Anal. Chem. **2000,** *72,* 552−558

The Characteristics of Peptide Collision-Induced Dissociation Using a High-Performance MALDI-TOF/TOF Tandem Mass Spectrometer

Katalin F. Medzihradszky,[†,‡,#] Jennifer M. Campbell,[§] Michael A. Baldwin,[†,‡,||,⊥] Arnold M. Falick,[§] Peter Juhasz,[§] Marvin L. Vestal,[§] and Alma L. Burlingame*[,†,‡,#]

Mass Spectrometry Facility, Department of Pharmaceutical Chemistry, Department of Neurology, Institute for Neurological Diseases, and the Liver Center, University of California, San Francisco, California 94143, and PE Biosystems, 500 Old Connecticut Path, Framingham, Massachusetts 01701

A new matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometer is described for sequencing peptides. This instrument combines the advantages of high sensitivity for peptide analysis associated with MALDI and comprehensive fragmentation information provided by high-energy collision-induced dissociation (CID). Unlike the postsource decay technique that is widely used with MALDI-TOF instruments and typically combines as many as 10 separate spectra of different mass regions, this instrument allows complete fragment ion spectra to be obtained in a single acquisition at a fixed reflectron voltage. To achieve optimum resolution and focusing over the whole mass range, it may be desirable to acquire and combine three separate sections. Different combinations of MALDI matrix and collision gas determine the amount of internal energy deposited by the MALDI process and the CID process, which provide control over the extent and nature of the fragment ions observed. Examples of peptide sequencing are presented that identify sequence-dependent features and demonstrate the value of modifying the ionization and collision conditions to optimize the spectral information.

During the 1980s, the discoveries of several soft ionization techniques provided the technical basis that allowed mass spectrometric detection and analysis of free, chemically polar, labile biopolymers and their degradation products for the first time. Because of the relatively low deposition of internal energy during these ion formation processes, these soft ionization techniques yield primarily direct measures of the molecular weight of biomolecules in the form of singly or multiply protonated or deprotonated pseudomolecular ions. During sputtering and ionization from viscous liquids (FAB/LSIMS), mobile liquids (electrospray), and solids (matrix-assisted laser desorption/ionization, MALDI), varying degrees of peptide fragmentation may be observed. However, optimization of fragmentation processes to provide comprehensive sequence and structural characterization requires vibronic activation of molecular ions in order to induce the extensive unimolecular bond dissociation desired. Of these three ionization techniques, MALDI usually deposits more internal energy than the other two methods through the use of "hot" matrixes. The actual degree of unimolecular decomposition thus induced, called postsource decay (PSD), varies with amino acid sequence in unpredictable ways and often yields mass spectra that provide only a partial amino acid sequence of the peptide being analyzed. Conversely, MALDI-generated molecular ions from "cold" matrixes can yield comprehensive sequence information from high-energy collisional activation, as established previously in this laboratory using EBE-oaTOF instrumentation.[1,2] In addition, it has been observed that when air is introduced into the collision cell of a MALDI reflectron-type instrument, PSD spectra derived using "cold" matrixes may be augmented with more abundant immonium ions and fragment ions arising from high-energy fragmentation processes.[3]

The most commonly available tandem mass spectrometers in current use employ quadrupoles or ion traps for the initial mass analysis, which are optimized for use with ions of low kinetic energies, i.e., <100 eV. Multiple collisions of such ions with neutral gas atoms or molecules can induce extensive fragmentation, but the resulting MS/MS spectra are dominated by ions formed by breaking the weakest bonds and by low-energy rearrangement pathways, whereas they are devoid of "high-energy" fragments that provide more complete structural information. By contrast, time-of-flight (TOF) analyzers are optimized for transmission of ions of high kinetic energies, typically up to 20 keV. Collisions of ions of even 1 keV give the high-energy fragments lacking in quadrupoles and ion traps. Therefore, there have been a number of instruments that have employed TOF analyzers in tandem mass spectrometers, sometimes as hybrids combined with sectors,[1,2,4−6] but often as TOF/TOF combinations.

† Mass Spectrometry Facility, University of California, San Francisco.
‡ Department of Pharmaceutical Chemistry, University of California, San Francisco.
§ PE Biosystems.
|| Department of Neurology, University of California, San Francisco.
⊥ Institute for Neurological Diseases, University of California, San Francisco.
Liver Center, University of California, San Francisco.

(1) Medzihradszky, K. F.; Adams, G. W.; Bateman, R. H.; Green, M. R.; Burlingame, A. L. *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 1−10.
(2) Medzihradszky, K. F.; Maltby, D. A.; Qiu, Y.; Yu, Z.; Hall, S. C.; Chen, Y.; Burlingame, A. L. *Int. J. Mass Spectrom. Ion Processes* **1997**, *160*, 357−369.
(3) Stimson, E.; Truong, O.; Richter, W. J.; Waterfield, M. D.; Burlingame, A. L. *Int. J. Mass Spectrom Ion Processes* **1997**, *169/170*, 231−240.

10.1021/ac990809y CCC: $19.00
Published on Web 12/22/1999

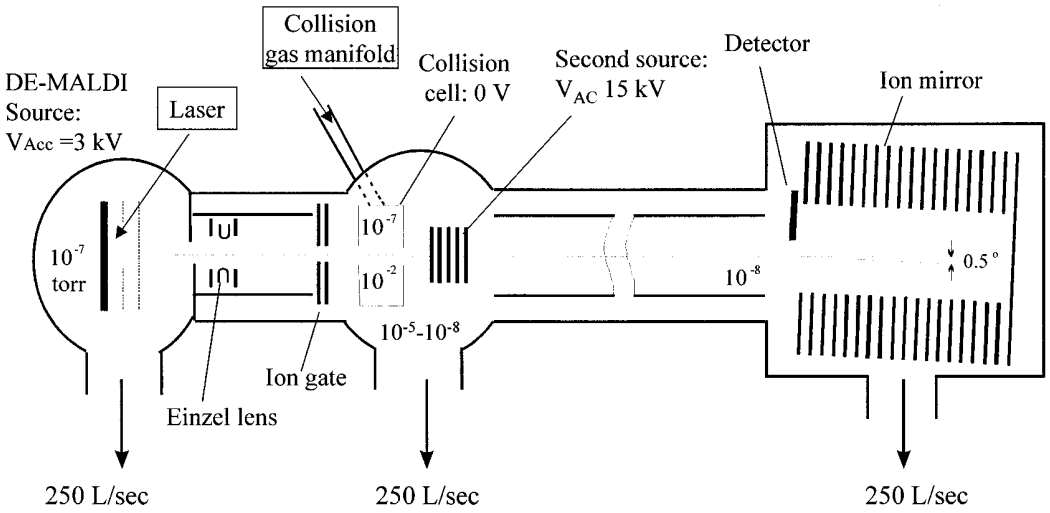

Figure 1. MALDI-TOF/TOF instrument (not to scale). The collision cell gas pressure is not measured directly but it is believed to increase from a background value of $<1 \times 10^{-7}$ Torr in the absence of gas to approximately 1×10^{-2} Torr when gas is introduced.

Schey et al. described a TOF/TOF instrument that was used to monitor fragment ions produced by ion/surface collisions, although the collision energy was limited to 750 eV. The second of the two orthogonal analyzers incorporated a reflectron, but the mass resolution was relatively low.[7] A TOF/TOF described by Boesl et al. focused the precursor ions to a second-order space focus point where they could be excited by laser radiation, the fragment ions then being mass analyzed in a reflectron TOF.[8] This instrument, which employed postacceleration of the fragment ions to reduce their energy spread, was used to explore laser ionization of a molecular beam, laser desorption of solid samples from a probe, and laser-induced fragmentation. Another TOF/TOF instrument that employed an excimer laser to obtain photodissociation achieved unit mass resolution for both precursor and fragment ions by using dual-stage reflectron analyzers.[9] Cornish and Cotter described an alternative dual-stage reflectron TOF/TOF equipped with a collision cell for collision-induced dissociation (CID).[10] This instrument employed a nonlinear or curved field reflectron that allowed focusing of fragment ions from PSD of peptides at a single reflectron voltage.[11] A similar strategy was employed in the design of a hybrid sector-TOF.[6]

In this paper, we describe a new TOF/TOF tandem mass spectrometer that permits improved precursor ion selection in comparison to conventional ion gates employed for PSD precursor ion selection, as well as collisional activation of the selected ion and recording of full fragment spectra at a single mirror voltage. In addition, we report observations of changes in peptide fragmentation patterns depending on the presence or absence of an Arg residue and the particular combination of matrix and collision gas.

EXPERIMENTAL SECTION

The TOF/TOF instrument shown in Figure 1 is based on the footprint of the standard Voyager DE STR mass spectrometer (PE Biosystems, Framingham, MA), with a total distance between the source plate (target) and the linear detector of 1.6 m. Adaptation of the standard instrument for MS/MS required a number of modifications and additions to the ion optics. However, in addition to recording MS/MS spectra, this instrument can still be operated in all conventional modes of a high-quality MALDI TOF mass spectrometer: i.e., linear, reflectron, and PSD. All MALDI spectra reported here were obtained with a nitrogen laser operating at a wavelength of 337.1 nm and at a frequency of 10 Hz rather than the standard 3 Hz. The components of the instrument can be divided into three sections based on their functionality: ion selection, collision region, and fragment detection.

The first mass analyzer, used for ion selection, is a dual-stage Wiley McLaren TOF mass spectrometer of length 26 cm, defined by the distance between the target plate and the center of the timed ion selector (TIS) described below, a higher resolution alternative to the Bradbury−Nielsen gate,[12] which is commonly used for precursor ion isolation. The operating parameters such as voltages and delayed extraction time are chosen such that the space focus plane of the accelerated ion beam is located at the TIS; thus, selection resolution should not be degraded by beam dispersion. This configuration eliminates the problem that ion beam dispersion inherently limits TOF-based ion isolation techniques. To achieve higher resolutions with the relatively short TOF geometry, the separation between the target plate surface and the grid has been increased from the standard 3 to 5 mm. The distance between the middle and final acceleration grids is 15 mm.

The energy of the ions in the first TOF defines the nature of the collisions between the precursor ions and the neutral gas in the collision cell. This instrument was designed for high-energy

(4) Strobel, F. H.; Solouki, T.; White, M. A.; Russell, D. H. *J. Am. Soc. Mass Spectrom.* **1990**, *2*, 91.
(5) Strobel, F. H.; Preston, L. M.; Washburn, K. S.; Russell, D. H. *Anal. Chem.* **1992**, *64*, 754.
(6) Davis, S. C.; Evans, S. European Patent, EP0551999, 1993.
(7) Schey, K. L.; Cooks, R. G.; Kraft, A.; Grix, R.; Wollnik, H. *Int. J. Mass Spectrom Ion Processes* **1989**, *94*, 1.
(8) Boesl, U.; Weinkauf, R.; Schlag, E. W. *Int. J. Mass Spectrom Ion Processes* **1992**, *112*, 121.
(9) Seeterlin, M. A.; Vlasak, P. R.; Beussman, D. J.; McLane, R. D.; Enke, C. G. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 751.
(10) Cornish, T. J.; Cotter, R. J. *Anal. Chem.* **1993**, *65*, 1043.
(11) Cordero, M. M.; Cornish, T. J.; Lys, I. A.; Cotter, R. J. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1356−1361.
(12) Bradbury, N. E.; Nielsen, R. A. *Phys. Rev.* **1936**, *49*, 388.

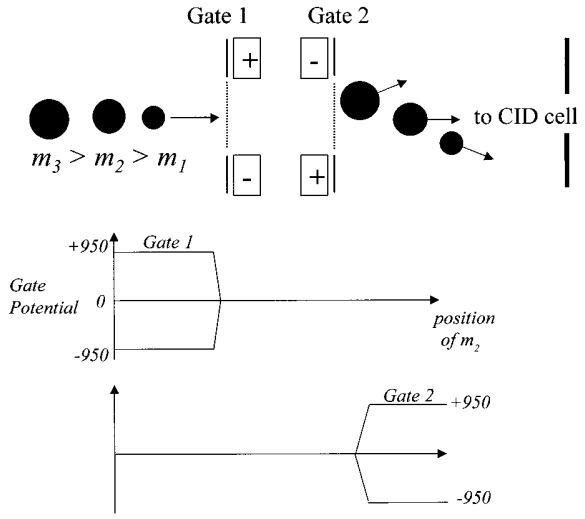

Figure 2. Schematic of the timed ion selector, showing the deflection of light ions (m_1) by gate 1 and heavy ions (m_3) by gate 2. Only ions of intermediate mass (m_2) are allowed to pass into the collision cell. Each gate is "open" when both electrodes are grounded and is "closed" by applying ±950 V.

collisions, i.e., in the kiloelectronvolt regime. All data presented here were recorded with 3 keV acceleration energy in the first TOF. As this is relatively low for MALDI, an einzel lens was added immediately after the TOF acceleration region to achieve high transmission efficiency for the lower energy ions from the ion source to the collision cell. For 3 keV acceleration energy, the voltage employed on the einzel lens was 1.4 kV. Further optimization of the collision conditions and determination of the nature of the collision dynamics is ongoing.

The TIS, which has been described previously,[13,14] is constructed from two tandem deflection gates (Figure 2). Gate 1 acts as a low-mass filter and is normally maintained in the "closed" state with ±950 V applied to the two electrodes, resulting in the deflection of ion trajectories through an angle sufficient to prevent transmission to the second source. Gate 2, which is separated from gate 1 by 6.7 mm, acts as a high-mass filter and is normally maintained in the "open" state with both electrodes at ground potential. The synchronization of the opening and closing of these deflection gates is under software control. At the calculated time of flight of the selected ion to gate 1, its voltage is rapidly switched from closed to open (0 V), allowing only ions with the selected mass and higher masses to be transmitted toward gate 2 without deflection. At the calculated time of arrival of the selected ion at gate 2, this is closed rapidly by applying ±950 V, deflecting the trajectories of all subsequent ions, i.e., those with masses higher than the selected ion. Therefore, the only ions that enter the collision cell must pass through the two gates within the short period between the opening of gate 1 and the closing of gate 2. Another high-resolution, dual-gate TIS has been described by Piyasada et al.[15]

The second section of the TOF/TOF is the collision cell region, including the collision cell itself and the ion optics necessary to transfer ions between mass analyzers. As the mechanism of the ion gate is such that nontargeted ions have trajectories deflected from the normal flight path, a separation of the gate and the cell is necessary to maximize the resolution of precursor selection; thus, the collision cell is located 8 cm behind the ion gate. The collision cell is 7.2 cm long and electrically connected to the flight tube and thus is maintained at ground potential. The collision gas is bled in through an adjustable leak valve, and the pressure is maintained via differential pumping through $^1/_8$ in. apertures at either end of the cell. No direct measure of the pressure in the collision cell is provided, but the amount of gas added is inferred from a combination of the increase in pressure measured by the ionization gauge in the source region of the first TOF and the CID spectrum obtained. For standard MS/MS operation, the indicated source pressure increases from 2.0×10^{-7} Torr (no collision gas) to approximately 1.0×10^{-6} Torr. Collision gases that have been tested to date include air, N_2, CH_4, He, Ar, and Xe. The collision cell is equipped with a guide wire maintained at a small negative potential of about −1 V to ensure high transmission of low-energy fragment ions.

The third section of the TOF/TOF is a second, pulsed TOF mass analyzer, which provides high-resolution spectra of the fragment ions. This mass spectrometer has a 3.75 cm long, single-stage acceleration region (see "Second Source" in Figure 1), a field free distance of 96.6 cm, and a 39.2 cm long single-stage ion mirror. The second source is connected to a rapid high-voltage power switch and is pulsed from ground to the accelerating voltage, typically 15 kV. Only ions that are in the volume of the second source at the time the pulse is applied will be accelerated toward the ion mirror. Hence, software is used to calculate the flight time of the precursor ion to the center of the second source. Fragment ions formed in the collision cell have the same velocities, and thus the same flight times to the second source as the selected precursor ion, and are similarly accelerated toward the ion mirror. The total energy of an intact precursor ion entering the reflectron is the sum of the energies gained in the two TOF accelerating pulses, which for the typical operating conditions and a singly charged ion is 18 keV. An ion guide running through the center of the field free region is usually maintained at −10 V, thereby improving transmission but significantly degrading the attainable resolution.

The two main factors affecting fragment ion resolution are the ion mirror voltage, which is inherent to any reflectron instrument, and the timing of the accelerating pulse to the second TOF, which is specific to the TOF/TOF. For an ion with 18 keV total acceleration energy, the mirror voltage that provides optimal precursor ion resolution is 28.8 kV. This large difference between the mirror potential and the ion energy indicates that the ions are only traveling approximately two-thirds of the depth of the reflector. As previously discussed, the TOF/TOF footprint is identical to that of a single MS instrument, and thus the mirror provides ideal focusing for ions accelerated from the first source region through a field free distance of 1.35 m. However, for the TOF/TOF data, the spectral quality reflects the focusing of the ions in TOF2, which has a field free distance of 96.6 cm. To maintain focused conditions for the shorter drift length, the total

(13) Vestal, M. L.; Juhasz, P.; Hines, W.; Martin, S. A. *Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics*, Orlando, Fl, 1998; p 35.

(14) Vestal, M. L.; Juhasz, P.; Hines, W.; Martin, S. A. In *Mass Spectrometry in Biology and Medicine*; Burlingame, A. L., Carr, S. A., Baldwin, M. A., Eds.; Humana Press: Totowa, NJ, 2000; pp 1−16.

(15) Piyasada, C. K. G.; Hakansson, P.; Ariyantane, T. R.; Barofsky, D. F. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1655−1664.

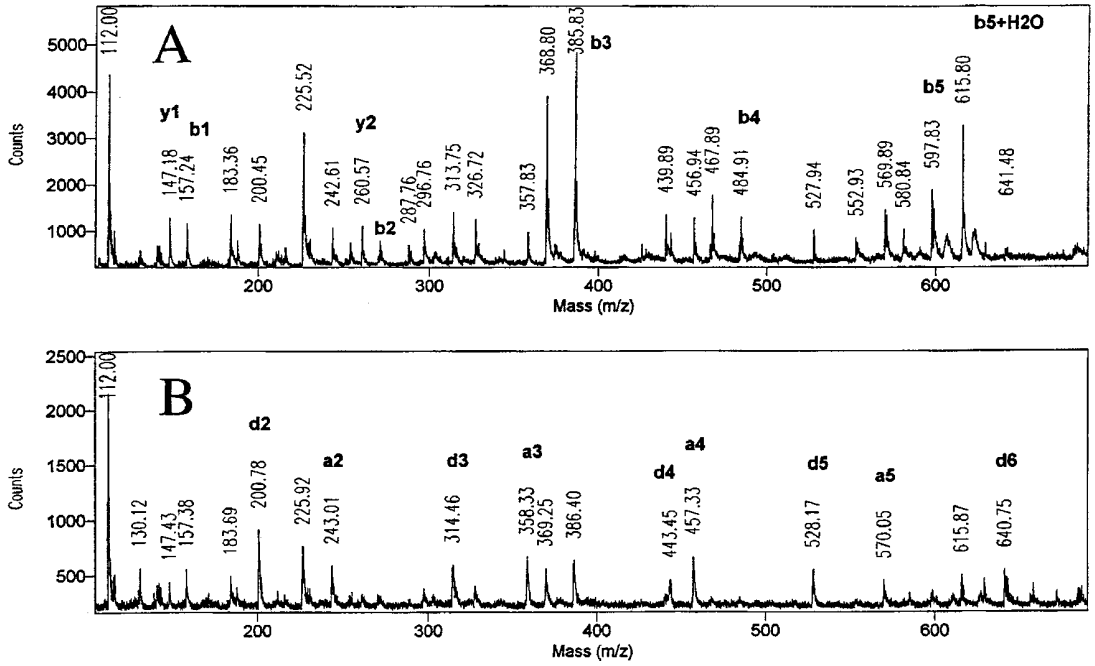

Figure 3. MALDI-TOF/TOF CID spectra of synthetic peptide Arg-Leu-Asp-Val-Leu-Gln (MH^+ at *m/z* 743.4). (A) Data acquired using CHCA as the matrix and He as the collision gas. (B) Data acquired using DHB as the matrix and Ar as the collision gas. The reflectron voltages during data acquisition were 27.0 and 28.8 kV, respectively.

distance ions travel in the mirror is lowered. When operated at this mirror voltage, the complete array of fragment ion masses can be recorded in a single spectrum. This ability to detect all fragments with the TOF/TOF at a single mirror voltage is a distinct advantage over PSD methods that are widely used for MS/MS in conventional MALDI-TOF mass spectrometers, although this problem is avoided by use of a curved field reflectron.[6,11] However, the correlation between fragment ion resolution and mass, while minimized relative to conventional PSD, is not completely eliminated. Consequently the fragment spectra may be "stitched" from those recorded at three mirror voltages: typically, 28.8, 25.9, and 24 kV. The use of three voltages ensures that all fragment ions are observed at high resolution and that low-intensity ions are rendered statistically discernible. Typical resolving power for the precursor ion is on the order of 5000 (fwhm) whereas fragment ions generally display a resolution of 2−3000. By optimizing the mirror voltage for the mass of an ion of interest, resolution of 4−5000 is achievable for fragment ions. The timing of the accelerating pulse to the second source also has a critical effect on fragment ion resolution. Typically, the pulse is applied when the ions of the target mass are in the center of the second source. Software calculates the optimum timing of the second TOF pulse for a given ion mass and accelerating voltage in the first TOF, based on the calculated distance traveled at the time of application of the pulse. A small degree of further optimization may be necessary to achieve the best sensitivity and resolution for each ion.

All spectra presented in this paper represent data acquired at a single mirror potential, as specified for each spectrum in the figure legend, without any requirement for spectrum "stitching". The calibration software is not yet fully developed, and all spectra were internally calibrated using the precursor ion and one of the identified immonium ions as calibration points. Correction was made for the initial velocity values in the calibration routine worked out for the Voyager DE STR.

All peptides were premixed with matrix, using 1 μL of sample solution and 1 μL of matrix solution, for deposition on the MALDI target plate and air-drying. α-Cyano-4-hydroxycinnamic acid (CHCA) solution was purchased from Hewlett-Packard. 2,5-Dihydroxybenzoic acid (DHB; Aldrich) was employed as a solution in 30% acetonitrile, 0.1% TFA. A two-component matrix was prepared containing a mixture of 2,6-dihydroxyacetophenone and diammonium hydrogen citrate (DHAP/DAHC) as described previously.[16]

RESULTS AND DISCUSSION

Combinations of different matrixes and collision gases were evaluated in an effort to optimize the quality of CID spectra. Matrixes tested were CHCA, DHB, and the two-component matrix. CHCA is the most commonly used matrix for peptide analysis, partly because it is relatively "hot" and induces unimolecular decomposition.[3,11,17] DHB is a "cooler" matrix[18] and is also suitable for the analysis of most peptides; higher molecular weight species usually yield more abundant signals from this matrix than from CHCA. The two-component matrix, DHAP/DAHC, is the "coolest" matrix currently known and its use has been recommended for species that readily decompose upon ionization, such as phospho- and sulfopeptides and glycopeptides with neuraminic acid.[16] Collision gases tested were He, Ar, air, and Xe.

The behavior of Arg-containing peptides was compared with that of peptides devoid of this basic residue. A series of peptides

(16) Gorman, J. J.; Ferguson, B. L.; Nguyen, T. B. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 529−536.
(17) Courchesne, P. L.; Luethy, R.; Patterson, S. D. *Electrophoresis* **1997**, *18*, 369−381.
(18) Karas, M.; Bahr, U.; Strupat, K.; Hillenkampf, F.; Tsarbopoulos, A.; Pramanik, B. N. *Anal. Chem.* **1995**, *67*, 675−679.

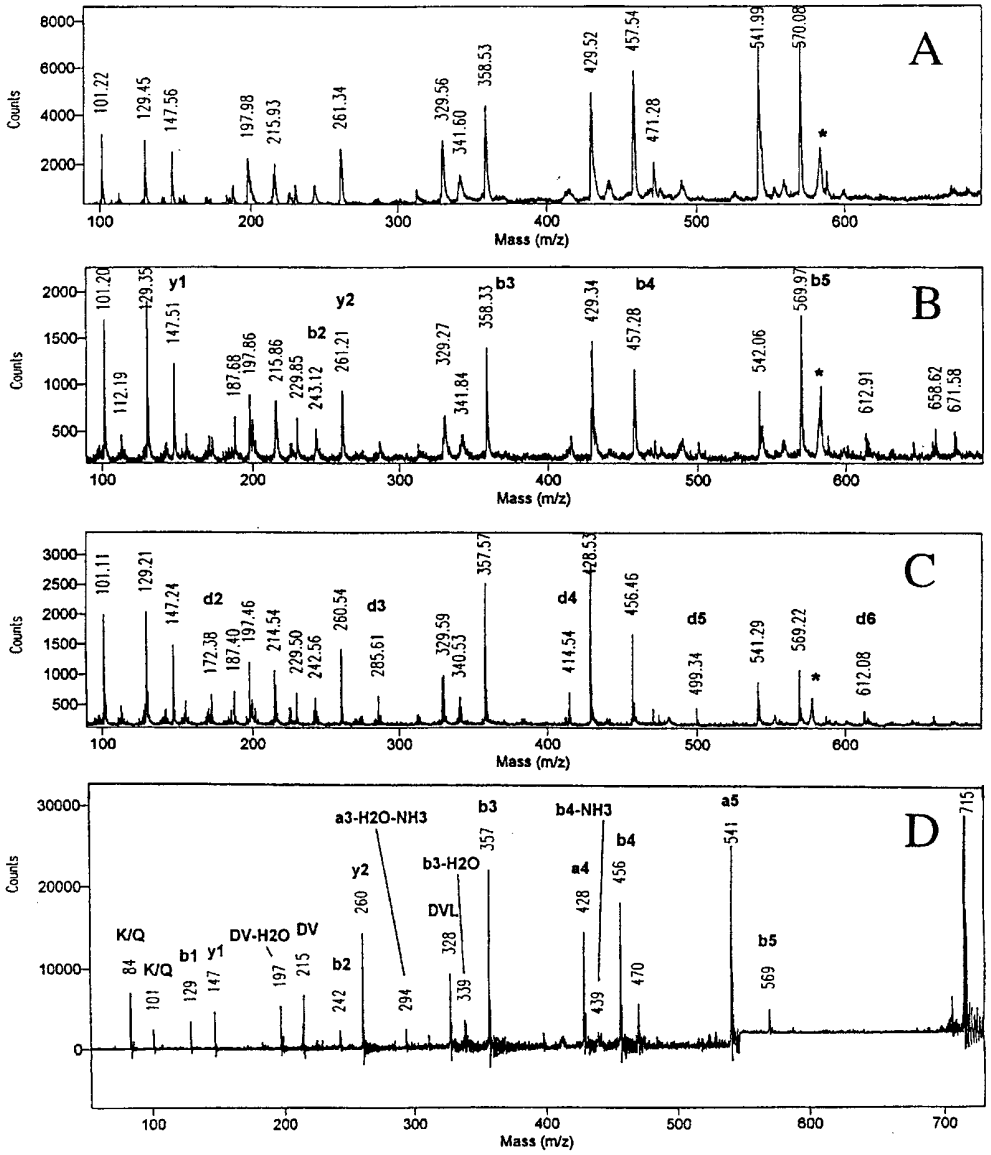

Figure 4. MALDI-TOF/TOF CID spectra of synthetic peptide Lys-Leu-Asp-Val-Leu-Gln (MH^+ at *m/z* 715.4). The reflectron voltage was 26.5 kV for each data acquisition. (A) Spectrum acquired using CHCA as the matrix and He as the collision gas. (B) Spectrum acquired using DHB/He. (C) Spectrum acquired using DHB/Ar. (D) MALDI-PSD spectrum of the same peptide acquired on a Voyager DE STR mass spectrometer with CHCA matrix, recorded in eight separate steps, lowering the reflectron voltage by 25% for each subsequent frame.

was synthesized differing only in the N-terminal basic residue, Arg, Lys, or His. The sequence Arg/Lys/His-Leu-Asp-Val-Leu-Gln was designed so that under appropriate conditions each residue would yield d ions, a characteristic product of remote charge fragmentation processes.[19] Figure 3 presents CID spectra of this peptide with Arg at its N-terminus. The data in panel A were acquired using CHCA as the matrix and He as the collision gas. The spectrum was dominated by N-terminal fragments as expected when the charge is preferentially retained there. Five complete ion series were detected: b, b-NH_3, a, a-NH_3, and d fragments. While all the expected side-chain fragments dependent upon high-energy collisional activation,[19] i.e., d ions, were detected, the spectrum was rather dominated by sequence ions and fragments due to ammonia loss from the Arg residue. In addition, an abundant $b_5 + H_2O$ ion was present as a result of a rearrangement reaction typical of peptides with basic N-termini. In summary, this CID spectrum exhibited ion types indicative of both high-energy CID and PSD fragmentation processes. A second CID spectrum of this compound in panel B acquired using DHB as the matrix and Ar as the collision gas was dominated by a and d ions, which is typical of high-energy CID or remote charge fragmentation of N-terminally charged peptides.[20] Only the cleavage at the C-terminus of the Asp residue yielded a b ion. As has been reported earlier for surface-induced dissociation experiments, the acidic hydrogen of the Asp side chain promotes charge-directed cleavage when the "ionizing" proton is retained at the Arg residue.[21] Thus, use of a "hot" matrix promotes metastable decomposition, while the collisional activation conditions determine the amount of

(19) Johnson, R. S.;. Martin, S. A.; Biemann, K. *Int. J. Mass Spectrom. Ion Processes* **1988**, *86*, 137−154.

(20) Medzihradszky, K. F.; Burlingame, A. L. *Methods: Companion Methods Enzymol.* **1994**, *6*, 284−303.

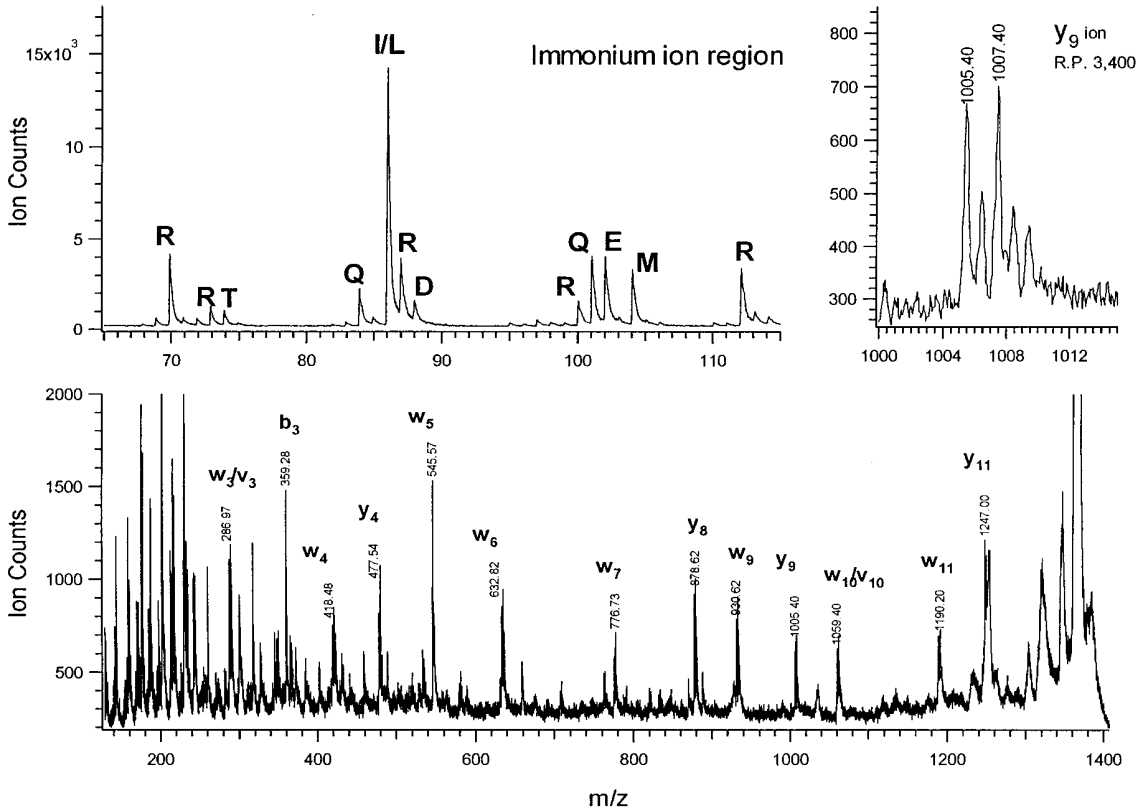

Figure 5. MALDI-TOF/TOF CID spectrum of an $^{16}O/^{18}O$-labeled tryptic peptide Asp-Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met-Gly-Arg, with expansions of the immonium ion region and of the y_9 ion. The HPLC-purified peptide was loaded in a CHCA matrix, Ar was used as the CID collision gas, and the reflectron voltage was 27.5 kV. For fragment ions containing $^{16}O/^{18}O$ isotopic doublets, only the ^{16}O-containing fragments are labeled. Peak intensities in the full spectrum in the lower panel are multiplied 8× relative to the immonium ion spectrum in the upper panel.

energy deposited using a "cooler" matrix.

Peptides that do not contain the highly basic Arg residue behave differently. The CID spectrum of Lys-Leu-Asp-Val-Leu-Gln acquired in the "hot" CHCA matrix with He as the collision gas (Figure 4A) was almost identical to a PSD spectrum (Figure 4D). Beside the immonium ions and some internal fragments, such as AspVal at m/z 215, complete a and b ion series were recorded along with some abundant C-terminal fragments (y_1 and y_2). Practically no d ions were observed. However, when a "cooler" matrix was employed along with the same collision gas (Figure 4B), some relatively low abundance side-chain d ion fragments appeared, and combining this "cooler" matrix with a heavier collision gas (Ar) further enhanced the high-energy fragmentation (Figure 4C). However, although all the d fragments were present in (C), the sequence ions were still dominant and every b ion was observed. The same trend was observed for the corresponding synthetic peptide containing His at its N-terminus. Use of the even "cooler" (two-component) matrix and heavier collision gas (Xe) did not improve the spectrum quality (data not shown). When samples were analyzed using the DHAP/DAHC mixture as the matrix, the spectra were PSD-like, while when Xe was the collision gas, the signals were generally suppressed, most likely due to scattering.

To evaluate this instrumentation for de novo sequencing, a C-terminally 1:1 $^{16}O/^{18}O$-labeled, HPLC-purified peptide from tryptic digestion in 50% labeled water was subjected to CID analysis (Figure 5). This peptide of M_r 1360.6, (^{16}O variant) was analyzed by employing CHCA as the matrix and Ar as the collision gas, this combination being chosen on the basis of our experience with standard peptides larger than those discussed above. An expansion of the low-mass region of the tandem spectrum revealed the presence of Arg, Thr, Lys/Gln, Ile/Leu, Asp, Glu, and Met residues in the form of immonium and related ions, thus providing valuable information on the peptide composition. The peptide was sequenced readily from the typical high-energy CID spectrum as Asp-Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met-Gly-Arg. C-Terminal ions were easily identified, since they exhibited doublets due to the isotope labeling, as in the expansion of y_9 illustrated. The identity of isomeric Leu and Ile residues was unambiguously established from the mass values of side-chain fragments.

The focusing conditions for this spectrum, which was recorded at a single fixed reflectron setting of 27.5 kV, were optimized for ions of approximately m/z 1000. Consequently the resolution in the immonium ion region was quite low and the peaks were distorted, whereas an expansion of the y_9 ion showed symmetrical peaks with a mass resolution of 3400 (fwhm). As the low-mass ions are more easily separated and identified, even when the instrument settings give low resolving power, it is generally more practical to optimize the focusing in the higher mass region, but any region may be selected for focusing as desired.

An indication of the sensitivity that can be achieved using this MALDI-TOF/TOF tandem mass spectrometer is illustrated in Figure 6. In this experiment, 100 fmol of a tryptic peptide was loaded onto the target plate with DHB matrix and subjected to

(21) Gu, C.; Somogyi, A.; Wysocki, V. H.; Medzihradszky, K. F. *Anal. Chim. Acta* **1999**, *397*, 247−256.

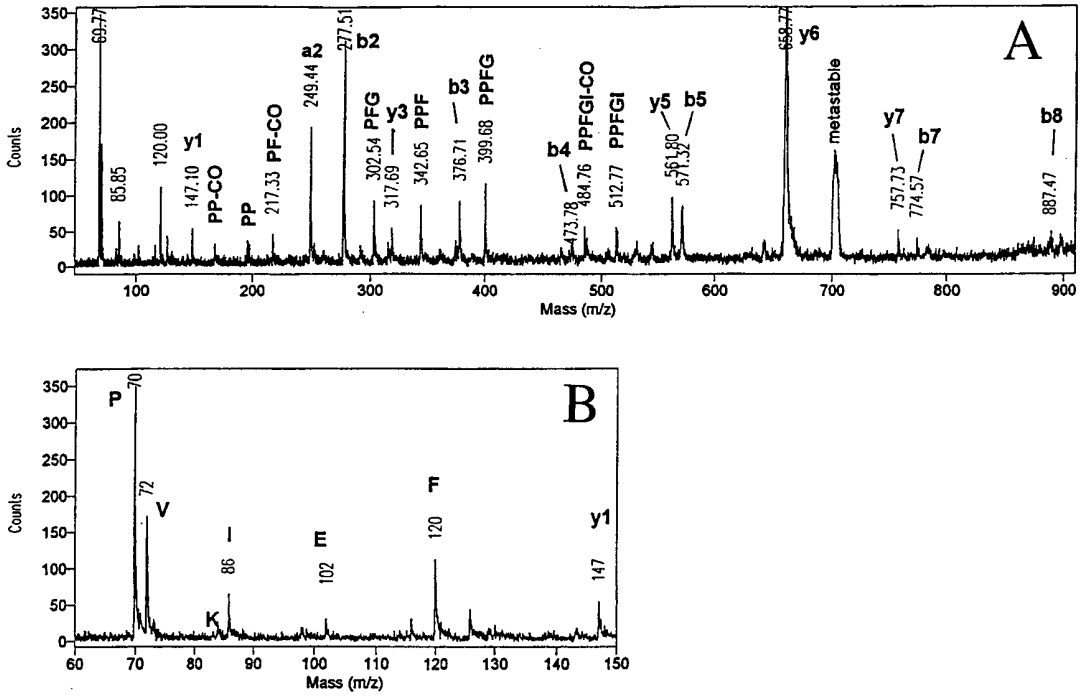

Figure 6. (A) MALDI-TOF/TOF CID spectrum of 100 fmol of tryptic peptide Glu-Phe-Val-Pro-Pro-Phe-Gly-Ile-Lys (MH^+ at *m*/*z* 1033.6), in DHB with He as the collision gas at a reflectron voltage of 28.8 kV. As shown here, the intensity of the strong y_6 ion for the favored cleavage at Val-Pro is off-scale by 3×. (B) An expansion of the immonium ion region.

CID analysis using He as the collision gas. The sequence ions and internal fragments are labeled in the full spectrum in panel A. Favored cleavage at Val-Pro gave a strong y_6 ion and several internal fragments. This spectrum contained all of the fragment ions that were observed in a spectrum obtained from 1 pmol of peptide (not shown), with no significant deterioration of the spectral quality at the lower sample level. Panel B shows an expansion of the immonium ion region, from which characteristic peaks could be recognized for every amino acid in this peptide other than Gly. From the low-mass ions obtained with He collision gas, the identification of Lys was not unambiguous, but air caused more fragmentation and gave much stronger low-mass ions (not shown). The high-quality data and excellent signal-to-noise ratio of the spectral data obtained for 100 fmol of this peptide indicate that the limit for obtaining comprehensive sequence information will be substantially lower.

CONCLUSIONS

The MALDI-TOF/TOF tandem mass spectrometer described here gives high-energy CID spectra of peptides that are highly superior to PSD spectra. Data can be obtained at a single mirror voltage although the focusing conditions in such spectra are not optimized for the entire mass range. Conditions can be selected to optimize high-, intermediate-, or low-mass ions. In general it is unnecessary to optimize low-mass ions, which tend to be strong and can be characterized more easily than the high-mass ions, even with instrument settings that give lower resolution. The nature and the extent of fragmentation can be controlled by the choice of matrix and collision gas, which determine the extent of the side-chain fragmentation, previously characterized for CID of ions formed by FAB or LSIMS in sector tandem instruments. For peptide sequencing, the sensitivity of this instrument is very high, comparable to that of conventional high-performance MALDI-TOF instruments, and highly superior to the earlier generation of sector-based high-energy CID tandem mass spectrometers using LSIMS or FAB ionization.

The nature of the MALDI-CID spectra of peptides is quite dependent upon the amino acid composition. Arginine-containing peptides yield side-chain fragment ions that are characteristic of high-energy CID under all conditions described here, whereas lysine-containing peptides give spectra more similar to PSD. The "cool" matrix DHB partially suppresses MALDI-induced decomposition compared with the "hotter" matrix CHCA. Peptide size also affects the CID spectrum. For heavier species (>1000 Da), the higher energy content of the precursor ions from CHCA can be advantageous. Similarly, although He collision gas induces relatively even fragmentation along the mass range, for bigger molecules, heavier Ar can be beneficial. Larger peptides give spectra dominated by sequence ions rather than charge-remote processes, even under conditions that would yield d and/or w satellite ions from smaller peptides. Fortunately, because of the high sensitivity of the MALDI method and extended lifetime of the sample on the target plate, different experimental conditions can be explored to obtain complementary information.

ACKNOWLEDGMENT

Support from NIH NCRR BRTP, Grant RR01614 to A.L.B. is gratefully acknowledged.

Received for review July 21, 1999. Accepted November 19, 1999.

AC990809Y