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Matrix-Assisted Laser Desorption Ion Trap Mass Spectrometry: Efficient Isolation and Effective Fragmentation of Peptide Ions

Jun Qin and Brian T. Chait*

The Rockefeller University, 1230 York Avenue, New York, New York 10021

Effective analysis of the sequence of peptides using matrix-assisted laser desorption/ionization (MALDI) tandem ion trap mass spectrometry requires efficient mass isolation and the ability to induce extensive sequence-specific fragmentation. The present paper describes a new excitation scheme, which we term red-shifted off-resonance large-amplitude excitation (RSORLAE), that can deposit higher amounts of internal energy in ions than is feasible with conventional resonant excitation. The new method provides an effective means for inducing fragmentation of MALDI-produced peptide ions with *m/z* values up to 3500. Prior to excitation, it is necessary to isolate ions of interest with high efficiency. We demonstrate that isolation efficiencies of >95% can be achieved by careful design of the rf scan functions used during ion isolation. In particular, sudden transitions in the amplitude of the rf field (from low to high amplitudes) must be avoided. The combined improvements in the efficiency for ion isolation and the efficacy of ion activation make MALDI tandem ion trap mass spectrometry a practical tool for the characterization of proteins with high sensitivity.

Effective analysis of the sequence of peptides using matrix-assisted laser desorption/ionization (MALDI) tandem ion trap mass spectrometry requires efficient mass isolation and the ability to induce extensive sequence-specific fragmentation.

The isolation of high-mass ions is normally achieved through a combination of reverse and forward radio frequency (rf) scans incorporating resonant ejection.[1] It is necessary to exercise care during this step since the isolation process can induce excitation and unwanted fragmentation of the isolated ion species.[2] Such excitation can be an especially serious problem for the internally hot ions produced by MALDI.[3] We show here by direct measurement that an isolation efficiency close to unity can be achieved for MALDI-generated peptide ions.

The production of informative fragment ions depends upon several factors, including the total amount of energy that can be deposited in the parent ions and the period of time over which fragmentation can occur prior to measurement of the fragments. In the ion trap, the deposition of energy is usually achieved by resonant excitation, sometimes referred to as "tickling".[4] Although the trap has a long time window for decomposition, it has proved challenging to supply sufficient internal energy by resonant excitation to produce extensive and informative fragmentation of high-mass species (e.g., singly charged peptide ions with m/z > 1000). More effective methods for producing fragments of large peptide ions are needed. This paper describes a new scheme for effective fragmentation of peptide ions in the ion trap.

EXPERIMENTAL SECTION

A full description of the instrument is described in a companion paper.[5] Briefly, the apparatus consists of an external MALDI source, an electrostatic lens, an ion trap, and a channeltron detector with a −10 kV conversion dynode. To improve the efficiency of ion transfer into the trap, we replaced the entrance end cap (containing a single hole) with a standard Finnigan ITMS detector end cap (containing seven holes). The rapid ramping of the rf field used in matched dynamic trapping[6] relies on the inherent time constant of the Finnigan ITMS apparatus, which takes about ∼170 μs to ramp from zero amplitude to any rf value specified in the control software. The firing of the laser is timed so that ions arrive at the entrance end cap on the rising slope of this fast rf ramp. Because large-amplitude ac fields are required for the new excitation scheme described in this paper, an ac amplifier with a gain of ∼3.5 was added to increase the amplitude of the supplemental ac field from the Finnigan frequency synthesizer. A custom data system, incorporating a LeCroy 9450 digital oscilloscope (LeCroy Corp., Chestnut Ridge, NY), was used for all the present experiments. This data system has the useful property that the oscilloscope can be triggered at an arbitrary time subsequent to the laser desorption event. Thus, we can monitor the fate of ions during the whole scan process of trapping, cooling, isolation, resonant excitation, and resonant ejection. Any ions that exit the detector end cap can be captured by the detector during the complex array of rf scan functions, allowing us to monitor the influence of any chosen scan function on the stability of ion trajectories in the trap. The detector ion extraction plate (bias −3 kV) is placed close to the exit end cap (∼0.5 cm), so that ions are detected with little delay upon exit from the ion trap. In all the experiments reported here, ions were accelerated to 25 eV for injection and He was used as the buffer gas. A matrix of 2,5-dihydoxybenzoic acid (DHB) was used for MALDI.

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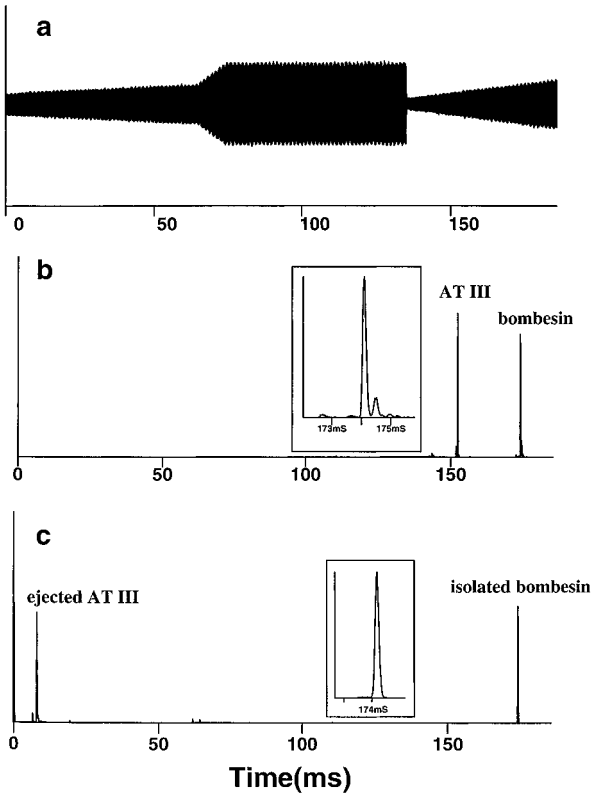

Figure 1. Measurement of the efficiency of mass isolation. (a) The rf waveform used for the efficiency measurement: (b) Measurement of the relative intensity of protonated bombesin and angiotensin III ions without mass isolation (ac modulation turned off). The inset shows a detail of the region surrounding the protonated bombesin ions prior to isolation. (c) Measurement of the relative intensity of protonated bombesin and angiotensin III ions after mass isolation of the bombesin species. The intensity of the ejected angiotensin III ions provides an internal reference. The inset shows a detail of the isolated bombesin ions. Masses higher than protonated bombesin are ejected at an ac frequency of 17 664 Hz (20 times mass extension) from 440 to 83.5 Da over a period of 45 ms [not shown in (a)]. Masses lower than protonated bombesin are ejected from 83.5 to 161.5 Da at 35 371 Hz (10 times mass extension) over 60 ms, followed by excitation for 30 ms and cooling at 312 Da for 30 ms.

RESULTS AND DISCUSSION

Mass Isolation. We have found that efficient mass isolation requires careful design of the isolating rf scan functions. Ions can be inadvertently ejected at the transition point of a sudden jump (discontinuity) in the rf field. A 10 ms duration linking scan that eliminates this sudden jump is needed to prevent ions from being inadvertently ejected (data not shown).

Due to the inherent shot-to-shot instability of the MALDI ion source, the isolation efficiency cannot be measured from different laser pulses. However, the isolation efficiency can be measured from a given laser shot by incorporating an internal standard. Figure 1 compares spectra of bombesin (MM = 1620 Da) obtained in the presence of an internal standard (angiotensin III, MM = 931 Da) either without mass isolation (b) or with mass isolation (c). For the spectrum obtained with mass isolation (Figure 1c), the electron multiplier was turned on during the mass isolation period to monitor the ejected angiotensin III ions. The early peak observed at ~10 ms corresponds to the ejected angiotensin III ions, while the late peak (~180 ms) corresponds to the isolated bombesin ions. Comparison of the ratio of the intensities of these two peaks with the ratio of the corresponding ion peaks in Figure 1b gives a measure of the absolute isolation efficiency. In the experiment shown, the efficiency was determined to be 105 ± 10%.

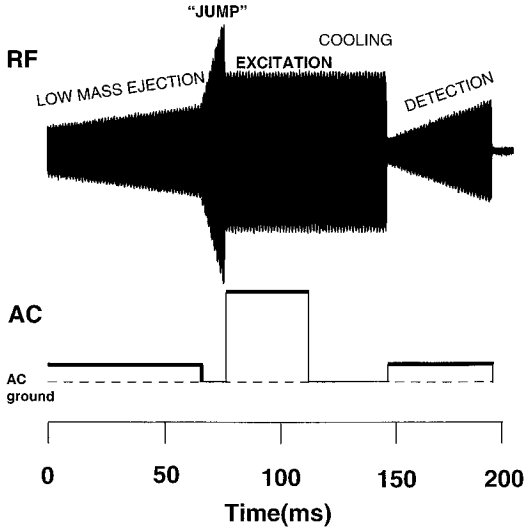

Figure 2. Scan function used for RSORLAE. After mass isolation, the amplitude of the rf field is raised to a higher level over a period of 10 ms (jump scan) and then dropped abruptly to a lower level for excitation. Excitation is performed with a large ac amplitude (21 V_{p-p}) at a frequency that is red-shifted ~ 5% from resonance.

Red-Shifted Off-Resonance Large-Amplitude Excitation (RSORLAE). To induce extensive fragmentation of large peptide ions (m/z > 1000), it is necessary to inject a large amount of energy into the ions. We have found that conventional resonant excitation does not always supply sufficient energy to bring about efficient dissociation of such species. To increase the level of internal energy deposition, we have devised a new scheme which we term red-shifted off-resonance large-amplitude excitation (RSORLAE). Figure 2 shows the scan function used in this procedure. After the ions of interest are isolated, a scan segment (the "jump scan") ramps the rf amplitude from a low to a high level over a period of 10 ms. Immediately after reaching the peak amplitude of the jump scan, the rf amplitude is reduced to a lower level for ion excitation. During this excitation period, ions are not excited resonantly—rather they are excited off-resonantly with a large amplitude (21 V_{p-p}) at a frequency that is red-shifted ~5% from the resonant frequency. The excitation time is varied in the range of 30 ms to 1 s to achieve the desired degree of fragmentation.

The detailed procedure for inducing fragmentation by RSORLAE is as follows. An excitation frequency in the range between 50 and 120 kHz is chosen and the rf level that brings about ejection of the ions of interest ($rf_{resonant}$) is measured at this chosen frequency. The rf level for RSORLAE is then set to a value that is 10−15 Da higher than $rf_{resonant}$, which has the effect of shifting the frequency of excitation ~5% to the red of the resonant frequency. Excitation is carried out at this red-shifted frequency with the largest amplitude available to us (21 V_{p-p}). Figure 3 compares the results of conventional resonant excitation of the undecapeptide substance P (a) with fragmentation of substance P produced by the RSORLAE procedure (b). The difference is dramatic. More extensive fragmentation is observed with the new procedure. In addition, while only y_n series fragments are

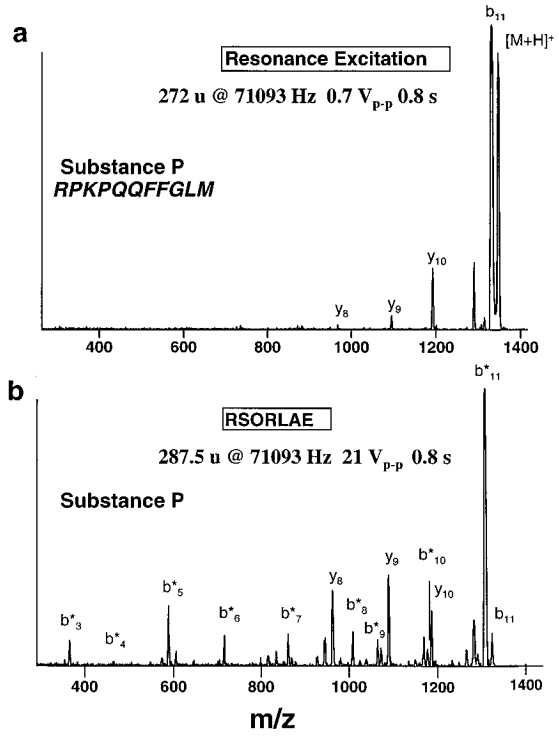

Figure 3. Comparison of the fragmentation (a measure of the internal energy input) of substance P using (a) conventional resonant excitation and (b) RSORLAE. The spectra in both (a) and (b) were obtained at an excitation frequency of 71 093 Hz over 800 ms using a jump scan from 134 to 550 Da over 10 ms. The low mass/charge exclusion limit was changed from 272 Da in (a) to 287.5 Da in (b) to produce an effective red shift in the excitation frequency. In addition, a large ac amplitude (21 V_{p-p}) was used for RSORLAE compared with that used for resonant excitation (0.7 V_{p-p}). b_n* designates the b_n series of fragment ions that have lost H_2O/NH_3.

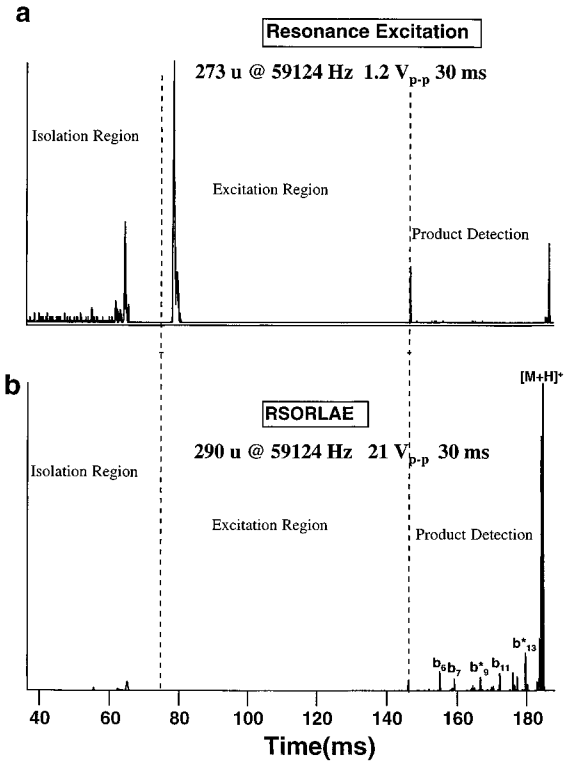

Figure 4. Comparison of the fate of bombesin ions during isolation, excitation, and product detection for (a) conventional resonant excitation and (b) RSORLAE. (a): rf level 273 Da, resonant frequency 59 124 Hz, amplitude 1.2 V_{p-p}, and excitation time 30 ms. (b): same conditions as for (a) except that the low mass/charge exclusion limit is shifted to 290 Da and the ac amplitude is increased to 21 V_{p-p}. No jump scan was used in these experiments. Rather, the final rf level for low mass ejection was connected with the rf level for excitation by a simple linking scan.

observed using conventional resonant excitation, the higher energy b_n series[7] are also present using RSORLAE.

The experiment shown in Figure 4 provides a clue to the reasons for the improved fragmentation obtained with RSORLAE. This experiment monitors the fate of bombesin ions during isolation, excitation, and product detection for (a) conventional resonant excitation and (b) RSORLAE. It is observed that resonant excitation at $q_z = 0.15$ leads to considerable loss of the protonated bombesin parent ion species during the excitation process (Figure 4a). In addition, only weak fragment ions are observed. By contrast, at a similar value of q_z, RSORLAE does not result in premature ejection of the bombesin parent ion species, and an extensive fragmentation pattern is observed (Figure 4b).

It is of interest to note that ejection of ions under resonant conditions only occurs after a time lag of ~4 ms subsequent to commencement of the excitatory ac signal (Figure 4a). This lag is the time required for ions to develop an oscillation amplitude that exceeds the *z* dimension of the ion trap and is crucial for fragmentation, being the effective time window for excitation and dissociation. Our new strategy for injecting higher amounts of internal energy is to increase this time lag so that the process of dissociation can compete more successfully with the process of ejection. The condition for optimum excitation occurs when ions are maintained in oscillations that are just within the ion trap inner boundary for a period that is long enough for ions to build up sufficient internal energy so that dissociation wins over ejection. Under these circumstances, it should be feasible to deposit a relatively high amount of energy because the trapping potential is of the order of a few hundred electronvolts[8] (i.e., potential energy that can be converted to kinetic energy when the ions oscillate back to the center of the trap).

The efficacy of RSORLAE has been tested and shown to be highly effective for peptide sequencing with more than 100 peptides ($1000 < m/z < 3500$). For example, it proves feasible to extensively fragment singly protonated dynorphin 1−13, a peptide containing five basic amino acid residues, which has been documented to have a high resistance to fragmentation.[9] Figure 5 shows that we can deduce fully 11 of the 13 amino acid residues in the sequence of dynorphin 1−13.

Evaluation of the Effect of Off-Resonance Excitation on the Fragmentation Efficiency. To systematically evaluate the effect of off-resonance excitation on fragmentation, we measured the relative dissociation efficiency of bombesin as a function of low mass/charge exclusion limit, keeping the ac excitation frequency constant. This is equivalent to changing the ac excitation frequency while keeping the low mass/charge exclusion limit constant. Figure 6 shows the relative dissociation efficiency of protonated bombesin as a function of the excursion of the rf

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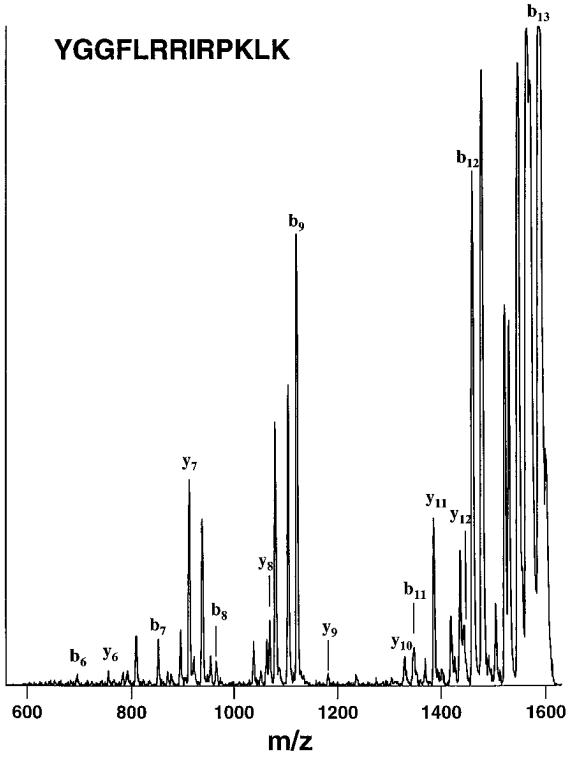

Figure 5. Collision-induced dissociation mass spectrum of dynorphin 1−13 obtained with RSORLAE in which the jump scan is from 160 to 640 Da, the excitation frequency is 119 936 Hz at a rf level of 556 Da, the excitation amplitude is 21 V_{p-p}, and the excitation time is 800 ms.

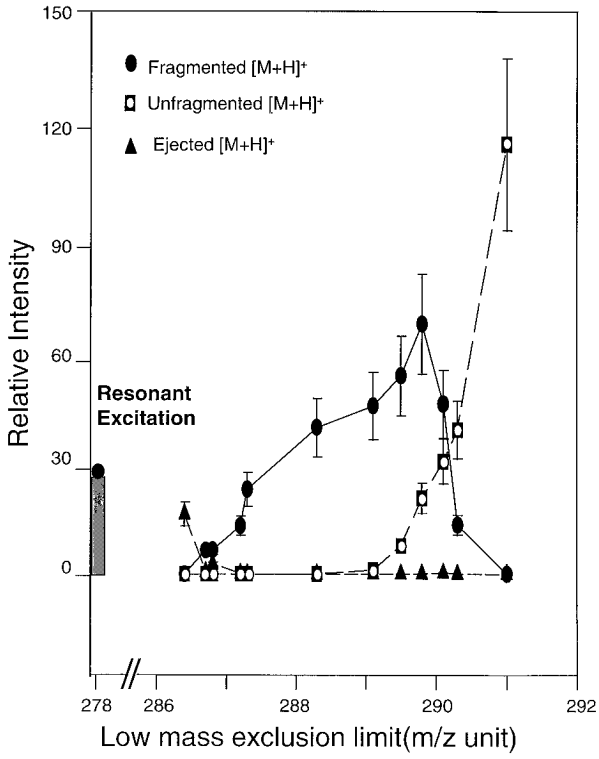

Figure 6. Relative intensities of protonated bombesin fragments as a function of the excursion of the rf setting from $rf_{resonant}$ (filled circles). The excitation frequency is set at 59 124 Hz and the amplitude at 21 V_{p-p} for 30 ms. Results for conventional resonant excitation is included for comparison (low mass/charge exclusion limit 278 Da, resonant frequency 59 124 Hz, and ac amplitude 0.7 V_{p-p} for 30 ms). Also provided are the relative intensities of unfragmented and ejected protonated bombesin. Note that the intensity scale for the bombesin fragments is a factor of 10 higher than that for unfragmented and ejected bombesin ions.

setting from $rf_{resonant}$. We define the dissociation efficiency as the sum of the intensities of five product ions (b_6, b^*_9, b^*_{12}, y_{12}, b^*_{13}) normalized to the intensity of the $[M + H - 17]^+$ species *recorded during the mass isolation period.* Because we carefully maintained conditions that yielded a constant $[M + H]^+/[M + H - 17]^+$ ratio prior to RSORLAE, such normalization to the intensity of the $[M + H - 17]^+$ ion is equivalent to a normalization to the intact $[M + H]^+$ ion.

For comparison, Figure 6 also provides the optimum fragmentation efficiency that we obtained under conventional resonant excitation conditions. Also shown are the relative intensities of unfragmented $[M + H]^+$ ions that are retained in the trap as well as $[M + H]^+$ ions that are ejected during excitation. The fragmentation efficiency was observed to peak at an optimum value of the separation of the rf setting from $rf_{resonant}$. At larger separations, the fragmentation efficiency drops and the intensity of unfragmented $[M + H]^+$ ions that are retained in the trap increases sharply. At the high ac amplitudes employed for RSORLAE, $[M + H]^+$ ions are rapidly ejected as the excitation frequency moves closer to the resonant frequency. The observed improvement in the fragmentation efficiency for RSORLAE over conventional resonant excitation is a factor of ∼2.5. Because high efficiency can be obtained over a range of more than 2 Da (Figure 6), there is no need for careful tuning of excitation conditions—in contrast to the requirements for resonant excitation. The "jump" scan segment also influences the efficacy of the RSORLAE process. Again, there exists an optimum value for the amplitude of the jump scan to yield the highest dissociation efficiency (data not shown). We have observed that the "jump scan" can add as much as ∼30% to the dissociation efficiency.

It is interesting to point out that off-resonance excitation failed to work when the frequency was blue-shifted. Either no ions were fragmented (when the frequency was shifted too far to the blue) or virtually all the ions were ejected when the frequency approached the resonant frequency.

A Model for RSORLAE. We do not yet have a complete understanding of the detailed physics of RSORLAE. However, the experiments illustrated in Figures 4 and 6 provide insights into a possible mechanism for the effective excitation process. To deposit large amounts of energy in the ions of interest, we believe that it is crucial for these ions to be sustained inside the ion trap for a sufficiently long period of time—i.e., long enough to allow the appropriate buildup of internal energy.

We believe that the high-order fields present in the "stretched" Finnigan ion trap[10] play a key role in RSORLAE. It has previously been shown that these high-order fields cause the resonant frequency to be shifted to the blue when large supplementary ac fields are applied.[11] Thus, under the conditions of RSORLAE, the resonant frequency will undergo a progressive shift away from the excitation frequency as a function of time (as the ions increase their oscillation amplitudes and sample progressively more nonquadrupolar components of the field). The shift slows the buildup

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of the maximum excursion of the ion oscillation amplitude compared with that observed using conventional resonant excitation, wherein the maximum excursion of the oscillation increases approximately linearly with time. This reduction in the rate of amplitude buildup constitutes a negative feedback that allows sustained excitation at large oscillation amplitudes. Collisional excitation is most effective for ions undergoing such large-amplitude oscillations. The initial ~5% red shift in frequency simply assures that ions are not ejected too quickly by the large excitation amplitude used in RSORLAE.

The above considerations also provide an explanation for the failure of blue-shifted off-resonance excitation. Here again, the secular frequency of the ions is shifted to the blue under the influence of the higher-order fields. However, in this case, the shifted frequency runs into the excitation frequency—i.e., the condition for resonant excitation whereupon ions are rapidly ejected. We can also understand the role of the "jump" scan segment by considering the effects of the higher-order fields. As the rf amplitude rises, the trapping volume shrinks. When the rf amplitude suddenly drops to a lower level, the trapping volume increases and the cloud of ions expands into a larger volume. Under these conditions, the cloud of ions occupies space that is, on average, further away from the center of the trap, where the effects of the higher-order field are more profound. Thus, the effect of the "jump" segment is to set the stage for the RSOLRAE process.

RSORLAE is different from both conventional resonant excitation and sustained off-resonance irradiation (SORI) as applied in Fourier transform ion cyclotron resonance mass spectrometry.[12] From a mechanistic point of view, RSORLAE takes advantage of the higher-order fields present in the Finnigan ion trap. From an operational point of view, RSORLAE can be applied at much lower values of q_z and much higher values of the ac excitation amplitude than can be used for effective fragmentation of peptide ions in conventional resonant excitation. Thus, in RSORLAE we use q_z values as low as 0.15 and ac amplitudes of ~21 V_{p-p}, whereas conventional resonant excitation normally requires $q_z >$ 0.3 and ac amplitudes of <4 V_{p-p}. The frequency shift (~5%) is much greater than that employed in SORI ($\ll$1%).[12] In addition RSORLAE can only be used with red-shifted frequencies, whereas SORI can be applied with both red-shifted and blue-shifted frequencies.[12,13]

CONCLUSIONS

We have devised a new excitation scheme, RSORLAE, that can deposit higher amounts of internal energy in ions than conventional resonant excitation. RSORLAE proved effective in fragmenting peptide ions produced by matrix-assisted laser desorption with m/z values up to 3500.[5]

Prior to excitation, it is important to isolate ions of interest with high efficiency. We show here that isolation efficiencies as high as 100% can be achieved with careful design of the rf scan functions used during ion isolation.

The combined improvements in the efficiency for ion isolation and the efficacy of ion activation make MALDI tandem ion trap mass spectrometry a practical tool for the characterization of proteins with high sensitivity.[5]

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