Use of Combined Mass Spectrometry Methods for the Characterization of a New Variant of Human Hemoglobin: The Double Mutant Hemoglobin Villeparisis $\beta 77(\text{EF1}) \text{His} \rightarrow \text{Tyr}$, $\beta 80(\text{EF4}) \text{Asn} \rightarrow \text{Ser}$

Danielle Promé, Catherine Deon, and Jean-Claude Promé

Laboratoire de Pharmacologie et Toxicologie Fondamentales du CNRS, 31077 Toulouse, France

Henri Wajcman and Frédéric Galacteros

INSERM U91, Hôpital Henri Mondor, 94010 Creteil, France

Yves Blouquit

INSERM U350, Institut Curie, Faculté des Sciences, Orsay, France

Hemoglobin Villeparisis was found during a systematic measurement of glycosylated hemoglobin. Electrospray mass spectra of the globin indicate an apparently unchanged molecular weight within the error range (0.01%). The tryptic digest of the β chain shows a chromatographically abnormal $\beta\text{T-9}$ peptide. The mass-to-charge ratio value of its $[\text{M} + \text{H}]^+$ ion, as measured by liquid secondary ionization mass spectrometry, is one mass unit lower than that of the normal $\beta\text{T-9}$. However, the electrospray mass spectrum of this peptide exhibits mainly a doubly charged ion, whereas the normal $\beta\text{T-9}$ gives a triply charged ion. None of the allowed single amino acid substitutions for a 1-u shift down (Glu $\rightarrow$ Gln, Asp $\rightarrow$ Asn, or Asn $\rightarrow$ Ile) can explain the suppression of one protonation site. This can be due only to the replacement of the internal histidine by a nonbasic residue. Thus at least two amino acid exchanges occur within the same peptide: one involves the internal histidine, and the sum of the mass shifts is -1 u. Consideration of the $\beta\text{T-9}$ sequence and taking account for the genetic code rules, the only possibility was $^{11}\text{His} \rightarrow \text{Tyr}$ ($+26$ mass shift) associated with $^{14}\text{Asn} \rightarrow \text{Ser}$ (-27 mass shift). This conclusion was consistent with the tandem mass spectrum of the $[\text{M} + \text{H}]^+$ ion and was further confirmed by chemical microsequencing. (*J Am Soc Mass Spectrom* 1996, 7, 163–167)

In the past decade, a noteworthy success of mass spectrometry in determination of protein primary structures has been its application for the characterization of human hemoglobin variants [1–6]. The strategy used for their structural identification requires several steps, in most of which mass spectrometry may be utilized conveniently. The initial detection of the variant classically is done by electrophoresis or chromatography. The second step consists in a presumptive identification that requires a battery of electrophoretic and functional tests [7], and most recently a mass measurement of the intact protein has been added to these procedures [6]. When, at this point of the investigation, the variant remains unidentified,

protein chemistry methods may be used to determine the change in primary structure. Peptide mapping that uses reverse-phase high-performance liquid chromatography (RP-HPLC) may reveal an abnormal peptide after enzymic digestion. Analysis of the amino acid composition of this peptide or mass measurement by electrospray or fast-atom bombardment mass spectrometry (FAB-MS) may provide further information. Finally, the location of the amino acid exchange may be determined by Edman degradation and microsequencing or by tandem mass spectrometry [8–10]. Assuming that the point mutation is due to a single base substitution in a codon triplet of the DNA, all the amino acid exchange possibilities are not allowed. According to the genetic code, there is a strong limitation in the number of possible amino acid exchanges that are compatible with a measured mass shift [11, 12].

Among the more than 650 hemoglobin variants described, only a few are due to mechanisms other than

Address reprint requests to Dr. Jean-Claude Promé, Laboratoire de Pharmacologie et Toxicologie Fondamentales du CNRS, 205 Route de Narbonne, 31077 Toulouse Cedex, France.

point mutations in the coding sequence. These variants include fusion proteins, elongated or shortened chains, or subunits that have more than one amino acid change [13]. For instance, 16 variants, which include the one described here, carry two point mutations within the same polypeptide chain. By using the mass spectrometry strategy described in the foregoing text, it is difficult to detect double mutations that are closely separated within the polypeptide chain, because the observed mass shift is the cumulative sum of the shifts induced by each mutation. In this article we show how electrospray ionization used in combination with liquid secondary ionization mass spectrometry (LSIMS) and tandem mass spectrometry pinpoints a change in the number of protonation sites. This information was highly useful to characterize and locate the two amino acid exchanges present in the same tryptic peptide of hemoglobin Villeparisis.

Experimental

Purification

Washed red blood cells were hemolyzed with four volumes of water and 0.5 volumes of toluene and centrifuged to remove membranes. Structural studies were performed as previously described [14]. The abnormal hemoglobin component was isolated by preparative isoelectric focusing on a granulated flat bed. Globin was dehemoglobinized by the acid acetone method. The polypeptide subunits were separated by RP-HPLC by using an Aquapore RP 300 column (25 × 0.46 cm) (Brownlee Labs, Santa Clara, CA) eluted with a gradient of isopropanol in 0.2% trifluoroacetic acid (TFA) [13].

Trypsin Cleavage and Analysis of the Resulting Peptides

After aminoethylation, the chains were digested with TPCK-treated trypsin in 25-mM ammonium bicarbonate pH 8.8 by using an enzyme to substrate ratio of 3:100 (w/w). The tryptic peptides were separated by RP-HPLC at a flow rate of 1.5 mL/min by using a Vydac C8 column (25 × 0.46 cm). Elution was obtained in 70 min by developing a curvilinear gradient from 0 to 75% acetonitrile in 0.2% TFA. Peptides were collected manually in Eppendorf tubes and dried under vacuum. The amino acid compositions of the peptides were determined after hydrochloric acid hydrolysis and pre-column derivatization into phenylthiocarbonyl amino acids [15]. Sequencing of the abnormal peptides was performed by use of an Applied Biosystems (Foster City, CA) model 470A gas-phase sequencer.

Mass Spectrometry

Electrospray analysis was performed on a Trio 2000 instrument (Fisons Biotech, Manchester, U.K.). The

needle-to-skimmer voltage was set to 3 kV. The cone-to-skimmer potential was 50 V. Ten microliters of the peptides or the globin solution in a 1:1 acetonitrile:water mixture that contained 2% formic acid were injected into the continuous flow of the same solvent running at 10 μ L/min. Ten-second scans at 1-u resolution were recorded and averaged. The VG MaxEnt program was used to improve both the accuracy of molecular weight determination and the resolution of the protein spectra.

Liquid secondary ion mass spectroscopy was performed on an Autospec instrument (Fisons, VG Analytical, Manchester, UK). The cesium gun worked at 30 kV; the ion source voltage was 8 kV. Samples were dissolved in a few microliters of 20% aqueous acetic acid and 1 μ L was mixed on the target with 1 μ L of a 1:1 glycerol thioglycerol mixture acidified by 1 μ L of 1% trichloroacetic acid in water. Tandem mass spectra were measured in the constant B/E linked scan mode to detect decompositions that occurred in the first field-free region. The helium pressure in the collision cell was adjusted to reduce the intensity of the ion beam by 50% of its original value.

Results and Discussion

Purification of Hemoglobin Villeparisis

Hemoglobin Villeparisis was found during a systematic quantification of glycosylated hemoglobins (performed by ion exchange HPLC) in a Caucasian French patient without hematologic or clinical features. The electrophoretic study showed* that the abnormal hemoglobin had a lower isoelectric point than hemoglobin A, but migrated very close to it in cellulose acetate at alkaline pH. Electrophoresis of the chain in 6-M urea under various experimental conditions (pH 6.0, pH 9.0 or in the presence of Triton X) suggested that a histidine was replaced by a neutral and more hydrophobic residue. A structural study was performed because the pattern of electrophoretic mobilities of this variant differed in detail from the more than 200 that are available in the Data Bank of the Hemoglobin Laboratory of Hôpital Henri Mondor (Créteil, France).

The tryptic digest was analyzed first by RP-HPLC in our standard system, with a final acetonitrile concentration of 30%. This procedure showed that the height of the peak that normally contains β T-5 and β T-9 was halved, but failed to provide evidence of any additional peak. This result suggested that, under these experimental conditions, the abnormal peptide was not eluted from the column. The tryptic digest was therefore analyzed by using a Vydac C8 column, which allowed separation of large hydrophobic peptides. This analysis showed that the normal β T-9 peptide was missing. In addition, a very hydrophobic abnormal peak, that eluted at the end of the chromatogram was observed (Figure 1). The structure of this peptide was therefore determined by mass spectrometry.

Figure 1. RP-HPLC elution pattern of the aminoethylated β chain of hemoglobin Villeparisis. Experimental conditions: 0.2-mg tryptic digest was applied on a Vydac C8 column (25×0.46 cm) eluted at a flow rate of 1.5 mL/min by developing in 70 min a curvilinear gradient from 0 to 75% acetonitrile in 0.2% TFA.

Mass Spectrometry

Electrospray mass spectrometry was performed on the intact globin by using the MaxEnt program to increase the resolution of the deconvolution process. This analysis showed that the molecular weights of the α and β chains of hemoglobin Villeparisis were $15,126.3 \pm 1$ and $15,865.5 \pm 1$ u, respectively (compared with 15,126.3 and 15,867.4 u for normal α and β chains).

The above-mentioned abnormal β T-9 was examined by LSIMS. It exhibited a $[M + H]^+$ ion at m/z 1668.8 $\pm$ 0.2, that is, one mass unit lower than normal β T-9. Assuming a single amino acid substitution, a 1-u shift downward corresponded to either Glu $\rightarrow$ Gln, Asp $\rightarrow$ Asn, or Asn $\rightarrow$ Ile exchange. Another allowed exchange was Glu $\rightarrow$ Lys, but because lysine is a cleavage site for trypsin, this proposal very likely has to be ruled out [11]. None of these amino acid exchanges was consistent with the electrophoretic behavior of the variant. In addition, when electrospray mass spectrometry of the β T-9 peptide was performed, a change in the number of the protonation sites was detected. The normal β T-9 gave mainly a triply charged ion at m/z 557.3, whereas the mutated β T-9 gave a doubly charged ion at m/z 835.0 (Figure 2). In the normal β T-9, the triply charged ion was due to protonation at the three basic sites, namely, the amino terminus, the lysine side chain, and the internal histidine residue. The only possible explanation of the suppression of one protonation site by mutation was to postulate that the internal histidine residue was replaced by a neutral or acidic one.

Thus, a single amino acid exchange was not consistent with these observations, but two exchanges that occur on the same peptide could be present: the measured one mass shift downward should correspond to the sum of two mass shifts, one of them due to an amino acid exchange that involves the internal histidine. Fortunately, only a few possibilities have to be considered because of the selection requirements of

Figure 2. Electrospray mass spectra of (a) normal β T-9 and (b) mutated β T-9.

genetic rules. Histidine can be exchanged only by Pro, Leu, Asn, Gln, Arg, Tyr, or Asp. An exchange of His $\rightarrow$ Arg is ruled out because it would modify the electrophoretic behavior of the chain and introduce a new cleavage site for trypsin. Table 1 shows the calculated mass shifts for a second mutation when the other remaining exchange possibilities for His are considered. Examination of Table 1 shows that the only possibility was His $\rightarrow$ Tyr (+26 mass shift) associated with Asn $\rightarrow$ Ser (−27 mass shift). The two other alternatives, His $\rightarrow$ Asn associated with Asp $\rightarrow$ His or His $\rightarrow$ Leu associated with Asn $\rightarrow$ His, could not induce changes in the number of protonation sites.

Examination of the normal β T-9 sequence revealed that a single Asn residue was present in this peptide. Thus the previously suggested double mutation is reasonable and the mutated β T-9 peptide would be the result of the simultaneous exchange of the eleventh and the fourteenth residues, namely, His and Asn, by Tyr and Ser, respectively.

This hypothesis was confirmed by interpretation of the collision-induced dissociation spectra of the MH^+ ions of normal and abnormal peptides at m/z 1669.7 and 1668.8, respectively (Figure 3). From the eleventh to the thirteenth position, the ions that contain the

Table 1. Amino acid exchange possibilities for a double mutation that involves the His residue and allows a -1 -u cumulative mass shift of the β T-9 peptide (⁶⁷Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-Ala-⁷⁷His-Leu-Asp-⁸⁰Asn-Leu-⁸²Lys, where the exchanged residues under consideration are underlined)

Exchanges of His allowed by the genetic code	Mass difference	Calculated mass difference for a second exchange	Corresponding mutation	Change in the number of protonable sites
Arg ^a	+19	20	None	—
Asn	23	+22	Asp → His	0
Asp	22	+21	None	—
Gln	9	+8	None	—
Leu	24	+23	Asn → His	0
Pro	40	+39	None	—
Tyr	+26	27	Asn → Ser	1

^a Not considered; new tryptic cleavage.

N-terminus (A_{11} , B_{11} , A_{12} , B_{12} , A_{13} , B_{13}) were shifted upward 26 mass units, because of the presence of Tyr instead of His at the eleventh residue. In contrast, the mass of the B_{14} fragment was shifted downward one mass unit. This locates the second amino acid exchange at the fourteenth position from the N-terminus. Ions that contain the C-terminus were shifted down

one mass unit from Y_{14}'' to W_8 (Scheme I). The Y_6'' ion also was shifted downward one mass unit. This result confirms that amino acid exchange occurs at the first six residues from the C-terminus, which agrees with the Asn → Ser exchange. No sequence ions from the carboxyl-containing series were detected to confirm once more the exchange of the His residue. However, the sequence of the modified β T-9 peptide was difficult to deduce reliably from the tandem mass spectrum alone, because of the relatively large width of the fragment peaks. In particular, differentiation between A fragments ($B - 28$ ions) and B ions shifted downward 27 mass units by the first mutation.

Finally, because of its biological significance, the assignment of this double mutation was verified both by amino acid analysis and chemical microsequencing. Amino acid analysis of the chromatographic peak showed that it was indeed a β T-9 peptide, but one that had more than one abnormality: one His and one Asn were missing and there was an additional Tyr and Ser. By microsequencing, Tyr instead of His was found at the eleventh position and Ser instead of Asn at the fourteenth position of peptide β T-9. These results confirm definitively the reliability of the mass spectrometry determinations.

Figure 3. Collision-induced dissociation spectra of $[M + H]^+$ ions generated by LSIMS (B/E scans): (a) normal β T-9 at m/z 1669; (b) mutated β T-9 at m/z 1668.

Scheme I. Sequence ions from the decomposition of protonated normal and abnormal β T-9 peptides.

Acknowledgments

We thank Jean Riou (Service de Biochimie, Hôpital Henri Mondor) for his expertise in the electrophoretic study of the hemoglobin variants.

References

1. Matsuo, T.; Matsuda, H.; Katakuse, I.; Wada, Y.; Fujita, T.; Hayashi, A. *Biomed. Mass. Spectrom.* **1981**, *8*, 25-30.
2. Pucci, P.; Caresta, C.; Fioretti, G.; Mastrobuoni, A. M. *Biochem. Biophys. Res. Commun.* **1985**, *130*, 84-90.
3. Rahbar, S.; Lee, T. D.; Baker, J. A.; Rabinowitz, L. T.; Asmerom, Y.; Legesse, K.; Ranney, H. M. *Hemoglobin*, **1986**, *10*, 379-400.
4. Promé, D.; Promé, J. C.; Blouquit, Y.; Lacombe, C.; Rosa, J.; Robinson, J. D.; *Spectrosc. Int. J.* **1987**, *5*, 157-170.
5. Wada, Y.; Matsuo, T.; Sakurai, T. *Mass Spectrom. Rev.* **1989**, *8*, 379-434.
6. Shackleton, C. H. L.; Green, B. N.; Falick, A. M.; Witkowska, H. E. *J. Chromatogr.* **1991**, *562*, 175-190.
7. Schneider R. G.; Barwick R. C. In *CRC Handbook Series in Clinical Laboratory Science, Section I: Hematology*, Vol. IV; Schmidt, R. M.; Fairbanks, V. F., Eds.; CRC Press: Boca Raton, FL, 1986; p 125.
8. Wajcman, H.; Kister, J.; Promé, D.; Galacteros, F.; Gilzang, F. *Biochem. Biophys. Acta* **1993**, *1225*, 89-94.
9. Wada, Y.; Nishikawa, A.; Okatomo, N.; Inui, K.; Tsukamoto, H.; Okada, S.; Taniguchi, N. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 832-836.
10. Falick, A. M.; Shackleton, C. H. L.; Green, B. N.; Witkowska, H. E. *Rapid Commun. Mass Spectrom.* **1990**, *4*, 396-400.
11. Lacombe, C.; Promé, D.; Blouquit, Y.; Bardakdjian, J.; Arous, N.; Bost, M.; M'rad, A.; Galacteros, F.; Promé, J. C.; Rosa, J. *Hemoglobin* **1990**, *14*, 529-548.
12. Promé, D.; Promé, J. C.; Pratbernou, F.; Blouquit, Y.; Galacteros, F.; Lacombe, C.; Rosa, J.; Robinson, J. D. *Biomed. Environ. Mass Spectrom.* **1988**, *16*, 41-44.
13. International Hemoglobin Information Center variant list. *Hemoglobin* **1994**, *18*, 77-161.
14. Wajcman, H.; Bardakdjian, J.; Ducrocq, R. *Ann. Biol. Clinique* **1993**, *50*, 867-870.
15. Heinrikson, R. L.; Meredith, S. C. *Anal. Biochem.* **1984**, *136*, 65-74.