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Combined Mass Spectrometric Methods for the Characterization of Human Hemoglobin Variants Localized Within αT9 Peptide: Identification of Hb Villeurbanne α89 (FG1) His → Tyr

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Mutation-induced amino acid exchanges occurring on the large T9 peptide of the α-chain of human hemoglobin (residues 62–90) are difficult to identify. Despite their high *m/z* value (around *m/z* 3000), collision-induced dissociation spectra of liquid secondary ion mass spectrometrically generated protonated αT9 peptides were performed successfully. In parallel electrospray mass spectrometry (MS) was used both to measure the molecular mass of the intact proteins and to determine the number of protonatable sites in the αT9 peptides. Peptide ladder sequencing using carboxypeptidase digestions and analysis of the truncated peptides by matrix-assisted laser desorption ionization time-of-flight MS confirmed the interpretation. This set of methods allowed the characterization of three hemoglobin variants, with amino acid exchanges located in the αT9 part of the sequence. Two of them, Hb Aztec [α76(EF5) Met → Thr] and Hb M-Iwate [α87(F8) His → Tyr] were already known. The third [α89(FG1) His → Tyr] was novel and named Hb Villeurbanne. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

In the Hemoglobin Laboratory (Department of Biochemistry, Henri Mondor Hospital, Créteil, France) a presumptive identification of abnormal human hemoglobins is routinely performed by comparing several pieces of information with those in a databank. Electrophoretic mobilities, chromatographic retention times, functional properties, ethnic distribution and clinical presentation are taken into account.[1–3] Those variants which remain unknown or doubtful after passing this screening test are examined by electrospray mass spectrometry (ESMS) for molecular mass measurement and compared again. Most of the hemoglobins that remained unidentified may be either new variants or already described ones[4] that have not yet been encountered in our laboratory or variants for which several possibilities are offered. In these cases, a structural study involving tryptic peptide mapping of the modified chains is performed by reversed-phase high-performance liquid chromatography (RP-HPLC), accompanied, when needed, by molecular mass measurements by fast atom bombardment (FAB) or ESMS followed by sequence determination of abnormal tryptic peptides (for reviews, see Refs 5 and 6). This last information is routinely obtained by collision-induced dissociation (CID) of FAB-generated MH^+ ions. When the rules of the genetic code are used as an additional criterion for selecting the allowed amino acid exchanges, an univocal identification of the variant is generally made.[7] This procedure is more difficult to perform, however, when the exchanged amino acid residue is localized in a large tryptic peptide. The fragmentation yield of singly protonated peptides under CID decreases rapidly when their mass rises above 2000 Da and the number of amino acid exchange possibilities fitting within a given mass shift increases with the number of amino acids. For these reasons, modifications arising within the αT9 peptide (M_r = 2996 Da) are not easy to characterize. Since this peptide contains several cleavage sites for *Staphylococcus* endoprotease V8, its digestion yields an inconstant number of fragments and the consecutive use of this endoprotease does not always give satisfactory results. In this paper, we show that the identification of the nature and location of amino acid exchanges within the αT9 peptide is possible by mass spectrometry, using a combination of peptide ladder sequencing and CID.

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EXPERIMENTAL

Purification of globin chains and separation of the tryptic peptides

Electrophoretic studies. The methods for the electrophoretic analysis of Hbs included electrophoresis on cellulose acetate at alkaline pH, citrate agar electrophoresis, isoelectric focusing (IEF) of Hb, electrophoresis of globin chains in 6 M urea in Tris–EDTA buffer at pH 6.0 and 9.0 or in the presence of Triton X-100.[1–3] The amount of the abnormal Hb was estimated by cation-exchange HPLC using the Bio-Rad Variant system and the β Thalassemia Short program.[7]

Characterization of the structural abnormality. Hb X1 was selectively precipitated from the hemolyzate using the instability propan-2-ol test of Carrel and Kay.[8] Globin was prepared immediately after propan-2-ol precipitation by the acid acetone method.

Hb X2 and Hb X3 were separated, as described previously,[9] using a miniaturized flat-bed preparative IEF system modified from the method of Radola.[10]

The polypeptide chains were then separated by RP-HPLC using an Aquapore RP 300 column (250 mm × 4.6 mm i.d.) (Brownlee Labs, Santa Clara, CA, USA) eluted with a gradient of propan-2-ol in a solution of 0.2% trifluoracetic acid (TFA) in water. After aminoethylation,[11] the α-chains were digested with trypsin and the resulting peptides were separated by RP-HPLC using a Vydac C_8 column (Separation Group, Hesperia, CA, USA) (250 mm × 4.6 mm i.d.) in a 60 min concave curvilinear gradient from 0 to 42% acetonitrile in 0.2% TFA.[8]

ESMS of abnormal globins and αT9 peptides

Electrospray mass spectra of the intact globins and of αT9 peptides were measured on a TRIO-2000 instrument (Micromass VG-Biotech, Manchester, UK). A 10 μl volume of a 10 pmol μl^{-1} solution of peptides or globins in CH_3CN–H_2O (1:1) containing 2% formic acid were injected into a 10 $\mu l\ min^{-1}$ flow of the same solvent. The capillary voltage was 4 kV. For high-mass analysis, the cone-to-skimmer voltage was set to 60 V and myoglobin was used for external calibration. After the characterization of the normal chains by their molecular masses (accuracy ± 2 Da), the globin spectra were recalibrated by using the normal α-chains as an internal calibrant. The VG MaxEnt program was then used to process the spectra, thus improving both the accuracy of molecular mass determinations and the resolution of the experiments. The accuracy was estimated to be better than 1 Da.

Tandem MS of the tryptic peptides

An AUTOSPEC sector instrument (Micromass, Altrincham, UK) with EBE configuration and fitted with a liquid secondary ion mass spectrometry (LSIMS) ion source was used. The energy of the Cs^+ ions was 25 kV and the accelerating voltage was set to 8 kV. The matrix was dithiothreitol–dithioerythritol (5:1) acidified with 1% trichloroacetic acid in water. CID spectra were measured in the constant B/E linked scan mode to detect decompositions that occurred in the first field-free region.[12] The slits were wide open to obtain maximum of sensitivity. The helium pressure in the collision cell was adjusted to reduce the intensity of the ion beam by 50% of its original value. The scan rate was 5 s per decade and about ten scans were averaged. The MaxEnt Re program was used to improve the signal-to-noise ratio and to extract reliably ion signals from the important background.

Peptide digestion by carboxypeptidases B and Y

Digestions were performed in an Eppendorf tube at room temperature as follows: 1 μl each of carboxypeptidase Y[13] and carboxypeptidase B[14] solutions (10 $\mu g\ ml^{-1}$) (Aldrich, Milwaukee, WJ, USA) were added to 20 μl of a solution of an αT9 peptide (1–2 pmol μl^{-1}) in 10 mM HEPES buffer (pH 7.3). Aliquots of 1 μl were taken at different time intervals and mixed immediately with the matrix solution (2 μl of α-cyano-4-hydroxycinnamic acid at 10 mg ml^{-1} in CH_3CN–H_2O (2:1) containing 0.1% TFA). This stopped both enzymatic digestions simultaneously. A 1 μl of this mixture was applied to the stainless-steel target of the matrix-assisted laser desorption/ionization time-of-flight (MALDI/TOF) instrument (Micromass TofSpec, Manchester, UK) fitted with a single-stage reflectron and a 337 nm pulsed nitrogen laser. The samples were dried in a desiccator before use. The accelerating voltage was 18 kV. The instrument was externally mass calibrated in the reflectron mode using a mixture of reference peptides of known mass. Spectra were recorded in the positive-ion mode, summing 20–30 laser shots.

RESULTS AND DISCUSSION

Detection of the mutations

Three abnormal hemoglobins, called Hb X1, Hb X2 and Hb X3, were detected because of various circumstances. X1 was found during a systematic Hb screening performed on a subject originating from Cape Verde, X2 was studied because of the presence of an additional peak during measurement for glycated Hb and Hb X3 in the investigation of a cyanotic patient for which the diagnosis of Hb M Iwate has been proposed. The electrophoretic mobilities of these hemoglobins are given in Table 1. With the Bio-Rad Variant system and the β Thalassemia Short program, Hb X2 was eluted at 1.6 min and amounted to 28% and HB X3 at 3.9 min, but Hb X1 was eluted as Hb A. All three were α-chain mutants, as detected by electrophoresis and electrospray. In addition, the HPLC pattern of the tryptic digest indicated in all the presence of an abnormal αT9 peptide.

The ES mass spectrum measured on the intact globin of X1 showed two proteins with $M_r = 15126.7 \pm 1$ Da, corresponding to the normal α-chain (expected 15126.3), and $M_r = 15096.5 \pm 1$ Da, corresponding to an abnormal α-chain [Fig. 1(a)].

Table 1. Electrophoretic parameters of the three α-chain variants

Hb	IEF[a]	Cellulose acetate[b] (pH 8.6)	Citrate–agar[c]	Urea[d] pH 9.0	Urea[d] pH 6.0	Urea[d] Triton X-100
Hb A	0.0	0.0	0.0	10	10	10
Hb X1	−0.7	0.0	0.0	10	10	10
Hb X2	0.7	0.0	0.0	10	10	13.5
Hb X3	−4.7	2.4	8.0	10	10	15.1

[a] Migration in IEF is expressed in mm from the position of HbA in the reference map.[2]
[b] Migration in cellulose acetate is expressed according to a scale in which HbA, HbI Texas and HbC are 0, +8.5 and −10, respectively.[1]
[c] Migration in citrate–agar is expressed according to a scale in which HbF, HbA, HbS and HbC are −4.4, 0, +5.8 and+10, respectively.
[d] Migration of chains in urea is expressed according to a scale in which normal α-chain is 10 and normal β-chain is 20. Values are given only for the α-chains.

The deconvoluted ES mass spectra of the X2 and the X3 globins demonstrated identical molecular masses. They showed the presence of several proteins. The most abundant ones corresponded to normal α- and β-chains with $M_r = 15\,126.6 \pm 1$ Da (expected 15 126.3) and $M_r = 15\,867.5 \pm 1$ Da (expected 15 867.4). They were accompanied by their phosphate adducts (+98 Da) at $M_r = 15\,224.9$ and 15 965.4, respectively, as the samples still contained phosphate buffer. The peak at $M_r = 15\,152.3 \pm 1$ Da corresponded to an abnormal α-chain [Fig. 1(b)].

The Hb X1 variant

The abnormal α-chain was aminoethylated and digested with trypsin. The HPLC elution pattern of the tryptic peptides revealed an abnormal αT9 peptide [Fig. 2(a)]. Its LSI mass spectrum exhibited an MH^+ ion at *m/z* 2966.5 instead of *m/z* 2996.5 for normal αT9. The 30 u shift down was identical with the mass shift measured on the α-chain. Assuming a single point mutation and according to the genetic code rules, this shift could correspond to either Thr → Ala, Ser → Gly or Met → Thr exchanges[7] since the two other possibilities involving Glu and Trp must be ruled out as these amino acids were not present in αT9 peptide. Thus, as the αT9 peptide possesses one threonine, two serine and one methionine residues, four possible exchange sites have to be differentiated.

When performing CID on the MH^+ ion of the normal αT9 peptide, despite its high *m/z* value, several fragment ions emerged from the background. The signal-to-noise ratio was then greatly improved by running the MaxEnt program and several sequence ions were identified unambiguously [Fig. 3(a)]. Most of these ions, mainly of the y series, emerged above *m/z* 1400. When running the CID spectrum of the MH^+ ion from the abnormal αT9 peptide X1 [Fig. 3(b)], it was

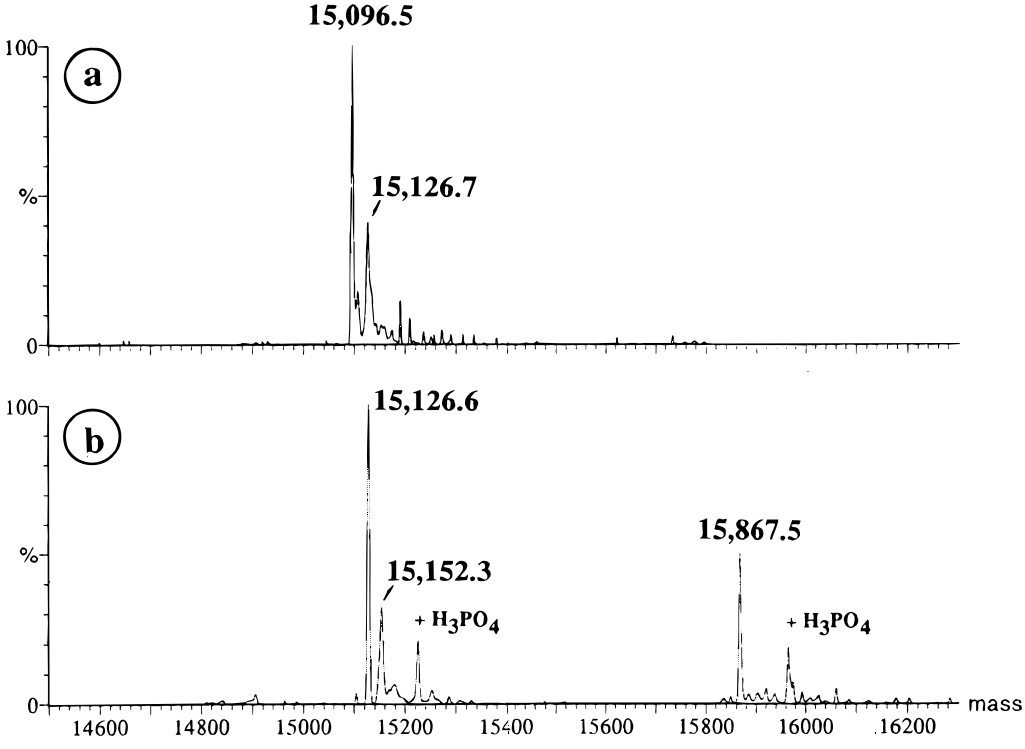

Figure 1. Deconvoluted ES mass spectra of the intact globins. For experimental conditions, see text. (a) Purified abnormal α-chain from Hb X1; (b) Hb X2. The spectrum of Hb X3 is identical.

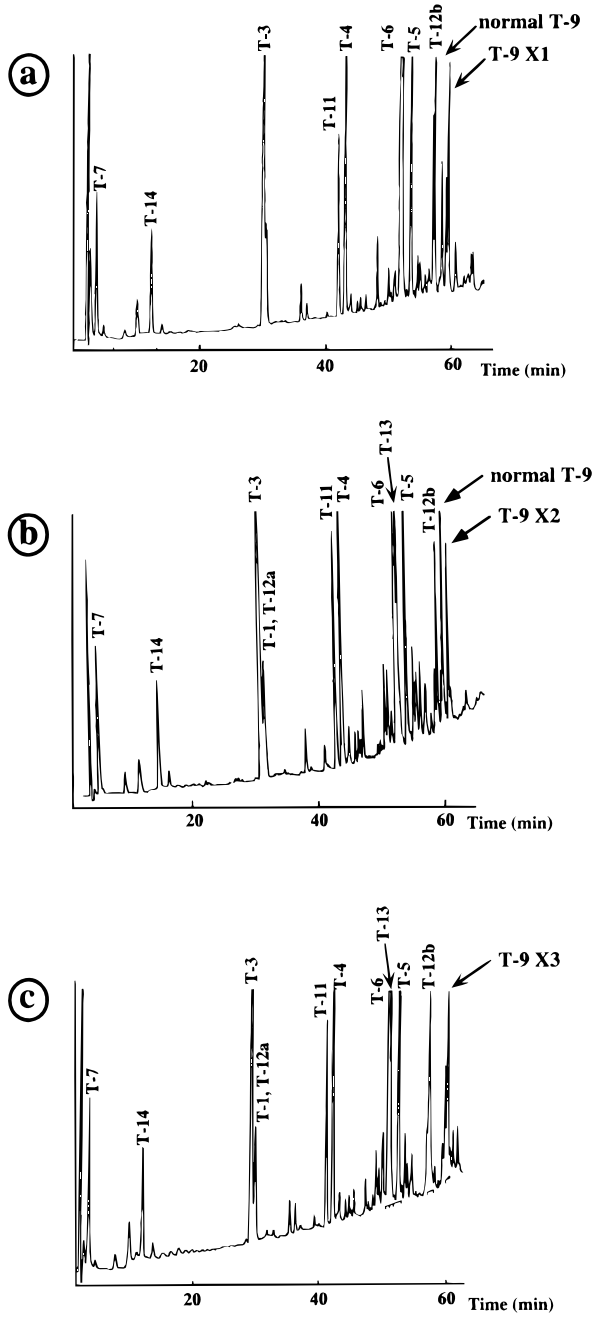

Figure 2. Elution pattern of the tryptic peptides derived from partly purified abnormal aminoethylated α-chains: (a) Hb X1; (b) Hb X2; (c) Hb X3.

observed that the y_{14} ion at m/z 1474 remained unchanged whereas both the y_{15} and y_{16} ions exhibited a 30 u shift down. Hence the exchange site was located on the fifteenth residue from the carboxyl end, which is the methionine residue. Therefore, the mutation corresponded to a Met → Thr exchange. The additional ion at m/z 1557 confirmed this assignment as the loss of water was consistent with a threonine residue placed at the terminal amino end of the y_{15} ion.

The X2 and X3 variants

As above, the α-chains were purified, aminoethylated and digested with trypsin. The resulting peptides were separated by RP-HPLC. Their elution patterns revealed abnormal αT9 peptides with a retention time increased relative to normal αT9 [Fig. 2(b) and (c)]. Both abnormal αT9 peptides showed an MH^+ ion at the same m/z value when analyzed by LSIMS (m/z 3022.5 instead of m/z 2996.5 for the normal αT9). Hence the 26 u shift up was the same as measured by ESMS of the intact globins. Assuming a single point mutation and according to the genetic code rules, the 26 u shift up might correspond to either Ala → Pro, His → Tyr or Ser → Leu or Ile exchanges.[7] If the amino acid exchange involved the histidine residue, it should induce the suppression of one protonable site in the peptide. Therefore, an ES mass spectrum was measured on each αT9 peptide. The normal αT9 peptide gave mainly a quintuply charged ion, as expected for protonation of five sites: the amino terminus, the lysine side-chain and the three histidine imidazole groups [Fig. 4(a)]. In contrast, both abnormal αT9 peptides gave a predominant quadruply charged ion [Fig. 4(b)]. This result fits only with the replacement of one of the three histidine residues by tyrosine, thus ruling out the other two possibilities.

To characterize which histidine residue was exchanged, the CID spectrum of each MH^+ ion was recorded. Several y ions, namely y_{14}, y_{15}, y_{16} and y_{27}, were clearly identified in the CID spectrum of the MH^+ ion of the normal αT9 peptide and some ions bearing the amino end, identified as b_{24} and a_{26}, were also seen [Fig. 3(a)]. Because of the low resolution needed in a search for maximum sensitivity, several assignments may be proposed for other ions. The most probable ones were indicated.

For the X2 variant [Fig. 3(c)], all the y series was shifted up by 26 u, whereas b_{24} remained unchanged. A weak a_{26} ion was seen at the same m/z value as for the normal peptide. Hence the His → Tyr exchange seemed to be located between amino acids 24 and 29, and probably at the histidine at position 28 if the assignment of a_{26} was correct [because of the smaller amount of sample, the signal-to-noise ratio in Fig. 3(c) was poorer than in others].

For the X3 variant [Fig. 4(d)], the above series of y ions was also shifted up by 26 u and the b_{24} ion was not changed. However, the a_{26} ion was shifted up by 26 u. Hence the exchange site seemed to be located at the histidine at position 26, as expected from presumptive diagnosis.

As the two histidine residues were included in the 24–29 sequence, only one ion (a_{26}) could differenciate exchanges occurring on the two sites. Hence another sequencing method was needed for an univocal location of these mutations. This was achieved by *C*-terminal ladder sequencing of the αT9 peptides by carboxypeptidase digestion and MALDI/TOF mass measurement of the truncated peptides.[15,16] A mixture of two carboxypeptidases was used: carboxypeptidase B to release the *C*-terminal lysine residue and carboxypeptidase Y to remove stepwise other amino acids from the carboxyl end of the $(n-1)$ peptide. The masses of the truncated peptides were measured during a time-course study.

The spectra of samples taken up at different time intervals are shown in Fig. 5. For the αT9 peptide of X2 [Fig. 5(a)], after a 5 min digestion the spectrum showed a mixture of three peptides: (i) intact αT9 at m/z 3024,

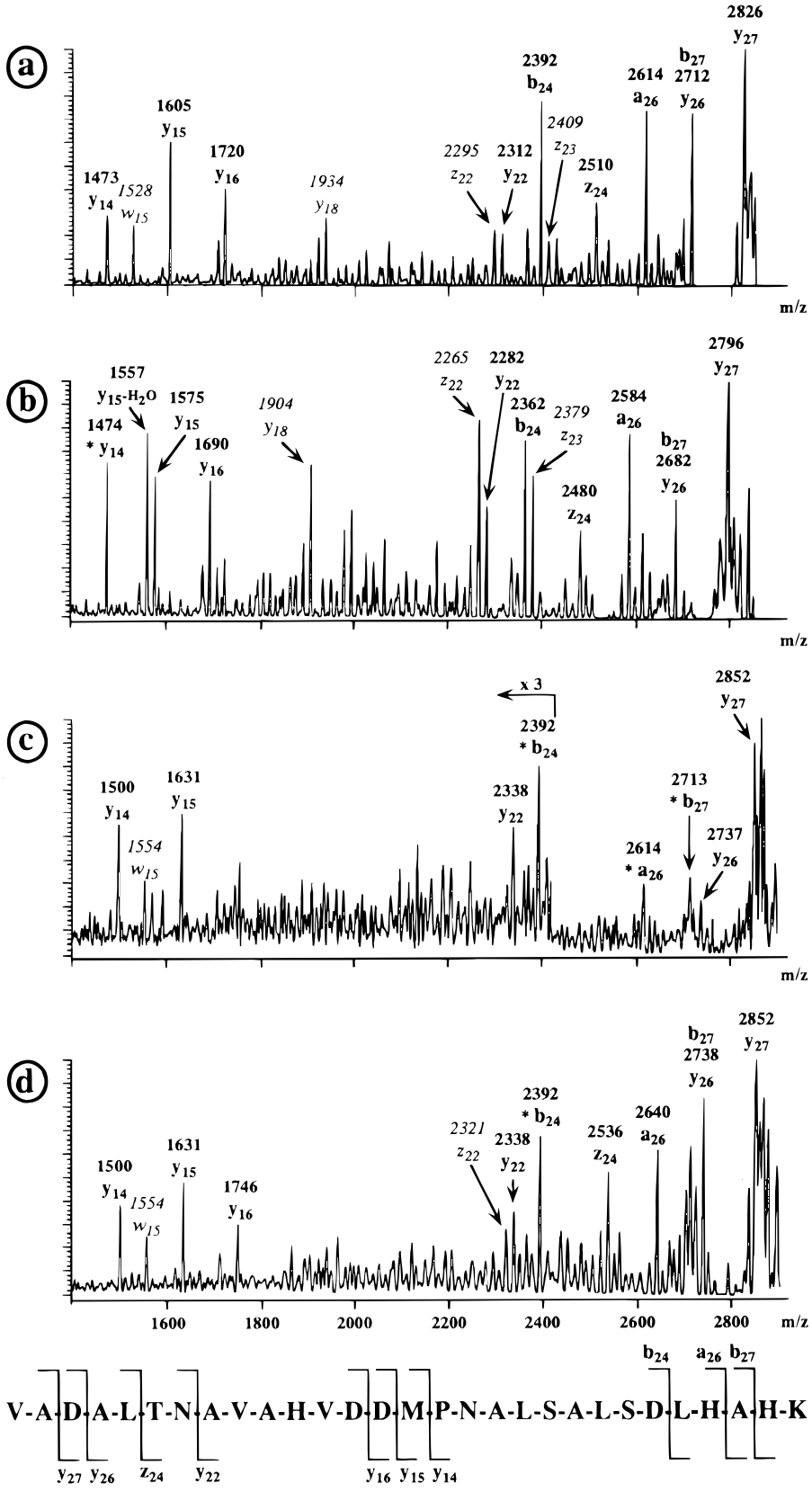

Figure 3. CID mass spectra of protonated αT9 peptides (constant *B*/*E* scans): (a) from normal Hb at *m*/*z* 2998; (b) from Hb X1 at *m*/*z* 2968; (c) from Hb X2 at *m*/*z* 3024; (d) from Hb X3 at *m*/*z* 3024. Reliable assignments are indicated by bold characters, possible assignments of ions having different origins are indicated in italics and non-shifted fragment ions by mutation are marked with asterisks.

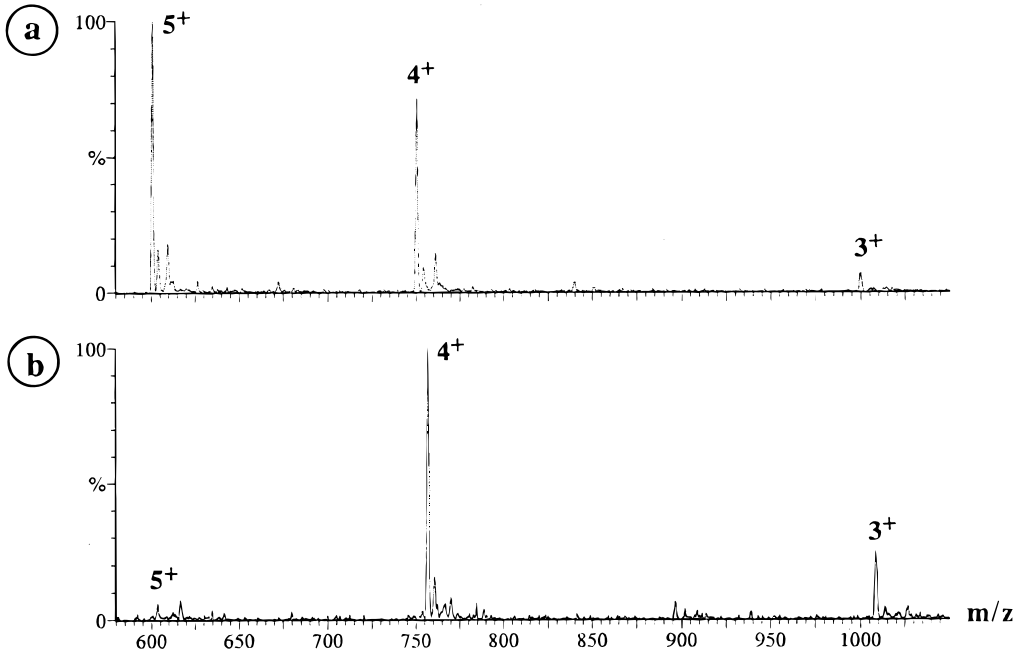

Figure 4. ES mass spectra of αT9 peptides: (a) from normal Hb; (b) from Hb X2. The spectrum from Hb X3 is identical.

(ii) a truncated peptide resulting from the loss of the *C*-terminal lysine residue and (iii) a truncated peptide corresponding to the loss of an additional tyrosine residue. This result confirmed the previous assignment of the His → Tyr exchange at position 28 detected by CID. After 1 h, the spectra showed the presence of a mixture of peptides corresponding to the digestion of the first 15 amino acids from the *C*-terminus. This confirmed that the sequence below alanine at position 27 was identical with that of normal αT9.

In parallel, the αT9 peptide isolated from the variant X3 lost first the terminal lysine, then successively histidine, alanine and tyrosine [Fig. 5(b)]. Before ^{25}Leu, the molecular masses of the truncated peptides were identical with those of the normal αT9. This indicated that the His → Tyr exchange occurred at ^{26}His.

For both normal and modified peptides, either after a longer incubation time or after addition of further aliquots of carboxypeptidase, no other truncated peptide was detected. The presence of the two consecutive aspartic acid residues, known to be very slowly hydrolyzed by carboxypeptidase Y, explained the stop of the sequential degradation.

The use of combined mass spectrometric methods allowed the univocal identification of the abnormal αT9 peptides and the identification of the hemoglobin variants. Performing library searches, it turned out that two of the three variants we have investigated were already known, the third being a new one. Hb X1 was therefore identified as Hb Aztec [α76(EF5) Met → Thr][17] and Hb X3 as Hb M-Iwate [α87(F8) His → Tyr].[18] The new X2 variant [α89(FG1) His → Tyr] was named Hb Villeurbanne from the place where the patient lives. This variant is the second one known at this position, the other being Hb Luton, a high oxygen affinity Hb, in which the histidine is replaced by a leucine.[19]

In conclusion, despite the high mass of the αT9 peptides, CID of their MH^+ ion gives useful sequence information. These data, associated with those given by electrospray and peptide ladder sequencing, have been informative enough to identify clearly and rapidly three variants, without the need for other time-consuming methods.

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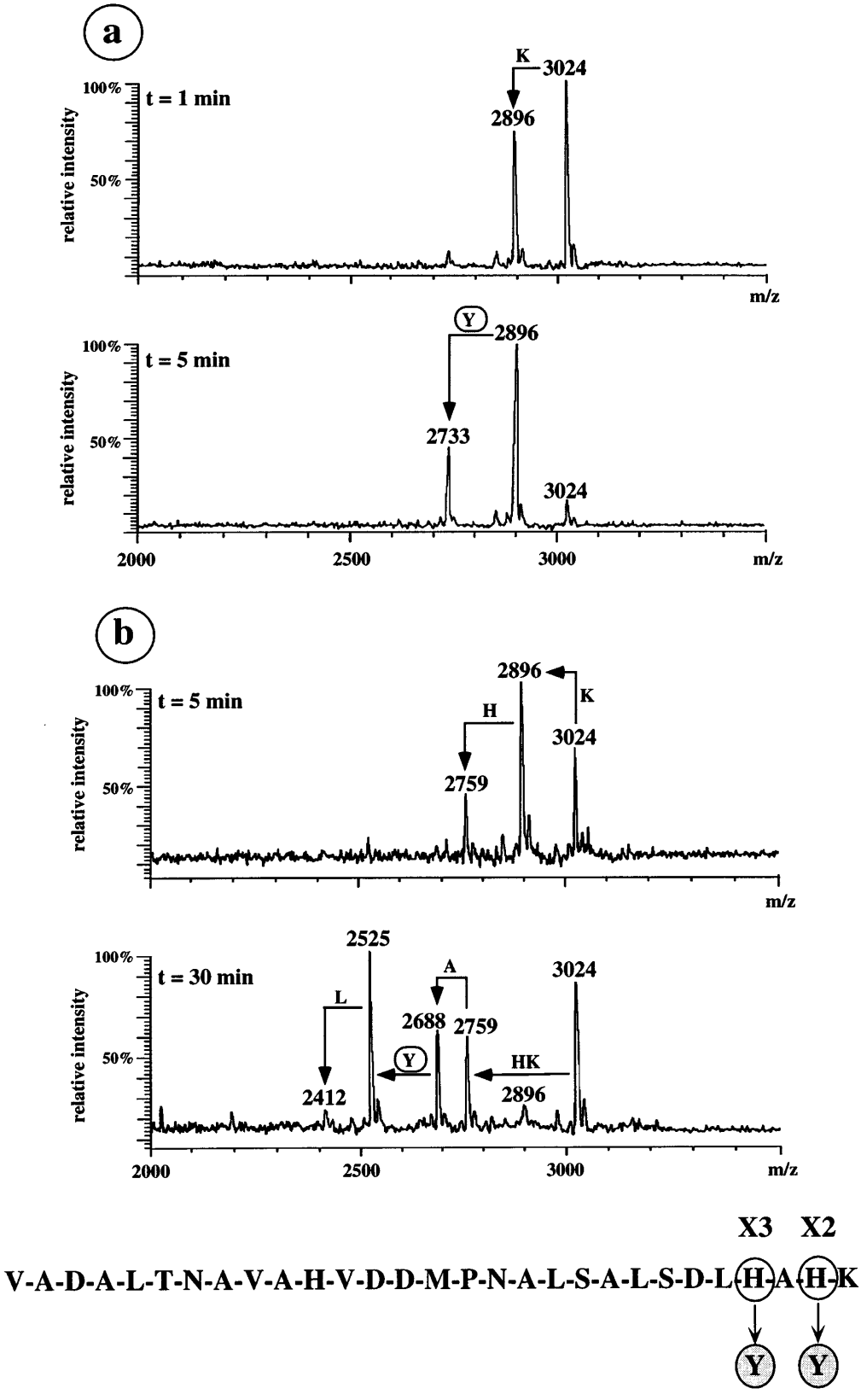

Figure 5. MALDI/TOF spectra of the truncated peptides formed during time-course hydrolysis of αT9 peptides by carboxypeptidases: (a) from Hb X2; (b) from Hb X3.

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