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In-Source Fragmentation of Very Labile Peptides in Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Synthetic acidic proline-rich peptides devoid of basic chemical groups were studied by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). Their ion mass spectra recorded in reflector positive ion mode have shown unusual features, i.e., absence or very weak presence of protonated peptide together with a major peak associated with fragmentation at a site that corresponds to the amide bond N-terminal to the first proline of the XPP motif. In contrast, arginine-containing analogues were stable in MALDI-TOF, whereas peptides sharing a free N-terminal amino group were moderately subject to the same fragmentation. Effects of extraction delay time suggest that this process takes place very early (nanoseconds) at the beginning of the plume expansion. The effect of the nature of the matrix on the survival yield indicates a better correlation with the initial axial velocity than with the matrix proton affinity. All the data show some strong differences with the classical in-source decay (ISD). Our results suggest the role of the available protons in the close neighborhood of the peptide during the crystallization process and the prompt fragmentation induced by collisions in the first step of ablation. Undoubtedly, our study highlights that the MALDI-TOF analysis of peptides containing proline and no basic group should be carried out with extreme caution.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI) are usually considered as "soft" or "gentle" ionization techniques for producing intact analyte ions in the gas phase.[1−3] For moderately labile analytes such as peptides and proteins, these two ionization sources usually allow the formation of intact protonated molecules. In fact, fragmentation of peptides should only be achieved through collision-activated dissociations (CAD) with a gas, at low or high energy.

To our knowledge, only a few authors have observed nonintact protonated molecules under ESI or MALDI conditions. The first observation, reported in 1993 by Yu et al.,[4] dealt with the MALDI-TOF analysis of an endoproteinase Asp-N digest of human interleukin 11. These authors have observed the production of unexpected ions in the reflector positive ion mode. Characterized by broad and unresolved peaks, they were attributed to the post source decay (PSD) fragmentation of the fragile D−X backbone amide bonds (the one-letter code will be used for the amino acids throughout the manuscript, X being any of the 20 amino acids). Other peptides, containing proline-rich sequence(s) such as, for example, the APPP motif, have shown an abundant unexpected ion corresponding to a matrix-dependent fragmentation between the alanine and the following proline.[5] Likewise, ESI mass spectrometry (MS) analysis of peptides containing the DP[6] or EEPP[7] motifs have shown C-terminus y ion fragments resulting from peptide backbone cleavage between the aspartate and the proline or between the second glutamate and the following proline, respectively. One of these peptides, SEEPPAFGLK−NH_2, was further analyzed by atmospheric pressure (AP)-MALDI. The protonated molecule and the fragment issued from the cleavage of the X−PP bond were also detected. The survival yield of this peptide was found to be correlated with the proton affinity (PA) of the MALDI matrix.[8]

In the present work, we report new results concerning fragmentation of proline-containing peptides that are extremely labile in MALDI-TOF. Briefly, the starting peptide (Ac−DAEPPILYSEY−NH_2), namely, peptide 1 (Table 1), was initially synthesized to explore certain aspects of the biological functions associated to the estrogen receptor.[9] It is totally devoid of basic chemical group. The recorded MALDI-MS spectra (positive reflector mode) showed an unexpected absence of signal or a signal of very weak intensity corresponding to the protonated

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(1) Fenn, J.; Mann, M.; Meng, C.; Wong, S.; Whitehouse, C. *Science* **1989**, *246*, 64–71.

(2) Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F. *Int. J. Mass Spectrom.* **1987**, *78*, 53–68.

(3) Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299–2301.

(4) Yu, W.; Vath, J. E.; Huberty, M. C.; Martin, S. A. *Anal. Chem.* **1993**, *65*, 3015–3023.

(5) Gorman, J. J.; Ferguson, B. L.; Nguyen, T. B. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 529–536.

(6) Skribanek, Z.; Mezo, G.; Mak, M.; Hudecz, F. *J. Pept. Sci.* **2002**, *8*, 398–406.

(7) Maux, D.; Enjalbal, C.; Martinez, J.; Aubagnac, J. L. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1470–1475.

(8) Schulz, E.; Karas, M.; Rosu, F.; Gabelica, V. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1005–1013.

(9) Kapitan, J.; Gallo, D.; Goasdoué, N.; Niçaise, M.; Desmadril, M.; Hecht, L.; Leclercq, G.; Barron, L. D.; Jacquot, Y. *J. Pept. Sci.* **2009**, *15*, 455–464.

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Table 1. Name and Primary Structure (One-Letter Code) of the Studied Peptides

type of study	pept. name	primary structure	$[M + H]^+$	$[M + Na]^+$ $[M + K]^+$	main frag. m/z	seq. of the main frag.[a]	$[M - H]^{-b}$
reference:	pept. 1	Ac−DAEPPILYSEY−NH_2	n.o.	+	980	PPILYSEY−NH_2	+
effect of acidic residues:	pept. 2	Ac−DAEPPILYS**A**Y−NH_2	n.o.	+	922	PPILYS**A**Y−NH_2	+
	pept. 3	Ac−DA**A**PPILYSEY−NH_2	n.o.	+	980	PPILYSEY−NH_2	+
	pept. 4	Ac−**A**AEPPILYSEY−NH_2	n.o.	+	980	PPILYSEY−NH_2	+
	pept. 5	Ac−**A**AAPPILYS**A**Y−NH_2	n.o. or v.w.	+	922	PPILYS**A**Y−NH_2	+
	pept. 6	Ac−DA**D**PPILYSEY−NH_2	n.o.	+	980	PPILYSEY−NH_2	+
	pept. 7	Ac−DAE**A**PILYSEY−NH_2	n.o.	+	833	PILYSEY−NH_2	+
effect of P residues:	pept. 8	Ac−DAE**AA**ILYSEY−NH_2	n.o.	+	n.o.		+
	pept. 9	**H**−DAEPPILYSEY−NH_2	+	+	980	PPILYSEY−NH_2	+
effect of basic sites:	pept. 10	**H**−DAEPPILYSEY**R**−NH_2	+	n.o.	n.o.		+
	pept. 11	**H**−DAEPPILYSE**R**−**OH**	+	v. w.	n.o		+
	pept. 12	Ac−DAEPPILYSE**R**−NH_2	+	v. w.	y v. w.		+

[a] The sequence of the main fragment was verified by MS/MS. [b] $[M - H]^-$ was observed as a unique ion. Peptide 1 is the reference peptide. Pept. stands for peptide, frag. for fragment, seq. for sequence, Ac− for acetylated, $-NH_2$ at the C-terminal extremity of the peptide for amidation, and H− at the N-terminus of the peptide for a primary amine. Modified position(s) compared to peptide 1 are bolded and underlined. The presence of the ions is indicated with a "+". The abbreviations n.o. and v.w. stand for not observed and very weak, respectively.

$[M + H]^+$. In contrast, a signal of moderate intensity was recorded in the case of Na and K cationized molecules. Moreover, a very large signal (base peak) corresponding to the y_8 ion fragment was systematically observed. To our knowledge, literature data on fragile peptide report that the protonated molecule is still observed besides a partial fragmentation in the ion source, depending or not upon the nature of the matrix. Our work evidences more labile peptides with no detection or negligible production of protonated molecules. Thus, the effects of different parameters on the stability of this peptide during MALDI-TOF analysis such as the effect mediated by (i) peptide sequence (Ala-scan on specific positions, free or substituted NH_2-terminus, arginine insertion), (ii) ion production (nature of the matrix, laser energy, and delay time for ion extraction), and (iii) instrument [positive and negative ion mode, linear, reflector, tandem mass spectrometry (MS/MS)] were explored. The results indicate that the very prompt fragmentation is sequence-dependent. In addition, this fragmentation appears as an atypical in-source decay (ISD). This suggests that fragmentation occurs in the early step of ablation/ionization or on the MALDI target. Upon the basis of quasi-preformed ions during crystallization, new insights of ion production and fragmentation in MALDI will be discussed to explain this unexpected behavior.

EXPERIMENTAL SECTION

Solid-Phase Peptide Synthesis, Modification, and Purification. All the peptides have been synthesized under N-ter acetylated and C-ter carboxamide, but peptides 9, 10, and 11, which have been synthesized with a free N-terminus. Peptide synthesis was carried out by using the standard solid-phase Fmoc chemistry procedure on an Applied Biosystems 433A automated peptide synthesizer (Courtaboeuf, France). Details of synthesis are given in the Supporting Information. High purity was evidenced by high-performance liquid chromatography (HPLC) and confirmed by MALDI-TOF mass spectrometry (Voyager DE Pro, Applied Biosystems) and micro- and nanoLC−ESI-MS/MS.

MALDI Sample Preparation. Samples were prepared according to the "dried droplet" method[3] by using different matrixes. CHCA (α-cyano-4-hydroxycinnamic acid, Applied Biosystems), SA (sinapinic acid or 3,5-dimethoxy-4-hydroxycinnamic acid, Fluka), and THAP (2′,4′,6′-trihydroxyacetophenone, Aldrich) were used at 5 mg/mL in ACN/aqueous 0.1% TFA (1:1).

DHB (2,5-dihydroxybenzoic acid, Aldrich-Sigma) was used at 10 mg/mL in ACN/H_2O (1:1) or in H_2O.

DHAP (2,5-dihydroxyacetophenone, Sigma) was prepared in two different ways: (i) at 3 mg/mL in ethanol/diammonium hydrogen citrate (DAHC) (1:1) or (ii) in ACN/aqueous 0.1% TFA/DAHC (0.5:0.5:1). The DAHC solution was prepared at 1 mg/mL in H_2O.

Some matrixes were prepared without adding 0.1% aqueous TFA.

Peptide Desalting. Microcolumns packed with Poros reversed-phase 20 R2 resin (Poros R2, Applied Biosystems) were prepared using GELoader tips as described previously to desalt peptides.[10] After a washing step with 20 μL of 0.1% aqueous TFA, the bound peptides were eluted from the columns onto the MALDI target using 0.5 μL of a matrix solution consisting of CHCA (5 mg/mL) in 50% ACN, 0.1% aqueous TFA.

MALDI-TOF Experiments. MALDI-TOF experiments were essentially performed using a 4700 proteomic analyzer apparatus (Applied Biosystems, Courtaboeuf, France) fitted with a pumped diode laser Nd:YAG ($\lambda = 355$ nm, pulse duration of 4 ns, repetition rate of 200 Hz). All modes (reflector, linear, MS/MS with optimized delayed extraction) were used in positive and negative ion modes. MALDI-TOF-TOF experiments were performed under pure metastable decomposition or CAD (N_2, 5.3×10^{-5} Pa) at 1 keV (collision energy). The time ion selector was adjusted to make sure that only the selected ion was considered. MS and MS/MS experiments were externally calibrated using classical mixtures of peptide references. In linear and reflector modes, the ion source accelerating voltage was set to 20 kV, whereas it was set to 8 kV for MS/MS.

Complementary results were obtained from a MALDI-TOF DE-Pro (Applied Biosystems, Courtaboeuf, France) fitted with a nitrogen laser ($\lambda = 337$ nm, pulse duration of 3 ns, repetition rate value of 10 Hz) in linear mode with continuous or delayed extraction (20 kV for accelerating voltage). Laser energy measured

(10) Gobom, J.; Nordhoff, E.; Mirgorodskaya, E.; Ekman, R.; Roepstorff, P. *J. Mass Spectrom.* **1999**, *34*, 105–116.

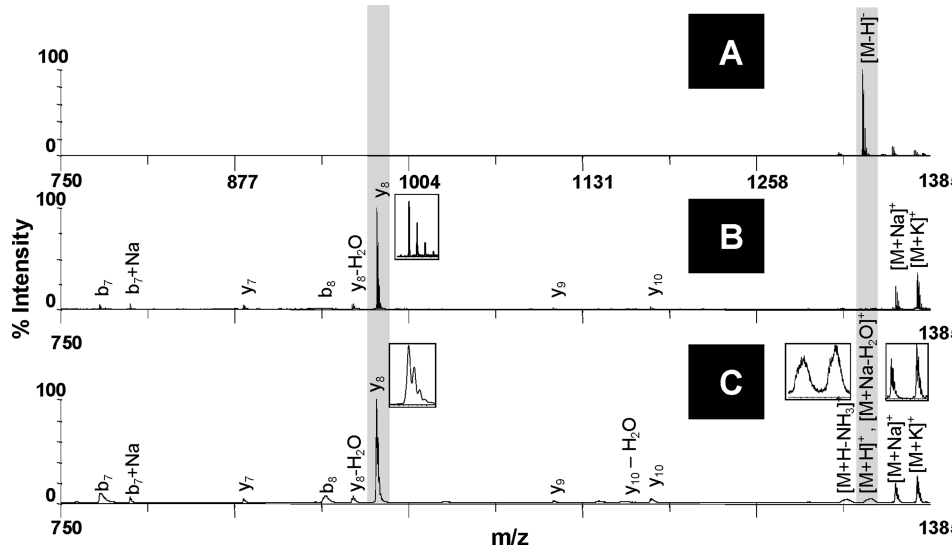

Figure 1. MALDI-TOF-MS mass spectra of peptide 1 acquired in negative ion reflector mode (A), in positive ion reflector mode (B), and in positive ion linear mode (C). Fragment ions observed in both positive reflector and linear modes are labeled as the y and b ion series. Magnifications of isotope patterns of several fragment ions are presented on the spectra. In both spectra, the base peak corresponding to the y_8 ion and the theoretical region of the mass spectrum where the molecular ion (theoretical *m/z* of the $[M + H]^+$ at 1337.63) should be are highlighted in gray.

after the neutral filter by using a power meter (Coherent Inc.) for both MALDI-TOF apparatus was adjusted in the range of 0.2−10 μJ/pulse. The size of the laser spot was determined to be ~70 μm × 50 μm and ~100 μm × 67 μm at the energy of 0.5 and 1.16 μJ/pulse, respectively.

Micro- and NanoLC−ESI-MS and MS/MS. LC−ESI-MS and MS/MS were performed by using an online coupling between a Dionex LC (nano LC, 240 nL/min using a C18 Acclaim pepmax 100, 3 Å, length 15 cm, inner diameter 75 μm, and microLC, 20 μL/min using a C18 Acclaim, 3 μm, 120 Å, length 15 cm, inner diameter 1 mm) and a Q-Trap (Applied Biosystems−Sciex). Peptide analyses were also performed in ESI-MS and MS/MS by using different declusterization potentials and collision energies.

RESULTS AND DISCUSSION

Characterization of the Extreme Fragility of the Peptide Ac−DAEPPILYSEY−NH_2 (Peptide 1, Table 1) by MALDI-TOF and NanoLC−ESI-MS Analysis. *MALDI-TOF, Reflector Mode.* Peptide 1 (Ac−DAEPPILYSEY−NH_2) was analyzed both in positive and negative ion reflector modes in MALDI-TOF by using the CHCA matrix. The analysis in the negative ion reflector mode exhibited only the expected deprotonated molecule $[M - H]^-$ at *m/z* 1335.57 (base peak) as well as $[M - 2H + Na]^-$ and $[M - 2H + K]^-$ at *m/z* 1357.55 and 1373.53, respectively (Figure 1A). In contrast, analysis in positive ion reflector mode exhibited unusual results in that no or very weak signal for the protonated molecule at *m/z* 1337.63 was detected. Nevertheless, cationized molecules $[M + Na]^+$ (*m/z* 1359.61) and $[M + K]^+$ (*m/z* 1375.59) were observed (Figure 1B). The most abundant and well-resolved ion was detected at *m/z* 980.51. We also noted the presence of less abundant ions at *m/z* 778.39, 800.42, 883.45, 941.45, 962.49, 1109.54, and 1180.58. Following sequence analysis by MALDI-TOF-TOF, these ions appeared in good agreement with those expected for some y- and b-derived fragments associated to the intact peptide 1. In this context, it is noteworthy that the most abundant ion at *m/z* 980.51 corresponded to the y_8 fragment that results from E−P peptide bond cleavage. Further sample desalting by a Poros reversed-phase 20 R2 microcolumn (analogue to a C18 Zip-Tip) did not improve the signal of the protonated molecule $[M + H]^+$ nor reduce the cationization of the peptide; the y_8 fragment remained the major ion. All attempts to reduce the fragmentation of peptide 1 by adding salts (NaCl and KCl, 10^{-2} to 10^{-5} M) were unsuccessful. Actually, despite increase of cationized molecule amount in function of salt concentration, the major ion remained the y_8 fragment. It should be noted that such a fragment was never observed as a cationized ion. MALDI-TOF-TOF studies of the cationized molecules confirmed their very high stability.

Micro- and NanoLC−ESI-MS and MS/MS. The purity of peptide 1 was further assessed by using micro- and nanoLC−ESI-MS/MS. Only one elution peak was found in the conventional total ion current (TIC) analysis showing singly charged ions at *m/z* 980.5 and 1337.6 $[M + H]^+$ and a doubly charged ion at *m/z* 669.8 $[M + 2H]^{2+}$. MS/MS analysis of *m/z* 980.5 confirmed the sequence PPILYSEY−NH_2. Remarkably, these ions were observed even using the lowest values of the declustering potential (10−90 V, data not shown). However, the ion abundance at *m/z* 980.5 decreased with decreasing declustering potential, suggesting that the y_8 fragment was produced in the ion source and not from in-solution conditions. Chemical acidolysis in solution of the D−P amide bonds was reported but in much harsher conditions[6] than those used in the present work. We clearly confirmed that peptide 1 was not degraded in solution by studying in microLC−ESI-MS and MS/MS the 1:1 mixture of peptide 1 with the synthetic peptide PPILYSEY−NH_2 miming the y_8 fragment. The peptide PPILYSEY−NH_2 eluted at a shorter elution time (R_t = 24.2 min) compared to peptide 1 (R_t = 26.5 min) (loading buffer A, 0.1% aqueous FA; elution buffer B, ACN, 0.1% FA; gradient 0−60% B in 60 min). Thus, the in-solution degradation of

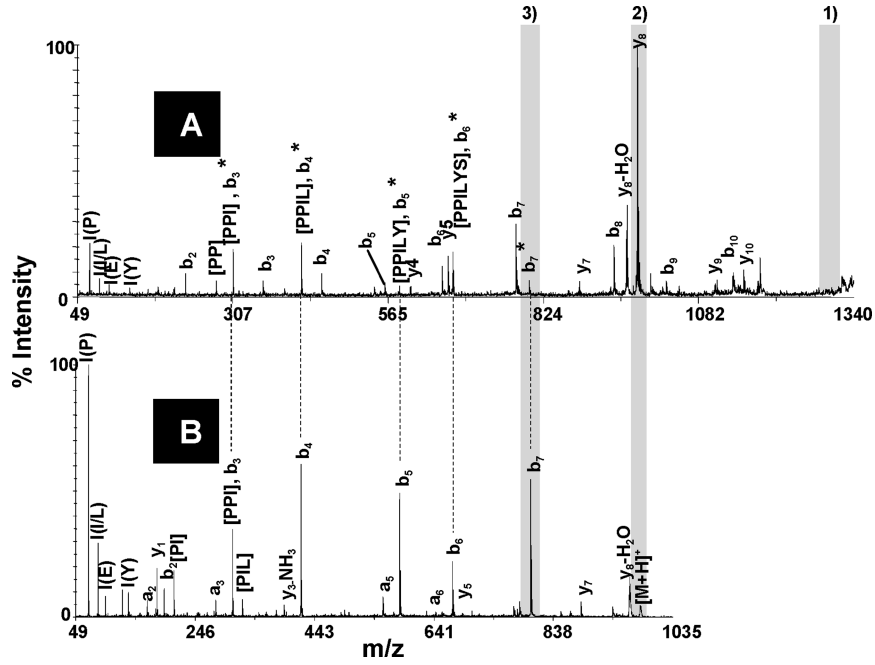

Figure 2. MALDI-TOF-TOF mass spectra recorded in CAD condition using a time ion selector centered either on *m/z* 1337 (theoretical *m/z* of peptide 1) (A) or on the main fragment ion at *m/z* 980 (B). The gray areas, namely, 1, 2, and 3, highlight the region at *m/z* 1337, the y_8 ion at *m/z* 980.51, and the b_7* specific fragment ion of the precursor at *m/z* 980.51, respectively. The a, b, and y ion series are reported. Immonium ions of proline I(P), leucine or isoleucine I(L/I), glutamic acid I(E), and tyrosine I(Y) are indicated. The b ions labeled with stars in spectrum A correspond to b fragment ions issued from the fragmentation of the ion at *m/z* 980.51 as indicated by the dashed lines. Internal fragments are mentioned, and their sequences appear in brackets.

peptide 1 is excluded and the y_8 fragment is clearly a fragment of peptide 1 produced in the ESI source.

MALDI-TOF, Linear Mode. The MALDI analysis of peptide 1 in linear mode (Figure 1C) displayed the same features than those in reflector mode (i.e., presence of the most abundant ion at *m/z* 980 and of the cationized peptide peaks $[M + Na]^+$ and $[M + K]^+$, all as well-resolved ions. However, a metastable peak around the *m/z* value of the $[M + H]^+$ ion was observed, interfering with a metastable peak shifted by around 4 u toward higher *m/z*. Accordingly, if the protonated molecule of the intact peptide 1 partly survives the acceleration in the source, its stability in the field free region is not high enough to allow detection in the reflector mode.

MALDI-TOF-TOF. As shown above, the absence of $[M + H]^+$ in positive ion reflector mode and its possible presence as a metastable peak in linear mode suggested that protonated molecules survive the extraction source and could therefore be studied in MALDI-TOF-TOF. Careful study of the selected ions was performed using gradual shifts of the precursor *m/z* by step of 2 u using a window of (−1u, 3u) for the time ion selector (TIS). The precursor was scanned from *m/z* 1330 to *m/z* 1346 allowing us to discard any contribution of the ion fragments forming the second metastable peak. (Figure S-1, see the Supporting Information).

As shown in Figure 2A, the MS/MS spectrum using CAD conditions and a TIS centered on *m/z* 1337 exhibited the expected b and y fragments of the protonated molecule, internal fragments and the y_8-derived fragments of the previously major y_8 ion observed in reflector positive ion mode (Figure 2B). In fact, this observation is not surprising since y_8 results from the metastable decomposition of the protonated molecule between the source and the TIS, and therefore, this latter is also selected and then fragmented in the collision cell. Similar results were observed in metastable conditions except the absence of immonium ions.

It should be noted that for the different modes, the peptide positive ion TOFs are quite different. In the reflector and linear modes, the TOFs are of 63.73 and 37.76 μs, respectively; it is much smaller in MS/MS, i.e., 11.6 μs for the flight from the ion source to the TIS. In fact, peptide 1 ion lifetime is not high enough to allow detection at time ≥37 μs in linear and reflector modes ($[M + H]^+$ not observed or very weak), but it is sufficient for some of these ions to survive up to the TIS at ∼11 μs. The results suggest that ions derived from peptide 1 fragmentation are mainly formed by a major and fast fragmentation (y_8) and only few protonated molecules survive at time of ∼10 μs.

Effects of the Primary Structure on the Stability of the Peptide (Table 1). *Effects of the Acidic Residues (E, D).* In a previous study, it has been shown in MALDI-MS that a selective facile gas-phase cleavage at the peptide bond C-terminal to aspartic acid (D−P and D−X) in fixed-charge aspartyl-containing peptides[4] could occur, leading to metastable ion peaks (PSD). In this example, the labile proton on the carboxylic group of the aspartic acid has been shown to be necessary for decomposition. Therefore, we studied the effect of the successive replacement by A or D of the E residues (peptides 2, 3, 6), the substitution of the D residue with A (peptide 4) or the substitution of the three acidic amino acids by A residues (peptide 5).

The modified peptides 2, 3, 4, 5, and 6 behaved similarly to peptide 1. Diminishing the number of acidic residues or even removing all of them did not reduce fragmentation. Thus, fragmentation did not depend on the presence of acidic residues. Such specific cleavage, adjacent to acidic residues, is usually reported in CAD experiments ("mobile proton" model, as a

review[11]) when the number of ionizing protons equals the number of basic residues in a peptide.[12,13] This cleavage is referred as "charge remote" cleavage (as a review[14]) to indicate that it occurs when the added proton is not mobile. In the case of peptides 1−6, the cleavage is already observed in the primary MS spectrum (without any CAD, except collisions in the plume) although the absence of basic site required for proton sequestration.

Effects of the Proline Residues (P). The role of the proline(s) in this fragmentation process was also explored by replacing the latter by alanine residues (peptides 7 and 8). The substitution of the N-terminal proline (E−P → E−A) led to the y_7 fragment (PILYSEY−NH_2). When both prolines were substituted (E−PP → E−AA, peptide 8), protonated molecule remained absent and no fragmentation was observed. The Na and K cationized molecules were only detected. These results are relevant to a proline-induced fragmentation, the presence of only one proline being sufficient to observe this process. This behavior that we observe in the MS spectra is usually described as the "proline effect" in the literature from CAD studies mainly using ESI[14] and sometimes MALDI[15] sources. We should note that, as already reported in the ESI-CAD studies,[16] fragmentation at the C-terminal side of proline is very unusual. Our result on the very low fragmentation at the P−P amide bond (y_7) is in good agreement with those already reported in ESI-CAD.[16]

Effects of the Basic Sites. In absence of a strong basic residue in a protonated peptide, the location of the proton cannot be necessarily pinpointed, and it is supposed that the multiple C=O amide oxygens solvate the proton.[17] Thus, we studied the effects induced by a weak or a strong basic site within peptide 1, i.e., in the N-terminal tail (peptide 9, with a free primary amine, pK_a of approximately 9), in the C-terminal tail (peptide 12, with an arginine residue, pK_a (guanidinium) of approximately 12), or both (peptides 10 and 11). In each case, the protonated molecule $[M + H]^+$ was observed in reflector positive ion mode MALDI-TOF. The intensity of the signal corresponding to the fragmentation was marginal except for peptide 9 for which the intensity of the signal associated to y_8 was similar to that of the $[M + H]^+$. Even if the presence of a free amine group at the N-terminal extremity is a prerequisite for protonated molecule production in MALDI (peptide 9), it cannot totally prevent the very prompt fragmentation of the E−P peptide bond, likely due to a large mobility of the proton. In contrast, the presence of a basic residue (R) in the sequence of the peptides (peptides 10, 11, and 12) produced protonated molecules without fragmentation due to a higher stabilization of the sequestered proton.

Four distinct behaviors emerge from peptide sequence: (i) no or very low abundant protonated ions as well as strong specific fragmentation at the E/A−PP or A−P bonds are observed in presence of prolines and in absence of a basic site (peptides 1, 2, 3, 4, 5, 6, and 7), (ii) no protonated molecule and no fragmentation but only cationized molecules are observed in absence of proline and basic site (peptide 8), (iii) both protonated and fragmented ions are observed with similar relative intensities when the N-terminal NH_2 is free (peptide 9), and (iv) only stable protonated molecules are observed when a basic residue is present in the sequence (peptides 10, 11, and 12).

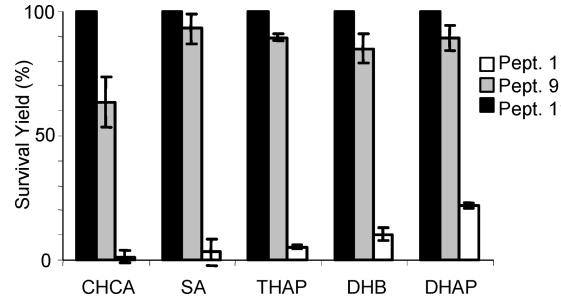

Figure 3. Experimentally measured survival yields (%) of the $[M + H]^+$ ion for peptide 1 (Ac−DAEPPILYSEY−NH_2, white bars), peptide 9 (H−DAEPPILYSEY−NH_2, gray bars), and peptide 11 (H−DAEPPILYSER−NH_2, black bars) at the threshold of the laser fluence, for different MALDI matrixes: CHCA, SA, DHB, DHAP, and THAP. Experimental errors were calculated over five different samples. $SY_{exp} = I(M^+)/[I(M^+) + I(F^+)]$, with I being the relative intensity of the ion and the approximation that $I(F^+) \approx I(y_8)$.

Effect of the Nature of the Matrix on the Stability of the Peptide. Effects induced by different matrixes were explored with peptides 1, 9, and 11. Experimental survival yields (SY_{exp}) associated to protonated molecules (M) and fast fragment ions (F) (formula 1) were evaluated in reflector mode using the usual matrixes CHCA, SA, DHB, DHAP, and THAP at the laser threshold energy of ion production (S/N ∼ 3−5).

$$SY_{exp} = I(M^+)/[I(M^+) + I(F^+)] \quad (1)$$

For each matrix/analyte combination, the averaged SY_{exp} value and standard deviation were determined from at least five different samples of the same tested peptide. Because the y_8 fragment is the major fragment, representing at least 90% of the total fragment intensity ($I(F^+)$), only its intensity was taken into account in formula 1.

As shown in Figure 3, the SY_{exp} value for peptide 11 (free NH_2 group at the N-terminus and an R residue at the C-terminus) is 100% whatever the matrix. In the case of peptide 1 (with no basic site), a significant SY_{exp} of only 20% was measured for the DHAP matrix, whereas it was particularly low with CHCA. The results on peptide 1 using DHAP are in good agreement with those reported on fragile peptides.[5] An intermediate behavior is associated with peptide 9 (free NH_2 group at the N-terminus). Indeed, the lowest SY_{exp} was observed with CHCA (60%), whereas it was close to 90% with other matrixes. The variation of the experimental SY for the peptides 1, 9, and 11 (Figure 3) showed a strong effect of the matrix.

As previously studied in AP-MALDI,[8] a possible correlation with the matrix PA was explored. To our knowledge, there is no published data concerning the PA value for the DHAP matrix.

(11) Paizs, B.; Suhai, S. *Mass Spectrom. Rev.* **2005**, *24*, 508–548.
(12) Gu, C.; Tsaprailis, G.; Breci, L.; Wysocki, V. H. *Anal. Chem.* **2000**, *72*, 5804–5813.
(13) Tsaprailis, G.; Nair, H.; Somogyi, A.; Wysocki, V. H.; Zhong, W.; Futrell, J. H.; Summerfield, S. G.; Gaskell, S. J. *J. Am. Chem. Soc.* **1999**, *121*, 5142–5154.
(14) Cheng, C.; Gross, M. L. *Mass Spectrom. Rev.* **2000**, *19*, 398–420.
(15) Fernandez Ocana, M.; Jarvis, J.; Parker, R.; Bramley, P. M.; Halket, J. M.; Patel, R. K.; Neubert, H. *Proteomics* **2005**, *5*, 1209–1216.
(16) Breci, L. A.; Tabb, D. L.; Yates, J. R., III; Wysocki, V. H. *Anal. Chem.* **2003**, *75*, 1963–1971.
(17) Nair, H.; Wysocki, V. H. *Int. J. Mass Spectrom. Ion Processes* **1998**, *174*, 95–100.

We used the kinetic method[18] and the PA values of the other matrixes[19] to determine the PA of DHAP. This measure was performed in MALDI-TOF-TOF, indicating a value of 870 kJ/mol (Figure S-2, parts A and B, see the Supporting Information). The effect of the matrix on the SY is reported in Figure 3. Contrary to the results reported on less fragile molecules,[8] no clear SY dependence on the nature of the matrix was observed for the very fragile peptide. SY_{exp} is ranking in the order CHCA < SA < THAP < DHB < DHAP when the PA is ranking in the following order CHCA (840 kJ/mol) < DHB (854 kJ/mol) < DHAP (870 kJ/mol) < SA (874 kJ/mol) < THAP (891 kJ/mol).

Effects of the Delay Time in Delayed Extraction and Laser Fluence on the Stability of Peptide 1. In delayed extraction, ions move with their initial velocity before being accelerated in the extracting electrostatic field applied at a delay time (Td) triggered by the laser shot. Varying this Td should allow exploring the plume expansion through the initial axial velocity and its possible effect(s) on the peptide stability. Systematic experiments were performed on peptide 1 (Ac−DAEPPILYSEY−NH_2) using the CHCA matrix (data not shown). Major features of mass spectra (linear and reflector modes) remained unchanged when the Td was adjusted in the 50−2000 ns range. Even in continuous extraction mode (DE-Pro MALDI-TOF), the y_8 fragment remained the major ion indicating that y_8 is formed at the early step of ablation and not in the diluted plume.

Likewise, increasing the laser fluence from the threshold (S/N ∼ 3−5) did not influence the features noted above, and y_8 remained the base peak representing 75−80% of all other fragments. These results evidence that the peptide 1 fragmentation, from which results y_8, is relevant to a very fast mechanism(s) triggered by UV absorption and not from delayed fragmentation through metastable decomposition.

Initial Velocity. The effects of the mean axial initial velocity v_{in}, which has been proposed to correlate with the internal energy of the analyte,[8,20,21] was also explored. The real value of v_{in} is difficult to measure.[22] Thus, using two acceleration stages (DE-Pro MALDI-TOF) in linear mode we only compared for a given analyte (ACTH 18−39 or chain B of insulin) and for the different matrixes the slope of the TOF curve versus delay time (Td = 50−600 ns).[23] As shown in Figure S-3 (see the Supporting Information), the slope is proportional to the initial velocity when the same extraction geometry and voltages are applied. The lowest slope was found for CHCA. When the initial velocity was normalized to 1 for CHCA, slope values associated with other matrixes were 1.16 for THAP, 1.38 for SA, 2.3 for DHB, and 2.46 for DHAP in the case of ACTH 18−39 (Supporting Information Figure S-3). The matrix property that best correlates with the fragmentation extent (SY) of the analyte is the initial velocity (v_{in}) and not the PA. The comparison with the survival yield for the very fragile peptide 1 shows a better correlation, i.e., the lower initial velocity, the lower survival yield indicating the influence of the collision rate[22] in the early step of ablation in the fragmentation process. For the moderately fragile peptide 9, the correlation between SY_{exp} and v_{in} shows a threshold when v_{in} is higher than for CHCA. For the very stable peptide 11, the constant stability is independent of the matrix initial velocity.

A striking feature of the peptide 1 was the absence of protonated molecule and a very intense y_8 fragment. All these data cannot be explained using the fragmentation framework through classical ISD mechanisms[24,25] or proton transfer in the gas phase. ISD fragmentation is characterized by the following properties: (i) it mainly depends on the nature of the matrix.[24,25] In this work, the main fragment (y_8) was found for all the matrix specially for CHCA and DHB which were expected to produce y and (c, z) ISD fragments, respectively. In addition CHCA was found a poor matrix for ISD when DHB was found the matrix of choice in contrast with our results; (ii) ISD fragmentation was not observed in continuous extraction[24] when y_8 fragmentation for peptide 1 was detected in our experiments; (iii) ISD fragmentation was also observed in negative ion mode but not in these experiments; (iv) ISD fragmentation is generally observed at high laser fluence but not at the fluence threshold.[24,25] All these points highlight that the prompt fragmentation (y_8) mentioned in this work cannot be understood as a pure ISD fragmentation and the in-source decay mediated by hydrogen radicals cannot be invoked.[26]

We have highlighted that the presence of proline is of prime importance for producing a very prompt (nanosecond scale) and strong fragmentation. The "proline effect" is generally observed in MS/MS and seldom in MS. However, the mechanism should be the same in MALDI prompt fragmentation. Proton-mediated fragmentation being very fast, the origin of the fragmentation must be found in the protonation state of the peptide, when it is on the target or in the early step of ablation.

CONCLUSION

To rationalize our results, we must assume that during solvent evaporation and crystallization, a balance of proton transfer might occur between the peptide, matrix molecules, the solvents and the associated counterions. From this pseudoequilibrium, it would result that one proton could be solvated by the various C=O amide oxygens along the backbone[17] or that this proton could be directly linked to the more basic site, i.e., the first proline residue in the sequence of peptide 1.[27] After laser irradiation, the peptide molecules are entrained by the matrix movement induced by the pressure pulse. The small differential movements between the peptide and matrix molecules during the early step of ablation should be similar to low-energy collision effects. Then, these collisions could promote the proton transfer (if necessary) and could activate the fragmentation of the peptide similarly to what happens in CAD. The "proline effect" also remains valid with a major cleavage at the X−P bond. The effect of the nature of the matrix on the survival yield (peptide 1) can be understood from

(18) Cooks, R. G.; Wong, P. S. H. *Acc. Chem. Res.* **1998**, *31*, 379–386.
(19) Mirza, S. P.; Raju, N. P.; Vairamani, M. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 431–435.
(20) Glückmann, M.; Karas, M. *J. Mass Spectrom.* **1999**, *34*, 467–477.
(21) Karas, M.; Bahr, U.; Fournier, I.; Glückmann, M.; Pfenninger, A. *Int. J. Mass Spectrom.* **2003**, *226*, 239–248.
(22) Spengler, B.; Kirsch, D. *Int. J. Mass Spectrom.* **2003**, *226*, 71–83.
(23) Juhasz, P.; Vestal, M. L.; Martin, S. A. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 209–217.
(24) Brown, R. S.; Carr, B. L.; Lennon, J. J. *J. Am. Soc. Mass Spectrom.* 1996.
(25) Brown, R. S.; Feng, J.; Reiber, D. C. *Int. J. Mass Spectrom.* **1997**, *169/170*, 1–18.
(26) Kocher, T.; Engstrom, A.; Zubarev, R. A. *Anal. Chem.* **2005**, *77*, 172–177.
(27) Piszkiewicz, D.; Landon, M.; Smith, E. L. *Biochem. Biophys. Res. Commun.* **1970**, *40*, 1173–1178.

the initial velocity since the lowest initial velocity is associated to a higher collision rate[22] to produce the higher y_8 fragmentation. For the highest initial velocity with a smaller collision rate, the fragmentation is reduced and some protonated peptides can survive even if their lifetime is very short for TOF analysis. It should be noted that the protonation could also involve other sites of the backbone, but the quasi-equilibrium during the crystallization process implies a more efficient trapping at the proline site.[6,27] This could explain why the y_8 fragment is principally formed during the MS experiments, whereas others of less abundance are also detected. A consequence of this model of quasi-preformed ions is that the number of available protons should be very low in the close neighborhood of the peptide in the target. It is difficult to readily correlate the observed results with the pH of the matrix solution. The fast fragmentation (y_8) and the absence of protonated molecules for peptide 1 were similarly detected in various conditions of pH: (i) CHCA dissolved in ACN/0.1% aqueous TFA (pH = 1.2), (ii) CHCA dissolved in ACN/H_2O (pH = 2.3), (iii) DHAP dissolved in ACN/0.1% aqueous TFA (1:1) (pH = 2.8), and (iv) DHAP dissolved in nonacidic solution with DHAC (pH = 4.7). The influence of the pH, mainly by adding 0.1% aqueous TFA, was noticeably observed on more classical peptides, showing an increasing ion yield for the protonated species.

The low proton density in this model is also consistent with the low production of multiply charged ions in MALDI. Furthermore, because matrix crystallization is not an instantaneous process (a few minutes for the solvent evaporation), embedded peptide could have different environments. These environments involving a variable low density of protons which is also consistent with the "sweet spot" phenomenon observed in MALDI.

For peptide 1, the formation of the low abundant protonated molecule with a short lifetime can be understood as resulting from delayed proton transfer during the plume expansion.

This model is also valuable to describe the behavior of the moderately fragile peptide 9 with an N-terminal amino group. Indeed, during the crystallization process, an available proton can be located preferentially onto the N-terminal[17] tail and also onto the proline. The former case should promote the formation of the protonated molecule with possible fragmentations especially at X−P bond ("proline effect") depending on the collision rate in the plume and the proton transfer from the N-terminal $-NH_2$. Similarly to peptide 1, if there is no available proton in the *close neighborhood* of peptide 9 when it is on the target, a proton transfer in the plume or in gas phase between protonated matrix and neutral peptide can occur.

The deprotonated peptide which was found very stable (no fragmentation) for all the studied peptides would result from a different local environment compared to that producing protonated molecule and fast fragmentation.

The above results including the effect of the sequence, the laser energy, the delay time, the nature of the matrix, and the mean axial velocity suggest that the ionization and fragmentation of a fragile peptide is mainly due to (i) the proton availability at or in the close neighborhood of the peptide during the crystallization process and (ii) the very prompt fragmentation induced by the collisions in the first step of ablation. Noticeably, extremely prompt fragmentation initially observed in MS can be useful in proteomics studies implying proline-rich peptides, especially when trypsin is not used as the proteolytic enzyme. At least, the analysis of proline-rich peptides in MALDI-TOF should be carried out with extreme cautions.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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