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PROTEIN FINGERPRINT BY FAST ATOM BOMBARDMENT MASS SPECTROMETRY: CHARACTERIZATION OF NORMAL AND VARIANT HUMAN HAEMOGLOBINS

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SUMMARY: A procedure using fast atom bombardment mass spectrometry was developed for mapping the proteolytic digest of proteins. The procedure was successfully applied to the tryptic peptides of the human β-globin chain. Almost all the expected peptides were identified by direct analysis of the peptide mixture on the mass spectrometer. Peptide recognition along the β-globin chain sequence was easily made on the basis of their molecular weight. The general applicability of this mapping procedure in the analysis of haemoglobinopathies was demonstrated by its use for the structural characterization of a variant β-globin chain.

High resolution procedures for peptide mapping play a fundamental role in protein sequencing and other structural studies.

In the past, peptide separations have been accomplished by ion-exchange chromatography, gel filtration, paper and thin-layer electrophoresis or chromatography, but the low power resolution of these techniques does not allow the separation of all the components of complex mixtures of peptides produced by chemical or enzymic cleavage of proteins (1).

The introduction of high pressure liquid chromatography (HPLC), especially in the reverse phase mode, has greatly improved peptide separation; the high power resolution and the speed of this technique has provided a new dimension in protein structural studies (2-7).

Unfortunately, it has not always been possible to obtain complete peptide mapping of proteins by HPLC. Each peptide mixture requires different chromatographic conditions, and setting up the appropriate system increases the length of the procedure. Moreover, peptide identification, performed by aminoacid analysis, causes further delay.

Since its introduction, mass spectrometry has been regarded as the best technique to acquire structural information on peptide mixtures, but the

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problem of sample derivatization and the low intensity of molecular ions, and often their absence, has greatly limited its use in protein structural studies.

Recently, a new soft ionization method has been developed: fast atom bombardment (FAB) (8) that has proved to be the ideal ionization technique for polar molecules such as peptides, carbohydrates, vitamins, hormones, etc.; high intensity quasi-molecular ions (MH^+) have been obtained by using non-derivatized samples.

FAB has already been used for sequencing highly-purified peptides (9), while peptide mixture analyses have proved to be most useful for acquiring protein structural information (10-11) or controlling protein sequences obtained by DNA sequencing (12-13).

The present study reports the fingerprint of the tryptic digest of the human β-globin chain by FAB mass spectrometry. The general applicability of this mapping procedure in the analysis of haemoglobinopathies was demonstrated by using this method to study a haemoglobin variant, identified as an O-Arab haemoglobin.

MATERIALS AND METHODS

Normal and variant β-globin chains were purified on a CM-52 cellulose ion-exchange column eluted with a linear gradient of phosphate buffer (5 mM pH 6.7 to 20 mM, pH 7.2) containing 8 M urea plus 50 mM β-mercaptoethanol (14). Samples were desalted by gel filtration on a Sephadex G-50 column in 10% acetic acid.

Tryptic digestions were carried out in 0.4% ammonium bicarbonate pH 8.5 at 37°C overnight. Samples were lyophilized, resuspended in 5% acetic acid and directly analyzed by mass spectrometry.

Edman degradation was performed directly on the tryptic digest of the variant β-globin chain using 5% phenylisothiocyanate (PITC) in pyridine as coupling agent. The peptides were suspended in 150 ul of water and treated with 150 ul of the reagent solution. The coupling reaction was allowed to proceed at 45°C for 1 hour, then the sample was dried under nitrogen. The cleavage step was performed by adding 150 ul of anhydrous trifluoroacetic acid (TFA) at 45°C for 10 min. The sample, dried under vacuum, was washed twice with water, redissolved in 5% acetic acid and loaded onto a glycerol-coated probe tip, after which 1 ul of α-thioglycerol was added.

FAB spectra were recorded on a VG ZAB HF double-focusing instrument equipped with a FAB source (from the same suppliers) and an M-Scan gun. Typical experimental conditions used Xenon as a primary ionizing beam (current at 20 uA) at 8 KeV accelerating potential.

Trypsin, glycerol and α-thioglycerol were from Sigma Chemical Co., PITC and trifluoroacetic acid of sequanal grade were from Rathburn. Cellulose CM-52 was from Whatman, Sephadex G-50 was from Pharmacia. All other chemicals were Baker reagents of analytical grade.

RESULTS AND DISCUSSION

Figure 1 shows the FAB mass spectrum in the 400-2,200 range of atomic mass units of the tryptic digest of the human β-globin chain. Thirteen intense signals were recorded with weak satellite peaks occurring 15 mass

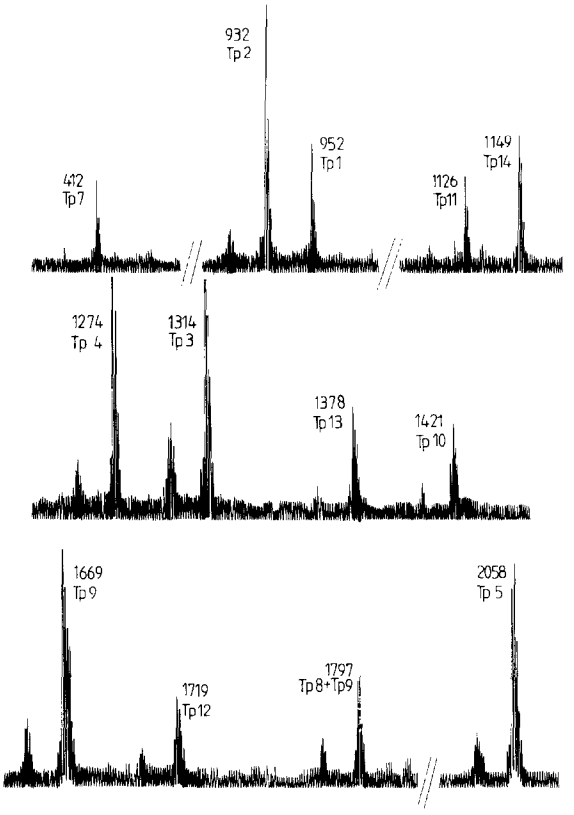

Fig. 1 : FAB mass spectrum of tryptic digest of human β-globin chain.

units lower than the individual parent peak . These weaker signals were presumably caused by the loss of an amino group. Out of the 13 intense signals, 12 showed to have an atomic mass corresponding to the molecular weight of known tryptic peptides; the Table reports the found quasi-molecular ions related to the tryptic peptides with their position in the sequence.

The thirteenth signal at m/z = 1,797 corresponds to peptide Tp9 plus an extra lysine residue at the N-terminus; this is due to the incomplete cleavage between Lys 66 and Val 67 because of the presence of a Lys residue in position 65. The peptide bond between the two lysine residues is the preferential site for tryptic cleavage. Subsequently, the release occurs of lysine 66 to form peptide Tp9.

All the expected peptides deriving from the tryptic cleavage of the β-globin chain were recognized with the exception of the two di-peptides Val-Lys (60-61) and Tyr-His (145-146), Tp6 and Tp15 respectively. The quasi-molecular ions of these dipeptides should have occurred in the low mass region of the spectrum, at m/z = 246 and m/z = 319, respectively, where the signals might have been suppressed by the intense background peaks due to the matrix.

TABLE 1

Observed mass values, sequence and position in the sequence of tryptic peptides of human β-globin chain

Quasi-molecular ion (MH^+)	Peptide	Sequence	Position in the sequence
952	Tp1	V-H-L-T-P-E-E-K	1-8
932	Tp2	S-A-V-T-A-L-W-G-K	9-17
1,314	Tp3	V-N-V-D-E-V-G-G-E-A-L-G-R	18-30
1,274	Tp4	L-L-V-V-Y-P-W-T-Q-R	31-40
2,058	Tp5	F-F-E-S-F-G-D-L-S-T-P-D-A-V-M-G-N-P-K	41-59
412	Tp7	A-H-G-K	62-65
1,797	Tp8+Tp9	K-V-L-G-A-F-S-D-G-L-A-H-L-D-N-L-K	66-82
1,669	Tp9	V-L-G-A-F-S-D-G-L-A-H-L-D-N-L-K	67-82
1,421	Tp10	G-T-F-A-T-L-S-E-L-H-C-D-K	83-95
1,126	Tp11	L-H-V-D-P-E-N-F-R	96-104
1,719	Tp12	L-L-G-N-V-L-V-C-V-L-A-H-H-F-G-K	105-120
1,378	Tp13	E-F-T-P-P-V-Q-A-A-Y-Q-K	121-132
1,149	Tp14	V-V-A-G-V-A-N-A-L-A-H-K	133-144

To illustrate the general application possibilities of this mapping procedure, we used it for the structural characterization of a variant β-globin chain.

The variant protein was purified by ion-exchange chromatography on a CM-cellulose column; its elution time was higher than that of the normal β-globin chain, showing a more basic character. The tryptic digest of the variant protein was directly analyzed by FAB mass spectrometry.

The spectra were compared with those recorded in the case of the normal β-globin chain; all the signals were associated to the already identified peptides listed in Table 1, with the exception of peptide Tp13. Figure 2 shows the FAB spectra in the range from 1,200 to 1,500 mass units of both variant and normal tryptic digests. It is important to note the absence of the peak at m/z = 1,378 corresponding to peptide Tp13, and the presence of two new signals at m/z = 1,249 and m/z 1,377, respectively.

The absence of the peptide Tp13 peak clearly indicated the occurrence of the genetic mutation in the sequence from 121 to 132. The new signal at m/z = 1,249 corresponds to peptide Tp13 minus a mass of 129, which can be attributed to the loss of the N-terminal glutamic acid. The signal at

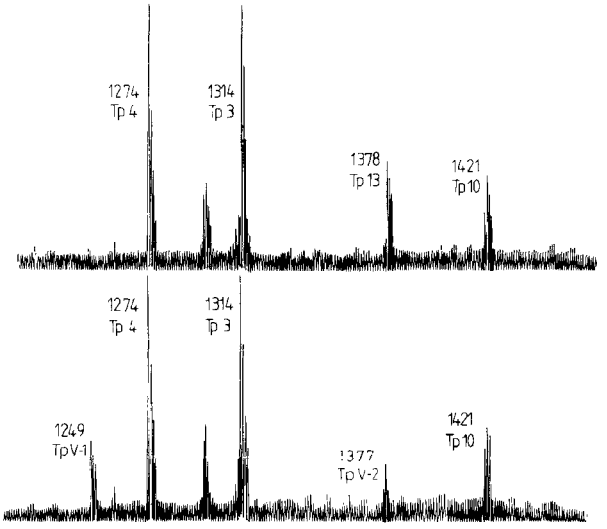

Fig. 2 : FAB spectra of tryptic digest of normal (top) and variant (bottom) β-globin chain in the 1,200-1,500 mass unit range. Tp V-1 and Tp V-2 are the two new peptides deriving from the variant globin.

m/z = 1,377 corresponds to the first new signal with an additional mass of 128, corresponding to the mass of a lysine or glutamine residue.

These two observations suggest that a lysine residue substituted the glutamic acid at the N-terminus of peptide Tp13, thus creating a new site for tryptic cleavage in position 121. As previously described for peptide Tp9 in the normal β-globin chain, the peptide bond between the two lysine residues in position 120-121 is preferentially cleaved by trypsin with regard to the following Lys-Phe bond. This incomplete cleavage produced the two new peptides.

In order to give more support to this interpretation, the tryptic digest was subjected to a single step of Edman degradation without separating the individual peptides. The mixture was then subjected to mass measurement; the region from 1,100 to 1,400 mass units of the spectrum is shown in Fig. 3.

The signal at m/z = 1,249 was shifted to m/z = 1,102 by the loss of phenylalamine 122 (residue molecular weight 147), confirming the above reported assignment. Similarly, the signal at m/z = 1,377 changed to m/z = 1,249 by losing the N-terminal lysine 121 (residue molecular weight 128). It should be pointed out that both these signals were associated to satellite peaks occurring 135 mass units higher than the parent signals. This was because the C-terminal lysine residues in these peptides were in part modified by PITC during Edman degradation.

Thus, it is concluded that the variant haemoglobin has a lysine in position 121 of its β-globin chain sequence instead of glutamic acid. Such a

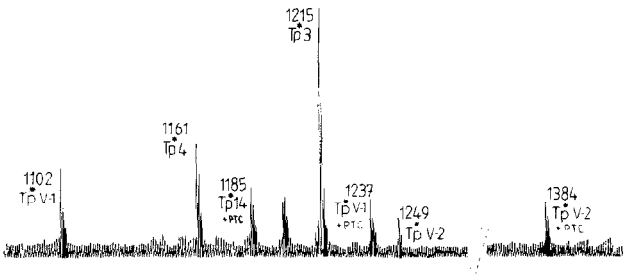

Fig. 3 : FAB mass spectrum of tryptic peptides from variant β-globin chain after Edman degradation. Tp* indicates the truncated peptides, while Tp* + PTC refers to peptides partially modified by PITC during Edman degradation.

variant has already been described and it is classified as an O-Arab haemoglobin (15).

These results demonstrate that FAB mass spectrometry can be used for obtaining a reproducible fingerprint of proteins; the only limitation of this method is the failure of identifying very short peptides under the used conditions. The mapping procedure does not depend on the chromatographic behaviour of peptide mixtures but only on an intrinsic property of peptides. Furthermore, it is possible to acquire structural information on proteins by subjecting peptide mixtures to simple chemical and/or enzymic reactions without separating the individual peptides.

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