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Characterization of Hydrophobic Peptides by Atmospheric Pressure Photoionization-Mass Spectrometry and Tandem Mass Spectrometry

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The use of photoionization at atmospheric pressure shows great potential for the mass analysis of large apolar or hydrophobic peptides. Mass spectra that were obtained using this technique showed mainly singly charged ions. While polar peptides spectra do not produce fragment ions, others lead to B-type or C-type in-source fragmentation. These dissociation reactions, which could involve electron capture dissociation processes in the case of the C-type ions, are observed for hydrophobic peptides. Both the compatibility of this ionization mode with reversed- or normal-phase liquid chromatographic separation and its sensitivity allow liquid chromatography coupling to both mass spectrometry and tandem mass spectrometry for the analyses of hydrophobic peptide mixtures. Atmospheric pressure photoionization seems to be an interesting alternative method to study hydrophobic peptides that are not easily ionizable by more classical ionization techniques such as electrospray ionization and matrix-assisted laser desorption/ionization.

Over the last twenty years, mass spectrometry (MS) has become a valuable method for biomolecular analysis.[1] The advent of electrospray and matrix-assisted laser desorption/ionization (MALDI) ion sources in the mid 1980s allowed the broadening of the field of mass spectrometry applications to compounds of biological interest. Both the sensitivity and mass accuracy of these techniques turned mass spectrometry into a very efficient tool that is nowadays complementary to biological and biochemical methodologies in the fields of structural biology, proteomics, or noncovalent complexes studies.[1]

However, further improvement of MS techniques remains a challenge in order to study membrane proteins and hydrophobic peptides.[2] Among existing ionization modes, MALDI has already proven to be able to measure the molecular weight of several monomeric or oligomeric membrane proteins. This can be illustrated by the characterization of the monomeric porin from *Rhodobacter capsulatus*[3] or the monomeric and trimeric porins from *Escherichia coli*.[4] This method is able to give information about subunits composing membrane complexes. It can be used in the presence of detergents, even though they can substantially lower the overall spectral quality. In general, elimination of detergents is preferable before mass measurement.[5] It is commonly known that electrospray ionization (ESI) is less tolerant to detergents than MALDI, and several protocols were developed to eliminate those additives before introducing the sample into the mass spectrometer.[6−8] Mass spectra of different molecular weight membrane proteins (≤50 000) have thus been obtained.[8−10] In alternative approaches, difficulties in the preparation and analysis of membrane proteins were avoided by studying the peptides after enzymatic digestion of the proteins of interest.[11,12] However, combined problems of solubility and digestion together with the high hydrophobicity of some peptides can make this sample preparation step relatively tedious, without guaranteeing successful MS analysis.

The underlying problems of the approaches described above limit the efficiency of mass spectrometry for the analysis of hydrophobic proteins and peptides. Atmospheric pressure photoionization source appeared to us as a potential alternative to ionize these compounds. The photoionization of a model peptide (gramicidin S) had previously been described. In that approach,

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the analyte was desorbed as a neutral species by laser irradiation and subsequently ionized by VUV photoionization. Although this method allowed the photoionization of relatively large peptides (>1000 Da), it did not prove to be very efficient in terms of ion yield and reproducibility.[13,14] According to Becker and Wu,[15] the reasons for this are the excessively high internal energy of laser-desorbed species and the propensity of vibrationnally excited radical cations to fragment rapidly. The first problem to solve was thus to reduce the internal energy of ions in order to observe intact peptides.

Two atmospheric pressure ionization sources were concomitantly developed by Agilent and Applied Biosystems/MDS Sciex and recently commercialized under the brand names Photomate and Photospray, respectively. A research version of these sources were first described by Syage et al.[16] and Koster and Bruins, respectively.[17] These authors described these ion sources as a new tool for ionizing low-polarity compounds after (optional) liquid chromatographic (LC) separation. The concept is mainly based on previously obtained results from different fields such as photoionization detection of compounds separated by gas chromatography or LC. As far as mass spectrometry analysis was concerned, no commonly used ionization technique using photoionization was previously described. At that time, besides several examples of the use of photoionization coupled to ion mobility spectroscopy, only two publications could be found dealing with MS analysis of atmospheric pressure-generated photoions.[18]

From these results, Bruins designed its new ion source analyses (which will be used in its commercialzed version in the present study) for performing liquid chromatography (LC)/MS. The principle is surprisingly simple. First, the LC eluent is nebulized and next vaporized in a heated quartz tube ($T = 250-500$ °C). The gaseous mixture is transferred toward a krypton lamp (positioned at the end of the heated quartz tube) generating a beam of photons of mainly 10 eV ($\lambda = 123$ nm). A reactive compound (called "dopant" in the following text) is added to the mobile phase, coaxially to the LC eluent at a 10−15% flow rate ratio. The analyte ionization occurs by reaction with the ionized dopant either by charge exchange or by proton transfer. When low ionization potential solvents are used (for example, heptane or isooctane, which are commonly used in normal-phase LC), no dopant is required, as the solvent itself acts as the dopant. Actually, the use of a dopant to boost the analyte ionization allows drawing of a parallel between atmospheric pressure photoionization and atmospheric pressure chemical ionization (APCI). This could also be related to the pioneering work of Dawkins et al., who first introduced small peptides in solution phase in a chemical ionization source.[19]

Originally designed for the analysis of small, apolar molecules, the Photospray source has been used so far for the analysis of several compound classes such as flavonoids[20] and naphthalenes[21] or for the identification of metabolites in complex biological samples.[22] In addition to the extended range of compounds that can be readily ionized by atmospheric pressure photoionization, very often increased sensitivity is observed for compounds that can also be ionized by the more traditional techniques such as APCI and ESI. The goal of this paper is to demonstrate the potential of atmospheric pressure photoionization for the ionization and mass spectrometric analysis of hydrophobic peptides. All attempts to obtain reliable spectra with APCI on the model hydrophobic peptides involved in the present study failed, with the exception of gramicidin S. For that reason, the discussion will only be focused on the potency of atmospheric pressure photoionization compared with electrospray ionization.

EXPERIMENTAL SECTION

Materials. Gramicidin S and poly(ethylene glycol) (PEG) were purchased from Sigma (Saint Quentin Fallavier, France). Gramicidin A was purchased from Fluka (Saint Quentin Fallavier, France). Tetrapeptide YPLG-NH_2 was purchased from Interchim (Montluçon, France). Calibration solution PepMix 4 used for MALDI experiments was purchased from LaserBio Labs (Sophia-Antipolis, France). Hydrophobic peptides E1-*N*ter and TME1 were kindly provided by Dr. François Penin (IBCP, Lyon, France). All solvents were HPLC grade.

Hydrophobicity of the peptides was estimated by the grand average of hydropathicity method (GRAVY), using the "protparam" software available on expasy website (http://www.expasy.org/). GRAVY values: bradykinin (−1.060), angiotensin I (-0.325), E1-*N*ter (0.595), gramicidin S (1.060), gramicidin A (2.107), and TME1 (1.370).

Photospray Ionization-Mass Spectrometry (PSI-MS). Photoionization was realized using the Photospray source (Applied Biosystems, Les Ulis, France). This source is fitted with a krypton PKS 100 lamp (Cathodeon, Cambridge, England) that generates a continuous flow of mainly 10-eV photons with a minor presence of 10.6-eV photons. Mass spectra were recorded using an hybrid quadrupole/time-of-flight (TOF) Qstar Pulsar i mass spectrometer (Applied Biosystems). Samples were diluted at a final concentration of 10 μM in a H_2O/MeOH 1:1 (v/v) mixture prior to injection. Hydrophobic peptide E1-*N*ter was diluted in trifluoroethanol (TFE) at a final concentration of 900 μM. For each experiment, the sample was injected into the mass spectrometer by flow injection (FIA): 1 μL of the sample was loaded into an injection loop and next eluted with a suitable solvent (called "LC solvent" in analogy with liquid chromatographic separations). The LC solvents were introduced into the photospray ionization source using a HPLC pump P1000XR from Thermo Separation Product at a flow rate of 200 μL/min. When needed, a dopant (toluene or acetone) was introduced using a Harvard syringe pump model

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22 (Harvard, Les Ulis, France). Mass spectrometric instrumental parameters were adjusted such that the best signal-to-noise ratio for each compound were obtained. Typical parameters were respectively for PEG, YPLG-NH_2, and E1-*N*ter: DP1 (declustering potential 1) = 75, 10, 150 V; FP (focusing potential) = 185, 30, 80 V; DP2 (declustering potential 2) = 15, 20, 30 V. The TOF analyzer was calibrated using a mixture of various molecular weight PEGs generating ions up to m/z 3000 with good signal-to-noise ratio (Figure 1A). For MS/MS spectra, the collision energy and the collision gas (Ar) pressure were chosen such that good fragmentation coverage across the mass range was achieved. For the fragmentation of E1-*N*ter, typical parameters were a collision energy of 50 eV and a residual gas pressure of 3.3×10^{-5} Torr, corresponding to a CAD gas setting of 3.

ESI-MS. The E1-*N*ter electrospray mass spectrum in the direct infusion mode was recorded on a hybrid quadrupole/time-of-flight Qstar Pulsar i mass spectrometer fitted with an IonSpray source. The sample was diluted in 70% formic acid to a final concentration of 10 μM. The instrument was calibrated using a 3 mM solution of cesium iodide in a 2-propanol/H_2O mixture (50/50, v/v).

MALDI-MS. The peptides were diluted in a solution of dihydroxybenzoic acid (20 mg/mL) in a mixture CH_3CN/H_2O/TFE (70/30/0.1, v/v/%) to a final concentration of 9 μM. A 1-μL sample of this solution was spotted on a MALDI plate according to the so-called dry-droplet method. Mass spectra were obtained in reflectron mode using an accelerating voltage of 20 000 V and a grid ratio of 70%. The standard solution of peptides, PepMix 4, was used for instrument calibration.

Liquid Chromatography. Liquid chromatographic separation of E1-*N*ter was realized with a reversed-phase C_8 2.1 × 30 mm column, using a gradient elution of 30−80% acetonitrile in H_2O in 35 min, at a flow rate of 200 μL/min.. The collected fractions were lyophilized and dissolved in a mixture CH_3CN/H_2O/HCOOH (70:30:5, v/v/v) before their analysis by MALDI-MS.

LC/MS Experiments. Liquid chromatographic separation of a peptide mixture of bradykinin (0. 22 μg/μL), angiotensin II (0.23 μg/μL), gramicidin A (0.43 μg/μL), and E1-*N*ter (0.24 μg/μL) was realized with a reversed-phase C_4 2.1 × 150 mm column, using a gradient elution of 5−40% acetonitrile in H_2O in 25 min, at a flow rate of 200 μL/min. A total of 99% of the flow rate was diverted toward the PSI source and the rest to UV detector.

RESULTS AND DISCUSSION

After mass calibration of the TOF analyzer, several parameters that were thought to influence the mass spectral quality were examined. To achieve this, a small hydrophobic peptide was used as a model compound (YPLG-NH_2, monoisotopic mass, 447.2 Da). The goal was to find optimal operating conditions for mass spectrometric analysis of hydrophobic biological compounds (i.e., instrumental parameters, solvent flow rate and nature, dopant type and use). A solution of the hydrophobic tetrapeptide at a concentration of 10 μM in H_2O/MeOH 1:1 (v/v) was used, with H_2O/MeOH 1:1 (v/v) as the LC solvent. A mass spectrum of this peptide displayed $[M + H]^+$, $[M + Na]^+$, and $[M + Na + H_2O]^+$ ions (Figure 1B).

Influence of the Probe Position. The position of the heated nebulization probe appeared not to be a very critical parameter. It was observed, however, that the signal level improved when

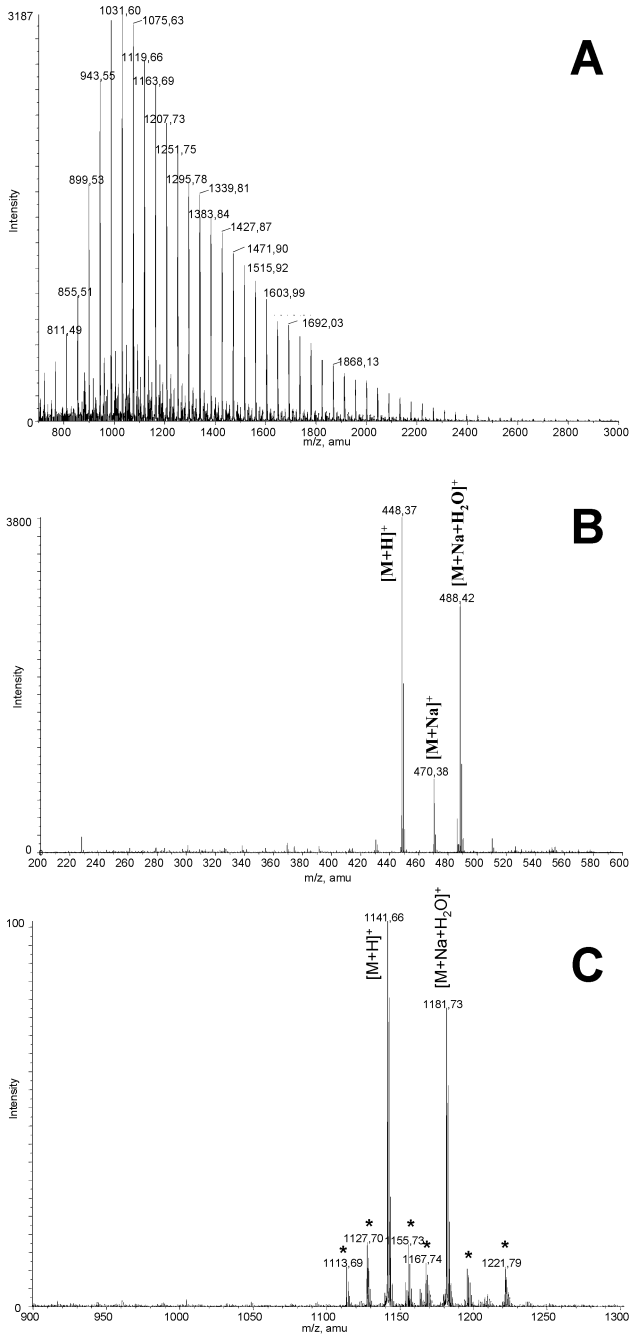

Figure 1. (A) Photoionization mass spectrum of PEG mixture solution dissolved in H_2O/MeOH (50/50; v/v) with heptane/2-propanol (70/30; v/v) as LC solvent. (B) Photoionization mass spectrum of YPLG-NH_2 peptide dissolved in H_2O/MeOH (50/50; v/v) with H_2O/MeOH (50/50; v/v) as LC solvent. Theoretical monoisotopic mass, 447.2 Da. Peaks labeled by an asterisk correspond to contaminant compounds. (C) Photoionization mass spectra of gramicidin S dissolved in H_2O/MeOH (50/50; v/v) with heptane/2-propanol (70/30; v/v) as LC solvent. Theoretical monoisotopic mass, 1140.77 Da.

the probe was slightly remote from the orifice. Interestingly, the relative intensities of the ions $[M + H]^+$, $[M + Na]^+$, and $[M + Na + H_2O]^+$ depended on the probe position. This observation can best be explained by the differences in ion desolvation and ion beam sampling. However, the relative intensities of the different species in the resulting spectrum remain more or less similar. Maximum intensity was observed when the probe was in position +3; in this position, ions have to follow a longer

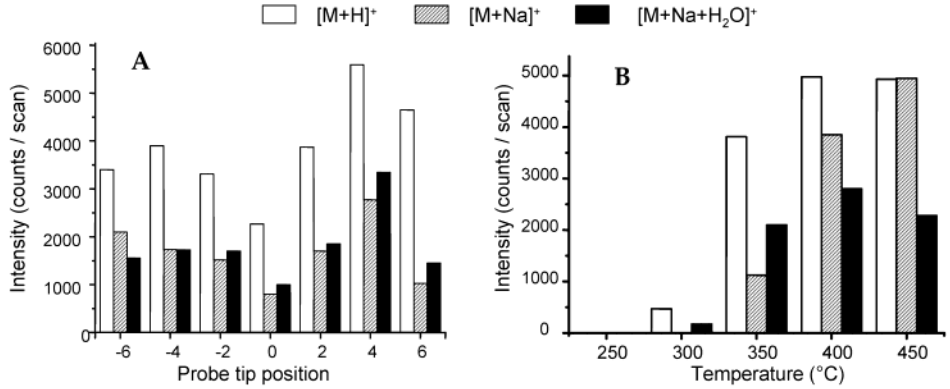

Figure 2. (A) Effect of the probe tip position versus signal intensity and (B) of the probe temperature versus signal intensity.

trajectory before entering the mass spectrometer (Figure 2A). Under these conditions, the potential difference applied between the probe tip and the curtain plate (offset voltage) is critical for the signal quality. This point will be discussed later.

Influence of Probe Temperature. The temperature of the heated nebulization probe plays a role in the ion desolvation yield (Figure 2B). With regard to the protonated molecules, more solvated species and adducts, such as $[M + Na]^+$ ions, appeared as expected in the spectra at higher temperatures. The intensity ratio between the $[M + Na + H_2O]^+$ and $[M + Na]^+$ ions dropped when the temperature was changed from 350 to 450 °C. A higher probe temperature allows a better desolvation and consequently a significant signal increase of these species. Addition of water to the cationized molecules pointed out the penetration of water vapor into the ion source. Under the conditions used for recording this spectrum, only a small increase of the curtain gas pressure was possible, which did not significantly decrease the relative abundance of the water adducts, by comparison with the temperature effect.

More generally, the signal intensity is weak at probe temperatures below 350 °C. This effective minimal temperature that still governs acceptable ion yield seems to be affected by the solvent flow rate rather than the solvent vaporization efficiency. The boiling points of the solvents were indeed much lower than the probe temperature. The best results were obtained for a temperature of 400 °C.

Influence of the Offset Voltage. The so-called "offset voltage" is the potential difference applied between the probe tip and the curtain plate. This parameter is critical for the sensitivity as this voltage difference is used to drag ions into the mass spectrometer, and was adjusted by varying the ion source voltage (ISV) value, which in the case of the Photospray source corresponds to the potential applied on the heated nebulization probe. The minimal ISV value required to observe a signal was 1100 V. Under these conditions, almost no ions are transported into the mass spectrometer. The offset voltage has to be adjusted for each compound in order to attain the best sensitivity. In the case of YPLG-NH_2, this was obtained at an ISV value of 1500 V. In the case of E1-*N*ter (vide infra) the optimal value was 2000 V, which should not be exceeded in order to avoid a discharge in the source.

Nature of the Reactive Compound (Dopant). The effect of the dopant nature was not extensively tested; however, it was observed that addition of a dopant in a ratio of 10−15% of the solvent flow greatly enhanced the analyte's ion yield. These observations are in good agreement with those of Bruins et al.[18] When a heptane/2-propanol mixture was used as the solvent, the dopant role was minor. This can be explained by the fact that the ionization potential of heptane (9.93 eV) is lower than the energy of the photons emitted by the krypton lamp. This means that a so-called "autodoping" effect occurs and that the solvent itself acts as the dopant. For this reason, adding a dopant such as toluene or acetone to a solvent mixture containing heptane has only minor effect on the ionization yield.

To compare the atmospheric pressure photoionization with the laser postionization method described by Becker and Wu,[15] gramicidin S (the first ever photoionized biological compound) was subjected to analysis under various conditions. Regardless of the nature of the solvent (either H_2O/MeOH 1:1 v/v or heptane/2-propanol 80:20 v/v was used), the mass spectra contained the $[M + H]^+$ ion at m/z 1141.66 (Figure 1C). This is in agreement with the theoretical calculated value of m/z 1141.70. This demonstrates that the ion formed by photoionization results from protonation of the peptide. At the same time, $[M + Na + H_2O]^+$ and $[M + 2Na + H_2O-H]^+$ ions were observed at m/z 1181.73 and 1221.79. As indicated above, an increase of the curtain gas pressure did not reduce the abundance of the water adduct ions. Other peaks were attributed to contaminant compounds. No in-source fragmentation of gramicidin S was observed.

Origin of the $[M + Na]^+$ Ions. According to the ionization mechanism proposed for photoionization,[18] $[M + Na]^+$ ions are unexpected in PSI. Thus, $[M + Na]^+$ ions could be formed from thermospray-like ionization processes. To discriminate ions originating from thermal ionization−desolvation or photoionization mechanisms, the mass spectra of gramicidin A (HCO-VGALAV-VVWLWLWLW-NH$(CH_2)_2$OH) were recorded by switching off the UV lamp (Figure 3A) or under photoionization conditions (lamp on) (Figure 3B). In the absence of the photon beam, only sodium adducts were observed under their singly ($[M + Na]^+$) and doubly charged forms ($[M + 2Na]^{2+}$). By contrast, photoionization of gramicidin A solution led to abundant $[M + H]^+$ ions generating a series of B-type fragment ions. Thus $[M + Na]^+$ ions are expected to be generated mainly by a thermospray-like ionization mechanism, the protonated species arising only from photoionization reactions. It is noteworthy that switching the lamp off and on leads to complementary structural data, the stable cationized molecules being useful for mass measurements, and fragments arising from the protonated molecules providing sequence identification.

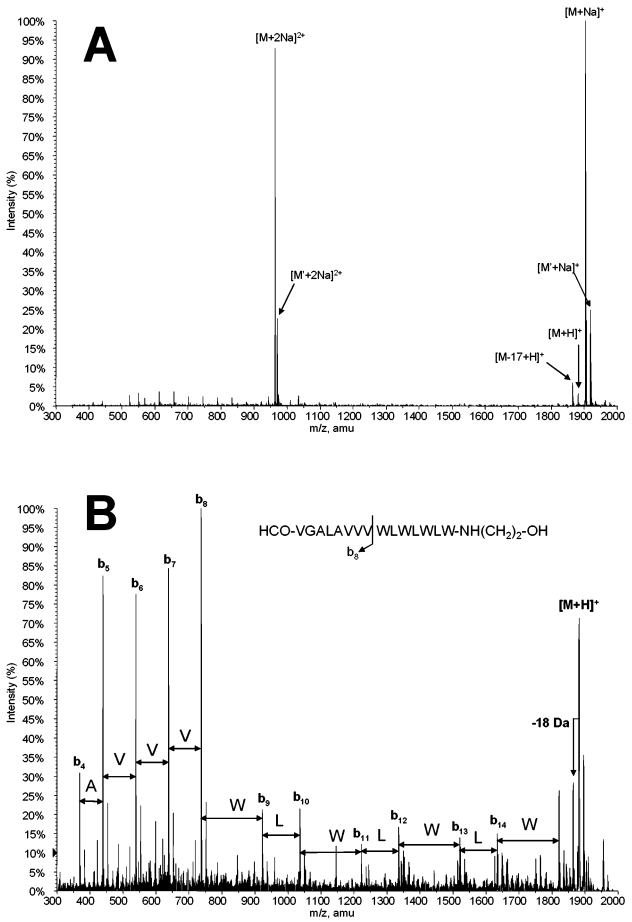

Figure 3. (A) Photoionization mass spectrum of the gramicidin A peptide dissolved in H_2O/MeOH 50:50 (v/v) with heptane/2-propanol (70:30; v/v) as LC solvent and the UV lamp off; (B) photoionization mass spectrum of the gramicidin A peptide dissolved in H_2O/MeOH 50:50 (v/v) with heptane/2-propanol (70:30; v/v) as LC solvent and the UV lamp on. Inset: drawing of the gramicidin A peptide sequence. Theoretical monoisotopic mass, 1881.03 Da. Each peak is associated with the one corresponding to the Ile isoform of gramicidin A, with a mass increment of 14 Da.

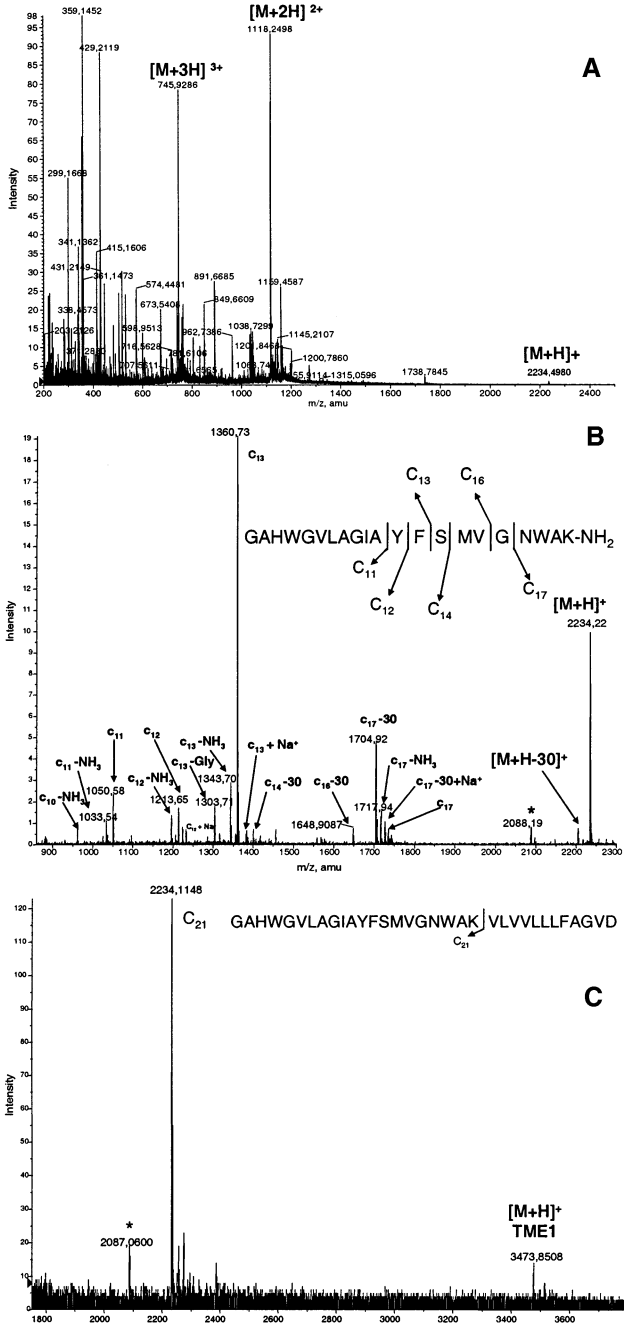

Figure 4. (A) ESI mass spectrum of the E1-*N*ter peptide dissolved in 70% HCOOH. (B) Photoionization mass spectrum of the E1-*N*ter peptide dissolved in TFE with heptane/2-propanol (70:30; v/v) as LC solvent. Inset: drawing of the E1-*N*ter peptide sequence and C-type cleavage sites. Theoretical monoisotopic mass, 2233.11 Da. Peak labeled with an asterisk corresponds to the E1-*N*ter peptide lacking the F13 due to a decrease of synthesis yield at this step. (C) Photoionization mass spectrum of TME1 peptide dissolved in TFE with heptane/2-propanol (70:30; v/v) as LC solvent. Inset: TME1 peptide sequence and C-type cleavage site. Theoretical monoisotopic mass, 3472.87 Da.

Next, a larger hydrophobic peptide, E1-*N*ter, was studied. This peptide is the *N*-terminal transmembrane fragment of a membrane glycoprotein from hepatitis C virus. Its amino acid sequence is GAHWGVLAGIAYFSMVGNWAK-NH_2.[23] This peptide is insoluble in water without the use of a detergent. It was thus stocked in 0.5% TFE, at a molar concentration of 900 μM. For electrospray ionization, obtaining a mass spectrum in the direct infusion mode required the use of a 70% formic acid solution. This spectrum is shown in Figure 4A. Much better results were obtained by using LC/MS as discussed below. The photoionization mass spectrum was recorded by FIA of 1 μL of this solution. The spectrum contained—among other ions—a peak at m/z 2234.22 corresponding to the $[M + H]^+$ ion (calculated theoretical value, m/z 2234.13) (Figure 4B). To evaluate the sensitivity of the ionization process, the signal was recorded as a function of the sample concentration using the continuous direct introduction mode. The intensity was plotted versus the peptide concentration (data not shown). It showed that the signal intensity increased strongly between 10 and 20 μM concentrations and more moderately at higher concentrations. These results indicate that a suitable sample concentration would correspond to 20 μM.

No abundant peak corresponding to cationized species was observed in the E1-*N*ter mass spectra. Unexpectedly, when

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compared to gramicidin S experiments, other ions were observed at m/z 2087.28, 1704.92, 1360.73, and 1050.58 (and some additional less abundant fragment ions), respectively. Ammonia loss was observed from most of these ions. Another interesting observation was the loss of 30 Da from the ions at m/z 2234.22 (2204.20) and 1734.93 (1704.92), corresponding to the loss of the serine side chain (loss of CH_2O, labeled "-S" in Biemann's nomenclature).[24] The fragments formed in the ion source that do not contain the serine residue did not show this fragmentation pathway. MS/MS experiments confirmed this interpretation: (i) absence of 30-Da loss from the parent ions at m/z 1050.6 and 1360.7, (ii) presence on the MS/MS spectrum of the ion m/z 1704.9, and (iii) loss of 30 Da from all B-ions containing the serine residue.

Finally, a loss of the N-terminal glycine was observed for the ions at m/z 1360.73 and below.

To achieve the structural assignment of the more intense fragment ions, their MS/MS spectra were recorded (Figure 5). All these spectra display an abundant loss of an ammonia molecule and a B-ions series,[24] which distribution is centered on a major B-ion corresponding to a fragmentation from the central position in the peptide chain. It is also noteworthy that many A-ions are observed, whereas no Y-ions are present. It was thus proposed that in-source produced ions result from C-type fragmentations.[25] MS/MS experiments allowed us to identify quite large sequence pieces: WGVLAGIA for the ion at m/z 1050.6, WGVLAGIAYF for the ion m/z 1360.7, and WGVLAGIAYFSMVG for the ion at m/z 1704.9. These data confirm the identity of the ions at m/z 1050.6, 1360.7, and 1704.9 as C_{11}, C_{13}, and C_{17} fragment ions, respectively.

According to Downard and Biemann,[25] C-type fragmentations would be sequence-dependent, especially in the vicinity of residues such as T, W, K, or S and to a lower extent Q, C, D, N, or Y, with respective fragmentation probabilities of 54, 44.8, 29.1, 23, 20.4, 12.5, 10.5, 10.3, and 7.3%. All fragments generated through E1-*N*ter photoionization correspond to expected C-type fragmentation sites. C_{11}, C_{13}, and C_{17} fragments would indeed result from fragmentations from the N-side of residues Y12 (fragmentation probability, 7.3%), S14 (23%), and N18 (10.3%), respectively. Using these probabilities, fragmentations from the N-side of residues W4 and W18 (fragmentation probability, 44.8%) and F13 (7.3%) are expected. Spectral analysis partially confirmed this expectation, since the C_{12} fragment was observed (m/z 1213.6) corresponding to a fragmentation before residue F13. The intensity of this peak was also in agreement with the fragmentation probability. On the other hand, no C-type fragmentation involving residues W4 and W18 was observed. It is very remarkable that the fragmentation probability upstream from these residues is quite high (44.8%). The lack of these fragmentations could be explained by the presence of a basic amino acid near the cleavage site, thus disabling these processes.[25] Finally, C_{14} and C_{16} fragmentations were also observed with very low yields on the mass spectrum, that being again in agreement with Downard's and Biemann's results.

C-type fragmentations were shown recently by McLafferty and co-workers to occur preferentially from multiply charged ions under electron capture dissociation (ECD) conditions.[26,27] Such

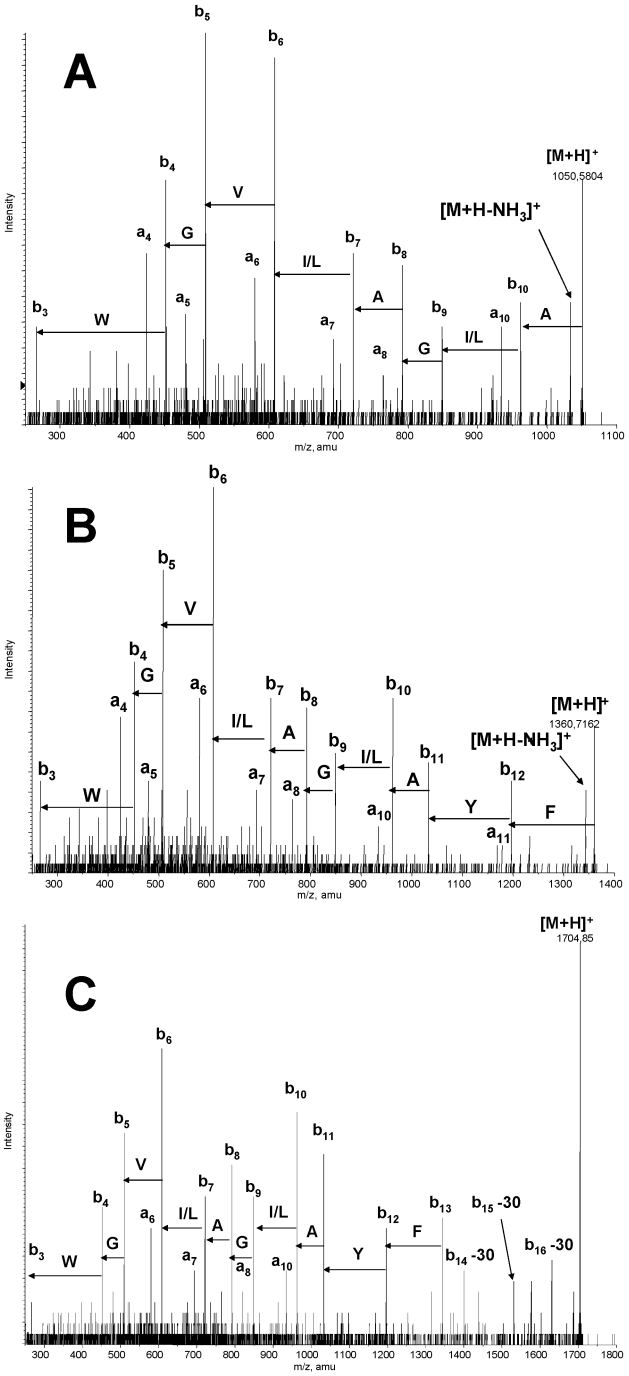

Figure 5. (A) MS/MS mass spectra of C_{11} ion at m/z 1050.6, (B) C_{13} ion at m/z 1360.7, (C) C_{17} ion at m/z 1704.8.

type of reactions could thus occur in the photoionization source between sample molecules and low-energy electrons released from the photoionized solvent and dopant molecules. Although electron capture has been recently described in negative ion mode photoionization,[28] no evidence for such process has been provided so far in the positive ion mode. Further, gramicidin A, as indicated

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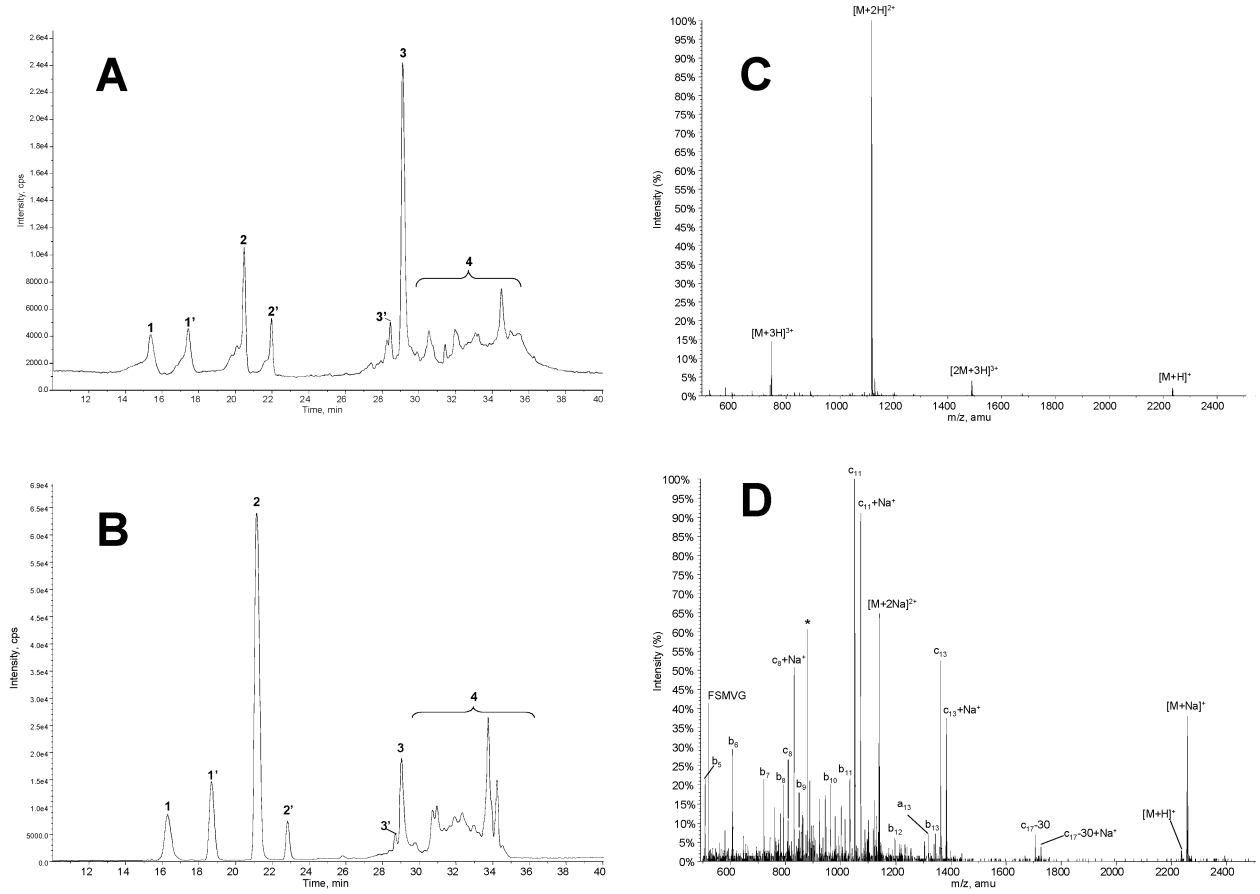

Figure 6. (A) Total ion current chromatogram of the peptide mixture in electrospray ionization mode, (B) total ion current chromatogram of the peptide mixture in photospray ionization mode, (C) electrospray mass spectrum of the E1-*N*ter peptide, and (D) Photospray ionization mass spectrum of the E1-*N*ter peptide. Peaks 1, 2, 3, 3′, and 4 correspond to bradykinin, angiotensin II, El-*N*ter peptide, El-*N*ter peptide lacking F13, and gramacidin A, respectively. Peaks 1′ and 2′ correspond to methylated forms of bradykinin and angiotensin II, respectively.

above (Figure 3), led to B-type ions series and did not show any C-type fragments. Thus, more detailed investigations on the occurrence of electron capture dissociation processes in atmospheric pressure photoionization, which are out of the scope of the present paper, are strongly needed.

The same C-type fragmentation was observed with the larger TME1 peptide (a complete transmembrane crossing from a membrane glycoprotein from hepatitis C virus), which yielded a major C_{21} fragment ion (Figure 4C).

LC/ESI-MS versus LC/PSI-MS. To broaden the application field of photoionization, coupling of reversed-phase liquid chromatography with photospray ionization was investigated. Comparison of LC/ESI-MS with LC/PSI-MS was realized under identical chromatographic conditions by use of a mixture of polar and hydrophobic peptides (bradykinin, angiotensine I, E1-*N*ter, gramicidin A). The two corresponding total ion current chromatograms are shown in Figure 6. At first sight, the ionization efficiencies of the different peptides are quite similar for the two ionization methods. However, a significant improvement of the signal-to-noise ratio is observed in the photoionization mode with regard to electrospray ionization. The differential behavior of the peptides under ES or PS ionization conditions will be exemplified by the E1-*N*ter peptide in the following discussion (Figure 6C and D). LC coupling led to a much cleaner electrospray spectrum of E1-*N*ter (Figure 6C) than that obtained by direct infusion (see Figure 3A). Doubly and triply charged (protonated) species dominated the spectrum without significant fragment ion peaks. By contrast, the mass spectrum obtained under LC/PSI-MS conditions displayed both protonated and cationized molecules from which a large number of fragment ions arose. As discussed above in the case of gramicidin A sodium adducts, the use of water as coeluent could explain the high intensity of the $[M + Na]^+$ ion peak as well as the doubly charged $[M + 2Na]^{2+}$ ion peak (m/z 1139.6). C-Type fragment ions were the major species whereas some B-type ions could also be observed. Even though the fragmentation events involved mainly the $[M + H]^+$ precursor ions, some sodium-cationized fragments can be observed originating likely from electron capture dissociations of the $[M + Na]^+$ ions in the ion source. Comparison of both spectra is an illustration of the complementarity of electrospray and photospray ionization modes, the sequence information arising from the PSI mass spectra whereas the multiply protonated ions generated by electrospray correspond to stable gas-phase species.

Further studies are needed for evaluating the ability of the photoionization source to be combined with a normal-phase liquid chromatography for which apolar solvents compatible with the ionization method can be used. This coupling would be of great interest for the separation of complex hydrophobic compound mixtures containing detergents or other chemicals that could be undesirable with ESI-MS. These aspects are currently under investigation in our laboratory with synthetic peptide mixtures and digests of membrane proteins.

CONCLUSION

This set of results shows that photoionization is a very suitable alternative method to ionize biological compounds. It is highly interesting that this method can be applied to hydrophobic peptides especially as these compounds are usually difficult to analyze (including the sample preparation). Moreover, photoionization sensitivity is close to electrospray sensitivity (10 μM solutions of peptide samples). This appears clearly in the LC/MS experiments, which showed ionization rates in the same order of magnitude for both methods. All the presented results clearly show that the favored process for the photoionization of these peptides involves proton-transfer reactions. No molecular ion $M^{\bullet+}$ formed by charge exchange reaction was detected during our studies.

The photoionization spectrum of E1-*N*ter peptide displayed highly specific fragmentation pathways, i.e., an unusual C-type fragmentation. The correlation of the relative intensities of these C-ions with C-type fragmentation probabilities reported by Downard and Biemann and the absence of C-terminal ions (Y or Z) suggest that photoionized peptides have a very narrow internal energy distribution.

In-source fragmentations, which could be related to ECD reactions, provide valuable structural information. However, these reactions decrease the sensitivity of this method by multiplying the number of ion peaks present in the spectrum and limit the available information when complex mixtures are studied.

This problem could be resolved by coupling the photoionization source with liquid chromatography. The present study, done under reversed-phase chromatographic conditions, could be extended to normal-phase LC, as apolar eluents have been shown to be compatible with the atmospheric pressure ionization method. This coupling would be of great interest for the separation of complex hydrophobic compound mixtures containing detergents or other chemicals unsuitable for ESI-MS. Moreover, the good quality of MS and MS/MS spectra allows us to consider LC/MS and LC/MS/MS experiments while maintaining a good sensitivity.

Another objective is to limit the internal energy of the ions in order to minimize and control the fragmentation reactions. Depending on the sequence and polarity of the peptides under investigation, the photoionization spectra show great differences. Some of them exhibit abundant sodium-cationized molecules, whereas others show mainly protonated species. In some cases, the most abundant fragment ions belong to the B-series, and in other cases, the C-type ions dominate the spectrum. Further experiments should be undertaken for a better understanding of the intimate processes occurring in an atmospheric pressure ion source.

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