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# Structural analysis of biologically active peptides and recombinant proteins and their modified counterparts by mass spectrometry

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ABSTRACT

The structural characterization of the *Escherichia coli*-expressed human interferon α-2b (rh-IFN α-2b) was carried out by employing the fast atom bombardment (FAB) and plasma desorption (PD) mapping methods. The mass spectral data of the rh-IFN α-2b and the trypsin-generated peptide mixture allowed rapid and facile confirmation of the cDNA-derived sequence and determination of the existing disulfide pattern in the protein molecule. The same PD/FAB mapping approach was successfully employed in the structural determination of the iodination reaction product of rh-IFN α-2b and the potent vasoconstrictor peptide endothelin.

## INTRODUCTION

In recent years, the major efforts of the biotechnology industry have been directed towards the development of novel therapeutic agents through the production of biologically active proteins by recombinant DNA techniques. The successful isolation, cloning and sequencing of the gene coding for a protein has made it possible to produce biologically active proteins and their genetically or chemically modified variants in large quantities. That, in turn, has led to the development of new drugs and better diagnostic kits, thus opening up new avenues for the treatment of diseases. In 1987, human α-2 interferon became the first genetically engineered protein to be approved and marketed as a drug for the treatment of hairy-cell leukemia. Since then, it has found wider applications in the treatment of genital warts, AIDS-related Kaposi's sarcoma, and it was recently reported to be effective against hepatitis B and C viruses [1].

In this highly competitive area of biotechnology and pharmaceutical research, it is important to check whether the gene product is indeed the same as that of the wild type, as well as for the presence of any genetically or post-translationally

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introduced alterations. Nevertheless, the rapid and accurate protein structural characterization is hampered by the sheer complexity of these molecules. One of the conventional methods for the determination of protein sequences is the indirect approach through the translation of the gene base sequence coding for the protein. Inaccuracies in protein sequences can result, however, either from mistakes in reading DNA-sequencing gels or from post-translational modifications that cannot be inferred from the DNA sequence. On the other hand, the automated stepwise Edman degradation usually requires time-consuming fragmentation of the sample and isolation of its constituent peptides. Furthermore, the sequencing process is slow (approximately 1 h per amino acid), unable to sequence N-blocked proteins and does not reveal information regarding the presence of modified or unusual amino acid side-chains in the protein sample.

There is a need, therefore, for an alternative method to provide rapid and accurate information on the primary structure of recombinant proteins. Mass spectrometry (MS) assisted by developments in particle-induced desorption ionization methods, as well as recent advances in instrumentation, offers the most promising approach to the solution of these problems. Fast atom bombardment (FAB) [2] and $^{252}$Cf plasma desorption time-of-flight mass spectrometry (PD-MS) [3,4] allowed allowed the ionization of peptides and small proteins (up to 30 kDa) previously not amenable to MS, and led to the development of new peptide and protein "mapping" approaches, often referred to as "FAB mapping" [5,6] and "PD mapping" [7]. Additional sequence information on peptides and enzyme-generated peptide fragments of proteins can be obtained by FAB-MS analysis followed by tandem mass spectrometry (FAB-MS–MS) [8–11]. Moreover, the recently introduced methods of matrix-assisted UV-laser desorption [12–14] and electrospray ionization [15,16] added a new dimension to the analysis of large peptides and proteins due to the higher sensitivity, mass accuracy and mass range.

In this report, we discuss the use of FAB and PD time-of-flight MS to extensively characterize recombinant human interferon α-2b (rh-IFN α-2b) and its chemically modified counterparts. The characterization of the biologically important peptide endothelin and its iodinated derivatives will also be described.

## EXPERIMENTAL

### *Materials*

rh-IFN α-2b was purified from *Escherichia coli* (Schering-Plough Research, Bloomfield, NJ, U.S.A.). Endothelin and big endothelin were obtained from Peninsula Labs. (Belmont, CA, U.S.A.). Ammonium bicarbonate, 4-vinylpyridine and tri-*n*-butylphosphine were purchased from Aldrich (Milwaukee, WI, U.S.A.) while dithiothreitol (DTT) and dithioerythritol (DTE) were obtained from Sigma (St. Louis, MO, U.S.A.). L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was purchased from Worthington (Freehold, NJ, U.S.A.). High-performance liquid chromatography (HPLC)-grade trifluoroacetic acid (TFA)

was obtained from Pierce (Rockford, IL, U.S.A.), and water and acetonitrile were HPLC grade purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All the other chemicals were of the highest purity commercially available and were used without further purification.

*FAB-MS analysis*

FAB-MS analysis of peptides and protein digest mixtures was carried out on a VG ZAB-SE double-focusing mass spectrometer operating at an accelerating voltage of 8 kV. Approximately 1 nmol of the peptide sample, dissolved in 2 $\mu$l of 0.1% aqueous TFA, was deposited onto a stainless-steel probe tip containing 1–2 $\mu$l of matrix (thioglycerol or 5:1 DTT/DTE mixture) and then ionized by bombardment with a beam of 7–8 keV xenon atoms.

*PD-MS analysis.*

PD mass spectra were acquired over a 1–4 h period on a BIOION 20 $^{252}$Cf PD time-of-flight mass spectrometer with a 10-$\mu$Ci sample of $^{252}$Cf emitting *ca.* 2000 fission fragments per s and using an accelerating voltage of 18 kV. The mass resolution was approximately 300 at 50% of peak base. Protein (1–2 nmol) dissolved in a 1:2 mixture of ethanol–0.1% aqueous TFA was applied on a nitrocellulose-coated aluminum foil [17] and spin-dried [18,19] before its insertion into the source of the mass spectrometer. The electrospray method [20] was used for coating the aluminum foil with a nitrocellulose film. In the case of salt-contaminated samples, the nitrocellulose-adsorbed peptide and protein samples were washed with 0.1–1.0 ml of 0.1% TFA in order to remove the salt contaminants. All PD mass spectra shown in this paper have been previously subjected to background subtraction.

*Enzymatic digestion*

The protein was dialyzed against several changes of 0.1 *M* ammonium bicarbonate buffer (pH 7.8) followed by a single dialysis against deionized water and lyophilization. A 200-$\mu$g aliquot of rh-IFN $\alpha$-2b was dissolved in 1% ammonium bicarbonate buffer (pH 8.4 adjusted with 10% ammonium hydroxide). TPCK-treated trypsin or *Staphylococcus aureus* V8 protease was added at an enzyme-to-substrate ratio of 1:50 (w/w) and 1:25 (w/w), respectively, and the solution was incubated at 37°C for 18–24 h. The reaction was stopped by flash-freezing followed by lyophilization, and the crude digest mixture was subjected to FAB- and PD-MS analysis. Reduction of the disulfide bonds was carried out by adding 30-fold molar excess of DTT over the cysteine present and reacting under nitrogen at 40°C for 1 h.

*Iodination reaction*

Iodination reaction of rh-IFN $\alpha$-2b was carried out by the oxidative iodine monochloride iodination technique [21]. Lyophilized protein sample was dis-

solved in 20 m*M* phosphate buffer (pH 8.5 adjusted with 2.25 *M* sodium hydroxide) to a final concentration of 4 mg/ml. Iodine monochloride reagent (a mixture of NaI, NaCl, $NaIO_3$ and HCl) was added to the protein at a 2:1 molar ratio, and the reaction mixture was stirred for 2 min at room temperature. Endothelin and endothelin 22-31 were iodinated following the chloramine T iodination procedure [22].

*Cysteine modification*

Complete reductive pyridylethylation of cysteine was performed by reacting 100 μg of rh-IFN α-2b with 4-vinylpyridine in the presence of tri-*n*-butylphosphine [23,24].

*Chromatography*

The iodinated rh-IFN α-2b was applied onto a Sephadex G-50 (Pharmacia, Uppsala, Sweden) superfine gel column (40 cm × 1.6 cm) and eluted with 20 m*M* phosphate buffer, while the UV absorbance of the eluate at 280 nm was monitored throughout the run. The fractions whose elution volume corresponded to that of the uniodinated protein were collected, pooled and lyophilized, and then dissolved in 0.1% aqueous TFA and subjected to PD-MS analysis. In the case of endothelin, the iodination reaction products were separated by reversed-phase HPLC on a μBondapak $C_{18}$ column (30 cm × 0.39 cm, 10 μm; Waters, Millipore, Milford, MA, U.S.A.) using a linear gradient of 0.1% aqueous TFA and 0.1% TFA in acetonitrile (15–70% acetonitrile in 30 min), then dissolved in 0.1% aqueous TFA and subjected to FAB-MS analysis. The reductive pyridylethylation reaction product of rh-IFN α-2b was dissolved in 0.1% aqueous TFA containing 27% (v/v) acetonitrile, applied onto a reversed-phase Vydac $C_4$ guard column (25 mm × 4.6 mm, 5 μm; The Separations Group, Hesperia, CA, U.S.A.) and eluted with a linear gradient of 0.1% aqueous TFA and 0.1% TFA in acetonitrile (B) with UV detection at 215 nm. The gradient for the separation was 27–63% B in 15 min at a flow-rate of 1.0 ml/min. The modified protein appeared as a sharp, single peak with a retention time of 10.1 min, eluting at an acetonitrile composition of *ca.* 50%. Fractions containing the protein product were collected, pooled and evaporated to dryness in a Savant Speed-Vac. The sample was resuspended in 1% ammonium bicarbonate buffer and digested with trypsin under similar conditions to those described for rh-IFN α-2b. After digestion, the sample was concentrated to 20 μl and analyzed by FAB-MS.

## RESULTS AND DISCUSSION

In the case of rh-IFN α-2b (MW 19269), a leukocyte protein that has potent antiviral, antiproliferative and immunomodulating properties, the molecular weight (MW) was determined by PD-MS analysis [25]. The isotopically averaged MW as determined from the singly and multiply charged protonated PD signals

was 19 238 (mass measurement accuracy of 0.16%), which is sufficient for providing an initial indication of the correctness of the gene product. In the next step of the rh-IFN α-2b "mapping" procedure, the protein was cleaved at the C-terminal side of the lysine and arginine residues with trypsin. The amino acid sequence and the mass value of the expected tryptic peptide fragments from the cDNA-derived sequence of rh-IFN α-2b are shown in Table I. FAB-MS analysis of the crude digest mixture gave signals due to all the predicted tryptic peptides within the mass range scanned, *i.e.* 200–4800 Da (Fig. 1). The low-MW tryptic mono- and dipeptides could not be observed due to the high background usually present in the low mass region of the FAB mass spectrum. Several peptide signals arising from either incomplete or unexpected cleavages were also observed in the FAB mass spectrum, as shown in Fig. 1 and Table I. We have found that incomplete cleavage by trypsin occurs where there are two contiguous cleavage sites in the polypeptide chain, *i.e.* R–R, K–K, R–K and K–R. The presence of these products in the rh-IFN α-2b tryptic digest was also confirmed with the Edman-derived sequences (data not shown). Unexpected cleavages may have resulted from residual chymotryptic activity in the enzyme preparation.

The interferon molecule contains four cysteines at positions 1, 29, 98 and 138 of the protein sequence, which are contained in the tryptic peptides $T_1$, $T_5$, $T_{10}$ and $T_{17}$, respectively (Table I). The six possible combinations of disulfide pairings along with the expected $MH^+$ mass values of the resulting disulfide-linked peptide fragments are shown in Table II. The only peptide signals detected in the FAB mass spectrum of the unreduced tryptic digest mixture were at *m/z* 4616 and 2118 (Fig. 2A), which corresponded to the disulfide-linked peptides $T_1$–S–S–$T_{10}$ and $T_5$–S–S–$T_{17}$, respectively (Fig. 3). These signals at *m/z* 4616 and 2118 disappeared upon treatment of the tryptic digest mixture with DTT, whereas the signal intensities of the released peptides $T_1$, $T_5$, $T_{10}$ and $T_{17}$ increased dramatically due to the reduction of the disulfide bonds (Fig. 2B). The presence of the signal at *m/z* 2118 in Fig. 2B is due to an incomplete reduction of the disulfide bond between Cys(29) and Cys(138) over a period of 20 min; however, this peptide signal disappeared completely upon extending the reaction time to 60 min. Similar results were obtained from our "PD mapping" experiments, thus indicating that Cys(1) and Cys(29) were linked to Cys(98) and Cys(138), respectively, by a disulfide bond, which agreed with the previously reported results of disulfide bond mapping by HPLC [26].

The presence of cysteine-containing peptides was also confirmed by FAB-MS analysis of a tryptic digest of rh-IFN α-2b, in which the cystine residues of the protein were S-alkylated with 4-vinylpyridine in the presence of tri-*n*-butylphosphine [23,24]. The tryptic peptides containing one S-pyridylethyl cysteine (PEC) residue will have mass values 106 Da higher than those of their unmodified counterparts. The PEC-containing tryptic peptides $T_1$, $T_5$ and $T_{17}$ gave strong signals at *m/z* 1418, 1016 and 1315, respectively, while the PEC-$T_{10}$ peptide fragment did not give any detectable signal. It is notable that no signal at *m/z* 2118 was observ-

TABLE I

FAB-MS ANALYSIS OF α-2 INTERFERON TRYPTIC PEPTIDES

| Peptide | Sequence | Molecular weight | Ions observed as $MH^{+a}$ | |
|---|---|---|---|---|
| | | | Untreated | PEC derivative |
| $T_1$ | CDLPQTHSLGSR | 1313.6 | + | +[b] |
| $T_2$ | R | 174.2 | | |
| $T_3$ | TLMLLAQMR | 1076.5 | + | |
| $T_4$ | R | 174.2 | | |
| $T_5$ | ISLFSCLK | 910.2 | + | +[b] |
| $T_6$ | DR | 289.3 | | |
| $T_7$ | HDFGFPQEEFGNQFQK | 1955.3 | + | |
| $T_8$ | AETIPVLHEMIQQIFNLFSTK | 2460.2 | + | |
| $T_9$ | DSSAAWDETLLDK | 1450.7 | + | |
| $T_{10}$ | FYTELYQQLNDLEACVIQGVGVTETPLMK | 3304.2 | + | N.D.[c] |
| $T_{11}$ | EDSILAVR | 902.1 | + | |
| $T_{12}$ | K | 146.2 | | |
| $T_{13}$ | YFQR | 612.7 | + | |
| $T_{14}$ | ITLYLK | 750.0 | + | |

| | | | | |
|---|---|---|---|---|
| $T_{15}$ | EK | 275.3 | | |
| $T_{16}$ | K | 146.2 | | |
| $T_{17}$ | YSPCAWEVVR | 1209.5 | + | +[b] |
| $T_{18}$ | AEIMR | 618.8 | + | |
| $T_{19}$ | SFSLSTNLQESLR | 1481.8 | + | |
| $T_{20}$ | SK | 233.3 | | |
| $T_{21}$ | E | 147.1 | + | |
| *Other cleavage products* | | | | |
| $T_{20,21}$ | SKE | 362.2 | + | |
| $T'_1$ | SLGSR | 518.3 | + | |
| $T'_8$ | AETIPVLHEMIQQIFNLF | 2144.0 | + | |
| $T''_8$ | NLFSTK | 708.4 | + | |
| $T'_{5,17}$ | $T_5$-SS-$KT_{17}$ | 2245.8 | + | |
| $T'_{13}$ | $KT_{13}$ | 740.8 | + | |
| $T_{5,17}$ | $T_5$-SS-$T_{17}$ | 2117.7 | + | |
| $T_{1,10}$ | $T_1$-SS-$T_{10}$ | 4615.8 | + | |
| $T'_{1,10}$ | ($T_1$-$T'_1$)-SS-$T_{10}$ | 4115.5 | + | |

[a] Present in FAB-MS analysis.

[b] Present as PE-Cys adduct.

[c] N.D., Not determined or outside of mass range scanned.

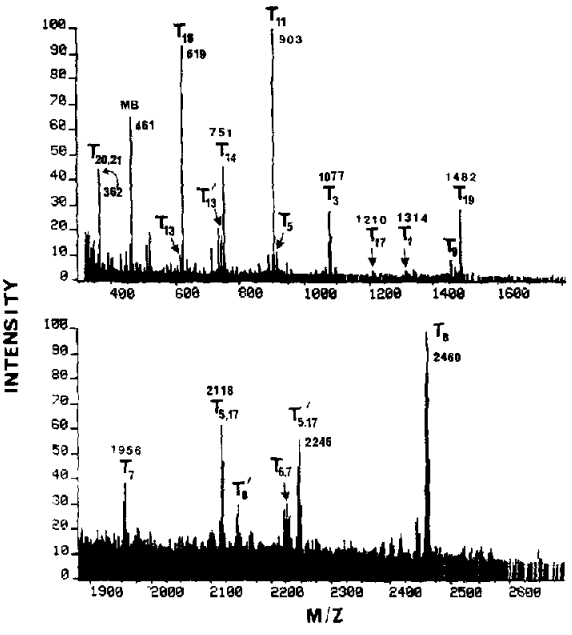


Fig. 1. Positive-ion FAB mass spectrum of the tryptic digest of rh-IFN α-2b. Tryptic peptides $T_{6,7}$ and $T_{20,21}$ arise from an incomplete cleavage at residues R(33) and K(164), respectively (see also Table I).

ed in this digest, thus indicating complete reduction and alkylation of the disulfide bond between Cys(29) and Cys(138).

We have used the PEC derivatization procedure successfully for several other proteins in our laboratory. Unlike alkylation with iodoacetic acid or iodoacetamide, which can react with other residue side-chains (*e.g.* histidine), S-alkylation with 4-vinylpyridine is highly specific for cystine residues and is not known to

TABLE II

POSSIBLE COMBINATIONS OF DISULFIDE-LINKED PEPTIDES IN rh-IFN α-2b TRYPTIC DIGEST

| S–S Bond linkage | Expected peptide | $MH^+$ |
|---|---|---|
| 1–29 | (1–12) + (24–31) | 2222.8 |
| 98–138 | (84–112) + (135–144) | 4512.7 |
| 1–98 | (1–12) + (84–112) | 4616.8[a] |
| 29–138 | (24–31) + (135–144) | 2118.7[a] |
| 1–138 | (1–12) + (135–144) | 2522.1 |
| 29–98 | (24–31) + (84–112) | 4213.4 |

[a] Ions detected by FAB-MS.

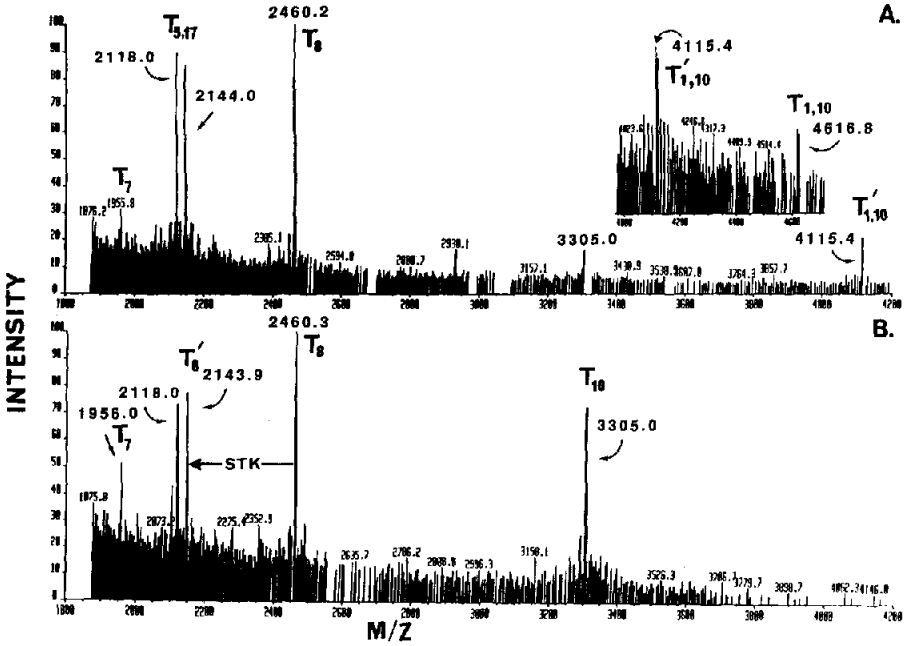


Fig. 2. Upper mass range of the positive-ion FAB mass spectrum of the rh-IFN α-2b tryptic digest before (A) and after (B) a 20-min DTT reaction.

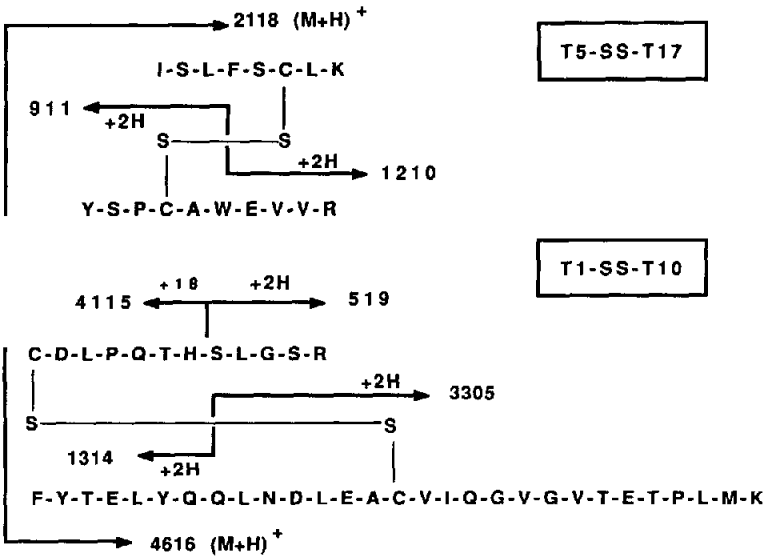


Fig. 3. Assignment of the disulfide bonds in rh-IFN α-2b from the observed FAB-MS signals in the analysis of the unreduced and reduced tryptic digest mixture.

modify other side-chains. Another reason for using this modification is to improve the sensitivity of mass spectral detection of some cysteine-containing peptides, which tend to give relatively weak FAB signals, even in the presence of a reducing FAB matrix such as thioglycerol. The PEC-containing peptides may provide more intense molecular ion signals due to the ability of the pyridine ring to stabilize positive charge. An additional advantage of pyridylethylation is the denaturation of the protein, rendering a greater portion of the protein sequence susceptible to enzymatic cleavage.

Similarly, sequence and disulfide mapping of human granulocyte–macrophage colony stimulating factor (rh-GM-CSF) and a series of modified rh-GM-CSF has been successfully carried out using the FAB/PD mapping approach [25].

Site-directed covalent modification of protein amino acid residues is currently used for a variety of applications, including metabolic studies and identification of enzyme active sites [27]. In an effort to prepare a suitable derivative of rh-IFN α-2b for its metabolic studies, a synthetic scheme was proposed in which an important part was the preparation of a monoiodinated derivative of rh-IFN α-2b, by introducing a iodine atom on one of the four tyrosine residues in the molecule. The macromolecular synthesis involved the treatment of rh-IFN α-2b (MW 19 269) with iodine monochloride following the method of Doran and Spar [21]. The PD mass spectrum of the reaction product gave a signal at $m/z$ 19 505 corresponding to the singly charged protonated molecular ion ($MH^+$) of the modified interferon molecule (Fig. 4), thus indicating the presence of a diiodinated derivative of rh-IFN α-2b. That was further supported by the presence of the $MH_2^{2+}$, $MH_3^{3+}$ and $MH_4^{4+}$ multiply charged protonated molecular ions at $m/z$ 9743, 6494 and 4866, respectively (Fig. 4). Thus, the synthetic modification reaction of rh-IFN α-2b and the extent of covalent modification were rapidly monitored by PD-MS analysis. Further determination of the iodination site(s) is cur-

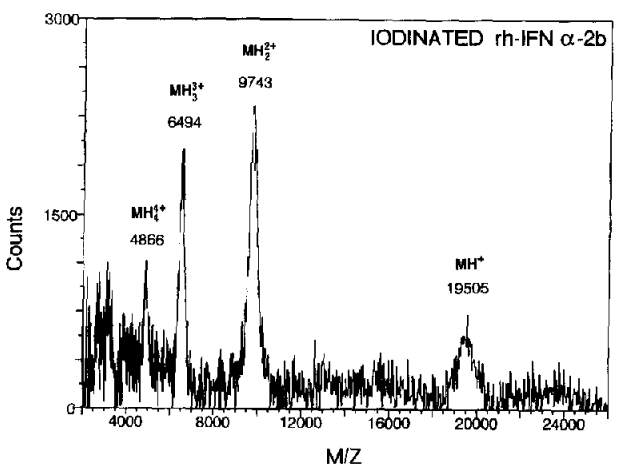


Fig. 4. Positive-ion $^{252}Cf$ PD mass spectrum of iodinated rh-IFN α-2b.

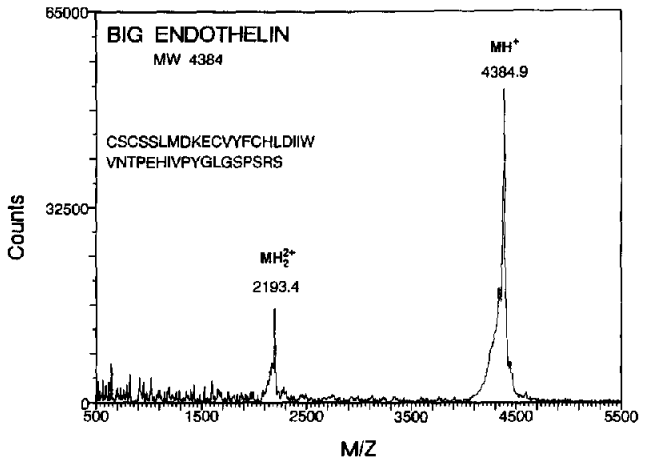


Fig. 5. Positive-ion $^{252}$Cf PD mass spectrum of big endothelin.

rently underway by a combination of chromatographic isolation of the product(s), tryptic degradation of each protein product followed by MS analysis of the resulting peptide mixture.

MS analysis has also been used to monitor synthetic modification reactions of biologically active peptides, such as the iodination reaction of endothelin. Both the 21-residue endothelin peptide, a potent vasoconstrictor peptide of endothelial origin [28], and its circulating precursor 39-residue form termed big endothelin [28] were subjected to FAB- and PD-MS analysis, which provided MW and sequence information. The PD mass spectrum of big endothelin along with a series of C-terminal sequence ions [10,29] is shown in Figs. 5 and 6, respectively.

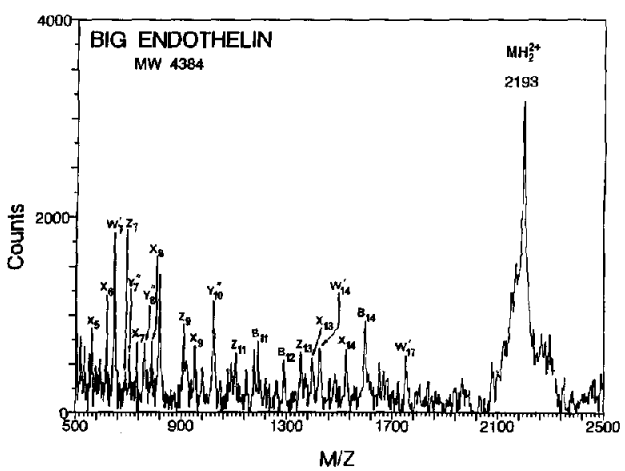


Fig. 6. Positive-ion $^{252}$Cf PD mass spectrum of big endothelin. The doubly charged molecular ion region with the C-terminal ions denoted.

The iodination reaction products of endothelin and endothelin 22-31 were separated by reversed-phase HPLC. FAB-MS analysis of the isolated fractions showed the presence of a major and a minor product corresponding to mono- and disubstituted iodotyrosine residues, respectively. It should be noted that the FAB mass spectra of both mono- and diiodinated derivatives exhibited loss of iodine. The iodinated peptides apparently undergo a FAB-induced radical dehalogenation with concomitant incorporation of hydrogen, leading to deiodinated products.

## CONCLUSION

This work demonstrates that MS is a powerful analytical tool in structure characterization studies of peptides and proteins. The accuracy of the MW determination of proteins by PD-MS is sufficient for quality control of genetically engineered proteins and for rapid indication of any modifications [30,31]. In addition, a mapping strategy combining proteolytic and/or chemical cleavage reactions of the protein with FAB/PD-MS analysis of the resulting peptide mixture can provide sequence and disulfide bond information, as well as the location of any modification site(s). The detection efficiency of the cysteine-containing enzymatic fragments can be improved by a reductive S-alkylation modification reaction. Furthermore, this mapping approach is invaluable in structural studies of chemically induced modifications of peptides and proteins, providing rapid monitoring of the synthetic modification reaction, as well as the extent and the location of the modification(s).

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## REFERENCES

1 G. L. Davis, L. A. Balart, E. R. Schiff *et al.*, *N. Engl. J. Med.*, 321 (1989) 1501.
2 M. Barber, R. S. Bordoli, R. D. Sedgwick and A. N. Tyler, *J. Chem. Soc. Chem. Commun.*, (1981) 325.
3 R. D. Macfarlane and D. F. Torgerson, *Science*, 191 (1976) 920.
4 B. Sundqvist and R. D. Macfarlane, *Mass Spectrom. Rev.*, 4 (1985) 421.
5 H. R. Morris, M. Panico and G. W. Taylor, *Biochem. Biophys. Res. Commun.*, 117 (1983) 299.
6 B. W. Gibson and K. Biemann, *Proc. Natl. Acad. Sci. U.S.A.* 81 (1984) 1956.
7 A. Tsarbopoulos, G. W. Becker, J. L. Occolowitz and I. Jardine, *Anal. Biochem.*, 171 (1988) 113.
8 K. Biemann and H. A. Scoble, *Science*, 237 (1987) 992.
9 K. Biemann and S. A. Martin, *Mass Spectrom. Rev.*, 6 (1987) 1.
10 K. Biemann, *Biomed. Environ. Mass Spectrom.*, 16 (1988) 99.
11 S. A. Carr, *Adv. Drug Deliv. Rev.*, 4 (1990) 113.

12 M. Karas and F. Hillenkamp, *Anal. Chem.*, 60 (1988) 2299.
13 M. Karas, A. Ingendoh, U. Bahr and F. Hillenkamp, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 841.
14 R. Beavis and B. T. Chait, *Rapid Commun. Mass Spectrom.*, 3 (1989) 233.
15 C. M. Whitehouse, R. N. Dreyer, M. Yamashita and J. B. Fenn, *Anal. Chem.*, 57 (1985) 675.
16 J. A. Loo, H. R. Udseth and R. D. Smith, *Anal. Biochem.*, 179 (1989) 404.
17 G. P. Jonsson, A. B. Hedin, P. L. Håkansson, B. U. R. Sundqvist, B. G. S. Säve, P. F. Nielsen, P. Roepstorff, K.-E. Johansson, I. Kamensky and M. S. L. Lindberg, *Anal. Chem.*, 58 (1986) 1084.
18 I. Jardine, G. F. Scanlan, A. Tsarbopoulos and D. J. Liberato, *Anal. Chem.*, 60 (1988) 1086.
19 P. F. Nielsen, K. Klarskov, P. Hojrup and P. Roepstorff, *Biomed. Environ. Mass Spectrom.*, 17 (1988) 355.
20 C. J. McNeal, R. D. Macfarlane and E. L. Thurston, *Anal. Chem.*, 51 (1979) 2036.
21 D. M. Doran and J. L. Spar, *J. Immunol. Methods*, 39 (1980) 155.
22 H. Zola, *Monoclonal Antibodies: A Manual of Techniques*, CRC Press, Boca Raton, FL, 1987, Ch. 6, p. 163.
23 M. Friedman, L. H. Krull and J. F. Cavins, *J. Biol. Chem.*, 245 (1970) 3868.
24 P. C. Andrews and J. E. Dixon, *Anal. Biochem.*, 161 (1987) 524.
25 A. Tsarbopoulos, B. N. Pramanik, P. Bartner, P. Reichert, J. Labdon, T. L. Nagabhushan and P. P. Trotta, in A. Hedin, B.U.R. Sundqvist and A. Benninghoven (Editors), *Ion Formation from Organic Solids IFOS V*, Wiley, Chichester, 1990, p. 101.
26 N. B. Lydon, C. Favre, S. Bove, O. Neyret, S. Benureau, A. M. Levine, G. F. Seelig, T. L. Nagabhushan and P. P. Trotta, *Biochemistry*, 24 (1985) 4131.
27 J. F. Riordan and M. Sokolovsky, *Acc. Chem. Res.*, 4 (1971) 353.
28 M. Yanagisawa, H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazahi, V. Goto and T. Masahi, *Nature*, 332 (1988) 411.
29 P. Roepstorff and J. Fohlman, *Biomed. Mass Spectrom.*, 11 (1984) 601.
30 A. Tsarbopoulos, *Peptide Res.*, 2 (1989) 258.
31 P. Roepstorff, *Acc. Chem. Res.*, 22 (1989) 421.