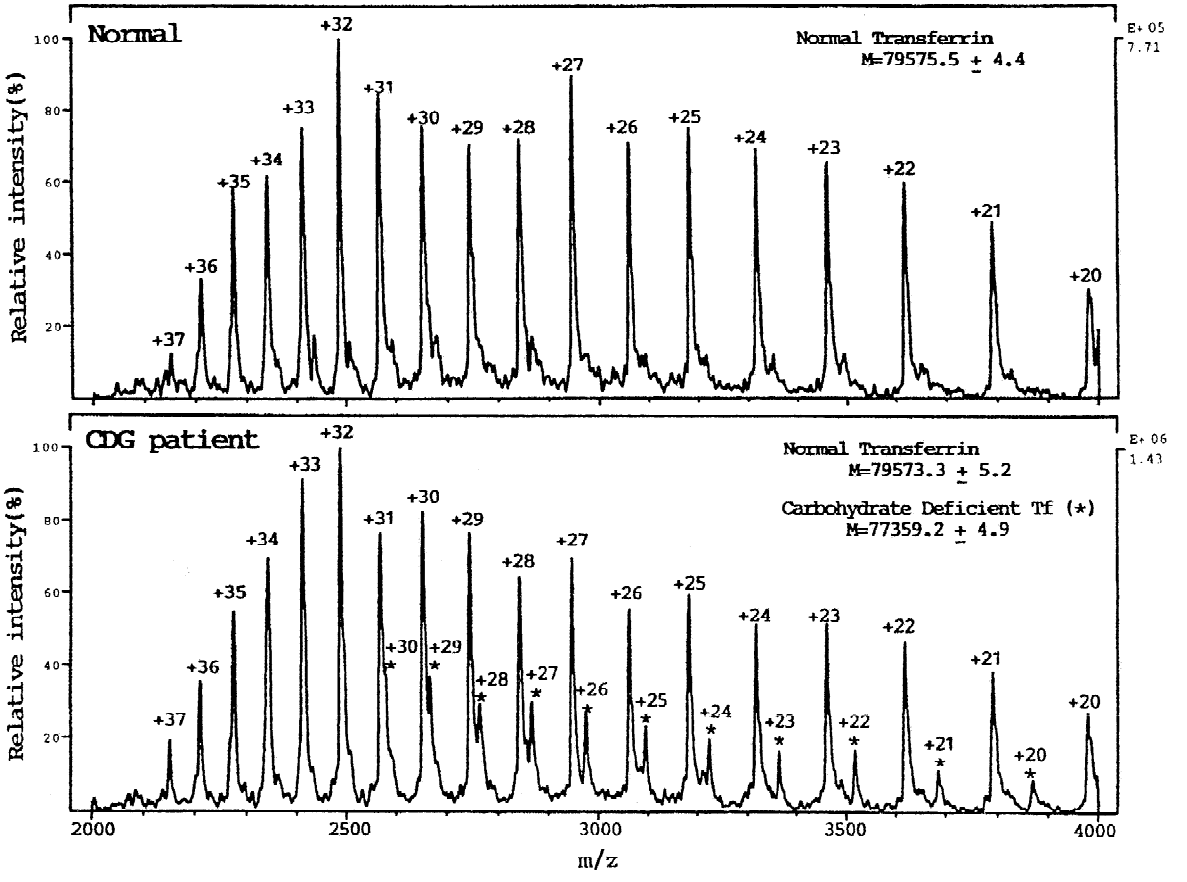

Fig. 6. ESI mass spectra of immunoprecipitated Trf from a control (top), and that from a patient with carbohydrate-deficient glycoprotein syndrome (bottom). Taken from Nakanishi et al. [25] with permission.

3.4. Transthyretin variants [21,22]

Analysis of TTR variants is important for the diagnosis of familial amyloidotic polyneuropathy (FAP), a hereditary neurodegenerative disease characterized by protein deposits that ultimately lead to organ failure and death. It is hypothesized that amino acid substitutions alter the stability of the tetramer, presumably leading to aggregation or polymerization of TTR monomers to form amyloid fibrils. The structures causing amyloidogenesis have not been clarified; some TTR variants are not amyloidogenic and the penetration of the disease of a variant is different among ethnic groups. More information on the clinical findings and chemical analysis of TTR is needed to elucidate the molecular mechanism of amyloidogenesis.

The spectra of TTR from a normal control (Fig. 7a) revealed peaks with a molecular mass corresponding to free monomer, TTR monomer conjugated with cysteine, cysteinyl glycine, and glutathione by disulfide linkages (marked by A, B, and C, respectively). After reduction with dithiothreitol or other reducing reagents, these adducts changed to free TTR. TTR prepared from patients with variant TTR showed additional peaks of variant. Fig. 7b shows the analysis of a variant TTR in which the 101st Gly is substituted by Ser. Doublet peaks are observed both in free TTR and in each adduct TTR. The mass difference of the doublet peaks corresponds to the difference between glycine and serine, i.e., 30. Using this procedure, we analyzed 52 cases of TTR variants including 12 different variants (Table 2). Three of these (38Asp→Ala), (49Thr→Ile), and (101Gly→Ser) were new. These substitutions were determined by the analysis of LC–ESI-MS–MS using a tryptic peptide mixture of the patient's TTR. These sequences were confirmed by DNA analysis.

In Fig. 8, the transformed ESI-MS spectrum of immunoprecipitated TTR from an FAP patient (the 30th valine was substituted by methionine in the TTR of this patient), before and after liver transplantation. In this experiment, TTR was reduced

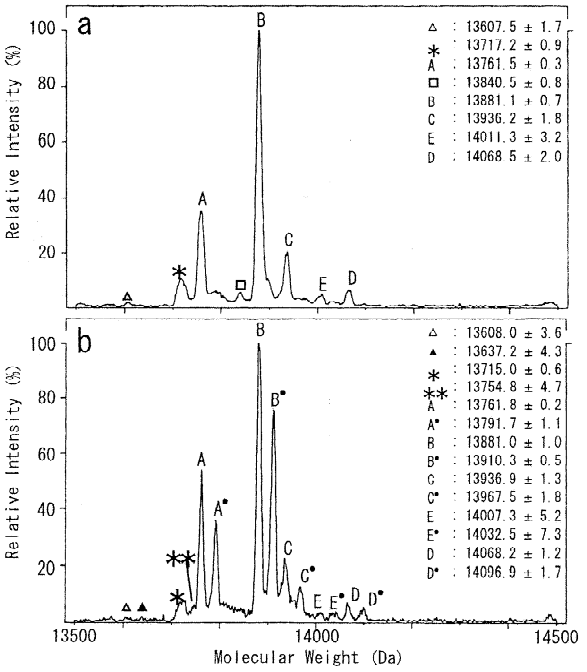

Fig. 7. The transformed ESI mass spectra of non-reduced TTR prepared by immunoprecipitation from a normal control and from a patient with familial amyloidotic polyneuropathy. (a) A control; (b) a case with a variant TTR(G101S) [21]. (A) Unmodified normal monomer TTR; (B), (C), (D), (E) monomer TTR conjugated with cysteine, cysteinylglycine, glutathione, and glutamyl cysteine, respectively.

Table 2
Transthyretin and SOD-1 variants analyzed in our group (1994–2001)

Variant	Substitution and mass differences	Case No.	Ref.
TTR	18Asp→Glu (+14 Da)	2	
	30Val→Met (+32 Da)	26	
	36Ala→Pro (+26 Da)	1	
	38Asp→Ala (−44 Da)	2*	[22]
	47Gly→Ala (+14 Da)	2	
	49Thr→Ile (+12 Da)	2*	[43]
	50Ser→Ile (+26 Da)	3	
	50Ser→Arg (+69 Da)	1	
	60Thr→Ala (−30 Da)	4**	
	101Gly→Ser (+30 Da)	1*	[21]
	109Ala→Ser (+16 Da)	2	
	114Tyr→Cys (−60 Da)	6	
SOD-1	4Ala→Ser (+16 Da)	1*	[23]
	37Gly→Arg (+99 Da)	1	
	46His→Arg (+19 Da)	1	
	86Asn→Ser (−27 Da)	1	
	111Cys→Tyr (+60 Da)	1*	[23]

*First case in the world.
**First case in Japan.

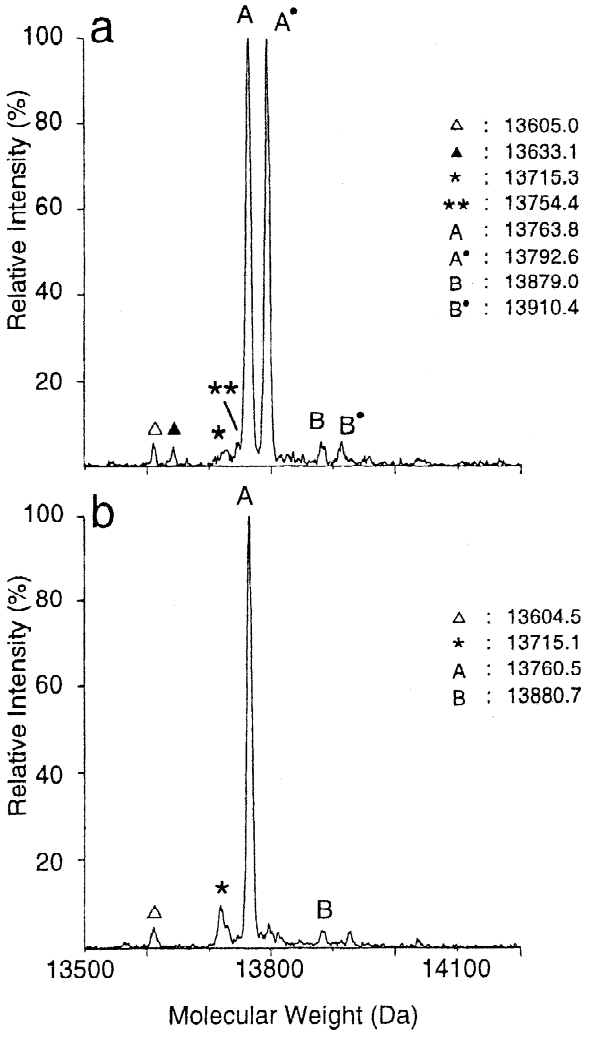

Fig. 8. The change in TTR after liver transplantation. The transformed ESI mass spectra of reduced TTR prepared by immunoprecipitation from a patient with familial amyloidotic polyneuropathy, before and after transplantation. (a) Before; (b) after. This patient was a heterozygote of 30Val→Met. Taken from Shimizu et al. [26] with permission.

before analysis. After reduction, the adduct peak lowered, and a simple profile was observed. A clearly separated abnormal doublet pattern is observed in the upper panel, and the variant ion peaks marked by closed circles disappeared after liver transplantation (lower panel). The TTR of the FAP patient before and after liver transplantation shown in this figure was analyzed at the request of Dr. Y. Ando (Kumamoto University, Japan), whom we thank for collaborating with us in this study.

3.5. Transthyretin S-sulfonate [41]

In addition to detecting variant proteins causing various diseases, we detected various covalent adducts of Hb, TTR and SOD-1. Here, we show one of the interesting adducts we found. An adduct peak 80 Da larger than free intact TTR, was revealed to be TTR conjugated with sulfite. This isoform was observed prominently in serum from a molybdenum cofactor-deficient patient, as shown in Fig. 9. The spectrum in Fig. 9A shows TTR from a control infant, and Fig. 9B is that from a patient with molybdenum cofactor deficiency. Several peaks of free and adduct TTR are observed in the spectrum from the control, as shown in the previous figure. In contrast, the spectrum of TTR from the patient showed a prominent peak at 80 Da larger than free TTR. After reduction, TTR+80 disappeared, and free TTR increased (Fig. 9C). Molybdenum cofactor is the obligate activator of sulfite oxydase, and a defect in the cofactor impairs sulfite oxidase activity. Since in patients with this disease the presence of elevated levels of sulfite leads to formation of S-sulfonated cysteine, the isoform was assigned to TTR S-sulfonate. This peak was observed at low levels in TTR from control sera. Human sulfite oxidase deficiency is a severe disease characterized by neurological abnormalities, seizures, mental retardation, and dislocation of the ocular lenses that often leads to death in infancy. The phenotype of sulfite oxidase deficiency can arise from a mutation either in the sulfite oxidase gene (isolated sulfite oxidase deficiency) or in any of several genes involved in the synthesis of molybdopterin (molybdenum cofactor deficiency). This adduct ion peak was significantly elevated (ca. 80% versus less than 10% in control individuals) in three cases of molybdenum cofactor deficiency (Table 3). This marked peak could be a marker for screening patients with this disease.

TTR obtained from rabbit serum, human TTR purchased from a commercial source, and TTR prepared from some individuals for analysis of

cessfully detected five different variants of SOD-1 in erythrocytes (10 μl hemolysate), and 1 variant in the spinal cord (20 mg wet tissue) using the immunoprecipitation method from patients with familial amyotrophic lateral sclerosis (Table 2). Fig. 10A shows the transformed mass spectra of SOD-1 prepared by immunoprecipitation from a normal individual. The molecular mass of the main peak, 15 844.8 Da, coincides with the theoretical average molecular mass of the intact monomer SOD-1, 15 844.6 Da. The values of minor peaks, 15 964.1 and 16 150.0 Da, coincide with the theoretical average molecular mass of intact monomer SOD-1 conjugated with cysteine and glutathione by a disulfide linkage: 15 963.7 and 16 149.9 Da, respectively.

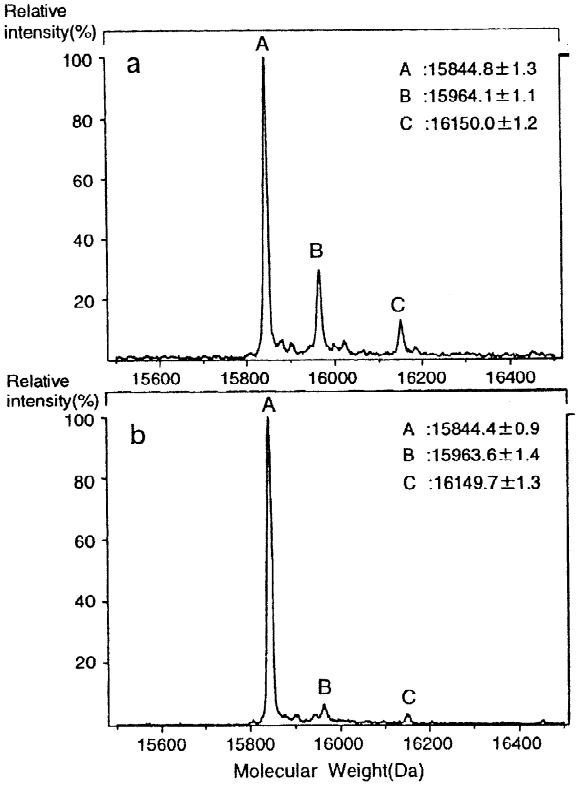

Fig. 10. The transformed ESI mass spectra of SOD-1 prepared by immunoprecipitation from a normal individual. (a) Not reduced; (b) reduced; (A) unmodified normal monomer SOD-1; (B) monomer SOD-1 conjugated with cysteine by disulfide bond; (C) monomer SOD-1 conjugated with glutathione. Taken from Nakanishi et al. [23] with permission.

The spectrum shown in Fig. 10b is from normal SOD-1 prepared by immunoprecipitation and reduced by addition of DTT. On reduction, the relative abundance of the ion peak of free monomer SOD-1 increased, and peaks corresponding to SOD-1 conjugated with cysteine or glutathione decreased. Therefore, we concluded that the three peaks marked in Fig. 10a correspond to free monomer SOD-1 (marked by A), SOD-1 conjugated with cysteine (B) and glutathione (C). SOD-1 from patients with FALS prepared by immunoprecipitation, followed by reduction, showed ion peaks of mutant SOD-1 in addition to normal peaks (Fig. 11). In the spectrum shown in Fig. 11a, an extra peak (marked with a dot) was observed at the molecular mass of 15 944.5 Da. The mass difference, 99.3 Da, between the normal and extra peak of free SOD-1 (A and A˙, respectively) coincides with the difference between glycine and arginine residues, 99.14 Da. SOD-1 from the other two patients also showed ion peaks of mutant SOD-1 in addition to normal peaks (Fig. 11b and c). The mass difference between the normal and extra peak of free SOD-1 (A and A˙, respectively) coincided with the difference in molecular mass between normal and substituted residues. The analysis can be completed within 2 h, including preparation of SOD-1 and MS measurement. This method provides rapid diagnosis and screening of the FALS associated with the SOD-1 mutant. The amino acid substitutions determined by DNA analyses can be proved by this method. The abundance of ion peaks corresponds to the approximate ratio of each component. The ratio of mutant/normal SOD-1 in erythrocytes may reflect the instability of the mutants, and may relate to the pathogenesis of the disease. MS measurement using immunoprecipitated material will be useful in studies to elucidate the role of mutant SOD-1 also in an animal model [31].

4. New trends

As the sequencing of the human genome progresses, interest is shifting to the role of genetic variation in phenotype. Studies have begun to target the identification of single nucleotide polymorphisms (SNIPs) at the genomic level, as well as to identify

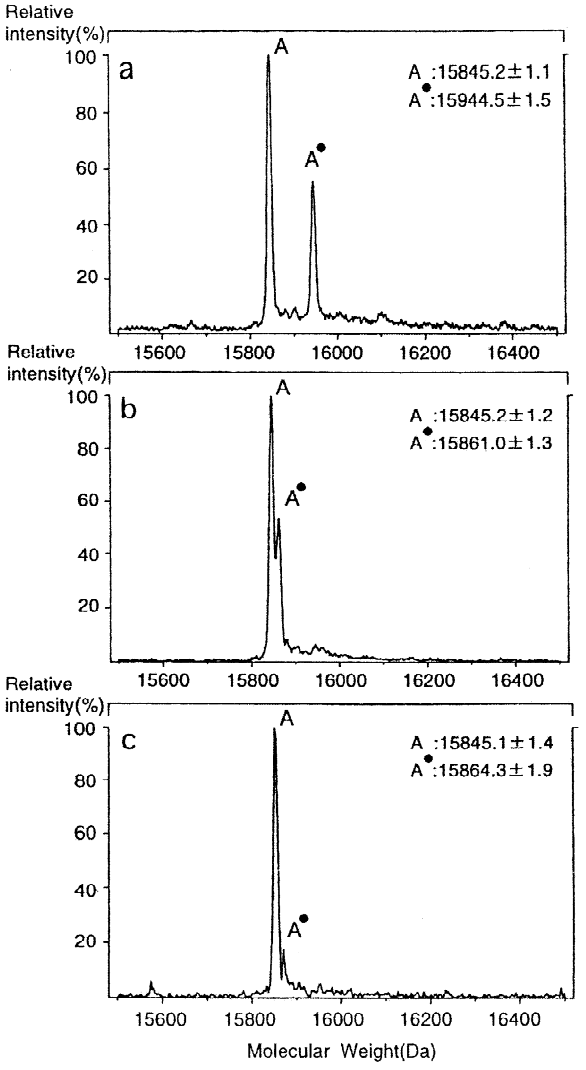

Fig. 11. The transformed ESI mass spectra of reduced SOD-1 prepared by immunoprecipitation from patients with FALS [23]. (a) [G37R]; (b) [A4S]; (c) [H46R]. (A) Unmodified normal monomer SOD-1; (A•) unmodified mutant monomer SOD-1. Taken from Nakanishi et al. [23] with permission.

specific polymorphisms in genes suspected of a role in disease. Various DNA technologies are applied to identify these polymorphisms. However, protein analysis to detect the variants resulting from SNIPs may give more direct information. With protein analysis, protein levels, modified structures, and fragmented structures can be detected. For these purposes, modern MS technology is quite suitable. The sensitivity of MS analysis may be lower than that of DNA analysis at the present time. General use of advanced MS technology, for example, Fourier transform ion cyclotron resonance MS, may allow more sensitive analysis. For Hb, TTR, and SOD-1, protein analyses to detect SNIPs are possible as the protein contents are high enough for this purpose. By cleavage of abnormal components (separated by isoelectric focusing) by trypsin, and by a mixture of proteinase, Glu-C and Asp-N, followed by analysis of peptides by LC–ESI-MS–MS using LCQ (Thermoquest San Jose), we detected amino acids covering all sequences of α- and β-chains, including the region of small tryptic peptides, and that of the core in one analysis. Y series ions of CID spectra of peptides having no basic amino acid (some of peptides generated by Glu-C and Asp-N digestion) were insufficient by positive ionization, but sufficient by negative ionization.

Another post-genomics project is preparation of a protein catalogue for body fluid, each cell and various tissues. Two-dimensional polyacrylamide gel electrophoresis, enzyme digestion of the spots, and MS are often used; this is called proteomics. To identify specifically increased or decreased proteins in disease, this technique will be useful and popular. We started the analysis of protein profiles of human vitreous humor to elucidate the pathogenesis of various retinopathies [45].

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References

[1] H. Wajcman, Human Hemoglobin Variants, The Electric Versions, http://globin.cse.psu.edu
[2] L. Bry, P.C. Chen, D.B. Sacks, Clin. Chem. 47 (2001) 153.
[3] F.E. Dwulet, M.D. Benson, Biochem. Biophys. Res. Commun. 114 (1983) 657.
[4] S. Tawara, M. Nakazato, K. Kangawa, H. Matsuo, S. Araki, Biochem. Biophys. Res. Commun. 116 (1983) 880.

[5] L.H. Conners, A.M. Richardson, R. Théberge, C.E. Costello, Amyloid 7 (2000) 54.
[6] D.R. Rosen et al., Nature 362 (1993) 59.
[7] J. Jacobsson, P.A. Johnsson, P.M. Andersen, L. Forsgren, S.L. Marklund, Brain 124 (2001) 1461.
[8] Y. Wada, A. Hayashi, T. Fujita, T. Matsuo, I. Katakuse, H. Matsuda, Biochim. Biophys. Acta 667 (1981) 233.
[9] M. Karas, F. Hillenkamp, Anal. Chem. 60 (1988) 2299.
[10] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.
[11] T. Nakanishi, M. Kishikawa, A. Shimizu, A. Hayashi, F. Inoue, J. Mass Spectrom. 30 (1995) 1663.
[12] T. Nakanishi, A. Miyazaki, M. Kishikawa, A. Shimizu, T. Yonezawa, J. Am. Soc. Mass Spectrom. 7 (1996) 1040.
[13] A. Miyazaki, T. Nakanishi, M. Kishikawa, A. Shimizu, Y. Ohba, A. Tanaka, H. Sasaki, J. Mass Spectrom. 31 (1996) 1311.
[14] A. Miyazaki, T. Nakanishi, M. Kishikawa, A. Shimizu, K. Jozaki, T. Yonezawa, Intern. Med. 36 (1997) 365.
[15] Y. Ohba, Y. Hattori, S. Sakata, Y. Yamashiro, N. Okayama, T. Hirano, T. Nakanishi, A. Miyazaki, A. Shimizu, Hemoglobin 21 (1997) 179.
[16] T. Nakanishi, A. Miyazaki, M. Kishikawa, A. Shimizu, Y. Aoki, M. Kikuchi, Hemoglobin 22 (1998) 23.
[17] T. Nakanishi, A. Miyazaki, M. Kishikawa, A. Shimizu, Y. Aoki, M. Kikuchi, J. Mass Spectrom. 33 (1998) 565.
[18] T. Nakanishi, A. Miyazaki, M. Kishikawa, A. Shimizu, O. Kishida, S. Sumi, T. Tsubakio, K. Imai, Hemoglobin 22 (1998) 355.
[19] A. Miyazaki, T. Nakanishi, M. Kishikawa, T. Nakagawa, A. Shimizu, A.H. Mohammed Mawjood, K. Imai, Y. Aoki, M. Kikuchi, Hemoglobin 23 (1999) 267.
[20] T. Nakanishi, A. Miyazaki, K. Iguchi, A. Shimizu, Clin. Chem. 46 (2000) 1689.
[21] M. Kishikawa, T. Nakanishi, A. Miyazaki, M. Hatanaka, A. Shimizu, S. Tamoto, N. Ohsawa, H. Hayashi, M. Kanai, Hum. Mutat. 12 (1998) 363.
[22] M. Kishikawa, T. Nakanishi, A. Miyazaki, A. Shimizu, H. Kusaka, M. Fukui, T. Nishiue, Amyloid: Int. J. Exp. Clin. Invest. 6 (1999) 278.
[23] T. Nakanishi, M. Kishikawa, A. Miyazaki, A. Shimizu, Y. Ogawa, S. Sakoda, T. Ohi, H. Shoji, J. Neurosci. Methods 81 (1998) 41.
[24] T. Nakanishi, N. Okamoto, K. Tanaka, A. Shimizu, Biol. Mass Spectrom. 23 (1994) 230.
[25] T. Nakanishi, A. Shimizu, N. Okamoto, A. Ingendoh, M. Kanai, J. Am. Soc. Mass Spectrom. 6 (1995) 854.
[26] A. Shimizu, T. Nakanishi, M. Kishikawa, A. Miyazaki, in: E.J. Karjalainen et al. (Ed.), Advances in Mass Spectrometry, Vol. 14, Elsevier, 1998, p. 405.
[27] M. Kishikawa, T. Nakanishi, A. Miyazaki, A. Shimizu, M. Nakazato, K. Kangawa, H. Matsuo, J. Mass Spectrom. 31 (1996) 112.
[28] R. Théberge, L. Connors, M. Skinner, J. Skare, C.E. Costello, Anal. Chem. 71 (1999) 452.
[29] Y. Ando, P.L. Ohlsson, O. Surh, N. Nilhin, T. Yamashita, G. Holmgren, A. Danielson, O. Sandgren, M. Uchino, M. Ando, Biochem. Biophys. Res. Commun. 228 (1996) 480.
[30] S. Ikeda, T. Tokuda, A. Nakamura, I. Ueno, T. Taketomi, N. Yanagisawa, Y. Li, Int. J. Exp. Clin. Invest. 4 (1997) 104.
[31] K. Fukada, S. Nagano, M. Satoh, C. Tohyama, T. Nakanishi, A. Shimizu, T. Yanagihara, S. Sakoda, Eur. J. Neurosci., in press.
[32] M. Kishikawa, T. Nakanishi, A. Shimizu, Proceedings of the IX International Symposium on Amyloidosis 2001, in press.
[33] M. Kishikawa, T. Nakanishi, A. Shimizu, M. Yoshino, Pediatr. Res. 47 (2000) 492.
[34] U. Kobold, J.-O. Jeppsson, T. Dülffer, A. Finke, W. Hoelzel, K. Miedema, Clin. Chem. 43 (1997) 1944.
[35] T. Nakanishi, A. Shimizu, J. Mass. Spectrom. Jpn. 47 (1999) 389.
[36] T. Nakanishi, A. Shimizu, J. Chromatogr. B 746 (2000) 83.
[37] A. Shimizu, A. Hayashi, Y. Yamamura, A. Tsugita, K. Kitayama, Biochim. Biophys. Acta 97 (1965) 472.
[38] A. Finke, U. Kobold, W. Hoelzel, C. Weykamp, K. Miedema, J.-O. Jeppsson, Clin. Chem. Lab. Med. 36 (1998) 299.
[39] N.B. Roberts, B.N. Green, M. Morris, Clin. Chem. 43 (1997) 771.
[40] N.B. Roberts, A.B. Amara, M. Morris, B.N. Green, Clin. Chem. 47 (2001) 316.
[41] T. Nakanishi, A. Miyazaki, M. Kishikawa, M. Yasuda, Y. Tokuchi, Y. Kanada, A. Shimizu, J. Mass Spectrom. 32 (1997) 773.
[42] M. Kishikawa, T. Nakanishi, A. Miyazaki, A. Shimizu, Amyloid: Int. J. Exp. Clin. Invest. 6 (1999) 48.
[43] M. Nakazato, M. Kishikawa, A. Shimizu, presented at the VIIIth International Symposium on Amyloidosis, Rochester, MN, 7–11 August 1998.
[44] M. Yoshioka, Z. Tamura, Chem. Pharm. Bull. (Tokyo) 19 (1971) 178.
[45] T. Nakanishi, R. Koyama, T. Ikeda, A. Shimizu, J. Chromatogr. B, submitted for publication.