Pseudo-MS^3 in a MALDI Orthogonal Quadrupole-Time of Flight Mass Spectrometer

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Both the matrix selected and the laser fluence play important roles in MALDI-quadrupole/time of flight (QqTOF) fragmentation processes. "Hot" matrices, such as α-cyano-4-hydroxycinnamic acid (HCCA), can increase fragmentation in MS spectra. Higher laser fluence also increases fragmentation. Typical peptide fragment ions observed in the QqTOF are a, b, and y ion series, which resemble low-energy CID product ions. This fragmentation may occur in the high-pressure region before the first mass-analyzing quadrupole. Fragment ions can be selected by the first quadrupole (Q1), and further sequenced by conventional MS/MS. This allows pseudo-MS^3 experiments to be performed.

For peptides of higher molecular weight, pseudo-MS^3 can extend the mass range beyond what is usually accessible for sequencing, by allowing one to sequence a fragment ion of lower molecular weight instead of the full-length peptide. Peptides that predominantly show a single product ion after MS/MS yield improved sequence information when this technique is applied. This method was applied to the analysis of an in vitro phosphorylated peptide, where the intact enzymatically-generated peptide showed poor dissociation via MS/MS. Sequencing a fragment ion from the phosphopeptide enabled the phosphorylation site to be unambiguously determined. (J Am Soc Mass Spectrom 2002, 13, 1034–1041) © 2002 American Society for Mass Spectrometry

After the matrix-assisted laser desorption/ionization (MALDI) technique was first developed [1–5], there were extensive studies on the selection of matrices optimized for various types of lasers [6–9]. Although most researchers are now primarily using 337 nm nitrogen lasers and only a few selected matrices, there has been much discussion of MALDI matrices with regard to mechanisms of ion formation [10–24].

In-source decay (ISD [25, 26]) and post-source decay (PSD [27]) in MALDI/TOF-MS have been studied for the purpose of determining the mechanism of MALDI ionization, and have led to the classification of matrices such as α-cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxybenzoic acid (DHB) as "hot" or "cold" based originally on the amount of glycoprotein fragmentation they induce [28].

The differences in fragmentation observed with various matrices have been attributed to differences in initial velocities of the ions generated [29–31], with higher initial velocities leading to cooling and less fragmentation. It has been noted that, for a given matrix, all analyte ions below 25 kDa appear to have the same initial velocity irrespective of mass, but the initial velocity depends on the matrix [31]. An early hypothesis that the internal energy and velocity of both analyte and matrix are a function of matrix sublimation temperatures has found empirical support [32]. Zenobi and co-workers found, however, that the internal energy distributions of analyte ions did not correlate with matrix sublimation temperatures, but instead depended on gas-phase proton transfer reactions [20, 21, 23]. Increasing laser fluence has been shown to increase analyte ion dissociation via in-source decay (ISD) [33–38]; nevertheless, Gluckmann and Karas have questioned whether an increase in laser fluence is an accurate measure of the amount of energy transferred to the analyte [31].

Lavine and Allison [39] demonstrated that MS^3-type experiments could be performed on a reflectron MALDI-TOF using a combination of ISD and PSD. A mixture of bumetanide and HCCA was used to control the extent of in-source fragmentation. This combination of ISD and PSD has not become widely utilized, however, probably because of the inherent difficulties in generating and interpreting the poorly resolved PSD mass spectra.

With the recent development of MALDI QqTOF instruments, experiments involving two stages of mass spectrometry, including true MS/MS with collision-induced dissociation (CID), are possible [40]. We have

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found that an additional fragmentation step can be created through the use of a "hot" matrix and/or increased laser fluence. This "pseudo-MS^3" is the combination of non-selective fragmentation followed by true MS/MS in a MALDI QqTOF mass spectrometer. The pseudo-MS^3 technique, which makes it possible to isolate and dissociate source-generated fragments, increases the mass range accessible for peptide sequencing. A proteomics application is shown where the pseudo-MS^3 technique has allowed unambiguous identification of a phosphorylation site in an *in vitro* phosphorylated peptide.

Experimental

Materials

The peptide [Glu-1]-fibrinopeptide-B (G1FB) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. A stock solution was prepared at a concentration of 1 μg/μL in HPLC-grade water, and then was serially diluted with HPLC-grade water.

The *in vitro* phosphorylated protein was digested with *Streptococcus aureus* V8 from Sigma Chemical Co. The kinase used was a serine/threonine kinase. The resulting peptides were separated on a 2-D cellulose phosphopeptide mapping plate [41]. It was estimated that ca. 2 pmol was applied to the plate. The radiolabeled spot was scraped off the plate, eluted sequentially with two 100-μL washes of pH 1.9 buffer containing 88% formic acid: acetic acid:water (25:78:897, vol:vol:vol), followed by one wash with 100 μL water. The eluate was cleaned up with a Millipore Zip-Tip (Bedford, MA), and spotted on the MALDI target. The overall recoveries through the entire clean-up procedure could not be determined, but the amount spotted on the MALDI target was below 2 pmol.

Mass Spectrometry

MALDI-MS and MS/MS were performed on an Applied Biosystems Div., Perkin-Elmer Corp. (Foster City, CA) API QSTAR-Pulsar (QSTAR), with argon as the collision gas. The instrument was equipped with a nitrogen laser operating at 337 nm. Matrices used were premixed DHB and HCCA solutions (Agilent Technologies Inc., Palo Alto, CA). In all cases, a 0.5 μL aliquot of the peptide solution was spotted on the target, followed by 0.5 μL of the matrix solution. The spots were allowed to dry at room temperature. Once ions with sufficient S:N (10:1 for precursor ions, 3:1 for product ions) were observed, data acquisition was terminated to conserve sample.

Results and Discussion

Matrix and Laser Fluence Effects on the MS Spectra

Before the advent of MALDI-QqTOF, PSD was the primary method used with TOF instruments to generate sequence data from MALDI-generated ions. Because HCCA is classified as a "hot" matrix, it is often selected for PSD analysis because desorption from HCCA crystals increases the internal energy of the ions and promotes increased fragmentation. Using G1FB, a model tryptic peptide, increased fragmentation of precursor ions was observed in MS mode with HCCA. On the QqTOF, at the threshold laser fluence for DHB (35% laser power, ~27 microjoules) (Figure 1a), no fragmentation of the protonated molecule was observed. At an increased laser fluence (40% laser power), there was a small amount of fragmentation, and the spectrum contained only a few, low relative-abundance y ions (Figure 1b). However, two y ions were observed when HCCA was used as the matrix (Figure 1c), even at the threshold laser fluence for HCCA (15% laser power). At a slightly increased laser fluence (20% laser power), a more complete series of y ions (y_6–y_9) was observed (Figure 1d). These results show that an increased amount of fragmentation is observed when HCCA is used as the matrix, compared to DHB. To ensure that a statistically significant number of ions were recorded, a relatively large amount of peptide (30 pmol), compared to typical proteomic studies, was deposited on the MALDI target.

To investigate whether the peptide concentration applied to the MALDI target had an effect on fragmentation, MALDI-QqTOF-MS spectra of 300 fmol of G1FB were acquired at several different laser energies, using HCCA as the matrix. As can be seen in Figure 2, the extent of fragmentation can be controlled by varying the laser fluence regardless of the amount of peptide applied to the target. At threshold laser fluence (15%), both the protonated molecule and the y_6 product ion were observed. At 25% laser fluence, the y_9 ion was also observed. When the laser fluence was increased to 50%, a complete y ion series from y_6 to y_9 was observed in addition to the protonated molecule. By examining the ratio of the y_6 and the y_9 product ions to the protonated molecule intensity, (Table 1), the effect of increasing the laser power is apparent. Both ratios increase with higher laser power, indicating that more fragmentation has occurred. The data shown in Figure 2 were acquired with only 300 fmol spotted on the MALDI target, demonstrating that fragmentation can be increased, even when only subpicomole amounts of sample are available for analysis.

With less peptide applied to the MALDI target, a relatively higher laser fluence was necessary to obtain the same degree of fragmentation as that obtained with a larger amount of peptide applied to the target. In Figure 1d, with 30 pmol applied to the MALDI target,

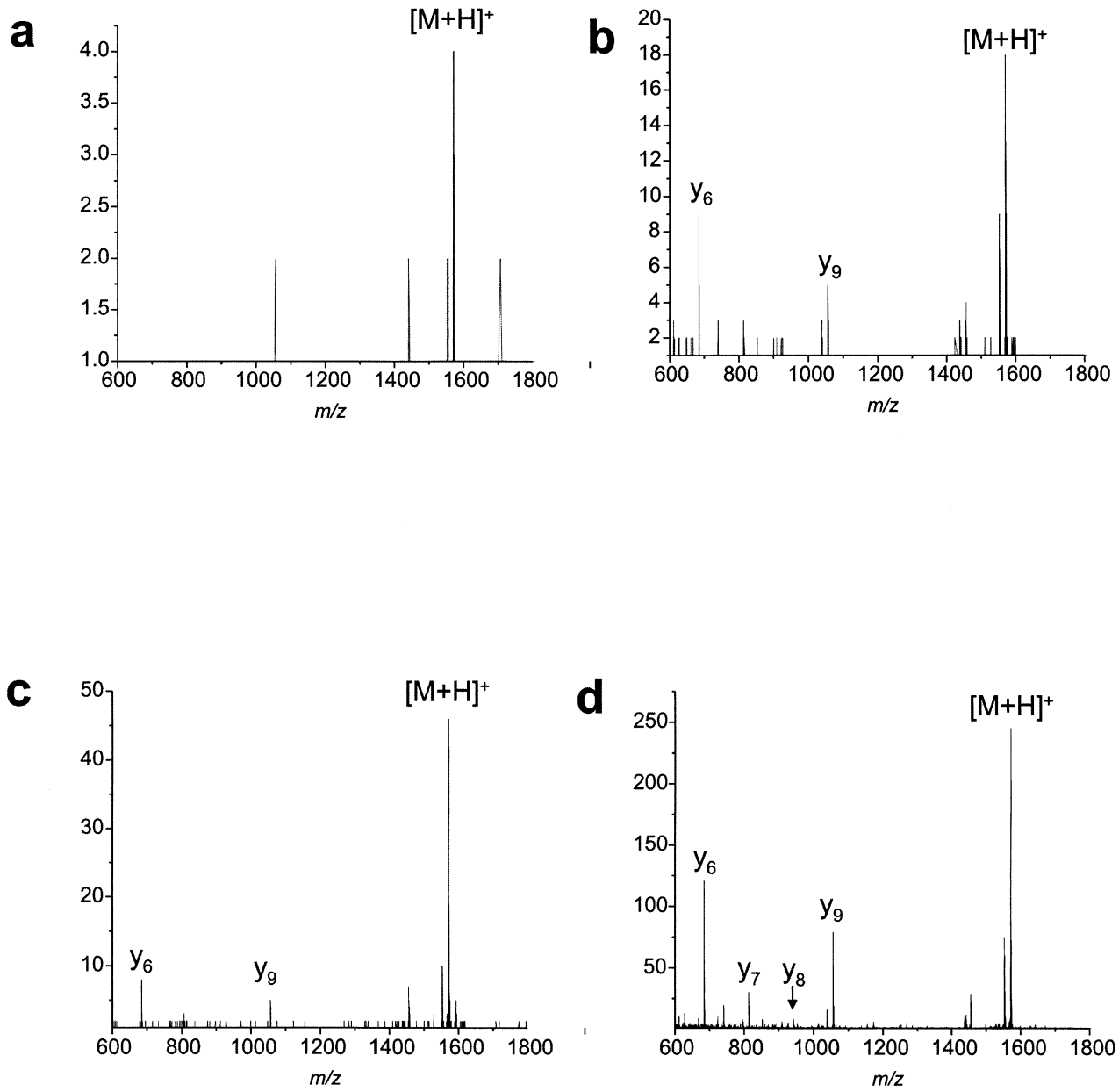

Figure 1. The effect of matrix on the MALDI-MS spectra of G1FB in DHB and HCCA, 30 pmol on target. (**a**) QqTOF MALDI-MS spectrum of G1FB in DHB at 35% laser fluence (threshold laser power); (**b**) QqTOF MALDI-MS spectrum of G1FB in DHB at 40% laser fluence; (**c**) QqTOF MALDI-MS spectrum of G1FB in HCCA at 15% laser fluence (threshold laser power); (**d**) QqTOF MALDI-MS spectrum of G1FB in HCCA at 20% laser fluence.

the y_6–y_9 ion series was observed with 20% laser fluence. In Figure 2c, with only 300 fmol applied to the target, 50% laser fluence was necessary to produce a complete y_6–y_9 series. Thus, with less sample applied to the MALDI target, more laser fluence is necessary for more complete sequence coverage.

At threshold laser power, with a "cold" matrix such as DHB, MALDI-QqTOF-MS spectra are obtained which show strong signals from the protonated mole-

Table 1. Ratio of product ion intensities for G1FB compared to the Intensity of the Protonated Molecule of G1FB

Laser power	Ratio of y_6 to $[M + H]^+$ intensity	Ratio of y_9 to $[M + H]^+$ intensity
15%	1.33	y_9 ion not observed
25%	1.45	0.73
50%	1.74	1.00

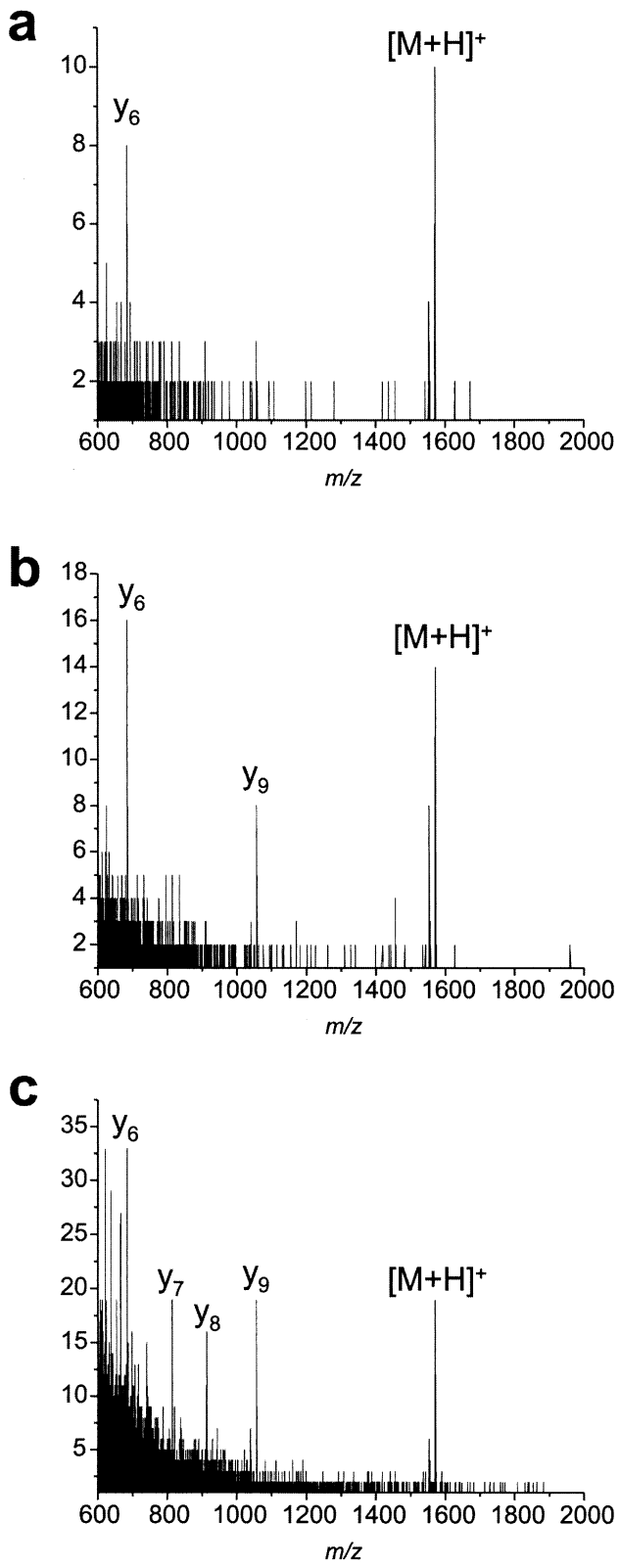

Figure 2. The effect of laser fluence on the MALDI-MS spectra of G1FB in HCCA, 300 fmol on target. (**a**) QqTOF MALDI-MS spectrum of G1FB in HCCA at 15% laser fluence; (**b**) QqTOF MALDI-MS spectrum of G1FB in HCCA at 20% laser fluence; (**c**) QqTOF MALDI-MS spectrum of G1FB in HCCA at 50% laser fluence.

cules with little fragmentation observed, similar to MALDI/TOF-MS spectra. With a "hot" matrix, such as HCCA, even at threshold laser fluence, both fragment ions and the protonated molecule are observed in the MALDI-QqTOF-MS spectra. With increased laser fluence, the relative abundance of higher mass fragments decreases, and the relative abundance of lower-mass fragments increases, suggesting that consecutive dissociation may be occurring (data not shown).

Pseudo-MS^3

On a QqTOF, MALDI-MS/MS experiments on an ion selected by Q1 can be performed. If the ion selected for MS/MS is a fragment ion instead of a precursor ion, MALDI pseudo-MS^3 experiments can be performed.

For the peptide G1FB, a series of y ions was produced in the MS/MS spectrum with HCCA (Figure 3a). MS/MS spectra of two of these y ions, y_6 and y_9 (*m/z* 684.339 and 1056.477, respectively), are shown in Figure 3b and c. The resulting pseudo-MS^3 spectra contained mainly b and y-NH_3 ions. Even with only 300 fmol spotted on the target, and only 100 shots acquired, pseudo-MS^3 spectra with a signal to noise ratio of 4:1 were obtained.

In conventional MALDI-TOF instruments, ions that fragment in the source before they are extracted into the TOF produce a series of z and c ions. In MALDI-QqTOF-MS at high laser fluence, however, these types of ions are not observed. Instead, y and b ion series are observed, which are characteristic of low energy CID [42]. When the QqTOF is used in the MALDI-MS mode, Q1 is set to transmit all ions. When the QqTOF is used in the MALDI-MS/MS mode, however, it is possible to set Q1 to transmit either a protonated molecule or a fragment ion. Hence these ions must be formed prior to Q1.

The design of the QSTAR incorporates an rf-only quadrupole (Q0), flushed with N_2 gas, which serves to collisionally cool the ions by reducing their kinetic energy to near thermal, and to focus the ion beam before it enters Q1 [40]. Typical operating pressures in Q0 (4-8 mTorr), however, are sufficient to cause CID in other instruments [43]. Hence, it is possible that the collisions with background nitrogen, which cool the ions, also cause some of the translational energy to be converted to internal energy, thus leading to CID.

Application of Pseudo-MS^3 to the Localization of a Phosphorylation Site

The utility of the QqTOF MALDI pseudo-MS^3 for obtaining sequence and phosphorylation-site information from a biological sample was demonstrated with a peptide isolated from an *in vitro* phosphorylated protein that contained an unknown phosphorylation site. Since the peptide was known to be phosphorylated,

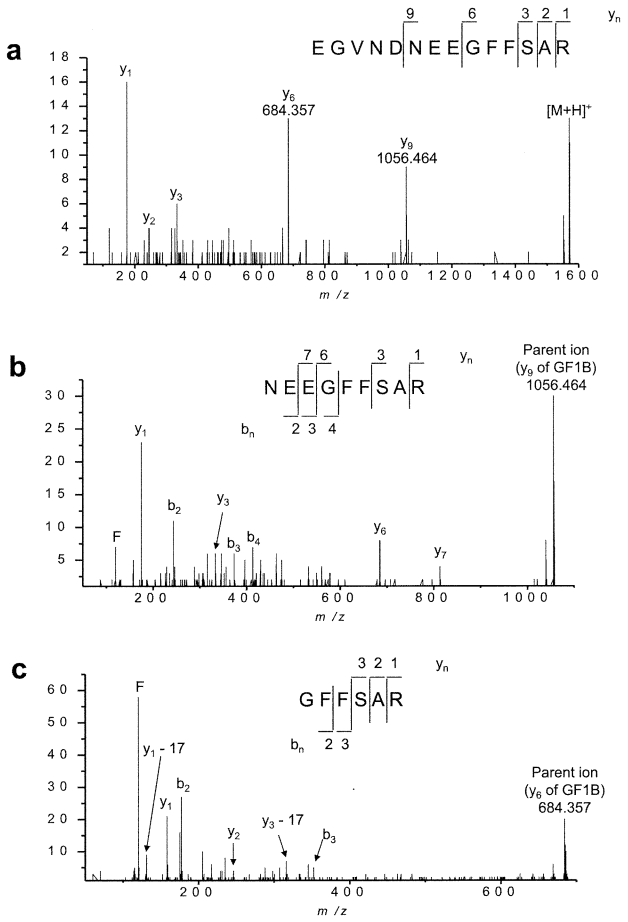

Figure 3. QqTOF MALDI mass spectra of G1FB in HCCA, 300 fmol on target. (**a**) MALDI-MS/MS spectrum of the protonated molecule of G1FB (*m/z* 1570.691) in HCCA at 40% laser fluence; (**b**) Pseudo-MS^3 of the y_9 product ion (*m/z* 1056.464) from G1FB in HCCA at 40% laser fluence. (**c**) Pseudo-MS^3 of the y_6 product ion (*m/z* 684.357) from G1FB in HCCA at 40% laser fluence.

DHB was used as the matrix because it does not promote loss of H_3PO_4 as readily as HCCA does [44].

At low laser fluence, a single enzymatic fragment, at *m/z* 1945.873 was observed. This enzymatic fragment was difficult to dissociate, and no peaks that localize the phosphorylation site to a single amino acid residue were observed. At higher laser fluence, increased fragmentation was observed (Figure 4a). Fragment ions, at *m/z* 1279.624 and 1586.810, were produced, along with losses of 98 Da from these fragment ions. The 98 Da loss from both the precursor and fragment ions confirmed the presence of a phosphoryl group.

MS/MS of the most abundant fragment ion, at *m/z* 1279.624, was performed. Loss of 98 Da from this precursor ion is a prominent product ion in the MALDI-MS/MS spectrum, indicating that the phosphoryl group is still present on the fragment ion (Figure 4b). A sequence-specific series of ions was observed. Seven residues of the peptide were unambiguously identified from the sequence ions. In addition, the mass difference between the ions at *m/z* 339.208 and *m/z* 408.227 was 69.019 Da, which corresponds to dehydroalanine, the residue remaining after β-elimination of a phosphoryl group from phosphoserine [45]. An internal fragment ion was observed which contained a non-phosphorylated tyrosine. Additionally, ions were not observed at *m/z* 216.043, which would correspond to the immonium ion of a phosphorylated tyrosine residue [46]. Thus, the serine residue is unambiguously identified as the only phosphorylation site in this peptide.

Conclusion

Using a MALDI-QqTOF in the MS mode, fragmentation can be increased with higher laser power at the expense of an increased rate of sample consumption. Fragmentation can also be increased in the MS mode by using a "hot" matrix, such as HCCA. In contrast to ISD, the fragment ions observed are predominantly sequence-specific y and b ions. These b and y ions can be isolated and subjected to a further stage of MS/MS, thus generating pseudo-MS^3 experiments. Using pseudo-MS^3, a phosphorylation site on a biological peptide, which showed poor dissociation by direct MS/MS, was successfully identified.

The MALDI pseudo-MS^3 experiments reported here are analogous to skimmer-induced dissociation-MS/MS experiments in electrospray ionization. Like skimmer-induced dissociation, this initial fragmentation step is non-selective in that all ions generated in the source can undergo fragmentation. This additional fragmentation step should, however, prove useful for obtaining an additional level of sequence information from previously separated peptides or for simple mixtures. MALDI-QqTOF-MS/MS on the $[M + H - 98]^+$ ion from a phosphorylated peptide has recently been demonstrated for determining the phosphorylation site at the picomole level [47, 48]. Because these experiments were done in DHB, not HCCA, and because the loss of H_3PO_4 from a phosphorylated peptide can occur in the source of a conventional MALDI-TOF, it cannot be determined whether the reported spectra were actually produced by MS/MS or pseudo-MS^3. Our results on both phosphorylated and unphosphorylated peptides demonstrate that the pseudo-MS^3 technique is of general applicability for obtaining sequence information from b and y ions, even when only low levels of sample are available, such as those encountered in proteomic studies.

Using this technique, it is possible to obtain sequence information in those instances where a single cleavage from the protonated molecule dominates the MS/MS spectrum. This is accomplished while maintaining the high mass accuracy necessary for database searching. This technique should also be applicable to other cases where MS/MS does not provide sufficient sequence and structural information, such as glycopeptides, cross-linked peptides, and covalently bound peptide-ligand complexes.

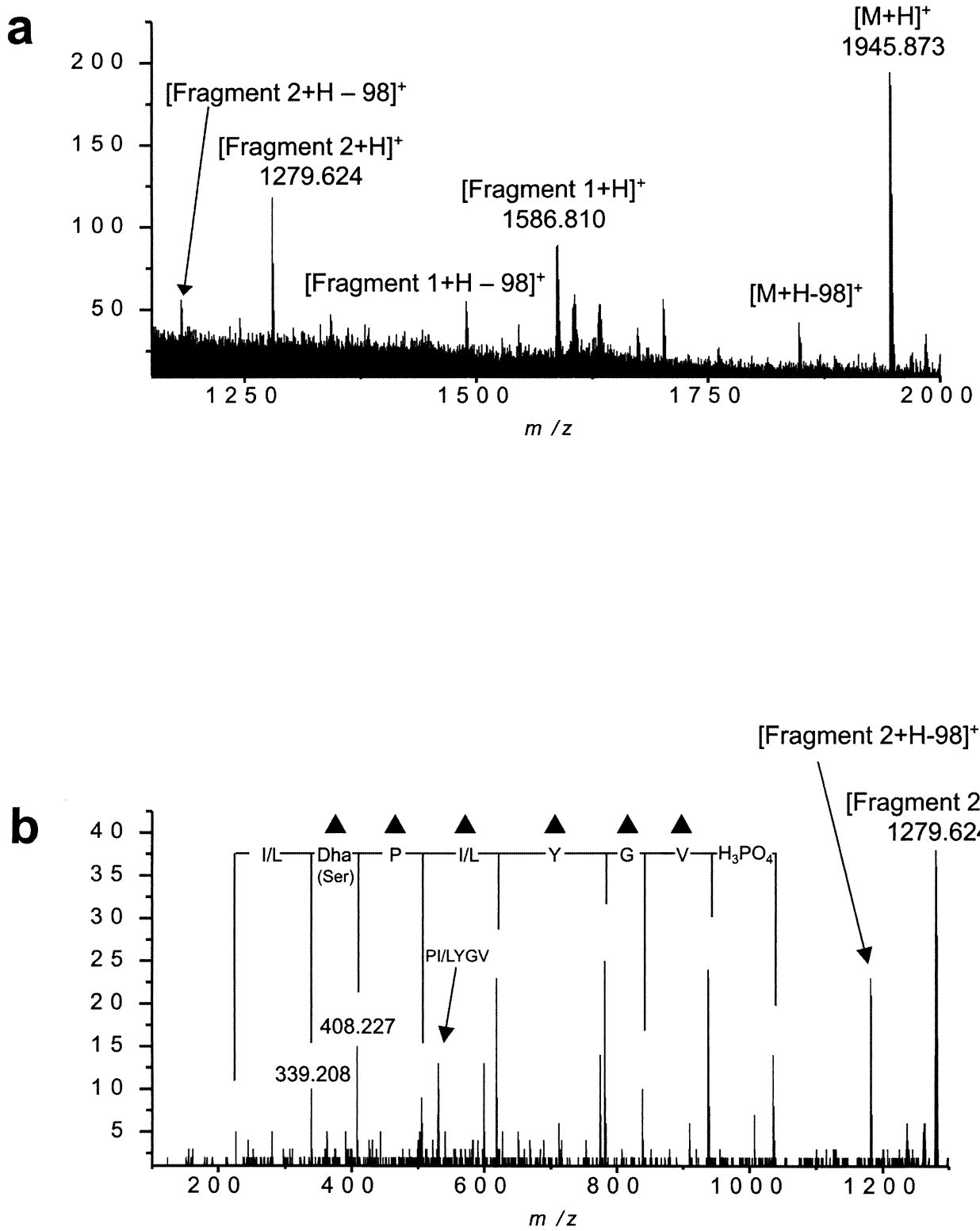

Figure 4. QqTOF MALDI mass spectra of a phosphopeptide in DHB. (**a**) MS of a phosphopeptide, with fragment ions shown. (**b**) Pseudo-MS^3 of fragment 2 (*m/z* 1279.624) selected from Figure 4(**a**), localizing the phosphorylation site. Product ions indicated with a filled triangle formerly contained the phosphoryl group.

References

1. Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F. Matrix-Assisted Ultraviolet Laser Desorption of Non-Volatile Compounds. *Int. J. Mass Spectrom. Ion Processes* **1987,** *78,* 53–68.
2. Hillenkamp, F.; Karas, M. Mass Spectrometry of Peptides and Proteins by Matrix-Assisted Ultraviolet Laser Desorption/Ionization. *Methods Enzymol.* **1990,** *193,* 280–295.
3. Hillenkamp, F.; Karas, M.; Beavis, R. C.; Chait, B. T. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Biopolymers. *Anal. Chem.* **1991,** *63,* 1193A–1203A.
4. Strupat, K.; Karas, M.; Hillenkamp, F. 2,5-Dihydroxybenzoic Acid: A New Matrix for Laser Desorption-Ionization Mass Spectrometry. *Int. J. Mass Spectrom. Ion Processes* **1991,** *111,* 89–102.
5. Chan, T. W. D.; Colburn, A. W.; Derrick, P. J. Matrix-Assisted Laser Desorption/Ionization Using a Liquid Matrix: Formation of High-Mass Cluster Ions From Proteins. *Org. Mass Spectrom.* **1992,** *27,* 53–56.
6. Beavis, R. C.; Chait, B. T. Matrix-Assisted Laser-Desorption Mass Spectrometry Using 355 nm Radiation. *Rapid Commun. Mass Spectrom.* **1989,** *3,* 436–439.
7. Beavis, R. C.; Chait, B. T. Factors Affecting the Ultraviolet Laser Desorption of Proteins. *Rapid Commun. Mass Spectrom.* **1989,** *3,* 233–237.
8. Zhao, S.; Somayajula, K. V.; Sharkey, A. G.; Hercules, D. M.; Hillenkamp, F.; Karas, M.; Ingendoh, A. Novel Method for Matrix-Assisted Laser Mass Spectrometry of Proteins. *Anal. Chem.* **1991,** *63,* 450–453.
9. Beavis, R. C.; Chaudhary, T.; Chait, B. T. a-Cyano-4-Hydroxycinnamic Acid as a Matrix for Matrix-Assisted Laser Desorption Mass Spectrometry. *Org. Mass Spectrom.* **1992,** *27,* 156–158.
10. Scott, C. T. J.; Kosmidis, C.; Jia, W. J.; Ledingham, K. W. D.; Singhal, R. P. Investigations of desorbed species from matrix materials used in MALDI. *AIP Conf. Proc.* **1995,** *329,* 64–67.
11. Kosaka, T.; Kinoshita, T.; Takayama, M. Ion Formation and Fragmentation of Sinapinic Acid in Electron Ionization, Liquid Secondary Ion and Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **1996,** *10,* 405–408.
12. Kinsel, G. R.; Edmondson, R. D.; Russell, D. H. Profile and Flight Time Analysis of Bovine Insulin Clusters as a Probe of Matrix-Assisted Laser Desorption/Ionization Ion Formation Dynamics. *J. Mass Spectrom.* **1997,** *32,* 714–722.
13. Lehmann, E.; Knochenmuss, R.; Zenobi, R. Ionization Mechanisms in Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry: Contribution of Pre-formed Ions. *Rapid Commun. Mass Spectrom.* **1997,** *11,* 1483–1492.
14. Tang, W.; Nelson, C. M.; Zhu, L.; Smith, L. M. Positive Ion Formation in the Ultraviolet Matrix-Assisted Laser Desorption/Ionization Analysis of Oligonucleotides by Using 2,5-Dihydroxybenzoic Acid. *J. Am. Soc. Mass Spectrom.* **1997,** *8,* 218–224.
15. Huang, Y.; Russell, D. H. Photochemistry and Proton Transfer Reaction Chemistry of Selected Cinnamic Acid Derivatives in Hydrogen Bonded Environments. *Int. J. Mass Spectrom. Ion Processes* **1998,** *175,* 187–204.
16. Knochenmuss, R.; Karbach, V.; Wiesli, U.; Breuker, K.; Zenobi, R. The Matrix Suppression Effect in Matrix-Assisted Laser Desorption/Ionization: Application to Negative Ions and Further Characteristics. *Rapid Commun. Mass Spectrom.* **1998,** *12,* 529–534.
17. Land, C. M.; Kinsel, G. R. Investigation of the Mechanism of Intracluster Proton Transfer from Sinapinic Acid to Biomolecular Analytes. *J. Am. Soc. Mass Spectrom.* **1998,** *9,* 1060–1067.
18. Lehmann, E.; Zenobi, R. Investigations of the Cationization Mechanism in Matrix-Assisted Laser Desorption/Ionization. *Adv. Mass Spectrom.* **1998,** *14,* 1–11.
19. Zenobi, R.; Knochenmuss, R. Ion Formation in MALDI Mass Spectrometry. *Mass Spec. Reviews* **1998,** *17,* 337–366.
20. Breuker, K.; Knochenmuss, R.; Zenobi, R. Proton Transfer Reactions of Matrix-Assisted Laser Desorption/Ionization Matrix Monomers and Dimers. *J. Am. Soc. Mass Spectrom.* **1999,** *10,* 1111–1123.
21. Breuker, K.; Knochenmuss, R.; Zenobi, R. Gas-Phase Basicities of Deprotonated Matrix-Assisted Laser Desorption/Ionization Matrix Molecules. *Int. J. Mass Spectrom.* **1999,** *184,* 25–38.
22. Kinsel, G. R.; Gimon-Kinsel, M. E.; Gillig, K. J.; Russell, D. H. Investigation of the Dynamics of Matrix-Assisted Laser Desorption/ionization Ion Formation Using an Electrostatic Analyzer/Time-of-Flight Mass Spectrometer. *J. Mass Spectrom.* **1999,** *34,* 684–690.
23. Stevenson, E.; Breuker, K.; Zenobi, R. Internal Energies of Analyte Ions from Different Matrix-Assisted Laser Desorption/Ionization Matrices. *J. Mass Spectrom.* **2000,** *35,* 1035–1041.
24. Gross, J.; Hillenkamp, F.; Wan, K. X.; Gross, M. L. Metastable Decay of Negatively Charged Oligodeoxynucleotides Analyzed with Ultraviolet Matrix-Assisted Laser Desorption/Ionization Post-Source Decay and Deuterium Exchange. *J. Am. Soc. Mass Spectrom.* **2001,** *12,* 180–192.
25. Brown, R. S.; Lennon, J. J. Mass Resolution Improvement by Incorporation of Pulsed Ion Extraction in a Matrix-Assisted Laser Desorption/Ionization Linear Time-of-Flight Mass Spectrometer. *Anal. Chem.* **1995,** *67,* 1998–2003.
26. Brown, R. S.; Carr, B. L.; Lennon, J. J. Factors that Influence the Observed Fast Fragmentation of Peptides in Matrix-Assisted Laser Desorption. *J. Am. Soc. Mass Spectrom.* **1996,** *7,* 225–232.
27. Spengler, B.; Kirsch, D.; Kaufmann, R. Fundamental Aspects of Postsource Decay in Matrix-Assisted Laser Desorption Mass Spectrometry. 1. Residual Gas Effects. *J. Phys. Chem.* **1992,** *96,* 9678–9684.
28. Karas, M.; Bahr, U.; Strupat, K.; Hillenkamp, F.; Tsarbopoulos, A.; Pramanik, B. N. Matrix Dependence of Metastable Fragmentation of Glycoproteins in MALDI TOF Mass Spectrometry. *Anal. Chem.* **1995,** *67,* 675–679.
29. Claereboudt, J.; Claeys, M.; Geise, H.; Gijbels, R.; Vertes, A. Laser Microprobe Mass Spectrometry of Quaternary Phosphonium Salts: Direct Versus Matrix-Assisted Laser Desorption. *J. Am. Soc. Mass Spectrom.* **1993,** *4,* 798–812.
30. Kinsel, G. R.; Preston, L. M.; Russell, D. H. Fragmentation of Vitamin B12 During 337 nm Matrix-Assisted Laser Desorption Ionization. *Biol. Mass Spectrom.* **1994,** *23,* 205–211.
31. Gluckmann, M.; Karas, M. The Initial Ion Velocity and Its Dependence on Matrix, Analyte, and Preparation Method in Ultraviolet Matrix-Assisted Laser Desorption/Ionization. *J. Mass Spectrom.* **1999,** *34,* 467–477.
32. Thierolf, M.; Bahr, U.; Karas, M. *Proceedings of the 45th ASMS Conference on Mass Spectrometry*, Palm Springs, CA, June, 1997, p 856.
33. Brown, R. S.; Feng, J.; Reiber, D. C. Further Studies of In-Source Fragmentation of Peptides in Matrix-Assisted Laser Desorption-Ionization. *Int. J. Mass Spectrom. Ion Processes* **1997,** *169/170,* 1–18.
34. Schlüter, H.; Mentrup, D.; Gross, I.; Meyer, H. E.; Spengler, B.; Kaufmann, R.; Zidek, W. Intification of Endogenous des-arg9-[hyp3]-bradykinin in Human Plasma with Post-Source-Decay Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *Anal. Biochem.* **1997,** *246,* 15–19.
35. Spengler, B. Post-Source Decay Analysis in Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Biomolecules. *J. Mass Spectrom.* **1997,** *32,* 1019–1036.
36. Reiber, D. C.; Brown, R. S.; Weinberger, S.; Kenny, J.; Bailey, J. Unknown Peptide Sequencing Using Matrix-Assisted Laser

Desorption/Ionization and In-Source Decay. *Anal. Chem.* **1998,** *70,* 1214–1222.
37. Szilágyi, A.; Varney, J. E.; Derrick, P. J.; K., V. Dependence of Matrix-Assisted Laser Desorption/Ionization Post-Source Decay Spectra on Laser Power. *Rapid Commun. Mass Spectrom.* **1998,** *12,* 489–492.
38. Chaurand, P.; Lützenkirchen, F.; Spengler, B. Peptide and Protein Identification by Matrix-Assisted Laser Desorption Ionization (MALDI) and MALDI-Post-Source Decay Time-of-Flight Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **1999,** *10,* 91–103.
39. Lavine, G.; Allison, J. Evaluation of Bumetanide as a Matrix for Prompt Fragmentation Matrix-Assisted Laser Desorption/Ionization and Demonstration of Prompt Fragmentation/Post-Source Decay Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *J. Mass Spectrom.* **1999,** *34,* 741–748.
40. Loboda, A. V.; Krutchinsky, A. N.; Bromirski, M.; Ens, W.; Standing, K. G. A Tandem Quadrupole/Time-of-Flight Mass Spectrometer with a Matrix-Assisted Laser Desorption/Ionization Source: Design and Performance. *Rapid Comm. Mass Spectrom.* **2000,** *14,* 1047–1057.
41. Affolter, M.; Watts, J. D.; Krebs, D. L.; Abersold, R. Evaluation of Two-Dimensional Phosphopeptide Maps by Electrospray Ionization Mass Spectrometry of Recovered Peptides. *Anal. Biochem.* **1994,** *223,* 74–81.
42. Wu, Z.; Fenselau, C. Collision-Induced Ionization of Neutral Peptides. *Org. Mass Spectrom.* **1993,** *28,* 1035–1040.
43. Haller, I.; Mirza, U. A.; Chait, B. T. Collision Induced Decomposition of Peptides. Choice of Collision Parameters. *J. Am. Soc. Mass Spectrom.* **1996,** *7,* 677–681.
44. Larsen, M. R.; Sorenson, G. L.; Fey, S. J.; Larsen, P. M.; Roepstorff, P. Phospho-Proteomics: Evaluation of the Use of Enzymatic Dephosphorylation and Differential Mass Spectrometric Peptide Mass Mapping for Site Specific Phosphorylation Assignment in Proteins Separated by Gel Electrophoresis. *Proteomics* **2001,** *1,* 223–238.
45. Tholey, A.; Reed, J.; Lehmann, W. D. Electrospray Tandem Mass Spectrometric Studies of Phosphopeptides and Phosphopeptide Analogues. *J. Mass Spectrom.* **1999,** *34,* 117–123.
46. Steen, H.; Kuster, B.; Mann, M. Quadrupole Time-of-Flight Versus Triple-Quadrupole Mass Spectrometry for the Determination of Phosphopeptides by Precursor Ion Scanning. *J. Mass Spectrom.* **2001,** *36,* 782–790.
47. Baldwin, M. A.; Medzihradszky, K. F.; Lock, C. M.; Fisher, B.; Settineri, T. A.; Burlingame, A. L. Matrix-Assisted Laser Desorption/Ionization Coupled with Quadrupole/Orthogonal Acceleration Time-of-Flight Mass Spectrometry for Protein Discovery, Identification, and Structural Analysis. *Anal. Chem.* **2001,** *73,* 1707–1720.
48. Lee, C. H.; McComb, M. E.; Bromirski, M.; Jilkine, A.; Ens, W.; Standing, K. G.; Perreault, H. On-Membrane Digestion of β-casein for Determination of Phosphorylation Sites by Matrix-Assisted Laser Desorption/Ionization Quadrupole/Time-of-Flight Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2001,** *15,* 191–202.