

Fragmentation of Singly Protonated Peptides via a Combination of Infrared and Collisional Activation

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The coupling of matrix-assisted laser desorption/ionization (MALDI) to Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) provides an exceptionally capable platform for peptide analysis, but an important limitation of this approach is the difficulty in obtaining informative tandem mass spectra (MS/MS) of singly protonated peptides. This difficulty is especially pronounced with peptide ions containing basic amino acid residues (for example, tryptic peptides). While such ions can be fragmented in some instrument configurations, most FTICR instruments have comparatively little facility for high-energy fragmentation. Here, a novel MS/MS approach implemented with MALDI-FTICR-MS and specifically intended for enhanced fragmentation of singly protonated peptides is described. The method involves infrared irradiation in concert with the simultaneous application of sustained off-resonance irradiation collision-induced dissociation (SORI-CID). This form of MS/MS, described as a combination of infrared and collisional activation (CIRCA), is shown to provide a greater capacity for dissociation of singly charged model peptide ions as compared to infrared multiphoton dissociation (IRMPD) or SORI-CID alone. Overall, the CIRCA approach is demonstrated to be a feasible technique for accessing useful fragmentation pathways of singly charged peptides, including those harboring basic amino acid residues—a crucial feature in the context of proteomics.

Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) represents an excellent tool for bioanalytical mass spectrometry. Owing to a powerful combination of high resolving power ($m/\Delta m > 10^5$ with broadband detection) and unsurpassed mass accuracy (mass errors on the order of a few parts per million or less), analysis of complex biological samples can be accomplished with a remarkable level of detail.^{1–5} Another asset of

FTICR-MS is compatibility with several forms of tandem mass spectrometry (MS/MS or MSⁿ), including collision-induced dissociation (CID), surface-induced dissociation (SID), infrared multiphoton dissociation (IRMPD), and electron capture dissociation (ECD).^{6–15}

The implementation of matrix-assisted laser desorption/ionization (MALDI) with FTICR-MS provides a particularly capable tool for rapid, highly resolved, and mass-accurate analysis of protein tryptic digests; nevertheless, a significant shortcoming of MALDI-FTICR-MS in this regard is the inherent difficulty in performing effective MS/MS on the singly charged peptides produced by MALDI. In particular, singly protonated peptides containing basic amino acid residues are far more recalcitrant to dissociation than their multiply protonated counterparts. This is of special importance in proteomic approaches based on tryptic digestion. Trypsin produces peptides carboxy-terminal in either a lysine residue or an arginine residue, both of which have high gas-phase basicities.¹⁶ These residues sequester the charge-carrying proton, thereby arresting charge-directed fragmentation pathways. Since sequentially relevant peptide fragmentation channels are generally charge-directed in nature, singly protonated peptides with the charge isolated at a basic residue are not easily fragmented in a useful manner.^{17–20} In the absence of a mobile proton, any available charge-remote pathways (e.g., the aspartic acid effect and others)

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tend to predominate. Thus, low-energy MS/MS of singly protonated peptides is generally characterized by relatively uninformative fragmentation products, particularly in the presence of at least one basic residue.

For peptide analysis, MALDI is perhaps most commonly coupled to time-of-flight (TOF) MS. With the use of a MALDI-TOF/TOF instrument, for example, high-energy CID of singly protonated tryptic peptides can be accomplished with a reasonable yield of sequence information. Such an analyzer is designed to access these fragmentation channels by accelerating ions through hundreds of volts of potential. In FTICR-MS, CID is generally accomplished with the aid of sustained off-resonance irradiation (SORI). In addition to adjusting the duration of SORI, the amplitude of the SORI pulse can be optimized for a given precursor ion and is generally on the order of $10 V_{(b-p)}$. A typical SORI-CID experiment is capable of achieving fragmentation energies on the order of 10 eV.²¹ The upper limit of SORI-CID fragmentation energy in FTICR-MS is ultimately dictated by the magnitude of the magnetic induction—a fixed parameter for a given instrument.

IRMPD can also be implemented with MALDI-FTICR-MS and introduces some significant advantages over SORI-CID including higher duty cycle and the production of richer fragmentation spectra. The greater extent of fragmentation in IRMPD versus SORI-CID is due to the nonselective nature of IRMPD, which deposits energy into the precursor ion as well as the resulting product ions, thus leading to sequential fragmentations.²² By contrast, SORI excitation is specific to the precursor ion. IRMPD has been extensively used in this laboratory for the MS/MS analysis of oligosaccharides.^{23–25} However, attempts at IRMPD of MALDI-generated peptide ions have been less successful. This result is not entirely unexpected because IRMPD, like SORI-CID, accesses the lowest energy fragmentation pathways. IR photons supplied by a CO₂ laser at 10.6 μm have an energy of 0.12 eV, rendering IRMPD an unlikely candidate for achieving higher energy MS/MS.

There have been a number of reports in the literature detailing the combination of various MS/MS techniques in order to gain the favorable characteristics of each. These combinations include the tandem implementation of IRMPD and ECD for dissociation of electrosprayed ions^{26–28} and alternating cycles of CID and IRMPD to produce more abundant fragmentation information.^{29,30} Elevated temperatures have also been used to thermally assist CID and IRMPD processes.^{31,32}

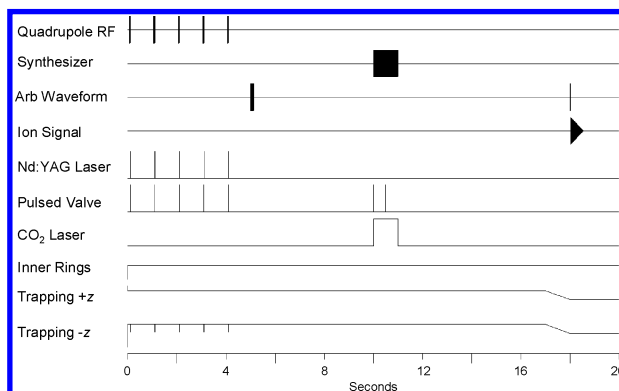


Figure 1. Generic pulse sequence for a MALDI-FTICR-MS/MS experiment with CIRCA. Individual SORI-CID and IRMPD experiments were performed by removing the unwanted pulses from the CIRCA sequence.

In an effort to obtain quality fragmentation spectra for singly protonated peptides using MALDI-FTICR-MS, an MS/MS approach has been developed whereby precursor ions are subjected to a SORI-CID event while concurrently being irradiated with infrared photons. The new tandem MS experiment, best described as a combination of infrared and collisional activation (CIRCA), was used to probe peptide ions produced by MALDI in an FTICR-MS instrument. MS/MS with CIRCA was compared to the individual implementation of SORI-CID and IRMPD on the same model peptides. Peptide fragmentation behavior with CIRCA is discussed with respect to the superimposition of CID and IRMPD energetics, and some limitations of CIRCA are also addressed.

EXPERIMENTAL SECTION

Chemicals. Model peptides including P₁₄R, bombesin, substance P, and angiotensin II were obtained from Sigma (St. Louis, MO). The MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was also acquired from Sigma and used without further purification.

Sample Preparation. Stock solutions of model peptides were spotted on a stainless steel MALDI target (1 μL) to provide approximately 1 pmol of peptide per spot. Each spot was treated with an equal volume of 50 μg/μL DHB in 50% acetonitrile containing 0.1% trifluoroacetic acid. The spots were allowed to dry under ambient laboratory conditions.

Mass Spectrometry. An IonSpec HiRes MALDI-FTICR-MS (Lake Forest, CA) served as the platform for all experiments described herein. The instrument configuration included an external ion source based on a third harmonic Nd:YAG laser (355 nm) and a 7.0 T actively shielded superconducting magnet. A CO₂ laser (10.6 μm, 20 W maximum power, Parallax, Waltham, MA) provided IR photons for IRMPD and CIRCA experiments. Modifications for addition of the IR laser to this instrument have been described in detail elsewhere.^{5,23–25,33}

The pulse sequence for tandem MS experiments with CIRCA is shown in Figure 1. Essentially the same pulse sequence was used in SORI-CID and IRMPD experiments, except that the unwanted pulses were removed from the CIRCA sequence as appropriate. Briefly, several MALDI laser pulses were each

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accompanied by an RF pulse on the quadrupole ion guide to direct the ions to the ICR cell. Concurrently, a pulsed valve event was triggered to leak argon into the ICR and ion guide regions of the instrument for vibrational cooling of the ions. A brief reduction of the source-side ($-z$ direction) trapping plate potential was made to allow ions to enter the ICR cell. Desired precursor ions were isolated in the ICR cell via stored waveform inverse Fourier transform (SWIFT) ejection provided by an arbitrary waveform generator. Isolated precursors were then interrogated by MS/MS using either SORI-CID, IRMPD, or CIRCA. SORI amplitudes ranged from 8 to 10 $V_{(b-p)}$, with a typical duration of 500–1000 ms. All SORI was carried out at a frequency offset of +1000 Hz relative to the precursor ion cyclotron frequency. The argon pulsed valve was triggered twice during each SORI event in order to temporarily elevate the pressure in the ICR analyzer to 10^{-5} torr. Prior to ion acceleration and mass analysis, a pressure 10^{-10} torr was reestablished. IRMPD was performed with the laser power set to 20 W and pulse widths of 2–3 s. The SORI amplitude and IR pulse width were each adjusted to produce maximum fragmentation without undue loss of signal. Once IRMPD and SORI-CID parameters were individually optimized, the same parameters were combined in the corresponding CIRCA experiment.

RESULTS AND DISCUSSION

Initial experiments to assess the CIRCA technique were conducted on $P_{14}R$. This synthetic peptide is prone to dissociation due to the tendency of peptides to fragment at proline residues.²⁰ This proline effect renders the $P_{14}R$ peptide somewhat labile and therefore a rather unrealistic model for fragmentation of singly charged peptide ions; however, an idealized peptide was chosen for these preliminary experiments in order to determine if the CIRCA approach merited further investigation. There was an initial concern that superimposition of the SORI-CID and IRMPD experiments would only serve to relax IR-energized ions through ion-neutral collisions due to the transiently elevated pressure in the ICR cell. If the ions were collisionally cooled at a greater rate than the absorption of IR photons, no improvement in dissociation would be experienced. This phenomenon is particularly important when carrying out IRMPD in ion trap mass analyzers, which operate at a much higher optimum pressure than FTICR mass spectrometers.³¹ As shown in Figure 2, these concerns were not substantiated by experiment. The MS/MS of $P_{14}R$ with IRMPD produced four y-type ions, and the SORI-CID experiment produced five y ions. In the CIRCA experiment, eight y-series sequence ions were observed, with significantly increased overall abundance of fragment ions. Clearly, execution of CIRCA allows for deposition of more energy into the ion population than can be introduced by IRMPD or SORI-CID alone. Notably, even in the case of a relatively labile peptide, the observed fragment ion series are dictated by the position of the basic residue. Thus, only y-type ions were observed for $P_{14}R$ due to the C-terminal arginine residue.

Having established the capability of CIRCA to enhance peptide fragmentation as compared to SORI-CID or IRMPD alone, it was next determined what effect the application of SORI would have on the interaction of trapped ions with the IR laser beam in the absence of collision gas. Since off-resonance excitation causes the cyclotron radius to oscillate, the exposure of precursor ions to IR irradiation can be manipulated using SORI. For example, if the

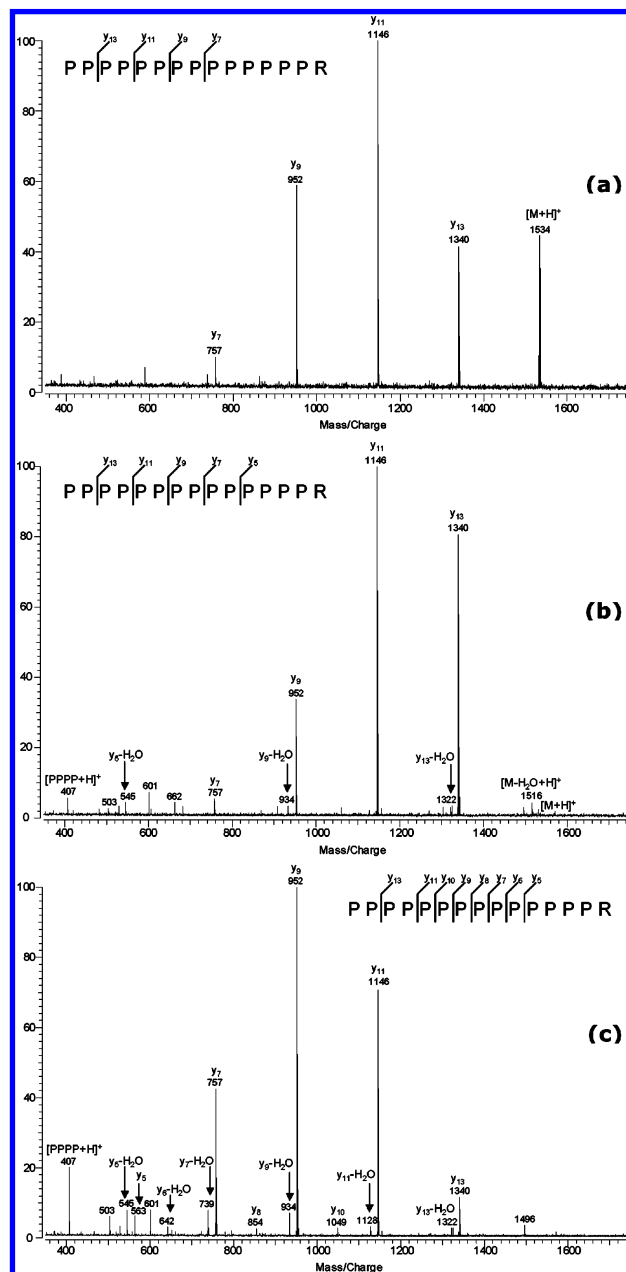


Figure 2. MALDI-FTICR-MS/MS of $P_{14}R$ with IRMPD (a), SORI-CID (b), and CIRCA (c). The SORI amplitude in (b) and (c) was 10 $V_{(b-p)}$; the IR pulse width in (a) and (c) was 2 s.

majority of the ion population were trapped on-axis with respect to the IR beam path, the application of a SORI pulse could be used to attenuate the deposition of energy, providing an additional means of tuning fragment ion yield.³³ Conversely, trapped ions not engaging in cyclotron motion at the center of the ICR cell (where the CO_2 laser is targeted) may not appreciably interact with the IR beam. In this case, improvement in IRMPD efficiency can be realized by application of a SORI pulse during IRMPD.³⁴

In order to determine whether either of these phenomena made a substantive contribution to the outcome of CIRCA experiments, the IRMPD of $P_{14}R$ was conducted with and without the inclusion of SORI, in both cases without the admission of

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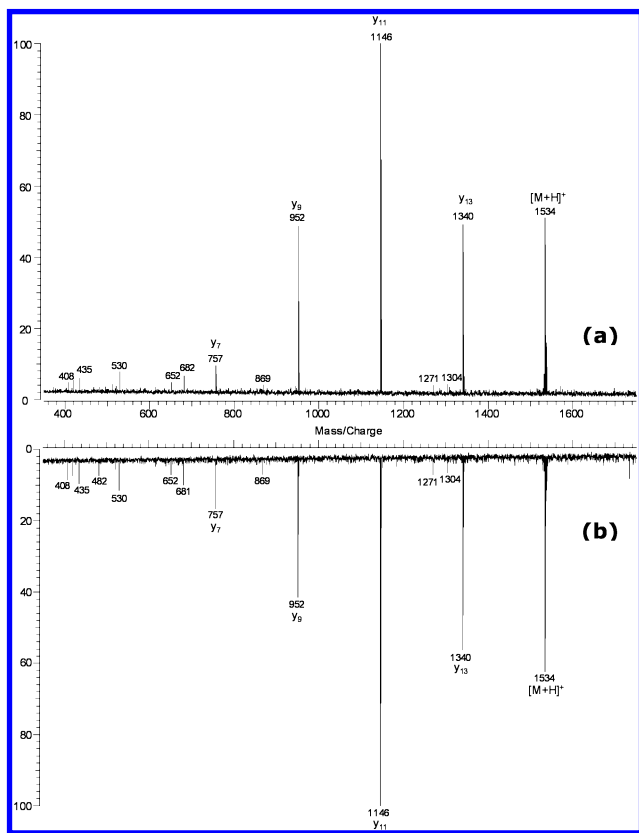


Figure 3. MALDI-FTICR-MS/MS of $P_{14}R$ with IRMPD. In (a), no SORI event was applied. In (b), SORI was performed with an amplitude of 8 V_(b-p) during the IR irradiation. The IR pulse width in both cases was 2 s.

collision gas. As compared in Figure 3, the addition of off-resonance excitation does not appear to have any measurable impact on the IRMPD outcome. Most likely, this is due to the expansion of the IR beam by dispersion optics (to a final diameter of 12 mm) and good alignment with the trapped ion population. Evidently, the dissociation experienced in a CIRCA experiment was not being significantly aided or hindered strictly as a consequence of SORI coinciding with IRMPD.

Subsequent experiments were directed toward more realistic peptides. The tandem mass spectra for singly charged bombesin obtained with IRMPD, SORI-CID, and CIRCA are shown in Figure 4. Notably, bombesin contains two basic residues: an arginine and a histidine. Every effort was made to achieve fragmentation with IRMPD; however, no detectable sequence ions were produced (perhaps partly due to the presence of both an arginine and a histidine residue). SORI-CID of $[M + H]^+$ bombesin precursors produced a number of b-series ions and a single y-type ion. All of the observed ions retained the arginine residue. When probed with CIRCA, fragmentation products representing all but one peptide bond cleavage were obtained, with 10 b cleavages and 5 y cleavages observed. Interestingly, the CIRCA experiment produced three y-type cleavages that did not include the arginine residue. In the higher energy CIRCA MS/MS, the arginine-exclusive ions were most likely protonated at the histidine residue. The observed preference for arginine-containing fragments over histidine-containing fragments is consistent with the relative gas-phase basicities of these residues.¹⁶ No product ions lacking both basic residues were detected.

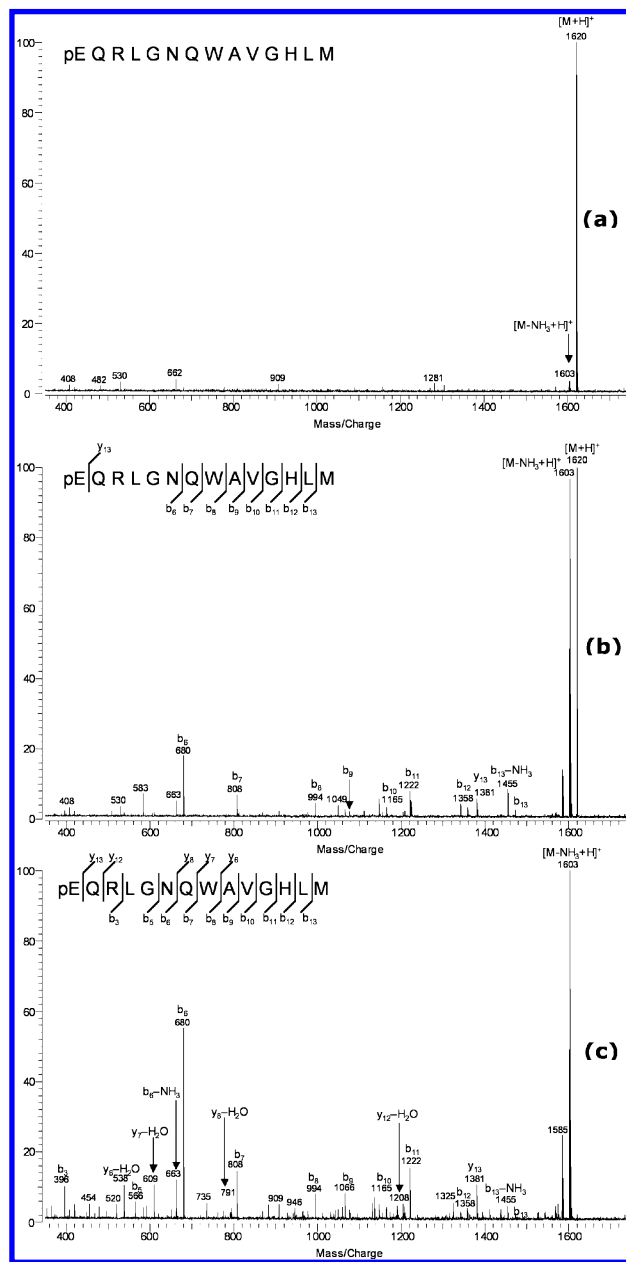


Figure 4. MALDI-FTICR-MS/MS of bombesin with IRMPD (a), SORI-CID (b), and CIRCA (c). The SORI amplitude in (b) and (c) was 10 V_(b-p); the IR pulse width in (a) and (c) was 3 s.

Figure 5 presents the MALDI-FTICR-MS/MS spectra for substance P using IRMPD, SORI-CID, and CIRCA. In the case of SORI-CID and IRMPD individually, only a few sequence fragments were produced; however, in the CIRCA fragmentation spectrum all but two possible peptide bond cleavages were provided. Again, in the case of substance P, multiple basic amino acid residues were present and only fragment ions containing at least one of the basic sites were observed.

Fragmentation of angiotensin II by the three activation methods produced a noteworthy result. As depicted in Figure 6, the IRMPD fragmentation produced only the y_7 ion, a product of charge-remote fragmentation C-terminal of the aspartic acid residue (the aspartic acid effect). While the y_7 remained the major product of SORI-CID, a number of other products were also observed with all ions produced harboring the arginine residue,

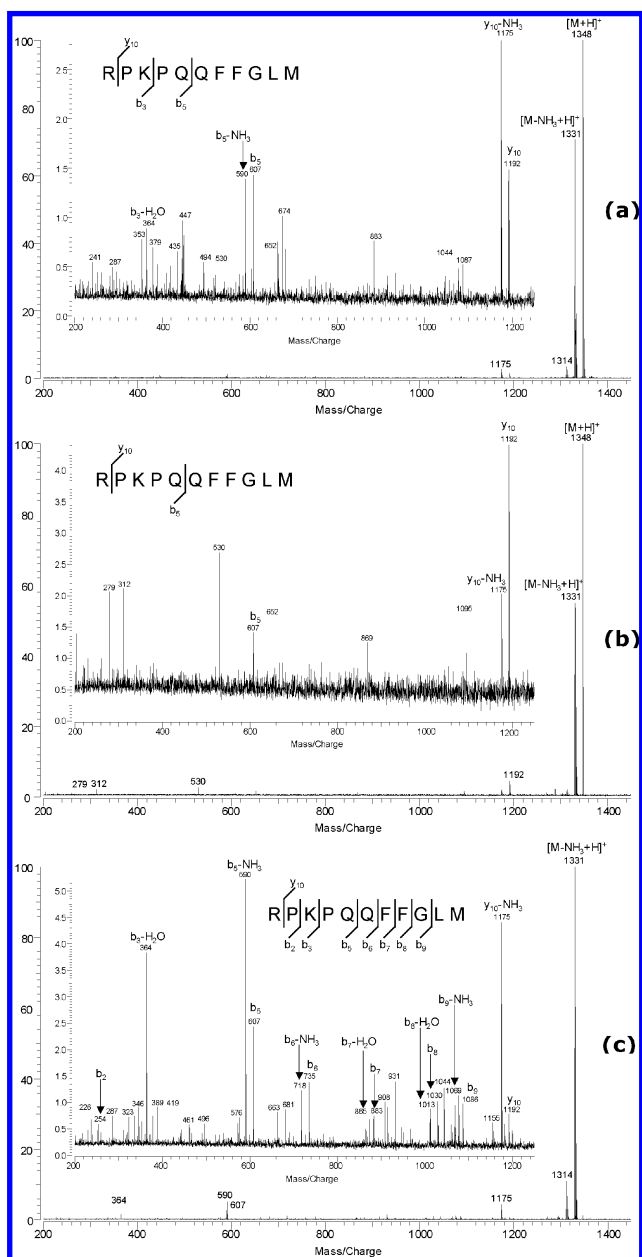


Figure 5. MALDI-FTICR-MS/MS of substance P with IRMPD (a), SORI-CID (b), and CIRCA (c). The SORI amplitude in (b) and (c) was 8 $V_{(b-p)}$; the IR pulse width in (a) and (c) was 3 s. Expanded views of m/z 200–1200 are inset.

the histidine residue, or both. All but one of the peptide bonds was cleaved. Importantly, the CIRCA experiment yielded an almost identical result to the SORI-CID, with the exception that CIRCA did not produce the y_4 ion (although this was not a particularly abundant signal in the SORI-CID experiment). As angiotensin II had the lowest molar mass among the model peptides investigated, the corresponding ion would have a lower density of vibrational modes as compared to the peptide ions of higher molecular weight. With a less efficient reservoir of vibrational energy, the addition of IR irradiation to the SORI-CID event would be expected to offer less improvement in fragment yield as opposed to ions of higher molar mass. The result for angiotensin II was in agreement with this prediction, suggesting that with decreasing mass comes diminishing returns for implementation of CIRCA as opposed to SORI-CID alone. A similar result was also obtained for the

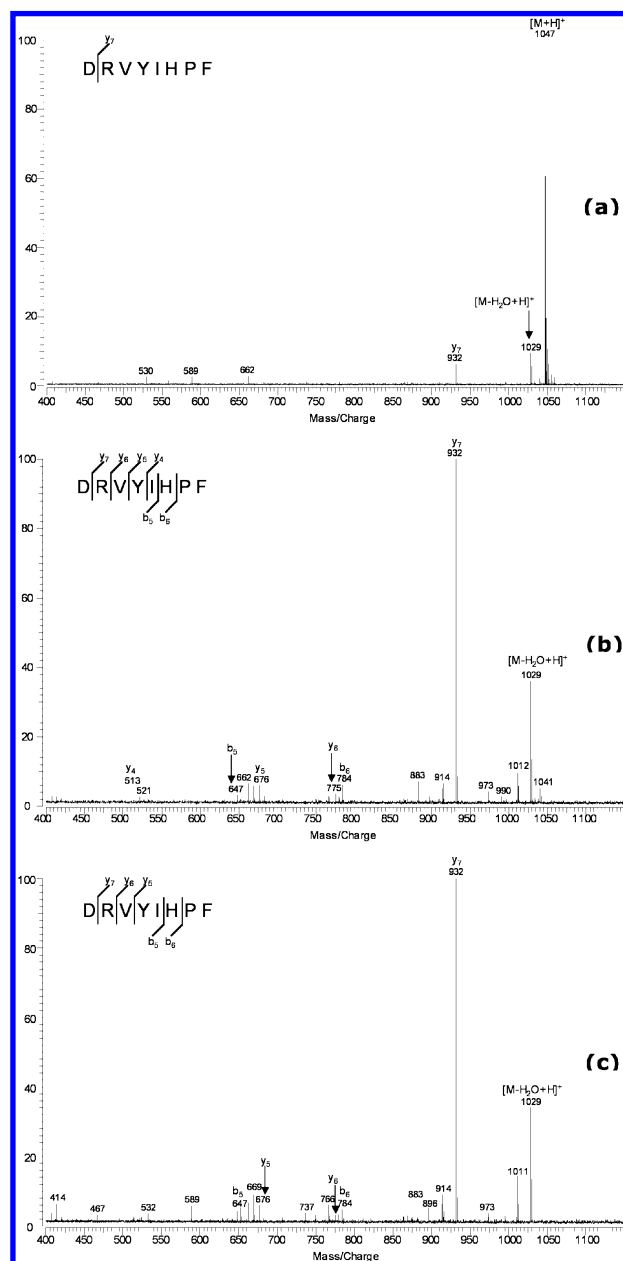


Figure 6. MALDI-FTICR-MS/MS of angiotensin II with IRMPD (a), SORI-CID (b), and CIRCA (c). The SORI amplitude in (b) and (c) was 8 $V_{(b-p)}$; the IR pulse width in (a) and (c) was 2 s.

bradykinin fragment, an even smaller peptide with a nominal mass of 757 Da (data not shown).

Collectively, these initial experiments suggest that the CIRCA sequence is capable of depositing more energy into a population of precursor ions than SORI-CID or IRMPD alone. Importantly, IRMPD is inherently a slower heating process than SORI-CID. Each IR photon at $10.6 \mu\text{m}$ carries an energy of approximately 0.12 eV, while low-energy CID collisions deposit approximately 0.5–10 eV.²¹ On the basis of these values and in light of the present results, the energetic basis for improved fragmentation in CIRCA can be proposed. As illustrated in Figure 7, vibrationally excited ions heated by the absorption of multiple IR photons can then be readily dissociated by a single ion-neutral collision. That is, the improvement in fragmentation efficiency experienced with the use of CIRCA is primarily attributed to the collision-induced

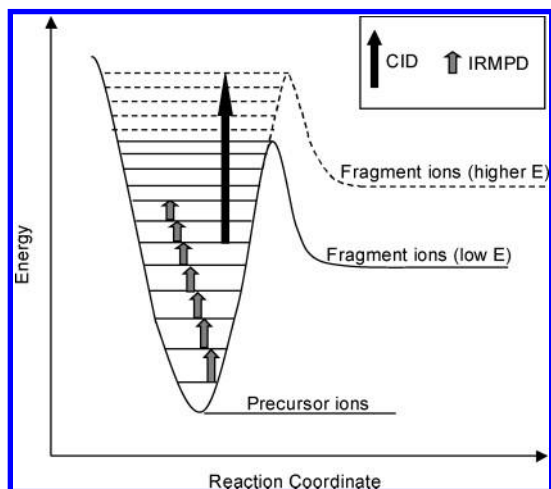


Figure 7. Proposed energetics of the CIRCA process. Improvement in fragmentation efficiency and the ability to access higher energy fragmentations are primarily attributed to the collision-induced dissociation of ions already residing in excited vibrational states due to IR irradiation.

dissociation of ions already residing in excited vibrational states due to IR irradiation. In the case of singly charged peptides with basic residues, this additional energy allows higher energy fragmentation channels to be accessed (i.e., charge-directed fragmentation), even when lower energy dissociation channels are also available (i.e., charge-remote fragmentation). The proposed energetics are also consistent with the inability of CIRCA to enhance the fragmentation of smaller peptides when compared to CID alone.

An alternative explanation for the improved fragment yield provided by CIRCA might be that IR irradiation further fragments the products of SORI-CID. While this process probably occurs to some extent, it is most likely a minor contributor to the improved

fragment yield based on the energetic arguments above. Because IR deposits significantly more energy into the precursor ion (due to a greater density of vibrational modes), it appears most probable that CIRCA improves fragment yield through SORI-CID of the IR-activated precursor ion.

CONCLUSIONS

CIRCA yields improved fragmentation of singly protonated peptides by virtue of increased energy input compared to that of SORI-CID or IRMPD alone. This was shown to hold true in the presence of basic amino acid residues. CIRCA seems to offer no improvement in the case of smaller ions, although this is not a major limitation because sequencing of larger peptides is generally preferred in proteomic experiments. While CIRCA can produce informative sequence ions, the fragment series observed are still primarily dictated by the location of any basic residues, and therefore, the technique is still subject to some limitations related to the lack of a readily mobile proton. Nonetheless, the CIRCA technique has been shown to provide a viable approach to fragmentation of MALDI-produced peptide ions in FTICR-MS despite the presence of basic amino acid residues. Due to this capability in particular, CIRCA assumes considerable gravity in the context of MS-based proteomics, a field almost entirely based on the analysis of peptides terminated by basic residues.

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