Fast-atom-bombardment mass spectrometry

A new technique for the determination of molecular weights and amino acid sequences of peptides

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A detailed study of the mass spectra of peptides produced by the new technique of fast-atom bombardment is reported. Molecular weights of unmodified peptides containing up to 21 amino acids have been determined. In favourable cases, the molecular-weight determination may be made on as little as 0.1 nmol of sample. Positive-ion and negative-ion spectra are obtained with equal facility. With sample sizes in the range 2–50 nmol, sequence information can be obtained in many cases. The technique represents an important contribution to structural studies on peptides, since (i) blocked peptides may be studied, (ii) no prior formation of chemical derivatives is necessary (except for distinction between lysine and glutamine), (iii) sensitivity is good, (iv) large peptides, and, in some cases, mixtures of peptides, can be studied, and (v) the technique is easy to operate, with ions being produced over a long period (5–30 min).

The sequencing of peptides by mass spectrometry has made important contributions to the study of peptides and proteins during the last decade. Two approaches have been employed with wide success. In one, oligopeptides (most commonly four to nine residues) are acetylated and permethylated, and sequenced after volatilization, and ionization by electron impact; sequence determination of simple mixtures is possible (Morris *et al.*, 1971; Morris, 1980). In the other, mixtures of small peptides are reduced and silylated, and sequenced with the aid of computer interpretation, after separation of components by g.l.c. and ionization by electron impact (Khorana *et al.*, 1979). An alternative technique is that of chemical-ionization mass spectrometry (Gray *et al.*, 1970). This method may complement electron-impact studies, since the ions characteristic of molecular weight (usually MH^+) are more stable than the odd-electron molecular ions produced by electron impact. Hence information on molecular weight is frequently more clearly obtained. Additionally, sequence ions that contain the C-terminal portion of the peptide are often produced, and these complement sequence information obtained from the N-terminus (by either electron impact or chemical ionization). Although these methods are powerful, they suffer from the disadvantages that (i) large peptides are not amenable to study directly, and (ii) chemical modifications of peptides are necessary

before sequence determination. In some cases, field-desorption mass spectrometry has permitted molecular-weight determination of unmodified peptides in the molecular-weight range 1000–2000 (Wada *et al.*, 1981). Unfortunately, this method has enjoyed less-widespread use, partly owing to practical difficulties in its application. However, Barber *et al.* (1981) have pioneered the technique of fast-atom-bombardment (FAB) mass spectrometry. In their paper on the subject, they report the mass spectrum of Met-Lys-bradykinin, obtained without prior chemical modification; the spectrum contains ions that permit the sequence determination. Additionally, our own group has reported the sequence determination of a mixture of tetradeca- and pentadeca-peptides from a *Paracoccus* cytochrome *c*-550 by FAB mass spectrometry (Williams *et al.*, 1981*a*). We now report a study of a wide range of peptides by FAB mass spectrometry.

Experimental

Mass spectrometry

Mass spectra were obtained on a Kratos MS 50 instrument, operating at accelerating voltages in the range 5–8 kV. Samples were introduced into the ion source on a copper probe tip via a conventional vacuum lock. A thin layer of glycerol was applied to the copper probe tip, and solid samples were dissolved in this layer before insertion into the source. Peptides from enzymic digests (2–100 nmol)

Abbreviation used: FAB mass spectrometry, fast-atom-bombardment mass spectrometry.

were dissolved in a small quantity of glycerol, and these solutions were then transferred to the copper probe tip. The area of the probe tip lay in the range 0.1–0.25 cm²; typically, 10–50% of the area of the tip was covered by the glycerol matrix containing the sample.

Ions from the sample were obtained in the gas phase by bombardment of the glycerol solution with 4–6 keV argon atoms at a pressure approx. 1.3 mPa. These argon atoms were obtained by charge exchange of 4–6 keV argon ions, with a standard Kratos (Urmston, Manchester, U.K.) FAB source and associated fast-atom gun. Spectra were scanned from high mass to low mass, at rates lying in the range 100–1000 s per decade.

Peptides

The peptides studied were either (i) commercially available (Peninsula Laboratories, San Carlos, CA, U.S.A., and Cambridge Research Biochemicals, Huntingdon, Cambs., U.K.), (ii) obtained by enzymic digests of proteins (kindly provided by Dr. R. P. Ambler, Department of Molecular Biology, University of Edinburgh, Edinburgh, U.K., and Dr. A. D. Auffret, Department of Biochemistry, University of Leeds, Leeds, U.K.), or (iii) synthetic samples (kindly provided by Dr. R. C. Sheppard, M.R.C. Laboratory for Molecular Biology, Cambridge, U.K.). All samples were used as obtained, except as noted below.

Chymotryptic digest of a *Paracoccus* cytochrome *c*-550 fragment

The peptide (approx. 10 nmol) was digested under standard conditions of a 50:1 molar ratio of substrate to chymotrypsin in 0.2 M-ammonium acetate buffer, pH 8.5. The solution was incubated at 37°C overnight, and the mixture of products was isolated by freeze-drying.

Results

The work of Barber *et al.* (1981) has already established that FAB mass spectrometry is well suited to obtain mass spectra of polar molecules and salts. In our own preliminary work (Williams *et al.*, 1981b) we have proposed that an important process in the production of gaseous ions is momentum transfer from the impacting argon atoms to sample molecules. Our data show that the production of gaseous positive ions from peptides is facilitated by the presence of basic sites in the peptide. Thus arginine and lysine residues (and, presumably, to a smaller extent, histidine residues) facilitate the production of a protonated molecular ion (MH⁺) in positive-ion spectra. In principle, the effect should work well when the peptide has a net single positive charge at neutral pH, and indeed excellent positive-

ion spectra are produced with high sensitivity in such cases. Conversely, acidic residues, such as aspartic acid and glutamic acid, promote the production of negative ions. Again, we find that excellent negative-ion spectra [(M–H)[–] giving molecular-weight information] are produced when peptides have a net single negative charge at neutral pH. However, the charge of ions that appear in FAB mass spectra is not solely dictated by the above factors. Good positive-ion spectra (containing MH⁺) may be obtained from peptides having a net negative charge at neutral pH, and good negative-ion spectra [containing (M–H)[–]] from peptides having a net positive charge at neutral pH. Nevertheless, our recommendation is that, if sample size is limited (< or ≪ 1 nmol), the spectrum first obtained should correspond to the sign of the net charge established by electrophoresis. The production of multiply charged ions is not normally significant for structural studies on peptides. This fact presumably reflects the relatively high heat of formation of multiply charged ions in the gas phase, where the charges cannot be stabilized by intermolecular solvation.

We now consider the most important features of positive-ion and negative-ion FAB mass spectra of peptides. First, abundant MH⁺ and/or (M–H)[–] ions are produced from unmodified peptides, in favourable cases up to molecular weights in excess of 2000. The spectra may be counted from low mass by virtue of the presence of a low-abundance ion at each *m/z* value. The calibration of the count may be checked by the presence of ions due to protonated glycerol oligomers at *m/z* 93, 185, 277 ... 1197 in positive-ion spectra, and at *m/z* 91, 183, 275 ... 1195 in negative-ion spectra. The sensitivity of the technique varies somewhat from sample to sample, but molecular-weight determination can often be made on 0.1–1.0 nmol of sample. For example, abundant (M–H)[–] ions are obtained from gastrin-17 (structure 1) at *m/z* 2077 (Fig. 1a), and from human insulin C-peptide 40–60 (structure 2) at *m/z* 1904 (Fig. 1b). Note that negative-ion spectra of these molecules were obtained in view of the net charges existing at neutral pH (indicated by the signs in structures 1 and 2).

Comparison of Figs. 1(a) and 1(b) shows a more abundant (M–H)[–] ion in the latter case, even though similar sample loadings and multiplier settings were used. This observation presumably reflects the more favourable lower net negative charge in structure 2. The peptide 2 is sufficiently ionic to produce a sensitive negative-ion FAB mass spectrum, but apart from these features is relatively hydrophobic. In contrast, a mono-anion of structure 1 will be a high-energy species, and therefore is produced in lower abundance.

The MH⁺ or (M–H)[–] ions are normally the

Fig. 1. Molecular-ion regions in the negative-ion FAB mass spectra of (a) gastrin-17 (1) and (b) human insulin C-peptide 40-60 (2)

Structure 1 (gastrin-17)

Structure 2 (human insulin C-peptide 40-60)

most abundant in FAB mass spectra of peptides (excluding low-mass background), and a sample size of 2–3 nmol was normally adequate for molecular-weight determination. In favourable cases, this sample size can be lowered by an order of magnitude. Sequence information is frequently obtained with approx. 20 nmol samples, although again approx. 2 nmol is adequate in favourable cases. In positive-ion spectra, the three most common and useful fragmentations that we have observed are summarized in structure 3 → structure 4 (Scheme 1), and structure 5 → structure 6 and structure 5 → structure 7 (Scheme 2). In the structure 3 → structure 4 fragmentation a $C_{(\alpha)}H-N$ bond is broken with a hydrogen migration to the *N*-terminal position, which retains the charge. Although the positive charge is most conveniently represented as arising via protonation of the *N*-terminus, it may be held at other sites. Indeed, this fragmentation is particularly favoured when arginine and/or lysine are present near the *N*-terminus.

Thus, in the spectrum (Fig. 2) of the neuropeptide substance P (structure 8), sequence ions of this type

occur at m/z 752, 899, 1046, 1103 and 1216. These establish the sequence X-Phe-Phe-Gly-Leu-MetNH₂. As with chemically modified peptides, leucine is not differentiated from isoleucine. In addition, lysine and glutamine have the same nominal mass, but may be distinguished by acetylation of the former (see below). Other features of this spectrum are discussed subsequently.

The remaining two positive-ion fragmentations that we discuss (structure 5 → structure 6 and structure 5 → structure 7) occur with charge retention by the *C*-terminal fragments 6 and 7. Each fragmentation occurs with an associated hydrogen migration, one nominally giving rise to an *N*-terminal amino group (structure 6) and the other to a terminal alkyl group (structure 7). Each of these fragments carries a proton at some site. These types of fragmentation are promoted when a basic amino acid (e.g. arginine, which provides a site to carry the proton) is contained in the *C*-terminal portion of the peptide. Recognition of the sequence ions 6 and 7 is aided by their characteristic separation by 15 mass units. We propose the names given with structures 4,

Fig. 2. Positive-ion FAB mass spectrum of substance P (8)

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH₂

Structure 8 (neuropeptide substance P)

Scheme 2

Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg

Structure 9 (human fibrinopeptide A)

6 and 7 for the sequence ions, and subsequently use the corresponding abbreviations that are given.

Ions of type 6 and 7 are evident in the positive-ion spectrum (Fig. 3) of human fibrinopeptide A (structure 9).

The alkyl sequence ions 7 occur at m/z 259, 316,

373, 430, 559, 630, 743, 890, 1005, 1062, 1191, 1248 and 1335, and the amino sequence ions 6 at m/z 274, 331, 388, 445, 574, 645, 758, 905, 1020, 1077, 1206, 1263 and 1350. Thus the sequence of this peptide excepting the two *C*-terminal and two *N*-terminal amino acid residues can be deduced from

Fig. 3. Positive-ion FAB mass spectrum of human fibrinopeptide A (9)

Structure 10

the one spectrum. However, the possibilities for the unknown pairs of amino acids at each terminus are limited, since the sums of their masses are known.

It is important to note that the sequence ion designated +CAIk cannot form when R represents a proline residue. The presence of a +CA ion corresponding to the mass difference of proline in the absence of the corresponding +CAIk ion supports the inference that the mass difference is due to a proline residue. This point is illustrated by reference to the spectrum (Fig. 2) of substance P (8). The +CA ions (6) occur at m/z 613, 741, 869, 966, 1094 and 1191, and the +CAIk ions (7) at m/z 598, 726, 854 and 1079. Together, these series of ions and MH^+ indicate the sequence Arg-Pro-Lys-Pro-Gln-Gln-Y, with the expected absence of the +CAIk ions m/z 951 and 1176 at the proline residues. We have also noted that, when the amino acid residue at the *N*-terminus in ion 6 is proline, there is frequently an ion of similar abundance at an m/z value 2 units lower. For example, in the spectrum of substance P (Fig. 2) these ions are observed m/z 1189 and 964. This appears likely to be due to an enhanced stability of proline iminium ions (structure 10).

We emphasize that in some spectra the formation of all three types of sequence ion (+NA, +CA, +CAIk) enables the complete sequence of the peptides to be determined. For example, this is the case in the spectrum (Fig. 2) of substance P (8), which has been discussed. It is also true in the spectrum of physalaemin:

An additional fragmentation, observed less generally, occurs on occasions in positive-ion spectra. This fragmentation leads to the formation of acylium ions ($R-C\equiv O^+$), exactly as in the spectra of acetylated and permethylated peptides (Morris *et al.*, 1971). As with these chemically modified peptides, the resulting ions allow sequence information to be derived from fragments containing the *N*-terminus of the peptide. For example, in the spectrum of physalaemin (sequence given above), acylium sequence ions are observed at m/z 1117, 1004, 947, 784 and 637, and these indicate the sequence X-Phe-Tyr-Gly-Leu-MetNH₂.

In the negative-ion mode, the presence of a

Structure 11 (negative-ion *N*-terminal amide sequence ion; -NA)

Scheme 3

carboxylate anion appears to be important in the generation of the relatively stable sequence ions. However, it is not a prerequisite. The three main types of sequence ions found in negative-ion spectra are formed by cleavage of the same bonds as those (structures 4, 6 and 7) found in positive-ion spectra. However, the products (structures 11, 12 and 13; Schemes 3 and 4) now contain a net single negative charge, most generally due to the presence of a carboxylate anion. For convenience, in the formation of structure 11, this charge is shown in the *N*-terminal amino acid, although this is not necessarily the case.

For example, in the negative-ion spectrum of the decapeptide 14 [$(M-H)^-$ at m/z 1110], the presence of a glutamic acid residue in the *C*-terminal region favours the formation of relatively abundant -CA (m/z 429, 558, 615, 778, 906 and 1053) and -CAIk (m/z 414, 543, 600, 763, 891 and 1038)

ions, which establish the sequence Gly-Phe-Lys-Tyr-Gly-Glu-Y. However, the presence of a net negative charge in the *C*-terminal portion of the peptide is not mandatory for the observation of -CA and -CAIk ions, as is evident from the negative-ion spectrum (Fig. 4) of human fibrinopeptide A (9). The -CA ions (12) occur at m/z 903, 1018, 1075, 1204, 1261, 1348 and 1463, and the -CAIk ions (13) at m/z 888, 1003, 1060, 1189, 1246, 1333 and 1448. These ion series establish, in conjunction with $(M-H)^-$, the sequence Ala-Asp-Ser-Gly-Glu-Gly-Asp-Y. In addition, *C*-terminal sequence information is obtained from the -NA ions at m/z 1377, 1278 and 1221, establishing

Scheme 4

Gly-Phe-Lys-Tyr-Gly-Glu-Gly-Ile-Leu-Glu

Structure 14

Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Leu-Asp-PheNH₂

Structure 15 (gastrin-13)

the sequence X-Gly-Val-Arg. Thus information derived from the positive-ion spectrum (Fig. 3) is supplemented and confirmed.

Sequence ions of the type -NA (11) are frequently observed in the negative-ion spectra of peptides that contain acidic amino acids close to the *N*-terminus; however, the presence of such acidic residues is not essential for the observation of -NA ions. For example, in the negative-ion spectrum (Fig. 5) of the gastrointestinal peptide fragment gastrin-13 (15), sequence ions are observed at *m/z* 1479, 1364 and 1251; these establish the sequence X-Leu-Asp-PheNH₂. In all cases where the sequence determination is not total, restrictions are of course placed on the nature of X and Y, since the atomic masses of X and Y are determined.

With only 5–20 nmol of sample, a positive-ion spectrum may be obtained, the instrument switched to the negative-ion mode and a negative-ion spectrum obtained on the same sample. When peptides are *N*-acetylated and permethylated before examination by electron impact, arginine must be modified before these chemical modifications (Morris *et al.*, 1973). Arginine may be handled directly by FAB mass spectrometry. Additionally, we find that peptides containing disulphide bridges, methionine sulphone or cysteic acid do not pose problems. For example, somatostatin, oxytocin and

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂

Structure 16 (oxytocin)

vasopressin (all containing disulphide bridges) produce MH⁺ ions in their positive-ion spectra. Sequence ions have been observed for these peptides, but generally appear to be of a lower abundance than those formed from non-cystine-containing peptides. Thus, in the positive-ion spectrum of oxytocin (16), +NA ions (4) indicate the sequence X-Leu-GlyNH₂, and +CA ions (6) show the sequence

Cys-Tyr-Leu(or Ile)-Gln-Y

In this case, since formation of the +CA ions requires cleavage of the disulphide bridge, the +CA ions are not formed with the normal single hydrogen rearrangement (structure 5 → structure 6), but rather with a double hydrogen transfer. The second hydrogen is presumably transferred to convert a sulphur radical into a more stable thiol group.

Apart from the representative results already discussed, it was thought desirable to obtain FAB mass spectra of a series of peptides produced via enzymic digestion of a protein. A series of 13

Fig. 4. Negative-ion FAB mass spectrum (m/z 800–1550) of human fibrinopeptide A (9)

Fig. 5. Negative-ion FAB mass spectrum (m/z 1220–1650) of gastrin-13 (15)

peptides obtained by enzymic digestion of an amidase (R. P. Ambler, personal communication), and each isolated as a pure component, were chosen for study. These were studied 'blind', the spectra being scanned downwards from a region somewhat higher in mass than that estimated from the roughly known size of the peptides. Sample sizes were in the range 10–50 nmol, permitting molecular weights to be determined, and checked for consistency, by running both positive-ion and negative-ion spectra. When this was done, the MH^+ and $(M - H)^-$ ions differed by the expected 2 mass units. Only one sample failed to give information on the molecular weight. In all the other cases, a subsequent comparison with sequences determined by classical solution methods showed perfect agreement between expected and observed molecular weights, which lay in the range 588–1617. The spectra also gave some sequence information. Similar further quantities of the peptides were *N*-acetylated [acetic anhydride/methanol (1:4, v/v), room temperature, 3 h] to determine the number of basic amino acids in each sample. An increment of 42 in the molecular weight occurs for each acetyl group introduced. The spectra showed that, although these conditions are commonly used for *N*-acetylation, acetylation was often

incomplete when the peptides contained more than one amino group. Additionally, carboxy groups were partially converted into methyl ester groups. Incomplete acetylation and methyl ester formation was avoided if acetylation was performed with acetic anhydride/water (1:1, v/v) for $\frac{1}{2}$ h at room temperature. *N*-Acetylation of the ϵ -amino group of lysine removes the mass degeneracy of lysine and glutamine residues.

In FAB mass spectrometry, sample volatilization is achieved by momentum transfer from the impacting argon atoms, rather than thermally. Hence components of a mixture that have different thermal volatilities frequently produce signals in a single scan. Thus in favourable cases the technique may be used in the analysis of peptide mixtures, as illustrated by the following examples. In mixture analysis, molecular ions can normally be distinguished from fragment ions because of the appreciably larger abundance of the former.

A sample obtained by enzymic digestion, and subsequent purification of peptides, from *Azotobacter vinelandii* cytochrome c_4 was deduced from dansyl-Edman sequencing to have the sequence 17.

The molecular-ion region of the positive-ion mass

Fig. 6. Partial FAB mass spectra (m/z 740–960) of a chymotryptic digest of peptide 18 in (a) the positive-ion mode and (b) the negative-ion mode

Ala-Gly-Asp-Ala-Ala-Ala-Gly-Glu-Gly-Lys-Ala-Ala

Structure 17

Structure 18

spectrum of the sample showed three ions of similar abundance at m/z 846, 917 and 988. These MH^+ ions differ by 71 mass units, and indicate that the sample is a mixture of similar amounts of three peptides terminating in ...Gly-Lys, ...Gly-Lys-Ala and ...Gly-Lys-Ala-Ala. The two lower-mass ions are clearly not due to sequence ions, as (i) they are as abundant as m/z 988 and (ii) loss of a C-terminal amino acid to give a +NA (4) sequence ion results in loss of the amino acid mass +1, and not loss of the amino acid mass (see the Appendix).

The peptide 18, obtained from a *Paracoccus* cytochrome *c*-550 and sequenced by dansyl-Edman techniques (R. P. Ambler, personal communication), has a molecular weight of 2501, and a FAB mass spectrum could not be obtained from the intact peptide. The peptide (approx. 10 nmol) was therefore subjected to a chymotryptic digestion. Both positive-ion and negative-ion FAB mass spectra of the product were obtained. In the former, the most abundant MH^+ ion is m/z 842 (Fig. 6a); in the latter, the most abundant $(\text{M-H})^-$ ions are m/z 743 and 951 (Fig. 6b). These three products are obtained by the anticipated cleavages at tryptophan and tyrosine residues. The two peptides with a net negative charge show up most strongly in the negative-ion spectrum, and the neutral peptide shows up most strongly in the positive-ion spectrum.

The spectra reported in the present paper were obtained with the use of two instruments with magnetic sectors that limit their mass ranges to either 1700 or 1000 at full accelerating voltage (8 kV). Comparison of results obtained with these two instruments indicates a marked loss in sensitivity when the accelerating voltage is lowered to increase the mass range. It is therefore possible that the molecular weights of peptides that can be successfully determined by FAB mass spectrometry extend to appreciably higher values than those reported here.

Conclusion

Fast-atom-bombardment mass spectrometry clearly represents an important advance in struc-

tural studies on peptides. It will aid structural studies that rely on 'wet chemistry', since molecular-weight and sequence information can be obtained irrespective of the nature of blocked *N*- or *C*-termini. In this respect, its application in the study of novel neuropeptides should be advantageous, and should complement the use of the electron-impact method in the study of blocked peptides. Additionally, FAB mass spectrometry has the potential to determine directly the in-chain masses of novel amino acids. This is especially useful where these novel amino acids might be changed by hydrolytic procedures, or by the permethylation procedure used in electron-impact mass spectrometry.

The method should prove to be a powerful adjunct to electron-impact mass spectrometry, since it has strengths precisely where the electron-impact technique has weaknesses (e.g. relatively poor in molecular-weight determination, and in mass range). Electron-impact mass spectrometry is often powerful in determining the sequence of, say, the first six or seven amino acids from the *N*-terminus. In FAB mass spectrometry, the +NA or -NA ions, which would give the same information, may be obscured by background peaks or those from glycerol. However, the FAB mass spectrum frequently gives the higher-mass sequence ions, and almost always determines the molecular weight (up to 1600, and frequently up to 2000). Since momentum transfer produces volatilization, thermally labile substances can now be studied with much greater ease. Finally, the technique is relatively simple to operate, and much less liable to mechanical failure than is field-desorption mass spectrometry. At the present stage of development of the technique, a limitation that has to be considered is that sequence ions are not always of sufficient abundance to give extensive sequence information.

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APPENDIX

Table 1. *Integral masses of amino acid residues*

The mass of terminal amino acids may be deduced from MH^+ or $(M-H)^-$ values of the masses shown in the Table are modified as follows. (i) Following a series of $+CA$ or $-CA$ ions, use the values in the Table, (ii) following $+Calk$ or $-Calk$ ions, add 15 mass units to the values; (iii) following $+NA$ or $-NA$ ions, add 1 mass unit to the values; (iv) following $+NA$ or $-NA$ ions, differences corresponding to those in the Table may indicate a C-terminal amide.

Gly	57	Asp	115	Lys	128
Ala	71	Asn	114	His	137
Val	99	Glu	129	Pro	97
Leu	113	Gln	128	Met	131
Ile	113	Phe	147	Arg	156
Ser	87	Tyr	163	Cys	103
Thr	101	Trp	186		