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Review

Advanced analytical methods for hemoglobin variants

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Abstract

Hemoglobin variants are the protein mutations most often encountered in the clinical scene. They have been useful for developing methods to analyze mutant proteins because of their size and ease of collection in large amounts. Improvements in analytical methods have been directed toward higher resolution in electrophoresis and shorter elution times in chromatography. More importantly, in the last 20 years, hemoglobin variants have been used in the development of mass spectrometric strategies for analyzing protein mutations. This approach consists of a series of steps: measurement of the molecular mass of globins to detect or confirm the presence of mutations, peptide mass mapping or peptide mass fingerprinting of an enzymatic digest to identify mutated peptides, and tandem mass spectrometry to determine or confirm the site and type of mutation. The mass spectrometric strategy has enabled rapid analysis and demonstrated a superb ability to detect a number of hemoglobin variants, particularly those without a change in electrophoretic or chromatographic properties. Even with the recent advances in DNA analysis, protein analysis is still essential, because post-translational modifications following amino acid substitutions can occur including N-terminal acetylation, deamidation and oxidation-mediated processes.

Keywords: Reviews; Peptide mass fingerprinting; Post-translational modification; Hemoglobin variants; Protein mutations

Contents

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1. Introduction

Human hemoglobin is a tetrameric protein composed of two different globin subunits, each of which non-covalently binds a heme molecule. The subunits are α- and β-globins consisting of 141 and 146 amino acid residues, respectively, for the major hemoglobin Hb A (or Hb A_0). The α-globin also constitutes the adult minor species, Hb A_2, and the fetal species, Hb F, with δ- and γ-globins, respectively, in place of β-globin. The vast majority of hemoglobin variants have single amino acid substitutions in one of the globin molecules and are derived from single nucleotide substitutions in the corresponding gene [1,2]. Most cases of hemoglobin mutations are heterozygous except for sickle cell hemoglobin in the high-incidence region. Hemoglobin with abnormal α-globin is present at a smaller percentage (10–25% of the total hemoglobin) than hemoglobin with mutant β-globin (35–50%). This is explained by the duplication of the α-globin gene locus. For either subunit, the amounts of the variants are reduced owing to precipitation or catabolism in the cases of structurally unstable variants that cause hemolysis.

To date, 700 different hemoglobin variants have been identified [3,4]. Some are associated with hematological disorders such as anemia or polycythemia (see below), while others are asymptomatic and were found by population-based screening studies using electrophoresis. More recently, hemoglobin variants have been collected in laboratory measurements of glycated hemoglobin for diabetes by means of ion-exchange chromatography [5]. The hemoglobin variants with altered stability or function and thus clinical significance cause the following manifestations (Table 1). Homozygous Hb S causes hemolytic anemia (sickle cell anemia) and vasoocclusion. Sickle cell trait, or heterozygous Hb S, is asymptomatic except for an uncommon but highly distinctive symptom of painless hematuria. Unstable hemoglobin variants cause non-immune hemolytic anemia. High-oxygen-affinity variants are often asymptomatic and should be suspected in patients with erythrocytosis. Low-affinity variants are found in patients with cyanosis or a low hematocrit without apparent causes after thorough evaluation. Methemoglobin should be suspected in the cyanotic patients whose blood does not ''pink up'' when equilibrated with room air.

Structural analysis of hemoglobin variants consists of two steps, detection and structure elucidation. Electrophoresis is used for the detection of variants, and chromatography is used to separate the peptides containing amino acid substitutions that will later be determined by Edman-based sequence analysis [6]. However, this strategy has changed drastically in the last two decades through the development of mass spectrometry (MS) [7]. In 1981, MS was first used for the analysis of a complex mixture of peptides from hemoglobin variants [8,9]. A few years later, another ionization method, fast-atom bombardment, contributed to the establishment of a new basic strategy, called peptide mass mapping, that allowed both the detection and the characterization of hemoglobin variants [10]. At the beginning of the 1990s, MS joined the pool of detection methods that could analyze whole globin molecules of M_r (relative molecular mass) 1.5×10^4 [11,12]. This was first brought about by the advent of electrospray ionization (ESI), which allows proteins with molecular masses previously considered to be beyond the capability of MS to be analyzed by using multiply charged species that bring the mass-to-charge ratio (m/z) down to within the limits of the available mass analyzers. Currently, matrix-assisted laser desorption/ionization (MALDI) in combination with time-of-flight (TOF) mass analyzers enables the measure-

Table 1
Representative hemoglobin variants with clinical significance

Name	Mutation	Population	Major clinical abnormalities
S	β6E→V	African	Anemia, ischemia, infarcts
Köln	β98V→M	Sporadic	Hemolytic anemia
Linkoping	β36P→T	North Europe, sporadic	Polycythemia
Kansas	β102N→K	Sporadic	Mild anemia
M Iwata	α87H→Y	Sporadic	Methemoglobinemia

ment of singly charged molecular ions of intact proteins. Sequence analysis by Edman degradation is replaced by a mass spectrometric sequencing technique that is based on collision-induced dissociation (CID) [13]. This review summarizes the electrophoretic and chromatographic methods for detecting hemoglobin variants and describes the strategy involving the modern mass spectrometric techniques.

2. Detection of hemoglobin variants

2.1. Electrophoretic methods

The usual method for screening hemoglobin variants is electrophoresis, because hemoglobin is the most abundant protein in blood and does not require staining for visualization. Isoelectric focusing in polyacrylamide slab gels is the most widely used electrophoretic method because of its good resolution [14] (Fig. 1). In principle, electrophoresis is applicable to detect mutations that alter the charge of the hemoglobin molecule, but some mutations, which should be silent with respect to charge, can still cause alterations in the higher-order structure that lead to a change in the overall charge of the molecule and thus become detectable. Mutations with such alterations tend to be associated with abnormal function or oxygen affinity and thus are often clinically significant.

After electrophoresis, the variant hemoglobin can be recovered from the gel. The band corresponding to the mutant hemoglobin is cut out and dipped in 2% (v/v) acetic acid. After standing for 2 h, the hemoglobin that has been eluted from the gel is collected [15]. This is a useful purification technique for subsequent structural analysis.

Isoelectric focusing under denaturing conditions is another method of analyzing hemoglobin variants [16]. In this case, the hemoglobin is incubated in a denaturing solution of 6 *M* urea and 1 *M* 2-mercaptoethanol and analyzed in an acrylamide slab gel containing 6 *M* urea. The two globin subunits are separated, and the mutant globins that differ from the normal chain by their charge are readily distinguished (Fig. 2). This method can be applied to dried blood samples. A large-scale screening of fetal hemoglobin variants has been carried out using this method [17].

2.2. Chromatographic methods

Native hemoglobin is fractionated by ion-exchange chromatography. The chromatographic system using cation-exchange monobead resins results in good resolution, and typical examples of hemoglobin analysis are presented in the application notes provided by the manufacturer. More recently, a

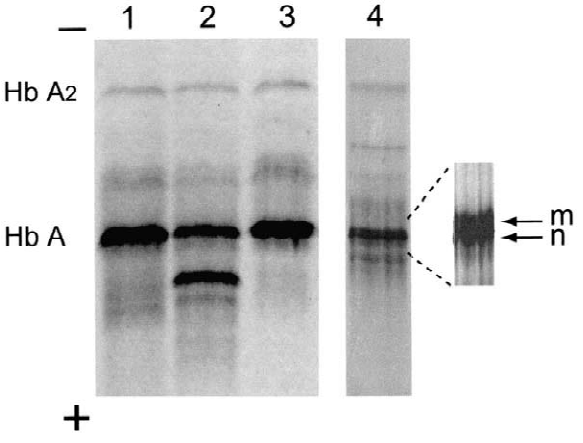

Fig. 1. Isoelectric focusing of hemoglobin. Lane 1, normal hemolysate; lane 2, Hb Moriguchi β97H→Y; lane 3, Hb Miyazono β79D→E (unseparated); lane 4, Hb Linkoping (Meilahti) β36P→V. A very small difference between the isoelectric points of Hb Linkoping (m) and normal HbA (n) is detectable.

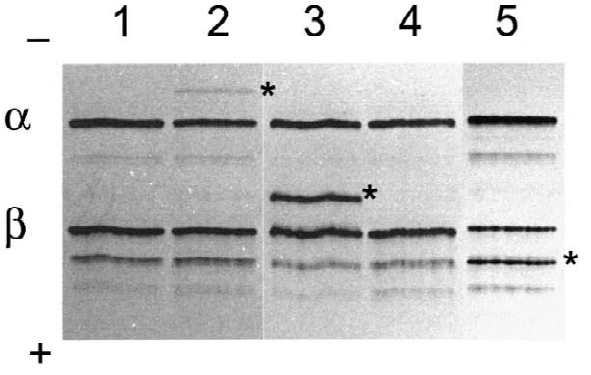

Fig. 2. Isolectric focusing of globins in 6 *M* urea. Lane 1, normal globins from adult blood; lane 2, Hb Waimanalo α64D→N; lane 3, Hb S β6E→V; lane 4, Hb Rainier β145Y→C (unseparated); lane 5, Hb Tacoma β30R→S. The mutated globins are marked by asterisks.

porous silica medium improved the elution time of hemoglobin analysis while still maintaining the high resolution required to meet the demands for both screening and confirmatory purposes [18].

Reverse-phase chromatography detects a change in the hydrophobicity of the globin subunits. In this technique, the hemoglobin is dissolved in 0.1% (v/v) trifluoroacetic acid and injected onto a reversed-phase C_4 column followed by elution with a gentle acetonitrile gradient [19,20]. The heme is dissociated and the globin subunits as well as their variants are separated (Fig. 3).

A large amount of globin subunits can be separated using an open column packed with cation-exchange resin in the presence of 8 *M* urea and 50 m*M* 2-mercaptoethanol. This method allows the purification of variant globins with an altered charge.

2.3. Mass spectrometry

MS is another choice for detecting hemoglobin variants. The molecular mass of proteins can be determined currently by ESI- or MALDI–MS. The average mass of α- and β-globins is M_r 15126.4 and 15867.3, respectively. The difference in the molecular mass between amino acids ranges from 0 to 129 mass units, and thus most amino acid substitutions of hemoglobin are efficiently analyzed by MS except for the substitutions involving Leu/Ile (Δ 0 mass unit) and Lys/Gln (Δ 0.036 mass units), which are silent in nominal mass [12].

The resolution of the mass measurement is a critical factor for successfully detecting the mutation. The resolution of the most widely used mass spectrometers is within a range from a few to ten thousand. For small proteins or peptides, it is possible to correctly identify the ^{12}C monoisotopic species in the molecular ion clusters, and, therefore, the molecular mass of these molecules is expressed by the monoisotopic unit called ''exact mass'' (Fig. 4). On the other hand, for larger molecules over M_r 10 000 including globins, the ''average mass'', or so-called ''molecular mass'', is used. This kind of ''mass'' is read from the value corresponding to the center of molecular ion clusters in the mass spectrum. A resolution of at least a few thousand is necessary to measure the molecular mass of purified globin molecules to an accuracy of 1 mass unit, and even higher resolution is required to recognize the presence of a heterozygous variant representing a mass change of less than 10 mass units [7].

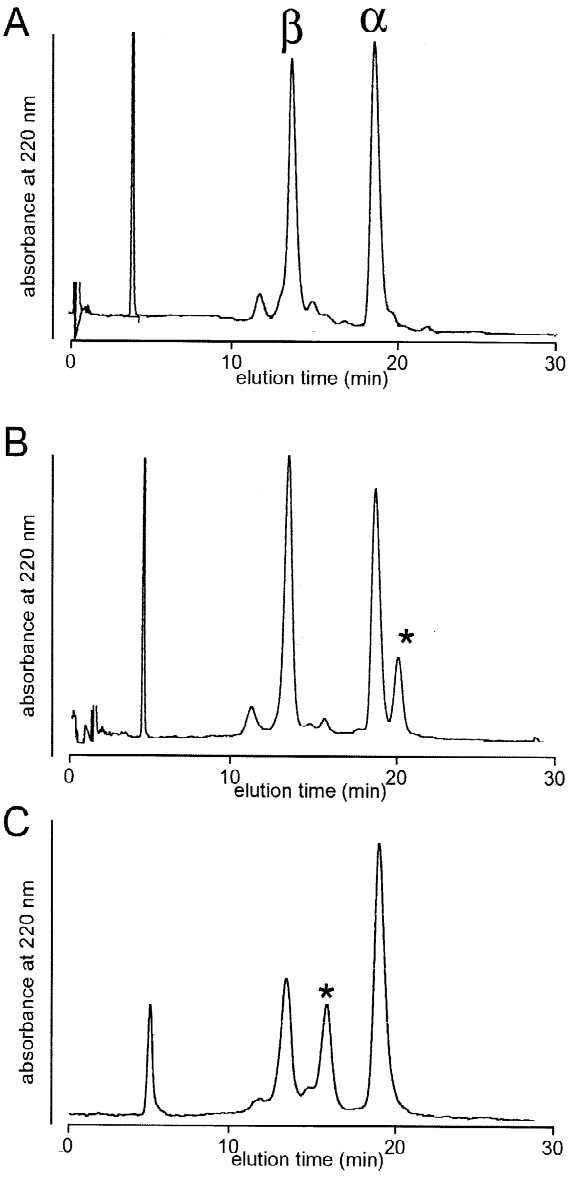

Fig. 3. Reverse-phase chromatography of globins on a C_4 column. The globins were eluted by increasing concentrations (38–50%) of acetonitrile in 0.1% trifluoroacetic acid. (A) Normal chromatogram; (B) Hb Tokyo-II α89H→N; (C) Hb Moriguchi β97H→Y. The mutated globins are marked by asterisks.

The ESI generates multiply charged (protonated) ions of the component molecules, and the resulting

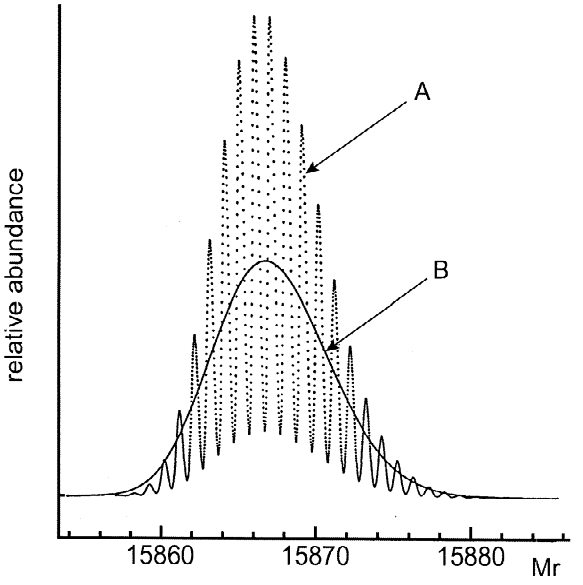

Fig. 4. Isotopic distributions of β-globin. Theoretical drawing at resolutions of 15 000 (A) and 2000 (B) are presented. The monoisotopic mass (^{1}H, ^{12}C, ^{14}N, ^{16}O and ^{32}S), average mass and nominal mass of β-globin are 15 857.3, 15 867.2, and 15 849, respectively. The unit-mass spacing of peaks at a resolution of 15 000 is primarily due to ^{13}C isotopic distributions.

spectrum is complicated (Fig. 5). In contrast, MALDI produces mainly singly charged species and the mass spectrum is simple (Fig. 6).

The sample preparation of hemoglobin for the MALDI is very simple. The hemolysate at a hemoglobin concentration of 1% (w/v) is mixed with 5 vol. of matrix solution, or saturated 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) dissolved in 30% acetonitrile and 0.1% trifluoroacetic acid, and dried on the MALDI sample plate.

2.4. Advanced MS performance

Superb resolving power is achieved by Fourier transform ion cyclotron resonance (FTICR) MS. The combination of ESI with FTICR shows very good potential for the characterization of biopolymers with part-per-million mass accuracy and mass resolution exceeding 10^6 [21]. The on-line integration of ESI-FTICR with capillary zone electrophoresis (CZE) is currently an excellent approach for microsample analyses. In fact, this combination produced a mass spectrum of hemoglobin from a single erythrocyte corresponding to ~450 amol with mass resolution in excess of 45 000 [22], and allowed the direct identification of a variant globin differing in molecular mass by 1 mass unit from the normal one [23] (Fig. 7).

3. Characterization of hemoglobin variants

Determination of the primary amino acid sequence of proteins resembles the challenge of solving a complex but ultimately logical jigsaw puzzle. However, the analysis of hemoglobin variants is much less formidable, since these generally represent single amino acid substitutions in the known structure of normal globins. Structural analysis of protein variants requires two steps, peptide-mapping and sequence analysis [7]. However, a considerable proportion of hemoglobin variants can be characterized by peptide-mapping alone, as described below.

The primary purpose of peptide-mapping is to find the mutated peptide derived from the enzymatic digest of the variant globin. Enzymatic cleavage of intact globins leaves an uncleaved portion or ''core''. The alkylation of cysteine thiols solves this problem. It also prevents intra- or inter-molecular disulfide formation during sample preparation, which is necessary even though these linkages do not occur in the native hemoglobin. Carboxy- or carbamide-methylation with iodoacetic acid or iodoacetamide, respectively, after reduction is usually employed for this purpose, and is carried out in a denaturing solution such as 6 *M* guanidine. Alternatively, the oxidation of the cysteine residues by performic acid is used to prevent disulfide formation.

The standard enzyme for cleaving globins into peptides is trypsin. In some cases, other enzymes such as endoproteinase Asp-N are used to generate moderately sized peptides, especially for α-globin which generates large tryptic peptides [24].

The removal of salts (desalting) prior to MS is necessary because the formation of adduct ions with alkali metals such as sodium and potassium will reduce the abundance of the protonated molecular ion $[M+H]^+$ species, which are the informative ions for the mass measurement of proteins and peptides. Adducts also unnecessarily complicate mass spectra.

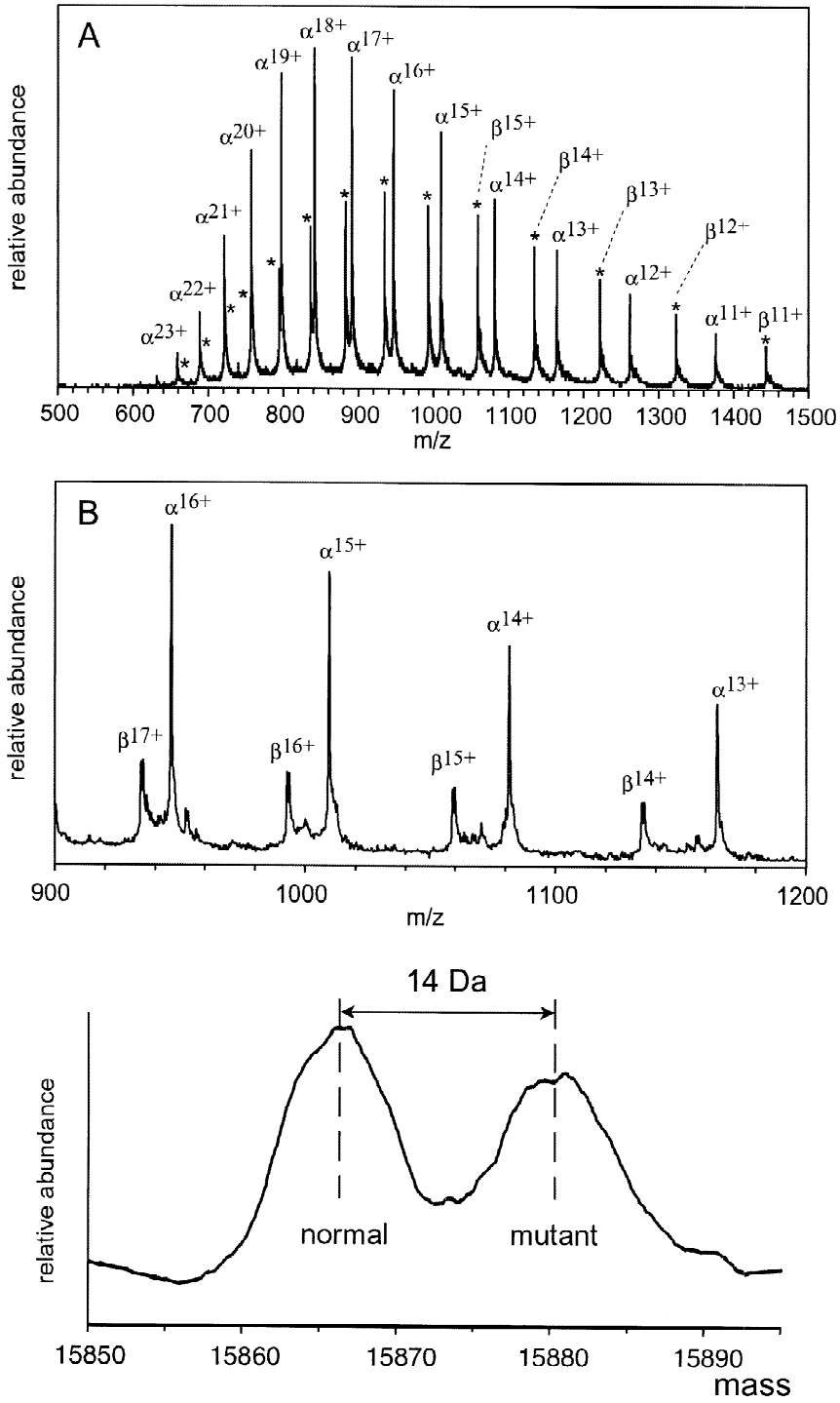

Fig. 5. ESI mass spectrum of globins. (A) Normal globins. The ions for β-globin are indicated by asterisks. The ions are protonated species, and the number of charges are given above the signals. (B) Globins from Hb Miyazono β79D→E. The signals for β-globin are doublets. (C) Deconvoluted peaks resulting from the transformation of the mass spectrum shown in B. The difference in molecular mass is determined to be 14 mass units.

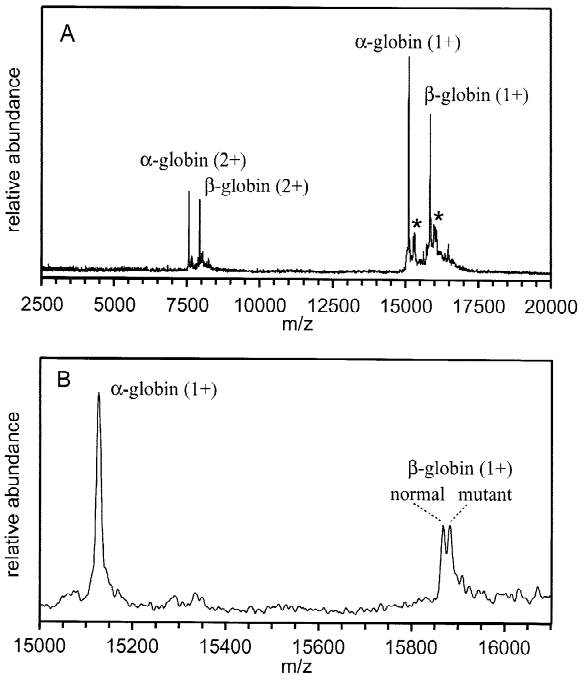

Fig. 6. MALDI–TOF mass spectra of globins. (A) Normal globins. Hemolysate was directly mixed with the matrix solution and then dried on the MALDI sample plate. Single and double charge ions of α- and β-globins are observed. Glycated species (asterisks) are identified. Heme is detected at m/z 616.2 (data not shown). (B) Globins from Hb Miyazono β79D→E. The mutant β-globin with a molecular mass 14 mass units larger than normal is detected.

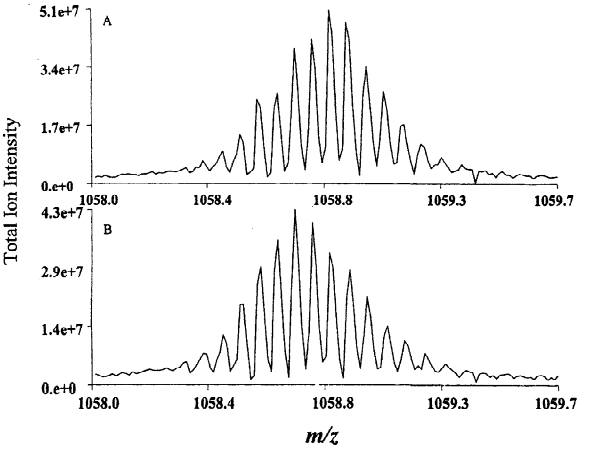

Fig. 7. High-resolution FTICR spectra of the 15+ charge state of the β-globins in (A) Hb A and (B) Hb C β6E→K. The change in molecular mass is 0.948 mass units. The unit-mass spacing of isotopic constituents is resolved. (Reproduced from Ref. [22] under permission of the publisher)

Recently, desalting can generally be effectively performed at a small but sufficient scale using a device such as Ziptip™ (Millipore) or Poros™ (Applied Biosystems), packed with C_{18} or C_4 medium.

3.1. Peptide mapping by chromatographic methods

Conventionally, reversed-phase chromatography using a C_{18} column and a gradient of acetonitrile or methanol is widely used for separating peptides [25,26]. Any alterations, such as a shift in retention time, or new or missing signals indicating the mutated peptides, are easily recognized when compared with the chromatograms of normal samples. The mutated peptides are then isolated and subjected to sequencing or amino acid analysis.

3.2. Peptide mass fingerprinting

Peptide mapping by MS is called ''peptide mass fingerprinting'' or ''peptide mass mapping'', because the individual peptides in a mixture are characterized in terms of their molecular mass. MS of peptides is carried out by ESI or MALDI. ESI produces a rather complicated mass spectrum due to the multiply charged ions (Fig. 8). The use of ESI in combination with tandem mass spectrometry (MS/MS) permits the amino acid sequencing of mutated peptides without first isolating them from peptide mixtures, as described below. ESI also allows on-line LC–MS configuration. This hyphenation method facilitates the detection of mutated peptides with a good signal-to-noise ratio compared with the mixture analysis. However, it is not an exclusive choice for globins, from which only 15 peptides are generated. In addition, MALDI produces a lucid mass spectrum of singly charged ions (Fig. 9).

In many cases, the molecular mass of the mutated peptides allows for the amino acid substitutions to be determined without any further analyses [7]. For example, the site and type of the mutation can be determined when it involves an enzymatic cleavage site (Fig. 10). In other cases, the molecular mass of a

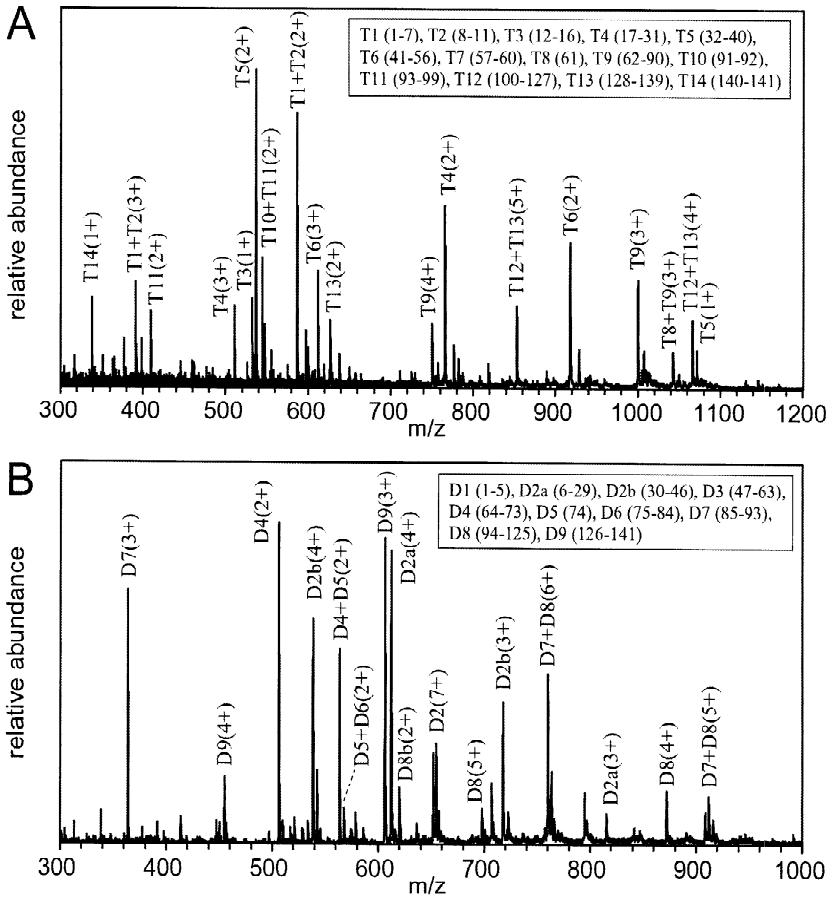

Fig. 8. ESI mass spectra of enzymatic digests of carboxymethylated α-globin. (A) Tryptic digest. (B) Endoproteinase Asp-N digest. The number above each peak is that of the predicted peptide starting from the N-terminus. The residue numbers of each peptide are indicated in parentheses in the inset.

mutated peptide will limit the number of possible amino acid substitutions it contains.

3.3. Sequence analysis by MS

In MS–MS, collision with a neutral gas between two MSs is presently the most widely used way to achieve collision-induced dissociation (CID). The resulting fragment ions (product ions) are then mass analyzed in a second mass spectrometer. It is generally easy to interpret the tandem mass spectrum of mutated peptides, because the spectrum can be compared with that from the normal counterpart [13] (Fig. 11). However, CID does not always produce any key fragments in a quantity sufficient to define the mutation. This occurs when the mutation is present in a large peptide or when there is inefficient dissociation at the mutated residue. The use of different kinds of enzymes would circumvent the former difficulty by generating a peptide with a size amenable to CID analysis [24].

The sequencing of intact proteins by CID may become powerful as the technology improves, but, at present, the sequence that can be analyzed by this method is confined to only a small part of the globin molecule [27].

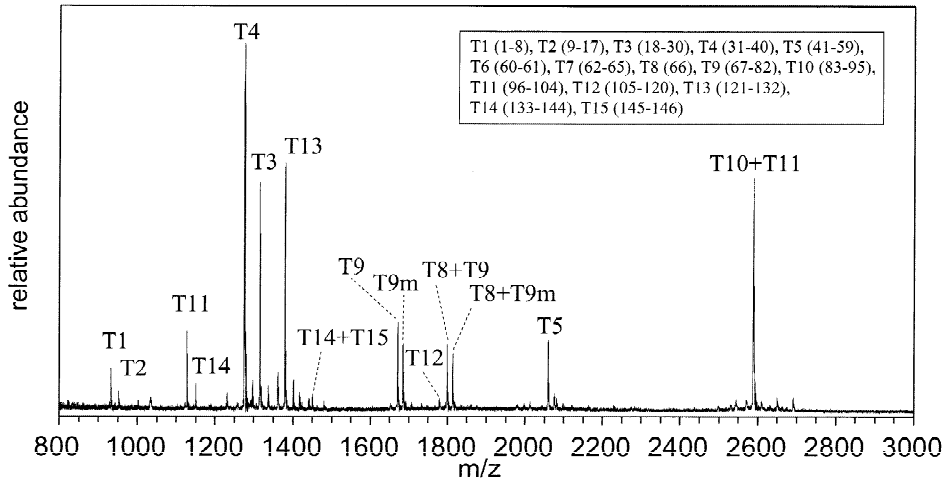

Fig. 9. MALDI mass spectrum of a tryptic digest of the β-globin from Hb Miyazono. The protonated molecular ion of the mutated peptide T9m is detected at m/z 1683.9, which is 14 mass units larger than that of normal peptide T9. A combined peptide T8+T9m is also detected.

4. DNA analysis

The globin mRNAs are comprised of less than 500 nucleotide bases, and they are easily isolated from peripheral blood. Therefore, for detecting variants, the genetic approach is a practical alternative to the protein-based methods. In particular, DNA analysis is required when the mutation leads to severe instability of the protein, such that the hemoglobin variant is readily broken down after it is synthesized. This occurs in the cases of hemolytic anemia. However, the hemoglobin variants are heterozygous in the globin gene locus, and, moreover, the α-globin gene is duplicated. Therefore, direct sequencing of the amplified products may give an ambiguous result, especially for α-globin variants.

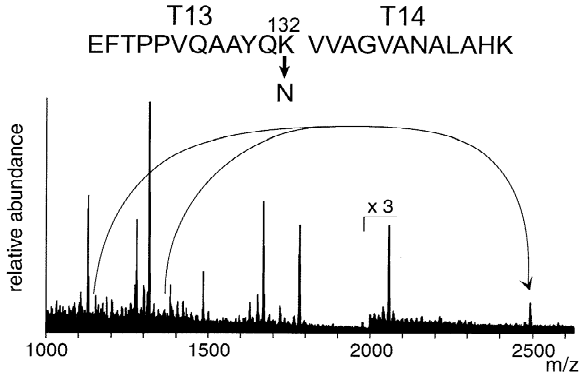

Fig. 10. An example of a missing cleavage site. In this example of a β-globin variant, a mutation at the lysine residue at position 132 is readily recognized by the lack of tryptic peptides 13 (T13) and 14 (T14). The new peptide is observed at m/z 2495.3, indicating the replacement of lysine by asparagine. These data were generated by fast atom bombardment MS.

Post-translational modification is known to occur following amino acid substitutions. Four categories of such changes have been described in hemoglobin variants. Native hemoglobin is not N-terminally acetylated except for the γ-globin of fetal hemoglobin, because the N-terminal valine of α-, β-, and δ-globins inhibits the acetylation. However, mutations involving the N-terminal amino acids can disrupt this process, resulting in either the initiator methionine being retained, or acetylation, or both [28,29]. Deamidation of an asparaginyl residue to an aspartate has been reported at several but specific sites of α- and β-globins in a number of cases.

Two other types of post-translational modification are associated with severe hemolysis and may be explained by an oxidation-based mechanism. The

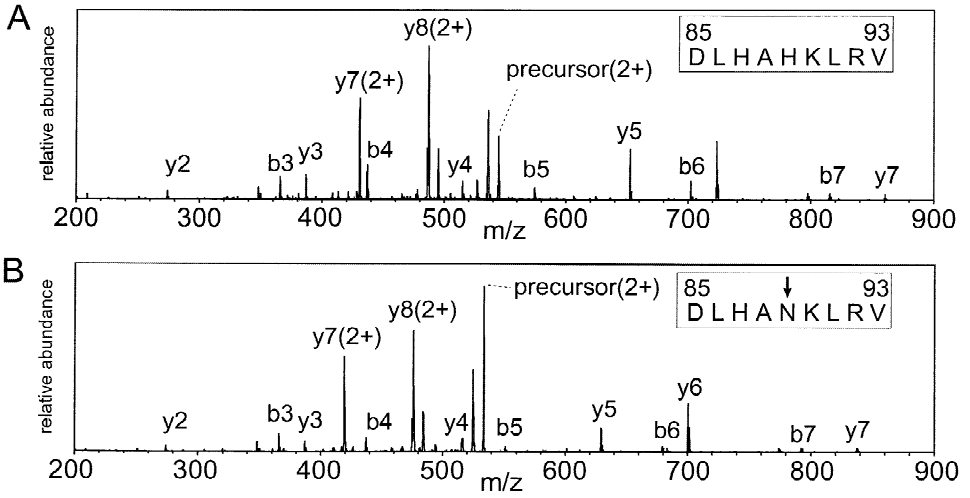

Fig. 11. CID mass spectra of the peptides from endoproteinase Asp-N digestion of α-globin. (A) As a reference, the $[M+2H]^{2+}$ ion for the normal peptide at m/z 544.8 was selected as the precursor. (B) The $[M+2H]^{2+}$ ion for the mutated peptide at m/z 533.3 was selected as the precursor. Note the difference in m/z values for the b4, b5, y4, and y5 product ions between the two spectra, indicating the replacement of histidine by asparagine at position 89.

amino acid substitution of Hb Bristol is β67 Val→Asp, while DNA analysis predicts Met at this site [30]. An oxidation-mediated process may be involved in the conversion of methionine to aspartate. The last example is the modification of the normal β141 residue, leucine, to, most likely, hydroxyleucine [31]. The same change has been reported in association with amino acid substitutions at other sites. These modifications show the importance of characterizing hemoglobin variants completely at both the protein and DNA levels.

5. Concluding remarks

More than 40 years ago, the sickle cell hemoglobin mutation was characterized by a fingerprinting method that was a two-dimensional analysis combining paper chromatography and electrophoresis [32]. The mutated peptide in the aberrant spot was then subjected to Edman analysis. The methods that were used to characterize hemoglobin variants for 30 years thereafter were basically dependent on the chemical properties of peptides. On the other hand, the mass spectrometric methods available today characterize proteins and peptides in terms of their physical properties, and allow rapid analysis [9]. As a result of the recent enthusiasm for proteomics, mass spectrometers competent for protein analysis have become part of many laboratories and institutions. The down-sizing of the instruments, while retaining their resolution and sensitivity, will further popularize the MS-based strategy for characterizing hemoglobin variants.

References

[1] G. Stamatoyannopoulos, P.W. Majerus, R.M. Perlmutter, H. Varmus, The Molecular Basis of Blood Diseases, 3rd ed, Saunders, Philadelphia, PA, 2001.
[2] C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, The Metabolic and Molecular Bases of Inherited Disease, 8th ed, McGraw-Hill, New York, 2001.
[3] International Hemoglobin Information Center, Hemoglobin 20 (1996) 215.
[4] On the web (http://globin.cse.psu.edu/)
[5] E. Bisse, N. Zorn, A. Eigel, M. Lizama, P. Huaman-Guillen, W. Marz, A. Van Dorsselaer, H. Wieland, Clin. Chem. 44 (1998) 2172.
[6] E. Antonini, L. Rossi-Bernardi, E. Chiancone (Eds.), Methods Enzymol., Vol. 76, 1981.
[7] Y. Wada, in: J.M. Walker (Ed.), The Protein Protocols Handbook, 2nd ed, Humana Press, Totowa, NJ, 2002, pp. 681–692.

[8] Y. Wada, A. Hayashi, T. Fujita, T. Matsuo, I. Katakuse, H. Matsuda, Biochim. Biophys. Acta 667 (1981) 233.
[9] Y. Wada, A. Hayashi, T. Fujita, T. Matsuo, I. Katakuse, H. Matsuda, Int. J. Mass Spectrom. Ion Phys. 48 (1983) 209.
[10] Y. Wada, T. Matsuo, T. Sakurai, Mass Spectrom. Rev. 8 (1989) 379.
[11] C.H. Shackleton, A.M. Falick, B.N. Green, H.E. Witkowska, J. Chromatogr. 562 (1991) 175.
[12] Y. Wada, Biol. Mass Spectrom. 21 (1992) 617.
[13] A.M. Falick, C.H. Shackleton, B.N. Green, H.E. Witkowska, Rapid Commun. Mass Spectrom. 4 (1990) 396.
[14] P. Basset, Y. Beuzard, M.C. Garel, J. Rosa, Blood 51 (1978) 971.
[15] R. Kaneko, Y. Wada, M. Hisada, H. Naoki, T. Matsuo, J. Chromatogr. B Biomed. Sci. Appl. 731 (1999) 125.
[16] K. Altland, M. Kaempfer, H. Granda, Hum. Genet. 53 (1979) 97.
[17] Y. Wada, T. Fujita, K. Kidoguchi, A. Hayashi, Hum. Genet. 72 (1986) 196.
[18] C.-N. Ou, C.L. Rognerud, Clin. Chim. Acta 313 (2001) 187.
[19] L.F. Cognote, H.P.J. Bennett, S. Solomon, Biochem. Biophys. Res. Commun. 89 (1979) 851.
[20] J.B. Shelton, J.R. Shelton, W.A. Schroeder, J. Liq. Chromatogr. 7 (1984) 1969.
[21] K.D. Henry, J.P. Quinn, F.W. McLafferty, J. Am. Chem. Soc. 113 (1991) 5447.
[22] S.A. Hofstadler, J.C. Severs, R.D. Smith, F.D. Swanek, A.G. Ewing, Rapid Commun. Mass Spectrom. 10 (1996) 919.
[23] L. Yang, C.S. Lee, S.A. Hofstadler, L. Pasa-Tolic, R.D. Smith, Anal. Chem. 70 (1998) 3235.
[24] T. Nakano, Y. Wada, J. Mass Spectrom. Soc. Jpn. 48 (2000) 341.
[25] W.A. Schroeder, J.B. Shelton, J.R. Shelton, D. Powars, J. Chromatogr. 174 (1979) 385.
[26] J.B. Wilson, H. Lam, P. Pravatmmuang, T.H.J. Huisman, J. Chromatogr. 179 (1979) 271.
[27] K.J. Light-Wahl, J.A. Loo, C.G. Edmonds, R.D. Smith, H.E. Witkowska, C.H. Shackleton, C.S. Wu, Biol. Mass Spectrom. 22 (1993) 112.
[28] J.P. Boissel, T.J. Kasper, S.C. Shah, J.I. Malone, H.F. Bunn, Proc. Natl. Acad. Sci. USA 82 (1985) 8448.
[29] C. Vasseur, Y. Blouquit, J. Kister, D. Prome, J.S. Kavanaugh, P.H. Rogers, C. Guillemin, A. Arnone, F. Galacteros, C. Poyart, J. Rosa, H. Wajcman, J. Biol. Chem. 267 (1992) 12682.
[30] D.C. Rees, J. Rochette, C. Schofield, B. Green, M. Morris, N.E. Parker, H. Sasaki, A. Tanaka, Y. Ohba, J.B. Clegg, Blood 88 (1996) 341.
[31] S.O. Brennan, J. Shaw, J. Allen, P.M. George, Br. J. Haematol. 81 (1992) 99.
[32] V.M. Ingram, Nature 178 (1956) 792.