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Metastable Decay of Peptides and Proteins in Matrix-assisted Laser-desorption Mass Spectrometry

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Fragmentation of protein and peptide ions generated by matrix-assisted laser desorption has been investigated using a modified LAMMA 1000 reflecting time-of-flight (TOF) mass spectrometer. Whereas fragmentation of covalent bonds prior to ion acceleration (i.e., within several ns after the laser pulse) in general is not observed using the matrix technique, extensive fragmentation on a longer time scale can be studied in our instrument. The high mass resolution ($M/\Delta M \approx 1200–1800$ for insulin and peptides) permits the investigation of even small mass losses from parent molecular ions (occurring in the first section of the field-free drift region) by measuring flight time differences of daughter ions acquired during passage through a two-stage reflectron. The kind and extent of this metastable decay has been found to depend strongly on the substance under investigation. Typical fragmentations are loss of ammonia and parts of the amino acid side-chains. The large abundances of peaks due to such metastable fragmentation, observed for most of the peptides and proteins investigated, may, at least in part, explain peak broadening (and, hence, poor mass resolution) typical in matrix-assisted laser-desorption TOF mass spectra.

One of the main goals in matrix-assisted laser desorption of high-mass biopolymers, a technique first described by Karas and Hillenkamp,[1] is the achievement of high mass resolution and high mass accuracy for the full mass range (up to ca 300 000 Da) accessible to date. For several proteins having molecular weights below 30 000 Da, Beavis and Chait reported a mass resolving power, $m/\Delta m$, of 300–500 leading to an accuracy in mass measurement of about $\pm 0.01\%$ by means of internal calibrants.[2] However, mass spectra of proteins in the very high mass region as reported in the literature have degraded mass resolutions, reaching values of not higher than ca 50 in the range above ca 100 000 Da. Since, in this mass range, the detection of the ions is governed by processes of secondary-ion rather than secondary-electron formation at the instrument's target surfaces (such as a conversion dynode, an ion detector or field grids[3–5]), spread in flight times of these secondary species has been suspected to cause substantial peak broadening.[3]

However, even under instrumental conditions that carefully avoid such secondary-ion formation at or near the detector, mass resolution remains much lower than expected. We report here on another mechanism that contributes to peak broadening, resulting from the lack of stability of high-mass ions during their flight through the time-of-flight (TOF) mass spectrometer. It is shown that a substantial number of ions decay after acceleration and prior to detection, even under threshold conditions of laser irradiation. This leads to peak broadening by two mechanisms: (i) flight-time differences between neutrals, parent ions and fragment ions resulting from their passage through electric fields (e.g., ion lenses, fringe fields in front of channel-plate detectors, ion converters etc.), and (ii) kinetic energy release. The fact that protein or peptide ions tend to decay in the flight tube is especially notable, since in general no immediate fragmentation from the desorption process can be observed in normal mass spectra under the same instrumental conditions.

Different approaches have been used in our laboratory to determine the amount and kind of metastable decay of laser-desorbed protein and peptide ions.[6] In this paper we describe the detection of fragments using a two-stage reflectron instrument, which allows us to observe even small mass losses of a few daltons, in contrast to using linear acceleration or deceleration fields, by which the amount of neutralization can be determined.[6] Metastable decay affects time-of-flight mass spectra in a very different way, depending on the basic type of instrument. Fragment ions formed by decay in the field-free drift region keep moving with almost the same velocity as their parent ions. In a *linear* TOF instrument they are thus detected at basically the same time as the parent ions, although kinetic energy release from the fragmentation process leads to peak broadening.

Almost the same behavior is expected for the case when *ion mirrors* are used in the flight path.[7] An ion mirror is simply a very short ion reflectron, unable to perform energy compensation. (The short first stage of a two-stage reflectron can be used as an ion mirror.) As a result of their loss of kinetic energy, fragment ions from metastable decay have shorter flight paths (and thus shorter flight times) in reflecting electric fields, compared to their parent ions. However, in the case of ion mirrors, the time spread between parent ions and metastable fragments is very small, especially for the case of small mass losses, since the resulting flight-path difference in the mirror is very small compared to the complete flight path through the instrument.

In a *single-stage reflectron* the relation between mass

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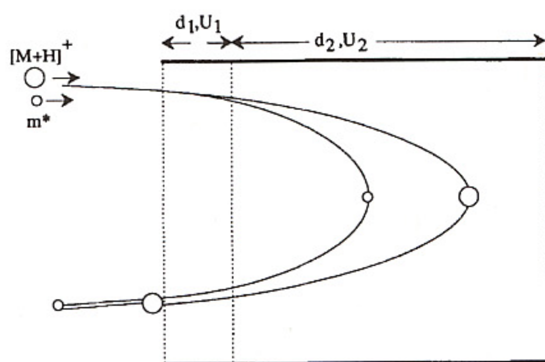

Figure 1. Scheme of the two-stage reflectron. The fragment ions from metastable decay (small circles) take a shorter path through the reflectron than the parent ions (large circles) and thus have shorter flight times.

loss, ΔM, and flight-time difference

$$\Delta t = t_{\text{parent}} - t_{\text{metastable fragment}}$$

is linear.[7,8] Use of a single-stage reflectron for detection of metastable ions in TOF mass spectrometry has been described by several authors.[7-10] The flight-time difference between the parent ion and a metastable fragment is always smaller than the difference between the parent ion and the corresponding fragment formed immediately at desorption. A more complicated relation between mass loss and flight-time difference holds for the case of a *two-stage reflectron*. The flight-time deviation Δt between parent ion m and fragment ion m^* formed in the field-free drift region is determined by the flight times of the different species through the two electric fields of the reflectron. The flight time t^* of a fragment ion from metastable decay is described by

$$t^* = 2f m^{1/2}[d_1(2/(U_1 e))^{1/2}(a^{1/2} - (a - 1/f)^{1/2}) + d_2/U_2(2U_1(a - 1/f)/e)^{1/2}] \quad (1)$$

where $f = m^*/m$, $a = U_0/U_1$, d_1, d_2 are the lengths of stages 1 and 2, U_1, U_2 are the voltages across stages 1 and 2 and U_0 is the acceleration voltage.

In our experiments we used a two-stage reflectron (Fig. 1) for two reasons: (i) In contradistinction to the case of a single-stage reflectron the flight-time difference between parent ion and a metastable fragment ion is always *larger* compared with the difference between a parent ion and corresponding fragment ion formed immediately at desorption, especially when the fi" deceleration-field strength is increased. Thus, the mass resolution for detecting small mass losses due to metastable decay can be considerably improved by employing a two-stage system. From experiments using linear acceleration or deceleration fields we concluded, that such decay of laser-desorbed peptides mainly leads to small mass losses.[6] The two-stage reflectron therefore appeared to be the better instrument for studying the fragmentations. (ii) Another advantage of the two-stage reflectron over the single-stage reflectron, is that metastable fragments can be distinguished more easily from desorbed fragments or parent ions. This can be achieved by tuning the acceleration voltage without changing the potentials on the field grids of the reflectron. The two-stage reflectron is able to compensate shifts in ion kinetic energy over a range of about 10%, so that the flight time of parent ions and desorbed fragments stays constant. The flight-time deviation of fragments from metastable decay, on the other hand, depends upon the acceleration voltage (at constant reflectron potentials) in the case of a two-stage reflectron. In order to increase this effect, the reflectron has been operated in an unusual way, where the ions use only a small part of the second stage. Figure 2 shows the variation of the peak pattern of metastable ions from melittin (MW = 2847 Da) by changing the acceleration voltage. (This spectrum is discussed further in the Results section.)

EXPERIMENTAL

The mass spectrometer used was a modified Leybold-Heraeus (Cologne, Germany) model LAMMA 1000 laser-desorption time-of-flight mass spectrometer,[12] equipped with a Laser Science Inc. (Cambridge, MA, USA) model VSL-337 ND nitrogen laser ($\lambda = 337$ nm, 250 μJ/pulse, $\tau = 3$ ns) for desorption. The instrument has been modified to enable ion acceleration up to 20 keV kinetic energy to be achieved. The ion reflectron is a two-stage instrument with a first deceleration stage, d_1, of 25 mm and a second stage, d_2, of 123 mm. Typical operating voltages were 10 930 V (sample), 0 V (flight tube and reflectron entrance grid), 9965 V (reflectron second grid) and 15 050 V (reflectron third grid). These unusual settings were used for the reasons described above. Spectra were taken in the positive-ion mode at irradiances close to the threshold of peptide-ion detection ($E_{th} \approx 10^6$–10^7 W/cm^2). Instrument pressure was 10^{-6} mBar.

Samples were obtained from Serva Feinbiochemica GmbH and Co. (Heidelberg, Germany). Samples were dissolved in 0.1% trifluoracetic acid in water. The protein mixture was prepared by mixing equimolar amounts of peptide solution. Sinapinic acid (3,5-dimethoxy 4-hydroxy cinnamic acid) was used as

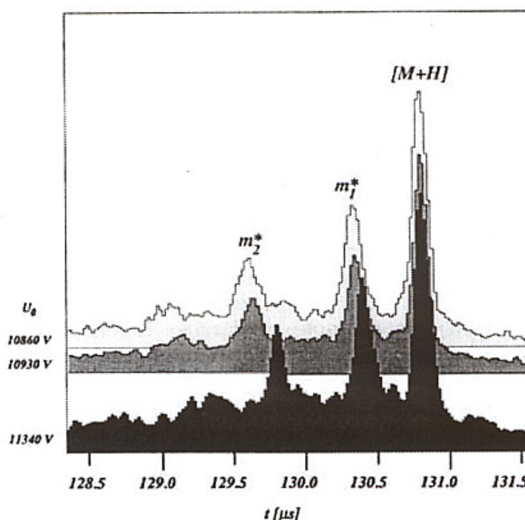

Figure 2. Variation of the peak pattern by changing the acceleration voltage U_0. The position of the parent-ion signal stays constant, while the metastable-fragment signals move with varying kinetic energy.

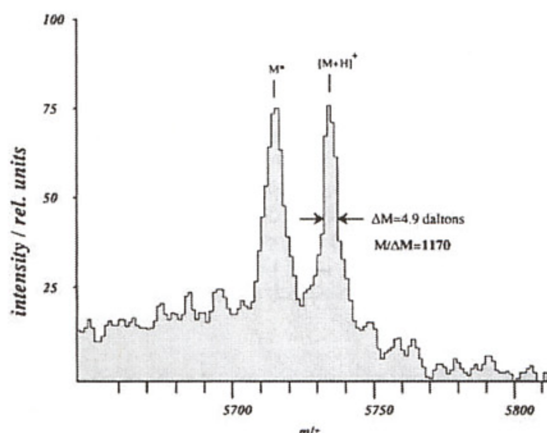

Figure 3. Matrix-assisted laser desorption mass spectrum of bovine insulin in sinapinic acid matrix. The laser ($\lambda = 337$ nm) irradiance used was at the threshold for ion detection; sampling time 20 ns; total flight time of $[M+H]^+$ parent ion 186 218 ns.

matrix. The molar concentration ratios of matrix and analyte were 350:1 (peptide mixture) and 1350:1 (single-compound analysis), respectively.

RESULTS AND DISCUSSION

A typical mass resolving power ($M/\Delta M$) of 1100 to 1800 has been reached for the proteins and peptides tested. Figure 3 shows the molecular ion region of a mass spectrum of bovine insulin ($MW_{average} = 5733.85$ Da). The signal from protonated molecules shows a resolution of $M/\Delta M = 1170$. For insulin, as well as for the other substances tested, one or more additional peaks were detected. Such peaks have been identified as resulting from decay of metastable ions rather than from the formation of fragments during desorption by employing three different tests: (a) variation of the acceleration voltage leads to a shift of daughter-ion peaks from metastable decay, but leaves flight times of stable ions unchanged (within the reflectron limits of energy compensation). (b) Reflecting the ions in the short first stage of the reflectron leads to vanishing flight-time deviations between parent and metastable ions, since the lengths of their flight paths become almost equal. (c) Employing a linear TOF mode makes metastable ion peaks disappear. Figure 4 shows the molecular ion region of melittin using these three different modes of operation. Additional peaks due to metastable decay are observed in the two-stage reflectron mode only.

Patterns of metastable decay have been found to differ in the group of peptides investigated. While bombesin, for example, undergoes metastable fragmentation only to a very low extent, up to 6 rather intense fragment ion signals can be observed in the case of ACTH. Figure 5 shows a typical mass spectrum of a mixture of 4 peptides, using the two-stage reflectron mode. The molecular ion regions are displayed in detail to demonstrate the varying fragmentation pattern. The broad peaks (labelled 'SF') preceding the signals of ACTH and melittin are most probably due to small fragment ions, which do not reach the second stage of the reflectron. For the two-stage reflectron mode, flight-time differences $\Delta t = t_{parent} - t^*$ can be calculated according to Eqn (1) as a function of mass differences $\Delta M = M_{parent} - M_{fragment}$. Figure 6 shows a diagram of the relationship between Δt and ΔM for fragment ions of insulin, melittin, ACTH and substance P, calculated (solid lines) for the operational conditions in our two-stage reflectron. From the Δt data measured for the various daughter ions, corresponding mass losses ΔM due to metastable decay can be determined. Data points indicated in Fig. 6 are values averaged over several experiments. For all of the substances under investigation the most abundant daughter ion is generated by a mass loss of 17 mass units (most probably loss of NH_3). In the case of bombesin the peak intensity of this fragment ion was usually low (less than 10% of parent-ion peak intensity). In the case of insulin, on the other hand, the corresponding daughter ion reaches the same intensity as the parent molecular ion (Fig. 3).

Most of the mass losses observed can be attributed to reasonable fragmentations, that have been described in the literature, e.g., for collision-induced dissociation (CID).[13] Losses of 72, 57 and 44 mass units were interpreted as loss of part of the arginine side-chain, loss of the leucine side-chain and loss of formamidine from the arginine side-chain, respectively. Loss of 86 mass units can be attributed to loss of part of the arginine side-chain, and loss of 17 and 34 mass units is interpreted as due to loss of one and two ammonia molecules.[13] It appears particularly noteworthy that the intensities of metastable peaks are rather high (usually in the range of 10 to 100% that of the parent ion). This means that the efficiency of fragmentation must be high for peptides formed by matrix-assisted laser desorption, although no fragmentation occurs immediately at desorption.

The mechanism of metastable fragmentation of larger peptides is still under investigation in our laboratory. Observation of small mass losses from large molecules is in good agreement with the RRKM model, assuming rapid internal energy relaxation.[14] Besides the unimolecular decay on a relatively long time-scale, there is another possible pathway of a bimolecular

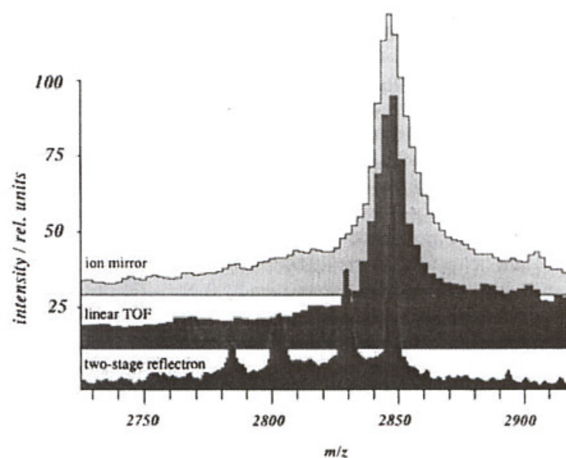

Figure 4. Molecular-ion region of melittin, recorded in the two-stage reflectron mode, the linear-time-of-flight mode and the ion-mirror mode (reflection in the first stage of the reflectron), respectively.

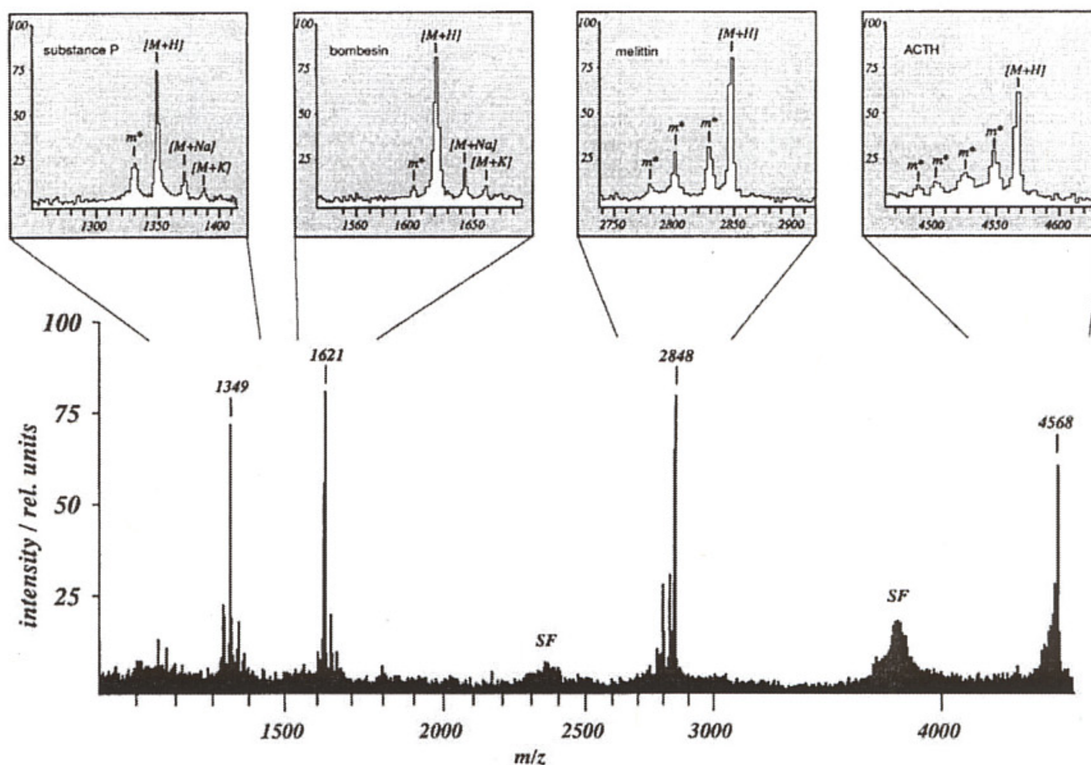

Figure 5. Mass spectrum of a mixture of the peptides substance P, bombesin, melittin and ACTH, using 337 nm laser wavelength and sinapinic acid matrix. The spectrum has been averaged over 50 laser shots. Fragment peaks from metastable decay are marked as m* in the sections. Broad peaks preceding signals of melittin and ACTH are due to small fragments formed in metastable dissociations or secondary ions formed at the second reflector grid.

mechanism, driven by collisions of the parent ions with residual gas molecules. The second mechanism would explain the similar fragmentation pattern observed in collision-induced dissociation and in our experiments.

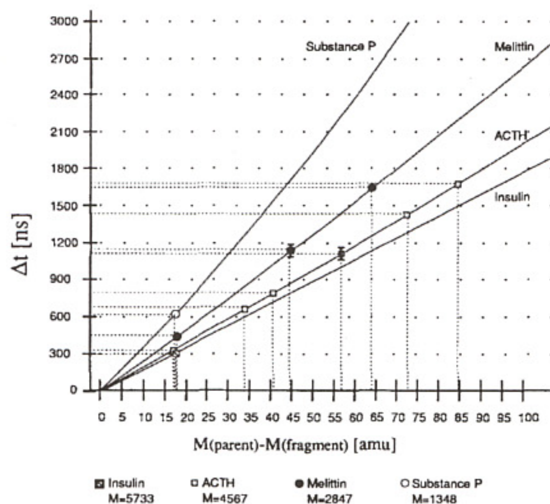

Figure 6. Calculated (solid lines) and observed flight-time deviations as a function of mass losses for substance P, melittin, ACTH and bovine insulin. The acceleration voltage was set to 10 930 V, U_1 was 9965 V and U_2 was 5085 V. Data points are values averaged over 3–5 experiments.

CONCLUSION

The experiments described demonstrate that peptide and protein ions generated by matrix-assisted laser desorption undergo intense fragmentation via metastable decay, although desorption of fragment ions of these substances is usually not observed with this ionization technique. Mass losses, as neutral species, from such decays appear to be small, with the most abundant ΔM amounting to 17 mass units (loss of NH_3). The 'susceptibility' to such decay and the pattern of daughter ions vary to a rather large extent between different parent molecules. Abundance of daughter ions and spread of ΔM appears to increase with increasing parent-ion mass. In the case of cytochrome *c* (the largest molecule measured in this investigation in the two-stage reflectron) the intensity of the daughter ions exceeds that of the parent molecular ions by a factor of ca 3. The more practical aspects of these results are: (i) One has to be aware, that metastable decay will limit mass resolution especially in linear instruments, since the kinetic energy release necessarily leads to substantial peak broadening. But also a reflectron instrument featuring a mass resolution of e.g., $M/\Delta M = 2000$ will not be able to separate e.g., $[M+H]$ and $[M+H-NH_3]$ in the mass range above 34 000 Da. Thus, under such conditions, metastable decay will also finally lead to peak broadening and poor effective mass resolution, especially in one-stage reflectrons. (ii) The similarity of the fragmentation pattern observed here and in CID experiments seen in conjunction with the

pressure dependence of such fragmentation[6] raises the question as to whether metastable decay of laser desorbed larger peptides is mainly induced by collisions with residual gas molecules. This would mean that typical vacua of 10^{-6} to 10^{-7} mBar are just not good enough for TOF mass spectrometry of very high-mass ions, as was suspected from cluster experiments.[15] (iii) On the other hand, metastable decay has to be considered as a possible tool for structure analysis (as described in references 7 and 8) of biopolymers, which under normal conditions do not show useful fragmentation. Analytical information has been increased in plasma desorption and liquid secondary-ion mass spectrometry (LSIMS) of peptide ions by lowering the acceleration voltage (increasing source residence time) or by delayed ion extraction.[16] Getting structural information from matrix-assisted laser desorption mass spectra will be a basic goal in the project of extending the analytical features of mass spectrometry into the very high-mass range.

REFERENCES

1. M. Karas, F. Hillenkamp, *Anal. Chem.* **60**, 2288 (1988).
2. R. C. Beavis, B. T. Chait, *Anal. Chem.* **62**, 1836 (1990).
3. B. Spengler, D. Kirsch, R. Kaufmann, M. Karas, F. Hillenkamp, U. Giessmann, *Rapid Commun. Mass Spectrom.* **4**, 301 (1990).
4. R. Kaufmann, B. Spengler, D. Kirsch, *Proceedings of the NATO Advanced Research Workshop Methods and Mechanisms for Producing Ions From Large Molecules*, Minaki, Canada (1990).
5. B. Spengler, D. Kirsch, R. Kaufmann, M. Karas, F. Hillenkamp, U. Giessmann, *Proceedings of the 38th Conference on Mass Spectrometry and Allied Topics*, Tucson, Arizona, p. 162, ASMS East Lansing (1990).
6. B. Spengler, R. Kaufmann, D. Kirsch (in preparation).
7. X. Tang, R. Beavis, W. Ens, F. Lafortune, B. Schueler, K. G. Standing, *Int. J. Mass Spectrom. Ion Processes.* **85**, 43 (1988).
8. S. Della-Negra, Y. Le Beyec, *Anal. Chem.* **57**, 2035 (1985).
9. X. Tang, W. Ens, F. Mayer, K. G. Standing, J. B. Westmore, *Rapid Commun. Mass Spectrom.* **3**, 443 (1989).
10. T. Leisner, O. Echt, O. Kandler, D. Kreisle, E. Recknagel, *Int. J. Mass Spectrom. Ion Processes.* **87**, R19 (1989).
11. U. Boesl, H. J. Neusser, R. Weinkauf, E. W. Schlag, *J. Phys. Chem.* **86**, 4857 (1982).
12. P. Feigl, B. Schueler, F. Hillenkamp, *Int. J. Mass Spectrom. Ion Phys.* **47**, 15 (1983).
13. G. M. Neumann, M. M. Sheil, P. J. Derrick. *Z. Naturforsch.* **39a**, 584 (1984).
14. D. L. Bunker, F. M. Wang, *J. Am. Chem. Soc.* **99**, 7457 (1977).
15. W. Aberth, *Anal. Chem.* **58**, 1221 (1986).
16. P. Demirev, J. K. Olthoff, C. Fenselau, R. J. Cotter, *Anal. Chem.* **59**, 1951 (1987).