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Establishment of a combined strategy of genetic and mass spectrometric analyses for characterizing hemoglobin mutations An example of Hb Hoshida (β43Glu→Gln)

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Abstract

Structural analysis of mutant hemoglobins has been efficiently accomplished by a consecutive mass spectrometric strategy: molecular mass measurement of the protein to detect mutation and to determine the molecular mass change, and fragmentation analysis with collision-induced dissociation to determine the site and type of mutation. A flaw of this method is an inherent inability to detect a mutation associated with no or little change of the molecular mass. In the present study, the strategy was improved by incorporating genetic analysis prior to mass spectrometry, which confirms the resulting amino acid change and searches for possible post-translational modifications. The method was applied to an unknown mutant, and elucidated a substitution of glutamine for glutamic acid at position 43 of β-globin subunit, the mutation of Hb Hoshida.

Keywords: Mutations; Hemoglobins

1. Introduction

Human hemoglobin is composed of two different globin molecules of 141 and 146 amino acid residues. To date, more than 670 mutations have been identified in hemoglobin [1]. They have been found by diagnostic tests for hematological abnormalities such as anemia and polycythemia, or in screening studies by means of electrophoresis. In addition, with the recent spread of chromatographic measurement of glycated hemoglobin for the screening of diabetic hyperglycemia, increasing numbers of mutants are incidentally found and submitted for structural characterization.

Since the early 1980s, hemoglobin mutants have contributed to the development of the mass spectrometric approach for structural elucidation of mutant proteins [2–4]. The strategy comprises a series of steps as follows. The presence of a mutation is confirmed by measuring the molecular mass of the whole molecule [5]. Subsequently, the mutant globin is cleaved by enzymatic digestion and subjected to mass spectrometry (MS) for identification of the peptide containing the mutation [2]. Finally, collision-induced dissociation (CID) or other enzymatic digestion is used to determine the site and type of mutation [5,6]. This strategy has demonstrated incomparable characterization ability in a number of

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mutants, especially those without a change in electrophoretic or chromatographic properties, as in neutral→neutral amino acid substitutions [7,8].

However, MS often presents a difficulty inherent in its analytical principle, with respect to mutations involving isobaric residues such as leucine and isoleucine and those giving minimal change in mass, e.g. lysine and glutamine differing by 0.036 u from each other. In the present study, we incorporated genetic analysis prior to MS to eliminate the problem, and applied the improved strategy to an unknown mutant. The established process allows reliable determination of a mutation and the related post-translational change.

2. Experimental

2.1. Sample preparation

Anticoagulated blood was obtained from a 57-year-old woman, who was incidentally found to have mutant hemoglobin by cation-exchange chromatography for measurement of glycated hemoglobin level.

After centrifuging at 3000 *g*, the buffy coat and red cell layers were separately collected. The red blood cells were washed once with phosphate-buffered saline, and then lysed with distilled water. Globin subunits, a mixture of α- and β-globin chains, were prepared by agitating the hemolysate in a solution of 0.01 vol.% hydrochloric acid in acetone.

2.2. Isoelectric focusing (IEF) and isolation of mutant hemoglobin

IEF was carried out with a polyacrylaimde slab gel containing a 1:1 mixture of pH 5–8 and pH 6.5–9 Pharmalyte solution (Pharmacia, Uppsala, Sweden). The band corresponding to the mutant hemoglobin was cut out and dipped in 2% acetic acid. After standing for 2 h at 4°C the hemoglobin that came out of the gel was collected.

2.3. Carboxymethylation and tryptic digestion

The globin sample was reduced with dithiothreitol in 6 *M* guanidine-HCl and carboxymethylated with iodoacetic acid for 3 h. Excess reagents were removed by directly applying the solution to reversed-phase liquid chromatography (RP-LC). The carboxymethylated globin was digested with trypsin.

2.4. Reversed-phase liquid chromatography (RP-LC)

Unmodified or carboxymethylated globins were purified by RP-LC carried out on a C4 column (Cosmosil 5C4 AR300, 4.6×150 mm) (Nacalai-tesque, Kyoto, Japan) using a linear gradient ranging from 0.1 vol.% trifluoroacetic acid (TFA) in water to 0.08 vol.% TFA in 70 vol.% acetonitrile at a flow-rate of 1 ml/min. This step allows separation of globin subunits.

For separation of peptides, a C18 column (Cosmosil 5C18 AR300, 4.6×250 mm) was used with the same gradient as described above.

2.5. Electrospray ionization (ESI) MS and fast atom bombardment (FAB) MS

ESI and FAB mass spectra were obtained using a JEOL SX102 double-focusing mass spectrometer with DA 7000 data system (JEOL, Akishima, Japan). For ESI-MS, samples were dissolved in a solvent of 75:24.8:0.2 methanol–H_2O–acetic acid (v/v/v) at a protein concentration of 10 μ*M*. An aliquot (20 μl) of the sample solution was directly introduced into the ESI ion source at a flow-rate of 0.8 μl/min with a syringe pump. Accelerating voltage was 5 kV. For FAB-MS, samples were ionized by bombardment with a xenon beam of 6 keV, and the secondary ions were accelerated at 7 kV. Glycerol was used as the sample matrix. FAB and ESI mass spectra were acquired at a resolution of 3000 and 2000, respectively (10% valley definition).

2.6. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS

MALDI mass spectra were acquired on a Voyager Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. For the sample matrix, α-cyano-4-hydroxycinnamic acid was dissolved in a solution containing 50% acetonitrile and 0.1% TFA at a

concentration of 10 g/l. Samples were dissolved in the same solution at a protein concentration of approximately 10 μ*M*, and then the sample solution was mixed with twice the volume of the matrix solution. The prepared sample solution (0.5 μl) was deposited on the sample plate and dried. Ions were accelerated at 20 kV. Argon was used as the collision gas in CID mode.

2.7. Genetic analysis

Total RNA was extracted from nucleated blood cells in the buffy coat layer by using a solution of phenol and guanidine thiocyanate, Isogen (Nippongene, Tokyo, Japan). The first strand of cDNA was synthesized using RTG You-prime First-Strand Beads (Pharmacia) and an antisense primer of 5′-CGAAAGAACGACAGGTTAAA-3′ complementary to the 3′-untranslated sequence of β-globin mRNA. Subsequently, polymerase chain reaction (PCR) was carried out using a high-fidelity DNA polymerase, KOD Polymerase (Toyobo, Tokyo, Japan) and a primer set of a sense primer, 5′-AGCAACCTCAAACAGACACC-3′, corresponding to the 5′-untranslated sequence and the antisense primer described above. The amplified DNA was inserted into a pT7Blue-T vector (Novagen, WI, USA), and the resulting clones were sequenced by a model 377 DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

3. Results and discussion

3.1. Isoelectric focusing of mutant hemoglobin

IEF of the hemolysate identified a mutant hemoglobin cathodic to normal Hb A ($\alpha_2\beta_2$) (data not shown). The amount of mutant was comparable with that of normal Hb A, and there were no additional bands cathodic to Hb A_2 ($\alpha_2\delta_2$), indicating that the mutant was derived from a β-globin subunit.

3.2. Genetic analysis

Globin mRNAs were isolated from peripheral blood cells, and the mutation was examined in the β-globin mRNA. The PCR aiming at amplification of a region including the full coding sequence (438 bases) of the β-globin gene was carried out following reverse transcription, and the amplified product was cloned into plasmids. Among ten clones sequenced, six had a mutation of GAG→CAG, corresponding to an amino acid substitution of glutamine for glutamic acid at the 43rd residue (data not shown). The frequency was reasonable, because the β-globin gene has a single locus unlike the duplicated α-globin gene and the patient was a heterozygote.

3.3. ESI-MS of intact globin molecule

The life of red blood cells is approximately 120 days. Hemoglobin has a similar life-span, considering that synthesis of the protein almost ceases in red blood cells at the time of appearance in the circulation. During life, hemoglobin undergoes post-translational modifications such as glycation, and some mutated residues are known to be specifically changed. For example, a considerable part of the substituted residue of asparagine in Hb Providence is changed to aspartic acid by deamidation [9]. Similarly, the mutated leucine at the N-terminal is partially acetylated [10]. It is thus required to verify that the protein isolated from the patient is not modified after translation from the mutated gene.

The substitution of glutamine for glutamic acid produces a decrease of a single mass unit in the globin molecule. ESI-MS of the β-globin, which was heterozygous for the mutation, did not disclose any abnormality, because the difference between normal and mutant proteins would be only 60 ppm in the molecular mass (Mr 15 867.2) of this protein (Fig. 1). The result, however, ruled out any gross abnormalities due to post-translational modification which occurred in the mutant.

3.4. FAB-MS of tryptic peptides

To facilitate further analyses, the mutant hemoglobin was purified. In the present study, the mutant hemoglobin separated on isoelectric focusing gel was successfully recovered from the gel, presenting a practical method of rapid isolation. The mutant β-globin was then purified by RP-LC following carboxymethylation, and digested with trypsin. The resultant peptides in the mixture were analyzed by FAB-MS (Fig. 2). The mass of the protonated

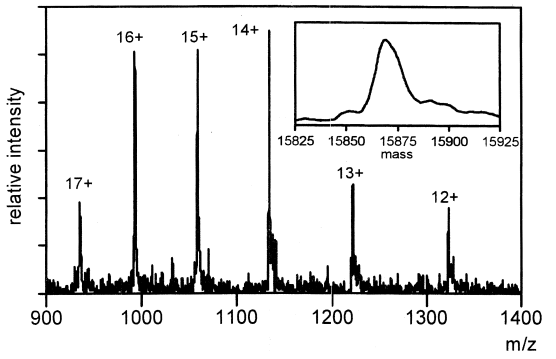

Fig. 1. ESI mass spectrum of β-globin from a patient with heterozygous hemoglobin mutation. A transformed spectrum is presented in the inset.

molecular ion for peptide T5 that contained the β43 residue was 2058.0 u (atomic mass unit), a single mass unit smaller than that of the normal peptide (Fig. 2b).

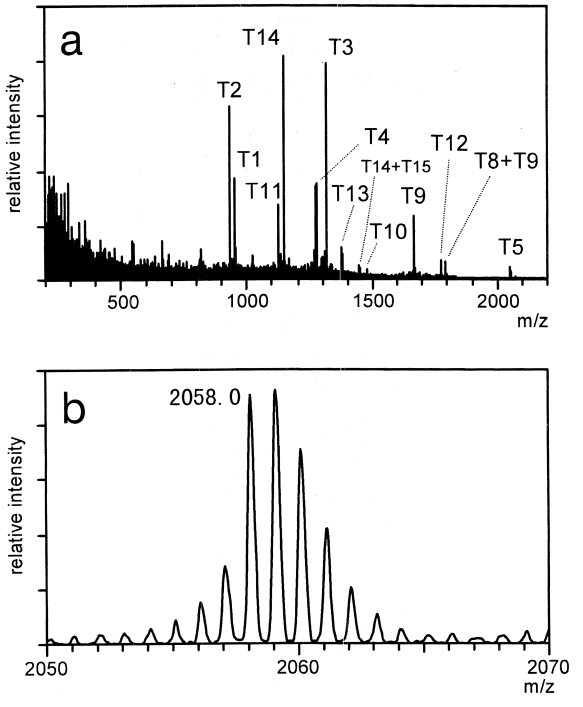

Fig. 2. FAB mass spectra of tryptic digest of mutant hemoglobin. (a) The peptide number from the N-terminus is shown above the signal. (b) The molecular ion region for T5 peptide is depicted. The monoisotopic mass of m/z 2058.0 for the protonated molecular ion of this peptide was smaller than that of the normal peptide by a single mass unit.

3.5. Sequencing by CID analysis

The mutation was verified by MALDI-TOF-MS of the isolated peptide T5. As shown in Fig. 3, a y-series of fragment ions was clearly observed in the CID mass spectrum. The signal at m/z 1764.2 in the mutant sample indicated glutamine at the 43rd residue, coinciding with the mutated code of CAG. The mutation of β43 Glu→Gln has been reported in Hb Hoshida [11,12].

3.6. Overall strategy and conclusion

The strategy is summarized in Fig. 4. A mutation of the genetic code is identified in the globin mRNA. In parallel, ESI-MS or MALDI-TOF-MS of whole molecules determines the presence of a mutated protein and the size of the molecular mass change as well. In the present study, however, the resolution of the standard mass spectrometer, approximately a few thousand, did not allow unit mass resolution of an isotopic cluster of a globin molecule of 15 kDa, and thus failed to detect a mutation giving a change of less than a few mass units [13]. The problem may be overcome by Fourier-transform MS, which enables analysis with a high resolution of over 100 000 [14].

Fragmentation into peptides allows discrimination of such a minute change even with measurements at a resolution of 3000, as shown in the present FAB-MS analysis. However, it is difficult for FAB-MS to identify small peptides, e.g. dipeptides that should be observed at m/z 246.2 and at m/z 319.1 in Fig. 2, in a digested mixture, due to chemical background signals derived from clusters of sample matrix or from small fragment ions generated during ionization. MALDI-TOF-MS is not free of the same difficulty (due to signals of sample matrix. In view of this problem, measurement of an undigested molecule described above is not redundant.

MS–MS or CID-MS finally verifies the structure (Fig. 4). In the present study a peptide containing a mutation was isolated from a tryptic digest and subjected to subsequent CID analysis, but tandem mass spectrometry (MS–MS) or on-line LC–MS–MS can replace these steps [5,6,12]. The strategy including genetic analysis and MS, presented herein facilitates high throughput characterization of mutant hemoglobins.

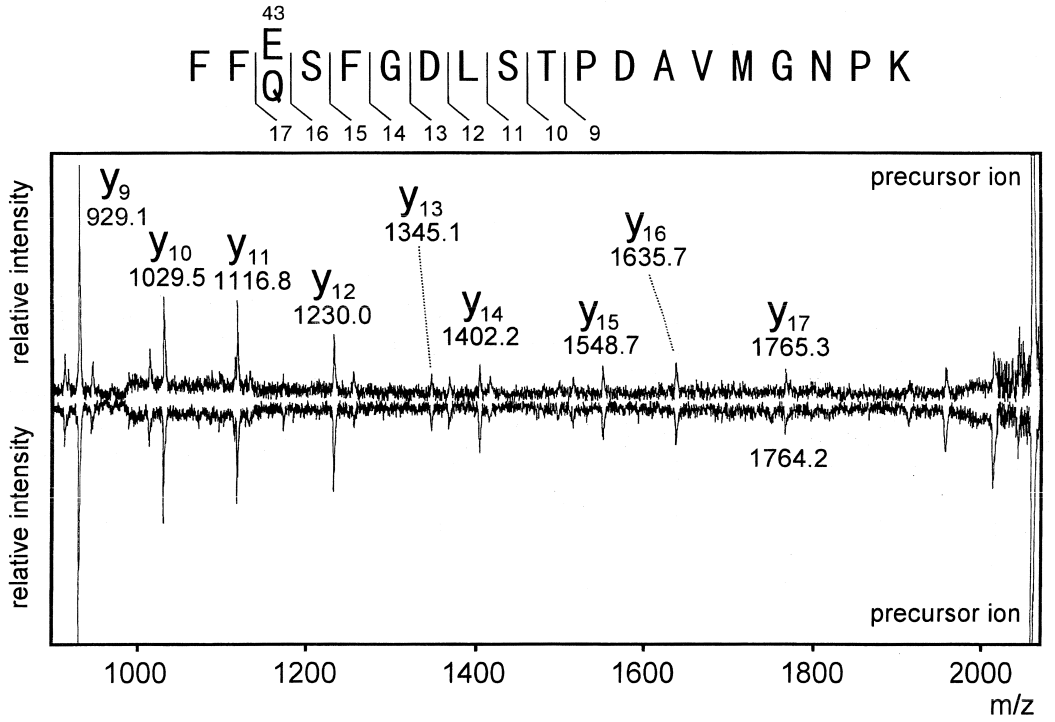

Fig. 3. MALDI-TOF mass spectra of ions produced by CID of T5 peptide, corresponding to residues 41–59 of β-globin. The normal (upper) and mutant (lower) samples are presented in mirror image. Diagrammatic representation of peptide bond fragmentations detected are shown above the spectrum. Glutamine at 43 is the residue substituted for glutamic acid in the mutant sample. The m/z values for the peak-top of ion signals are presented.

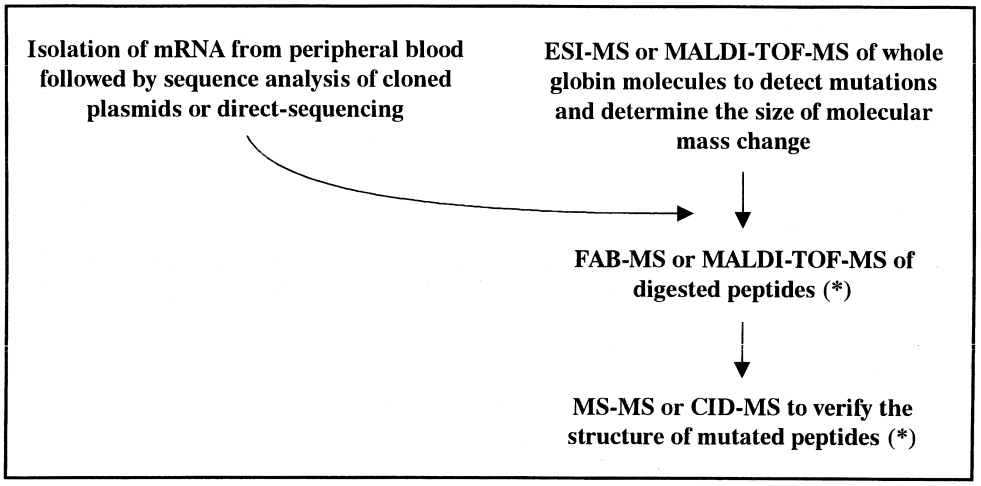

Fig. 4. Structural characterization of mutant hemoglobins. The steps indicated by asterisks can be combined with LC–MS–MS analysis.

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